

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/28765> holds various files of this Leiden University dissertation.

Author: Wissing, Michel Daniël

Title: Improving therapy options for patients with metastatic castrate-resistant prostate cancer

Issue Date: 2014-09-17

Improving therapy options for patients with metastatic castrate-resistant prostate cancer

Michel Daniël Wissing

Promotiecommissie:

Promotoren: prof. dr. A.J. Gelderblom
 prof. dr. P.J. van Diest (UMCU)
Overige leden: prof. dr. E. van der Wall (UMCU)
 prof. dr. J.J.M. van der Hoeven
 dr. G. van der Pluijm

The research presented in this thesis was performed at the departments of Clinical Oncology, Urology, and Pathology, Leiden University Medical Center (LUMC; Leiden, the Netherlands); at the department of Pathology, University Medical Center Utrecht (UMCU; Utrecht, the Netherlands); at the department of Oncology, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins Medical Institutions (JHMI; Baltimore, MD, USA); and at the Office of Hematology and Oncology Products, Center for Drug Evaluation and Research at United States Food and Drug Administration (OHOP, CDER, US FDA; Silver Spring, MD, USA). This research was sponsored amongst others by Leiden University, the Huygens Scholarschip Programme and the dr. Saal van Zwanenbergstichting.

Cover design J. Grashof & M.D. Wissing
Layout M.D. Wissing
Printed by Gildeprint drukkerijen
ISBN 978-94-6108-724-9

©2014 Michel D. Wissing

All rights reserved. No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or any information storage or retrieval system, without permission in writing from the author.

Financial support for printing of this thesis was kindly provided by: the department of Clinical Oncology LUMC, Bayer, Boehringer Ingelheim, Sanofi-Aventis and Amgen B.V.

Improving therapy options for patients with metastatic castrate-resistant prostate cancer

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 17 september 2014
klokke 13.45 uur

door

Michel Daniël Wissing

geboren te Tilburg
in 1988

Table of contents

Chapter 1	General introduction	7
Chapter 2	Under-representation of racial minorities in prostate cancer studies submitted to the FDA to support potential marketing approval, 1993-2013 <i>Cancer 2014 Jun 25. [Epub ahead of print]</i>	13
Chapter 3	Cabazitaxel in patients with metastatic castration-resistant prostate cancer: results of the compassionate use program in the Netherlands <i>Clin Genitourin Cancer 2013 Sep;11(3):238-250</i>	27
Chapter 4	CAST: A retrospective analysis of cabazitaxel and abiraterone acetate sequential treatment in patients with metastatic castrate-resistant prostate cancer previously treated with docetaxel <i>Submitted</i>	51
Chapter 5	Antimitotic agents for prostate cancer treatment a. Antimitotic agents for the treatment of patients with metastatic castrate-resistant prostate cancer [review] <i>Expert Opin Investig Drugs 2013 May;22(5):635-661</i> b. Tales of How Great Drugs Were Brought Down by a Flawed Rationale -- Letter <i>Clin Cancer Res 2013 Mar 1;19(5):1303</i>	71
Chapter 6	Analysis of the genomic response of human prostate cancer cells to histone deacetylase inhibitors <i>Epigenetics 2013 Sep;8(9):907-920</i>	119
Chapter 7	Targeting prostate cancer cell lines with polo-like kinase 1 inhibitors as a single agent and in combination with histone deacetylase inhibitors <i>FASEB J 2013 Oct;27(10):4279-4293</i>	139

Chapter 8	Combining the pan-aurora kinase inhibitor AMG 900 with histone deacetylase inhibitors enhances antitumor activity in prostate cancer <i>Cancer Med</i> 2014 Jul 3. [Epub ahead of print]	163
Chapter 9	Nuclear Eg5 (kinesin spindle protein) expression predicts docetaxel response and prostate cancer aggressiveness <i>Oncotarget</i> , in press	183
Chapter 10	Radium-223 chloride: extending life in prostate cancer patients by treating bone metastases <i>Clin Cancer Res</i> 2013 Nov 1;19(21):5822-5827	199
Chapter 11	NDRG1 in prostate cancer biology and treatment a. NDRG1 (N-myc downstream regulated gene 1) [gene card] <i>Atlas Genet Cytogenet Oncol Haematol</i> 2010;14(8):776-780 b. Identification of cetrimonium bromide and irinotecan as compounds with synthetic lethality against NDRG1 deficient prostate cancer cells <i>Cancer Biol Ther</i> 2013 May 1;14(5):401-410	209
Chapter 12	Small molecule screening of PC3 prostate cancer cells identifies tilorone dihydrochloride to selectively inhibit cells based on cyclin-dependent kinase 5 expression <i>Oncology Rep</i> 2014 Jul;32(1):419-424	237
Chapter 13	Summarizing discussion - Future perspectives on treatment of patients with metastatic castrate-resistant prostate cancer	249
Appendices	Nederlandse samenvatting	258
	Publicaties	264
	Over de auteur	267
	Dankwoord	268

Chapter 1

General introduction

Prostate cancer is the second deadliest cancer in men in Western countries, being only surpassed by lung cancer.¹ Prostate tumors initially respond to hormonal therapy, radiotherapy and/or surgery, therefore, patients in early stages of disease have a five year overall survival rate of nearly 100%.² However, once the tumor becomes castrate-resistant and metastasizes, overall survival decreases, and treatment options are limited though expanding. Currently, first-line therapy in the Netherlands for patients with metastatic castrate-resistant prostate cancer (mCRPC) consists of docetaxel with prednisone.³ Second-line therapies consist of cabazitaxel (with prednisone), abiraterone acetate (with prednisone) and enzalutamide.⁴⁻⁷ Other therapies, such as tasquinimod, are in advanced stages of clinical development.⁸ These improved therapies have contributed to the steady increase in the five year overall survival rate of prostate cancer patients from 63% to 86% in the Netherlands over the past two decades.⁹ Nevertheless, the incidence and absolute mortality of prostate cancer are rising, attributed to ageing of the population and the introduction of PSA screenings.^{10, 11} Therefore, research aimed at further improving mCRPC treatment in order to increase survival and/or quality of life for mCRPC patients, remains of crucial importance.

The mCRPC population is viewed as a homogenous population; all patients receive similar treatment. However, patient and/or disease characteristics, such as race, may influence the patient's response to mCRPC treatment.¹² Until such determinants are identified, it is important that clinical trials assessing novel prostate cancer treatment recruit participants who adequately reflect the population that will receive this therapy in clinical practice. **Chapter 2** addresses whether enrollment of racial minorities in prostate cancer studies reflects the racial distribution in the average prostate cancer population. Underrepresentation of racial minorities may question the scientific justification of use of studied therapies in minorities.

Despite the introduction of various targeted therapies, taxane chemotherapy remains crucial in the palliative treatment of mCRPC. Taxanes consist of docetaxel or cabazitaxel, and act on cancer cells by targeting and stabilizing microtubules.¹³ The incorrect formation of microtubules leads to mitotic arrest in cancer cells, as mitotic spindles cannot be formed correctly.¹⁴ Taxanes have other antitumor functions as well, such as a nuclear accumulation of p53, resulting in enhanced p53-induced apoptosis.¹⁵

Cabazitaxel has been used in Dutch clinics for mCRPC patients who progressed during or after docetaxel treatment since June 2011. Between June 2010, when the drug received approval from the United States (US) Food and Drug Administration (FDA), and May 2011, 51 patients were treated with cabazitaxel in the Netherlands via a compassionate use program. Such programs enable patients, for whom no good treatment alternative is currently available, to receive therapy when it is not yet reimbursed by insurance companies, but expected to be in the near future. Patients included in the compassionate use program are being monitored

to assess efficacy and safety of the therapy. These results were collected, analyzed and reported, and it was tested whether certain subgroups of mCRPC patients have a better outcome (**Chapter 3**).

In addition to cabazitaxel, abiraterone was recently approved as a second-line therapy in mCRPC patients after docetaxel treatment. The optimal treatment sequence has not been studied. Therefore, treatment decisions are currently based on the preference of physicians and/or patients.¹⁶ In the CAST-study, the clinical outcome of mCRPC patients treated sequentially with cabazitaxel and abiraterone after docetaxel was retrospectively evaluated (**Chapter 4**).

The success of taxanes spurred the search for other compounds that target cancer cells in mitosis. As reviewed in **Chapter 5 section A**, this resulted amongst others in the development of Eg5-inhibitors, polo-like kinase 1 (Plk1-)inhibitors and aurora kinase (AK-)inhibitors. Early clinical studies with such inhibitors did not show significant antitumor activity, resulting in skeptical views from some researchers. However, improved inhibitors do show clinical potential, and are currently being tested in clinical trials involving mCRPC patients (**Chapter 5 section B**).

Similar to other cancers, the concept that prostate cancer can be controlled or even cured by a single agent is being abandoned. This idea is being replaced by the hypothesis that it requires targeting of multiple cancer-related pathways with multiple therapies to control prostate cancer disease. Although testing of combination therapies in prostate cancer patients has steadily been growing, results of these studies have been disappointing.^{17, 18} Most combination therapies were tested based on trial-and-error, and lacked a solid rationale. In **Chapter 6**, Analysis of Functional Annotation (AFA) was used to provide a rational combination strategy with histone deacetylase inhibitors (HDACIs) in prostate cancer. Histone deacetylases deacetylate lysine residues in N-terminal tails of histones, and overexpression of these enzymes results amongst others in decreased expression of tumor-suppressor genes and increased expression of oncogenes in prostate cancer.¹⁹ Two HDACIs have been approved for their use in cutaneous T cell lymphomas, vorinostat (SAHA) and romidepsin; in solid tumors, HDACIs are not used outside the research setting yet. As HDACIs target many important cancer-related pathways, such as the DNA damage pathway,²⁰ this group of drugs has high potential for combination therapy. Therefore, HDACIs were used to identify potential novel combination therapies in the AFA study.

Based on the AFA results, it was hypothesized that combining HDACIs with agents that target mitotic enzymes such as Plk1- and AK-inhibitors may result in enhanced antitumor efficacy. Considering the potential of mitotic inhibitors in prostate cancer, combinations of HDACIs with Plk1- and AK-inhibitors were explored in preclinical studies (**Chapter 7** and **Chapter 8**,

respectively).

The development of inhibitors of the mitotic enzyme Eg5 was halted for prostate cancer treatment after a phase II trial with the Eg5-inhibitor ispinesib did not have significant effects on mCRPC patients.²¹ Immunohistochemistry of tumor samples from these patients, who all had disease progression during or after docetaxel treatment, revealed that 15 out of 16 included patients did not have overexpression of Eg5 in the tumor. Therefore, it was concluded that Eg5-inhibitors would not be effective in mCRPC patients due to the lack of Eg5 overexpression.

However, a more recent study found that Eg5 is overexpressed in about 50% of all prostate cancers.²² These results and the fact that Eg5 is mainly responsible for the separation of microtubules,²³ led to the rationale that Eg5 expression may be decreased upon docetaxel resistance, and may therefore be a marker for docetaxel response. This rationale is explored in **Chapter 9**.

Most prostate cancer-related deaths are contributed to cancer metastases.² Hence, treating metastases may be beneficial for mCRPC patients, reducing both morbidity and mortality of the disease. Radium-223 chloride is the first agent that has been shown to extend overall survival in cancer patients by exclusively targeting bone metastases.²⁴ It has been approved for the treatment of mCRPC patients with bone metastases by the US FDA, and has recently been introduced in Dutch clinical practice. In **Chapter 10**, the results leading to FDA approval of radium-223 are discussed, as well as the radionuclide's limitations.

Due to the clinical relevance of metastases, targeting genes involved in the formation and/or growth of prostate tumor metastases may result in significant clinical benefit for mCRPC patients. Two genes that play a crucial role in the formation, maintenance and/or growth of metastases, are N-myc downregulated gene 1 (NDRG1) and cyclin-dependent kinase 5 (CDK5). While NDRG1 is a metastasis suppressor gene in amongst others prostate cancer (**Chapter 11 section A**),²⁵ CDK5 is necessary for metastasis formation in prostate and pancreatic cancer.^{26, 27} In the final two chapters of this thesis, the Johns Hopkins Drug Library (JHDL) was used to perform a synthetically lethal high-throughput screen, thereby identifying small molecules that may target cancer cells based on the expression of NDRG1 (**Chapter 11 section B**) or CDK5 (**Chapter 12**).

In conclusion, this thesis handles various aspects of research involved in the development of novel therapies to treat mCRPC patients. The scope of drug development ranges from basic research, by performing AFA on HDACI-treated cells, to clinical research, such as the CAST-study.

References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;64(1):9-29.
2. <http://www.cancer.org/cancer/prostatecancer/detailedguide/prostate-cancer-survival-rates> [internet]. Survival rates for prostate cancer. American Cancer Society. Dublin, OH, US. 2013. Last accessed: November 1st, 2013.
3. Tannock IF, de Wit R, Berry WR et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351(15):1502-1512.
4. de Bono JS, Oudard S, Ozguroglu M et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* 2010;376(9747):1147-1154.
5. de Bono JS, Logothetis CJ, Molina A et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011;364(21):1995-2005.
6. Fizazi K, Scher HI, Molina A et al. Abiraterone acetate for treatment of metastatic castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301 randomised, double-blind, placebo-controlled phase 3 study. *Lancet Oncol* 2012;13(10):983-992.
7. Scher HI, Fizazi K, Saad F et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med* 2012;367(13):1187-1197.
8. A Study of Tasquinimod in Men With Metastatic Castrate Resistant Prostate Cancer. Bethesda, MD: US National Institute of Health, 2013. Available at: <http://clinicaltrial.gov/ct2/show/NCT01234311>.
9. http://www.cijfersoverkanker.nl/selecties/Dataset_1/img5102c95ec2307 [Internet]. Mortality rate of cancers in men in the Netherlands. [in Dutch] Nederlandse Kankerregistratie (NKR), integraal kankercentrum Nederland (IKNL). Utrecht, NL. 2013. Last accessed: November 1st, 2013.
10. Strobe SA, Andriole GL. Prostate cancer screening: current status and future perspectives. *Nat Rev Urol* 2010;7(9):487-493.
11. Ilic D, O'Connor D, Green S, Wilt TJ. Screening for prostate cancer: an updated Cochrane systematic review. *BJU Int* 2011;107(6):882-891.
12. Martin DN, Starks AM, Ambis S. Biological determinants of health disparities in prostate cancer. *Curr Opin Oncol* 2013;25(3):235-241.
13. Stein CA. Mechanisms of action of taxanes in prostate cancer. *Semin Oncol* 1999;26(5 Suppl 17):3-7.
14. Vaishampayan U, Parchment RE, Jasti BR, Hussain M. Taxanes: an overview of the pharmacokinetics and pharmacodynamics. *Urology* 1999;54(6A Suppl):22-29.
15. Giannakakou P, Nakano M, Nicolaou KC et al. Enhanced microtubule-dependent trafficking and p53 nuclear accumulation by suppression of microtubule dynamics. *Proc Natl Acad Sci USA* 2002;99(16):10855-10860.
16. http://www.uroweb.org/gls/pdf/08%20Prostate%20Cancer_LR%20March%2013th%202012.pdf [internet]. EAU guidelines on Prostate Cancer. European Association of Urology. EU. February 2012. Last accessed: November 1st, 2013.
17. Galsky MD, Vogelzang NJ. Docetaxel-based combination therapy for castration-resistant prostate cancer. *Ann Oncol* 2010;21(11):2135-2144.
18. Maitland ML, Hudoba C, Snider KL, Ratain MJ. Analysis of the yield of phase II combination therapy trials in medical oncology. *Clin Cancer Res* 2010;16(21):5296-5302.

19. Kortenhorst MS, Carducci MA, Shabbeer S. Acetylation and histone deacetylase inhibitors in cancer. *Cell Oncol* 2006;28(5-6):191-222.
20. Kachhap SK, Rosmus N, Collis SJ et al. Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. *PLoS One* 2010;5(6):e11208.
21. Beer TM, Goldman B, Synold TW et al. Southwest Oncology Group phase II study of ispinesib in androgen-independent prostate cancer previously treated with taxanes. *Clin Genitourin Cancer* 2008;6(2):103-109.
22. Xing ND, Ding ST, Saito R et al. A potent chemotherapeutic strategy in prostate cancer: S-(methoxytrityl)-L-cysteine, a novel Eg5 inhibitor. *Asian J Androl* 2011;13(2):236-241.
23. Kapitein LC, Peterman EJ, Kwok BH et al. The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. *Nature* 2005;435(7038):114-118.
24. Parker C, Nilsson S, Heinrich D et al. Alpha emitter radium-223 and survival in metastatic prostate cancer. *N Engl J Med* 2013;369(3):213-223.
25. Bandyopadhyay S, Pai SK, Gross SC et al. The Drg-1 gene suppresses tumor metastasis in prostate cancer. *Cancer Res* 2003;63(8):1731-1736.
26. Strock CJ, Park JI, Nakakura EK et al. Cyclin-dependent kinase 5 activity controls cell motility and metastatic potential of prostate cancer cells. *Cancer Res* 2006;66(15):7509-7515.
27. Feldmann G, Mishra A, Hong SM et al. Inhibiting the cyclin-dependent kinase CDK5 blocks pancreatic cancer formation and progression through the suppression of Ras-Ral signaling. *Cancer Res* 2010;70(11):4460-4469.

Chapter 2

Under-representation of racial minorities in prostate cancer studies submitted to the FDA to support potential marketing approval, 1993-2013

Michel D. Wissing, Paul Kluetz, Yang-Min Ning, Jonca Bull, Christine Merenda, Anthony J. Murgo, Richard Pazdur

Cancer. 2014 Jun 25. [Epub ahead of print]

Abstract

Background: United States (US) Food and Drug Administration (FDA) approval of new drugs depends on results from clinical trials that must be generalized to the US population. However, racial minorities are frequently under-represented in clinical studies. Enrollment of racial minorities was compared in key clinical studies submitted to the FDA in the last 10 years in support of potential marketing approval for prostate cancer (PCa) prevention or treatment.

Methods: Patient demographic data were obtained from archival datasets of large registration trials submitted to the FDA to support proposed PCa indications. Six countries/regions were analyzed: US, Canada, Australia, “Europe”, United Kingdom and Eastern Europe. Background racial demographics were collected from national census data.

Results: Seventeen key PCa clinical trials were analyzed. These trials were conducted in the past twenty years, comprising 39,574 patients with known racial information. A majority of patients were enrolled in the US, but there appears to be a trend towards increased non-US enrollment over time. In all countries/regions, racial minorities were generally under-represented. There was no significant improvement in racial minority enrollment over time. The US enrolled the largest non-white population (7.1%).

Conclusions: Over the past twenty years, racial minorities were consistently under-represented in key PCa trials. There is a need for effective measures that will improve enrollment of racial minorities. With increased global enrollment, drug developers should aim to recruit a patient population which resembles the racial demographics of the patient population to which drug use will be generalized upon approval.

Introduction

Prostate cancer (PCa) incidence and mortality rates vary among races. Between 2006 and 2010, the age-adjusted PCa incidence rates were 144.9, 228.5, 81.8 and 77.8 per 100,000 men in white, black, Asian and Native American men in the United States (US), respectively.¹ The age-adjusted PCa mortality rates were 21.2, 50.9, 10.1 and 20.7 per 100,000 men in the respective racial groups.¹ These results indicate that black people are most prone to PCa disease and death. In contrast, Asians are least likely to be diagnosed with and die from PCa. Comparing incidence rates to mortality rates, relative mortality is strikingly higher in black and Native American populations. In general, these patient groups present with more advanced disease and receive less aggressive treatment.²⁻⁶ Furthermore, black patients diagnosed with very low-risk PCa may have more advanced PCa than white patients with the same diagnosis.⁷ Multiple underlying factors may contribute to aforementioned epidemiological findings, such as genetic tumor alterations, differences in lifestyle, cultural and socioeconomic factors, distances needed to travel to the nearest hospital for treatment (which for multiple American Indian tribes can exceed 100 miles), variations in participation in population-based PSA screening, and non-conformity in clinical trial participation between racial groups.^{6, 8-15}

Because the presentation of PCa disease differs amongst racial groups, the response to PCa therapies may also differ. Therefore, the US Food and Drug Administration (FDA) strongly recommends conducting clinical trials testing novel PCa drugs in a population that adequately represents the racial distribution of the US population that would receive the drug in a clinical setting. Nevertheless, minority accrual in clinical trials has been a challenge, as minorities are traditionally under-represented in clinical trials.^{14, 15} Disparity between racial distribution in clinical trial populations compared to the average US population has been a factor in FDA decisions in the past. For example, during the Oncology Drug Advisory Committee meeting discussing finasteride and dutasteride for the chemoprevention of PCa in December 2010, the FDA noted that these studies lacked adequate enrollment of black patients, questioning how well the studies represented the average US PCa population. Therefore, minority accrual is of high importance in clinical studies with PCa patients.

In the current study, we report the enrollment of racial minorities in key clinical trials conducted in PCa patients submitted for FDA review in the past two decades. Minority accrual was compared across various countries/regions in the world. We further investigated whether minority accrual has changed over time.

Methods

Data collection

For the collection of demographic data, various statistical government resources, publicly available on the internet, were used. These included US 2010 census data, Canadian 2011

national household survey data, Australian 2011 census data, United Kingdom Census 2001 data, data from Institut Montaigne (French demographics) and data from the Czech Statistical Office (Český statistický úřad).

Key clinical trials submitted for FDA review in which patients were recruited between 1993 and 2013 were analyzed regardless of the marketing approval outcome. The proposed therapeutic indications were specific to PCa patients, i.e. drug or biologic products that sought approval for the treatment of bone metastases in cancer patients in general were not included in this study. Datasets from selected clinical trials were retrieved from FDA's archives. Anonymity was applied to non-public data.

Data analyses

Data were analyzed for the following regions: globally, US, Canada, Australia, "Europe", United Kingdom and Eastern Europe. Countries in "Europe" included the 27 countries in the European Union (excluding Croatia) and the 4 countries forming the European Free Trade Association. Eastern European countries consisted of all countries in Europe east of the former Berlin wall, including Russia, but excluding Turkey. Turkey was considered part of West-Asia (the Middle East).

Eighteen key, or "pivotal", PCa clinical trials were identified during the time period specified in our analysis, 1993-2013. Of these, the key study supporting FDA approval of mitoxantrone was excluded from our analysis, as racial demographics of these patients had not been collected. The remaining 17 clinical trials, all included in our analysis, included key studies for review of abiraterone acetate, atrasentan hydrochloride, cabazitaxel, degarelix, denosumab, docetaxel, dutasteride, enzalutamide, finasteride, leuprorelin (six-month formulation), radium-223 dichloride, satraplatin, sipuleucel-T and triptorelin (six-month formulation).

On the basis of the initiation of patient enrollment, the 17 selected clinical trials were divided into two major time periods. In group 1, clinical trials were included that initiated enrollment between 1993 and 2004 (n=7); trials that initiated enrollment after 2004 were included in group 2 (n=10). Group 1 marks clinical trials that were designed before FDA approval of docetaxel, while clinical trials in group 2 were designed after docetaxel had been approved for its use in patients with castrate-resistant PCa.

US FDA guidelines recommend that investigators separately collect demographics regarding race and ethnicity in clinical trials.¹⁶ In these guidelines, racial groups are divided into white, black, Asian, American Indian/Alaska Native, Pacific Islander, or other. Ethnicity is divided into Hispanic/Latino or non-Hispanic/Latino. In line with these guidelines, we divided race into white, black, Asian, native or other. White patients included people with European descent, the Middle East and Africa north of the Sahara. Black patients descended from sub-Saharan

Africa. Asian patients included all patients from Asian descent except for the Middle East and Russia. Natives were considered American Indians, Alaska Natives, Canadian Indians, Pacific Islanders and Aborigines. The race “other” primarily consisted of patients with a multiracial background.

A majority of studies in our analysis did not report Hispanics as a separate ethnicity, but as a race. All patients whose registered race was “Hispanic” were excluded from analyses, as only their ethnicity, but not their self-reported race, was known. Patients who did not have their race reported were also excluded from our analyses. In total, the race of $\geq 90\%$ of patients was retrieved for analysis.

We calculated enrollment estimates for racial minorities that reflect the incidence and mortality of PCa amongst racial populations in the US to further explore whether minority representation was adequate in the clinical trials analyzed. To estimate projected patient enrollment in the US based on PCa incidence, we multiplied the age-adjusted incidence of PCa in each racial group by their proportion in the US population based on 2010 US census data. For each race, the calculated number was divided by the sum of all calculated numbers. This resulted in estimates of the percentage of each racial group needed in a PCa clinical trial to reflect the racial distribution of patients diagnosed with PCa. A similar procedure was used to estimate the projected patient minority enrollment in the US based on PCa mortality, which would approximately represent the racial distribution of patients who die from PCa.

Results

In total, 40,912 patients had been enrolled in selected clinical trials between October 1993 and February 2011. The race of 1,338 patients was unknown and were excluded. Of the remaining 39,574 patients, 60.2% were enrolled in the US, 26.5% in “Europe” (4.6% in the United Kingdom), 6.6% in Eastern Europe, 4.8% in Canada and 1.6% in Australia (Table 1).

Table 1. The percentage of patients enrolled per nation/region in key prostate cancer clinical trials analyzed in the current study.

Country/Region	Percentage of patients enrolled
United States	60.2%
Canada	4.8%
Australia	1.6%
“Europe”	26.5%
United Kingdom	4.6%
Eastern Europe	6.6%

Patients from the 17 trials were pooled together. Note that not all percentages add up to 100% because some studies enrolled patients in other countries/regions than listed, and because patients from the United Kingdom and parts of Eastern Europe are also counted in “Europe”.

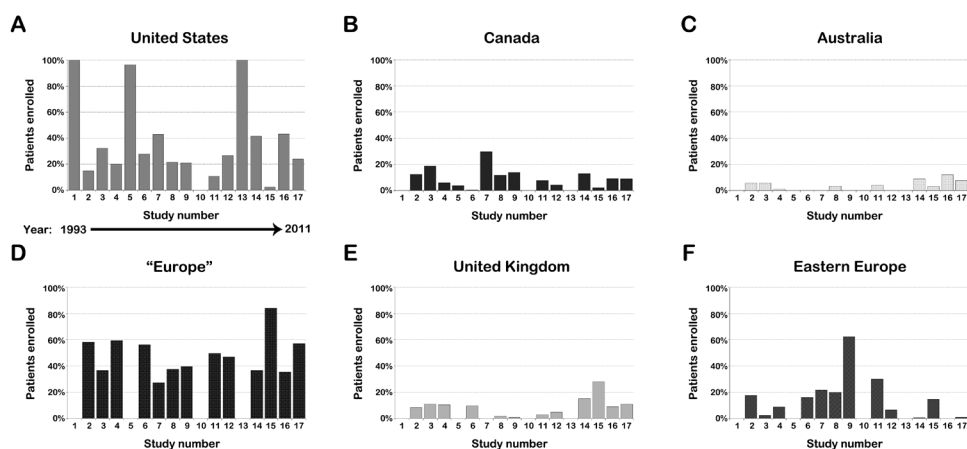


Figure 1. Percentages of patients enrolled from selected nations/regions in key prostate cancer clinical trials.

The percentage of patients enrolled in each analyzed country/region is displayed for all individual studies as shown in Figure 1, sorted by time of enrollment initiation. Patient enrollment varied widely between different studies: some studies completely enrolled patients in the US, while one recruited patients outside the US only.

Next we studied whether there was a difference in enrollment in countries between key PCa clinical trials initiated before or after 2005 (Table 2). Patient enrollment in the US had decreased. Enrollment in Canada was relatively low and slightly decreased post-2005. In contrast, patient enrollment in European countries was higher post-2005. Patient enrollment in Australia was also higher; however, this increase was driven mostly by the four most recent studies, in which up to 12% of patients were enrolled in Australia (Table 2, Fig. 1C). Although Eastern European enrollment was higher in group 2 compared to group 1, enrollment of Eastern Europeans decreased again in the most recent studies of group 2 (Fig. 1F).

Table 2. Average percentage of patients enrolled in key PCa clinical trials initiated before and after FDA docetaxel approval per analyzed nation/region.

Country/region	1993-2004	2005-2013
United States	47.7%	29.2%
Canada	10.1%	7.1%
Australia	1.8%	3.9%
"Europe"	34.0%	38.7%
United Kingdom	5.6%	7.4%
Eastern Europe	9.5%	13.5%

Percentages were calculated by dividing the sum of percentage enrollment in each individual study by the number of studies. Note that not all percentages add up to 100% because some studies enrolled patients in other countries/regions than listed, and because patients from the United Kingdom and parts of Eastern Europe are also counted in "Europe".

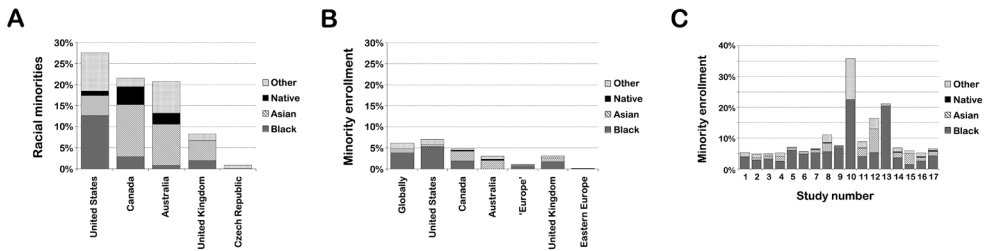


Figure 2. Racial minority demographics in Western nations/regions and enrollment of racial minorities in key PCa trials. A) Demographic distribution of racial minorities in a selection of Western nations. B) Global racial minority enrollment and racial minority enrollment in selected nations/regions. C) Global enrollment of racial minorities for each key PCa clinical trial.

In B), data was pooled from all 17 key PCa studies.

Our analyses further focused on enrollment of racial minorities in the selected PCa clinical trials in relation to national/regional racial minority population statistics. Studying national census data across the various countries, the non-white population was largest in the US (27.6%), while in Eastern European countries such as the Czech Republic less than 1% of the population consisted of non-whites (Fig. 2A). Pooling racial demographic data from all 17 studies and comparing enrollment between different nations/regions, enrollment of racial minorities was largest in the US (7.1%) (Fig. 2B); this population primarily consisted of black patients (5.3%). In other countries/regions, enrollment of racial minorities did not exceed 5%; minority accrual was virtually non-existent in Eastern Europe. Hence, comparing enrollment of racial minorities (Fig. 2B) to the expected pool of eligible patients based on census data (Fig. 2A), enrollment of racial minorities was appreciably lower across all nations/regions. Comparing global enrollment of racial minorities over time, enrollment of non-whites seemed to be consistent at approximately 5-10% (Fig. 2C). Exceptions were study 10, 12 and 13, which enrolled more than 15% of racial minorities. Study 10 was performed entirely outside the US. As this was a non-Western nation, it was not further analyzed. Study 13 was conducted completely in the US; study 12 had a large Asian population as this study had a higher enrollment in Asia compared to the other studies.

In the US, enrollment of racial minorities was higher across all studies as compared to global minority enrollment (compare Fig. 3A to Fig. 2C), possibly due to the large minority population in the US in general (Fig. 2A). Notably, enrollment of racial minorities was more than 20% in studies 9, 12 and 13 (Fig. 3A). In studies 12 and 13, Hispanic/Latino ethnicity was not collected as a separate category. Therefore, these patients may have been included in the racial groups 'black' and 'other', increasing minority enrollment as measured in this study compared to clinical trials in which the Hispanic population was excluded from analysis. The reported minority enrollment in study 9 excluded the 9% Hispanics from the analysis, as their race had not been collected separately from ethnicity.

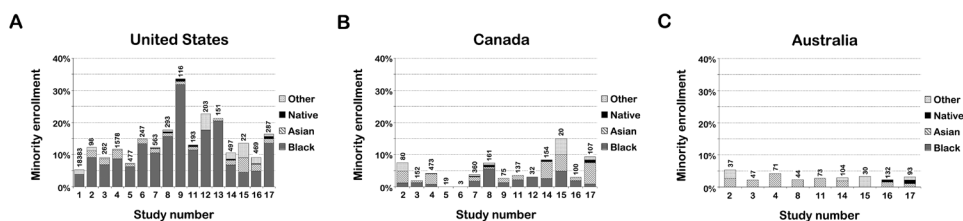


Figure 3. Enrollment of racial minorities in key PCa clinical trials in A) the United States, B) Canada and C) Australia.

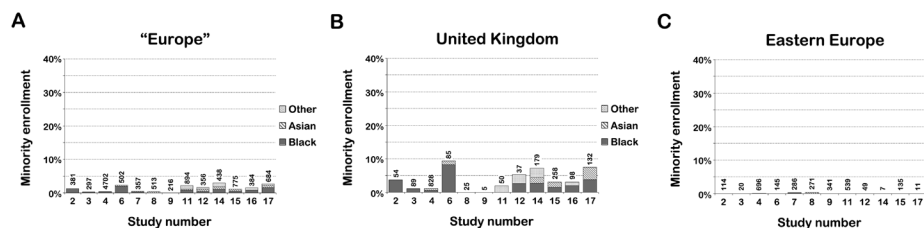


Figure 4. Enrollment of racial minorities in key PCa clinical trials in "Europe" (all countries of the European Union plus the European Free Trade Association), the United Kingdom, and Eastern Europe (all countries east of the former Berlin wall, including Russia but excluding Turkey).

Studies were excluded from graphs if no patient had been enrolled in the analyzed nation/region for that particular study. The total number of patients enrolled from the analyzed country/region (including white patients) is listed above each bar.

Racial minority enrollment did not exceed 15% in any Canadian (Fig. 3B) or Australian (Fig. 3C) study population. Comparing enrollment of racial minorities between Canada and Australia, which have a similar proportion of racial minorities in the population (Fig. 2A), Australia enrolled markedly fewer racial minorities.

In "Europe", enrollment of racial minorities was relatively low (Fig. 4A). We further specified enrollment in the United Kingdom (Fig. 4B), a Western European country with an extensive colonial history, and in Eastern European countries (Fig. 4C). As one would expect based on the demographics (Fig. 2A), enrollment of racial minorities was higher in the United Kingdom, while virtually all patients enrolled in Eastern Europe were white. Similar results were found when analyzing minority enrollment of France separately: in line with its demographics, racial minority enrollment was higher in France compared to Eastern Europe, but slightly lower compared to the United Kingdom (data not shown).

Discussion

Data from our study indicate that racial minorities are consistently under-represented in major clinical trials assessing novel PCa therapies, also relative to differences in population demographics by country. This finding is consistent with previous reports regarding PCa and other diseases.^{14, 15} Treatment benefit may differ between racial groups due to variations

in tumor characteristics.⁹ Therefore, the US FDA and other regulatory authorities strongly recommend registration of racial demographics for clinical studies.¹⁶ Furthermore, studies have indicated that oncology patients who participated in clinical trials had better survival compared to non-participants.^{14, 17, 18} These results may have been biased: patients with a worse prognosis may have been excluded from clinical trials, and physicians treating patients in a clinical trial may adhere more strictly to provided study protocols. For adjuvant breast cancer treatment, protocol adherence was strongly associated with improved survival.¹⁷ Therefore, PCa survival may improve among minorities with increased participation in clinical trials.

In this study, we compared racial minority enrollment in PCa studies to the expected accrual based on census data. Our analysis has several important limitations. First, census data are self-reported and are only representative of people who respond to the questionnaires. Second, demographics were collected inconsistently: while in some studies Hispanics were reported under a separate category for ethnicity as recommended by US FDA guidelines,¹⁶ in a majority of studies Hispanics were reported as a separate race. Third, to be able to adequately study the treatment effect in racial minorities, one could argue that enrollment of racial minorities should be increased even further. Fourth, data from the SEER database indicate that the incidence and mortality of PCa is higher in the black population than in the white population in the US.¹ Therefore, one could argue that adequate representation of the US PCa patients should include a higher percentage of black patients than reported in the census (12.6%). For example, we estimated that in studies performed in the general PCa population, evaluating a treatment in early stage disease (such as finasteride and dutasteride for PCa risk reduction), a representation of the US population would include 31.1% non-whites, and 18.9% black patients specifically. For therapies aimed at patients with late stage disease (such as enzalutamide for metastatic castrate-resistant PCa patients after docetaxel treatment), a stage at which most patients will eventually die from PCa, should include an estimated 26.1% black population due to the higher mortality rate in this racial group. The latter two examples further demonstrate that enrollment of racial minorities needs to be improved compared to current enrollment.

There are obvious limitations to grouping patients into broad phenotypic categories (white, black, Asian, native and other minorities). These racial groups are only very general predictors of biologic variations that may result in differences in PCa growth and therapy response. These biologic differences, in addition to environmental, socioeconomic and dietary variability, may be substantial between two populations categorized as the same race but from differing countries. E.g., while people from the Arabian Peninsula and North Africa are by consensus considered white in epidemiological studies, it is highly likely that substantial differences in environmental and genetic factors exist between these two

subgroups and Europeans within the same racial category. Similarly, significant differences may exist between Japanese patients living in Japan compared to those of Japanese ancestry who reside in the US. Despite these limitations, capturing racial demographics in clinical trials remains an important tool to ensure that the population enrolled more accurately approaches the general PCa population within a country. Furthermore, racial demographic data can identify potential challenges in clinical trial access, and reveal differences in the safety and efficacy of a therapy between racial subgroups, potentially aiding in the discovery of predictive biomarkers.

Although the need to increase minority accrual has universally been acknowledged, it remains a challenge as to how this goal can be achieved. Various efforts have had limited success in the past; for instance, efforts to increase enrollment of racial minorities in the PCPT study were fruitless.¹⁹ The limited success of efforts to improve enrollment of racial minorities is reflected in our study results, as no significant improvement in minority recruitment has been made over the past two decades.

Previous research indicated that white and non-white patients have an equal interest in learning about clinical trials.²⁰ However, the investigators also found that the media used to collect information about clinical trials differed between racial groups in the US. While white patients used the internet and physicians more often as an information source, racial minorities more often relied on information provided by other patients. This is seemingly in contrast with a study by Markman et al., which suggested that the internet may be useful as a source to recruit minorities for participation in clinical trials.²¹ Other studies have identified additional factors that may improve minority enrollment, such as approaching Asian patients by a more senior doctor, informing patients by staff and/or local leaders from the same ethnicity, and making use of written translations.²² Furthermore, while most clinical trials are performed in university hospitals in major cities, rural minorities, particularly Native Americans in the US, would benefit if patients in community/peripheral hospitals would be recruited for studies too. Most importantly, studies concluded that the main factor influencing enrollment of racial minorities was level of trust.^{20, 22, 23}

Despite the limited success of improving enrollment of racial minorities in oncology studies in general, some studies were more successful in recruiting minorities, such as study 9 in our analysis. Additional exploration indicated that enrollment of black patients was particularly high in one study site in Aventura, Florida. Here, 19 out of 29 patients enrolled in the study were classified as black; half of all black patients enrolled in this study were enrolled from this site. The population that this hospital served included a large population from the Caribbean, primarily indicated as “white/other, Hispanic” in the US census data. There seems to be discrepancy in reporting whether patients from the Caribbean are considered white (Hispanic), black or Native American (Puerto Rico). It is plausible that the investigator in that study registered patients from the Caribbean as black.

As a final note, our study results indicate that the majority of patients in PCa registration trials intended for US FDA review for marketing approval were enrolled in the US. However, our data also suggest a potential trend towards increased non-US enrollment. One key advantage of global clinical trial conduct is rapid enrollment, which can decrease the time needed to bring an effective treatment to the market. However, it also raises the question whether the results acquired from treating patients abroad properly reflect the efficacy and safety of the treatment in the average US population who will receive the treatment upon FDA approval. The current study underlines the importance of this question, as enrollment of racial minorities was lower in all studied nations/regions compared to the US, in particular in Eastern Europe. On the other hand, study 10, which globally had the highest enrollment of racial minorities, was conducted completely outside the US, in a country with a considerably higher non-white population. This indicates that adequate minority accrual is possible and there is not necessarily an objection to recruitment of patients outside the US or outside Western nations, provided that the rights, safety, and well-being of the subjects participating in those trials are ensured, there is compliance with good clinical practices, and that the results can be generalized to the US population. However, it must be acknowledged that biologic and socioeconomic characteristics of racial populations in the US may differ from such characteristics in the same racial population accrued in another country.

In conclusion, investigators and drug developers should take differences in racial demographics into account when selecting countries for clinical trials. In accordance with FDA's guidelines, sponsors are advised to classify racial demographics consistently and to consult the review division prior to study initiation about the estimated size of minority enrollment in their proposed trial.¹⁶ While acknowledging that biologic and environmental differences may exist within the same racial category between countries, researchers should aim to improve enrollment of racial minorities during international trial conduct to accurately reflect demographics in countries for which they seek approval of their therapy. Inadequate minority enrollment may prevent achieving a full understanding of the benefit-risk profile of novel treatments in racial minorities. The trend toward increasing the number of non-US countries, including countries with a large white population, entails a trade-off of enhancing enrollment and time to trial completion versus enrolling a population that adequately represents the racial demographics of the average US population. Improved strategies to enhance minority accrual in such large multinational studies are needed.

Acknowledgments

The authors wish to thank Frank H. Cross Jr., dr. Peter F. Bross and dr. Ian F. Tannock for their help collecting datasets.

References

1. seer.cancer.gov [Internet]. SEER Stat Fact Sheets: Prostate. Available at: <http://seer.cancer.gov/statfacts/html/prost.html>. Accessed: June 29, 2013.
2. Desantis C, Naishadham D, Jemal A. Cancer statistics for African Americans, 2013. *CA Cancer J Clin* 2013;63(3):151-166.
3. Newman LA, Lee CT, Parekh LP et al. Use of the National Cancer Data Base to develop clinical trials accrual targets that are appropriate for minority ethnicity patients: a report from the American College of Surgeons Oncology Group (ACOSOG) Special Population Committee. *Cancer* 2006;106(1):188-195.
4. Rogers D, Petereit DG. Cancer disparities research partnership in Lakota Country: clinical trials, patient services, and community education for the Oglala, Rosebud, and Cheyenne River Sioux tribes. *Am J Public Health* 2005;95(12):2129-2132.
5. Shavers VL, Brown M, Klabunde CN et al. Race/ethnicity and the intensity of medical monitoring under 'watchful waiting' for prostate cancer. *Med Care* 2004;42(3):239-250.
6. Taksler GB, Keating NL, Cutler DM. Explaining racial differences in prostate cancer mortality. *Cancer* 2012;118(17):4280-4289.
7. Sundi D, Ross AE, Humphreys EB et al. African American men with very low-risk prostate cancer exhibit adverse oncologic outcomes after radical prostatectomy: should active surveillance still be an option for them? *J Clin Oncol* 2013;31(24):2991-2997.
8. Freedman ML, Haiman CA, Patterson N et al. Admixture mapping identifies 8q24 as a prostate cancer risk locus in African-American men. *Proc Natl Acad Sci U S A* 2006;103(38):14068-14073.
9. Martin DN, Starks AM, Ambis S. Biological determinants of health disparities in prostate cancer. *Curr Opin Oncol* 2013;25(3):235-241.
10. Petereit DG, Rogers D, Govern F et al. Increasing access to clinical cancer trials and emerging technologies for minority populations: the Native American Project. *J Clin Oncol* 2004;22(22):4452-4455.
11. Petereit DG, Guadagnolo BA, Wong R, Coleman CN. Addressing Cancer Disparities Among American Indians through Innovative Technologies and Patient Navigation: The Walking Forward Experience. *Front Oncol* 2011;1:11.
12. Holm JE, Vogeltanz-Holm N, Poltavski D, McDonald L. Assessing health status, behavioral risks, and health disparities in American Indians living on the northern plains of the U.S. *Public Health Rep* 2010;125(1):68-78.
13. Weinrich SP. Prostate cancer screening in high-risk men: African American Hereditary Prostate Cancer Study Network. *Cancer* 2006;106(4):796-803.
14. Goyal J, Nuhn P, Huang P et al. The effect of clinical trial participation versus non-participation on overall survival in men receiving first-line docetaxel-containing chemotherapy for metastatic castration-resistant prostate cancer. *BJU Int* 2012;110(11 Pt B):E575-E582.
15. Movsas B, Moughan J, Owen J et al. Who enrolls onto clinical oncology trials? A radiation Patterns Of Care Study analysis. *Int J Radiat Oncol Biol Phys* 2007;68(4):1145-1150.
16. www.fda.gov [Internet]. Guidance for Industry - Collection of Race and Ethnicity Data in Clinical Trials. Available at: <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm126396.pdf>. Accessed: July 1, 2013.
17. Schwentner L, van Ewijk R, Kurzeder C et al. Participation in adjuvant clinical breast cancer

- trials: does study participation improve survival compared to guideline adherent adjuvant treatment? A retrospective multi-centre cohort study of 9,433 patients. *Eur J Cancer* 2013;49(3):553-563.
18. Chow CJ, Habermann EB, Abraham A et al. Does enrollment in cancer trials improve survival? *J Am Coll Surg* 2013;216(4):774-780.
 19. Moinpour CM, Atkinson JO, Thomas SM et al. Minority recruitment in the prostate cancer prevention trial. *Ann Epidemiol* 2000;10(8 Suppl):S85-S91.
 20. Wood CG, Wei SJ, Hampshire MK, Devine PA, Metz JM. The influence of race on the attitudes of radiation oncology patients towards clinical trial enrollment. *Am J Clin Oncol* 2006;29(6):593-599.
 21. Markman M, Petersen J, Montgomery R. An examination of the influence of patient race and ethnicity on expressed interest in learning about cancer clinical trials. *J Cancer Res Clin Oncol* 2008;134(1):115-118.
 22. Symonds RP, Lord K, Mitchell AJ, Raghavan D. Recruitment of ethnic minorities into cancer clinical trials: experience from the front lines. *Br J Cancer* 2012;107(7):1017-1021.
 23. Skinner CS, Schildkraut JM, Calingaert B et al. Factors associated with African Americans' enrollment in a national cancer genetics registry. *Community Genet* 2008;11(4):224-233.

Chapter 3

Cabazitaxel in patients with metastatic castration-resistant prostate cancer: results of a compassionate use program in the Netherlands

Michel D. Wissing, Inge M. van Oort, Winald R. Gerritsen, Alfons J.M. van den Eertwegh, Jules L.L.M. Coenen, Andries M. Bergman, Hans Gelderblom

Clin Genitourin Cancer. 2013 Sep;11(3):238-250

Abstract

Background: Cabazitaxel has been reimbursed as a second-line therapy for patients with metastatic castrate-resistant prostate cancer (mCRPC) in the Netherlands since 2011. Before reimbursement was available, cabazitaxel was provided through a compassionate use program (CUP). We report the results of the Dutch CUP, detailing the safety and efficacy of cabazitaxel in a routine clinical practice setting.

Methods: Safety and efficacy data of all five Dutch centers participating in the cabazitaxel CUP were collected. Safety data were collected prospectively using the National Cancer Institute Common Toxicity Criteria for Adverse Events version 3.0. Overall survival (OS) and progression-free survival (PFS), time to PSA progression (TTPP), and best clinical response were evaluated retrospectively.

Results: Fifty-one patients were registered in the CUP; 49 received cabazitaxel. Forty-two of 49 patients (85.7%) had ≥ 2 metastatic sites. Patients received on average 6 cabazitaxel cycles (range 1-21). A dose reduction or dose delay occurred in 13 (26.5%) and 20 (40.8%) patients, respectively. Prophylactic granulocyte colony-stimulating factor (G-CSF) was used in 8 patients (16.3%). Grade ≥ 3 adverse events were observed in 25 patients (51.0%); 16 patients (32.7%) discontinued treatment because of treatment-emergent adverse events. Serious adverse events (SAEs) occurred in 16 (32.7%) patients; the most frequent SAEs were hematuria (4 patients (8.2%)) and urosepsis (3 patients (6.1%)). Febrile neutropenia occurred twice; no patient had grade ≥ 3 neuropathy. No toxicity-related mortality occurred. Median follow-up was 24.1 months. Median OS was 8.7 months (interquartile range (IQR) 6.0-15.9 months); median TTPP was 2.8 months (IQR 1.7-5.9 months).

Conclusion: In the Dutch CUP, patients with advanced mCRPC had delayed tumor progression with acceptable toxicities using cabazitaxel treatment.

Introduction

Metastatic castrate-resistant prostate cancer (mCRPC) is the second deadliest cancer in men in the United States (US), being surpassed only by lung and bronchial carcinomas.¹ A decade ago, the main therapy for patients with mCRPC consisted of mitoxantrone. However, this anthracenedione increased quality of life but not patient survival.² Therapy options for patients with mCRPC evolved in 2004, when docetaxel received approval as first-line therapy for this group of patients, based on two phase III clinical trials which concluded that docetaxel improved survival in patients with advanced prostate cancer.^{3, 4} Although the introduction of docetaxel implemented a radical improvement in treatment options for patients with mCRPC, about 50% of these patients did not respond to docetaxel-treatment, and there were few objective responses.³ Furthermore, all tumors that were initially targeted by docetaxel eventually developed resistance against this taxane.⁵ Approximately 70% of patients with mCRPC treated with docetaxel had progressive disease during treatment or within 3 months after discontinuation. These observations required further development of therapy options for patients with docetaxel-resistant mCRPC and led to the discovery and approval of cabazitaxel as a second-line therapy in patients with mCRPC.

Cabazitaxel is a tubulin-binding taxane that suppresses microtubule dynamics in mitosis, resulting in mitotic arrest and apoptosis.^{6, 7} This second-generation taxane effectively inhibited a wide variety of human and murine tumors *in vitro* and *in vivo* and was well tolerated by mice.⁶ Mice with established DU-145 prostate tumors had a 100% complete regression after treatment with cabazitaxel at the maximum tolerated dose (MTD); 5 of 6 mice had a tumor-free survival of ≥ 133 days.⁶ Cabazitaxel has poor substrate affinity for the adenosine triphosphate-dependent drug efflux pump P-glycoprotein (activated by overexpression of multidrug-resistant protein 1), which may partly contribute to the fact that cabazitaxel effectively inhibits cell lines with acquired resistance against docetaxel.^{6, 7} In phase I/II studies, the recommended dose was established at 20 or 25 mg/m² through a 1 h intravenous infusion once every three weeks, the dose-limiting toxicity being neutropenia.^{8, 9} Of the eight patients with mCRPC who received cabazitaxel treatment at doses ≤ 25 mg/m² in the phase I study, two patients had an objective partial response for ≥ 6 cycles, and an additional patient with mCRPC had a minor reduction in tumor size.⁸

In the subsequent TROPIC study (an open-label randomized multicenter phase III clinical trial), 755 patients with mCRPC were randomized to either mitoxantrone (12 mg/m² intravenously over 15-30 minutes every 3 weeks) plus prednisone (10 mg oral daily) or cabazitaxel (25 mg/m² intravenously over 1 h every 3 weeks) plus prednisone (10 mg oral daily).¹⁰ All patients with mCRPC included in the study had documented disease progression during or after docetaxel treatment. Median overall survival (OS) was significantly increased in the cabazitaxel-treated group compared with the mitoxantrone-treated group (15.1 vs. 12.7 months, respectively; $p < 0.0001$). Median OS was significantly increased in the cabazitaxel-treated group independent of the duration of androgen-deprivation therapy, suggesting that

cabazitaxel has effect in both aggressive and non-aggressive prostate tumors.¹¹ Furthermore, progression-free survival (PFS), defined as the first occurrence of prostate-specific antigen (PSA), radiologic or clinical progression, or death, was 1.4 months longer in the cabazitaxel-treated group (2.8 vs. 1.4 months; $p < 0.0001$), and the median time to PSA progression (TTPP) was increased from 3.1 to 6.4 months. However, both grade ≥ 3 hematologic and non-hematologic adverse events had an increased incidence in cabazitaxel-treated patients. The most frequent grade ≥ 3 hematologic adverse events were neutropenia and leukopenia, which occurred in 303 (82%) and 253 (68%) cabazitaxel-treated patients, respectively, vs. 215 (58%) and 157 (42%) mitoxantrone-treated patients. Febrile neutropenia occurred in 28 (8%) cabazitaxel-treated patients and in five (1%) mitoxantrone-treated patients. The most frequent grade ≥ 3 non-hematologic adverse event was diarrhea, which occurred in 23 (6%) cabazitaxel-treated patients and in one ($< 1\%$) mitoxantrone-treated patient. In the TROPIC-study, nine (2.4%) mitoxantrone-treated patients died within 30 days of the last dose of the study drug (six of disease progression), whereas in the cabazitaxel-treated group, 18 (4.9%) patients died within 30 days of the last dose (none of disease progression). The most frequent causes of mortality within 30 days of the last dose of the study drug were related to neutropenia and its complications, cardiac events, and renal failure.

The clinical benefit for patients with mCRPC in the TROPIC study led to the approval of cabazitaxel for treatment of mCRPC in the US in June 2010.¹² The European Medicines Agency approved cabazitaxel for mCRPC treatment in March 2011; later in 2011, the taxane was reimbursed by insurance companies in the Netherlands. Pending final registration of cabazitaxel, a compassionate use program (CUP) was established in the Netherlands and 25 other countries in 2010 to allow access to cabazitaxel for patients with mCRPC and to record overall safety. These programs have been introduced to facilitate the availability of new treatments that are not yet reimbursed for patients with a severe disease when no satisfactory alternative is available and when it is expected that the new medicine will be approved by official authorities in the near future. Recruitment for this CUP was terminated in the Netherlands in June 2011 as cabazitaxel would be reimbursed. In this study we report the safety and efficacy of cabazitaxel in patients with mCRPC as recorded in the Dutch CUP to give an indication of the experience with cabazitaxel in a routine clinical setting.

Patients and Methods

Patients

Patients in five Dutch medical centers were included. Patients were eligible for cabazitaxel treatment if they had mCRPC and documented disease progression during or after treatment with a docetaxel-containing regimen. Patients needed to be surgically or medically castrated; be ≥ 18 years; have an Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1, or 2; have a life expectancy of ≥ 3 months; and have adequate bone marrow, liver, and

renal functions. Patients were excluded from participation if they had received previous radiotherapy to $\geq 40\%$ of the bone marrow, previous radionuclide therapy, or if they had received anticancer therapy within four weeks of enrollment. Patients were also excluded when they presented with grade ≥ 2 peripheral neuropathy, grade ≥ 2 stomatitis, an infection treated with systemic antibiotic or antifungal medication, or known brain or leptomeningeal involvement. Other criteria that excluded patients from participation were a history of a grade ≥ 3 hypersensitivity reaction to docetaxel, polysorbate 80-containing medications or predniso(lo)ne, an active cancer other than mCRPC, an uncontrolled severe illness or medical condition, concurrent or planned treatment with potent inhibitors or inducers of cytochrome P450 3A4/5, participation in a clinical trial with any investigational drug, and reproductive potential without implementation of an accepted and effective method of contraception.

Study design

This study is an analysis of the treatment of patients with mCRPC with cabazitaxel through the CUP. While patients are treated within the CUP, they are closely monitored to assess the safety of the new medicine. Because of the nature of the study, it was an ambispective multicenter observational study. Safety data were collected prospectively; efficacy data were collected retrospectively. The study was approved by the institutional ethics committees, and written informed consent was obtained from all participants.

Treatment

All patients initially received 25 mg/m² cabazitaxel for 1 h intravenously on day 1 of a 21-day cycle, as well as 10 mg oral prednisone or prednisolone daily. Primary prophylaxis with granulocyte colony-stimulating factor (G-CSF) was considered in patients with high-risk clinical features for febrile neutropenia as described by European Organisation for Research and Treatment of Cancer (EORTC) guidelines, such as previous episodes of (febrile) neutropenia, age > 65 years, poor performance and/or nutritional status, extensive previous radiation, and/or serious comorbidities.¹³ G-CSF was administered when the physician estimated the chance for febrile neutropenia to be $\geq 20\%$. Patients were pretreated intravenously with an antihistamine (clemastine 1 mg), a corticosteroid (dexamethasone 8 mg or equivalent), and an H2 antagonist (ranitidine or equivalent) at least 30 minutes before cabazitaxel treatment. Additional oral or intravenous anti-emetic prophylaxis was administered at the physician's discretion. The recommended additional anti-emetic prophylaxis was metoclopramide. Patients who experienced grade ≥ 3 nausea and/or vomiting received more aggressive anti-emetic prophylaxis, namely, ondansetron. However, physicians were allowed to diverge from this protocol. Therefore, four patients received granisetron as anti-emetic prophylaxis. Furthermore, ondansetron was administered immediately if limited effect was expected from metoclopramide in an individual patient or if (severe) nausea and/or vomiting was

expected based on previous toxicities from other chemotherapy (docetaxel).

The protocol required a treatment delay when patients had an absolute neutrophil count of $\leq 1500/\text{mm}^3$, a thrombocyte count of $\leq 75000/\text{mm}^3$, grade ≥ 3 non-hematologic toxicities (except alopecia and nail changes) that had not recovered to the baseline, an aspartate aminotransferase or alanine aminotransferase concentration >1.5 times the upper limit of normal, and/or a bilirubin concentration higher than the upper limit of normal. If patients had not recovered from these toxic effects after two weeks of treatment delay, treatment was terminated. The protocol required a dose reduction to 20 mg/m^2 after an episode of grade ≥ 3 neutropenia and/or febrile neutropenia, grade 4 thrombocytopenia, grade ≥ 3 vomiting despite appropriate anti-emetic prophylaxis, grade ≥ 3 diarrhea or persisting diarrhea despite appropriate medication, grade ≥ 3 stomatitis, grade 2 peripheral neuropathy (patients with grade ≥ 3 peripheral neuropathy were withdrawn from treatment), liver abnormalities as described earlier, a creatinine clearance between 40 and 60 ml/min (patients with a creatinine clearance <40 ml/min were withdrawn from treatment), and any other grade ≥ 3 toxicity (except for alopecia and nail changes) that had improved to grade 2 or better. Dose re-escalation or further dose reductions were not allowed.

Although cabazitaxel treatment was discontinued after a maximum of ten cycles in the TROPIC study, physicians and patients were allowed to decide to continue treatment beyond ten cycles in the CUP if patients responded well to cabazitaxel treatment. Treatment was discontinued based on the patient's or physician's decision, adverse events, disease progression, and/or death. Patients were allowed to discontinue treatment at any time for any reason.

Outcome measures

Every patient underwent an extensive medical assessment before initiation of cabazitaxel treatment. This assessment included the collection of data regarding demographics (date of birth), vital signs, height, weight, ECOG performance status, history of prostate cancer, findings during physical examination, and hematologic (neutrophil and thrombocyte count, hemoglobin) and biochemical laboratory diagnostics (liver function, kidney function, and serum PSA concentration, among others). Furthermore, CT and bone scans were obtained if no recent test results were available. Before each cabazitaxel administration, new and existing symptoms were assessed and graded, physical examinations were performed, liver and renal functions were checked, a hematologic assessment was done, the serum PSA level was determined, and when clinically indicated, other diagnostic tests (e.g., CT, MRI, radiography, bone scans, and electrocardiograms) were performed. When cabazitaxel treatment was terminated, vital signs (weight, ECOG performance status) were registered and blood tests were performed. Prostate cancer progression, subsequent treatments, and OS were followed up until death or until the last date the patient was known to be alive before February 21, 2013.

OS was calculated as the number of days between the first day of cabazitaxel treatment and death or censoring. Other efficacy parameters were TTPP, PFS, best clinical response, and PSA response. TTPP and PSA response were considered the most reliable efficacy parameters, because serum PSA levels had been determined in patients every three weeks, whereas other diagnostic tests, such as radiologic assessments, were performed at the physician's discretion at random time points.

TTPP was calculated from the first day of cabazitaxel treatment according to Prostate Cancer Clinical Trials Working Group 2 (PCWG2) recommendations.¹⁴ In patients who had an initial PSA decrease, PSA progression was defined as an increase of at least 25% over the nadir PSA concentration. In patients with no decline from the baseline PSA level, PSA progression was defined as an increase of at least 25% over the nadir PSA concentration for a duration of ≥ 12 weeks of treatment. Furthermore, a patient's TTPP was not determined if a different treatment was started before PSA progression was measured or if >3 months elapsed between two subsequent PSA measurements.

To report PSA-based outcomes, waterfall plots were used as recommended by the PCWG2.¹⁴ First, the maximum PSA decrease during cabazitaxel treatment was assessed; if the PSA did not decrease at all, the maximum PSA level during cabazitaxel treatment was assessed instead. Second, the PSA change after 4 cycles was assessed.

PFS was defined as the number of months between initiation of cabazitaxel treatment and the first date of progression as measured by PSA progression (using the same criteria as for TTPP), tumor progression (either from increased measurable lesions or from increased lesions on CT/MRI/X-ray/bone scans), symptomatic progression, and/or death. Because tumor measurements were performed at the physician's discretion, in no patient was PFS based solely on tumor progression, i.e., patients with radiologic disease progression always had clinical progression or PSA progression as well. Furthermore, some patients discontinued cabazitaxel treatment because of symptoms and PSA progression, whereas, according to the definition of PCWG2, this progression may have been caused by a flare.¹⁴ Therefore, the PFS could not be determined in these patients. The best clinical response was considered progressive disease when both serum PSA levels were continuously increased compared with the baseline serum PSA level, PSA levels had a rising trend, and overall, patients did not have an improved condition. A partial response was defined as a PSA decrease of $\geq 50\%$ compared with the baseline in at least two separate PSA measurements three weeks apart and an improvement in the patient's symptoms. Furthermore, if measurable lesions had decreased in size, it was considered a partial response as well, regardless of serum PSA levels or a change in symptoms.

Patients were intensively monitored for adverse events throughout the study by physician visits and diagnostic tests such as blood tests and electrocardiograms. Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0.¹⁵ Adverse events could result in the addition of medication to

treat or prevent the adverse events, dose reduction, dose delay, or withdrawal from the study. All adverse events from the onset of treatment until 30 days after the last cabazitaxel administration were recorded.

Statistical analyses

All analyses were performed with the study population that received at least one dose of cabazitaxel. Microsoft Excel was used to calculate the median, interquartile range (IQR), range, mean and standard deviation (SD) for patient characteristics, treatment characteristics and G-CSF use. IBM SPSS Statistics (version 20) was used for the statistical analyses of efficacy parameters. OS, TTPP, and PFS were analyzed using the Kaplan-Meier method. OS data were censored at the last date the patient was known to be alive; PFS data were censored at the last date the disease state of the patient was assessed if no disease progression had occurred. Log-rank tests were used to calculate differences in TTPP and OS between groups that had been stratified based on age, body mass index (BMI), time between prostate cancer and mCRPC diagnosis, initial Gleason score, ECOG performance score at the start of cabazitaxel treatment, PSA levels at the start of cabazitaxel treatment, previous docetaxel therapy, and pretreatment with abiraterone/enzalutamide, as well for calculating the significance of the difference between median received cabazitaxel cycles.

Role of outside organizations

Sanofi-Aventis provided a database with all treatment-emergent adverse events (TEAEs) registered. The authors had full access to safety data collected in the CUP by Sanofi-Aventis; analyses were performed independently from Sanofi-Aventis. The decision to submit the report for publication was made by the chief investigators (MDW and HG), who wrote the manuscript with input from the other authors. Sanofi-Aventis reviewed the final manuscript before submission.

Results

Patients and treatment

Between July 28, 2010 and April 27, 2011, cabazitaxel treatment was initiated in 49 of 51 patients selected in five hospitals to participate in the CUP. Two patients withdrew from the CUP between selection and treatment initiation, as the result of being unable to visit the hospital because of a deteriorating condition. Data from these two patients were not included in the analyses, because the aim of our study was not to perform an intention-to-treat analysis but to determine the safety and efficacy of cabazitaxel in Dutch clinics. Median age of the 49 patients who received at least one administration of cabazitaxel was 64.6 years (IQR 58.6-70.0); three patients were older than 75 years (Table 1). Most patients (71.4%) had an ECOG performance status of 1 during selection; 12 patients (24.5%) had an ECOG

performance status of 2. A majority of patients (85.7%) had at least 2 sites of metastases; the two most frequent metastatic sites were bone and lymph nodes. Lung and liver metastases had been diagnosed in 6 (12.2%) and 7 (14.3%) patients, respectively. Twenty-four patients (49.0%) had received 2 or more chemotherapy regimens, and 10 patients (20.4%) had received abiraterone (10.2%), enzalutamide (8.2%), and/or immunotherapy (ipilimumab/CNTO95) (4.1%) before cabazitaxel. Patients had received a median dose of 750 mg/m² (IQR 450-900 mg/m²) docetaxel during the last docetaxel regimen. For patients whose disease progressed after the last docetaxel dose, median time from last docetaxel administration to disease progression was 3.22 months (IQR 1.36-6.87 months); 9 patients (18.4%) had disease progression during docetaxel treatment, whereas 11 patients (22.4%) had progressive mCRPC >6 months after the last dose of docetaxel. Before treatment initiation, the median serum PSA level was 355.5 ng/ml (IQR 123.0-1515.4 ng/ml) (Table 1). All but 1 patient (98.0%) had an initial PSA concentration ≥20 ng/ml.

Patients completed a median of 6 cycles (range 1-21 cycles) of cabazitaxel treatment in 126 days (range 21-469 days) (Fig. 1, Table 2). Nine patients (18.4%) completed 10 cycles of cabazitaxel treatment. Twelve patients (24.5%) required a dose reduction during the first 10 cycles; 1 additional patient required a dose reduction at the 20th cycle (Table 2). Seven patients needed dose reduction only at cycle 8 or higher; furthermore, the majority of patients (n=9) who needed a dose reduction had a dose reduction during their last or second to the last cycle (data not shown). Twenty patients (40.8%) required a dose delay (Table 2).

G-CSF was administered for prophylactic use to 8 patients (16.3%) for a total of 49 cycles. These eight patients completed a median number of 9.0 cabazitaxel cycles (IQR 7.5-10.0); the median number of cabazitaxel cycles in patients not treated with G-CSF was 5.0 (IQR 4.0-8.0).

After discontinuation of cabazitaxel treatment, 26 patients started other second-line systemic therapies. Twenty-three patients were treated with abiraterone acetate, two patients with docetaxel, and three patients with mitoxantrone. Three patients were treated with ipilimumab or placebo in a study setting, and four patients were treated with enzalutamide. Finally, three patients received a second cabazitaxel regimen after treatment with abiraterone acetate.

Safety

All patients reported TEAEs during treatment; 46 (93.9%) patients had adverse events possibly related to cabazitaxel treatment, as assessed at the start of each cabazitaxel cycle (Table 3). Although a serious adverse event (SAE) occurred in 16 (32.7%) patients, none of these adverse events resulted in patient death. Grade ≥3 events occurred in 25 (51.0%) patients; grade 4 events occurred in five (10.2%) patients. Sixteen (32.7%) patients discontinued treatment because of TEAEs.

Table 1. Baseline characteristics of patients treated with cabazitaxel via the Dutch CUP (n=49)

Age		
Mean [years (SD)]	63.6	(8.1)
Median [years (IQR)]	64.6	(58.6-70.0)
Patients <65 years	26	(53.1%)
Patients ≥75 years	3	(6.1%)
Performance status at the start of therapy		
ECOG 0	2	(4.1%)
ECOG 1	35	(71.4%)
ECOG 2	12	(24.5%)
Extent of metastatic disease		
Number of metastatic lesions		
0	0	(0%)
1	7	(14.3%)
≥2	42	(85.7%)
Localization of metastases		
Local recurrence	23	(46.9%)
Regional lymph node	17	(34.7%)
Distant lymph node	24	(49.0%)
Bladder	6	(12.2%)
Pelvis	13	(26.5%)
Bone	47	(95.9%)
Lung	6	(12.2%)
Liver	7	(14.3%)
Bone marrow	4	(8.2%)
Mediastinum	4	(8.2%)
Other	7	(14.3%)
Previous mCRPC therapy		
Number of chemotherapy regimens		
1	25	(51.0%)
≥2	24	(49.0%)
Other mCRPC therapy (abiraterone acetate, enzalutamide and/or immunotherapy)	10	(20.4%)
Docetaxel use		
Number of previous docetaxel regimens		
Mean (SD)	1.1	(0.3)
Median (range)	1.0	(1-3)
Cumulative dose of last docetaxel administration (mg/m ²)		
Mean (SD)	742.50	(358.58)
Median (IQR)	750	(450-900)
Disease progression relative to docetaxel administration		
<0 (during treatment)	9	(18.4%)
<3 months since last dose	17	(34.7%)
≥3-<6 months since last dose	12	(24.5%)
≥6 months since last dose	11	(22.4%)
Median time from last docetaxel dose to disease progression [months (IQR)]	3.22	(1.36-6.87)
Serum PSA concentration (ng/ml)		
Median (IQR)	355.5	(123.0-1515.4)
≥20 ng/ml	48	(98.0%)

Data are number of patients (%) if not specified otherwise. ECOG, Eastern Cooperative Oncology Group; IQR, interquartile range; PSA, prostate-specific antigen; SD, standard deviation

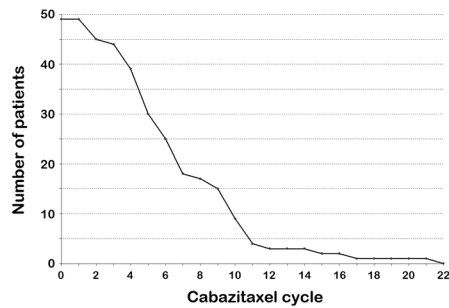


Figure 1. Cabazitaxel treatment in the Dutch CUP population. The graph displays the number of patients treated at cycle n.

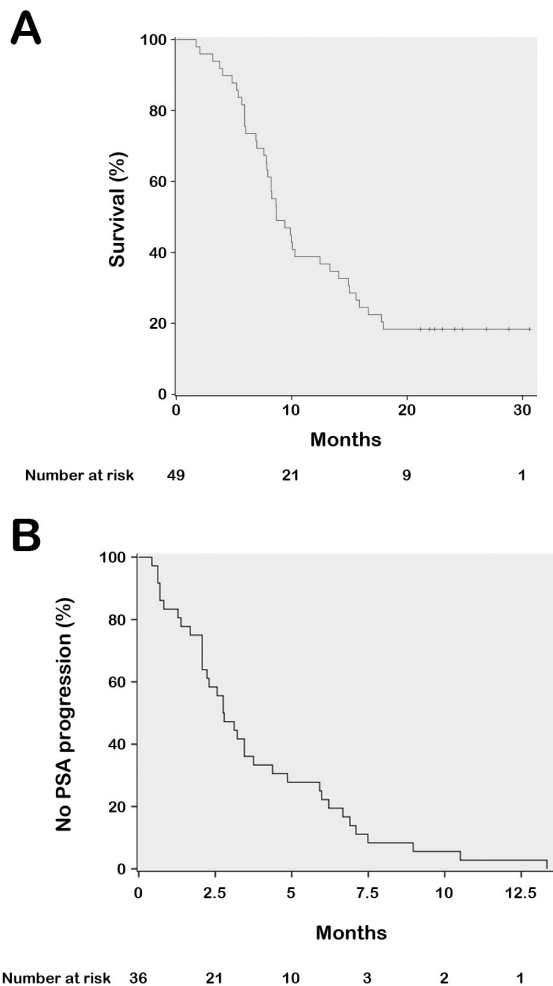


Figure 2. Kaplan-Meier estimates of (A) overall survival (OS) and (B) time to PSA progression (TTPP) in the Dutch cabazitaxel treated CUP population.

Vertical bars on the curves display censored observations.

All grade ≥ 3 TEAEs and SAEs, as well as grade 1 or 2 events that occurred in ≥ 2 patients, are listed in Table 3. The most frequent TEAE was fatigue, which occurred in 30 (61.2%) patients; grade ≥ 3 fatigue occurred in five (10.2%) patients. Other non-hematologic grade ≥ 3 TEAEs that were reported in at least two patients, were urosepsis (6.1%), bone pain (6.1%), paraplegia (4.1%), pulmonary embolism (4.1%), urinary tract infections (4.1%) and a decreased appetite (4.1%). The most frequent reported non-hematologic SAEs were hematuria and urosepsis, which occurred in 4 (8.2%) and 3 (6.1%) patients, respectively. One patient experienced grade 3 hematuria; this patient had received multiple fractions of radiation ($3 \times 8\text{Gy}$ and, $4 \times 5\text{Gy}$) to the pelvic region before cabazitaxel therapy. Two of four patients with grade 2 hematuria had received radiation therapy at an earlier stage as well. A grade ≥ 3 cardiac disorder (myocardial infarction) and diarrhea each occurred in one patient. Other frequently reported non-hematologic adverse events (all grades) were nausea (44.9%), diarrhea (40.8%), vomiting (26.5%) and malaise (20.4%) (Table 3). Grade 1 or 2 peripheral neuropathy was reported in nine patients (18.4%). Eleven patients (22.4%) had a weight loss of $\geq 5\%$ of their total body weight.

Hematologic adverse events occurred in 17 (34.7%) patients. Of all cabazitaxel-treated patients with mCRPC, six patients (12.2%) experienced grade ≥ 3 hematologic adverse events, of which grade ≥ 3 (febrile) neutropenia and anemia occurred most frequently (4.1%). Seven hematologic SAEs were reported: anemia (twice), febrile neutropenia (twice), neutropenic infection, neutropenic sepsis, and hemorrhagic anemia.

Efficacy

Median follow-up was 24.1 months (IQR 22.4-26.9 months). At the cutoff date for the final analysis, 40 patients had died. Kaplan-Meier analysis of OS is displayed in Figure 2A. Median OS was 8.7 months (IQR 6.0-15.9 months); mean OS was 12.9 months (95% Confidence Interval (CI) 10.3-15.5 months) (Fig. 2A, Table 4). Fourteen patients (28.6%) had continuous progressive disease despite cabazitaxel treatment; nine patients (18.4%) had a partial response. Hence, disease control (partial response plus stable disease) was established in 35 patients (71.4%). In these 35 patients, median OS was 13.3 months (IQR 7.9 months-undetermined); mean OS was 15.6 months (95% CI 12.5-18.8 months).

TTPP was determined in 36 patients (Fig. 2B, Table 4). Mean TTPP was 3.8 months (95% CI 2.8-4.7 months); median TTPP was 2.8 months (IQR 1.7-5.9 months). Strikingly, the two patients with the longest TTPP (13.3 and 10.5 months) had received the most cabazitaxel cycles (21 and 14, respectively). The two patients, in whom cabazitaxel treatment was discontinued after ten cycles solely because of completion of ten cycles, had a TTPP of 9.0 and 7.5 months. This suggests that it might be clinically beneficial to continue treatment beyond ten cycles when there are no other indicators to stop cabazitaxel treatment; this needs to be investigated in more detail.

Predictive and prognostic factors for response to cabazitaxel treatment were determined.

Table 2. Treatment characteristics in cabazitaxel-treated patients (n=49)

Duration of treatment		
Number of treatment cycles		
Mean (SD)	6.39	(3.96)
Median (range)	6	(1-21)
Number of patients that completed ≥10 cycles	9	(18.4%)
Treatment time (days)		
Mean (SD)	144	(88)
Median (range)	126	(21-469)
Number of patients with a treatment delay	20	(40.8%)
Number of patients with a dose reduction		
dose reduction ≤cycle 10	13	(26.5%)
	12	(24.5%)

Data are number of patients (%) unless specified otherwise. SD, standard deviation

Table 3. Adverse events reported during cabazitaxel treatment

Patients with possibly related TEAE	46	(93.9%)		
Patients who discontinued treatment due to TEAE	16	(32.7%)		
Patients with hematological grade ≥3 adverse event	6	(12.2%)		
Patients with ≥5% weight loss	11	(22.4%)		
Patients with any grade 4 adverse event	5	(10.2%)		
Patients with any grade 5 adverse event	0			
	grade 3 or 4	SAE	all grades	
Any adverse event	25 (51.0%)	16 (32.7%)	49	(100%)
HEMATOLOGICAL ADVERSE EVENT				
Anemia	2 (4.1%)	2 (4.1%)	14	(28.6%)
Hemorrhagic anemia	1 (2.0%)	1 (2.0%)	1	(2.0%)
Neutropenia	2 (4.1%)	2 (4.1%)	3	(6.1%)
Febrile neutropenia	2 (4.1%)	2 (4.1%)	2	(4.1%)
Leukopenia	1 (2.0%)	0	3	(6.1%)
Thrombocytopenia	1 (2.0%)	0	2	(4.1%)
NON-HEMATOLOGICAL ADVERSE EVENT				
Fatigue	5 (10.2%)	0	30	(61.2%)
Bone pain	3 (6.1%)	1 (2.0%)	12	(24.5%)
Urosepsis	3 (6.1%)	3 (6.1%)	3	(6.1%)
Decreased appetite	2 (4.1%)	0	7	(14.3%)
Urinary tract infection	2 (4.1%)	0	5	(10.2%)
Paraplegia	2 (4.1%)	2 (4.1%)	2	(4.1%)
Pulmonary embolism	2 (4.1%)	2 (4.1%)	2	(4.1%)
Nausea	1 (2.0%)	0	22	(44.9%)
Diarrhea	1 (2.0%)	0	20	(40.8%)
Vomiting	1 (2.0%)	0	13	(26.5%)
Back pain	1 (2.0%)	0	6	(12.2%)
Hematuria	1 (2.0%)	4 (8.2%)	5	(10.2%)
Spinal cord compression	1 (2.0%)	2 (4.1%)	2	(4.1%)
Myocardial infarction	1 (2.0%)	1 (2.0%)	1	(2.0%)
Hypocalcemia	1 (2.0%)	0	1	(2.0%)
Duodenal ulcer hemorrhage	1 (2.0%)	1 (2.0%)	1	(2.0%)
Colitis	1 (2.0%)	0	1	(2.0%)
Hydronephrosis	1 (2.0%)	0	1	(2.0%)
Malaise	0	0	10	(20.4%)

Pyrexia	0	1 (2.0%)	8 (16.3%)
Dehydration	0	1 (2.0%)	1 (2.0%)
Diplopia	0	1 (2.0%)	1 (2.0%)
Peripheral neuropathy	0	0	9 (18.4%)
Pain in extremity	0	0	7 (14.3%)
Arthralgia	0	0	6 (12.2%)
Constipation	0	0	5 (10.2%)
Headache	0	0	5 (10.2%)
Muscle spasms	0	0	4 (8.2%)
Cough	0	0	4 (8.2%)
Rectal hemorrhage	0	0	3 (6.1%)
Dysgeusia	0	0	3 (6.1%)
Peripheral sensory neuropathy	0	0	3 (6.1%)
Peripheral oedema	0	0	3 (6.1%)
Abnormal hepatic function	0	0	2 (4.1%)
Nasopharyngitis	0	0	2 (4.1%)
Dyspnoea	0	0	2 (4.1%)
Epistaxis	0	0	2 (4.1%)
Oropharyngeal pain	0	0	2 (4.1%)
Groin pain	0	0	2 (4.1%)
Muscular weakness	0	0	2 (4.1%)
Musculoskeletal chest pain	0	0	2 (4.1%)
Musculoskeletal pain	0	0	2 (4.1%)
Musculoskeletal stiffness	0	0	2 (4.1%)
Urinary retention	0	0	2 (4.1%)
Influenza-like illness	0	0	2 (4.1%)

Data are number of patients (%). Toxic effects were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (v 3.0). All adverse events that occurred in at least two patients are listed, as well as all grade 3 or 4 adverse events and SAEs. TEAE, treatment-emerging adverse event; SAE, serious adverse event

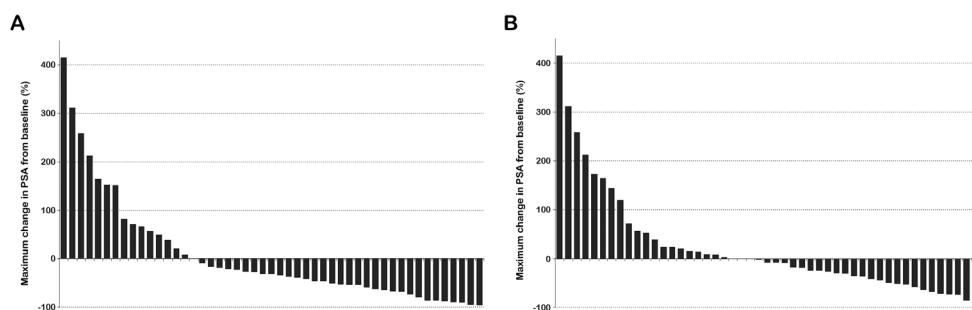


Figure 3. Waterfall plots showing (A) the maximal change in serum PSA levels from baseline during/after cabazitaxel therapy before initiation of another mCRPC treatment; (B) the change in serum PSA levels from baseline after four cabazitaxel cycles.

If patients had been treated for less than 4 cycles, the serum PSA level during/after the last cabazitaxel cycle was used.

Age, BMI, time between prostate cancer and mCRPC diagnosis, initial Gleason score, and ECOG performance score did not significantly influence the clinical outcome for cabazitaxel-treated patients (Table 4). However, patients with a PSA <500 ng/ml at the start of treatment had a longer OS than patients with an initial PSA level of ≥ 500 ng/ml (10.1 months vs. 7.9 months; $p=0.016$). Patients who had received <10 cycles of docetaxel treatment had a significantly decreased median TTPP (2.8 vs. 3.5 months) and OS (7.8 vs. 10.0 months) compared with patients who had received ≥ 10 docetaxel cycles ($p=0.049$ and $p=0.015$, respectively) (Table 4). Furthermore, ten of the twelve patients (83.3%) who had a TTPP >4 months had received ≥ 10 cycles of docetaxel before cabazitaxel treatment, whereas four of the nine patients (44.4%) who had a TTPP <2 months, had received ≥ 10 docetaxel cycles before cabazitaxel treatment (data not shown). The number of cabazitaxel cycles received was not significantly different between patients who had received <10 cycles and those who had received ≥ 10 docetaxel cycles, because the median number of cabazitaxel cycles was 5 and 6, respectively ($p=0.163$). Similarly, the percentage of patients who discontinued treatment because of adverse events did not differ between the two groups (30.0% and 27.6%, respectively; $p=0.858$). Patients pretreated with abiraterone and/or enzalutamide had a decreased OS (5.9 vs. 10.0 months; $p=0.027$) (Table 4). The median TTPP tended to differ significantly as well (2.1 vs. 3.2 months; $p=0.052$). Between these groups of patients, the median number of cabazitaxel cycles was not significantly different (4 vs. 6; $p=0.065$). PFS was similar to TTPP, because disease progression was first indicated by a rising PSA level in most patients. PFS was determined in 46 patients. Median PFS was 2.8 months (IQR 1.7-4.9 months), and mean PFS was 3.8 months (95% CI 2.8-4.7 months) (Table 4).

Figure 3 displays waterfall plots of the two analyses of PSA progression as recommended by the PCWG2.¹⁴ Fifteen patients had a continuous increase in PSA levels. One patient had a PSA measurement at the start of the cabazitaxel treatment only because this patient had SAEs during the first cycle and did not have his PSA measured afterwards. Six patients had an initial PSA decrease, but had their serum PSA levels increase to the baseline PSA level or higher during the first four cycles. The remaining 27 patients had a decrease in PSA levels that was sustained during the first four cycles of cabazitaxel treatment. Of these patients, 19 had a PSA decrease of $\geq 25\%$ for at least four cycles. The maximum decrease in PSA was 92.9%; this patient's PSA level decreased from 3669 ng/ml to 172.4 ng/ml during six cabazitaxel cycles. Despite the PSA decrease, the patient discontinued treatment because of a deteriorating condition. Seven months after discontinuation of cabazitaxel, his PSA level had increased to 5000 ng/ml.

Finally, we studied whether patients who had a decrease in PSA levels by at least 25% and 50% compared with the baseline PSA serum concentration, had an increased OS and TTPP compared with patients who did not have such a PSA response. Patients who had at least a 25% decrease in PSA after four cycles of cabazitaxel had a median TTPP and OS of 6.2 (IQR 4.4-7.5) and 16.6 (9.4-undetermined) months, respectively, compared with 2.1 (0.8-

Table 4. Efficacy parameters of cabazitaxel treatment

Overall survival (OS) (n=49)					
Mean [months (95% CI)]	12.9	(10.3-15.5)			
Median [months (IQR)]	8.7	(6.0-15.9)			
Time to PSA progression (TTPP) (n=36)					
Mean [months (95% CI)]	3.8	(2.8-4.7)			
Median [months (IQR)]	2.8	(1.7-5.9)			
Progression-free survival (PFS) (n=46)					
Mean [months (95% CI)]	3.8	(2.8-4.7)			
Median [months (IQR)]	2.8	(1.7-4.9)			
Best response (n=49)					
Progressive disease (%)	14	(28.6%)			
Partial response (%)	9	(18.4%)			
OS in patients who responded to cabazitaxel (n=35)					
Mean [months (95% CI)]	15.6	(12.5-18.8)			
Median [months (IQR)]	13.3	(7.9-N/A)			

Patient characteristic	TTPP			#	OS			
	#	Median (IQR)			#	Median (IQR)		
age <65 years	20	3.2	(1.4-4.9)	p=0.458	26	8.3	(5.9-17.8)	p=0.731
age ≥65 years	16	2.6	(2.1-5.9)		23	9.9	(7.6-15.9)	
BMI <25	10	2.8	(2.1-5.9)	p=0.972	15	9.9	(6.9-N/A)	p=0.616
BMI 25-30	20	2.6	(1.4-4.9)		24	8.2	(5.7-14.9)	
BMI >30	5	3.5	(2.1-4.4)		9	13.3	(8.7-15.9)	
time to mCRPC <12 months	8	2.1	(0.7-3.1)	p=0.282	11	7.6	(5.9-10.1)	p=0.121
time to mCRPC ≥12 months	28	2.8	(2.1-6.0)		38	9.9	(6.0-17.8)	
Gleason score <8	10	2.8	(2.2-3.5)	p=0.407	12	12.5	(6.0-15.6)	p=0.750
Gleason score 8-10	16	2.5	(2.1-6.7)		22	9.4	(8.2-17.9)	
ECOG <2	28	2.8	(2.1-6.0)	p=0.118	37	10.0	(7.6-16.6)	p=0.347
ECOG ≥2	8	1.7	(0.7-3.1)		12	7.0	(4.8-9.4)	
PSA <500 ng/ml	23	3.1	(0.8-5.9)	p=0.655	26	10.1	(7.9-N/A)	p=0.016
PSA ≥500 ng/ml	13	2.6	(2.1-6.0)		23	7.9	(5.7-14.1)	
Docetaxel <10 cycles	15	2.8	(1.4-3.5)	p=0.049	19	7.8	(5.9-10.3)	p=0.015
Docetaxel ≥10 cycles	21	3.5	(2.1-6.9)		30	10.0	(7.9-N/A)	
prior treatment with abiraterone/ enzalutamide	7	2.1	(1.3-3.1)	p=0.052	8	5.9	(5.4-8.3)	p=0.027
no prior treatment with abiraterone/ enzalutamide	29	3.2	(2.1-6.2)		41	10.0	(7.6-17.8)	

The IQR could not be determined if >25% of patients were alive at the cutoff date (N/A). #, number of patients; IQR, interquartile range; BMI, body mass index; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen.

2.8) and 7.9 (IQR 5.9-10.0) months in the rest of the patients ($p<0.001$). Patients who had at least a 50% decrease in PSA had a median TTPP and OS of 6.9 (IQR 5.9-9.0) and 16.6 (15.0-undetermined) months, respectively, compared with 2.3 (1.4-3.5) and 8.3 (IQR 5.9-

13.3) months in the rest of the patients ($p=0.017$ and $p=0.024$, respectively). However, between patients who had a 25% to 50% decrease and those who had a >50% decrease in PSA, the TTP and OS did not differ significantly ($p=0.854$ and $p=0.644$, respectively).

Discussion

In the TROPIC study, patients with mCRPC treated with cabazitaxel had an increased PFS and OS compared with mitoxantrone-treated patients irrespective of the aggressiveness of the tumor.^{10, 11} However, both grade ≥ 3 hematologic and non-hematologic adverse events had an increased incidence in cabazitaxel-treated patients. Since the completion of this phase III study, several studies with cabazitaxel reported fewer high-grade adverse events.¹⁶⁻¹⁸ In the TROPIC study, grade ≥ 3 adverse events occurred in 82% of all patients; adverse events were assessed on a weekly basis.¹⁰ Of all patients included in the German CUP, grade ≥ 3 TEAEs occurred in 30.6% of patients.¹⁹ In our study, grade ≥ 3 adverse events occurred in 51.0% of patients. Similarly, grade ≥ 3 neutropenia, leukopenia, and diarrhea were reported in 82%, 68%, and 6% of patients in the TROPIC study, respectively.¹⁰ Grade ≥ 3 neutropenia, leukopenia, and diarrhea were reported in 7.2%, 9.0%, and 0.9% of patients of the German CUP, respectively.¹⁹ Preliminary data of cabazitaxel-use in patients with mCRPC through a CUP in Italy reported grade ≥ 3 neutropenia, leukopenia, and diarrhea in 48.9%, 25.6%, and 1.1% of patients, respectively.¹⁶ In an expanded access program (EAP) in Spain grade ≥ 3 neutropenia and diarrhea occurred in 24% and 1.5% of cabazitaxel-treated patients with mCRPC, respectively.¹⁷ All three studies assessed adverse events once every three weeks. In line with these results, grade ≥ 3 neutropenia, leukopenia and diarrhea occurred in 4.1%, 2.0%, and 2.0% of patients who participated in the Dutch CUP, respectively. The decreased number of hematologic adverse events may be partially result from the use of prophylactic G-CSF in high-risk patients according to EORTC guidelines¹³, whereas in the TROPIC study no prophylactic G-CSF was allowed. Furthermore, in the TROPIC study TEAEs were assessed weekly, whereas in the CUPs and docetaxel phase III study, TEAEs were assessed once every three weeks, simulating the clinical practice setting.³ Most of the TEAEs that were missed by doing an assessment every three weeks instead of weekly, were asymptomatic TEAEs that disappeared within three weeks (such as neutropenia without fever), and thus were not clinically relevant. Finally, patients and/or doctors could have decided to discontinue cabazitaxel treatment at an earlier stage because of the availability of abiraterone acetate as an alternative drug for patients with mCRPC, preventing the onset of grade ≥ 3 adverse events. This latter is confirmed by the lower percentage of patients completing ten cycles compared with the TROPIC study (18% vs. 28%). Nevertheless, these data indicate that SAEs such as febrile neutropenia are relatively well controlled in a clinical setting in which physicians administer prophylactic G-CSF and other preventive medicine to patients who are at high risk for the development of SAEs.

In the cabazitaxel-treated arm of the TROPIC study five patients died of cardiac problems within 30 days of cabazitaxel treatment.¹⁰ According to the investigators, none of these cardiac events were related to cabazitaxel. A subsequent study, which directly investigated the relationship between cabazitaxel use and cardiac disorders, concluded that cabazitaxel had no significant effect on the QTc interval in patients with advanced solid tumors.¹⁸ It is generally thought that the increased number of mortal cardiac events in the cabazitaxel-treated group of the TROPIC study was not related to cabazitaxel. In the Dutch CUP, one patient had a myocardial infarction between cabazitaxel courses; grade 5 TEAEs did not occur in participating patients.

Collected efficacy parameters in our study were OS, TTPP, and PFS. Time to radiologic or clinical progression was not determined, because clinical progression was not reported in a standardized format, and radiologic assessments had been performed based on the physician's decision. In general, most physicians performed radiologic tests only when other tools to measure disease progression, such as PSA measurements and clinical assessments, were inconclusive.

Median OS was considerably lower in the Dutch CUP population compared with OS in the cabazitaxel-treated population of the TROPIC study (8.7 vs. 15.1 months). The median TTPP was lower as well: 2.8 vs. 6.4 months. In the German CUP, the mean biochemical PFS and OS were 3.8 and 13.9 months, respectively. This was comparable to our results, in which the mean TTPP and OS were 3.8 and 12.9 months, respectively. Our results were also similar to preliminary results from the Spanish EAP; the median PFS in this study was 4.4 months.¹⁷ The Italian CUP did not report efficacy data. The most likely explanation for this discrepancy between the TROPIC study and our study is a difference in the patient population: in general, patients in the Dutch CUP had more advanced prostate cancer than patients in the TROPIC study. Only 31% of cabazitaxel-treated patients in the TROPIC study had received two or more chemotherapy regimens; in the Dutch CUP 49.0% of patients had received two or more chemotherapy regimens. Furthermore, ten patients (20.4%) had received abiraterone (five patients), enzalutamide (four patients) and/or immunotherapy (ipilimumab/CNTO95) (two patients) before cabazitaxel, whereas patients enrolled in the TROPIC study had no previous treatment with these agents. Recent research concludes that treating patients with abiraterone or enzalutamide before taxane therapy may reduce the efficacy of taxanes.^{20, 21} In a retrospective study of 35 chemonaïve patients treated with abiraterone who subsequently received docetaxel at progression, a median OS of 12.5 months and a PSA response in 9 patients (25.7%) were reported with docetaxel, which is significantly lower than figures reported in the TAX-327 trial (19.8 months and 45%, respectively).^{3, 20} Another evident difference in the patient population is the number of metastatic sites in patients: 85.7% of patients in the Dutch CUP had ≥ 2 metastatic sites, whereas only 61% of patients in the TROPIC study had ≥ 2 metastatic sites.¹⁰ Furthermore, patients in our study had a median PSA of 355.5 ng/ml at the start of cabazitaxel treatment, whereas the median PSA was 143.9

ng/ml in patients who would be treated with cabazitaxel at the start of the TROPIC study. These observations strengthen the need for observational studies as presented in this chapter: the current clinical situation does not necessarily comply with the study population of the phase III registration study.

Recently, hormonal therapy with abiraterone acetate has been approved by the US Food and Drug Administration (FDA) and in the Netherlands as a second-line therapy for patients with mCRPC, based on the results of the COU-AA-301 study.²² Thus, both cabazitaxel and abiraterone acetate are therapeutic options for patients with symptomatic mCRPC who progressed during or after docetaxel treatment. Enzalutamide has just been approved by the US FDA as well but is awaiting approval in the European Union; a phase III study has indicated prolonged OS when administered as second-line therapy in patients with mCRPC.²³ CUPs with enzalutamide are ongoing. Finally, docetaxel could be re-introduced after an initial response and a substantial docetaxel-free interval. There is no scientific evidence for the most preferred treatment strategy in patients with symptomatic mCRPC after docetaxel-based therapy. Therefore, a wide variety of clinical studies is being performed to create a scientific basis for the optimal treatment strategy for this group of patients.

To further improve the tolerability of cabazitaxel in patients with mCRPC without compromising efficacy, three phase II studies are assessing different dosing schedules, such as weekly cabazitaxel at 10 mg/m² or biweekly cabazitaxel at 16 mg/m².²⁴⁻²⁶ Furthermore, a phase III study (PROSELICA) is comparing the efficacy of 20 mg/m² cabazitaxel to 25 mg/m² cabazitaxel, both administered once every three weeks.^{27, 28} The CABARESC study is a phase II study in which budesonide is added to cabazitaxel to prevent cabazitaxel-induced diarrhea, the most frequent grade ≥ 3 non-hematologic adverse event in the TROPIC study.²⁹ In another clinical study, octreotide is added to cabazitaxel to prevent diarrhea as well.³⁰ However, considering the low percentage of grade ≥ 3 diarrhea reported in this CUP and other CUPs, one can question whether these studies are still needed, because it seems that diarrhea is already well controlled in a regular clinical setting.

Therapy efficacy and/or tolerability may be further improved by combining cabazitaxel with other treatments, such as the combination of cabazitaxel with custirsen (OGX-011)³¹, abiraterone acetate³², tasquinimod³³, carboplatin³⁴, or bavituximab³⁵. Other studies are investigating the use of cabazitaxel in patients with less advanced prostate cancer, such as the FIRSTANA study, which compares the efficacy of cabazitaxel (25 or 20 mg/m²) to docetaxel as first-line therapy in patients with mCRPC.^{36, 37} Further clinical benefit could be achieved by selecting a subgroup of patients with mCRPC that is most likely to respond to cabazitaxel. Since fourteen patients (28.6%) in the Dutch CUP did not respond to cabazitaxel treatment at all, and patients who initially responded exhibited a wide variation in the duration of response, a marker predicting cabazitaxel response would prevent unnecessary treatment of patients, thereby cutting costs, reducing adverse events, and preventing delays in initiating other therapies that are targeting the tumor. No such marker has been identified

yet. The initial Gleason score has been identified as a predictive factor in abiraterone-treated patients, an initial Gleason score of 8 to 10 resulting in a lesser response to the agents.³⁸ A short time (<12 months) between the time of prostate cancer and mCRPC diagnosis was a prognostic factor for a lower PFS in patients treated with abiraterone and other endocrine-manipulating agents.³⁹ Since PFS in docetaxel-treated patients was not associated with the time to castration resistance, this may be a predictive factor in abiraterone-treated patients as well. The time to castration-resistance and the initial Gleason score were not significantly predictive or prognostic for the cabazitaxel response (TTPP/OS) in the Dutch CUP.

Our study and other studies suggest that pretreatment with abiraterone or enzalutamide may compromise the efficacy of cabazitaxel.^{20, 21} However, patients who received this pretreatment may have had more aggressive or more advanced prostate cancer. The ECOG performance status at the start of cabazitaxel treatment did not differ significantly though ($p=0.294$, data not shown). Abiraterone and enzalutamide are currently being assessed as first-line therapy in patients with mCRPC.^{40, 41} Considering the results from our study and other studies,^{20, 21} we think potential cross-resistance needs to be assessed more thoroughly in prospective randomized drug sequence studies.

The results of the Dutch CUP further suggest that if patients had received <10 docetaxel cycles, indicating they had disease progression or SAEs during docetaxel treatment, they are likely to have a lesser response to cabazitaxel treatment compared with patients who received ≥ 10 docetaxel cycles. This observation was not confounded by the number of cabazitaxel cycles received, because these numbers were similar between the two groups. Therefore, cabazitaxel may have a higher efficacy in patients who received at least 10 docetaxel treatments, suggesting that some patients are particularly sensitive to taxanes and reach a significant survival benefit with this therapy. In summary, the relationship between previous docetaxel, abiraterone, or enzalutamide treatment and cabazitaxel response needs further study, and more specific predictive markers need to be identified.

With the introduction of cabazitaxel as a second-line therapy in patients with mCRPC, treatment options for this group of patients have expanded. Results from the Dutch CUP study indicate that cabazitaxel has effect in patients with advanced mCRPC in a clinical setting, delaying disease progression and/or improving symptoms, while resulting in moderate toxicity. However, we are still at the beginning stage of the expansive research that is needed to optimize the treatment algorithm for patients with mCRPC.

Acknowledgments

The authors wish to thank Hans de Witte for his input and support, and Roeleke Koops for assisting with the collection of follow-up survival data. The authors had full access to the safety data collected by Sanofi-Aventis. Cabazitaxel (Jevtana) was provided by Sanofi.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012;62(1):10-29.
2. Tannock IF, Osoba D, Stockler MR et al. Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points. *J Clin Oncol* 1996;14(6):1756-1764.
3. Tannock IF, de Wit R, Berry WR et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351(15):1502-1512.
4. Petrylak DP, Tangen CM, Hussain MH et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* 2004;351(15):1513-1520.
5. Sartor AO. Progression of metastatic castrate-resistant prostate cancer: impact of therapeutic intervention in the post-docetaxel space. *J Hematol Oncol* 2011;4:18.
6. Bissery MC. Preclinical evaluation of new taxoids. *Curr Pharm Des* 2001;7(13):1251-1257.
7. Paller CJ, Antonarakis ES. Cabazitaxel: a novel second-line treatment for metastatic castration-resistant prostate cancer. *Drug Des Devel Ther* 2011;5:117-124.
8. Mita AC, Denis LJ, Rowinsky EK et al. Phase I and pharmacokinetic study of XRP6258 (RPR 116258A), a novel taxane, administered as a 1-hour infusion every 3 weeks in patients with advanced solid tumors. *Clin Cancer Res* 2009;15(2):723-730.
9. Pivot X, Koralewski P, Hidalgo JL et al. A multicenter phase II study of XRP6258 administered as a 1-h i.v. infusion every 3 weeks in taxane-resistant metastatic breast cancer patients. *Ann Oncol* 2008;19(9):1547-1552.
10. de Bono JS, Oudard S, Özgüroglu M et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* 2010;376(9747):1147-1154.
11. Oudard S, de Bono JS, Özgüroglu M, et al. Impact of cabazitaxel (Cbz) + prednisone (P; CbzP) on overall survival (OS) at 2 yrs and in patients (pts) with aggressive disease: post-hoc analyses of TROPIC trial. [abstract]. *Ann Oncol*, 2012 (suppl; abstr 933P).
12. cancer.gov [Internet]. FDA Approval for Cabazitaxel. Available at: <http://www.cancer.gov/cancertopics/druginfo/fda-cabazitaxel>. Accessed: August 13, 2012.
13. Aapro MS, Bohlius J, Cameron DA et al. 2010 update of EORTC guidelines for the use of granulocyte-colony stimulating factor to reduce the incidence of chemotherapy-induced febrile neutropenia in adult patients with lymphoproliferative disorders and solid tumours. *Eur J Cancer* 2011;47(1):8-32.
14. Scher HI, Halabi S, Tannock I et al. Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. *J Clin Oncol* 2008;26(7):1148-1159.
15. cancer.gov [Internet]. Cancer Therapy Evaluation Program, Common Terminology Criteria for Adverse Events, Version 3.0. Published August 9, 2006. Available at: http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaeV3.pdf. Accessed: August 13, 2012.
16. Bracarda S, Di Lorenzo G, Gasparro D et al. Updated safety result of a large Italian early access program (EAP) with cabazitaxel plus prednisone (CbzP) in metastatic castration-resistant prostate cancer (mCRPC) patients who progressed during or after docetaxel (D) therapy. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr e15185).

17. De Velasco G, Aparicio LA, Esteban E et al. Cabazitaxel in patients with advanced CRPC after docetaxel failure: Results of expanded access program (EAP) in Spain: Safety and efficacy. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr e15149).
18. Wade JL, Dakhil SR, Baron AD et al. A QTC study of cabazitaxel in patients with advanced solid tumors. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr e15115).
19. Heidenreich A, Scholz HJ, Rogenhofer S et al. Cabazitaxel Plus Prednisone for Metastatic Castration-resistant Prostate Cancer Progressing After Docetaxel: Results from the German Compassionate-use Programme. *Eur Urol* 2013;63(6):977-82.
20. Mezynski J, Pezaro C, Bianchini D et al. Antitumour activity of docetaxel following treatment with the CYP17A1 inhibitor abiraterone: clinical evidence for cross-resistance? *Ann Oncol* 2012.
21. Fitzpatrick JM. Management of castration-resistant prostate cancer: a call to urologists. *BJU Int* 2012;110(6):772-774.
22. de Bono JS, Logothetis CJ, Molina A et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011;364(21):1995-2005.
23. Scher HI, Fizazi K, Saad F et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med* 2012;367(13):1187-1197.
24. Clinicaltrials.gov [Internet]. Study of Weekly Cabazitaxel for Advanced Prostate Cancer. Available at: <http://clinicaltrials.gov/ct2/show/NCT01518283>. Accessed: August 13, 2012.
25. Clinicaltrials.gov [Internet]. A Study Looking at Novel Scheduling of Cabazitaxel for Patients With Metastatic Prostate Cancer (ConCab). Available at: <http://clinicaltrials.gov/ct2/show/NCT01541007>. Accessed: August 13, 2012.
26. Clinicaltrials.gov [Internet]. Second-line Chemotherapy in Castration Resistant Prostate Cancer (Prostyll). Available at: <http://clinicaltrials.gov/ct2/show/NCT01558219>. Accessed: August 13, 2012.
27. Clinicaltrials.gov [Internet]. Cabazitaxel at 20 mg/m² Compared with 25 mg/m² With Prednisone for the Treatment of Metastatic Castration Resistant Prostate Cancer (PROSELICA). Available at: <http://clinicaltrials.gov/ct2/show/NCT01308580>. Accessed: August 13, 2012.
28. Eisenberger MA, Hardy-Bessard A, Mourey L et al. Comparison of two doses of cabazitaxel plus prednisone in patients (pts) with metastatic castration-resistant prostate cancer (mCRPC) previously treated with a docetaxel (D)-containing regimen. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr TPS4692[^]).
29. stichtingduos.nl [Internet]. Een fase II studie in mHRPC naar de farmacodynamische effecten van budesonide op cabazitaxel (Jevtana®): Een gerandomiseerde, open-label multicenter studie: CABARESC. [in Dutch]. Available at: <http://www.stichtingduos.nl/studies/cabaresc-nl/>. Accessed: August 13, 2012.
30. Clinicaltrials.gov [Internet]. Cabazitaxel Plus Prednisone With Octreotide For Castration-Resistant Prostate Cancer (CRPC) Previously Treated With Docetaxel. Available at: <http://clinicaltrials.gov/ct2/show/NCT01469338>. Accessed: August 13, 2012.
31. Clinicaltrials.gov [Internet]. Comparison of Cabazitaxel/Prednisone Alone or in Combination With Custirsen for 2nd Line Chemotherapy in Prostate Cancer (AFFINITY). Available at: <http://clinicaltrials.gov/ct2/show/NCT01578655>. Accessed: August 13, 2012.
32. Clinicaltrials.gov [Internet]. Cabazitaxel and Abiraterone Acetate in Patients With Metastatic Castrate-Resistant Prostate Cancer. Available at: <http://clinicaltrials.gov/ct2/show/NCT01511536>. Accessed: August 13, 2012.
33. Clinicaltrials.gov [Internet]. The CATCH Prostate Cancer Trial: Cabazitaxel And Tasquinimod

- in Men With Prostate Cancer. Available at: <http://clinicaltrials.gov/ct2/show/NCT01513733>. Accessed: August 13, 2012.
34. Clinicaltrials.gov [Internet]. Cabazitaxel With or Without Carboplatin in Patients With Metastatic Castration-Resistant Prostate Cancer Previously Treated With Docetaxel-Based Therapy. Available at: <http://clinicaltrials.gov/ct2/show/NCT01505868>. Accessed: August 13, 2012.
 35. Clinicaltrials.gov [Internet]. Study of Cabazitaxel Plus Bavituximab as Second-line Chemotherapy for Patients With Castration-resistant Prostate Cancer. Available at: <http://clinicaltrials.gov/ct2/show/NCT01335204>. Accessed: August 13, 2012.
 36. Clinicaltrials.gov [Internet]. Cabazitaxel Versus Docetaxel Both With Prednisone in Patients With Metastatic Castration Resistant Prostate Cancer (FIRSTANA). Available at: <http://clinicaltrials.gov/ct2/show/NCT01308567>. Accessed: August 13, 2012.
 37. Oudard S, Sengelov L, Mainwaring PN et al. First-line use of cabazitaxel in chemotherapy-naïve patients with metastatic castration-resistant prostate cancer (mCRPC): A three-arm study in comparison with docetaxel. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr TPS4696[^]).
 38. Azria D, Massard C, Tosi D et al. An ambispective observational study in the safety and efficacy of abiraterone acetate in the French temporary authorizations for use (ATU): Predictive parameters of response. [abstract]. *J Clin Oncol* 30, 2012 (suppl 5; abstr 149).
 39. Loriot Y, Massard C, Albiges L et al. Personalizing treatment in patients with castrate-resistant prostate cancer: A study of predictive factors for secondary endocrine therapies activity. [abstract]. *J Clin Oncol* 30, 2012 (suppl 5; abstr 213).
 40. Ryan CJ, Smith MR, de Bono JS et al. Abiraterone in metastatic prostate cancer without previous chemotherapy. *N Engl J Med* 2013;368(2):138-148.
 41. Clinicaltrials.gov [Internet]. A Safety and Efficacy Study of Oral MDV3100 in Chemotherapy-Naïve Patients With Progressive Metastatic Prostate Cancer (PREVAIL). Available at: <http://clinicaltrials.gov/ct2/show/NCT01212991>. Accessed: February 26, 2013.

Chapter 4

CAST: A retrospective analysis of cabazitaxel and abiraterone acetate sequential treatment in patients with metastatic castrate-resistant prostate cancer previously treated with docetaxel

Michel D. Wissing, Jules L.L.M. Coenen, Pieter van den Berg, Hans M. Westgeest, Alfons J.M. van den Eertwegh, Inge M. van Oort, Monique M. Bos, André M. Bergman, Paul Hamberg, Bert-Jan ten Tije, Maartje Los, Martijn P.J.K. Lolkema, Ronald de Wit, Hans Gelderblom

Submitted

Abstract

Background: Cabazitaxel and abiraterone have both received approval for treating metastatic castrate-resistant prostate cancer (mCRPC) patients after first-line docetaxel therapy. In the CAST-study, the clinical outcome of docetaxel-treated mCRPC patients treated sequentially with both cabazitaxel and abiraterone was evaluated, to study whether treatment sequence could influence clinical outcome.

Methods: Data were collected retrospectively from mCRPC patients at twelve hospitals across the Netherlands who initiated cabazitaxel and/or abiraterone before December 2012. Primary outcome measure was overall survival (OS); secondary measures were progression-free survival (PFS), biochemical PFS, and best clinical and PSA response. Hospital admission data during treatment were collected, as well as toxicities resulting in treatment discontinuation or patient death.

Results: Sixty-three and 69 patients received Cab→Abi (cabazitaxel prior to abiraterone) and Abi→Cab (abiraterone prior to cabazitaxel) before July 10th, 2013, respectively. Median OS was 19.1 months and 17.0 months in Cab→Abi and Abi→Cab treated patients, respectively ($p=0.369$). Median PFS and biochemical PFS were significantly extended in Cab→Abi treated patients: 8.1 versus 6.5 ($p=0.050$) and 9.5 versus 7.7 months ($p=0.024$), respectively. Although partial responses to cabazitaxel occurred in both groups, Abi→Cab treated patients had a significantly decreased antitumor response from cabazitaxel than Cab→Abi treated patients (median PFS 5.0 versus 2.6 months, $p<0.001$). Minor differences in toxicities were observed based on therapy sequence; generally, toxicity from cabazitaxel could be severe, while abiraterone toxicity was milder.

Conclusions: This retrospective analysis indicates that primary progression on cabazitaxel or abiraterone did not preclude a response to the other agent in mCRPC patients. However, tumor response of both agents, particularly cabazitaxel, was lower when administered as higher-line therapy in the selected study population.

Introduction

Metastatic castrate-resistant prostate cancer (mCRPC) is the second deadliest cancer in men in the Western world.^{1, 2} Recently, novel therapeutic agents have emerged for the systemic treatment of these patients. Two such therapies are cabazitaxel and abiraterone acetate, which received United States Food and Drug Administration (US FDA) and European Medicines Agency (EMA) approval between 2010 and 2011 for use in mCRPC patients following disease progression during or after docetaxel. Although abiraterone also received FDA and EMA approval for use in docetaxel-naïve mCRPC patients, it has not been registered for this indication in the Netherlands.

Abiraterone acetate (Zytiga®, Johnson&Johnson) selectively inhibits the enzyme cytochrome P450 c17 (CYP17), thereby blocking testosterone biosynthesis.³ In a double-blind phase III registration study (COU-AA-301), patients treated with abiraterone plus prednisone had an increased median overall survival (OS) (15.8 versus 11.2 months), median radiologic progression-free survival (PFS) (5.6 versus 3.6 months), and median biochemical PFS (8.5 versus 6.6 months) as compared to patients treated with placebo plus prednisone.^{4, 5} Mineralocorticoid-related adverse events (fluid retention, hypokalemia, hypertension) and urinary tract infections were more frequently reported in the abiraterone-treated group than in patients receiving placebo.

Cabazitaxel (Jevtana®, Sanofi-Aventis) is a second generation taxane which exerts its antitumor activity by targeting microtubule dynamics.⁶ In preclinical studies, cabazitaxel was capable of inhibiting cancer cell lines with acquired resistance against docetaxel.^{7, 8} In a phase III registration study (TROPIC), patients treated with cabazitaxel plus prednisone had an increased median OS (15.1 versus 12.7 months), median PFS (2.8 versus 1.4 months) and median biochemical (6.4 versus 3.1 months) as compared to patients treated with mitoxantrone plus prednisone.⁹ Grade ≥ 3 hematological adverse events such as (febrile) neutropenia occurred more frequently in cabazitaxel-treated patients. Frequently reported grade ≥ 3 non-hematological adverse events included diarrhea, fatigue and asthenia.

Aforementioned registration studies were conducted parallel to each other; both agents resulted in a significant survival benefit. Direct comparison of cabazitaxel with abiraterone based on these studies is not possible. Therefore, both agents are approved in the post-docetaxel setting, but regulatory agencies such as the EMA and US FDA have made no mention of treatment sequence.¹⁰⁻¹² However, therapy sequence may influence clinical outcome¹³⁻¹⁷: resistance to one therapy may result in resistance to another therapy by similar mechanisms of action, or progression of mCRPC may result in decreased sensitivity to therapy. No clinical study has studied cabazitaxel and abiraterone treatment sequence. For this reason the CAST-study was conducted. In this retrospective study, the clinical

outcome is reported of (subgroups of) Dutch mCRPC patients treated with both cabazitaxel and abiraterone after receiving docetaxel as first-line therapy, evaluating antitumor activity and safety of both agents.

Patients and Methods

Patients

Twelve Dutch hospitals participated in this study, comprising the Netherlands Cancer Institute, five university hospitals and six regional hospitals across the nation. Eligible patients had confirmed mCRPC, for which they had received docetaxel at least once; all patients were medically or surgically castrated. Patients who had received abiraterone and/or cabazitaxel prior to docetaxel were excluded. All other mCRPC patients receiving cabazitaxel and/or abiraterone, as registered in electronic patient files, were included, including patients who received other treatments for their mCRPC before cabazitaxel and/or abiraterone therapy.

Study design and ethics

This study was a retrospective, multicenter, observational study. All patients had been informed before initiation of mCRPC treatment that data could be used anonymously for research purposes and were able to object at any time without consequences. Oral informed consent was acquired from patients who were still under treatment; data from other patients were collected if patients had not objected to data collection for research purposes. This is in accordance with the Dutch code of conduct for medical research and is in compliance with all Dutch and international laws regarding research with human data. Medical ethics committee approval was obtained before data collection.

Treatment

Standard treatment consisted of 1000 mg oral abiraterone acetate daily plus 10 mg oral prednisone daily or intravenous cabazitaxel 20 or 25 mg/m² every 21 days plus 10 mg oral prednisone daily. However, physicians were allowed to deviate from this protocol, for dose reductions, to initiate treatment at a lower dose, or to delay treatment. Discontinuation of treatment was based on disease progression, adverse events, patient's or physician's decision, and/or death. This decision was made entirely by the physician and patient; participation in the CAST-study did not influence this decision.

Medication to reduce or prevent side effects was allowed during cabazitaxel or abiraterone therapy, as well as additional symptomatic treatment against mCRPC, such as radiotherapy or denosumab. Although cabazitaxel and abiraterone are mentioned as second- and third-line therapies in this study, patients were allowed to receive alternative mCRPC therapies in between docetaxel, cabazitaxel and abiraterone. All such treatment decisions were made independent of participation in this study. Hence treatment regimens represented an

average clinical setting in the Netherlands.

Outcome measures

The primary outcome measure for patients who received both cabazitaxel and abiraterone was OS, defined as the number of days between start of second-line therapy (cabazitaxel or abiraterone) and death or censoring, regardless of therapies afterwards. Secondary outcome measures were PFS, biochemical PFS, and best clinical and prostate-specific antigen (PSA) response.

To determine PSA progression and response, guidelines from the Prostate Cancer Clinical Trials Working Group (PCWG) 2 were followed.¹⁸ Hence, PSA progression was defined as an increase of $\geq 25\%$ and ≥ 2 ng/ml over nadir PSA concentration. In general, PSA serum levels were measured in patients every three to four weeks. For determining the nadir PSA level, potential PSA flares were taken into account (patients with a continuous PSA rise from baseline within 12 weeks, not confirmed with imaging). Biochemical PFS was calculated from therapy start until PSA progression. Patients whose tumors responded to another systemic treatment before PSA progression, patients with PSA progression after more than three months between the last PSA assessment, and patients who died before PSA progression, were considered lost-to-follow-up.

PFS was calculated from therapy start until progression or censoring. Progression was established when PSA progression, radiological progression (CT/MRI/X-ray/bone scans), symptomatic progression (pain or other clinical symptoms) and/or death had occurred, following PCWG2 criteria. One form of progression sufficed to consider the patient's disease to be progressive, except for symptomatic progression. Symptomatic progression without radiological or PSA progression was not considered disease progression as this could be the subjective opinion of a patient unrelated to mCRPC progression, unless symptoms resulted in therapy adjustments (e.g. increased analgesic use or discontinuation of treatment). Radiological imaging was performed at the physician's discretion, and reviewed locally. 'Total (biochemical) PFS' is the sum of the separate (biochemical) PFS for cabazitaxel and abiraterone.

A patient's best response was defined as progressive disease (PD) when serum PSA levels were continuously increased compared to baseline for at least 12 weeks since treatment initiation (to exclude patients with PSA flares), and/or an increase in lesion size in radiologic imaging methods within three months of initiation of therapy. A partial response (PR) was defined as a PSA decrease of $\geq 50\%$ compared to baseline in at least two separate PSA measurements three weeks apart or a decrease in lesion size (when no new lesions had occurred and no other lesions had increased in size). All patients who did not fit the criteria for PR or PD were considered to have stable disease as the best response. If results were in contrast with each other, it was considered stable disease, except when physicians reported an increased tumor lesion size: in that case the disease was always considered progressive.

Adherence to this protocol was similar to PCWG2 recommendations.¹⁸

For safety data, adverse events had been collected inconsistently between physicians and hospitals. Therefore, collection of adverse event data according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) was not possible. Instead, all hospital admissions between treatment initiation and 30 days after the last administration of cabazitaxel or abiraterone were registered. These were registered consistently in all hospitals. Adverse events, regardless of severity, resulting in discontinuation of treatment or patient death were recorded separately.

Statistical analyses

Microsoft Excel was used to calculate the mean, median, interquartile range (IQR), range and standard deviation (SD) for patient characteristics, treatment characteristics, best response and adverse events. SPSS (version 20) was used for the Kaplan-Meier analyses of treatment duration, follow-up, OS and (biochemical) PFS. If patient death had not occurred or its date was unknown, OS data were censored at the last date the patient was known to be alive. If no disease and/or PSA progression had occurred, (biochemical) PFS data were censored at the last date the disease state of the mCRPC patient was assessed. Log-rank tests were used to compare durations (OS etc.) between patient groups. For comparison of other parameters between patient groups, student's t-tests were conducted. Cox regression analyses were performed to calculate hazard ratios (HRs).

Results

Patients

Between January 15th, 2009 and November 30th, 2012, 326 patients initiated abiraterone and/or cabazitaxel treatment at one of the twelve participating Dutch centers. The database was closed on July 10th, 2013. At this time, 44.5% and 15.0% of patients had only received abiraterone or cabazitaxel treatment, respectively. Sixty-three patients (19.3%) had received cabazitaxel followed by abiraterone (Cab→Abi), and 69 patients (21.2%) had received abiraterone followed by cabazitaxel (Abi→Cab). These percentages were similar when evaluating patients who had passed away before database cutoff (data not shown).

Baseline characteristics of Cab→Abi and Abi→Cab treated patients

Baseline patient and tumor characteristics of Cab→Abi and Abi→Cab treated patients at the start of second-line therapy are listed in Table 1. Patients treated with Cab→Abi were younger (65.6 vs. 69.8 years; $p<0.001$) and had a higher median PSA level at baseline (291 vs 130 ng/ml; $p=0.022$). Prior treatment between groups was similar, except for the percentage of patients having undergone surgical castrations (11.1% (Cab→Abi) vs. 1.4%

(Abi→Cab); $p=0.020$). While patients had received a similar median number of docetaxel cycles (10 vs. 9; $p=0.552$), patients receiving Abi→Cab had discontinued docetaxel more frequently due to toxicity (36.2% vs. 12.7%; $p=0.002$), whereas a higher percentage of Cab→Abi treated patients had completed all planned cycles (usually ten) (52.4% vs. 33.3%; $p=0.027$). Although not reaching statistical significance, patients in the Cab→Abi group had in general a shorter duration from diagnosis to second-line mCRPC therapy, but a longer duration between mCRPC diagnosis and second-line mCRPC therapy, and docetaxel and second-line mCRPC therapy. Other baseline characteristics were similar between groups.

Treatment characteristics of Cab→Abi and Abi→Cab treated patients

Treatment characteristics from second-line mCRPC therapy onwards are listed in Table 2. Of note, second-line therapy was considered cabazitaxel or abiraterone, third-line therapy the other agent, regardless of therapies between docetaxel, cabazitaxel and abiraterone. While there was no difference in second-line therapy duration (mean 159.9 versus 152.9 days; $p=0.955$), patients treated with Cab→Abi received significantly longer third-line therapy (mean 138.8 versus 100.8 days; $p=0.021$). Indeed, patients receiving cabazitaxel in the third-line (Abi→Cab) received significantly less cabazitaxel cycles (median 4 versus 7 cycles; $p<0.001$). Abiraterone was primarily discontinued due to disease progression; toxicity played a role in about one-third of patients discontinuing cabazitaxel, both in the second- and third-line.

Efficacy of Cab→Abi and Abi→Cab therapy

Median time to follow-up was 23.7 months and 21.8 months in Cab→Abi and Abi→Cab treated patients, respectively ($p=0.068$). Median OS was slightly greater in Cab→Abi treated patients: 19.1 months versus 17.0 months; however, this difference did not reach statistical significance ($p=0.369$) (Fig. 1A). When stratifying all patients based on second-line therapy, including patients that received cabazitaxel ($n=112$) or abiraterone ($n=214$) only, mCRPC patients that received abiraterone after docetaxel had a slightly greater median OS (13.2 versus 12.5 months); this difference was not statistically significant either ($p=0.386$). Median total PFS and total biochemical PFS were 8.1 versus 6.5 months ($p=0.050$) and 9.5 versus 7.7 months ($p=0.024$) in Cab→Abi versus Abi→Cab treated patients, respectively (Fig. 1B-C). Assessing (biochemical) PFS during second and third-line therapy separately, it was observed that (biochemical) PFS differed between Cab→Abi and Abi→Cab treated patients during second-line therapy, but were similar in the third-line (Table 3). When assessing best clinical and PSA responses, it was noted that during third-line therapy fewer patients had PRs, while an increased percentage of patients had PD. This was particularly evident when comparing cabazitaxel responses between second- and third-line therapy. Patients receiving cabazitaxel in the third-line also had a significantly shorter treatment duration than in the second-line (data not shown). Waterfall plots depicting maximum change in PSA from

Table 1. Baseline patient and tumor characteristics of patients treated with both cabazitaxel and abiraterone

	Cab→Abi (n=63)	Abi→Cab (n=69)	p-value
Age			
Median [years (range)]	65.6 (44-79)	69.8 (52-88)	<0.001
Gleason score			
Unknown	7 (11.1%)	8 (11.6%)	0.080
Median (range)	8 (6-10)	8 (6-10)	
Gleason ≥8	37 (66.1%)	34 (55.7%)	
Metastatic disease			
Number of metastatic lesions			0.928
1	11 (17.5%)	8 (11.6%)	
2	35 (55.6%)	43 (62.3%)	
≥3	17 (27.0%)	18 (26.1%)	
Prior treatment			
Radical prostatectomy	11 (17.5%)	5 (7.2%)	0.073
TUR-P	12 (19.0%)	11 (15.9%)	0.642
Surgical castration	7 (11.1%)	1 (1.4%)	0.020
Androgen-deprivation therapy	62 (98.4%)	69 (100%)	0.297
Lymph node dissection	13 (20.6%)	14 (20.3%)	0.961
Radiotherapy:			
- prostate	26 (41.3%)	27 (39.1%)	0.804
- metastases	32 (50.8%)	24 (34.8%)	0.064
Samarium-153/strontium-89	3 (4.8%)	3 (4.3%)	0.910
Ipilimumab/placebo	4 (6.3%)	6 (8.7%)	0.614
Enzalutamide/placebo	1 (1.6%)	1 (1.4%)	0.949
Docetaxel:			
# cycles [median (range)]	10 (3-20)	9 (2-33)	0.552
Reason to stop:			
- End of treatment	33 (52.4%)	23 (33.3%)	0.027
- Progressive disease	22 (34.9%)	23 (33.3%)	0.849
- Patient's decision	2 (3.2%)	1 (1.4%)	0.510
- Physician's decision	5 (7.9%)	7 (10.1%)	0.662
- Toxicity	8 (12.7%)	25 (36.2%)	0.002
- Unknown	0 (0.0%)	1 (1.4%)	0.341
- Other	0 (0.0%)	2 (2.9%)	0.176
Docetaxel rechallenge	9 (14.3%)	12 (17.4%)	0.413
Other	9 (14.3%)	15 (21.7%)	0.401
Time in months [median (IQR)] between:			
PCa diagnosis and 2 nd line therapy	49.7 (27.6-73.1)	61.1 (29.3-89.1)	0.195
mCRPC diagnosis and 2 nd line therapy	17.8 (12.0-24.2)	16.5 (10.1-29.0)	0.427
last docetaxel and 2 nd line therapy	5.6 (3.0-9.3)	3.7 (1.8-6.9)	0.051
Serum PSA concentration (ng/ml) at start second-line therapy			
Median (IQR)	291 (98-635)	130 (50-293)	0.022
<20 ng/ml	2 (3.2%)	9 (13.0%)	
ECOG performance status at start second-line therapy			
0-1	53 (84.1%)	58 (84.1%)	0.302
2	9 (14.3%)	11 (15.9%)	
3	1 (1.6%)	0 (0.0%)	

Data are number of patients (%) if not specified otherwise. Abi, abiraterone; Cab, cabazitaxel; CNS, central nervous system; ECOG, Eastern Cooperative Oncology Group; IQR, interquartile range; mCRPC, metastatic castrate-resistant prostate cancer; PCa, prostate cancer; PSA, prostate-specific antigen; SD, standard deviation; TUR-P, transurethral resection of the prostate

Table 2. Treatment characteristics of patients treated with both cabazitaxel and abiraterone

	Cab→Abi (n=63)	Abi→Cab (n=69)	p-value
Second-line treatment			
Mean treatment duration (days) (SD)	159.9 (70.9)	152.9 (89.1)	0.955
Patients with dose reduction (%)	23 (36.5%)	0 (0.0%)	
Median cumulative dose (range)	150 (60-515)	129 (25-392)	
Patients with additional therapy during treatment:			
- Radiotherapy	7 (11.1%)	8 (11.6%)	
- TUR-P/surgery	1 (1.6%)	1 (1.4%)	
Reason to discontinue:			
- End of treatment	13 (20.6%)	0 (0.0%)	
- Progressive disease	37 (58.7%)	67 (97.1%)	
- Toxicity	20 (31.7%)	4 (5.8%)	
- Other	15 (23.8%)	7 (10.1%)	
Patients without other therapy between Cab and Abi (%)	48 (76.2%)	51 (73.9%)	0.765
Patients with systemic therapy between Cab and Abi (%)	1 (1.6%)	10 (14.5%)	0.007
Enzalutamide/placebo (AFFIRM participants)	0 (0.0%)	5 (7.2%)	0.029
Docetaxel rechallenge	1 (1.6%)	6 (8.7%)	0.069
Ipilimumab	0 (0.0%)	1 (1.4%)	0.341
Mitoxantrone	0 (0.0%)	1 (1.4%)	0.341
Third-line treatment			
Mean treatment duration (days) (SD)	138.8 (97.3)	100.8 (59.8)	0.021
Patients with dose reduction	3 (4.8%)	11 (15.9%)	
Median cumulative dose (range)	109 (69-187)	100 (19-250)	
Patients with additional therapy during treatment:			
- Radiotherapy	19 (30.2%)	4 (5.8%)	
- TUR-P/surgery	0 (0.0%)	0 (0.0%)	
Reason to discontinue:			
- 3 rd line therapy still ongoing	3 (4.8%)	8 (11.6%)	
- End of treatment	0 (0.0%)	5 (7.2%)	
- Progressive disease	52 (82.5%)	42 (60.9%)	
- Toxicity	5 (7.9%)	23 (33.3%)	
- Death	3 (4.8%)	3 (4.3%)	
- Other	10 (15.9%)	8 (11.6%)	
Number of Cab cycles			
Mean (SD)	7.3 (3.2)	4.6 (2.7)	<0.001
Median (range)	7 (3-21)	4 (1-10)	
Patients who initiated therapy at 20 mg/m ²	6 (9.5%)	9 (13.0%)	0.528
Patients who completed ≥10 cycles	18 (28.6%)	6 (8.7%)	
Treatment duration of Cab and Abi combined (days)			
Mean (SD)	298.7 (133.8)	253.8 (114.1)	0.172
Median (IQR)	275 (215-344)	238 (160-322)	
Treatment after Cab and Abi			
Patients who had discontinued third-line therapy	60	61	
Patients with treatment after Cab and Abi	29 (48.3%)	34 (55.7%)	0.712
Enzalutamide/placebo	8 (13.3%)	13 (21.3%)	0.340
Radiotherapy	15 (25.0%)	20 (32.8%)	0.505
Samarium-153	3 (5.0%)	2 (3.3%)	0.579
Mitoxantrone	5 (8.3%)	6 (9.8%)	0.876
Cabozantinib/placebo	8 (13.3%)	4 (6.6%)	0.171
Cab rechallenge	3 (5.0%)	4 (6.6%)	0.793
Abi rechallenge	1 (1.7%)	4 (6.6%)	0.209
Other	9 (15.0%)	6 (9.8%)	0.405

Cumulative dose: Cab, mg/m²; Abi, g. Abi, abiraterone; Cab, cabazitaxel; IQR, interquartile range; SD, standard deviation; TUR-P, transurethral resection of the prostate

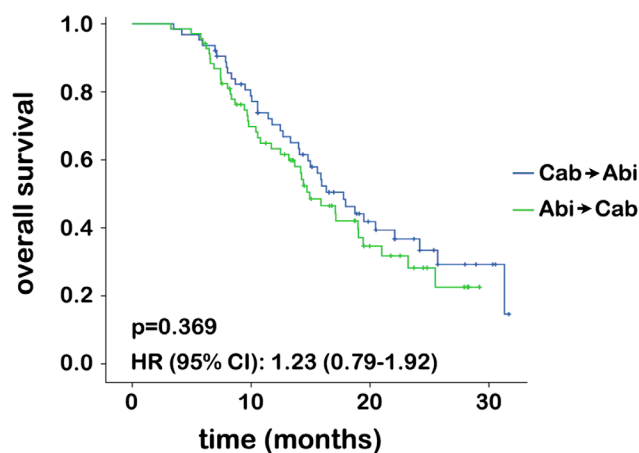
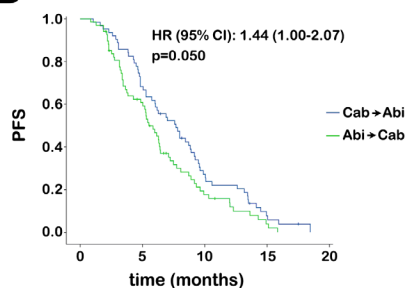
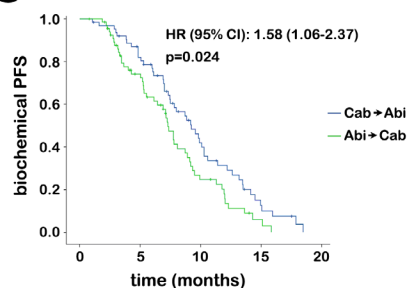
A**B****C**

Figure 1. Overall survival (OS) (A), progression-free survival (PFS) (B) and biochemical PFS (C) in metastatic castrate-resistant prostate cancer (mCRPC) patients treated with both cabazitaxel and abiraterone. CI, confidence interval; HR, hazard ratio.

Table 3. Efficacy of Cab→Abi and Abi→Cab treatment

	Cab→Abi (n=63)	Abi→Cab (n=69)	p-value
Second-line treatment			
PFS			
Mean [months (95% CI)]	5.3 (4.5-6.1)	3.2 (2.7-3.8)	<0.001
Median [months (IQR)]	5.0 (2.1-7.8)	2.7 (1.4-4.6)	
Biochemical PFS			
Mean [months (95% CI)]	6.0 (5.1-6.9)	3.4 (2.9-4.0)	<0.001
Median [months (IQR)]	6.2 (3.0-8.4)	2.8 (1.5-5.0)	
Best clinical response			
Progressive disease (%)	8 (12.7%)	19 (27.5%)	
Partial response (%)	29 (46.0%)	18 (26.1%)	
Best PSA response			
Progressive (%)	5 (7.9%)	21 (30.4%)	
Partial response (%)	32 (50.8%)	16 (23.2%)	

Third-line treatment

PFS	Mean [months (95% CI)]	2.9 (2.2-3.6)	3.2 (2.5-3.8)	0.451
	Median [months (IQR)]	2.4 (0.9-3.5)	2.6 (1.3-4.5)	
Biochemical PFS	Mean [months (95% CI)]	3.6 (2.7-4.5)	4.0 (3.3-4.7)	0.389
	Median [months (IQR)]	2.7 (1.4-4.7)	4.1 (1.4-5.1)	
Best clinical response	(n=62)	(n=68)		
Progressive disease (%)	24 (38.7%)	21 (30.9%)		
Partial response (%)	10 (16.1%)	18 (26.5%)		
Best PSA response	(n=61)	(n=66)		
Progressive (%)	17 (27.9%)	14 (21.2%)		
Partial response (%)	11 (18.0%)	21 (31.8%)		

Patients with stable disease as the best response, are not reported in the table. CI, confidence interval; IQR, interquartile range; OS, overall survival; PFS, progression-free survival; PSA, prostate-specific antigen

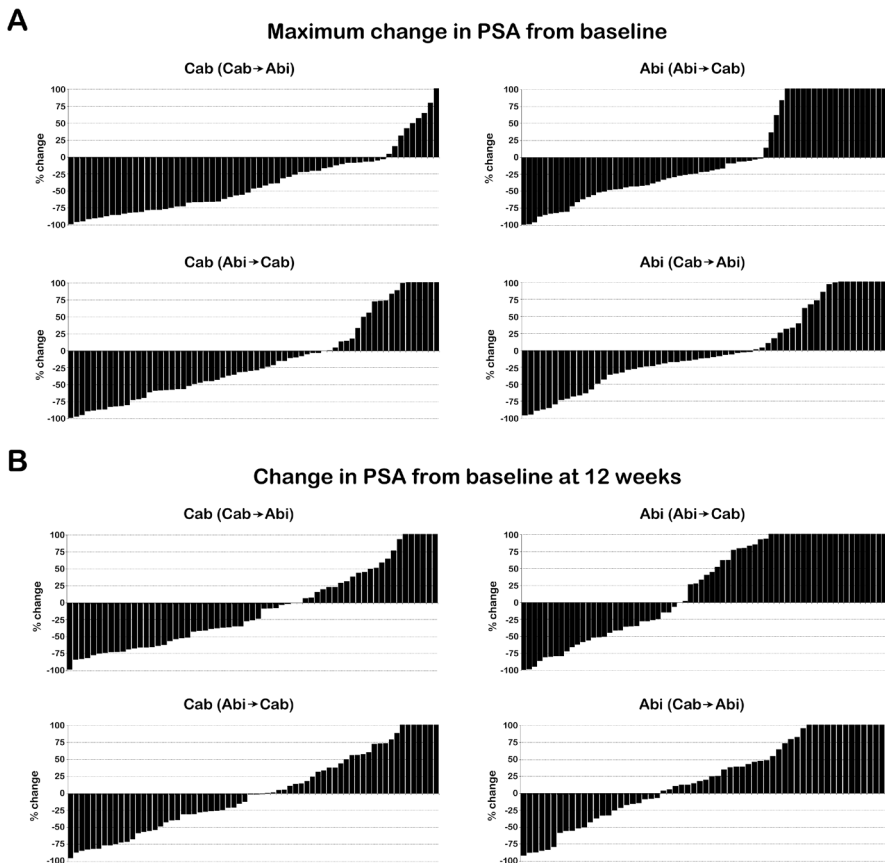


Figure 2. Waterfall plots depicting maximum prostate-specific antigen (PSA) changes from baseline (A) and change in PSA from baseline at 12 weeks (B) in mCRPC patients treated with cabazitaxel and abiraterone. Abi, abiraterone; Cab, cabazitaxel.

baseline and change in PSA from baseline at 12 weeks are displayed in Figure. 2. Cab→Abi treated patients had more frequently continuously rising PSA levels during cabazitaxel than Abi→Cab treated patients (Fig. 2A). This difference did not occur in abiraterone-treated patients; however, when assessing change in PSA from baseline at 12 weeks, patients receiving abiraterone after docetaxel had a slightly worse PSA outcome (Fig. 2B).

Subsequent performed subgroup analyses did not indicate that a subpopulation had a significantly improved OS when treated with one of the treatment sequences (Fig. 3A). However, a trend towards favorable median OS in Cab→Abi treated patients was observed in all subpopulations (HR>1; $p>0.05$ though), except for patients treated with less than 10 cycles and patients who discontinued docetaxel due to PD (HR 0.90 and 0.76, respectively). Similar results were found when performing subgroup analyses for PFS (Fig. 3B). Patients aged below 65, patients with a Gleason 8-10, patients who received ≥ 10 docetaxel cycles and patients who had not received enzalutamide before abiraterone had a significantly better PFS when treated with Cab→Abi (HR 2.64, 1.64, 1.85 en 1.44, respectively, $p\leq 0.05$).

Safety of Cab→Abi and Abi→Cab therapy

In total, about 60% of patients needed hospitalizations during cabazitaxel or abiraterone treatment (Table 4). In the second-line, 15.9% of cabazitaxel-treated patients required ≥ 2 hospitalizations, while only 7.2% of abiraterone patients required at least two hospitalizations (Abi→Cab group). No difference in hospitalizations was evident in the third-line. The primary adverse event causing hospitalization during cabazitaxel was febrile neutropenia (9.5% in Cab→Abi treated patients; 14.5% in Abi→Cab treated patients). Intriguingly, patients in the Abi→Cab group required more hospitalizations due to pain during both treatments (31.9% vs. 17.5% of Cab→Abi patients), while patients treated with Cab→Abi required more hospitalizations due to urinary tract infections (15.9% vs. 8.7%) and urinary obstruction during both treatments (7.9% vs. 1.4%).

Discontinuation of treatment due to toxicity occurred primarily during cabazitaxel therapy, with 21 patients (16.9%) having discontinued entirely because of toxicities (Table 4). For both therapies, main reasons to discontinue treatment were fatigue, malaise, nausea and vomiting. In cabazitaxel-treated patients, polyneuropathy was another frequently occurring toxicity resulting in treatment discontinuation.

Fifteen patients (11.4%) died within 30 days of the last administration of abiraterone or cabazitaxel (Table 5). Two-thirds of these patients were treated with Cab→Abi. The greater number of deaths in this patient group most likely reflects the general trend of physicians to continue abiraterone treatment longer as compared to cabazitaxel, e.g. when patients have a lower ECOG performance status. Three patients (60.0%) in the Abi→Cab group died due to toxicities; two of these had febrile neutropenia, the third had a non-neutropenic infection.

Discussion

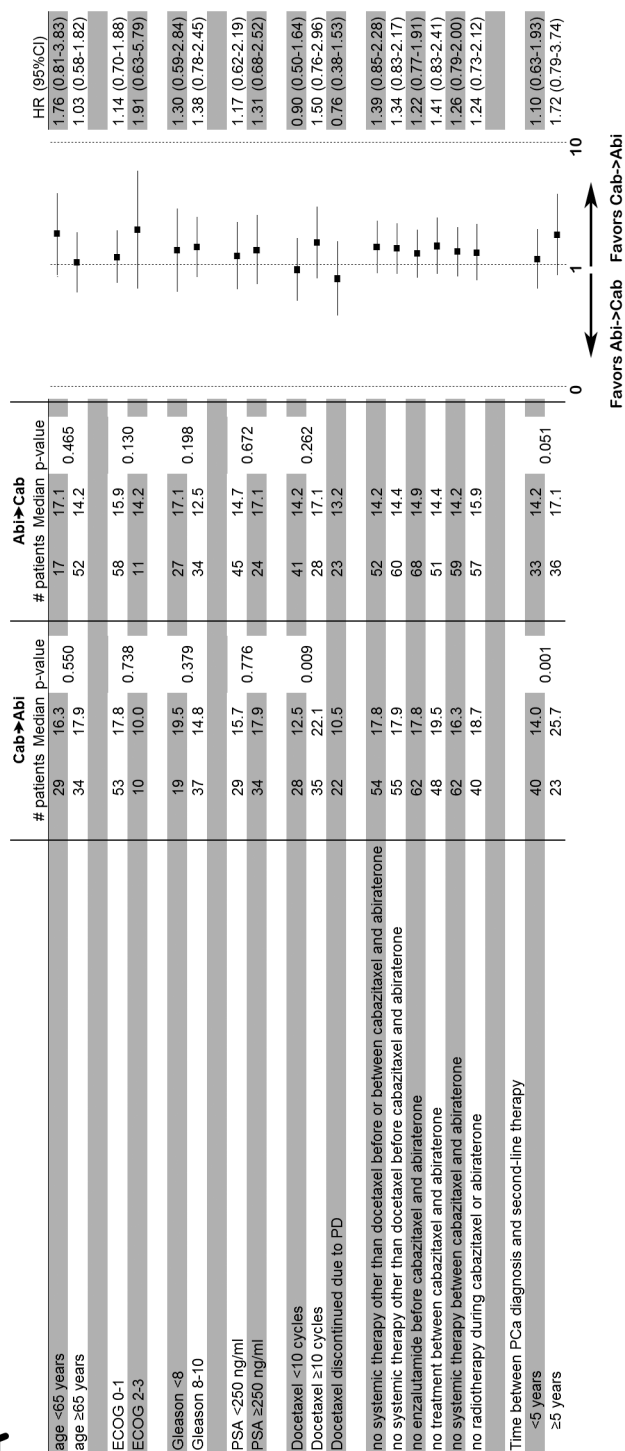
In recent years, cabazitaxel, abiraterone, enzalutamide, sipuleucel-T and radium-223 have been introduced to the clinic for mCRPC treatment. This rapidly expanding variety of available therapies has significantly improved survival of mCRPC patients.¹⁹ However, many questions remain how these therapies should be deployed in clinic to maximize clinical benefit.

Treatment with one agent may influence the efficacy of another therapy. Recent studies have suggested decreased antitumor effects of mCRPC therapies when administered later in the disease. In a retrospective analysis of a small group of abiraterone pretreated mCRPC patients, docetaxel seemed to have lower antitumor efficacy than normally observed.¹³ No responses to docetaxel were evident in abiraterone-refractory patients. In other retrospective analyses, abiraterone had limited antitumor activity in patients treated with docetaxel and enzalutamide, although some patients still benefited from abiraterone therapy.^{16, 17} Similar modest antitumor activity was reported when enzalutamide was administered to mCRPC patients after docetaxel and abiraterone.^{14, 15, 20} A recent preclinical study has suggested that cross-resistance may occur between taxanes and abiraterone.²¹

Despite suggestions of cross-resistance, multiple recent retrospective studies reported antitumor activity from cabazitaxel in mCRPC patients after docetaxel and abiraterone or enzalutamide, with partial responses occurring in 14% to 30% of patients.²²⁻²⁴ In line with these study results, we observed a similar percentage of mCRPC patients who responded to cabazitaxel therapy after docetaxel and abiraterone, confirming that progression on these agents does not prelude a response to cabazitaxel. Similarly, we observed partial responses with abiraterone in mCRPC patients who had received docetaxel and cabazitaxel prior to abiraterone. Hence, these results suggest that additional clinical benefit may be accomplished by treating patients sequentially with all three agents.

However, when comparing PFS and biochemical PFS of cabazitaxel in Cab→Abi and Abi→Cab treated patients, a significantly decreased PFS and biochemical PFS was observed in patients who had received cabazitaxel after abiraterone. Of note, when only selecting patients who discontinued second-line therapy due to PD, a similar decreased antitumor efficacy of particularly cabazitaxel as third-line therapy was observed (data not shown). Although PFS and biochemical PFS did not differ significantly in abiraterone treated patients based on prior therapy with or without cabazitaxel, the PSA response after twelve weeks of abiraterone treatment was slightly better in Abi→Cab treated patients as judged by the waterfall plots. Further research is needed to assess whether this observed decreased antitumor efficacy in higher-line therapy is due to cross-resistance or other factors, such as tumor mutations, differences in the patient populations, or a decreased tolerability of advanced mCRPC patients to aggressive therapies. Mechanisms for therapy resistance need to be unraveled

A



B

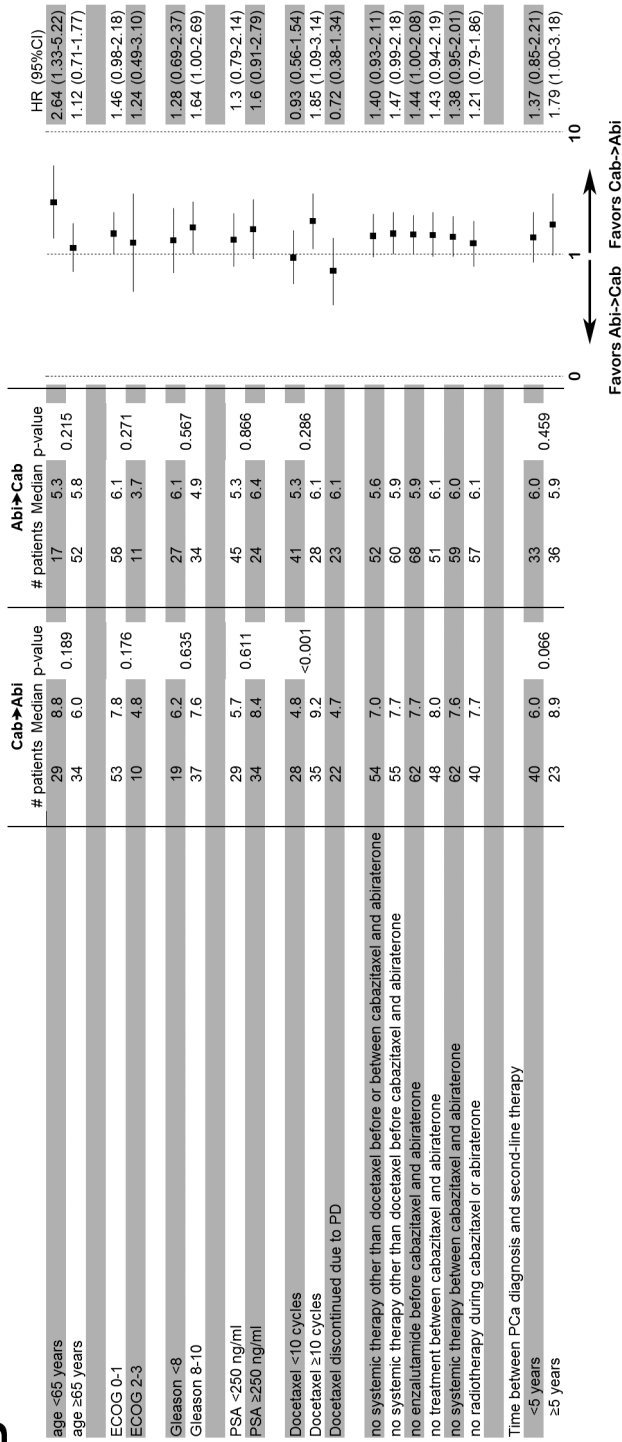


Figure 3. Analysis of overall survival (A) and progression-free survival (B) in subgroups of patients defined by baseline characteristics. Hazard ratios (HRs) higher than 1 favor the Cab→Abi group, lower than 1 favor the Abi→Cab group. #, number; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group performance status; PCa, prostate cancer; PD, progressive disease.

Table 4. Severe adverse events during cabazitaxel and abiraterone treatment

	Cab→Abi (n=63)			Abi→Cab (n=69)		
	During cab	During abi	Total	During abi	During cab	Total
Patients with ≥1 hospitalization	21 (33.3%)	28 (44.4%)	37 (58.7%)	19 (27.5%)	35 (50.7%)	42 (60.9%)
Patients with ≥2 hospitalizations	10 (15.9%)	11 (17.5%)	20 (31.7%)	5 (7.2%)	10 (14.5%)	21 (30.4%)
Duration of hospitalizations (days) [median (IQR)]	5 (2-8)	5 (2-9)	5 (2-8)	5 (2-7)	5 (4-9)	5 (3-9)
Adverse events causing hospitalization						
Anemia	1 (1.6%)	3 (4.8%)	4 (6.3%)	0 (0.0%)	3 (4.3%)	3 (4.3%)
Thrombocytopenia	1 (1.6%)	2 (3.2%)	3 (4.8%)	0 (0.0%)	1 (1.4%)	1 (1.4%)
Neutropenia	2 (3.2%)	0 (0.0%)	2 (3.2%)	0 (0.0%)	1 (1.4%)	1 (1.4%)
Febrile neutropenia	6 (9.5%)	3 (4.8%)	7 (11.1%)	0 (0.0%)	10 (14.5%)	10 (14.5%)
Pain	5 (7.9%)	6 (9.5%)	11 (17.5%)	11 (15.9%)	12 (17.4%)	22 (31.9%)
Urinary tract infection	5 (7.9%)	6 (9.5%)	10 (15.9%)	2 (2.9%)	5 (7.2%)	6 (8.7%)
Urinary obstruction	2 (3.2%)	3 (4.8%)	5 (7.9%)	0 (0.0%)	1 (1.4%)	1 (1.4%)
Hematuria	3 (4.8%)	1 (1.6%)	4 (6.3%)	1 (1.4%)	5 (7.2%)	6 (8.7%)
Renal function abnormalities	4 (6.3%)	2 (3.2%)	5 (7.9%)	2 (2.9%)	2 (2.9%)	4 (5.8%)
Pyrexia (not neutropenic)	3 (4.8%)	3 (4.8%)	6 (9.5%)	2 (2.9%)	5 (7.2%)	6 (8.7%)
Diarrhea	3 (4.8%)	1 (1.6%)	4 (6.3%)	0 (0.0%)	4 (5.8%)	4 (5.8%)
Nausea	3 (4.8%)	3 (4.8%)	4 (6.3%)	4 (5.8%)	4 (5.8%)	8 (11.6%)
Vomiting	4 (6.3%)	2 (3.2%)	6 (9.5%)	4 (5.8%)	4 (5.8%)	8 (11.6%)
Constipation	1 (1.6%)	2 (3.2%)	3 (4.8%)	1 (1.4%)	1 (1.4%)	2 (2.9%)
Dyspnoea	1 (1.6%)	2 (3.2%)	3 (4.8%)	3 (4.3%)	3 (4.3%)	5 (7.2%)
Pneumonia	2 (3.2%)	4 (6.3%)	6 (9.5%)	2 (2.9%)	2 (2.9%)	4 (5.8%)
Infection of the GI tract	0 (0.0%)	1 (1.6%)	1 (1.6%)	1 (1.4%)	2 (2.9%)	3 (4.3%)
Sepsis e.c.i.	1 (1.6%)	1 (1.6%)	2 (3.2%)	1 (1.4%)	3 (4.3%)	4 (5.8%)
Liver function test abnormalities	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (4.3%)	3 (4.3%)
Hypokalemia	0 (0.0%)	1 (1.6%)	1 (1.6%)	0 (0.0%)	2 (2.9%)	2 (2.9%)
Edema	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.4%)	1 (1.4%)
Allergic response	1 (1.6%)	0 (0.0%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Spinal cord compression	4 (6.3%)	4 (6.3%)	7 (11.1%)	1 (1.4%)	4 (5.8%)	5 (7.2%)
Pathological fracture	0 (0.0%)	2 (3.2%)	2 (3.2%)	0 (0.0%)	1 (1.4%)	1 (1.4%)
Hypotension	1 (1.6%)	0 (0.0%)	1 (1.6%)	0 (0.0%)	2 (2.9%)	2 (2.9%)
Vertigo	0 (0.0%)	1 (1.6%)	1 (1.6%)	3 (4.3%)	1 (1.4%)	4 (5.8%)
Other	4 (6.3%)	12 (19.0%)	16 (25.4%)	11 (15.9%)	12 (17.4%)	20 (29.0%)
Discontinuation due to toxicity						
# patients that discontinued therapy	63	60		69	61	
- toxicity sole reason to discontinue	9 (14.3%)	2 (3.3%)		1 (1.5%)	12 (19.7%)	
- toxicity important factor for discontinuation	11 (17.5%)	3 (5.0%)		3 (4.3%)	10 (16.4%)	
- adverse events resulting in discontinuation:						
- febrile neutropenia	1 (1.6%)	0 (0.0%)		0 (0.0%)	2 (3.3%)	
- other hematological toxicity	1 (1.6%)	1 (1.7%)		0 (0.0%)	2 (3.3%)	
- fatigue	8 (12.7%)	1 (1.7%)		2 (2.9%)	9 (14.8%)	
- nausea/vomiting/malaise	5 (7.9%)	1 (1.7%)		1 (1.4%)	6 (9.8%)	
- diarrhea	2 (3.2%)	0 (0.0%)		0 (0.0%)	3 (4.9%)	
- polyneuropathy	5 (7.9%)	0 (0.0%)		0 (0.0%)	4 (6.6%)	
- liver function abnormality	2 (3.2%)	1 (1.7%)		0 (0.0%)	2 (3.3%)	
- other non-hematological toxicity	6 (9.5%)	3 (5.0%)		1 (1.4%)	11 (18.0%)	

#, number; Abi, abiraterone; Cab, cabazitaxel; IQR, interquartile range

Table 5. Patient deaths within 30 days of last treatment

# patients reason for patient death	Cab→Abi (n=63) 10 (15.9%)	Abi→Cab (n=69) 5 (7.2%)
- disease progression	8 (80.0%)	2 (40.0%)
- febrile neutropenia	0 (0.0%)	2 (40.0%)
- Non-neutropenic infection	0 (0.0%)	1 (20.0%)
- ileus	1 (10.0%)	0 (0.0%)
- unknown	1 (10.0%)	0 (0.0%)

#, number; Abi, abiraterone; Cab, cabazitaxel

(mutations that occur, pathways that are circumvented). Some evidence exists that taxanes play a role in the transnuclear localization of the androgen receptor, suggesting that taxanes target tumor cells in the androgen receptor pathway too.²⁵ Such overlapping mechanisms of action may form a basis for cross-resistance.

In the CAST-study, we did not observe a statistically significant difference in median OS between Cab→Abi and Abi→Cab treated patients. However, a trend towards improved OS in Cab→Abi treated patients was noted, except for the subgroup of patients with rapid PD during docetaxel therapy. In a similar retrospective study, a better survival was reported in a cohort of patients treated with Cab→Abi compared to Abi→Cab treated patients.²⁶ However, analysis of all patients that received cabazitaxel and/or abiraterone after docetaxel, including patients who received only one therapy, indicated that patients receiving abiraterone as second-line therapy tended to have a survival advantage, particularly due to a subgroup of patients with an extensive response duration (>1 year) to abiraterone. Therefore, no clear benefit of one therapy sequence over the other was observed in terms of OS. Larger patient groups may be needed to observe significant differences between therapy sequence, but currently this is the largest retrospective study comparing such treatment sequence in mCRPC patients.

When evaluating the safety data of Cab→Abi and Abi→Cab treated patients, we observed more severe toxicity during cabazitaxel treatment than during abiraterone treatment, similar to toxicity reports in their respective phase III studies.^{4,9} Although results from cabazitaxel compassionate use programs reported fewer adverse events as compared to the TROPIC study,^{9,27,28} our results indicate that particularly febrile neutropenia can be serious and even life-threatening, contributing to patient death in 2-3% of cabazitaxel-treated patients. The potentially severe toxicity from cabazitaxel needs to be taken into account by physicians and patients when deciding treatment for their mCRPC.

This study has various limitations due to its retrospective nature. Patients' and treatments' characteristics were inevitably not completely equal between treatment groups and not all

potentially variable characteristics were collected in the clinical setting (such as baseline lactate dehydrogenase or circulating tumor cell counts). Treatment choices were not standardized but based on factors such as the physician's and patient's preference. E.g., patients who responded well to docetaxel, were most likely more interested in cabazitaxel as second-line therapy than patients who discontinued docetaxel early due to heavy toxicity. Differences in the use of tumor imaging methods were evident: while some patients received bone and/or CT scans multiple times during treatment, others had not received a CT scan at all. Serum PSA levels were consistently measured every 3 to 4 weeks, but this marker has its limitations too.²⁹ All such limitations occur with every retrospective study. Nevertheless, when several retrospective studies have a similar conclusion, this may be the best evidence we can get as a prospective randomized study may never be performed. Such a prospective study would need an extensive study duration and will be costly, whereas its clinical value will be limited due to the rapidly changing treatment landscape. E.g., patients had more advanced disease and had received extensive (experimental) chemotherapies in early cabazitaxel and abiraterone studies compared to mCRPC patients currently receiving these therapies. To a lesser extent the study population in our retrospective study is presumably not completely equal to the current mCRPC population, e.g. as some of these patients would receive enzalutamide these days. In the Netherlands, abiraterone is still only administered after docetaxel, while in other countries abiraterone is also administered before docetaxel.

In conclusion, this retrospective analysis of mCRPC patients treated with both cabazitaxel and abiraterone post-docetaxel found that progression on one agent did not preclude a response to the other agent, although antitumor efficacy of the agents, particularly cabazitaxel, was decreased when administered as higher-line therapy. Abi→Cab treated patients had more hospital admissions during cabazitaxel than Cab→Abi treated patients and discontinued cabazitaxel more frequently due to toxicity, while, as expected, abiraterone resulted generally in less severe toxicity as compared to cabazitaxel. These results should be used in the shared decision-making between patients and physicians, balancing the pros and cons of cabazitaxel or abiraterone after docetaxel. Further research is needed to provide more data regarding optimal treatment sequencing.

Acknowledgments

The authors wish to thank everyone who assisted in the collection of patient data for this study, such as the research nurses and secretaries who helped collecting all medical files. We would like to thank dr. M. Temizkan, dr. J.G. Haasjes, dr. C.G. Schaar, dr. W.E. Fiets, dr. J. Baars, dr. F.J.S. Netters and MSc. E.S. De Morrée for the provision of missing patient information. The authors further wish to thank prof. J.J.M. Van der Hoeven and dr. V.O. Dezentjé for their support and input.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013;63(1):11-30.
2. Malvezzi M, Arfe A, Bertuccio P, Levi F, La Vecchia C, Negri E. European cancer mortality predictions for the year 2011. *Ann Oncol* 2011;22(4):947-956.
3. Barrie SE, Potter GA, Goddard PM, Haynes BP, Dowsett M, Jarman M. Pharmacology of novel steroidal inhibitors of cytochrome P450(17) alpha (17 alpha-hydroxylase/C17-20 lyase). *J Steroid Biochem Mol Biol* 1994;50(5-6):267-273.
4. de Bono JS, Logothetis CJ, Molina A et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011;364(21):1995-2005.
5. Fizazi K, Scher HI, Molina A et al. Abiraterone acetate for treatment of metastatic castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301 randomised, double-blind, placebo-controlled phase 3 study. *Lancet Oncol* 2012;13(10):983-992.
6. Bissery MC. Preclinical evaluation of new taxoids. *Curr Pharm Des* 2001;7(13):1251-1257.
7. Mita AC, Denis LJ, Rowinsky EK et al. Phase I and pharmacokinetic study of XRP6258 (RPR 116258A), a novel taxane, administered as a 1-hour infusion every 3 weeks in patients with advanced solid tumors. *Clin Cancer Res* 2009;15(2):723-730.
8. Paller CJ, Antonarakis ES. Cabazitaxel: a novel second-line treatment for metastatic castration-resistant prostate cancer. *Drug Des Devel Ther* 2011;5:117-124.
9. de Bono JS, Oudard S, Ozguroglu M et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* 2010;376(9747):1147-1154.
10. Heidenreich A, Bastian PJ, Bellmunt J et al. Guidelines on prostate cancer, February 2012. European Association of Urology [internet]. Arnhem, the Netherlands. Available from http://www.uroweb.org/gls/pdf/08%20Prostate%20Cancer_LR%20March%2013th%202012.pdf. Accessed: October 17, 2013.
11. FDA Approves New Treatment for Advanced Prostate Cancer, June 17, 2010 [internet]. United States Food and Drug Administration, Silver Spring, MD, USA. Available from <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm216143.htm>. Accessed: January 24, 2014.
12. FDA approves Zytiga for late-stage prostate cancer; April 28 2011 [internet]. United States Food and Drug Administration, Silver Spring, MD, USA. Available from <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm253055.htm>. Accessed: January 24, 2014.
13. Mezynski J, Pezaro C, Bianchini D et al. Antitumour activity of docetaxel following treatment with the CYP17A1 inhibitor abiraterone: clinical evidence for cross-resistance? *Ann Oncol* 2012.
14. Loriot Y, Bianchini D, Ileana E et al. Antitumour activity of abiraterone acetate against metastatic castration-resistant prostate cancer progressing after docetaxel and enzalutamide (MDV3100). *Ann Oncol* 2013.
15. Noonan KL, North S, Bitting RL, Armstrong AJ, Ellard SL, Chi KN. Clinical activity of abiraterone acetate in patients with metastatic castration-resistant prostate cancer progressing after enzalutamide. *Ann Oncol* 2013;24(7):1802-1807.
16. Schrader AJ, Boegemann M, Ohlmann CH et al. Enzalutamide in Castration-resistant Prostate Cancer Patients Progressing After Docetaxel and Abiraterone. *Eur Urol* 2013.

17. Bianchini D, Lorente D, Rodriguez-Vida A et al. Antitumour activity of enzalutamide (MDV3100) in patients with metastatic castration-resistant prostate cancer (CRPC) pre-treated with docetaxel and abiraterone. *Eur J Cancer* 2013.
18. Scher HI, Halabi S, Tannock I et al. Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. *J Clin Oncol* 2008;26(7):1148-1159.
19. Chaumard-Billotey N, Aitichou M, Boyle H et al. Impact of new drugs in the median overall survival of patients with metastatic castration resistant prostate cancer (mCRPC). [abstract]. 17th European Cancer Congress; 2013 Sep 27 - Oct 1; Amsterdam, the Netherlands. Abstract 2910.
20. Badrising S, van der Noort V, van Oort IM et al. Clinical activity and tolerability of enzalutamide (MDV3100) in patients with metastatic, castration-resistant prostate cancer who progress after docetaxel and abiraterone treatment. *Cancer* 2014;120(7):968-75.
21. van Soest RJ, van Royen ME, de Morree ES et al. Cross-resistance between taxanes and new hormonal agents abiraterone and enzalutamide may affect drug sequence choices in metastatic castration-resistant prostate cancer. *Eur J Cancer* 2013;49(18):3821-3830.
22. Pezaro CJ, Le Moulec S, Albigen L et al. Response to cabazitaxel in CRPC patients previously treated with docetaxel and abiraterone acetate. [abstract]. *J Clin Oncol* 2013, 31 (suppl 6; abstr 155).
23. Pezaro CJ, Omlin AG, Altavilla A et al. Activity of Cabazitaxel in Castration-resistant Prostate Cancer Progressing After Docetaxel and Next-generation Endocrine Agents. *Eur Urol* 2013.
24. Sella A, Sella T, Peer A et al. Activity of cabazitaxel following docetaxel and abiraterone acetate in patients with castration-resistant prostate cancer. [abstract]. *J Clin Oncol* 2013, 31 (suppl 6; abstr 186).
25. Darshan MS, Loftus MS, Thadani-Mulero M et al. Taxane-induced blockade to nuclear accumulation of the androgen receptor predicts clinical responses in metastatic prostate cancer. *Cancer Res* 2011;71(18):6019-6029.
26. Sonpavde G, Bhor M, Hennessy D et al. Outcomes with different sequences of cabazitaxel and abiraterone acetate following docetaxel in metastatic castration-resistant prostate cancer (mCRPC). [abstract]. 17th European Cancer Congress; 2013 Sep 27 - Oct 1; Amsterdam, the Netherlands. Abstract 2905.
27. Heidenreich A, Scholz HJ, Rogenhofer S et al. Cabazitaxel plus prednisone for metastatic castration-resistant prostate cancer progressing after docetaxel: results from the German compassionate-use programme. *Eur Urol* 2013;63(6):977-982.
28. Wissing MD, van Oort IM, Gerritsen WR et al. Cabazitaxel in patients with metastatic castration-resistant prostate cancer: results of a compassionate use program in the Netherlands. *Clin Genitourin Cancer* 2013;11(3):238-250.
29. Collette L, Burzykowski T, Schroder FH. Prostate-specific antigen (PSA) alone is not an appropriate surrogate marker of long-term therapeutic benefit in prostate cancer trials. *Eur J Cancer* 2006;42(10):1344-1350.

Chapter 5

Antimitotic agents for prostate cancer treatment

Chapter 5a

Antimitotic agents for the treatment of patients with metastatic castrate-resistant prostate cancer

Michel D. Wissing, Paul J. van Diest, Elsken van der Wall, Hans Gelderblom

Expert Opin Investig Drugs. 2013 May;22(5):635-61

Abstract

Introduction: Metastatic castrate-resistant prostate cancer (mCRPC) is the second deadliest cancer in men. The group of taxanes, which target microtubules of mitotic cells, is currently the only chemotherapy which has proven to increase overall survival in mCRPC patients. Other mitotic inhibitors are being explored for their clinical potential in mCRPC treatment.

Areas covered: In this review, we summarize recent developments in the application of mitotic inhibitors for mCRPC from a clinical perspective. The four main groups of mitotic inhibitors currently being tested in clinical trials are microtubule inhibitors, polo-like kinase 1 inhibitors, aurora kinase inhibitors and kinesin-spindle protein inhibitors. Compounds of these groups of inhibitors that are in clinical development for mCRPC are discussed. For this extensive overview, relevant literature was searched in PubMed and clinicaltrials.gov, and in presentations at ASCO/AACR meetings.

Expert opinion: In general, mitotic inhibitors are clinically well tolerated but exert limited antitumor activity compared to preclinical study results. However, efficacy of mitotic inhibitors is improving, either by personalizing treatment, by introducing more active compounds, by decreasing resistance of cancer cells against mitotic inhibitors, or by using mitotic inhibitors in combination therapies.

Introduction

Metastatic castrate-resistant prostate cancer (mCRPC) is the second deadliest cancer in men, being only surpassed by lung cancer.¹ The five-year survival rate of mCRPC patients is only 30%.¹ Due to this high mortality, a major area of prostate cancer research focuses on the development of new therapies that are effective in mCRPC patients.

Despite clinical testing of a wide variety of compounds, only two groups of compounds have been implemented to treat patients with advanced prostate cancer: hormonal therapy, which targets androgen receptors and/or androgen synthesis, and antimitotic chemotherapeutic agents. This chapter focuses on the latter one.

The development of antimitotic drugs for prostate cancer treatment started in 1974, when patients with advanced prostate cancer were treated with estramustine.² In the 1980s, it became known that estramustine interferes with microtubules, thereby targeting cancer cells in mitosis. In 1981, another antimitotic chemotherapeutic agent was tested in mCRPC patients: vincristine.³ In this study, 15% of patients treated with vincristine monotherapy had an objective response (partial response (PR) or stable disease (SD)), the median response duration being 22 weeks. In the same study, an objective response was established in 26% of estramustine-treated patients for a median duration of 20 weeks. Addition of vincristine to estramustine monotherapy did not improve its efficacy.

Following this study, many other antimitotic agents have been tested in prostate cancer patients, with varying success. In this chapter, we will discuss the recent clinical development of different groups of mitotic inhibitors. We will focus on studies with mCRPC patients, but will also discuss results from clinical studies that tested antimitotic compounds in other advanced solid tumors with a potential impact on the treatment of mCRPC patients. Finally, we will briefly discuss results from recent studies, mostly preclinical, that are paving the way for future directions of clinical trials involving mitotic inhibitors in mCRPC patients.

Taxanes

Four Food and Drug Administration (FDA)-approved drugs have shown to increase progression-free survival (PFS) and overall survival (OS) in mCRPC patients: the hormonal therapies abiraterone acetate (Zytiga) and enzalutamide (Xtandi), and the taxanes docetaxel (Taxotere) and cabazitaxel (Jevtana). Generally, it is considered that taxanes act on cancer cells by targeting and stabilizing microtubules. Taxanes are thought to target cells in mitosis, as the incorrect formation of microtubules leads to mitotic arrest, and ultimately, apoptosis (Fig. 1). Although docetaxel was initially selected as an antitumor agent for its ability to cause mitotic arrest in cancer cells, it is now known that taxanes have other antitumor effects too, such as anti-angiogenic effects and p53 nuclear accumulation, the latter resulting in enhanced p53-induced apoptosis.^{4, 5} Furthermore, as reviewed by Thadani-Mulero et al.,⁶ taxanes inhibit nuclear accumulation of the androgen receptor, as its trafficking is

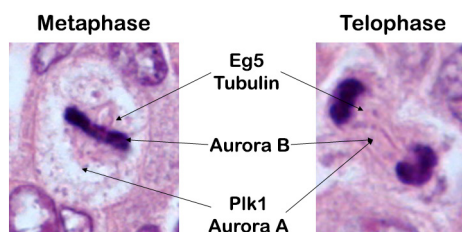


Figure 1. Overview of a cell in metaphase (left) and telophase (right), detailing the main localizations of mitotic targets during mitosis. Eg5 and tubulin are localized at microtubules throughout mitosis. Plk1 and aurora kinases mainly localize to the spindle poles during metaphase and to the midbody at the end of mitosis. While aurora B localizes to the midbody at the end of mitosis as well, during metaphase it mainly localizes to centromeres. It needs to be taken into account that these enzymes localize to a lesser extent to other mitotic structure too.

microtubule-dependent. These intracellular effects may also contribute to mCRPC inhibition.

Docetaxel

In the early 2000s, mCRPC patients were treated with mitoxantrone (Novantrone), an anthracenedione which exerts its antitumor effect by inhibition of type II topoisomerase and by DNA/RNA intercalation.⁷ This therapy was approved based on the results of a phase III study in which 160 mCRPC patients were treated with prednisone with or without mitoxantrone.⁸ The study concluded that mitoxantrone increased quality of life, but did not increase OS in the (relatively small) group of patients treated with mitoxantrone. The treatment scope for mCRPC patients radically changed with the introduction of docetaxel in 2004. Docetaxel binds to β -tubulin, stabilizing microtubules by polymerization.⁹ Therefore, mitosis is disrupted and cells arrest in G2/M phase, resulting in apoptosis, independent from p53. Docetaxel also counters the effect of expression of the anti-apoptotic oncogenes bcl-2 and bcl-xL.⁹ In 2004, the TAX-327 phase III study concluded that 335 mCRPC patients treated once every three weeks with docetaxel plus prednisone had an increased median OS compared to 337 mCRPC patients treated with mitoxantrone plus prednisone (18.9 vs. 16.5 months, respectively).¹⁰ Adverse events resulting in discontinuation of treatment were fatigue, musculoskeletal or nail changes, sensory neuropathy, and infections. Similarly, a phase III study from the Southwest Oncology Group found an increased OS of 1.9 months in mCRPC patients treated with docetaxel plus estramustine (median OS 17.5 months) compared to treatment with mitoxantrone plus prednisone (median OS 15.6 months).¹¹ These studies resulted in FDA approval of docetaxel as first-line therapy in mCRPC patients. About 50% of docetaxel-treated mCRPC patients had a PR. To improve its efficacy, studies combining docetaxel with other antitumor agents have been performed in mCRPC patients. A recent meta-analysis of twelve phase II studies indicated that docetaxel-based combination

therapy may be more effective in mCRPC patients, increasing survival with similar adverse events compared to docetaxel monotherapy.¹² However, treatment efficacy was not improved in any published phase III study in which high-dose calcitriol (vitamin D), bevacizumab (Avastin), risedronate, atrasentan (Xinlay), zibotentan, or GVAX immunotherapy was added to docetaxel treatment (Table 1).¹³⁻²⁰ Furthermore, combining docetaxel with aflibercept (Zaltrap) did not significantly increase median OS in mCRPC patients; the combination of lenalidomide (Revlimid) with docetaxel did not have a statistically significant treatment effect either.^{21, 22} Currently, phase III studies are being performed in which docetaxel is combined with dasatinib (Sprycel) or OGX-011 (Custirsen) in mCRPC patients (Table 1).²³⁻²⁶ Furthermore, multiple combination therapies have been tested with success in phase I/II clinical trials, such as the combination of docetaxel and temozolomide (Temodal) and the combination of docetaxel with zoledronic acid (Zometa).^{27, 28} Nevertheless, docetaxel plus prednisone remains standard first-line therapy.

Cabazitaxel

Recently, a second generation taxane, cabazitaxel (Jevtana), has received FDA approval as second-line therapy for mCRPC patients. Cabazitaxel is potentially superior to docetaxel, having low affinity with P-glycoprotein (P-gp), an ATP-dependent drug efflux pump.²⁹ For registration, a randomised phase III clinical trial was performed in 755 patients with mCRPC who had progression after docetaxel treatment (TROPIC).³⁰ In this study, patients received either 12 mg/m² mitoxantrone or 25 mg/m² cabazitaxel in combination with daily oral prednisone. Men treated with cabazitaxel had both increased OS and PFS, the median of the parameters being increased by 2.4 months (15.1 vs. 12.7 months) and 1.4 months (2.8 vs. 1.4 months), respectively. Time to tumor progression (TTP) and time to prostate-specific antigen (PSA) progression were increased after cabazitaxel treatment as well, and 39.2% of cabazitaxel-treated patients had a reduction in serum PSA concentration of ≥50%. Grade ≥3 neutropenia (82% vs. 58%), leukopenia (68% vs. 42%), anemia (11% vs. 5%) and diarrhea (6% vs. <1%) were increased in cabazitaxel-treated patients. Results of compassionate use programs have been published by Heck et al. and others.³¹⁻³³ These post-marketing studies report similar responses, but less adverse events in mCRPC patients. Other clinical studies, summarized in Table 2, are being conducted to further optimize cabazitaxel use in mCRPC patients.

Other taxanes

Besides aforementioned FDA-approved taxanes, other taxanes have been or are tested in prostate cancer as well, the most noteworthy being (nab-)paclitaxel and tesetaxel.

Paclitaxel (Taxol) is a taxane that has been approved by the FDA for its use in ovarian cancer, breast cancer, non-small-cell lung cancer (NSCLC), and AIDS-related Kaposi sarcoma. A phase II study concluded that paclitaxel only had minor antitumor activity in mCRPC patients, as

Table 1. Phase III studies testing combination therapy with docetaxel in mCRPC patients.

Docetaxel with	Main mode of action	DLT	Median OS	Compared to	Status	References
estramustine (Emcyt)	alkylating agent	GI disorders	17.5 months (vs. 15.6 months)	mitoxantrone	phase III published	11
high-dose calcitriol	vitamin D	hypercalcemia	17.8 months (vs. 20.2 months)	docetaxel	phase III published	13, 204
bevacizumab (Avastin)	angiogenesis inhibitor	hypertension, hemorrhages	22.6 months (vs. 21.5 months)	docetaxel	phase III published	14
risedronate	bisphosphonate	N/A	19.2 months (vs. 18.4 months)	docetaxel	phase III published	15
atrasentan (Xinlay)	endothelin A receptor antagonist	(febrile) neutropenia	18 months (phase III) / 17.6 months (phase II)	docetaxel	phase III published	16, 17
GVAX	immunotherapy	N/A	13 months in both arms (predicted survival)	docetaxel	phase III published	19
GVAX	immunotherapy	N/A	16 months in both arms (predicted survival)	docetaxel	phase III published	18
Zibotentan	endothelin A receptor antagonist	peripheral edema, headache, cardiac failure	24.5 months (vs. 22.5 months)	docetaxel	phase III published	20
Aflibercept (Zaltrap)	VEGF-inhibitor	neutropenia, dysphonia, hypertension	not significantly improved	docetaxel	phase III halted	21, 205, 206
lenalidomide	Antiangiogenesis, antineoplastic, immune modulation	N/A	N/A	docetaxel	phase III halted	22
dasatinib (Sprycel)	tyrosine kinase inhibitor	rash, lethargy	N/A	docetaxel	phase III ongoing	23, 25, 207
OGX-011 (Custirsen)	antisense clusterin inhibitor	N/A	23.8 months (vs. 16.9 months) (phase II)	docetaxel	phase III ongoing	24, 26

Patients in all treatment groups were treated with prednisone. mCRPC, metastatic castrate-resistant prostate cancer; OS, overall survival; VEGF, vascular endothelial growth factor.

Table 2. Ongoing clinical trials that aim to improve the efficacy of cabazitaxel in mCRPC patients.

Study name	Phase	Aim	Reference
PROSELICA	III	to compare cabazitaxel administered at 20 mg/m ² to standard cabazitaxel treatment	208
-	II	to evaluate the use of cabazitaxel administered weekly at 10 mg/m ²	209
ConCab	II	to compare cabazitaxel administered on day 1, 8, 15 and 22 at 10 mg/m ² in a 5-week cycle to standard cabazitaxel treatment	210
Prostlyl	II	to evaluate the use of cabazitaxel administered biweekly at 16 mg/m ²	211
-	II	to evaluate the reduction in diarrhea after addition of octreotide to standard cabazitaxel treatment	212
CABARESC	II	to evaluate the reduction in diarrhea after addition of budosonide to standard cabazitaxel treatment	213
FIRSTANA	III	to compare cabazitaxel to docetaxel as first-line therapy	214
AFFINITY	III	to compare standard cabazitaxel treatment with or without the addition of custirsen (OGX-011)	215
CATCH	I	to determine the recommended treatment dose of tasquinimod when added to standard cabazitaxel treatment	216
-	I	to evaluate the use of combination therapy with cabazitaxel and abiraterone acetate	217
-	I/II	to evaluate the use of combination therapy with cabazitaxel and bavituximab	218
-	I/II	to compare standard cabazitaxel treatment with or without the addition of carboplatin	219

a PR was established in only 4.3% of patients, while grade 4 toxicities such as leukopenia occurred in 61% of patients.³⁴ Since then, multiple phase II studies have been conducted in mCRPC patients combining paclitaxel with radiotherapy or other chemotherapeutic agents.³⁵⁻⁴⁷ Most phase II studies concluded that the results did not warrant a phase III study, as either toxicity was too high, or efficacy was insufficient.^{36, 39, 42-46} No phase III study has been initiated in mCRPC patients.

Recently, the FDA approved nanoparticle albumin-bound (nab)-paclitaxel (Abraxane) in combination with carboplatin for the initial treatment of advanced NSCLC patients. The *in vivo* antitumor activity of nab-paclitaxel was lower in prostate cancer models than in mice with lung cancer and for this reason less thoroughly explored.⁴⁸ However, nab-paclitaxel was evaluated in a phase II study as neoadjuvant therapy in high-risk prostate cancer patients before radical prostatectomy, with disappointing results.⁴⁹ In another phase II study, nab-paclitaxel was evaluated as a first-line therapy in 38 mCRPC patients.⁵⁰ SD for >8 weeks was established in 43% of evaluable patients, the drug being well tolerated, leading to the conclusion that nab-paclitaxel may be useful in patients who are not suitable for docetaxel-based therapy. No phase III studies have been initiated in mCRPC patients. Currently, a phase I study combining nab-paclitaxel with vandetanib, an inhibitor of tyrosine kinases, vascular endothelial growth factor receptor (VEGFR) and other kinases, is being conducted.⁵¹ Tesetaxel (DJ-927) is the first identified taxane which is administered orally.⁵² This taxane has a half maximal inhibitory concentration (IC₅₀) of 0.395 ng/ml in DU-145 prostate cancer cells, and exerts antitumor effects in P-gp-mediated multidrug resistant cell lines *in vitro* and *in vivo*.⁵² Phase I studies with tesetaxel have been conducted; no mCRPC patients were

included in these clinical trials. Patients with advanced solid tumor responded well to tesetaxel treatment (with or without capecitabine), 3.6% of patients having a PR or complete response, and 62.5% having SD.^{53, 54} The compound was well absorbed and had acceptable toxicities, the most frequent dose-limiting toxicity (DLT) being (febrile) neutropenia and gastrointestinal disorders. A phase II study with tesetaxel in patients with progressive mCRPC is ongoing.⁵⁵

Other microtubule inhibitors

Besides taxanes, other microtubule inhibitors have been developed for mCRPC patients. The development of most of these inhibitors, such as vincristine, has been discontinued, except for the epothilones.⁵⁶

Epothilones stabilize microtubules by binding to tubulin as well.⁵⁷ Despite their similar mechanism of action, the structure of epothilones is quite different from taxanes, and interestingly, epothilones have antitumor effects in taxane-resistant cancer cell lines.⁵⁷⁻⁵⁹ Furthermore, epothilones are effective in tumors that overexpress multidrug resistance proteins such as multidrug resistance protein 1 (MDR1) and multidrug resistance-associated protein 1 (MRP1).^{59, 60}

Ixabepilone (BMS-247550), an epothilone B analogue, is the only epothilone that has received FDA approval, namely for the treatment of advanced breast cancers. Its use is controversial, as OS was only extended in subset analyses of two phase III studies with patients with breast cancer.⁶¹⁻⁶⁵ Five phase I/II studies have been conducted with ixabepilone in mCRPC patients (Table 3).⁶⁶⁻⁷⁰ Because a phase II study comparing mitoxantrone to ixabepilone reported that the two compounds have similar efficacies and toxicities, it is unlikely that ixabepilone will have a future role in mCRPC treatment.⁶⁹ However, ixabepilone is currently being tested in a phase II study as neoadjuvant therapy in patients with high risk, clinically localized prostate cancer. Preliminary results indicate that ixabepilone effectively reduced the PSA levels in a majority (87.5%) of patients.⁷¹ However, as the clinical significance of changes in serum PSA levels after therapy is disputed, the prostate cancer working group (PCWG) recommends to focus on PSA progression instead of PSA response.⁷² Therefore, long-term follow up of these patients needs to be done to assess the clinical benefit for patients, especially as ixabepilone also had significant toxic effects, with only 31.3% of patients being able to finish treatment. Patupilone (EPO906), epothilone B, has been studied in mCRPC patients in two phase II clinical trials (Table 3).^{73, 74} In the phase II study by Hussain et al., patupilone hardly showed

Table 3 (right page). Clinical studies testing epothilones in metastatic castrate-resistant prostate cancer patients. Only studies have been included in which all patients had been diagnosed with mCRPC. # pts, number of patients; SD, stable disease; PR, partial response; PFS, progression-free survival; TTPP, time to PSA progression; TTP, time to progression; OS, overall survival; PSA, prostate-specific antigen; mCRPC, metastatic castrate-resistant prostate cancer; MTD, maximum tolerated dose; RP2D, recommended phase II dose; DLT, dose-limiting toxicity; AE, adverse event; IV, intravenous; Ref, reference.

Compound	Tumors	Dosing schedule	# pts	Most frequent DLT (phase I) or grade 3 or 4 AE (phase II)	Efficacy	Status	Ref.
Ixabepilone (BMS-247550)	mCRPC	3h IV (30-35 mg/m ²) on day 2 with oral estramustine (280 mg thrice daily for 5 days) in 3-week cycles	13	neutropenia, peripheral neuropathy	91.7% PSA decrease ≥50%, median TTPP 4.4 months. 14.3% CR, 42.9% PR, 14.3% SD	Pilot study published	⁶⁶
	mCRPC previously treated with docetaxel	½h IV mitoxantrone 8-12 mg/m ² (12 mg/m ² RP2D), 3h IV ixabepilone 20-35 mg/m ² (35 mg/m ² RP2D), pegfilgrastim 0/6 mg subcutaneously day 2, prednisone 5 mg twice daily in 3-week cycles	36	(febrile) neutropenia, diarrhea	30.6% PSA decrease ≥50%, 10.0% PR.	Phase I published	⁶⁷
	mCRPC	3h IV (35 mg/m ²) on day 2 with/without oral estramustine (280 mg thrice daily for 5 days) in 3-week cycles	92	(febrile) neutropenia, neuropathy, fatigue	68.9% PSA decrease ≥50% (vs. 47.7%), TTPP 5.2 months (vs. 4.4 months). 47.8% PR (vs. 32.0%), 34.8% SD (vs. 52.0%)	Phase II published	⁶⁸
docetaxel-refractory mCRPC	docetaxel-refractory mCRPC	3h IV (35 mg/m ²) or IV mitoxantrone (14 mg/m ²) with orally prednisone (5 mg) twice daily in 3-week cycles	82	(febrile) neutropenia, anemia	19.5% PSA decrease ≥50% (vs. 19.5%), 4.2% PR (vs. 9.5%)	Phase II published	⁶⁹
	mCRPC	1h IV (20 mg/m ²) day 1, 8 and 15 in 4-week cycles	109	neutropenia, neuropathy, fatigue	34.1% PSA decrease ≥50%, 9.9% PR. Median OS 12.8 months	Phase II published	⁷⁰
	high-risk, clinically localized prostate cancer	neoadjuvant IV weekly (20 mg/m ² /week) for 12 weeks before prostatectomy	16 (±30)	neuropathy, allergic response	87.5% PSA decrease (mean 46.8%)	Phase II ongoing	⁷¹
Patupilone	mCRPC previously treated with docetaxel	5 min. IV (2.5 mg/m ²) day 1, 8 and 15 in 4-week cycles	45	diarrhea, fatigue, neuropathy	13.3% PSA decrease ≥50%, 5.3% SD. Median OS 13.4 months	Phase II published	⁷³
	mCRPC previously treated with docetaxel	20 min. IV (8-10 mg/m ²) day 1 in 3-week cycles	83	diarrhea, fatigue, anorexia	46.8% PSA decrease ≥50%, 24.3% PR, 56.8% SD. Median TTPP & OS 6.1 & 11.3 months	Phase II published	⁷⁴
	chemonaive mCRPC patients	IV 10 mg/m ² or docetaxel 75 mg/m ² every 3 weeks, with twice daily 5 mg prednisone	67	diarrhea, nausea, vomiting, fatigue	55% confirmed ≥50% PSA decline	Phase II ongoing	²²⁰
KOS-862	mCRPC previously treated with docetaxel	IV (100 mg/m ²) day 1, 8 and 15 in 4-week cycles	38	fatigue, peripheral neuropathy, ataxia	5.3% PSA decrease ≥50%. Median TTP & OS 2.1 & 7.4 months	Phase II published	⁷⁵
Sagopilone (ZK-Epo)	mCRPC	3h IV (16 mg/m ²) day 1 with twice daily 5 mg prednisone in 3-week cycles	53	peripheral neuropathy, fatigue, pain in extremities	37.0% PSA decrease ≥50%, Median PFS 6.4 months	Phase II published	⁷⁶

any efficacy, as 15 out of 16 patients with measurable disease had continuous progressive disease (PD), and only 13% of all patients had a decrease in PSA of $\geq 50\%$. The second phase II study, performed by the Canadian Urologic Oncology Group, reported that 47% of patients had a PSA decline of $\geq 50\%$, and 24% of patients with measurable disease had a measurable PR. This improved response was most likely caused by the different patient population, as patients in the second study had more advanced prostate cancer. Intriguingly, patients in both studies did not have significant hematological toxicities, diarrhea and fatigue being the most frequent grade ≥ 3 adverse events.

KOS-862, epothilone D, was tested in 38 mCRPC patients who had progressed following docetaxel therapy.⁷⁵ This epothilone lacked antitumor activity, while toxicity was severe.

Finally, sagopilone (ZK-EPO), a fully synthetic epothilone, plus prednisone have recently been tested in chemo-naïve mCRPC patients in a phase II study.⁷⁶ Similar to patupilone, very few patients treated with this compound had hematological adverse events. Although 37.0% of patients had a PSA decrease of $\geq 50\%$, the measured efficacy did not warrant a comparison between sagopilone and docetaxel in a phase III study.

Kinesin spindle protein inhibitors

While taxanes were the first group of chemotherapeutic agents that successfully extended survival in mCRPC patients, a major disadvantage of these tubulin-targeting agents is that they target microtubules in healthy non-dividing cells too, where microtubules have both mechanical (structural) and transportational functions.⁷⁷ Due to this lack of specificity for cancer cells, administration of taxanes may result in serious side effects in patients, such as neurotoxicity.¹⁰ Therefore, mitotic inhibitors with improved specificity for tumor cells would provide benefit for patients. Kinesin spindle protein (KSP) inhibitors specifically target mitotic cells by inhibiting the mitotic kinesin Eg5, an enzyme of the kinesin-5 subfamily.^{78, 79} Eg5 is active during mitosis only, separating spindle poles during metaphase, as it crosslinks two antiparallel microtubules and moves to the plus-ends of both microtubules (Fig. 1).⁷⁹ Inhibition of Eg5 leads to mitotic arrest, ultimately resulting in apoptosis.⁸⁰ The exact mechanism by which apoptosis is induced is still disputed.⁸⁰⁻⁸⁴ Healthy cells that rapidly divide are inhibited by KSP inhibitors too. Therefore, the major side effects in patients treated with KSP inhibitors are of hematological origin, such as neutropenia and thrombocytopenia. Stimulating the bone marrow by addition of granulocyte colony-stimulating factor (G-CSF) can decrease the severity of some hematological adverse events.

Multiple KSP inhibitors have been identified, but only one KSP inhibitor has been tested specifically in prostate cancer patients, namely ispinesib.

Ispinesib (SB-715992, NSC-727990)

Ispinesib is a highly specific and potent inhibitor of the KSP ATPase.⁸⁵ This quinazolinone

Table 4. Clinical studies testing KSP inhibitors in patients with advanced solid tumors

Compound	Tumors	Dosing schedule	# pts	Most frequent DLT	Efficacy	Status	Refs.
Ispinesib (SB-715992)	Advanced solid tumors, majority (58.3%) mCRPC	Docetaxel (50–75 mg/m ² , MTD 60 mg/m ²) + ispinesib (6–12 mg/m ² , MTD 10 mg/m ²) IV day 1 of 3-week cycle	24	neutropenia	29.2% SD after 6 cycles	Phase I published	86
	Advanced solid tumors	IV day 1, 8 and 15 of 4-week cycle, 1–8 mg/m ² /week (RP2D 7 mg/m ² /week)	30	neutropenia	37.5% SD after 6 cycles	Phase I published	87
	Advanced solid tumors	IV day 1 of 3-week cycle, 1–21 mg/m ² (RP2D 18 mg/m ²)	42	neutropenia	9.5% SD after 4 cycles	Phase I completed	88
	Advanced solid tumors	IV day 1–3 of 3-week cycle, 1–8 mg/m ² /day (MTD 6 mg/m ² /day)	27	neutropenia / leukopenia	minor response in 3.7%, 7.4% SD after 4 cycles	Phase I completed	89
SB-743921	mCRPC progressed after docetaxel	IV day 1 of 3-week cycle, 18 mg/m ²	21	neutropenia	Median PFS 2.1 months, median OS 14 months	Phase II published	90
	Advanced solid tumors or relapsed/ refractory lymphoma	IV day 1 of 3-week cycle, 2–8 mg/m ² (MTD 4 mg/m ²)	44	neutropenia	2.3% PR (12 months), 13.6% SD after 4 cycles	Phase I published	92
MK-0731	Advanced solid tumors	IV day 1 of 3-week cycle, 6–48 mg/m ² (MTD 17 mg/m ²)	43	neutropenia	18.2% SD after 8 cycles	Phase I published	93
AZD-4877	Advanced solid tumors	IV day 1, 8 and 15 of 4-week cycle, 10–25 mg/dose	18	neutropenia / febrile	32.3% SD after 2 cycles	Phase I published	94
	Advanced solid tumors	IV day 1, 8 and 15 of 4-week cycle, 5–45 mg/dose (MTD 30 mg/dose)	43	neutropenia	17.1% SD after 3 cycles	Phase I published	95
ARRY-520	Advanced tumors	IV day 1, 4, 8 and 11 of 3-week cycle, 2–15 mg/dose (MTD 11 mg/dose)	29	neutropenia	12.5% SD after 12 weeks	Phase I published	221
	Advanced solid tumors	IV day 1 of 3-week cycle or IV day 1 and 2 of 2-week cycle, 2.5–3.3 mg/m ² (MTD 2.5 mg/m ² per cycle)	>34	neutropenia	8.8% SD after 13 weeks	Phase I completed	97

KSP, kinesin-spindle protein; mCRPC, metastatic castrate-resistant prostate cancer; SD, stable disease; PR, partial response; PFS, progression-free survival; OS, overall survival; MTD, maximum tolerated dose; RP2D, recommended phase II dose; DLT, dose-limiting toxicity; IV, intravenous; # pts, number of patients; Refs, references.

derivative has been successfully tested in phase I clinical trials in patients with advanced solid tumors (Table 4).⁸⁶⁻⁸⁹ In one phase I study combining docetaxel and ispinesib, the majority (58.3%) of patients had been diagnosed with mCRPC.⁸⁶ Of the 14 patients with mCRPC included in this trial, six patients had SD for ≥ 18 weeks, and one patient had a PSA decrease of $\geq 50\%$. Toxicity was acceptable. A phase II clinical trial was started by the Southwest Oncology Group in which ispinesib was administered to mCRPC patients who had progressed during/after docetaxel.⁹⁰ Patients were i.v. treated once every three weeks with 18 mg/m² ispinesib 1h. No response was seen in the first 21 patients included in this trial. Immunohistochemistry analysis on archival tumor tissue from 16 of the 21 patients indicated that 15 out of 16 patients did not have significant expression of KSP inside the tumor. Therefore, it was concluded that ispinesib is most likely not effective in primary prostate tumors due to the low mitotic index of these tumors, resulting in low KSP expression. However, a more recent study found that 50% of prostate tumor samples stained positive for KSP.⁹¹

Other KSP inhibitors

Four other potent and selective KSP inhibitors have been tested in patients with solid tumors in phase I studies: SB-743921, MK-0731, AZD-4877 and ARRY-520 (Table 4).⁹²⁻⁹⁷ Although none of these phase I studies focused on prostate cancer patients in particular, mCRPC patients were included in studies for MK-0731 and AZD-4877.

MK-0731, a compound that is thought to have an allosteric regulation of KSP, was administered to seven mCRPC patients.⁹³ Four mCRPC patients received MK-0731 at the maximum tolerated dose (MTD) of 17 mg/m². Although PSA reductions of $>50\%$ were observed, no mCRPC patient had SD for more than 5 months.

AZD-4877 has been tested in patients with advanced solid malignancies in three phase I studies (Table 4). In the study by Infante et al., two mCRPC patients were included.⁹⁵ Individual observations of these patients were not reported. In a Japanese phase I study with AZD-4877, one included patient had prostate cancer and one prostate/renal cancer. Both patients received 15 mg AZD4877 via a 1h i.v. infusion on days 1, 8 and 15 of a 28-day cycle.⁹⁴ The patient with prostate/renal cancer had SD for more than two cycles.

ARRY-520 is currently being tested in phase I/II studies in patients with multiple myeloma. The clinical development of ispinesib, SB-743921, MK-0731, or AZD-4877 have all been discontinued, as these KSP inhibitors had limited antitumor effects at the MTD in patients with advanced solid tumors and/or mCRPC in particular. New KSP inhibitors such as K858 and S-trityl-L-cysteine are in preclinical development and could potentially decrease adverse events and/or increase the efficacy of KSP inhibitors.^{91, 98, 99}

Aurora kinase inhibitors

Aurora kinases are a group of serine/threonine kinases characterized by an activation loop, a destruction box, and three conserved aurora boxes at the amino terminal domain.^{100, 101} The aurora family consists of three enzymes: aurora A, B and C. Aurora A localizes to the spindle poles during mitosis and to the midbody during cytokinesis (Fig. 1).¹⁰⁰ Its activity peaks during pro-metaphase, but it plays a role in regulation of the cell cycle from late S-phase to mitotic exit, including regulation of centrosome maturation, mitotic entry, centrosome separation, bipolar spindle assembly, chromosome alignment and cytokinesis.¹⁰¹⁻¹⁰⁵ Aurora B has a nuclear localization during mitosis, but during cytokinesis it localizes to the midbody as well (Fig. 1).¹⁰⁰ Aurora B is active during G2/M phase; its activity peaks from the end of metaphase until the end of cytokinesis.^{101, 106} It is necessary for mitotic entry, the correct regulation of spindle microtubule-kinetochore attachments, chromosome condensation, the mitotic spindle assembly checkpoint, chromosome segregation, and cytokinesis.¹⁰¹ Aurora C is hardly expressed in normal cells except germ line cells, and is involved in meiosis. Although it was thought that aurora C does not play a role in tumorigenesis, some recent preclinical studies indicate that aurora C may have oncogenic activity as well.^{107, 108} Aurora A and B are overexpressed in various cancers, including prostate cancer.¹⁰⁹ Overexpression of aurora A is associated with the transformation of prostate adenocarcinoma to treatment-related androgen-independent neuroendocrine prostate cancer.¹¹⁰⁻¹¹² Furthermore, preclinical studies have indicated that aurora A may aid in androgen-independent prostate cancer cell growth by phosphorylating and activating the androgen receptor.¹¹³ Inhibition of aurora kinases results in polyploidy and apoptosis.^{114, 115} Therefore, multiple aurora kinase inhibitors have been introduced in clinic. In the following sub-sections, aurora kinase inhibitors that have recently been studied in clinical trials with (amongst others) mCRPC patients will be discussed (Table 5).

Danuserib (PHA-739358)

Danuserib is a pan-aurora kinase inhibitor that binds to the ATP pocket. This pyrrolopyrazole targets other tyrosine kinases, most notably Abl, as well.^{116, 117} The IC_{50} s were 220 and 120 nM in DU-145 and PC3 prostate cancer cells, respectively, as determined by cell proliferation assays.¹¹⁷ In the *in vivo* transgenic adenocarcinoma of the mouse prostate model, danuserib inhibited tumor growth in 81.3% of mice.¹¹⁷ Results from two phase I studies with danuserib in patients with advanced solid tumors, amongst others mCRPC, have been published (Table 5).^{118, 119} None of the included mCRPC patients had a PR or prolonged (≥ 6 months) SD. In the phase II study conducted in mCRPC patients, 2 out of 81 evaluable patients (2.5%) had a PSA reduction of $\geq 50\%$ after three months.¹²⁰ Median PFS was 2.8 months; 13.6% (11/81) of patients had a PFS ≥ 6 months. The main grade ≥ 3 adverse event was neutropenia, the most frequent side effect of aurora kinase inhibitors in general. Due to the limited PSA response, danuserib was not further assessed in mCRPC patients.

Table 5. Clinical studies testing aurora kinase inhibitors in patients with advanced solid tumors.

Compound	Tumors	Dosing schedule	# pts	Most frequent DLT (phase I) / grade 3 or 4 AE (phase II)	Efficacy	Status	Refs.
Danusertib (PHA-739358)	Advanced/metastatic solid tumors	3h/6h IV day 1,8 and 15 of 4-week cycles, 45-400 mg/m ² (MTD 330 mg/m ² 6h IV)	50	(febrile) neutropenia	13.2% SD for ≥6 months.	Phase I published	118
	Advanced solid tumors	24h IV biweekly, 45-1000 mg/m ² , with or without G-CSF (MTD without G-CSF 500 mg/m ² , recommended dose 750 mg/m ² with G-CSF)	56	(febrile) neutropenia	2.4% PR, 9.5% SD for ≥6 months.	Phase I published	119
	mCRPC progressed after docetaxel	6h IV day 1, 8 and 15 of 4-week cycles 330 mg/m ² ; or 24h IV biweekly, 500 mg/m ²	81 (43 + 38)	(febrile) neutropenia	2.5% PSA response. 13.6% SD for ≥6 months. Median PFS 2.8 months	Phase II published	120
MLN8054	Advanced solid tumors	orally daily day 1-5:8-12 or 4x daily day 1-14 of 4-week cycles, 25-80 mg/day (MTD 4x daily 70 mg/day for 14 days)	43	somnolence, transaminitis	7.0% SD for ≥4 cycles	Phase I published	222
	Advanced solid tumors	orally (1x/4x) daily for 7/14/21 days followed by a 14-day treatment-free period, 5-80 mg/day (MTD4x daily 60 mg for 14 days)	61	reversible grade 3 benzodiazepine-like effects (somnolence, cognitive AEs, confusion, hallucination)	4.9% SD for >6 cycles	Phase I published	223
Alisertib (MLN8237)	Advanced solid tumors	orally 1x/2x daily for 7/14/21 days followed by a 14-day treatment-free period, (MTD 50 mg 2x daily for 7 days of 50 mg daily for 21 days)	59	neutropenia, stomatitis	10.2% SD for >6 months	Phase I published	224
	Advanced solid tumors	orally 1x/2x daily for 7/14/21 days followed by a 14-day treatment-free period, 5-150 mg (RP2D 50 mg 2x daily for 7 days)	87	neutropenia, thrombocytopenia, diarrhea	1.1% PR, 23.0% SD for ≥3 months	Phase I published	124
	Children with refractory/recurrent solid tumors	orally 1x/2x daily for 7 days of a 3-week cycle, 45-100 mg/m ² /day (MTD 80 mg/m ² /day 1x daily)	37	neutropenia, thrombocytopenia	3.0% PR, 18.2% SD for ≥6 cycles	Phase I published	225
	Platinum-resistant/-refractory epithelial ovarian, fallopian tube or primary peritoneal carcinoma	orally 2x daily (50 mg) for 7 days of a 3-week cycle	31	neutropenia, leukopenia, stomatitis, thrombocytopenia	19.4% SD for ≥3 months. Median PFS 1.9 months	Phase II published	226

Advanced solid tumors	orally 2x daily (50 mg) for 7 days of a 3-week cycle	100	neutropenia, leukopenia, fatigue, anemia, stomatitis, and thrombocytopenia	11.0% PR, 45.0% SD	Phase I/II ongoing	227
Platinum and taxane resistant/refractory advanced ovarian or breast cancer	orally 2x daily (10-50 mg) day 1-3, 8-10&15-18, with paclitaxel (60-80 mg/m ² day 1, 8&15) in 4-week cycles (MTD TBD)	28	diarrhea, febrile neutropenia, stomatitis	28.6% PR, 10.7% SD for ≥6 months	Phase I/II ongoing	228
Barasertib (AZD1152)	2h IV every 7/14 days, 100-650 mg (MTD 200/450 mg, respectively) 2h IV day 1&2 or 48h IV day 1	59	(febrile) neutropenia	25.4% SD after 6 weeks	Phase I published	129
Advanced solid tumors	2h IV day 1&2 or 48h IV day 1 every two weeks, 50-300 mg (MTD 110 mg/day and 150 mg, respectively)	35	(febrile) neutropenia	22.9% SD after 8 weeks	Phase I published	130
ENMD-2076	orally daily continuously, 60-200 mg/m ² (MTD 160 mg/m ²)	67	hypertension, neutropenia	3.4% PR, 19.0% SD for ≥5.7 months (25 weeks)	Phase I published	133
platinum-resistant recurrent epithelial ovarian cancer	orally daily continuously, 250-325 mg/day	64	hypertension, fatigue, increased liver enzymes	21.9% PFS ≥6 months, 7.8% PR (median duration 7 months), Median OS 12 months	Phase II published	134
tozasertib (MK-0457/VX-680)	24h IV every 3 weeks, 4-96 mg/m ² /h (MTD 64 mg/m ² /h). In 7 pts 100 mg orally 48h before MTD	27	neutropenia, herpes zoster	44.4% SD for ≥2 cycles	Phase I published	138
Advanced solid tumors	72h IV in 3-week cycles, 4.5-36mg/m ² /72h (MTD 27mg/m ² /72h)	40	febrile neutropenia	10.0% SD for ≥6 months	Phase I published	144
PF-03814735	orally daily day 1-5/1-10 of 3-week cycles, 5-100 mg (MTD 80 mg for 5 days/50 mg for 10 days)	57	(febrile) neutropenia	7.7% SD for ≥6 cycles	Phase I published	147
Advanced solid tumors	24h IV every three weeks, 13.5-300 mg (MTD 230 mg)	57	(febrile) neutropenia	33.3% SD	Phase I completed	145
Advanced solid tumors	24h IV every two weeks, 5-140 mg (MTD <140 mg)	52	neutropenia	29% SD	Phase I completed	146
AMG-900	orally daily day 1-4 of 2-week cycles, 1-30 mg (MTD 24mg)	19	(febrile) neutropenia, thrombocytopenia	N/A	Phase I ongoing	153

Studies that included prostate cancer patients are mentioned, as well as studies that included patients with other solid tumors of which (preliminary) results have been published since 2011. mCRP; metastatic castrate-resistant prostate cancer; SD, stable disease; PR, partial response; PFS, progression-free survival; PSA, prostate-specific antigen; N/A, not available; MTD, maximum tolerated dose; RP2D, recommended phase II dose; DLT, dose-limiting toxicity; AE, adverse event; IV, intravenous; # pts, number of patients; Refs, references.

Alisertib (MLN8237)

Alisertib is a second-generation aurora kinase A inhibitor. It is thought to be more potent than its predecessor, MLN8054, and has reduced benzodiazepine-like adverse events compared to MLN8054 (Table 5).¹²¹ It effectively inhibits a wide variety of tumor cell lines *in vitro*; in PC3 cells the IC_{50} was 54 nM.^{122, 123} *In vivo* administration of alisertib resulted in tumor growth inhibition, amongst others in CWR22 prostate cancer xenografts.¹²²

Multiple phase I/II studies have been conducted with alisertib in solid tumors. Only one study by Dees et al. included mCRPC patients; none of the four mCRPC patients had a PR.¹²⁴ In addition, one phase I study is currently ongoing to evaluate the safety and tolerability of MLN8237 in combination with docetaxel, which has a focus on mCRPC.¹²⁵

Barasertib (AZD1152)

Barasertib is the first identified highly selective aurora B inhibitor.¹²⁶ This 5-acetanilide-substituted 3-aminopyrazole effectively inhibited tumor growth of colorectal, leukemic and lung cancers *in vivo*.^{126, 127} A preclinical study in prostate cancer cells concluded that pretreatment of these cells with barasertib, administered at 60 nM for 48 h, increased DNA damage inflicted by radiotherapy, and impaired DNA repair mechanisms.¹²⁸

Two phase I studies with barasertib in patients with advanced solid tumors have been published.^{129, 130} In the study by Boss et al., two mCRPC patients were included and received weekly barasertib via a 2h i.v. infusion.¹²⁹ Of all patients in this treatment schedule, 36.8% of patients had SD after six weeks of treatment.

ENMD-2076

ENMD-2076, a tartrate salt, is a tyrosine kinase inhibitor with activity not only against aurora A (IC_{50} 14 nM) and B (IC_{50} 350 nM), but also against other enzymes, such as enzymes involved in angiogenesis (VEGFR, fibroblast growth factor receptor (FGFR), etc.) or the FMS-like tyrosine kinase-receptor 3 (Flt3) (IC_{50} 1.86 nM), a potential target for leukemic tumors.^{131, 132} The antiproliferative IC_{50} value for PC3 cells was established at 600 nM, for other tumor cell lines IC_{50} s were in the nanomolar range (25-700 nM) as well. Treating mice with doses ranging between 50 and 225 mg/kg resulted in effective tumor growth inhibition of a wide variety of tumors.¹³²

In clinical studies, the most frequent DLT/grade ≥ 3 treatment-emergent adverse event was hypertension instead of neutropenia.^{133, 134} No patient included in clinical trials had been diagnosed with mCRPC. Phase II studies with ENMD-2076 are ongoing in patients with metastatic sarcomas and advanced/metastatic triple-negative breast cancer.^{135, 136}

Tozasertib (MK-0457, VX-680)

Tozasertib is a pan-aurora kinase inhibitor, targeting the ATP-binding site of aurora kinases, and is most effective in inhibiting aurora A.¹³⁷ Its IC_{50} is in the lower nanomolar

range (<100nM) in a variety of cell lines, amongst others PC3 cells. As tozasertib has cross-reactivity with Flt-3, it has most extensively been studied in leukemia, but some phase I/II studies have been conducted with tozasertib in patients with solid tumors as well. In the published phase I study with tozasertib in patients with advanced solid tumors, two mCRPC patients were included, of which one had SD for six cycles.¹³⁸ This patient had been treated with a combination of tozasertib orally and i.v. at the MTD.

Other aurora kinase inhibitors

Various other aurora kinase inhibitors have been tested recently in patients with advanced solid tumors: (1) AT9283, an inhibitor of aurora A and B, as well as other serine/threonine kinases, such as Jak2, Jak3 and Abl; (2) PF-03814735, an orally bioavailable inhibitor of aurora A and B; (3) BI 811283, an aurora kinase B inhibitor; (4) MK-5108, an aurora kinase A inhibitor; and (5) SNS-314, a pan-aurora kinase inhibitor.¹³⁸⁻¹⁴³ SD was reported as the best response in phase I studies with these compounds in patients with advanced solid tumors.¹⁴⁴⁻¹⁴⁹ Due to the limited response, no further studies are conducted with these agents in adults with solid tumors.

New aurora kinase inhibitors are emerging from preclinical research, which potentially optimize the effect of aurora kinase inhibition, while minimizing adverse events. Examples of such inhibitors are HOI-07, AMG-900 and GSK1070916A, of which the latter two are currently studied in clinical trials.¹⁵⁰⁻¹⁵²

AMG-900 is a recently discovered, highly selective and potent orally bioavailable pan-aurora kinase inhibitor. It inhibits cell lines resistant against paclitaxel and the aurora kinase inhibitors AZD1152, MK-0457 and danusertib in low nanomolar ranges (<5 nM).¹⁵¹ Oral administration of AMG-900 at a concentration of 15 mg/kg (days 1 and 2 weekly) or 3 mg/kg/day *in vivo* inhibited tumor growth by 50-97% in nine different xenograft models, of which three were multidrug-resistant tumor models. A phase I study is ongoing in patients with advanced solid tumors, in which the MTD has been determined at 24 mg without G-CSF support when administered for four consecutive days every two weeks.¹⁵³

GSK1070916A is a selective ATP-competitive inhibitor of aurora B and C, with a high enzyme-inhibitor dissociation half-life of >8h for the aurora B-INCENP (inner centromere protein) enzyme.^{152, 154} In PC3, LNCaP and DU-145 prostate cancer cell lines, the IC₅₀ was ≤15 nM.¹⁵⁵ In *in vivo* studies, administration of GSK1070916A resulted in delayed tumor growth, and in tumor reductions in lung, colorectal and hematological cancer models.¹⁵⁵ A phase I study has been initiated in patients with advanced solid tumors.¹⁵⁶

Polo-like kinase 1 inhibitors

Polo-like kinase 1 (Plk1) is a serine/threonine kinase, which is a part of the family of five polo-like kinases.¹⁵⁷ The enzyme plays essential roles during mitosis: major functions include activation of Cdc2, bipolar spindle assembly, centrosome maturation, chromosome condensation and separation, regulation of the anaphase-promoting complex and initiation of cytokinesis (Fig. 1).^{158, 159} More recently, extra-mitotic roles of Plk1 have been discovered, such as downregulation of p53.^{160, 161} Plk1 is overexpressed in various tumors, amongst others in prostate cancer.^{158, 162} Its expression is increased in androgen-insensitive prostate cancer cells, suggesting that it plays a role in androgen-independent growth, similar to aurora kinases.¹⁶³ As Plk1 is overexpressed and contains two highly specific polo-box domains (PBDs) in the C terminus, agents that bind to these PBDs can highly selectively target cancer cells.¹⁵⁸ Multiple Plk1 inhibitors have been developed, of which volasertib, TAK-960, TKM-080301, NMS-1286937 and rigosertib are currently being studied in patients with solid tumors. Similar to aurora kinase inhibitors, neutropenia is the most frequent adverse event in patients treated with Plk1 inhibitors.

HMN-214

HMN-214, an oral prodrug of HMN-176, was the first Plk1 inhibitor tested in clinic.¹⁶⁴ HMN-176 is a stilbazole derivative cytotoxic to PC3 and DU-145 cells as well as cisplatin-, adriamycin-, vincristine-, etoposide- and taxol-resistant cancer cell lines.¹⁶⁴ HMN-214 had equal or superior antitumor activity in mice with established PC3 tumors compared to cisplatin, adriamycin, vincristine and tegafur-uracil (UFT).¹⁶⁴ In a phase I study, HMN-214 had limited antitumor effects.¹⁶⁵ No patient had been diagnosed with prostate cancer. From another phase I study, final results have not been reported.¹⁶⁶ No phase II trial has been initiated.

Volasertib (BI 6727)

BI 2536 and its successor volasertib are dihydropteridine derivatives, inhibiting Plk1 by competitively binding to its ATP-binding pocket.^{167, 168} The compounds have IC_{50} s in the low nanomolar range in amongst others prostate cancer cells, and exert *in vivo* antitumor activity as well.¹⁶⁷⁻¹⁶⁹ A summary of clinical trials with BI 2536 and volasertib in patients with solid tumors can be found in Table 6.

In one phase II and two phase I studies mCRPC patients were treated with BI 2536. None of the two mCRPC patients included in the phase I study by Mross et al. had SD for >3 months.¹⁷⁰ In the other phase I study, one of three mCRPC patients had SD for >3 months.¹⁷¹ In the phase II study, twenty mCRPC patients were treated with 200 mg BI 2536 via a 1h i.v. infusion.¹⁷² One-third of evaluable patients had SD after four courses. Grade 3 or 4 neutropenia was observed in 20% (4 out of 20) of patients. Due to the limited antitumor activity, it was decided not to assess BI 2536 any further as a single agent. Although BI

Table 6. Clinical studies testing polo-like kinase 1 (PLK1-)inhibitors in patients with advanced solid tumors.

Compound	Tumors	Dosing schedule	# pts	Most frequent DLT (phase I) / grade 3 or 4 AE (phase II)	Efficacy	Status	Refs
HMN-214	Advanced solid tumors	orally day 1-21 of 4-week cycles, 3-9.9 mg/m ² /day (MTD 8 mg/m ² /day)	33	myalgia/bone pain syndrome, hyperglycemia	24.1% SD, 3.4% SD >6 months	Phase I published	165
	Advanced tumors	orally day 1-5 of 4-week cycles, 6-48 mg/m ² (MTD 18 mg/m ²)	32	neutropenia, neutropenic sepsis, myalgia, electrolyte disturbances, neuropathy	3.1% minor response, ≥9.4% SD for >3 months	Phase I completed	166
BI 2536	Advanced solid tumors	1h IV day 1 of 3-week cycles, 25-250 mg (MTD 200 mg)	40	(reversible) neutropenia	3.2% PR, 22.6% SD for >3 months	Phase I published	170
	Advanced solid tumors	1h IV day 1 and 8 (MTD 200 mg/course) or 24h IV day 1 (MTD >225 mg) of 3-week cycles, 25-225 mg	70	neutropenia	11.4% SD for >3 months	Phase I published	171
	Advanced solid tumors-	1h IV day 1-3 of 3-week cycles, 150-210 mg/course (MTD 180 mg/course)	21	neutropenia, anemia, thrombocytopenia, fatigue, hypertension, elevated liver enzymes	28.6% SD for >3 months	Phase I published	229
	recurrent/advanced/metastatic NSCLC progressed after platinum based therapy	1h IV day 1 of 3-week cycles, 100-325 mg (MTD 300 mg), in combination with pemetrexed (500 mg/m ²)	41	neutropenia, pruritis, rash, elevated liver enzymes	5.1% PR, 53.8% SD after 2 cycles	Phase I published	230
	mCRPC	1h IV day 1 of 3-week cycles, 200-250 mg	20	neutropenia	25.0% SD after 4 cycles	Phase II completed	172
	advanced HNC, breast cancer, ovarian cancer, soft tissue sarcoma or melanoma	1h IV day 1 of 3-week cycles, 200-250 mg	71	(febrile) neutropenia, thrombocytopenia, anemia, pain	42.4% SD according to RECIST. Median PFS & OS 1.4 & 9.5 months	Phase II published	231
	stage IIIB/IV NSCLC progressed after chemotherapy	1h IV day 1 (200 mg) or day 1-3 (150/180 mg/course) of 3-week cycles	95	neutropenia, anemia, dyspnea	4.2% PR. Median PFS 1.6-2.5 months, median OS 5.9-8.0 months	Phase II published	232
	unresectable pancreatic adenocarcinoma	1h IV day 1 (200 mg) or day 1-3 (180 mg/course) of 3-week cycles	86	neutropenia, leukopenia, thrombocytopenia	2.3% PR, 24.4% SD after 4 cycles. 27.8% PFS >3 months, median PFS & OS 1.5 & 4.9 months	Phase II published	233

volasertib (BI 6727)	Advanced solid tumors	1h IV day 1 of 3-week cycles, 12-450 mg (MTD 400 mg, RP2D 300 mg)	65	(Febrile) neutropenia, thrombocytopenia	4.6% PR, 26.2% SD after 3 months	Phase I published	173
	Advanced solid tumors	2h IV day 1 of 3-week cycles in combination with cisplatin (100 mg/m ²) / carboplatin (AUC 6) (MTD 300 mg)	61	neutropenia, thrombocytopenia, fatigue TBD	8.2% PR, 18.0% PR/SD after 6 cycles	Phase I ongoing	174
	Advanced solid tumors	IV day 1 of 3-week cycles	±15	TBD	TBD	Phase I ongoing	179
	Advanced solid tumors	IV day 1 of 3-week cycles in combination with BIBW 2992 orally daily	±60	TBD	TBD	Phase I ongoing	178
	Advanced solid tumors	IV day 1 of 3-week cycles in combination with BIBF 1120 orally daily (200 mg)	±30	TBD	TBD	Phase I ongoing	176
	Advanced solid tumors	IV in 3-week cycles	±7	TBD	TBD	Phase I completed	234
	Advanced solid tumors	IV in 3-week cycles	±59	TBD	TBD	Phase I completed	235
	Locally advanced or metastatic urothelial cancers	2h IV day 1 of 3-week cycles, 300-350 mg	50	neutropenia, thrombocytopenia, anemia, hyponatremia	19.4% PR, 22.6% SD after 4 cycles	Phase II ongoing	175
	Recurrent platinum resistant/refractory ovarian cancer	IV day 1 of 3-week cycles	±110	TBD	TBD	Phase II ongoing	177
	advanced/metastatic NSCLC after platinum based therapy	IV day 1 of 3-week cycles in combination with pemetrexed (500 mg/m ²)	±143	TBD	TBD	Phase II completed	236
GSK-461364	Advanced solid tumors	4h IV Day 1, 8 and 15 (MTD 225 mg), or day 1, 2, 8, 9, 15 and 16 (MTD 75 mg) of 4-week cycles, 25-300 mg orally, dose escalation study	40	neutropenia, thrombocytopenia, pulmonary embolism	15% SD after 4 cycles	Phase I published	182
NMS- 1286937	Advanced solid tumors		±21	TBD	TBD	Phase I completed	183
TKM-080301	Advanced solid tumors	IV, dose escalation study	±42	TBD	TBD	Phase I ongoing	184
TAK-960	Advanced solid tumors	orally, dose escalation study	±84	TBD	TBD	Phase I ongoing	185

rigosertib (ON 01910. Na)	Advanced solid tumors	2h IV biweekly for 3 weeks of 4-week cycles, 80-3120 mg (MTD 3120 mg)	20	Abdominal pain/death	6.3% PR, 6.3% SD	Phase I published	237
	Advanced solid tumors	24h IV weekly (250-1350 mg/m ²) in combination with oxaliplatin 2h IV biweekly (85 mg/m ²)	13	none	6.7% PR, 13.3% SD for ≥12 weeks	Phase I published	238
	Advanced solid tumors	2h IV biweekly (600-1800 mg/m ² , MTD 1800 mg/m ²) in combination with gemcitabine weekly (750-1000 mg/ m ² , MTD 1000 mg/m ²) for 3 weeks of 4-week cycles	40	Death	5.3% PR, 57.9% SD (of the pancreatic cancer patients)	Phase I published	193
	Advanced solid tumors	orally twice daily, 70-700 mg (MTD 560 mg)	25	dysuria	4.0% PR, 24.0% SD for ≥24 weeks	Phase I completed	239
	Advanced tumors	24h IV weekly (250-2750 mg/m ²)	23	not reported	No PR, % SD not reported	Phase I completed	240
	Advanced tumors	72h IV in 2-week cycles, 50-250 mg/ m ² /day	5 (±29)	TBD	40.0% SD for >6 weeks	Phase I completed	241
	Advanced solid tumors	orally twice/thrice daily in 3-week cycles, 140-700 mg/day	2 to 72	TBD	TBD	Phase I ongoing	242
	ovarian cancer resistant to platinum based therapy	2h IV biweekly for 3 weeks of 4-week cycles, 3200 mg	N/A	TBD	TBD	Phase II completed	243
	previously untreated metastatic pancreatic cancer	2h IV biweekly (1800 mg/m ²) in combination with gemcitabine weekly (1000 mg/m ²) for 3 weeks of 4-week cycles, versus gemcitabine only	650	TBD	TBD	Phase II/III ongoing	244

All studies are mentioned, except for studies that were over a year old, had not been published in journals and did not include mCRPC patients. mCRPC, metastatic castrate-resistant prostate cancer; SD, stable disease; PR, partial response; PFS, progression-free survival; OS, overall survival; MTD, maximum tolerated dose; RP2D, recommended phase II dose; DLT, dose-limiting toxicity; AE, adverse event; IV, intravenous; # pts, number of patients; Refs, references; HNC, head and neck cancer; NSCLC, non-small cell lung cancer; TBD, to be determined.

2536 was well tolerated by patients, combination therapy was not pursued due to the introduction of volasertib.

Volasertib is thought to have superior efficacy and/or to be less toxic compared to BI 2536.¹⁶⁹ One phase I study in which patients with advanced solid tumors were treated with BI 6727 has been published.¹⁷³ In this study, four patients had been diagnosed with mCRPC. Three of 65 patients had a PR (4.6%), none of whom were mCRPC patients. SD for more than 3 months was established in 26.2% of patients. As volasertib was well tolerated, (pre) clinical research with this small molecule as a monotherapy and in combination with other therapies is ongoing.^{169, 174-179}

GSK-461364

GSK-461364, a thiophene amide, is an ATP-competitive Plk1 inhibitor, which has over a 100-fold greater potency for Plk1 than 50 other kinases, including Plk2 and Plk3.¹⁸⁰ In a panel of >120 cancer cell lines, GSK-461364 had IC₅₀s below 100 nM in 91% of cell lines, the inhibitor thus causing a prometaphase arrest.¹⁸¹ In a phase I study performed in patients with advanced solid malignancies (Table 6), one mCRPC patient was included.¹⁸² This patient did not respond to GSK-461364 treatment. No phase II studies have been initiated with this compound.

Other Plk1 inhibitors

Apart from aforementioned Plk1 inhibitors, three Plk1 inhibitors with different delivery methods have been developed: TAK-960, NMS-1286937 and TKM-080301. These compounds had promising results in preclinical studies and are currently being studied in patients with advanced solid tumors in phase I clinical trials (Table 6).¹⁸³⁻¹⁸⁵

NMS-1286937 (NMS-P937) is an orally bioavailable 4,5-dihydro-1H-pyrazolo[4,3-h]quinazoline derivative.¹⁸⁶ It targets highly selectively Plk1 (IC₅₀ 2 nM), and inhibits cell proliferation in a wide variety of cancer cell lines, both solid and hematological, at low nanomolar concentrations.¹⁸⁷ NMS-1286937 effectively inhibited tumor growth in established human colorectal and leukemic xenografts with minimal and reversible weight loss.^{186, 187}

TAK-960 is another orally bioavailable Plk1 inhibitor. It exhibited antitumor activity *in vitro* at low nanomolar concentrations, arresting tumor cells, including cells that express MDR1, in G2/M phase.¹⁸⁸ TAK-960 inhibited tumor growth amongst others in mice with established PC3 human prostate tumors.

TKM-080301 is a lipid nanoparticle (LNP) containing small interfering RNA against human Plk1 mRNA.^{189, 190} Due to its selective distribution, myelosuppression and other common side effects of Plk1 inhibitors may be reduced. Indeed, *in vivo* studies reported that tumor growth was inhibited and toxicity largely restricted to the liver and spleen.¹⁹⁰

Rigosertib (ON 01910.Na)

Rigosertib (Estybon) is a benzyl styryl sulfone analog, which inhibits both Plk1, by competitively binding to the substrate binding site, and the PI3K pathway.^{191, 192} IC₅₀ values of rigosertib in DU-145 and PC3 cells were 200 and 150 nM, respectively.¹⁹¹ The *in vivo* antitumor activity of rigosertib was increased when combined with other chemotherapeutic agents (oxaliplatin, doxorubicin or gemcitabine).¹⁹¹ Therefore, clinical studies have not been initiated in mCRPC patients, but mostly focus on tumors in which these chemotherapies are used, such as pancreatic cancer (Table 6).¹⁹³

Conclusions

Docetaxel and cabazitaxel are mitotic inhibitors and are currently used in clinic as first- and second-line therapies for mCRPC patients. Due to this success, and with new mitotic targets for cancer therapy being discovered, multiple mitotic inhibitors are being studied for their use in mCRPC treatment. These inhibitors can be divided into four groups: microtubule inhibitors, KSP inhibitors, Plk1 inhibitors and aurora kinase inhibitors. Of these last three groups, Plk1 inhibitors and aurora kinase inhibitors are currently most extensively studied in mCRPC patients. Currently, these inhibitors seem to have most clinical potential in hematological tumors. However, combination therapies and new compounds with increased potency and selectivity are being studied in patients with prostate cancer and other solid tumors as well.

Expert opinion

Recently, the treatment scope for mCRPC patients has expanded. Besides docetaxel, other life-extending therapies, such as the second-generation taxane cabazitaxel, have entered the market. Therefore, the need for a good biomarker to assess the response to therapy has become stringent. Although changes in serum PSA levels are now mostly used as an indicator for the response to mCRPC treatment, its clinical significance remains controversial. Increasing serum PSA levels during the first 12 weeks of treatment are considered unreliable; therefore, physicians are encouraged to continue treatment beyond 12 weeks despite rising PSA levels, unless PD is determined via other objective assessments.⁷² This recommendation would cause overtreatment and a treatment delay in patients who do have continuous PD. Overtreatment and treatment delays could be further reduced by introducing markers that predict the response to therapy. Such markers form the basis for targeted therapy in other cancers: e.g., breast cancer patients whose tumors express estrogen receptor or human epidermal growth factor receptor 2 will be treated with tamoxifen or trastuzumab, respectively.^{194, 195} Although mitotic inhibitors are targeted therapies against a single or group of enzyme(s), such as Plk1, it is intriguing that none of aforementioned clinical trials

determined *a priori* the expression of the targeted enzyme(s) in the tumor, while tissue is available from all mCRPC patients as biopsies have been taken. Not all tumors overexpress these enzymes: e.g., only about 50% of prostate tumors overexpress KSP or Plk1.^{91, 162} Future clinical studies need to determine whether the *a priori* expression of the targeted mitotic enzyme inside mCRPC cells is of influence of the antitumor efficacy of the mitotic inhibitor in humans. KSP inhibitors or Plk1 inhibitors may have no effect in 50% of mCRPC patients whose tumors do not overexpress the targeted enzyme. Aurora kinase inhibitors may exert most antitumor effect in aggressive tumors with a treatment-related neuroendocrine phenotype, as aurora A is more regularly overexpressed in these tumors.¹¹¹ If this is confirmed, treatment of mCRPC patients may be personalized based on this expression profile. Alternatively, if these inhibitors do exert an antitumor effect in tumors that do not express the targeted enzyme, off-target antitumor effects of these inhibitors need to be assessed.

With an increasing number of therapies to which only part of mCRPC patients respond, it is expected that personalized medicine, in which treatment is based on the expression profiles of tumors, will play an increasingly important role in mCRPC treatment.

The antitumor effect of mitotic inhibitors could be further improved by improving the specificity of the compound for cancer cells. This can be achieved by optimizing the compound itself and/or by improving its method of delivery, e.g. by delivery in LNPs which specifically bind to tumor cells. Furthermore, compounds could potentially be used in combination therapy to accomplish synergistic antitumor effects.

Preclinical studies suggest that mitotic inhibitors may be combined with docetaxel.^{112, 196, 197}

In a recently performed phase I study in patients with advanced solid tumors, combination of MK-5108 with docetaxel resulted in a PR in 2 out of 17 patients (no patient with MK-5108 mono-therapy had a PR), but also in more serious adverse events.¹⁴⁸ Other clinical trials combining aurora kinase inhibitors with docetaxel are being performed, such as a phase I study with MLN8237.¹²⁵ An important limitation of these clinical studies is that aurora kinase inhibitors are administered after docetaxel treatment, while a preclinical report suggests that this combination therapy is most effective when aurora kinase inhibitors are administered before docetaxel.¹⁴³

Recently, Feng et al. discovered that miR-100 (microRNA) resensitized docetaxel-resistant lung adenocarcinoma SPC-A1 cells to docetaxel.¹⁹⁸ Plk1 was identified as a direct target of miR-100, and knock-down of Plk1 or (over)expression of miR-100 resulted in increased sensitivity to docetaxel *in vitro* and *in vivo*, respectively.

These data indicate that the combination of mitotic inhibitors with docetaxel could potentially result in an enhanced antitumor effect in mCRPC patients.

Other combinations involving mitotic inhibitors should be tested as well. Multiple clinical trials are currently being performed in which mitotic inhibitors are combined with other compounds, such as the combination of volasertib with the triple angiokinase inhibitor

BIBF1120 (an inhibitor of the VEGF receptor, and PDGFR (platelet-derived growth factor receptor) and FGFR kinase activity in cancer cells).¹⁷⁶ With the increasing interest in combination therapies for cancer treatment, it is important to select combination therapies based on a rationale. Recently, a preclinical study from our group indicated that the combination of mitotic inhibitors with histone deacetylase (HDAC-)inhibitors may have clinical benefit in prostate cancer patients.^{169, 199, 200} This combination therapy targets the mitotic pathway from both a genetic and epigenetic perspective, while other cancer-related pathways are targeted as well. More (pre)clinical studies need to be performed to create and test such rational combination therapies.

Resistance of tumor cells to taxanes and other chemotherapies limits their use. Only 50% of mCRPC patients respond to docetaxel therapy, and eventually all tumors become docetaxel refractory.¹⁰ The response rate is similar or even lower after treatment with other mitotic inhibitors. A wide variety of intracellular alterations have occurred in docetaxel-resistant mCRPC cells, such as increased expression of the Notch and Hedgehog signaling pathways and increased expression of TGF- β , MDR1 and MRP1.^{201, 202} As summarized by Madan et al., these alterations result in increased drug efflux by transmembranic pumps such as P-gp, changes in the site to which taxanes bind to microtubules, increased dynamic activity of microtubules, and changed expression of subunits of microtubules such as β III.²⁰³ Research is ongoing in which intracellular changes leading to chemotherapy resistance are being utilized as markers for therapy response. Furthermore, other studies are assessing how these intracellular changes can be prevented, reverted or targeted, which would have significant clinical consequences.

As aforementioned therapy improvements – overcoming therapy resistance, and introducing personalized medicine, compounds with improved specificity, improved methods of delivery and combination therapy – are currently extensively studied, it is expected that in the next decade more mitotic inhibitors will find their way into the clinic for (prostate) cancer treatment.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012;62(1):10-29.
2. Muntzing J, Shukla SK, Chu TM, Mittelman A, Murphy GP. Pharmacoclinical study of oral estramustine phosphate (Estracyt) in advanced carcinoma of the prostate. *Invest Urol* 1974;12(1):65-68.
3. Soloway MS, de Kernion JB, Gibbons RP et al. Comparison of estramustine phosphate and vincristine alone or in combination for patients with advanced, hormone refractory, previously irradiated carcinoma of the prostate. *J Urol* 1981;125(5):664-667.
4. Belotti D, Vergani V, Drudis T et al. The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin Cancer Res* 1996;2(11):1843-1849.
5. Giannakakou P, Nakano M, Nicolaou KC et al. Enhanced microtubule-dependent trafficking and p53 nuclear accumulation by suppression of microtubule dynamics. *Proc Natl Acad Sci U S A* 2002;99(16):10855-10860.
6. Thadani-Mulero M, Nanus DM, Giannakakou P. Androgen receptor on the move: boarding the microtubule expressway to the nucleus. *Cancer Res* 2012;72(18):4611-4615.
7. Poirier TI. Mitoxantrone. *Drug Intell Clin Pharm* 1986;20(2):97-105.
8. Tannock IF, Osoba D, Stockler MR et al. Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points. *J Clin Oncol* 1996;14(6):1756-1764.
9. Khan MA, Carducci MA, Partin AW. The evolving role of docetaxel in the management of androgen independent prostate cancer. *J Urol* 2003;170(5):1709-1716.
10. Tannock IF, de Wit R, Berry WR et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351(15):1502-1512.
11. Petrylak DP, Tangen CM, Hussain MH et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* 2004;351(15):1513-1520.
12. Serpa NA, Tobias-Machado M, Kaliks R, Wroclawski ML, Pompeo AC, Del GA. Ten years of docetaxel-based therapies in prostate adenocarcinoma: a systematic review and meta-analysis of 2244 patients in 12 randomized clinical trials. *Clin Genitourin Cancer* 2011;9(2):115-123.
13. Scher HI, Jia X, Chi K et al. Randomized, open-label phase III trial of docetaxel plus high-dose calcitriol versus docetaxel plus prednisone for patients with castration-resistant prostate cancer. *J Clin Oncol* 2011;29(16):2191-2198.
14. Kelly WK, Halabi S, Carducci M et al. Randomized, double-blind, placebo-controlled phase III trial comparing docetaxel and prednisone with or without bevacizumab in men with metastatic castration-resistant prostate cancer: CALGB 90401. *J Clin Oncol* 2012;30(13):1534-1540.
15. Meulenbeld HJ, van Werkhoven ED, Coenen JL et al. Randomised phase II/III study of docetaxel with or without risnedronate in patients with metastatic Castration Resistant Prostate Cancer (CRPC), the Netherlands Prostate Study (NePro). *Eur J Cancer* 2012;48(16):2993-3000.
16. Armstrong AJ, Creel P, Turnbull J et al. A phase I-II study of docetaxel and atrasentan in men with castration-resistant metastatic prostate cancer. *Clin Cancer Res* 2008;14(19):6270-6276.
17. Quinn DI, Tangen CM, Hussain M et al. SWOG S0421: Phase III study of docetaxel (D) and

- atrasentan (A) versus docetaxel and placebo (P) for men with advanced castrate resistant prostate cancer (CRPC). [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr 4511).
18. Higano C, Saad F, Somer B et al. A phase III trial of GVAX immunotherapy for prostate cancer versus docetaxel plus prednisone in asymptomatic, castration-resistant prostate cancer (CRPC). [abstract]. In: *Genitourinary Cancers Symposium*, 2009 Feb 26-28, Orlando, FL (Abstr LBA150).
 19. Small E, Demkow T, Gerritsen R et al. A phase III trial of GVAX immunotherapy for prostate cancer in combination with docetaxel versus docetaxel plus prednisone in symptomatic, castration-resistant prostate cancer (CRPC). [abstract]. In: *Genitourinary Cancers Symposium*, 2009 Feb 26-28, Orlando, FL (Abstr 7).
 20. Nelson JB, Fizazi K, Miller K et al. Phase 3, randomized, placebo-controlled study of zibotentan (ZD4054) in patients with castration-resistant prostate cancer metastatic to bone. *Cancer* 2012;118(22):5709-5718.
 21. Sanofi and Regeneron Announce Regulatory and Clinical Update for Zaltrap® (aflibercept). Tarrytown, NY; Sanofi, 2012. Available at: http://en.sanofi.com/Images/30138_20120405_ZALTRAP_BLA_VENICE_en.pdf [Last accessed 11 December 2012].
 22. Lenalidomide Plus Docetaxel: Phase III Results Fall Short. Plainsboro, NJ: OncLive, 2012. Available at: <http://www.onclive.com/publications/oncology-live/2012/january-2012/Lenalidomide-Plus-Docetaxel-Phase-III-Results-Fall-Short> [Last accessed 11 December 2012].
 23. Randomized Study Comparing Docetaxel Plus Dasatinib to Docetaxel Plus Placebo in Castration-Resistant Prostate Cancer (READY). Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT00744497> [Last accessed 12 December 2012].
 24. Comparison of Docetaxel/Prednisone to Docetaxel/Prednisone in Combination With OGX-011 in Men With Prostate Cancer (SYNERGY). Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01188187> [Last accessed 12 December 2012].
 25. Araujo JC, Mathew P, Armstrong AJ et al. Dasatinib combined with docetaxel for castration-resistant prostate cancer: results from a phase 1-2 study. *Cancer* 2012;118(1):63-71.
 26. Chi KN, Hotte SJ, Yu EY et al. Randomized phase II study of docetaxel and prednisone with or without OGX-011 in patients with metastatic castration-resistant prostate cancer. *J Clin Oncol* 2010;28(27):4247-4254.
 27. Tamaskar I, Mekhail T, Dreicer R et al. Phase I trial of weekly docetaxel and daily temozolomide in patients with metastatic disease. *Invest New Drugs* 2008;26(6):553-559.
 28. Facchini G, Caraglia M, Morabito A et al. Metronomic administration of zoledronic acid and taxotere combination in castration resistant prostate cancer patients: phase I ZANTE trial. *Cancer Biol Ther* 2010;10(6):543-548.
 29. Bradshaw DM, Arceci RJ. Clinical relevance of transmembrane drug efflux as a mechanism of multidrug resistance. *J Clin Oncol* 1998;16(11):3674-3690.
 30. de Bono JS, Oudard S, Ozguroglu M et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* 2010;376(9747):1147-1154.
 31. Heck MM, Hoppner M, Horn T, Thalgott M, Gschwend JE, Retz M. Compassionate use of abiraterone and cabazitaxel: First experiences in docetaxel-pretreated castration-resistant prostate cancer patients. *Urologe A* 2012.

32. De Velasco G, Aparicio LA, Esteban E et al. Cabazitaxel in patients with advanced CRPC after docetaxel failure: Results of expanded access program (EAP) in Spain: Safety and efficacy. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr e15149).
33. Bracarda S, Di Lorenzo G, Gasparro D et al. Updated safety result of a large Italian early access program (EAP) with cabazitaxel plus prednisone (CbzP) in metastatic castration-resistant prostate cancer (mCRPC) patients who progressed during or after docetaxel (D) therapy. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr e15185).
34. Roth BJ, Yeap BY, Wilding G, Kasimis B, McLeod D, Loehrer PJ. Taxol in advanced, hormone-refractory carcinoma of the prostate. A phase II trial of the Eastern Cooperative Oncology Group. *Cancer* 1993;72(8):2457-2460.
35. Hudes GR, Nathan F, Khater C et al. Phase II trial of 96-hour paclitaxel plus oral estramustine phosphate in metastatic hormone-refractory prostate cancer. *J Clin Oncol* 1997;15(9):3156-3163.
36. Smith DC, Esper P, Strawderman M, Redman B, Pienta KJ. Phase II trial of oral estramustine, oral etoposide, and intravenous paclitaxel in hormone-refractory prostate cancer. *J Clin Oncol* 1999;17(6):1664-1671.
37. Smith DC, Chay CH, Dunn RL et al. Phase II trial of paclitaxel, estramustine, etoposide, and carboplatin in the treatment of patients with hormone-refractory prostate carcinoma. *Cancer* 2003;98(2):269-276.
38. Berry W, Friedland D, Fleagle J et al. A phase II study of weekly paclitaxel/estramustine/carboplatin in hormone-refractory prostate cancer. *Clin Genitourin Cancer* 2006;5(2):131-137.
39. Mackler NJ, Pienta KJ, Dunn RL et al. Phase II evaluation of oral estramustine, oral etoposide, and intravenous paclitaxel in patients with hormone-sensitive prostate adenocarcinoma. *Clin Genitourin Cancer* 2007;5(5):318-322.
40. Kelly WK, Halabi S, Elfiky A et al. Multicenter phase 2 study of neoadjuvant paclitaxel, estramustine phosphate, and carboplatin plus androgen deprivation before radiation therapy in patients with unfavorable-risk localized prostate cancer: results of Cancer and Leukemia Group B 99811. *Cancer* 2008;113(11):3137-3145.
41. Sanfilippo NJ, Taneja SS, Chachoua A, Lepor H, Formenti SC. Phase I/II study of biweekly paclitaxel and radiation in androgen-ablated locally advanced prostate cancer. *J Clin Oncol* 2008;26(18):2973-2978.
42. Sella A, Yarom N, Zisman A, Kovel S. Paclitaxel, estramustine and carboplatin combination chemotherapy after initial docetaxel-based chemotherapy in castration-resistant prostate cancer. *Oncology* 2009;76(6):442-446.
43. Sewak S, Kosmider S, Ganju V et al. Phase II study of paclitaxel and vinorelbine (PacI-Vin) in hormone-refractory metastatic prostate cancer: double tubulin targeting. *Intern Med J* 2010;40(3):201-208.
44. DiPaola RS, Chen YH, Stein M et al. A randomized phase II trial of mitoxantrone, estramustine and vinorelbine or bcl-2 modulation with 13-cis retinoic acid, interferon and paclitaxel in patients with metastatic castrate-resistant prostate cancer: ECOG 3899. *J Transl Med* 2010;8:20.
45. Beer TM, Ryan C, Alumkal J, Ryan CW, Sun J, Eilers KM. A phase II study of paclitaxel poliglumex in combination with transdermal estradiol for the treatment of metastatic castration-resistant prostate cancer after docetaxel chemotherapy. *Anticancer Drugs* 2010;21(4):433-438.

46. Smith DC, Tangen CM, van Veldhuizen PJJ et al. Phase II evaluation of early oral estramustine, oral etoposide, and intravenous paclitaxel combined with hormonal therapy in patients with high-risk metastatic prostate adenocarcinoma: Southwest Oncology Group S0032. *Urology* 2011;77(5):1172-1176.
47. Jeske S, Tagawa ST, Olowokure O, Selzer J, Giannakakou P, Nanus DM. Carboplatin plus paclitaxel therapy after docetaxel in men with metastatic castrate resistant prostate cancer. *Urol Oncol* 2011;29(6):676-681.
48. Desai N, Trieu V, Yao Z et al. Increased antitumor activity, intratumor paclitaxel concentrations, and endothelial cell transport of cremophor-free, albumin-bound paclitaxel, ABI-007, compared with cremophor-based paclitaxel. *Clin Cancer Res* 2006;12(4):1317-1324.
49. Shepard DR, Dreicer R, Garcia J et al. Phase II trial of neoadjuvant nab-paclitaxel in high risk patients with prostate cancer undergoing radical prostatectomy. *J Urol* 2009;181(4):1672-1677.
50. Kolevska T, Ryan CJ, Huey V et al. Phase II trial of nab-paclitaxel as first-line therapy of hormone refractory metastatic prostate cancer (HRPC). [abstract]. *J Clin Oncol* 27:15s, 2009 (suppl; abstr 5152).
51. Phase I Abiraxane Weekly and Three Weekly Schedule With Vandetanib. Bethesda, MD: US National Institute of Health, 2013. Available at: <http://clinicaltrials.gov/ct2/show/NCT00667147> [Last accessed 21 January 2013].
52. Shionoya M, Jimbo T, Kitagawa M, Soga T, Tohgo A. DJ-927, a novel oral taxane, overcomes P-glycoprotein-mediated multidrug resistance in vitro and in vivo. *Cancer Sci* 2003;94(5):459-466.
53. Baas P, Szczesna A, Albert I et al. Phase I/II study of a 3 weekly oral taxane (DJ-927) in patients with recurrent, advanced non-small cell lung cancer. *J Thorac Oncol* 2008;3(7):745-750.
54. Saif MW, Sarantopoulos J, Patnaik A, Tolcher AW, Takimoto C, Beeram M. Teseaxel, a new oral taxane, in combination with capecitabine: a phase I, dose-escalation study in patients with advanced solid tumors. *Cancer Chemother Pharmacol* 2011;68(6):1565-1573.
55. Teseaxel in Chemotherapy-naïve Patients With Progressive, Castration-resistant Prostate Cancer. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01296243> [Last accessed 12 December 2012].
56. Daliani DD, Assikis V, Tu SM et al. Phase II trial of cyclophosphamide, vincristine, and dexamethasone in the treatment of androgen-independent prostate carcinoma. *Cancer* 2003;97(3):561-567.
57. Sepp-Lorenzino L, Balog A, Su DS et al. The microtubule-stabilizing agents epothilones A and B and their desoxy-derivatives induce mitotic arrest and apoptosis in human prostate cancer cells. *Prostate Cancer Prostatic Dis* 1999;2(1):41-52.
58. Lee FY, Borzilleri R, Fairchild CR et al. BMS-247550: a novel epothilone analog with a mode of action similar to paclitaxel but possessing superior antitumor efficacy. *Clin Cancer Res* 2001;7(5):1429-1437.
59. Chou TC, O'Connor OA, Tong WP et al. The synthesis, discovery, and development of a highly promising class of microtubule stabilization agents: curative effects of desoxyepothilones B and F against human tumor xenografts in nude mice. *Proc Natl Acad Sci U S A* 2001;98(14):8113-8118.
60. Dorff TB, Gross ME. The epothilones: new therapeutic agents for castration-resistant prostate cancer. *Oncologist* 2011;16(10):1349-1358.
61. Hortobagyi GN, Gomez HL, Li RK et al. Analysis of overall survival from a phase III study

- of ixabepilone plus capecitabine versus capecitabine in patients with MBC resistant to anthracyclines and taxanes. *Breast Cancer Res Treat* 2010;122(2):409-418.
62. Sparano JA, Vrdoljak E, Rixe O et al. Randomized phase III trial of ixabepilone plus capecitabine versus capecitabine in patients with metastatic breast cancer previously treated with an anthracycline and a taxane. *J Clin Oncol* 2010;28(20):3256-3263.
 63. Roche H, Conte P, Perez EA et al. Ixabepilone plus capecitabine in metastatic breast cancer patients with reduced performance status previously treated with anthracyclines and taxanes: a pooled analysis by performance status of efficacy and safety data from 2 phase III studies. *Breast Cancer Res Treat* 2011;125(3):755-765.
 64. Jassem J, Fein L, Karwal M et al. Ixabepilone plus capecitabine in advanced breast cancer patients with early relapse after adjuvant anthracyclines and taxanes: a pooled subset analysis of two phase III studies. *Breast* 2012;21(1):89-94.
 65. Withdrawal of IXEMPRA, (ixabepilone) EMEA/H/C/000930. London, UK: European Medicines Agency, 2009. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Other/2010/01/WC500062430.pdf [Last accessed 12 December 2012].
 66. Smaletz O, Galsky M, Scher HI et al. Pilot study of epothilone B analog (BMS-247550) and estramustine phosphate in patients with progressive metastatic prostate cancer following castration. *Ann Oncol* 2003;14(10):1518-1524.
 67. Rosenberg JE, Ryan CJ, Weinberg VK et al. Phase I study of ixabepilone, mitoxantrone, and prednisone in patients with metastatic castration-resistant prostate cancer previously treated with docetaxel-based therapy: a study of the department of defense prostate cancer clinical trials consortium. *J Clin Oncol* 2009;27(17):2772-2778.
 68. Galsky MD, Small EJ, Oh WK et al. Multi-institutional randomized phase II trial of the epothilone B analog ixabepilone (BMS-247550) with or without estramustine phosphate in patients with progressive castrate metastatic prostate cancer. *J Clin Oncol* 2005;23(7):1439-1446.
 69. Rosenberg JE, Weinberg VK, Kelly WK et al. Activity of second-line chemotherapy in docetaxel-refractory hormone-refractory prostate cancer patients : randomized phase 2 study of ixabepilone or mitoxantrone and prednisone. *Cancer* 2007;110(3):556-563.
 70. Liu G, Chen YH, DiPaola R, Carducci M, Wilding G. Phase II trial of weekly ixabepilone in men with metastatic castrate-resistant prostate cancer (E3803): a trial of the Eastern Cooperative Oncology Group. *Clin Genitourin Cancer* 2012;10(2):99-105.
 71. Layton JL, Plette AM, Renzulli JF et al. The impact of neoadjuvant weekly ixabepilone for high-risk prostate cancer: A phase I/II clinical trial. [abstract]. *J Clin Oncol* 30, 2012 (suppl 5; abstr 158).
 72. Scher HI, Halabi S, Tannock I et al. Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. *J Clin Oncol* 2008;26(7):1148-1159.
 73. Hussain A, DiPaola RS, Baron AD, Higano CS, Tchekmedyan NS, Johri AR. Phase II trial of weekly patupilone in patients with castration-resistant prostate cancer. *Ann Oncol* 2009;20(3):492-497.
 74. Chi KN, Beardsley E, Eigl BJ et al. A phase 2 study of patupilone in patients with metastatic castration-resistant prostate cancer previously treated with docetaxel: Canadian Urologic Oncology Group study P07a. *Ann Oncol* 2012;23(1):53-58.
 75. Beer TM, Higano CS, Saleh M et al. Phase II study of KOS-862 in patients with metastatic androgen independent prostate cancer previously treated with docetaxel. *Invest New Drugs*

- 2007;25(6):565-570.
76. Beer TM, Smith DC, Hussain A et al. Phase II study of first-line sagopilone plus prednisone in patients with castration-resistant prostate cancer: a phase II study of the Department of Defense Prostate Cancer Clinical Trials Consortium. *Br J Cancer* 2012;107(5):808-813.
 77. Roberts K. Cytoplasmic microtubules and their functions. *Prog Biophys Mol Biol* 1974;28:371-420.
 78. Mayer TU, Kapoor TM, Haggarty SJ, King RW, Schreiber SL, Mitchison TJ. Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science* 1999;286(5441):971-974.
 79. Kapitein LC, Peterman EJ, Kwok BH, Kim JH, Kapoor TM, Schmidt CF. The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. *Nature* 2005;435(7038):114-118.
 80. Sarli V, Giannis A. Targeting the kinesin spindle protein: basic principles and clinical implications. *Clin Cancer Res* 2008;14(23):7583-7587.
 81. Vijapurkar U, Wang W, Herbst R. Potentiation of kinesin spindle protein inhibitor-induced cell death by modulation of mitochondrial and death receptor apoptotic pathways. *Cancer Res* 2007;67(1):237-245.
 82. Tao W, South VJ, Diehl RE et al. An inhibitor of the kinesin spindle protein activates the intrinsic apoptotic pathway independently of p53 and de novo protein synthesis. *Mol Cell Biol* 2007;27(2):689-698.
 83. Liu M, Aneja R, Liu C et al. Inhibition of the mitotic kinesin Eg5 up-regulates Hsp70 through the phosphatidylinositol 3-kinase/Akt pathway in multiple myeloma cells. *J Biol Chem* 2006;281(26):18090-18097.
 84. Chin GM, Herbst R. Induction of apoptosis by monastrol, an inhibitor of the mitotic kinesin Eg5, is independent of the spindle checkpoint. *Mol Cancer Ther* 2006;5(10):2580-2591.
 85. Lad L, Luo L, Carson JD et al. Mechanism of inhibition of human KSP by ispinesib. *Biochemistry* 2008;47(11):3576-3585.
 86. Blagden SP, Molife LR, Seebaran A et al. A phase I trial of ispinesib, a kinesin spindle protein inhibitor, with docetaxel in patients with advanced solid tumours. *Br J Cancer* 2008;98(5):894-899.
 87. Burris III HA, Jones SF, Williams DD et al. A phase I study of ispinesib, a kinesin spindle protein inhibitor, administered weekly for three consecutive weeks of a 28-day cycle in patients with solid tumors. *Invest New Drugs* 2011;29(3):467-472.
 88. Chu QS, Holen KD, Rowinsky EK et al. Phase I trial of novel kinesin spindle protein (KSP) inhibitor SB-715992 IV Q 21 days. [abstract]. *J Clin Oncol* 22:14S, 2004 (July 15 Suppl; abstr 2078).
 89. Heath EI, Alousi A, Eder JP et al. A phase I dose escalation trial of ispinesib (SB-715992) administered days 1-3 of a 21-day cycle in patients with advanced solid tumors. [abstract]. *J Clin Oncol* 24:18S, 2006 (June 20 suppl; abstr 2026).
 90. Beer TM, Goldman B, Synold TW et al. Southwest Oncology Group phase II study of ispinesib in androgen-independent prostate cancer previously treated with taxanes. *Clin Genitourin Cancer* 2008;6(2):103-109.
 91. Xing ND, Ding ST, Saito R et al. A potent chemotherapeutic strategy in prostate cancer: S-(methoxytrityl)-L-cysteine, a novel Eg5 inhibitor. *Asian J Androl* 2011;13(2):236-241.
 92. Holen KD, Belani CP, Wilding G et al. A first in human study of SB-743921, a kinesin spindle protein inhibitor, to determine pharmacokinetics, biologic effects and establish a recommended phase II dose. *Cancer Chemother Pharmacol* 2011;67(2):447-454.

93. Holen K, DiPaola R, Liu G et al. A phase I trial of MK-0731, a Kinesin Spindle Protein (KSP) inhibitor, in patients with solid tumors. *Invest New Drugs* 2011.
94. Esaki T, Seto T, Ariyama H et al. Phase I Study to Assess the Safety, Tolerability and Pharmacokinetics of AZD4877 in Japanese Patients with Solid Tumors. *Arch Drug Inf* 2011;4(2):23-31.
95. Infante JR, Kurzrock R, Spratlin J et al. A Phase I study to assess the safety, tolerability, and pharmacokinetics of AZD4877, an intravenous Eg5 inhibitor in patients with advanced solid tumors. *Cancer Chemother Pharmacol* 2012;69(1):165-172.
96. Stephenson JJ, Lewis N, Martin JC et al. Phase I multicenter study to assess the safety, tolerability, and pharmacokinetics of AZD4877 administered twice weekly in adult patients with advanced solid malignancies. [abstract]. *J Clin Oncol* 26, 2008 (May 20 suppl; abstr 2516).
97. Goncalves PH, Sausville EA, Edelman MJ et al. A phase I study of ARRY-520 in solid tumors. [abstract]. *J Clin Oncol* 28:15s, 2010 (suppl; abstr 2570).
98. Nakai R, Iida S, Takahashi T et al. K858, a novel inhibitor of mitotic kinesin Eg5 and antitumor agent, induces cell death in cancer cells. *Cancer Res* 2009;69(9):3901-3909.
99. Wiltshire C, Singh BL, Stockley J et al. Docetaxel-resistant prostate cancer cells remain sensitive to S-trityl-L-cysteine-mediated Eg5 inhibition. *Mol Cancer Ther* 2010;9(6):1730-1739.
100. Carmena M, Earnshaw WC. The cellular geography of aurora kinases. *Nat Rev Mol Cell Biol* 2003;4(11):842-854.
101. Dar AA, Goff LW, Majid S, Berlin J, El-Rifai W. Aurora kinase inhibitors--rising stars in cancer therapeutics? *Mol Cancer Ther* 2010;9(2):268-278.
102. Hirota T, Kunitoku N, Sasayama T et al. Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. *Cell* 2003;114(5):585-598.
103. Marumoto T, Hirota T, Morisaki T et al. Roles of aurora-A kinase in mitotic entry and G2 checkpoint in mammalian cells. *Genes Cells* 2002;7(11):1173-1182.
104. Marumoto T, Honda S, Hara T et al. Aurora-A kinase maintains the fidelity of early and late mitotic events in HeLa cells. *J Biol Chem* 2003;278(51):51786-51795.
105. Berdnik D, Knoblich JA. *Drosophila* Aurora-A is required for centrosome maturation and actin-dependent asymmetric protein localization during mitosis. *Curr Biol* 2002;12(8):640-647.
106. Terada Y, Tatsuka M, Suzuki F, Yasuda Y, Fujita S, Otsu M. AIM-1: a mammalian midbody-associated protein required for cytokinesis. *EMBO J* 1998;17(3):667-676.
107. Slattery SD, Mancini MA, Brinkley BR, Hall RM. Aurora-C kinase supports mitotic progression in the absence of Aurora-B. *Cell Cycle* 2009;8(18):2984-2994.
108. Khan J, Ezan F, Cremet JY et al. Overexpression of active Aurora-C kinase results in cell transformation and tumour formation. *PLoS One* 2011;6(10):e26512.
109. Chieffi P, Cozzolino L, Kisslinger A et al. Aurora B expression directly correlates with prostate cancer malignancy and influence prostate cell proliferation. *Prostate* 2006;66(3):326-333.
110. Beltran H, Rickman DS, Park K et al. Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. *Cancer Discov* 2011;1(6):487-495.
111. Mosquera JM, Beltran H, Park K et al. Concurrent AURKA and MYCN gene amplifications are harbingers of lethal treatment-related neuroendocrine prostate cancer. *Neoplasia* 2013;15(1):1-10.
112. Jeet V, Russell PJ, Verma ND, Khatri A. Targeting Aurora Kinases: A Novel Approach to Curb

- the Growth & Chemoresistance of Androgen Refractory Prostate Cancer. *Curr Cancer Drug Targets* 2011.
113. Shu SK, Liu Q, Coppola D, Cheng JQ. Phosphorylation and activation of androgen receptor by Aurora-A. *J Biol Chem* 2010;285(43):33045-33053.
 114. Jeong NY, Yoon YG, Rho JH et al. The novel resveratrol analog HS-1793-induced polyploid LNCaP prostate cancer cells are vulnerable to downregulation of Bcl-xL. *Int J Oncol* 2011;38(6):1597-1604.
 115. Moretti L, Niermann K, Schleicher S et al. MLN8054, a small molecule inhibitor of aurora kinase a, sensitizes androgen-resistant prostate cancer to radiation. *Int J Radiat Oncol Biol Phys* 2011;80(4):1189-1197.
 116. Fancelli D, Moll J, Varasi M et al. 1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazoles: identification of a potent Aurora kinase inhibitor with a favorable antitumor kinase inhibition profile. *J Med Chem* 2006;49(24):7247-7251.
 117. Carpinelli P, Ceruti R, Giorgini ML et al. PHA-739358, a potent inhibitor of Aurora kinases with a selective target inhibition profile relevant to cancer. *Mol Cancer Ther* 2007;6(12 Pt 1):3158-3168.
 118. Steeghs N, Eskens FA, Gelderblom H et al. Phase I pharmacokinetic and pharmacodynamic study of the aurora kinase inhibitor danusertib in patients with advanced or metastatic solid tumors. *J Clin Oncol* 2009;27(30):5094-5101.
 119. Cohen RB, Jones SF, Aggarwal C et al. A phase I dose-escalation study of danusertib (PHA-739358) administered as a 24-hour infusion with and without granulocyte colony-stimulating factor in a 14-day cycle in patients with advanced solid tumors. *Clin Cancer Res* 2009;15(21):6694-6701.
 120. Meulenbeld HJ, Bleuse JP, Vinci EM et al. Randomized phase II study of danusertib in patients with metastatic castration-resistant prostate cancer after docetaxel failure. *BJU Int* 2012.
 121. Sells T, Ecsedy JA, Stroud S et al. MLN8237: an orally active small molecule inhibitor of aurora A kinase in phase I clinical trials. [abstract]. *Proc Am Assoc Cancer Res (AACR)* 49, 2008 (abstr 2997).
 122. Manfredi MG, Ecsedy JA, Chakravarty A et al. Characterization of Alisertib (MLN8237), an investigational small-molecule inhibitor of aurora A kinase using novel in vivo pharmacodynamic assays. *Clin Cancer Res* 2011;17(24):7614-7624.
 123. Maris JM, Morton CL, Gorlick R et al. Initial testing of the aurora kinase A inhibitor MLN8237 by the Pediatric Preclinical Testing Program (PPTP). *Pediatr Blood Cancer* 2010;55(1):26-34.
 124. Dees EC, Cohen RB, von Mehren M et al. Phase I study of aurora A kinase inhibitor MLN8237 in advanced solid tumors: safety, pharmacokinetics, pharmacodynamics, and bioavailability of two oral formulations. *Clin Cancer Res* 2012;18(17):4775-4784.
 125. A Phase 1 Study of MLN8237 in Patients With Advanced Solid Tumors Including Castration-Resistant Prostate Cancer Receiving a Standard Docetaxel Regimen. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01094288> [Last accessed 12 December 2012].
 126. Mortlock AA, Foote KM, Heron NM et al. Discovery, synthesis, and in vivo activity of a new class of pyrazoloquinazolines as selective inhibitors of aurora B kinase. *J Med Chem* 2007;50(9):2213-2224.
 127. Wilkinson RW, Odedra R, Heaton SP et al. AZD1152, a selective inhibitor of Aurora B kinase, inhibits human tumor xenograft growth by inducing apoptosis. *Clin Cancer Res* 2007;13(12):3682-3688.

128. Niermann KJ, Moretti L, Giacalone NJ et al. Enhanced radiosensitivity of androgen-resistant prostate cancer: AZD1152-mediated Aurora kinase B inhibition. *Radiat Res* 2011;175(4):444-451.
129. Boss DS, Witteveen PO, van der Sar J et al. Clinical evaluation of AZD1152, an i.v. inhibitor of Aurora B kinase, in patients with solid malignant tumors. *Ann Oncol* 2011;22(2):431-437.
130. Schwartz GK, Carvajal RD, Midgley R et al. Phase I study of barasertib (AZD1152), a selective inhibitor of Aurora B kinase, in patients with advanced solid tumors. *Invest New Drugs* 2012.
131. Sawyers CL. Finding the next Gleevec: FLT3 targeted kinase inhibitor therapy for acute myeloid leukemia. *Cancer Cell* 2002;1(5):413-415.
132. Fletcher GC, Brokx RD, Denny TA et al. ENMD-2076 is an orally active kinase inhibitor with antiangiogenic and antiproliferative mechanisms of action. *Mol Cancer Ther* 2011;10(1):126-137.
133. Diamond JR, Bastos BR, Hansen RJ et al. Phase I safety, pharmacokinetic, and pharmacodynamic study of ENMD-2076, a novel angiogenic and Aurora kinase inhibitor, in patients with advanced solid tumors. *Clin Cancer Res* 2011;17(4):849-860.
134. Matulonis UA, Lee J, Lasonde B et al. ENMD-2076, an oral inhibitor of angiogenic and proliferation kinases, has activity in recurrent, platinum resistant ovarian cancer. *Eur J Cancer* 2012.
135. Study of ENMD-2076 in Patients With Advanced/Metastatic Soft Tissue Sarcoma. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01719744> [Last accessed 12 December 2012].
136. A Phase II Study of the Aurora and Angiogenic Kinase Inhibitor ENMD-2076 in Previously Treated Locally Advanced and Metastatic Triple-Negative Breast Cancer. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01639248> [Last accessed 12 December 2012].
137. Harrington EA, Bebbington D, Moore J et al. VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. *Nat Med* 2004;10(3):262-267.
138. Traynor AM, Hewitt M, Liu G et al. Phase I dose escalation study of MK-0457, a novel Aurora kinase inhibitor, in adult patients with advanced solid tumors. *Cancer Chemother Pharmacol* 2011;67(2):305-314.
139. Howard S, Berdini V, Boulstridge JA et al. Fragment-based discovery of the pyrazol-4-yl urea (AT9283), a multitargeted kinase inhibitor with potent aurora kinase activity. *J Med Chem* 2009;52(2):379-388.
140. Dawson MA, Curry JE, Barber K et al. AT9283, a potent inhibitor of the Aurora kinases and Jak2, has therapeutic potential in myeloproliferative disorders. *Br J Haematol* 2010;150(1):46-57.
141. Jani JP, Arcari J, Bernardo V et al. PF-03814735, an orally bioavailable small molecule aurora kinase inhibitor for cancer therapy. *Mol Cancer Ther* 2010;9(4):883-894.
142. Gürtler U, Tontsch-Grunt U, Jarvis M, et al. Effect of BI 811283, a novel inhibitor of Aurora B kinase, on tumor senescence and apoptosis. [abstract]. *J Clin Oncol* 28, 2010 (suppl; abstr e13632).
143. van der Porten EC, Taverna P, Hogan JN, Ballinger MD, Flanagan WM, Fucini RV. The Aurora kinase inhibitor SNS-314 shows broad therapeutic potential with chemotherapeutics and synergy with microtubule-targeted agents in a colon carcinoma model. *Mol Cancer Ther* 2009;8(4):930-939.
144. Arkenau HT, Plummer R, Molife LR et al. A phase I dose escalation study of AT9283, a small

- molecule inhibitor of aurora kinases, in patients with advanced solid malignancies. *Ann Oncol* 2011.
145. Mross KB, Scheulen ME, Frost A et al. A phase I dose-escalation study of BI 811283, an Aurora B inhibitor, administered every three weeks in patients with advanced solid tumors. [abstract]. *J Clin Oncol* 28:15s, 2010 (suppl; abstr 3011).
 146. Scheulen ME, Mross KB, Richly H et al. A phase I dose-escalation study of BI 811283, an Aurora B inhibitor, administered days 1 and 15, every four weeks in patients with advanced solid tumors. [abstract]. *J Clin Oncol* 28, 2010 (suppl; abstr e13065).
 147. Schoffski P, Jones SF, Dumez H et al. Phase I, open-label, multicentre, dose-escalation, pharmacokinetic and pharmacodynamic trial of the oral aurora kinase inhibitor PF-03814735 in advanced solid tumours. *Eur J Cancer* 2011;47(15):2256-2264.
 148. Minton SE, LoRusso P, Lockhart AC, et al. A phase I study of MK-5108, an oral aurora A kinase inhibitor, in both monotherapy and in combination with docetaxel in patients with advanced solid tumors. [abstract]. *J Clin Oncol* 28, 2010 (suppl; abstr e13026).
 149. Robert F, Verschraegen C, Hurwitz H et al. A phase I trial of sns-314, a novel and selective pan-aurora kinase inhibitor, in advanced solid tumor patients. [abstract]. *J Clin Oncol* 27:15s, 2009 (suppl; abstr 2536).
 150. Xie H, Lee MH, Zhu F et al. Identification of an Aurora kinase inhibitor specific for the Aurora B isoform. *Cancer Res* 2012.
 151. Payton M, Bush TL, Chung G et al. Preclinical evaluation of AMG 900, a novel potent and highly selective pan-aurora kinase inhibitor with activity in taxane-resistant tumor cell lines. *Cancer Res* 2010;70(23):9846-9854.
 152. Anderson K, Lai Z, McDonald OB et al. Biochemical characterization of GSK1070916, a potent and selective inhibitor of Aurora B and Aurora C kinases with an extremely long residence time¹. *Biochem J* 2009;420(2):259-265.
 153. Carducci MA, Shaheen MF, Paller CJ et al. First-in-human study of AMG 900, an oral pan-Aurora kinase inhibitor, in adult patients (pts) with advanced solid tumors. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr 3009).
 154. Adams ND, Adams JL, Burgess JL et al. Discovery of GSK1070916, a potent and selective inhibitor of Aurora B/C kinase. *J Med Chem* 2010;53(10):3973-4001.
 155. Hardwicke MA, Oleykowski CA, Plant R et al. GSK1070916, a potent Aurora B/C kinase inhibitor with broad antitumor activity in tissue culture cells and human tumor xenograft models. *Mol Cancer Ther* 2009;8(7):1808-1817.
 156. Aurora B/C Kinase Inhibitor GSK1070916A in Treating Patients With Advanced Solid Tumors. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01118611> [Last accessed 12 December 2012].
 157. de Cárcer G, Manning G, Malumbres M. From Plk1 to Plk5: functional evolution of polo-like kinases. *Cell Cycle* 2011;10(14):2255-2262.
 158. Chopra P, Sethi G, Dastidar SG, Ray A. Polo-like kinase inhibitors: an emerging opportunity for cancer therapeutics. *Expert Opin Investig Drugs* 2010;19(1):27-43.
 159. Strebhardt K. Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy. *Nat Rev Drug Discov* 2010;9(8):643-660.
 160. Takaki T, Trenz K, Costanzo V, Petronczki M. Polo-like kinase 1 reaches beyond mitosis--cytokinesis, DNA damage response, and development. *Curr Opin Cell Biol* 2008;20(6):650-660.
 161. Liu XS, Song B, Liu X. The substrates of Plk1, beyond the functions in mitosis. *Protein Cell*

- 2010;1(11):999-1010.
162. Weichert W, Schmidt M, Gekeler V et al. Polo-like kinase 1 is overexpressed in prostate cancer and linked to higher tumor grades. *Prostate* 2004;60(3):240-245.
 163. Deeraksa A, Pan J, Sha Y et al. Plk1 is upregulated in androgen-insensitive prostate cancer cells and its inhibition leads to necroptosis. *Oncogene* 2012.
 164. Takagi M, Honmura T, Watanabe S et al. In vivo antitumor activity of a novel sulfonamide, HMN-214, against human tumor xenografts in mice and the spectrum of cytotoxicity of its active metabolite, HMN-176. *Invest New Drugs* 2003;21(4):387-399.
 165. Garland LL, Taylor C, Pilkington DL, Cohen JL, Von Hoff DD. A phase I pharmacokinetic study of HMN-214, a novel oral stilbene derivative with polo-like kinase-1-interacting properties, in patients with advanced solid tumors. *Clin Cancer Res* 2006;12(17):5182-5189.
 166. Patnaik A, Forero L, Goetz A et al. HMN-214, a novel oral antimicrotubular agent and inhibitor of pololike- and cyclin-dependent kinases: Clinical, pharmacokinetic (PK) and pharmacodynamic (PD) relationships observed in a phase I trial of a daily x 5 schedule every 28 days. [abstract]. *J Clin Oncol* 22, 2003 (suppl; abstr 514).
 167. Steegmaier M, Hoffmann M, Baum A et al. BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. *Curr Biol* 2007;17(4):316-322.
 168. Rudolph D, Steegmaier M, Hoffmann M et al. BI 6727, a Polo-like kinase inhibitor with improved pharmacokinetic profile and broad antitumor activity. *Clin Cancer Res* 2009;15(9):3094-3102.
 169. Wissing MD, Rosmus N, Gonzalez M et al. Targeting prostate cancer through a combination of Polo-like kinase inhibitors and histone deacetylase inhibitors. [abstract]. *Proc Am Assoc Cancer Res (AACR)*, 2010 (abstr 5414).
 170. Mross K, Frost A, Steinbild S et al. Phase I dose escalation and pharmacokinetic study of BI 2536, a novel Polo-like kinase 1 inhibitor, in patients with advanced solid tumors. *J Clin Oncol* 2008;26(34):5511-5517.
 171. Hofheinz RD, Al-Batran SE, Hochhaus A et al. An open-label, phase I study of the polo-like kinase-1 inhibitor, BI 2536, in patients with advanced solid tumors. *Clin Cancer Res* 2010;16(18):4666-4674.
 172. Pandha HS, Protheroe A, Wylie J et al. An open label phase II trial of BI 2536, a novel Plk1 inhibitor, in patients with metastatic hormone refractory prostate cancer (HRPC). [abstract]. *J Clin Oncol* 26, 2008 (May 20 suppl; abstr 14547).
 173. Schoffski P, Awada A, Dumez H et al. A phase I, dose-escalation study of the novel Polo-like kinase inhibitor volasertib (BI 6727) in patients with advanced solid tumours. *Eur J Cancer* 2012;48(2):179-186.
 174. Dumez H, Gombos A, Schöffski P et al. Phase I trial of the polo-like kinase (Plk) inhibitor volasertib (BI 6727) combined with cisplatin or carboplatin in patients with advanced solid tumors. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr 3018^).
 175. Stadler WM, Vaughn DJ, Sonpavde G et al. Phase II study of single-agent volasertib (BI 6727) for second-line treatment of urothelial cancer (UC). [abstract]. *J Clin Oncol* 29, 2011 (suppl 7; abstr 253).
 176. Combination of BI6727 (Volasertib) and BIBF1120 in Solid Tumors. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrial.gov/ct2/show/NCT01022853> [Last accessed 12 December 2012].
 177. BI 6727 (Volasertib) Randomised Trial in Ovarian Cancer. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrial.gov/ct2/show/NCT01121406> [Last accessed

- 12 December 2012].
178. An Open Label Phase I Dose Escalation Trial of Intravenous BI 6727 (Volasertib) in Combination With Oral BIBW 2992 (Afatinib) in Patients With Advanced Solid Tumours. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01206816> [Last accessed 12 December 2012].
 179. BI 6727 (Volasertib) Monotherapy Phase I Trial in Japanese Patients With Advanced Solid Tumours. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01348347> [Last accessed 12 December 2012].
 180. Gilmartin AG, Bleam MR, Richter MC et al. Distinct concentration-dependent effects of the polo-like kinase 1-specific inhibitor GSK461364A, including differential effect on apoptosis. *Cancer Res* 2009;69(17):6969-6977.
 181. Laquerre S, Sung C, Gilmartin A et al. A potent and selective Polo-like kinase 1 (Plk1) Inhibitor (GSK461364) induces cell cycle arrest and growth inhibition of cancer cell. [abstract]. *Proc Am Assoc Cancer Res (AACR)*, 2007 (abstr 5389).
 182. Olmos D, Barker D, Sharma R et al. Phase I study of GSK461364, a specific and competitive Polo-like kinase 1 inhibitor, in patients with advanced solid malignancies. *Clin Cancer Res* 2011;17(10):3420-3430.
 183. Study of NMS-1286937 in Adult Patients With Advanced/Metastatic Solid Tumors. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01014429> [Last accessed 12 December 2012].
 184. Dose Escalation Study to Determine Safety, Pharmacokinetics, and Pharmacodynamics of Intravenous TKM-080301. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01262235> [Last accessed 12 December 2012].
 185. Study of Orally Administered TAK-960 in Patients With Advanced Nonhematologic Malignancies. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01179399> [Last accessed 12 December 2012].
 186. Beria I, Bossi RT, Brasca MG et al. NMS-P937, a 4,5-dihydro-1H-pyrazolo[4,3-h]quinazoline derivative as potent and selective Polo-like kinase 1 inhibitor. *Bioorg Med Chem Lett* 2011;21(10):2969-2974.
 187. Valsasina B, Beria I, Alli C et al. NMS-P937, an orally available, specific, small molecule Polo-Like Kinase 1 inhibitor with antitumor activity in solid and haematological malignancies. *Mol Cancer Ther* 2012.
 188. Hikichi Y, Honda K, Hikami K et al. TAK-960, a novel, orally available, selective inhibitor of Polo-like kinase 1, shows broad-spectrum preclinical antitumor activity in multiple dosing regimens. *Mol Cancer Ther* 2011.
 189. Judge AD, Robbins M, Tavakoli I et al. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J Clin Invest* 2009;119(3):661-673.
 190. Semple SC, Judge AD, Robbins M et al. Preclinical characterization of TKM-080301, a lipid nanoparticle formulation of a small interfering RNA directed against polo-like kinase 1. [abstract]. *Proc Am Assoc Cancer Res (AACR)*; *Cancer Res* 71, 2011 (suppl 8; abstr 2829).
 191. Gumireddy K, Reddy MV, Cosenza SC et al. ON01910, a non-ATP-competitive small molecule inhibitor of Plk1, is a potent anticancer agent. *Cancer Cell* 2005;7(3):275-286.
 192. Chapman CM, Sun X, Roschewski M et al. ON 01910.Na is selectively cytotoxic for chronic lymphocytic leukemia cells through a dual mechanism of action involving PI3K/AKT inhibition and induction of oxidative stress. *Clin Cancer Res* 2012;18(7):1979-1991.
 193. Ma WW, Messersmith WA, Dy GK et al. Phase I study of Rigosertib, an inhibitor of the

- phosphatidylinositol 3-kinase and Polo-like kinase 1 pathways, combined with gemcitabine in patients with solid tumors and pancreatic cancer. *Clin Cancer Res* 2012;18(7):2048-2055.
194. Riggs BL, Hartmann LC. Selective estrogen-receptor modulators -- mechanisms of action and application to clinical practice. *N Engl J Med* 2003;348(7):618-629.
 195. Valabrega G, Montemurro F, Aglietta M. Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Ann Oncol* 2007;18(6):977-984.
 196. Qi W, Liu X, Cooke LS et al. AT9283, a novel aurora kinase inhibitor, suppresses tumor growth in aggressive B-cell lymphomas. *Int J Cancer* 2011.
 197. Qi W, Cooke LS, Liu X et al. Aurora inhibitor MLN8237 in combination with docetaxel enhances apoptosis and anti-tumor activity in mantle cell lymphoma. *Biochem Pharmacol* 2011;81(7):881-890.
 198. Feng B, Wang R, Chen LB. MiR-100 resensitizes docetaxel-resistant human lung adenocarcinoma cells (SPC-A1) to docetaxel by targeting Plk1. *Cancer Lett* 2012;317(2):184-191.
 199. Kachhap SK, Rosmus N, Collis SJ et al. Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. *PLoS One* 2010;5(6):e11208.
 200. Paller C, Wissing M, Kim E, et al. Preclinical profile of AMG900 in combination with HDACis in prostate cancer. [abstract]. *Proc Am Assoc Cancer Res (AACR)*, 2012 (abstr 2049).
 201. Marin-Aguilera M, Codony-Servat J, Kalko SG et al. Identification of docetaxel resistance genes in castration-resistant prostate cancer. *Mol Cancer Ther* 2012;11(2):329-339.
 202. Domingo-Domenech J, Vidal SJ, Rodriguez-Bravo V et al. Suppression of acquired docetaxel resistance in prostate cancer through depletion of notch- and hedgehog-dependent tumor-initiating cells. *Cancer Cell* 2012;22(3):373-388.
 203. Madan RA, Pal SK, Sartor O, Dahut WL. Overcoming chemotherapy resistance in prostate cancer. *Clin Cancer Res* 2011;17(12):3892-3902.
 204. Beer TM, Javle MM, Ryan CW et al. Phase I study of weekly DN-101, a new formulation of calcitriol, in patients with cancer. *Cancer Chemother Pharmacol* 2007;59(5):581-587.
 205. Coleman RL, Duska LR, Ramirez PT et al. Phase 1-2 study of docetaxel plus aflibercept in patients with recurrent ovarian, primary peritoneal, or fallopian tube cancer. *Lancet Oncol* 2011;12(12):1109-1117.
 206. Isambert N, Freyer G, Zanetta S et al. Phase I dose-escalation study of intravenous aflibercept in combination with docetaxel in patients with advanced solid tumors. *Clin Cancer Res* 2012;18(6):1743-1750.
 207. Demetri GD, Lo RP, MacPherson IR et al. Phase I dose-escalation and pharmacokinetic study of dasatinib in patients with advanced solid tumors. *Clin Cancer Res* 2009;15(19):6232-6240.
 208. Eisenberger MA, Hardy-Bessard A, Mourey L, et al. Comparison of two doses of cabazitaxel plus prednisone in patients (pts) with metastatic castration-resistant prostate cancer (mCRPC) previously treated with a docetaxel (D)-containing regimen. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr TPS4692^).
 209. Study of Weekly Cabazitaxel for Advanced Prostate Cancer. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01518283> [Last accessed 21 December 2012].
 210. A Study Looking at Novel Scheduling of Cabazitaxel for Patients With Metastatic Prostate Cancer (ConCab). Bethesda, MD: US National Institute of Health, 2012. Available at: <http://>

- clinicaltrials.gov/ct2/show/NCT01541007 [Last accessed 21 December 2012].
211. Second-line Chemotherapy in Castration Resistant Prostate Cancer (Prostyl). Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01558219> [Last accessed 21 December 2012].
 212. Cabazitaxel Plus Prednisone With Octreotide For Castration-Resistant Prostate Cancer (CRPC) Previously Treated With Docetaxel. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01469338> [Last accessed 21 December 2012].
 213. Cabaresc NL. Rotterdam, NL: Stichting DUOS, 2012. Available at: <http://www.stichtingduos.nl/studies/cabaresc-nl/> [Last accessed 21 December 2012].
 214. Cabazitaxel Versus Docetaxel Both With Prednisone in Patients With Metastatic Castration Resistant Prostate Cancer (FIRSTANA). Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01308567> [Last accessed 21 December 2012].
 215. Comparison of Cabazitaxel/Prednisone Alone or in Combination With Custirsen for 2nd Line Chemotherapy in Prostate Cancer (AFFINITY). Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01578655> [Last accessed 21 December 2012].
 216. The CATCH Prostate Cancer Trial: Cabazitaxel And Tasquinimod in Men With Prostate Cancer. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01513733> [Last accessed 21 December 2012].
 217. Cabazitaxel and Abiraterone Acetate in Patients With Metastatic Castrate-Resistant Prostate Cancer. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01511536> [Last accessed 21 December 2012].
 218. Ph Ib/Ila Study of Cabazitaxel Plus Bavixumab in Castration-resistant Prostate Cancer. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01335204> [Last accessed 21 December 2012].
 219. Cabazitaxel With or Without Carboplatin in Patients With Metastatic Castration-Resistant Prostate Cancer Previously Treated With Docetaxel-Based Therapy. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01505868> [Last accessed 21 December 2012].
 220. De Souza PL, Mellado B, Pfister C, et al. Randomized phase II trial of patupilone plus prednisone versus docetaxel plus prednisone in patients with chemotherapy-naïve, metastatic, castrate-resistant prostate cancer (CRPC). [abstract]. *J Clin Oncol* 28:15s, 2010 (suppl; abstr 4553).
 221. Gerecitano JF, Stephenson JJ, Lewis NL et al. A Phase I trial of the kinesin spindle protein (Eg5) inhibitor AZD4877 in patients with solid and lymphoid malignancies. *Invest New Drugs* 2012.
 222. Macarulla T, Cervantes A, Elez E et al. Phase I study of the selective Aurora A kinase inhibitor MLN8054 in patients with advanced solid tumors: safety, pharmacokinetics, and pharmacodynamics. *Mol Cancer Ther* 2010;9(10):2844-2852.
 223. Dees EC, Infante JR, Cohen RB et al. Phase 1 study of MLN8054, a selective inhibitor of Aurora A kinase in patients with advanced solid tumors. *Cancer Chemother Pharmacol* 2011;67(4):945-954.
 224. Cervantes A, Elez E, Roda D et al. Phase I pharmacokinetic/pharmacodynamic study of MLN8237, an investigational, oral, selective aurora a kinase inhibitor, in patients with

- advanced solid tumors. *Clin Cancer Res* 2012;18(17):4764-4774.
225. Mosse YP, Lipsitz E, Fox E et al. Pediatric Phase I Trial and Pharmacokinetic Study of MLN8237, an Investigational Oral Selective Small-Molecule Inhibitor of Aurora Kinase A: A Children's Oncology Group Phase I Consortium Study. *Clin Cancer Res* 2012;18(21):6058-6064.
226. Matulonis UA, Sharma S, Ghamande S et al. Phase II study of MLN8237 (alisertib), an investigational Aurora A kinase inhibitor, in patients with platinum-resistant or -refractory epithelial ovarian, fallopian tube, or primary peritoneal carcinoma. *Gynecol Oncol* 2012;127(1):63-69.
227. Lee P, Alvarez RH, Melichar B, et al. Phase I/II study of the investigational aurora A kinase (AAK) inhibitor MLN8237 (alisertib) in patients (pts) with non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), breast cancer (BrC), head/neck cancer (H&N), and gastroesophageal (GE) adenocarcinoma: Preliminary phase II results.. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr 3010).
228. Falchook GS, Goff BA, Kurzrock R, et al. Phase I/II study of weekly paclitaxel with or without MLN8237 (alisertib), an investigational aurora A kinase inhibitor, in patients with recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer (OC), or breast cancer (BrC): Phase I results. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr 5021).
229. Frost A, Mross K, Steinbild S et al. Phase i study of the Plk1 inhibitor BI 2536 administered intravenously on three consecutive days in advanced solid tumours. *Curr Oncol* 2012;19(1):e28-e35.
230. Ellis PM, Chu QS, Leighl N et al. A Phase I Open-Label Dose-Escalation Study of Intravenous BI 2536 Together With Pemetrexed in Previously Treated Patients With Non-Small-Cell Lung Cancer. *Clin Lung Cancer* 2012.
231. Schoffski P, Blay JY, De Greve J et al. Multicentric parallel phase II trial of the polo-like kinase 1 inhibitor BI 2536 in patients with advanced head and neck cancer, breast cancer, ovarian cancer, soft tissue sarcoma and melanoma. The first protocol of the European Organization for Research and Treatment of Cancer (EORTC) Network Of Core Institutes (NOCI). *Eur J Cancer* 2010;46(12):2206-2215.
232. Sebastian M, Reck M, Waller CF et al. The efficacy and safety of BI 2536, a novel Plk-1 inhibitor, in patients with stage IIIB/IV non-small cell lung cancer who had relapsed after, or failed, chemotherapy: results from an open-label, randomized phase II clinical trial. *J Thorac Oncol* 2010;5(7):1060-1067.
233. Mross K, Ditttrich C, Aulitzky WE et al. A randomised phase II trial of the Polo-like kinase inhibitor BI 2536 in chemo-naïve patients with unresectable exocrine adenocarcinoma of the pancreas - a study within the Central European Society Anticancer Drug Research (CESAR) collaborative network. *Br J Cancer* 2012;107(2):280-286.
234. BI 6727 (Volasertib) Human ADME Trial in Various Solid Tumours. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrials.gov/ct2/show/NCT01145885> [Last accessed 21 December 2012].
235. Dose Finding Study of BI 6727 (Volasertib) in Patients With Various Solid Cancers. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrial.gov/ct2/show/NCT00969553> [Last accessed 21 December 2012].
236. Trial of BI 6727 (Volasertib) Monotherapy and BI 6727 in Combination With Pemetrexed Compared to Pemetrexed Monotherapy in Advanced NSCLC. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrial.gov/ct2/show/NCT00824408> [Last

- accessed 21 December 2012].
237. Jimeno A, Li J, Messersmith WA et al. Phase I study of ON 01910.Na, a novel modulator of the Polo-like kinase 1 pathway, in adult patients with solid tumors. *J Clin Oncol* 2008;26(34):5504-5510.
 238. Ohnuma T, Holland JF, Goel S, et al. Final results of a phase I dose-escalation study of ON 01910.Na in combination with oxaliplatin in patients with advanced solid tumors. [abstract]. *J Clin Oncol* 29, 2011 (suppl; abstr e13584).
 239. Bowles DW, Diamond JR, Lam ET, et al. Phase I study of oral rigosertib in patients with advanced solid tumors. [abstract]. *J Clin Oncol* 30, 2012 (suppl; abstr 3017).
 240. Vainshtein JM, Ghalib MH, Kumar M, et al. Phase I study of ON 01910.Na, a novel polo-like kinase 1 pathway modulator, administered as a weekly 24-hour continuous infusion in patients with advanced cancer. [abstract]. *J Clin Oncol* 26, 2008 (May 20 suppl; abstr 2515).
 241. Ohnuma T, Cho SY, ROboz J, et al. Phase I study of ON 01910.Na by 3-day continuous infusion (CI) in patients (pts) with advanced cancer. [abstract]. *J Clin Oncol* 24:18S, 2006 (June 20 suppl; abstr 13137).
 242. Safety, Pharmacokinetics and Clinical Activity of Oral Rigosertib in Solid Tumors. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrial.gov/ct2/show/NCT01168011> [Last accessed 21 December 2012].
 243. Effect of 2-h Infusion of ON 01910.Na in Ovarian Cancer Patients. Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrial.gov/ct2/show/NCT00856791> [Last accessed 21 December 2012].
 244. Gemcitabine and ON 01910.Na in Previously Untreated Metastatic Pancreatic Cancer (ONTRAC). Bethesda, MD: US National Institute of Health, 2012. Available at: <http://clinicaltrial.gov/ct2/show/NCT01360853> [Last accessed 21 December 2012].

Chapter 5b

Tales of how great drugs were brought down by a flawed rationale -- Letter

Michel D. Wissing, Michael A. Carducci, Hans Gelderblom, Paul J. van Diest

Clin Cancer Res. 2013 Mar 1;19(5):1303

Introduction

In January 2012, Komlodi-Pasztor and colleagues published a paper in which they explained why, in their opinion, the rationale used for these inhibitors was flawed.¹ They concluded there is a low likelihood that any inhibitor of mitotic enzymes would play a role in the management of solid tumors. In the letter to the editor presented below, we explain why their reasoning is incorrect, leading to a conclusion we consider preliminary.

Letter

In a recent article, Komlodi-Pasztor and colleagues argued that cancer researchers used a flawed rationale to develop inhibitors of mitotic enzymes, such as polo-like kinase 1 (PLK1) and aurora kinases.¹ Scientists had been misled by the success of microtubule-targeting agents (MTA) by assuming that MTAs act on cancer cells solely by mitotic inhibition, whereas their primary targets are microtubules in interphase. Inhibitors of enzymes solely expressed during mitosis would not have a significant antitumor effect on solid tumors in clinical studies, as most solid tumors have a mean mitotic index of less than 1%.

However, despite the low mitotic index, mitotic enzymes are frequently overexpressed in cancer; for example, Weichert and colleagues reported that 52.6% of all human prostate carcinomas have strong expression of PLK1.² This suggests that mitotic enzymes also play a role beyond mitosis. Indeed, PLK1 has been found to regulate among others p53 and microtubule dynamics in interphase as well.³

The authors describe that cancer cells have mitotic slippage after 1 to 2 days of treatment with antimitotic agents.¹ They fail to mention that such cells either become aneuploid or polyploid, or are arrested in interphase.⁴ Although cells may initially be viable, all these states eventually result in cell death.

The authors explain the discrepancy between *in vivo* results and clinical efficacy of antimitotic agents further by the (twice) daily administration in mice, whereas in humans intravenous administration is done at a weekly or longer interval.¹ With the discovery of orally bioavailable compounds, such as the aurora kinase-inhibitors alisertib (MLN-8237), ENMD-2076, PF-03814735, MK-5108, and AMG-900 and the PLK1-inhibitors HMN-214, TAK-960, and NMS-1286937, similar dosing frequencies can be achieved in humans as in mice.

The major advantage of inhibitors of mitotic enzymes compared with MTAs is the lack of irreversible neuropathy after treatment; adverse events are fewer and rarely irreversible. Research is ongoing to increase the efficacy of aurora kinase- and PLK1-inhibitors, which could be accomplished by increased bioavailability to the tumor, that is, oral bioavailability, an improved pharmacokinetic profile, or activation by tumor-specific markers. Furthermore, addition of granulocyte colony-stimulating factor suppresses the adverse effect of mitotic inhibitors on the bone marrow, thereby allowing higher dosing. Aurora kinase- and PLK1-inhibitors could also have clinical potential in combination therapy, as combination therapy

could result in equal/improved efficacy and decreased toxicity compared with current treatment protocols. Therefore, we think that these promising agents should be further developed and deserve thorough testing in clinical trials.

References

1. Komlodi-Pasztor E, Sackett DL, Fojo AT. Inhibitors targeting mitosis: tales of how great drugs against a promising target were brought down by a flawed rationale. *Clin Cancer Res* 2012;18(1):51-63.
2. Weichert W, Schmidt M, Gekeler V et al. Polo-like kinase 1 is overexpressed in prostate cancer and linked to higher tumor grades. *Prostate* 2004;60(3):240-245.
3. Liu XS, Song B, Liu X. The substrates of Plk1, beyond the functions in mitosis. *Protein Cell* 2010;1(11):999-1010.
4. Gascoigne KE, Taylor SS. How do anti-mitotic drugs kill cancer cells? *J Cell Sci* 2009;122(Pt 15):2579-2585.

Chapter 6

Analysis of the genomic response of human prostate cancer cells to histone deacetylase inhibitors

Michel D. Wissing[†], Madeleine S.Q. Kortenhorst[†], Ronald Rodriguez, Sushant K Kachhap, Judith J.M. Jans, Petra van der Groep, Henk M.W. Verheul, Anuj Gupta, Paul O. Aiyetan, Elsken van der Wall, Michael A. Carducci, Paul J. van Diest, Luigi Marchionni

[†]These authors contributed equally to this work.

Epigenetics. 2013 Sep 1;8(9):907-20

Abstract

Histone deacetylases (HDACs) have emerged as important targets for cancer treatment. HDAC-inhibitors (HDACis) are well tolerated in patients and have been approved for the treatment of patients with cutaneous T-cell lymphoma (CTCL). To improve the clinical benefit of HDACis in solid tumors, combination strategies with HDACis could be employed. In this study, we applied Analysis of Functional Annotation (AFA) to provide a comprehensive list of genes and pathways affected upon HDACi-treatment in prostate cancer cells. This approach provides an unbiased and objective approach to high-throughput data mining. By performing AFA on gene expression data from prostate cancer cell lines DU-145 (an HDACi-sensitive cell line) and PC3 (a relatively HDACi-resistant cell line) treated with HDACis valproic acid or vorinostat, we identified biological processes that are affected by HDACis and are for this reason potential treatment targets for combination therapy. Our analysis revealed that HDAC-inhibition resulted among others in upregulation of major histocompatibility complex (MHC) genes and deregulation of the mitotic spindle checkpoint by downregulation of genes involved in mitosis. These findings were confirmed by AFA on publicly available data sets from HDACi-treated prostate cancer cells. In total, we analyzed 375 microarrays with HDACi treated and non-treated (control) prostate cancer cells. All results from this extensive analysis are provided as an online research source (available at <http://luigimarchionni.org/HDACis.html>). By publishing these data, we aim to enhance our understanding of the cellular changes after HDAC-inhibition, and to identify novel potential combination strategies with HDACis for the treatment of prostate cancer patients.

Introduction

An important mechanism of cells to epigenetically regulate gene expression is by acetylating and deacetylating histones.¹ Histone deacetylases (HDACs) are a class of enzymes that deacetylate lysine residues in the N-terminal tails of histones, thereby blocking gene transcription.¹ HDACs are frequently overexpressed in cancer; their overexpression leads among others to epigenetic silencing of tumor suppressor genes.¹ Therefore, various HDAC-inhibitors (HDACis) have been developed for cancer therapy, of which vorinostat (SAHA) and Romidepsin are approved by the United States Food and Drug Administration (US FDA) for the treatment of cutaneous T-cell lymphomas (CTCL). HDACis arrest cells in G0/G1 or G2/M phase dependent on the dose of HDACi and/or cell type used.² Despite preclinical data showing great promise and their success in liquid tumors, the potential of HDACis as single agents against solid tumors, specifically prostate cancer (PCa), seems to be limited in clinical studies.²

It seems that improving DNA accessibility with HDACis is merely the first step in cancer treatment. Therefore, recent studies have focused on combination strategies involving HDACis, with success. Valproic acid (VPA) in combination with epirubicin/FEC (5-fluorouracil, epirubicin, cyclophosphamide) resulted in an objective response in 64% of patients with solid advanced malignancies.³ Combination therapy with the HDACi magnesium valproate and DNA demethylating agent hydralazine resensitized 80% of cancer patients to chemotherapy on which they had previously progressed.⁴ This combination was successfully added to doxorubicin and cyclophosphamide therapy in breast cancer patients as well.⁵ The addition of vorinostat to the mammalian target of rapamycin (mTOR) inhibitor temsirolimus improved anticancer activity against renal cell carcinoma *in vitro* and *in vivo*.⁶ Other recent preclinical studies indicated that HDACis such as VPA may sensitize cancer cells, among others PCa cells, to radiotherapy.^{7, 8} In non-small cell lung cancer studies it was found that cells may be sensitized for radiotherapy through acetyl p53-mediated downregulation of c-myc.⁹ The rationale for such combination studies with HDACis was that HDACis may reverse epigenetic changes made by the tumor, downregulate gene expression involved in DNA damage repair and/or upregulate apoptosis in cancer cells.

In this study, we apply analysis of functional annotation (AFA) to HDACi-treated PCa cells, thereby providing a rationale for novel combination strategies with HDACis. AFA is a high-throughput bioinformatics approach to identify sets of genes that are differentially expressed between conditions, such as cancer cells pre- and post-treatment. It is conceptually similar to gene set enrichment analysis (GSEA).¹⁰⁻¹⁴ This unbiased method enables the interpretation of large amounts of gene expression data generated by microarray analysis through superimposition, selection, analysis and visualization of information encompassing distinct biological concepts, such as cellular signaling pathways, protein-protein interaction

(PPI) networks, gene ontology (GO), gene expression regulation by transcription factors and microRNA (miRNA) targets. In our study, AFA was used to detect cellular processes that are affected by HDACis in PCa cell lines. We analyzed data from an elaborate microarray experiment with an HDACi-sensitive (DU-145) and a (relatively) HDACi-insensitive PCa cell line (PC3) treated with the HDACis VPA and vorinostat (SAHA).¹⁵ We further complemented this analysis with gene expression profiles from HDACi-treated LNCaP and PC3 cells using all available data from the public domain.^{16, 17} Overall, we analyzed 375 distinct microarray experiments involving HDACi-treated PCa cells, which to our best knowledge is the largest analysis of this kind to date. By creating an encyclopedia of expression changes induced by HDACis in PCa cell lines, we have implemented a resource publicly available to the research community for use in future research, both for improving our understanding of the cellular effects of HDACis on PCa cells, and for developing hypotheses to test drug combinations with HDACis in preclinical studies. Finally, based on the AFA results, we present a rationale for the combination of HDACis with mitotic spindle checkpoint inhibitors and immunotherapy. Further (pre)clinical evaluation is necessary to validate these rationales.

Materials and Methods

Ethics statement

Anonymized human PCa specimens were obtained from the University Medical Center Utrecht (UMCU), after it was ensured that enough material remained to serve the patient's and family's needs, and in accordance to the Dutch code of conduct for the use of leftover body material.¹⁸ All investigations involving human samples were performed in strict adherence to the Declaration of Helsinki.

Cell lines and chemicals

PCa cells were obtained from the American Type Culture Collection (ATCC) and maintained as described previously by our group.¹⁵ Stock solutions of 10 mM vorinostat (AtonPharma/Merck) dissolved in DMSO were used for all experiments; vorinostat was further diluted in complete RPMI-1640 media (Invitrogen). VPA (Sigma-Aldrich) was stored as a salt, and freshly dissolved in complete RPMI-1640 media (RPMI-1640 media supplemented with 10% fetal bovine serum (FBS)) before each experiment. Colcemid (Sigma-Aldrich) was acquired as a 10 µg/ml solution in Hank's balanced salt solution (HBSS) and stored at 4°C.

Microarray experiment

Microarray data were obtained from time course experiments in which PC3 and DU-145 cells were treated with VPA or vorinostat for 48 h, and are available from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (series GSE34452). Expression data were processed using the R-Bioconductor library

limma.¹⁹⁻²⁴ Differential gene expression data were acquired using a generalized linear model approach (“multiple-loop, double-cube” design) as described by Kortenhorst et al.¹⁵ A detailed explanation of all procedures and methods used for microarray data preprocessing, and differential gene expression analysis and detection has been described in detail previously.^{15, 25} Briefly, we applied a complex design based on a “multiple-loop, double-cube” cDNA microarray experiment in this previous study, to enhance our understanding of the molecular underpinnings of HDACi resistance in PCa cells. To this end, we analyzed gene expression data from a total of 22 dual-color microarray hybridizations comparing DU-145 and PC3 cells treated with vorinostat and VPA after an incubation of 48 and 96 h. Data were normalized using within-array “loess” normalization, and an ANOVA coupled with empirical Bayes standard errors shrinkage was used to identify differentially expressed genes between treated cells and controls. HDAC inhibition resulted in differential expression of 2.8% to 10% of genes (adjusted p-value < 0.001) across all conditions. Between 51% to 72% of such genes were upregulated, while 28% to 49% were downregulated. Larger differential gene expression was observed in DU-145 cells compared with PC3 cells, with VPA treatment compared with vorinostat, and with longer drug exposures.

Other public domain data were obtained from GEO and the Connectivity Map, publicly available from the NCBI GEO database, along with MIAME (minimum information about a microarray experiment) compliant information. Details regarding the source of gene expression data sets used, the platforms and annotation packages employed, the preprocessing procedures adopted for each data set, as well as the statistical details of the analysis performed, are described extensively at <http://luigimarchionni.org/HDACis.html>.

Analysis of Functional Annotation (AFA)

We applied Analysis of Functional Annotation (AFA), a gene set analysis approach, on differential gene expression data obtained from distinct comparisons and across different studies to identify biological processes and signaling pathways modulated by HDACis in PCa cells.^{10, 11, 26-28} Overall, this methodology extends gene set analysis procedures, such as gene set enrichment analysis (GSEA) or parametric analysis of gene set enrichment (PAGE), by investigating biological processes enrichment over multiple experimental conditions as briefly summarized below.¹²⁻¹⁴

Functional gene sets (FGS), recapitulating distinct and complementary biological concepts such as cellular signaling pathways, PPI networks, downstream transcriptional responses, gene expression regulatory networks orchestrated by transcription factors and microRNA targets, were retrieved in the form of gene lists from various publicly available databases (see <http://luigimarchionni.org/HDACis.html>). These collections included the Reactome, the Human Protein Reference Database (HPRD), GO, the Kyoto Encyclopedia of Genes and Genomes (KEGG), the Molecular Signature Database (MSigDB) and NCBI Entrez Gene

database.^{14, 29-37}

A one-sided Wilcoxon rank sum test was separately applied across all investigated comparisons to test whether any given FGS was differentially expressed, upregulated or downregulated, using the absolute and signed t-statistics to order genes (for details on the linear model analysis see <http://luigimarchionni.org/HDACIs.html> and Kortenhorst et al.¹⁵). The enrichment analysis was performed on all non-redundant genes present on the microarray, according to the NCBI Entrez Gene database annotation.³⁸ Filtering of redundant microarray features (i.e., probes mapping to the same NCBI Entrez Gene identifier) was achieved by retaining only the probes with the largest absolute t-statistics for further analysis. Correction for multiple hypotheses testing was applied separately for each FGS collection by applying the Benjamini and Hochberg method.³⁹ Differentially expressed FGS were visualized using heatmaps; an adjusted p-value < 0.05 was considered significant.

To validate the results from the AFA on our microarray data, we further performed differential gene expression analysis and AFA on publicly available datasets of HDACi-treated PCa cells. Three datasets were available (GSE8645, GSE31620, and Connectivity Map). In one dataset (GSE8645), LNCaP cells had been treated with either 7.5 μ M CG-1521 or 5 μ M Trichostatin A (both HDACi) for 24h, after which a microarray was performed.⁴⁰ In dataset GSE31620, LNCaP cells were treated for 36h with TSA and/or the DNA-methylating agent 5-Azacytidine after which miRNA microarrays were performed.¹⁶ In the Connectivity Map, PC3 cells had been treated with various HDACi at various dosages for 6 hours.¹⁷ A detailed description of these datasets is available at <http://luigimarchionni.org/HDACIs.html>.

Flow cytometry

DU-145 and PC3 cells were synchronized in S-phase by a double thymidine block (Fig. 1). Cells were plated in 100 mm dishes; at 30–50% confluency, cells were incubated in DMEM-10/thymidine media (DMEM (Invitrogen) supplemented with 10% FBS and 2 mM thymidine (Sigma-Aldrich)) for 12h. Subsequently, cells were washed twice in PBS and incubated in DMEM-10 media. After 16h, the media was replaced with DMEM-10/thymidine and cells were incubated for 12–14h. Cells were released from S-phase by removing the thymidine-containing media; three hours later DMEM-10 media containing an HDACi and/or colcemid was added. Cells were harvested immediately after the S-phase release, at the start of treatment, and 3, 6, 9, 11, 14 or 17h after treatment initiation.

Cells were prepared for flow cytometry analysis using the method of Vindelov.⁴¹ Flow cytometry was performed on a BD-LSR II flow cytometer (BD Biosciences); data were interpreted using CellQuest (BD Biosciences).

Tissue Microarrays (TMAs) and immunohistochemistry (IHC)

For TMAs, human PCa samples were obtained from archives of the Pathology Department of the UMCU. Samples from 71 patients who had undergone radical prostatectomy were

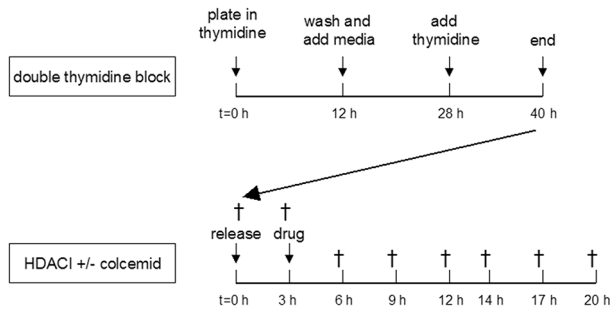


Figure 1. Summary of the flow cytometry protocol.

†, timepoints at which cells were harvested

collected; information concerning survival and PSA recurrence of these patients was collected. Tumors had been formalin-fixed and paraffin-embedded after resection.

From paraffin blocks four 1 mm cores were taken per specimen. These cores were transferred to a recipient composite paraffin block using a Beecher MT1 manual arrayer (Beecher Instruments). Four micron slides were prepared from each TMA. Spots that contained necrotic/dead tissue or fibrin instead of cancer cells were excluded.

IHC was performed with 4 μ m paraffin sections on the BOND-MAX (Leica) using the Bond Polymer Refine Detection™ (Leica) kit. With the exception of beta-2 microglobulin (B2M), antigen retrieval was performed in citrate buffer (pH 6.0) for 20 min at 100 °C. Sections were then incubated with anti-HLA-A antibody HCA2 (HCE kweek, UMCU) 1:200, anti-HLA-B antibody HC10 (HCE kweek) 1:400, anti-B2M (Dako) 1:600, anti-HLA-DR- α (Dako) 1:60 or anti-HLA-DR- β (Dako) 1:800, consecutively followed by incubation with Bond Polymer Refine Detection kit items (Leica) and incubation with DAB solution (Bond Polymer Refine Detection, Leica). Slides were counterstained with hematoxylin (Bond Polymer Refine Detection, Leica), and blindly examined and scored for protein expression by two investigators (MSQK, PID). Appropriate negative and positive controls were used.

Results

AFA after treatment with HDACIs

We previously used a “multiple-loop, double-cube” design to identify genes differentially expressed in PCa cell lines upon HDAC-inhibition with VPA or SAHA.¹⁵ In the current study we applied AFA, as previously described, to enable the interpretation of these results in the context of relevant cancer biology.^{10, 11, 26, 27, 42} To this end, we selected FGS from distinct databases, recapitulating different and complementary biological concepts: a) cellular

signaling pathways from Pathway Commons, b) PPI networks from the NCBI Entrez Gene database, c) downstream transcriptional responses, and d) gene expression regulatory networks orchestrated by transcription factors and microRNA targets. These collections included, among others, the HPRD, GO, KEGG, MSigDB, Pathway Commons and NCBI Entrez Gene databases.^{14, 29-37, 43} FGS whose expression altered most significantly upon HDAC-inhibition in DU-145 and PC3 cells across all conditions after correction for multiple testing (adjusted p-value < 0.05, top five FGS or more in case of ties), were retrieved and are displayed in Figure 2. This approach enabled the identification of biological themes that are differentially expressed upon HDAC-inhibition across all conditions irrespective of the gene expression direction change (Fig. 2A), as well as biological processes that are selectively up- or downregulated across all conditions (Figs. 2B and C, respectively). The complete lists with all AFA results, including FGS that were differentially expressed between cell lines, treatments and/or time-points, with all relevant statistics (e.g., fold-change, p-values, false discovery rates (FDR), log-odds), are available online at <http://luigimarchionni.org/HDACIs.html>.

We further explored the AFA results by analyzing the relationship among identified FGS in terms of gene overlap, identifying common and distinct sets of genes driving the enrichment. To this end, we assembled “gene to FGS” membership matrices for enriched FGS presented in Figure 2, and performed hierarchical clustering and social network analysis to group them based on gene overlap (for details see Figs S1-S6 at <http://luigimarchionni.org/HDACIs.html>).⁴⁴ This revealed both FGS groups whose enrichment was driven by similar, if not identical, genes, as well as FGS groups with minimal gene overlap, whose enrichment was hence due to separate and distinctive genes. Even more interesting, such divergent FGS groups pointed to complementary, yet distinct, biological concepts. For instance, a number of different FGS corresponding to specific signaling pathways and to their downstream targets were enriched, with minimal overlap of the underlying genes, providing a stronger rationale for pathway modulation upon HDACi-treatment. An example of such findings is the epidermal growth factor signaling, for which the FGS corresponding to both the pathway machinery (e.g., “ErbB1 receptor signaling network” from National Cancer Institute (NCI)-Pathway Commons) and the downstream targets (e.g., “EGFR Signaling Pathway” targets from HPRD) proved to be enriched (Fig. 2B and Figs. S2 and S5 at <http://luigimarchionni.org/HDACIs.html>).

Overall, treatment with HDACis altered gene expression programs related to biological processes that are well known to be involved in tumor development and progression, such as cell adhesion and cell cycle related FGS. In addition, well-established potential drug targets were modulated, such as the c-myc, the androgen receptor signaling, and the Epidermal Growth Factor Receptor 1 (EGFR1) signaling pathways. Furthermore, the interpretation of

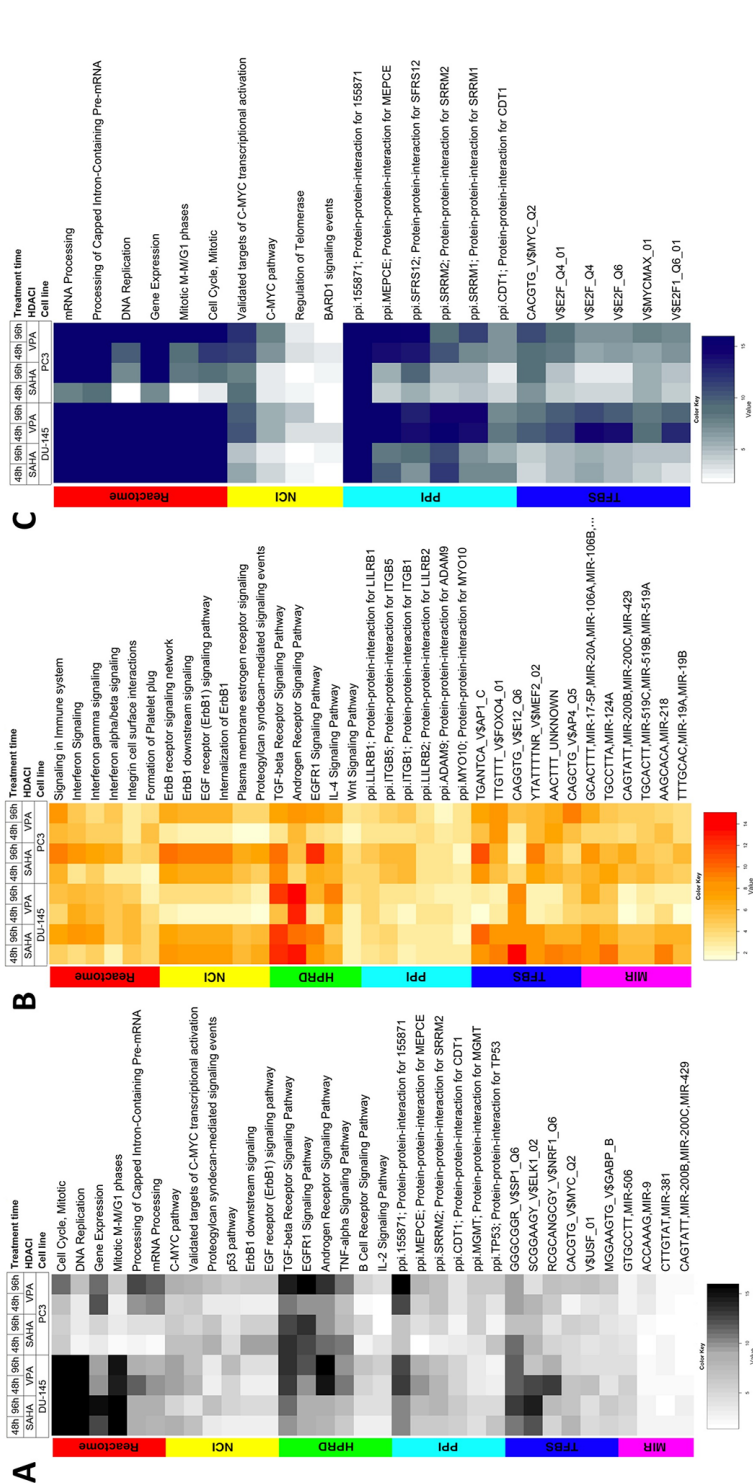


Figure 2. Heat maps visualizing differentially expressed functional gene sets (FGS) as determined by analysis of functional annotation (AFA) performed on DU-145 and PC3 cells treated with HDAC-inhibitors (HDACiS) VPA or vorinostat for 48 or 96 h. Each row represents a distinct FGS, while each column represents a distinct coefficient from our previous linear model analysis. The FGS that were most significantly differentially expressed across all comparisons performed are shown. Color scales correspond to the absolute adjusted p-values obtained from our analysis after base 10 logarithmic transformations (i.e., the number on the color scale increases with decreasing adjusted p-values). A) Differentially expressed FGS after ordering the genes based on absolute moderated t-statistics, thus irrespective of the direction of gene expression modulation upon HDAC-inhibition. B) FGS differentially expressed due to upregulated genes, as determined by signed t-statistics. C) FGS differentially expressed due to downregulated genes, as determined by signed t-statistics.

the AFA results attracted attention to two groups of functionally connected FGS that were up- or downregulated after HDAC-inhibition: major histocompatibility complexes (MHC) genes and mitotic spindle checkpoint genes, respectively (Fig. 2). These FGS will be further discussed below.

Treatment of PCa cells with HDACis results in upregulation of MHC genes

Interpretation of the AFA results indicated that HDACis may play a role in immuno-editing, as it transcriptionally modulated genes involved in this biological process. In fact, the “Signaling in Immune system” Reactome FGS proved to be most significantly upregulated across all studied conditions along with FGS related to IL-4 and Interferon signaling pathways (Fig. 2B). B2M, Human Leukocyte Antigen (HLA) class I molecules, and Interferon induced proteins were among the most upregulated genes driving this upregulation (see <http://luigimarchionni.org/HDACis.html>). Upregulation of FGS related to the immune system was observed in other collections as well: for example PPI networks with LILRB1 (Leukocyte immunoglobulin-like receptor B1) and LILRB2 were also upregulated upon HDAC-inhibition (Fig. 2B). Furthermore, the cAMP Response Element Binding protein (CREB) and the interferon-stimulated response element (ISRE) site gene sets were differentially expressed upon HDACi-treatment (see <http://luigimarchionni.org/HDACis.html>). Interestingly, CREB is part of an enhanceosome that increases transcription of MHC-I, B2M and MHC-II genes, and interferon is a major regulator of MHC-I transcription.^{45, 46}

We validated our data by performing differential gene expression analysis and AFA on an additional 353 microarray experiments from three publicly available data sets (GSE8645 and GSE31620, and Connectivity Map) involving PCa cells treated with HDACis.^{16, 17} We assessed the overall agreement of differential gene expression across all analyzed data sets using Correspondence At the Top (CAT) curves as described previously, which overall proved to be greater than expected by chance (Figs. S7-S16 at <http://luigimarchionni.org/HDACis.html>).^{10, 26, 47} We also compared and validated AFA results identifying the biological themes and processes which were consistently differentially expressed across the data sets, treatment conditions, and data points analyzed (Figs. S17-S19 at <http://luigimarchionni.org/HDACis.html>). Overall, FGS related to the immune system were significantly upregulated across most of the analyzed conditions, spanning distinct HDACis, cell lines and treatment time points (Fig. S18 at <http://luigimarchionni.org/HDACis.html>).

Based on the finding that FGS related to the immune system are differentially expressed upon HDACi-treatment, we hypothesized that PCa cells may alter the expression of genes related to the immune system, a change that is reverted by HDACis. To further explore this hypothesis, we assessed whether the expression of MHC proteins in human PCa tissue is deregulated compared with normal prostate tissue. For this purpose, a PCa Tissue

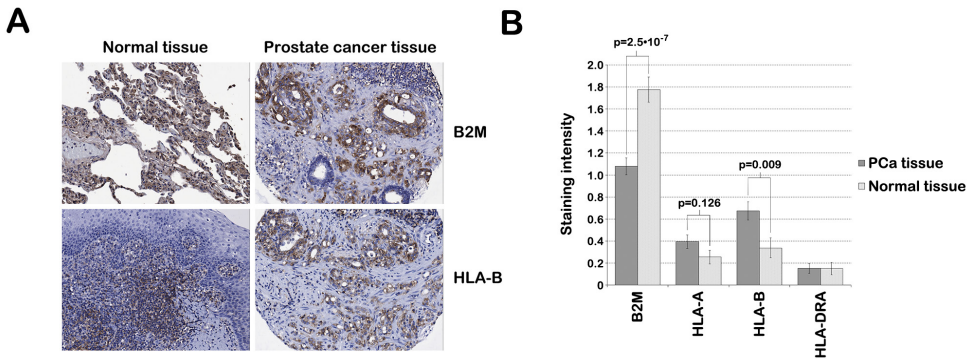


Figure 3. IHC staining of human prostate tissue in a TMA. A) Representative images of human PCa and normal prostate tissue stained for B2M (top) and HLA-B (bottom) show decreased expression of B2M and increased expression of HLA-B in PCa tissue compared with normal surrounding tissue in a PCa patient. B) Bar graph displaying the mean staining intensity of 71 cancerous and non-cancerous tissue samples for B2M, HLA-A, HLA-B and HLA-DRA protein expression.

Microarray (TMA) was performed using specimens from 71 patients who had undergone a radical prostatectomy. Protein expression of HLA-A, HLA-B, HLA-DRA and B2M was scored in PCa tissue and normal surrounding tissue (Fig. 3). Repeated measurement analysis using the panel of all four proteins revealed a significant combined change in MHC protein expression in human PCa tissue compared with normal surrounding tissue ($p < 0.001$). B2M and HLA-B were primarily responsible for this deregulation. B2M protein expression was downregulated compared with normal surrounding tissue ($p=2.5 \cdot 10^{-7}$), while HLA-B was upregulated ($p=0.009$), as determined by student's t-tests (Fig. 3). There was no eminent change in HLA-A and HLA-DRA protein expression. As a final note, B2M expression of non-cancerous tissue tended to be decreased in patients with PSA recurrence ($p=0.06$), possibly indicating that deregulation of B2M is correlated with PCa progression (data not shown).

Treatment of PCa cells with HDACIs results in downregulation of mitotic spindle checkpoint genes

Our AFA results indicated that HDACIs changed the expression of genes relevant to mitosis as well (Fig. 2, Figs. S17 and S19 at <http://luigimarchionni.org/HDACIs.html>). In the supplementary data (<http://luigimarchionni.org/HDACIs.html>) a summary can be found of genes contributing to altered expression of the "Cell cycle, mitotic" Reactome FGS in DU-145 and PC3 cells treated with VPA or vorinostat. Treatment of DU-145 cells with 1 mM VPA or 1 μ M vorinostat resulted in a transcriptional downregulation of genes coding for kinetochore and other mitotic proteins (adjusted p-value $< 10^{-9}$). In contrast, the more resistant PC3 cells showed less downregulation (adjusted p-value $< 10^{-9}$ only after 96h treatment with VPA) (Fig. 2 and <http://luigimarchionni.org/HDACIs.html>).

Without proper kinetochore formation, chromosomes will not divide equally across the two daughter cells. To prevent aneuploidy, cells activate the spindle assembly checkpoint, which prevents progression through mitosis if metaphase is not completed properly.⁴⁸ We hypothesized that treatment with HDACis VPA or vorinostat could result in a functional spindle checkpoint defect by downregulation of kinetochore proteins and other proteins involved in this checkpoint. In PC3 cells we expected the spindle checkpoint to be intact, as there is less transcriptional downregulation of mitotic spindle checkpoint and kinetochore genes. To test this hypothesis, DU-145 and PC3 cells were synchronized in S-phase by a double thymidine block (Fig. 1), released and subsequently treated with colcemid—a spindle poison that depolymerizes microtubules and limits microtubule formation in mitosis. Cells with an intact spindle checkpoint will recognize a defect in microtubule attachment through kinetochore signaling and be arrested in mitosis. In the absence of a functioning kinetochore, the spindle checkpoint is not intact and cells progress through mitosis in the absence of proper chromosome segregation by microtubules. Both PC3 and DU-145 cells accumulated in G2/M-phase after 18h of treatment with colcemid; a small sub-G0 population was measured as well. Therefore, it can be assumed that these cells have an intact spindle assembly checkpoint (Fig. 4A). Separate doses of colcemid were chosen for DU-145 and PC3 cells since the standard dose of 0.1 $\mu\text{g}/\text{ml}$ led to toxicity in DU-145 cells (Fig. 4A). Next, we treated synchronized PC3 and DU-145 cells after release from S-phase with SAHA or simultaneously with HDACis and colcemid. During the 20 h after administration of 9 μM SAHA, most PC3 cells were initially in the G2/M phase, but later in G1-phase as well. Treatment of DU-145 cells with SAHA resulted in an increased G1- and (later) sub-G0 population. After combination treatment, PC3 cells mainly accumulated in G2/M-phase after ≥ 9 h of treatment, most likely these cells are mitotically arrested by activation of the spindle checkpoint (Fig. 4B; Fig. S20 at <http://luigimarchionni.org/HDACis.html>). On the other hand, treatment of DU-145 cells for ≥ 9 h resulted in a time-dependent increase in a large sub-G0 population apart from a population in G2/M phase, the latter population getting smaller after 20 h of treatment (Fig. 4C; Fig. S21 at <http://luigimarchionni.org/HDACis.html>). This may indicate that part of the HDACi-sensitive cells (DU-145 cells) override the spindle checkpoint, leading to a G0/G1 arrest and eventually apoptosis, while cells that are relatively resistant to HDACis (PC3 cells) are arrested in mitosis by activation of the spindle assembly checkpoint.

Discussion

In the past decade, a growing number of scientists have concluded that single agents, often targeting a single pathway, are insufficiently effective for cancer treatment, as many complex signaling pathways are involved in cancer development and progression. Therefore, clinical trials have shifted focus to combination therapy to either target different pathways or target the same major pathway from different angles. However, many recent phase III studies have

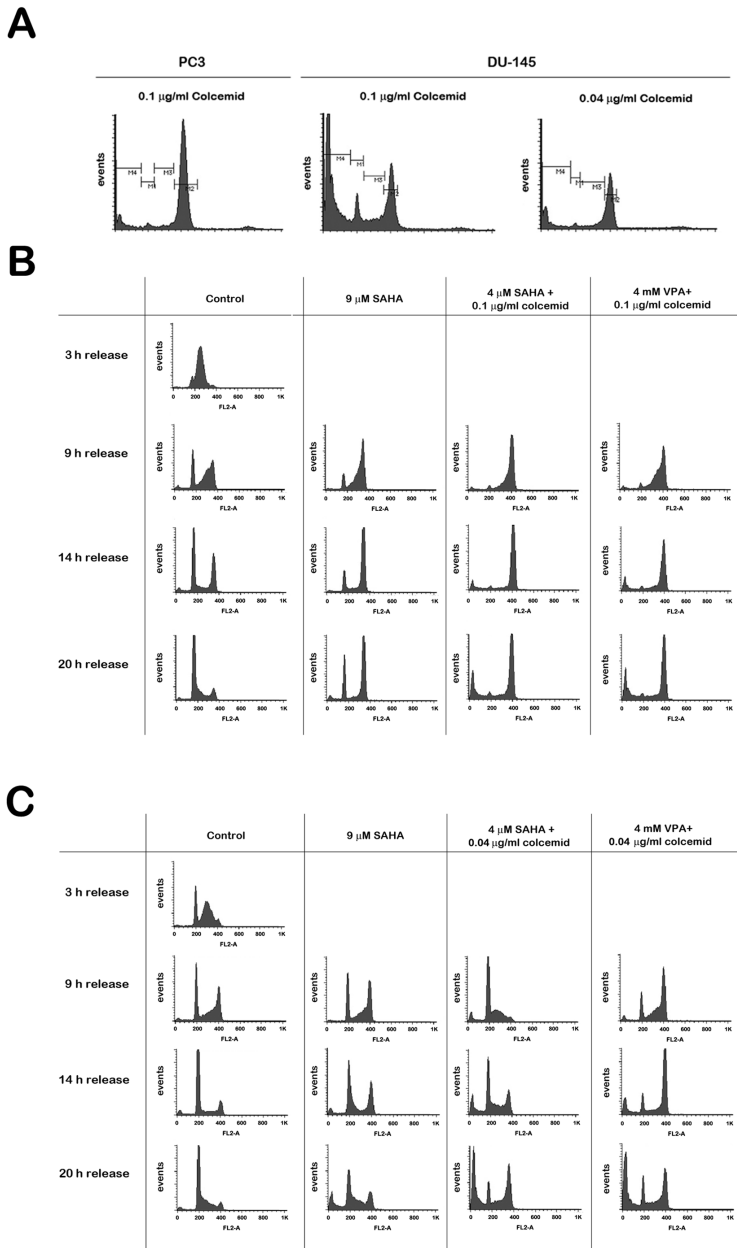


Figure 4. HDACis and spindle checkpoint activation in PCa cells. Simultaneous inhibition of PCa cells with HDACis and colcemid leads to accumulation of cells in mitosis in PC3 cells, but to a time-dependent increase of a sub-G0 population in DU-145 cells. A) DU-145 and PC3 cells were treated with 0.1 $\mu\text{g/ml}$ colcemid. This resulted in mitotic accumulation of PC3 cells (M2), but DU-145 cells exhibited an increased sub-G0 population (M4), indicating cytotoxicity. DU-145 cells were successfully arrested in mitosis with low toxicity at a final concentration of 0.04 $\mu\text{g/ml}$ colcemid. Combining HDACis with colcemid resulted in mitotic accumulation of PC3 cells (B), while it resulted in a time-dependent increase of a sub-G0 population in DU-145 cells (C).

concluded that clinical benefit of these combination therapies is limited, while toxicity is increased.⁴⁹⁻⁵¹ To speed up the implementation of combination therapies in clinic and to reduce costs of failing expensive clinical trials, there is a strong need to develop preclinical tools to identify successful combination strategies.

In this study, we show that AFA allows unbiased hypothesis generation for combination therapies. This systems biology approach successfully unraveled the mechanisms of action of HDACis, without the bias of assumptions based on previous literature or the preference of the researcher. We show major FGS expression changes after HDACi-treatment, induced either directly through regulation of histones or indirectly by regulating (multiple) genes in pathways. A major advantage of AFA is that it is able to identify and visualize modest gene expression changes when occurring across a predefined category, without prior arbitrary cut-offs for fold change, p-value, and/or false discovery rates (FDR). This is particularly relevant since previous studies have indicated that fluctuations in such basal gene expression patterns at levels less than what is considered significant (i.e., $FDR < 0.05$) do have a functional relationship.⁵² Furthermore, AFA combines biological information from different knowledge domains and different conditions and is thus equal to GSEA done over multiple contrasts. FGS representing similar biological concepts proved to be in agreement with each other, supporting the strength of AFA. In summary, AFA enables us to identify specific biological and molecular processes affected by chemotherapeutic agents in greater detail, as subtle but consistent changes can be identified, providing a framework for further investigation.

Hence, we performed differential gene expression analysis and applied AFA to four distinct data sets, ultimately analyzing over 42,475 distinct FGS over a total of 375 different samples. This analysis, which we made publically available online, is the largest performed on PCa cells treated with HDACis. Therefore, it constitutes an invaluable resource for the identification of FGS that are targeted by HDACis. Finally, we have investigated two of the identified molecular concepts for which multiple FGS proved affected upon HDAC-inhibition more into detail, namely, MHC genes and mitotic spindle checkpoint genes.

The immune system plays a prominent role in the development, progression and treatment of cancers, among others PCa.⁵³ Recognition of cancer cells by the immune system leads to eradication of these cells. Therefore, cancer cells need to escape this immune response (immune escape). This is accomplished by both genetic mutations and epigenetic modifications resulting in the loss of MHC class I and II molecules in some cancer cells (immuno-editing); these cells then get selected for further proliferation.⁵³ Indeed, we show in this study that in human primary PCa tissue samples MHC-related gene expression is deregulated. This is in line with earlier reports: for instance Sharpe et al. concluded that expression of MHC class I and II determinants (such as B2M) was downregulated in tissue samples from PCa patients.⁵⁴ While B2M expression is downregulated, HLA-A expression

does not differ significantly in our results. Tamura et al. concluded that expression of HLA-A is downregulated when PCa cells progress from hormone therapy sensitive to hormone resistant PCa.⁵⁵ Downregulation of HLA-A may not have occurred in our patients' samples yet, as most cancers are in an early stage when a radical prostatectomy is performed. Immunotherapy has become a common approach to treat cancer. It can be used to target specific pathways, such as the use of trastuzumab to target HER2 positive breast cancer cells,⁴³ or to modulate the immune system against cancer cells. The role of immunotherapy in PCa treatment is less well established. Although it is generally accepted that the immune system plays a role in PCa, the exact mechanism by which the immune system is involved in PCa initiation, progression and treatment remains unclear.^{54, 56} Sipuleucel-T has been approved by the US FDA as a cellular immunotherapy that improves overall survival in asymptomatic or minimally symptomatic metastatic castrate-resistant PCa patients, and other immunotherapies such as ipilimumab are in advanced stages of clinical development.^{57, 58} However, some have doubted its efficacy.^{59, 60} Therefore, immunotherapy is not yet widely used in PCa treatment.

As VPA and vorinostat upregulate FGS involved in the immune system in DU-145 and PC3 cells, treatment by HDACis may prompt a natural or induced immune response against tumor cells by the immune system or immunotherapy, respectively. Studies in other cancer types have also found that HDACis enhance the expression of immunologically important molecules and induce immune responses, strengthening our findings.⁶¹ As there currently is no tissue available from patients after treatment with HDACis, it is not possible to assess whether such immune responses occur in PCa patients. However, our results indicate that HDACis may revert the acquired B2M deficiency found in cancer cells resistant to immunotherapy.⁶² Considering the increasing importance of immunotherapy in PCa, it is imperative that future studies test the hypothesis that pretreatment with HDACis may sensitize PCa cells for immunotherapy. The clinical response to sipuleucel-T, ipilimumab and/or other immunotherapies may be increased through reversal of (epi)genetic modifications that lead to immune escape of the cancer cell.

Results from our study further indicate that posttranslational changes may not be solely responsible for the regulatory effects of HDACis during mitosis, but translational downregulation of genes involved in mitotic regulation may also play a role in creating mitotic defects. Treatment with high doses of SAHA seems to result in progression through mitosis in the absence of a mitotic spindle in HDACi-sensitive cells. This may lead to aneuploidy, a G0/G1 phase arrest and eventually to cell death.⁶³ Similar results have been reported by Noh et al.,⁶⁴ in which treatment of HeLa cells with Trichostatin A resulted in a transcription-dependent defective mitosis with loss of checkpoint control. This effect may be increased when treating PCa cells with agents that deregulate the spindle assembly checkpoint. Combination therapy of HDACis with one such spindle checkpoint deregulator,

aurora kinase inhibitors, has recently been tested in lymphoma cells *in vitro* and *in vivo*, with success.⁶⁵ Both results from this study and our results encourage testing combinations of HDACis with aurora kinase inhibitors or other agents that deregulate the spindle checkpoint, such as polo-like kinase 1 (Plk1-)inhibitors, in PCa and other solid tumors. Preliminary results of preclinical studies by our group involving combination therapies with HDACis (VPA/vorinostat) and an aurora kinase inhibitor (AMG 900) or Plk1-inhibitors (BI 2536/volasertib [BI 6727]) in PCa indicate that these combinations result in increased antitumor activity with acceptable toxicities.^{66, 67}

Our AFA results identified other FGS differentially expressed upon HDACi-treatment that may be of clinical importance too, such as upregulation of the androgen receptor signaling pathway. Increased sensitivity to androgen therapy after VPA treatment has been reported with other PCa cell lines.⁶⁸ Our data suggest that DU-145 and PC3 cells may have increased sensitivity to androgen therapy after HDACi-treatment as well, potentially by upregulation of androgen receptor expression.⁸ Further studies need to be performed to assess increased androgen sensitivity after HDACi-treatment in PCa.

In summary, in this study we successfully applied AFA. This created an expansive and complete library of biological processes modulated by HDACis in PCa cell lines, and hence is a valuable resource for researchers involved in research with HDACis. We further explored two pathways that were differentially expressed upon HDACi-treatment that may be of interest for future combination therapy. Novel studies combining HDACis with inhibitors of identified pathways are needed to assess clinical benefit for PCa patients. Furthermore, this study indicates that AFA can be used as an unbiased rational approach to identify novel combination strategies against cancer. Performing such analyses generates a comprehensive list of FGS differentially expressed upon cancer cell treatment.

Acknowledgments

The authors would like to thank Marianna Zahurak for her contribution to the statistical analysis for this project and René Medema for his support and discussion.

References

1. Villar-Garea A, Esteller M. Histone deacetylase inhibitors: understanding a new wave of anticancer agents. *Int J Cancer* 2004;112(2):171-178.
2. Prince HM, Bishton MJ, Harrison SJ. Clinical studies of histone deacetylase inhibitors. *Clin Cancer Res* 2009;15(12):3958-3969.
3. Munster P, Marchion D, Bicaku E et al. Clinical and biological effects of valproic acid as a histone deacetylase inhibitor on tumor and surrogate tissues: phase I/II trial of valproic acid and epirubicin/FEC. *Clin Cancer Res* 2009;15(7):2488-2496.
4. Candelaria M, Gallardo-Rincon D, Arce C et al. A phase II study of epigenetic therapy with hydralazine and magnesium valproate to overcome chemotherapy resistance in refractory solid tumors. *Ann Oncol* 2007;18(9):1529-1538.
5. Arce C, Perez-Plasencia C, Gonzalez-Fierro A et al. A proof-of-principle study of epigenetic therapy added to neoadjuvant doxorubicin cyclophosphamide for locally advanced breast cancer. *PLoS One* 2006;1:e98.
6. Mahalingam D, Medina EC, Esquivel JA et al. Vorinostat enhances the activity of temsirolimus in renal cell carcinoma through suppression of survivin levels. *Clin Cancer Res* 2010;16(1):141-153.
7. Chen X, Wong JY, Wong P, Radany EH. Low-dose valproic acid enhances radiosensitivity of prostate cancer through acetylated p53-dependent modulation of mitochondrial membrane potential and apoptosis. *Mol Cancer Res* 2011;9(4):448-461.
8. Alimirah F, Chen J, Basrawala Z, Xin H, Choubey D. DU-145 and PC-3 human prostate cancer cell lines express androgen receptor: implications for the androgen receptor functions and regulation. *FEBS Lett* 2006;580(9):2294-2300.
9. Seo SK, Jin HO, Woo SH et al. Histone deacetylase inhibitors sensitize human non-small cell lung cancer cells to ionizing radiation through acetyl p53-mediated c-myc down-regulation. *J Thorac Oncol* 2011;6(8):1313-1319.
10. Schaeffer EM, Marchionni L, Huang Z et al. Androgen-induced programs for prostate epithelial growth and invasion arise in embryogenesis and are reactivated in cancer. *Oncogene* 2008;27(57):7180-7191.
11. Daniel VC, Marchionni L, Hierman JS et al. A primary xenograft model of small-cell lung cancer reveals irreversible changes in gene expression imposed by culture in vitro. *Cancer Res* 2009;69(8):3364-3373.
12. Kim SY, Volsky DJ. PAGE: parametric analysis of gene set enrichment. *BMC Bioinformatics* 2005;6:144.
13. Mootha VK, Lepage P, Miller K et al. Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics. *Proc Natl Acad Sci U S A* 2003;100(2):605-610.
14. Subramanian A, Tamayo P, Mootha VK et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;102(43):15545-15550.
15. Kortenhorst MS, Zahurak M, Shabbeer S et al. A multiple-loop, double-cube microarray design applied to prostate cancer cell lines with variable sensitivity to histone deacetylase inhibitors. *Clin Cancer Res* 2008;14(21):6886-6894.
16. Hudson RS, Yi M, Esposito D et al. MicroRNA-1 is a candidate tumor suppressor and prognostic marker in human prostate cancer. *Nucleic Acids Res* 2012;40(8):3689-3703.

17. Lamb J. The Connectivity Map: a new tool for biomedical research. *Nat Rev Cancer* 2007;7(1):54-60.
18. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. *For. BMJ* 2002;325(7365):648-651.
19. Ihaka R, Gentleman RR. A language for data analysis and graphics. *Journal of Computational and Graphical Statistics* 1996;5:299-314.
20. Gentleman RC, Carey VJ, Bates DM et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004;5(10):R80.
21. Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* 2004;3:Article 3.
22. Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* 2005;21(9):2067-2075.
23. Smyth GK, Speed T. Normalization of cDNA microarray data. *Methods* 2003;31(4):265-273.
24. Smyth GK, Yang YH, Speed T. Statistical issues in cDNA microarray data analysis. *Methods Mol Biol* 2003;224:111-136.
25. Smyth GK. Limma: linear models for microarray data. In: Gentleman R, Carey RV, Dudoit S, Irizarry R, Huber W, editors. *Bioinformatics and Computational Biology Solutions using R and Bioconductor*. Springer; 2005:397-420.
26. Ross AE, Marchionni L, Vuica-Ross M et al. Gene expression pathways of high grade localized prostate cancer. *Prostate* 2011.
27. Ross AE, Marchionni L, Phillips TM et al. Molecular effects of genistein on male urethral development. *J Urol* 2011;185(5):1894-1898.
28. Benassi B, Flavin R, Marchionni L et al. c-Myc is activated via USP2a-mediated modulation of microRNAs in prostate cancer. *Cancer Discovery* 2012;2:236-247. [Published OnlineFirst January 5, 2012].
29. Croft D, O'Kelly G, Wu G et al. Reactome: a database of reactions, pathways and biological processes. *Nucleic Acids Res* 2011;39(Database issue):D691-D697.
30. Goel R, Harsha HC, Pandey A, Prasad TS. Human Protein Reference Database and Human Proteinpedia as resources for phosphoproteome analysis. *Mol Biosyst* 2012;8(2):453-463.
31. Ashburner M, Ball CA, Blake JA et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000;25(1):25-29.
32. Harris MA, Clark J, Ireland A et al. The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Res* 2004;32(Database issue):D258-D261.
33. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 2000;28(1):27-30.
34. Kanehisa M, Goto S, Hattori M et al. From genomics to chemical genomics: new developments in KEGG. *Nucleic Acids Res* 2006;34(Database issue):D354-D357.
35. Kanehisa M, Araki M, Goto S et al. KEGG for linking genomes to life and the environment. *Nucleic Acids Res* 2008;36(Database issue):D480-D484.
36. Mootha VK, Lindgren CM, Eriksson KF et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 2003;34(3):267-273.
37. Wheeler DL, Barrett T, Benson DA et al. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* 2008;36(Database issue):D13-D21.
38. Sayers EW, Barrett T, Benson DA et al. Database resources of the National Center for

- Biotechnology Information. *Nucleic Acids Res* 2012;40(Database issue):D13-D25.
39. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B* 1995;57(1):289-300.
 40. Roy S, Jeffrey R, Tenniswood M. Array-based analysis of the effects of trichostatin A and CG-1521 on cell cycle and cell death in LNCaP prostate cancer cells. *Mol Cancer Ther* 2008;7(7):1931-1939.
 41. Vindelov LL, Christensen IJ, Nissen NI. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* 1983;3(5):323-327.
 42. He X, Marchionni L, Hansel DE et al. Differentiation of a highly tumorigenic basal cell compartment in urothelial carcinoma. *Stem Cells* 2009;27(7):1487-1495.
 43. Cerami EG, Gross BE, Demir E et al. Pathway Commons, a web resource for biological pathway data. *Nucleic Acids Res* 2011;39(Database issue):D685-D690.
 44. Clauset A, Newman ME, Moore C. Finding community structure in very large networks. *Phys Rev E Stat Nonlin Soft Matter Phys* 2004;70(6 Pt 2):066111.
 45. van den Elsen PJ, Holling TM, Kuipers HF, van der SN. Transcriptional regulation of antigen presentation. *Curr Opin Immunol* 2004;16(1):67-75.
 46. Gobin SJ, Peijnenburg A, Keijsers V, van den Elsen PJ. Site alpha is crucial for two routes of IFN gamma-induced MHC class I transactivation: the ISRE-mediated route and a novel pathway involving CIITA. *Immunity* 1997;6(5):601-611.
 47. Irizarry RA, Warren D, Spencer F et al. Multiple-laboratory comparison of microarray platforms. *Nat Methods* 2005;2(5):345-350.
 48. Bolanos-Garcia VM. Assessment of the mitotic spindle assembly checkpoint (SAC) as the target of anticancer therapies. *Curr Cancer Drug Targets* 2009;9(2):131-141.
 49. Philip PA, Benedetti J, Corless CL et al. Phase III study comparing gemcitabine plus cetuximab versus gemcitabine in patients with advanced pancreatic adenocarcinoma: Southwest Oncology Group-directed intergroup trial S0205. *J Clin Oncol* 2010;28(22):3605-3610.
 50. Herbst RS, Ansari R, Bustin F et al. Efficacy of bevacizumab plus erlotinib versus erlotinib alone in advanced non-small-cell lung cancer after failure of standard first-line chemotherapy (BeTa): a double-blind, placebo-controlled, phase 3 trial. *Lancet* 2011;377(9780):1846-1854.
 51. Rosenthal SA, Bae K, Pienta KJ et al. Phase III multi-institutional trial of adjuvant chemotherapy with paclitaxel, estramustine, and oral etoposide combined with long-term androgen suppression therapy and radiotherapy versus long-term androgen suppression plus radiotherapy alone for high-risk prostate cancer: preliminary toxicity analysis of RTOG 99-02. *Int J Radiat Oncol Biol Phys* 2009;73(3):672-678.
 52. Hughes TR, Marton MJ, Jones AR et al. Functional discovery via a compendium of expression profiles. *Cell* 2000;102(1):109-126.
 53. Tomasi TB, Magner WJ, Khan AN. Epigenetic regulation of immune escape genes in cancer. *Cancer Immunol Immunother* 2006;55(10):1159-1184.
 54. Sharpe JC, Abel PD, Gilbertson JA, Brawn P, Foster CS. Modulated expression of human leucocyte antigen class I and class II determinants in hyperplastic and malignant human prostatic epithelium. *Br J Urol* 1994;74(5):609-616.
 55. Tamura K, Furihata M, Tsunoda T et al. Molecular features of hormone-refractory prostate cancer cells by genome-wide gene expression profiles. *Cancer Res* 2007;67(11):5117-5125.
 56. Josson S, Nomura T, Lin JT et al. Beta2-microglobulin induces epithelial to mesenchymal transition and confers cancer lethality and bone metastasis in human cancer cells. *Cancer*

- Res 2011;71(7):2600-2610.
57. Small EJ, Schellhammer PF, Higano CS et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. *J Clin Oncol* 2006;24(19):3089-3094.
 58. van den Eertwegh AJ, Versluis J, van den Berg HP et al. Combined immunotherapy with granulocyte-macrophage colony-stimulating factor-transduced allogeneic prostate cancer cells and ipilimumab in patients with metastatic castration-resistant prostate cancer: a phase 1 dose-escalation trial. *Lancet Oncol* 2012;13(5):509-517.
 59. Kantoff PW, Higano CS, Shore ND et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010;363(5):411-422.
 60. Huber ML, Haynes L, Parker C, Iversen P. Interdisciplinary critique of sipuleucel-T as immunotherapy in castration-resistant prostate cancer. *J Natl Cancer Inst* 2012;104(4):273-279.
 61. Khan AN, Tomasi TB. Histone deacetylase regulation of immune gene expression in tumor cells. *Immunol Res* 2008;40(2):164-178.
 62. Bernal M, Ruiz-Cabello F, Concha A, Paschen A, Garrido F. Implication of the beta2-microglobulin gene in the generation of tumor escape phenotypes. *Cancer Immunol Immunother* 2012;61(9):1359-1371.
 63. Ma X, Ezzeldin HH, Diasio RB. Histone deacetylase inhibitors: current status and overview of recent clinical trials. *Drugs* 2009;69(14):1911-1934.
 64. Noh EJ, Lim DS, Jeong G, Lee JS. An HDAC inhibitor, trichostatin A, induces a delay at G2/M transition, slippage of spindle checkpoint, and cell death in a transcription-dependent manner. *Biochem Biophys Res Commun* 2009;378(3):326-331.
 65. Kretzner L, Scuto A, Dino PM et al. Combining histone deacetylase inhibitor vorinostat with aurora kinase inhibitors enhances lymphoma cell killing with repression of c-Myc, hTERT, and microRNA levels. *Cancer Res* 2011;71(11):3912-3920.
 66. Wissing MD, Mendonca J, Kortenhorst MSQ et al. Targeting prostate cancer through a combination of Polo-like kinase inhibitors and histone deacetylase inhibitors [abstract]. In: Proceedings of the 101st Annual Meeting of the American Association for Cancer Research; 2010 Apr 17-21; Washington, DC. Philadelphia (PA): AACR; 2010. Abstract nr 5414.
 67. Paller CJ, Wissing MD, Kim E, et al. Preclinical profile of AMG 900 in combination with HDACIs in prostate cancer [abstract]. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Philadelphia (PA): AACR; 2012. Abstract nr 2049.
 68. Chou YW, Chaturvedi NK, Ouyang S et al. Histone deacetylase inhibitor valproic acid suppresses the growth and increases the androgen responsiveness of prostate cancer cells. *Cancer Lett* 2011;311(2):177-186.

Chapter 7

Targeting prostate cancer cell lines with polo-like kinase 1 inhibitors as a single agent and in combination with histone deacetylase inhibitors

Michel D. Wissing[†], Janet Mendonca[†], Madeleine S. Q. Kortenhorst, Nadine S. Kaelber, Matthew Gonzalez, Eunice Kim, Hans Hammers, Paul J. van Diest, Michael A. Carducci, Sushant K. Kachhap

[†]These authors contributed equally to this work.

FASEB J. 2013 Oct;27(10):4279-93

Abstract

Combinations of anticancer therapies with high efficacy and low toxicities are highly sought after. Therefore, we studied the effect of polo-like kinase 1 (Plk1) inhibitors on prostate cancer cells as a single agent and in combination with histone deacetylase (HDAC) inhibitors valproic acid and vorinostat. IC₅₀s of Plk1 inhibitors BI 2536 and BI 6727 were determined in prostate cancer cells by MTS assays. Morphological and molecular changes were assessed by immunoblotting, immunofluorescence, flow cytometry, real-time RT-PCR, and pulldown assays. Efficacy of combination therapy was assessed by MTS and clonogenic assays. IC₅₀ values in DU145, LNCaP, and PC3 cells were 50, 75, and 175 nM, respectively, for BI 2536 and 2.5, 5, and 600 nM, respectively, for BI 6727. Human prostate fibroblasts and normal prostate epithelial cells were unaffected at these concentrations. While DU145 and LNCaP cells were solely arrested in mitosis upon treatment, PC3 cells accumulated in G2 phase and mitosis, suggesting a weak spindle assembly checkpoint. Combining Plk1 inhibitors with HDAC inhibitors had synergistic antitumor effects *in vitro*. DMSO-treated prostate cancer cells were used as controls to study the effect of Plk1 and HDAC inhibition. Plk1 inhibitors decreased proliferation and clonogenic potential of prostate cancer cells. Hence, Plk1 may serve as an important molecular target for inhibiting prostate cancer. Combining HDAC inhibitors with BI 2536 or BI 6727 may be an effective treatment strategy against prostate cancer.

Introduction

Of the five known polo-like kinases (Plks), Plk1 has been studied most extensively. Plk1 is a serine-threonine kinase characterized by a N-terminal protein kinase domain and two highly specific C-terminal polo-box domains (PBDs).¹ It is crucial during cell division, playing an important role from G2/M transition through cytokinesis;² it may have functions beyond mitosis as well.^{3, 4} Elevated levels of Plk1 have been found in multiple cancers, including prostate cancer (PCa), colorectal cancer, breast cancer and non-small cell lung cancer.⁵ Plk1 overexpression is associated with a poor prognosis in various cancer types, making Plk1 a potential marker for cancer progression.^{2, 5, 6} Because of its highly specific PBD domains and overexpression in multiple cancers, several Plk1 inhibitors have been developed and are being tested in phase I and/or II clinical trials for treating both solid and non-solid tumors.² Two dihydropteridinone derivatives, BI 2536 and BI 6727 (Volasertib), act as Plk1 inhibitors by inhibiting the enzyme activity of Plk1 in an ATP-competitive way, resulting in significant antitumor activity *in vitro* and *in vivo* in a wide variety of cancer cell lines.^{2, 7, 8} In *in vitro* kinase assays, BI 2536 inhibits Plk1, as well as the two closely related kinases, Plk2 and Plk3, at lower nanomolar concentrations (half maximal inhibitory concentration (IC₅₀) values 0.83, 3.5, and 9 nM, respectively); similarly, BI 6727 potently inhibits Plk1, as well as Plk2 and Plk3 (IC₅₀ values 0.87, 5, and 56 nM, respectively), but it is ineffective against a panel of 50 known kinases, even at 10 μ M concentrations.⁷ Phase I and II studies conducted with BI 2536 as a single agent against various cancers, including metastatic castrate-resistant PCa, reported some antitumor effects in patients, while the compound was well tolerated.⁹⁻¹² BI 6727 is expected to be more potent against tumors due to its favorable pharmacokinetic properties, demonstrating sustained tumor exposure, a high volume of distribution, a long terminal half-life, and good oral bioavailability.⁸ A phase I study with BI 6727 in patients with advanced solid tumors confirmed these preclinical observations, the compound having a favorable pharmacokinetic profile, promising antitumor activity and manageable toxicities.¹³ Combining Plk1 inhibitors, which arrest cells in mitosis, with agents that arrest cells in other phases of the cell cycle could potentially further enhance cancer cell death.

In this study, we tested BI 2536 and BI 6727 in PCa cell lines both as a single agent and in combination with histone deacetylase (HDAC) inhibitors valproic acid (VPA) and vorinostat (suberoylanilide hydroxamic acid (SAHA)). HDACs deacetylate lysine residues in the N-terminal tails of histones, thereby blocking gene transcription; therefore, inhibition of HDACs changes the expression of a wide variety of genes in cancer cells, leading to growth arrest and/or apoptosis.^{14, 15} Although HDAC inhibitors were initially hypothesized to upregulate silenced genes only, we and others have found a significant number of genes silenced upon HDAC inhibition in PCa cell lines.¹⁶ Using analysis of functional annotation (AFA) we found multiple pathways downregulated by HDAC inhibitors, several of these being involved in mitosis and the cell cycle, such as Plk1.¹⁷ We speculated that combining Plk1

with HDAC inhibitors would have an additive and potentially synergistic effect in inhibiting PCa cells. Our rationale for combining the two inhibitors for treatment of PCa was 2-fold. First, building on our AFA data, we hypothesized that combining HDAC and Plk1 inhibitors might target Plk1 function through two different approaches. HDAC inhibition would lead to downregulation of Plk1 transcript and, hence, less Plk1 protein molecule per cell, which could be effectively inhibited at enzymatic level with the Plk1 inhibitor. Second, HDAC and Plk1 inhibitors inhibit cells in different stages of cell cycle. In an asynchronous culture, an HDAC inhibitor would effectively target cells in the G1/G2 phase of the cell cycle, while Plk1 inhibitors could target cells that are in the mitotic phase of the cell cycle. This could lead to an effective/enhanced inhibition in cell proliferation. Further, cells that are resistant to HDAC inhibition and progress through the interphase could be halted at mitosis by Plk1 inhibition and vice versa. To the best of our knowledge, this is the first report that combines HDAC inhibitors with Plk1 inhibitors for PCa treatment.

Materials and methods

Cell lines and treatment

PCa cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human prostate fibroblasts, kindly provided by dr. J. Isaacs (Johns Hopkins Medical Institutions, Baltimore, MD, US), were obtained from a prostate biopsy on a 62-year old patient with PCa having a Gleason score of 4. 957 E/human telomerase reverse transcriptase (hTERT) cells (hTERT-immortalized normal prostate epithelial cells),¹⁸ also kindly provided by dr. J. Isaacs, were grown in keratinocyte serum-free medium (K-SFM) supplemented with epithelial growth factor (EGF) and bovine pituitary extract (BPE) (Invitrogen, Carlsbad, CA, USA). All other cells were grown in Roswell Park Memorial Institute (RPMI)-1640 (Invitrogen) media supplemented with 10% fetal bovine serum (FBS). Cells were grown in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

Stock solutions of 10 mM of SAHA (AtonPharma, Lawrenceville, NJ, USA), BI 2536 and BI 6727 (Boehringer Ingelheim, Ingelheim am Rhein, Germany), made in dimethyl sulfoxide (DMSO), and stock solutions of 1M of sodium salt of VPA (Sigma-Aldrich, St. Louis, MO, USA), dissolved in complete RPMI media, were used for all experiments. For experiments, compounds were further diluted in complete RPMI media.

Proliferation assay

Cells were plated in 96-well plates containing 100 µl complete RPMI media and allowed to adhere overnight before treatment. Forty-eight hours after treatment, cell viability was measured using the CellTiter 96™ AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Absorption at 490nm was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Clonogenic assay

PCa cells were plated in 60 mm dishes and drugged at 50-60% confluency. Dishes were incubated for 48 h, after which 1×10^3 (DU145/PC3) or 2×10^3 cells (LNCaP) from each treated or control dish were plated in triplicate in 60 mm dishes and incubated for 12 days. Colonies were stained with a crystalviolet solution (Sigma-Aldrich) and counted manually.

Fluorescence microscopy

Cells were treated with BI 2536 or BI 6727 at 50-70% confluency. All cells, both floating and attached, were collected after 24 h of treatment, washed in phosphate-buffered saline (PBS) and fixed with 10% neutral buffered formalin (NBF). For visualization and quantification of polo-arrested cells, fixed cells were washed in PBS and their nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich). Samples (25 μ l) of the cell solution were mounted on slides. Cells were visualized with a Nikon Eclipse Ti inverted research microscope (Nikon Instruments, Linthicum, MD, USA). Multiple fields per slide were photographed, and the numbers of polo-arrested and non polo-arrested cells were counted manually.

For immunofluorescence, cells fixed in formalin were permeabilized with 0.125% Triton X-100 for 5 min. The permeabilized cells were incubated with primary antibodies for Plk1 (Antibody sc-5585, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or phosphorylated aurora kinases (Antibody #2914, Cell Signaling Technology). Cells were washed in PBS and probed with Alexa Fluor conjugated secondary antibodies (Molecular Probes, Invitrogen). Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich) and cells were mounted on slides. Confocal images were taken using the Zeiss LSM 510 meta-confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Plk1 activity assay

To assess Plk1 activity, glutathione S-transferase (GST)-PBD pulldown assays were performed according to Park et al.,¹⁹ with some modifications. The assay is based on the principle that active Plk1 phosphorylates the centromeric protein polo box interacting domain 1 (PBIP1) at T78 which creates a docking site resulting in a strong interaction between PBIP1 and a PBD domain of Plk1. Using tandemly linked PBIP1 motifs (6 repeats in our experiments) harboring the T78 phosphorylation site, expressed in bacteria as a GST fusion protein, active Plk1 can be pulled out from cells and tissue lysates, which can then be analyzed by Western blotting.

In brief, GST-PBIPtides were expressed and purified from *Escherichia coli* BL21 by using glutathione sepharose (GE Healthcare, Waukesha, WI, USA). Proteins bound to the beads were quantified by bicinchoninic acid (BCA) reagent (Pierce Biotechnology, Rockford, IL, USA). For GST-PBIPtide pulldown assays, PCa cells were lysed in lysis buffer (20 mM Tris-Cl (pH8.0), 150 mM NaCl, 0.5% Nonidet P-40, 1.5 mM EDTA, 1 X phosphoSTOP (Roche, Palo Alto, CA, USA), and 1X protease inhibitor (Roche)). The resulting 500 μ g of protein lysates

were clarified by centrifugation at 15,000 *g* for 20 minutes at 4 °C and incubated with bead-bound GST-PBIPtide (100 µg) to precipitate PBIPtide-bound Plk1. Bead-bound Plk1 was detected by Western blotting using a Plk1 antibody (Santa Cruz Biotechnology).

Flow cytometry

Cells were plated in 100 mm dishes and drugged at 50-70% confluency. After 24 h of treatment, both floating and attached cells were collected, washed in PBS and fixed in a solution containing 10% NBF. Cells were permeabilized in 90% methanol, probed with a primary antibody against phosphorylated H3, followed by an Alexa Fluor-488 conjugated secondary antibody. Nuclei were stained with Hoechst 33258 in PBS containing 10% FBS. Flow cytometry was performed on the LSRII 4-laser, 18-color benchtop flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analysed using BD FACSDiva™ software and FlowJo.

Immunoblotting and densitometry

Immunoblotting was performed as described previously.¹⁷ Total protein (10 µg) was used for electrophoresis and blotted on polyvinylidene difluoride (PVDF) membrane. Primary antibodies were diluted in blocking buffer (5% milk) to a 1:1000 dilution, except for vinculin and actin, which were diluted 1:4000. Secondary antibodies were used at a 1:4000 dilution. Blots were developed using enhanced chemoluminescence (ECL) (GE Healthcare) or Femto (Pierce Biotechnology) and density of bands was quantified by ImageJ software.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

PCa cells were treated at 50-70% confluency with BI 2536 or BI 6727 for 6 or 24 h. Cells were lysed using TRIzol (Invitrogen) and total RNA was extracted. Total RNA (1 µg) was used for complementary DNA (cDNA) synthesis using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). One tenth of the first strand cDNA reaction was used for RT-PCR amplification and analyzed further as described by Kachhap et al.¹⁷

Statistical methods

Studies to assess synergy in cell proliferation (i.e. in MTS assays) and clonogenic survival assays were performed utilizing CalcuSyn (Biosoft, Cambridge, UK) similar to previous preclinical studies testing combination therapies.^{20, 21} This software compares the antitumor effect of combination therapy at the provided doses to the (often extrapolated) doses of single agents required to produce an equal antitumor effect. To quantify levels of synergy, CalcuSyn calculates a combination index (CI) using the Chou-Talalay method.²² The calculated CI values were grouped in different levels of synergy (no synergy (CI>0.9), moderate synergy (0.7<CI≤0.9), synergy (0.3<CI≤0.7, +), strong synergy (0.1<CI≤0.3, ++), very strong synergy (CI≤0.1, +++)) according to the manufacturer's instructions.

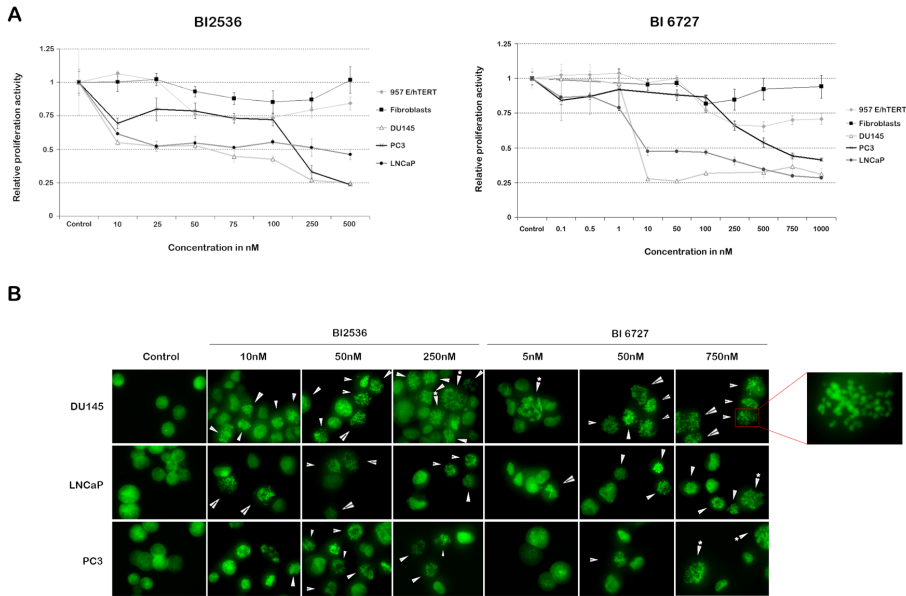


Figure 1. Plk1 inhibitors BI 2536 and BI 6727 reduce cell viability in PCa cells by inducing polo-arrest. A) MTS assays after treatment of PCa and normal human prostate cells with BI 2536 (left) or BI 6727 (right) for 48 h. B) Visualization of nuclei of PCa cells upon treatment for 24 h with BI 2536 or BI 6727 by staining with Hoechst 33258. Arrows point to polo-arrested cells, arrows with asterisk to enlarged polo-arrested cells.

Results

BI 2536 and BI 6727 cause defects in mitotic progression and inhibit proliferation of PCa cells.

Proliferation of PCa cells, as judged by MTS assays, was considerably reduced by both BI 2536 and BI 6727 (Fig. 1A). For BI 2536, the IC_{50} in DU145 cells was about 60nM, in LNCaP cells approximately 75 nM, and in PC3 cells about 175 nM (left graph, Fig. 1A). These IC_{50} values were below serum plasma levels as determined in patients treated with BI 2536, who had a C_{max} (maximum measured concentration in plasma) of 921 ng/ml (1.765 μ M).²³ It was noted that with increasing concentrations, proliferation of LNCaP cells was stably 50% lower compared to control cells, while in DU145 and PC3 cells proliferation activity decreased to 25% relative to controls. We inferred that this was most likely due to the slower proliferative nature of untreated LNCaP cells (doubling time 48 h) compared to DU145 (doubling time 35 h) and PC3 (doubling time 30 h) cells. BI 6727 effectively inhibited DU-145 and LNCaP cells: the IC_{50} in DU145 and LNCaP cells was in the low nanomolar range (<10 nM), but in PC3 cells, the IC_{50} was approximately 600 nM. Despite the relatively high IC_{50} in PC3 cells, this concentration was still comparable to serum plasma levels (C_{max} 2.346 μ M) established in patients treated with BI 6727.¹³

To explore whether the BI compounds have inhibitory effects on the proliferation of normal prostate cells, we treated 957 E/hTERT (doubling time 60 h), an immortalized normal prostate cell line, and human prostate fibroblast cells (doubling time 55 h) with the Plk1 inhibitors. As depicted in Fig. 1A, both cell lines were relatively unaffected by the inhibitors at concentrations that severely reduced the viability of cancer cells, a feature that may be attributed to the rapid proliferative nature of cancer cells compared to their normal counterparts, suggesting that at concentrations at which Plk1 inhibitors exhibit their effect in PCa cells, the inhibitors are not very toxic to healthy cells.

To investigate whether the reduction in cell proliferation was a result of Plk1 inhibition, we assessed the morphology of BI treated cells. Typical polo-arrested cells undergo mitotic dysregulation, exhibiting condensed chromosomes that fail to congress in the metaphase plate and appear randomly distributed through the cell, a feature attributed to monopolar spindles caused by Plk1 inhibition.² We visualized Hoechst 33258 stained nuclei of cells treated with Plk1 inhibitors for 24 h under a fluorescent microscope. Dysregulated mitosis was evident in DU145 and LNCaP cells after treatment with either BI compound at low nanomolar concentrations (≤ 50 nM), indicating that these cells were polo-arrested, while PC3 cells displayed mitotic dysregulation only at higher concentrations of the compounds (Fig. 1B, arrows). With an increase in concentration of the Plk1 inhibitors an increased number of enlarged cells, suggestive of polyploid cells or cells in G2/M phase, were also visible (Fig. 1B, arrows with asterisk and enlarged image). We next quantified these polo-arrested cells. Our data indicate that irrespective of the compound, DU145 cells exhibit most polo-arrested cells as compared to other PCa cell lines. This response cannot be solely attributed to a shorter doubling time of DU145 cells (± 24 h), as PC3 cells, which have a comparable doubling time, exhibited only 40-50% of polo-arrested cells when treated with BI 2536. This further suggested a differential response of PCa cells to both inhibitors.

BI 2536 and BI 6727 affect Plk1 enzyme activity in PCa cells differently.

Cancer cells are known to overexpress Plk1, which may provide a selective growth advantage.² As expected, both normal prostate cell lines had lower level of total Plk1 than their cancer counterparts. The differential response of PCa cells to Plk1 inhibitors could result from differences in dependence on Plk1 among the cell lines. Therefore, we first studied protein expression levels of total Plk1 after Plk1 inhibition by Western blot analysis (Fig. 2A). In general, cells treated with low nanomolar concentrations of the BI compounds did not exhibit a significant change in total Plk1 protein levels compared to untreated cells. However, with increasing concentrations of the BI compounds, an increase in total Plk1 was evident (Fig. 2A). BI 6727 treated DU145 cells were an exception: low nanomolar concentrations of the inhibitor increased the amount of Plk1 to its saturation level, as increasing concentrations did not demonstrate any further increase in Plk1 expression. This result may reflect the difference in response to BI 6727 as the compound is highly effective against DU145 cells

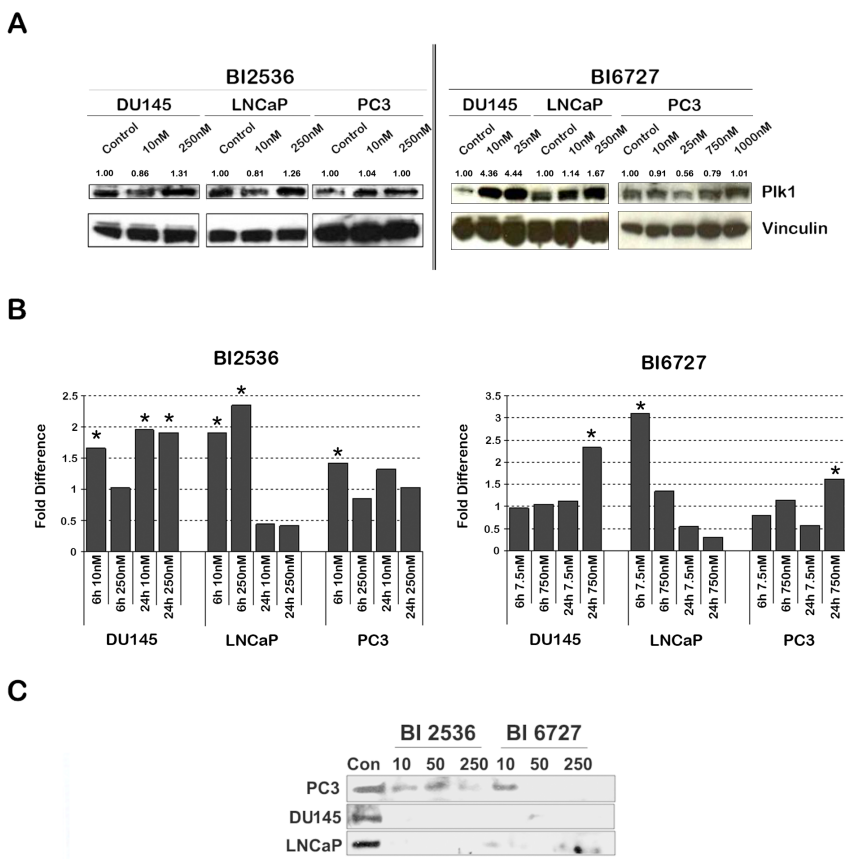


Figure 2. Plk1 inhibition leads to differential responses in Plk1 expression and activity among PCa cell lines. A) Western blot analyses for total Plk1 after treatment of PCa cells with Plk1 inhibitors for 24 h. B) Real time RT-PCRs performed after 6 and 24 h of BI treatment show mRNA levels of Plk1 in PCa cells after BI treatment. *, $p < 0.01$; concentrations in nM. C) Western blots indicating the levels of PBIPTide precipitated active Plk1 after GST-PBD pull down assays performed with BI treated PCa cell lysates. Numbers above Western blots indicate densitometric quantification of protein expression levels relative to untreated controls, normalized to the housekeeper vinculin.

even at lower nanomolar concentrations (as evident in Fig. 1A). To investigate whether the increase in Plk1 protein after treatment with the inhibitors was a direct effect of increased transcription, we quantified Plk1 transcripts by real-time RT-PCR. Messenger RNA (mRNA) levels of total Plk1 were measured after 6 h (early response) and 24 h (delayed response) of BI treatment. Plk1 expression was found to be induced in DU145 and LNCaP cells at different time points. Although LNCaP cells demonstrated an earlier response with both of the inhibitors, DU-145 cells exhibited a delayed response when treated with BI6727. Further, induction of Plk1 transcript in DU-145 cells was restricted to a higher concentration

of BI 6727. On the other hand, Plk1 expression in PC3 cells was modestly induced only when treated with a high concentration (750 nM) of BI 6727 (>1.5-fold increase; Fig. 2B). These results led us to infer that induction of Plk1 in DU145 and LNCaP cells could be a consequence of the cells being arrested in mitosis wherein Plk1 protein levels peak. We speculated that the increase in Plk1 protein after Plk1 inhibition seen in our Western blots may not reflect an increase in Plk1 activity. To ascertain this, Plk1 activity was determined by pulling down active Plk1 using the T78 PBIP1-GST peptide (see materials and methods). GST fusion PBIPtide proteins were expressed in BI21 cells and purified for the assay.¹⁹ Lysates of Plk1 inhibitor treated and control PCa cells were used to pull down active Plk1. Active Plk1 levels were assessed by Western blotting. As depicted in Fig. 2C, a marked reduction in Plk1

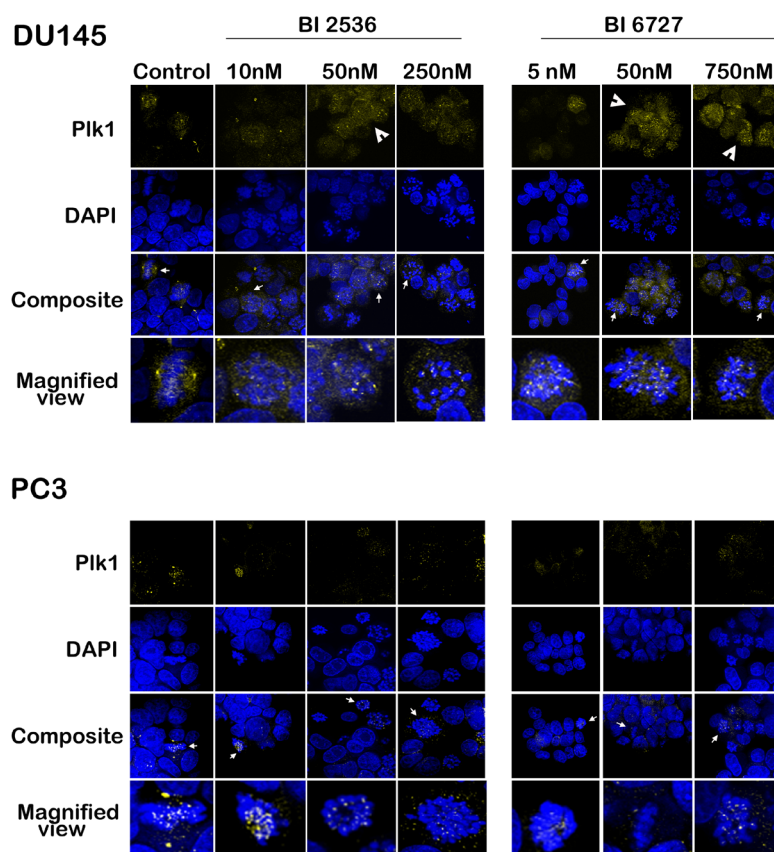


Figure 3. Plk1 inhibition results in a differential response in the cellular distribution of Plk1 (yellow) among PCa cell lines DU145 (top) and PC3 (bottom) as depicted by immunofluorescence.

Nuclei were stained with DAPI (blue). BI treatment led to an increase in cytoplasmic staining of Plk1 in DU145 cells (arrowheads). A representative cell in the composite image (arrow) is magnified below.

activity was evident in both DU145 and LNCaP cells after treatment with Plk1 inhibitors. Although Plk1 activity decreased after BI treatment compared to untreated cells in all PCa cells, Plk1 activity was markedly higher in BI treated PC3 cells compared to BI treated DU145 and LNCaP cells (Fig. 2C).

BI 2536 and BI 6727 affect the subcellular localization of Plk1 in PCa cells.

Because the subcellular localization of Plk1 changes relative to its function in different stages of mitosis and cytokinesis, we visualized Plk1 distribution by immunofluorescence after treating the cells with different concentrations of the BI compounds (Fig. 3). In untreated cells, Plk1 staining was most evident in mitosis where it seemed to localize to kinetochores as punctate dots, in the central spindle, in the centrosomal regions, and in the midbody during cytokinesis. This corroborates other reports in which Plk1 localized to the mitotic spindle poles and kinetochores during mitosis.^{24, 25} Consistent with earlier reports, we also observed centrosomal and faintly diffused cytoplasmic staining for Plk1 during interphase in untreated cells.²⁴ When BI 2536 and BI 6727 were administered, Plk1 localized to condensed chromosomes. Furthermore, an increase in cytoplasmic Plk1 staining was observed in DU145 cells, which seemed to be dose-dependent. However, PC3 cells exhibited this effect to a much lesser extent after similar treatment. Overall, PC3 cells demonstrated a distinct reduction in Plk1 staining and mitotic figures after BI treatment compared to DU145 cells. This observation is in accordance with our Western blot data, which indicated a larger increase in total Plk1 expression in DU145 cells compared to PC3 cells (Fig. 2A). Although there is an increase in Plk1 expression after treatment with the inhibitors, Plk1 activity is markedly reduced in DU145 cells, as demonstrated by the pulldown activity assay (Fig. 2C). Therefore, it is unlikely that the increased cytoplasmic localization of Plk1 seen through immunofluorescence is enzymatically active. The differences in subcellular localization of Plk1 further indicate a differential response of PCa cells to the inhibitors.

Expression and localization of active aurora kinases differ in PCa cells after BI 2536 and BI 6727 treatment

During mitosis, there is extensive crosstalk between Plk1 and aurora A and B kinases, two other key regulators of mitosis.²⁶ Aurora-A phosphorylates Plk1 during G2/M transition, while Plk1 controls aurora-A localization to the spindle poles through hBora.^{27, 28} Previous reports have indicated that Plk1 inhibition leads to mislocalization of aurora A.²⁷ Therefore, we assessed changes in aurora expression and localization after Plk1 inhibition. Because phosphorylation of aurora is necessary for the enzyme to exert its kinase activity, we determined active aurora A-C using an antibody that recognizes phosphorylated aurora A-C.²⁹ As indicated by Western blots, protein expression levels of phosphorylated aurora A-C in LNCaP cells were increased after treatment with high nanomolar concentrations of the BI compounds (Fig. 4A). Phosphorylated aurora A protein levels were elevated in DU145 cells

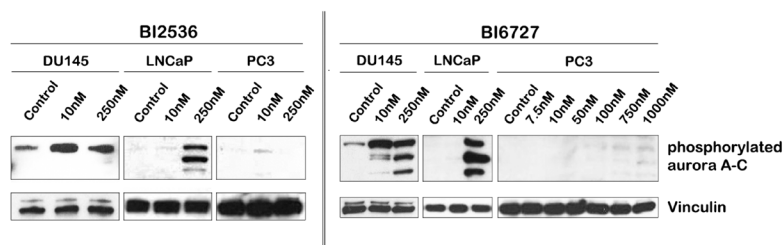
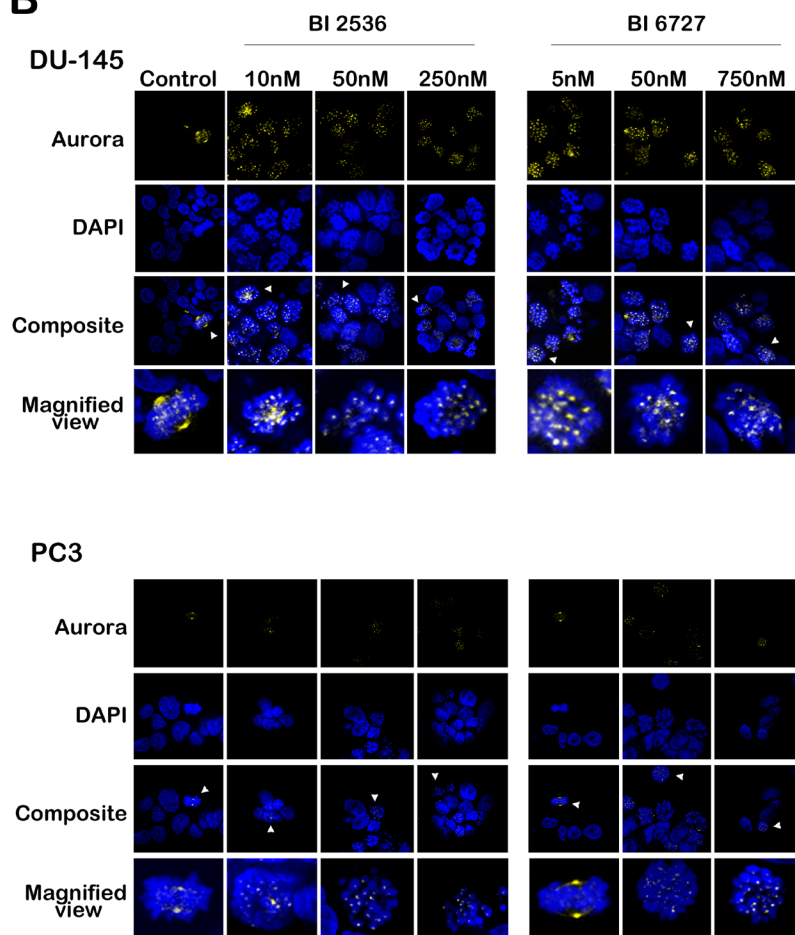
A**B**

Figure 4. Expression and localization of active aurora kinases differ between PCA cells after treatment with BI 2536 and BI 6727. A) Western blot analyses for phosphorylated aurora A-C (from high to low molecular weight: aurora A, B, C) after 24 h of Plk1 inhibition with BI 2536 and BI 6727 in PCA cell lines. B) Immunofluorescence for aurora (yellow) and DAPI (blue) in DU145 (top) and PC3 (bottom) cells treated for 24 h with BI 2536 (left) or BI 6727 (right). A representative cell (arrowhead) is magnified below.

when treated with BI 2536; in BI 6727 treated cells an increase in phosphorylated aurora A-C levels was observed with increasing dosage. Intriguingly, PC3 cells did not exhibit an increase in phosphorylated aurora proteins. To investigate whether this was not due to an aurora-null phenotype in PC3 cells, we performed a Western blot analysis in PC3 cells for total aurora kinase A and B (data not shown). Aurora A was the predominant isoform present in PC3 cells, which showed a slight increase on treatment with BI compounds. These data indicated that PC3 cells were not null for aurora protein kinase. To test whether PC3 cells lacked the ability to phosphorylate aurora kinase, protein expression levels of phosphorylated aurora A-C proteins were tested after nocodazole treatment. Phosphorylation of aurora kinases increased after nocodazole treatment in DU145, LNCaP, and PC3 cells, suggesting that the lack of phosphorylated aurora kinases in PC3 cells after treatment with the BI compounds was not due to their inability to phosphorylate aurora kinases. Thus, expression of phosphorylated aurora in response to treatment with the BI compounds differed among PCa cells.

As with Plk1, the localization of aurora kinases changes during progression through the cell cycle.²⁹ We visualized phosphorylated aurora A-C in untreated and BI treated PCa cells (Fig. 4B). Similar to previous reports, phosphorylated aurora A-C seemed to localize to kinetochores, poles, and spindles of mitotic cells in untreated controls.^{29, 30} PC3 cells arrested in mitosis after treatment with the compounds exhibited aurora localization similar to mitotically arrested BI treated DU145 cells. However, the total number of mitotic figures in PC3 cells was lower compared to DU145 cells, which may explain the lack of signal in the Western blot. Although we did not observe any marked increase in cytoplasmic aurora staining as observed in Plk1 after treatment, we did find a total lack of polar and spindle staining for aurora while kinetochore staining was retained. In this respect, our results differ from a previous report which found an increase in cytoplasmic aurora A after Plk1 knockdown.²⁷ One explanation could be the differences in antibodies used: while our observations are solely based on active/phosphorylated forms of aurora kinases, previous localization experiments were based on antibodies that recognized total aurora A. However, our results are in agreement with other reports that have observed impaired recruitment of aurora A to the centrosome and mitotic spindle.^{28, 30}

PCa cell lines exhibit variable degrees of mitotic arrest upon treatment with BI 2536 and BI 6727.

Next, we determined the effect of BI 2536 and BI 6727 on known markers of mitosis. Cells were treated for 24 h with the BI compounds at varying concentrations and protein expression levels were assessed by Western blots (Fig. 5A). At a final treatment concentration of 250nM with either compound, DU145 and LNCaP cells had a marked increase in phosphorylated histone H3, suggesting an increase in mitotic population. However, PC3 cells demonstrated only a slight increase in phosphorylated histone H3 when treated with higher nanomolar

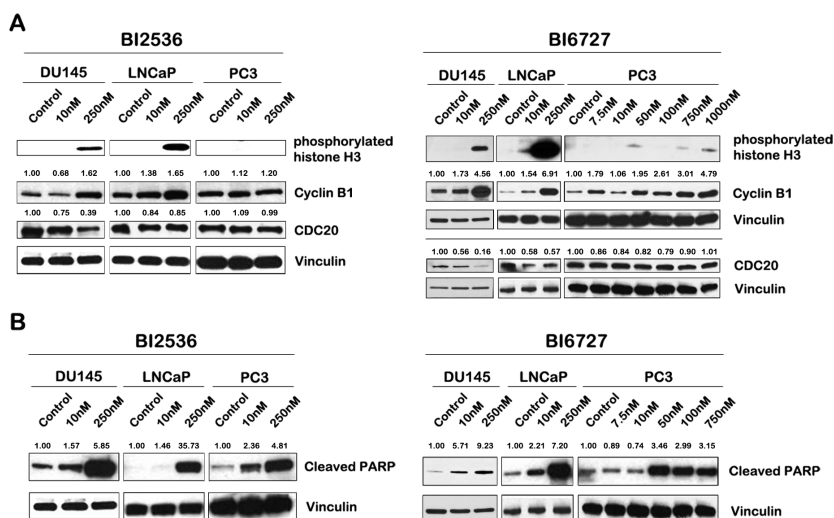


Figure 5. Mitotic markers (A), but not apoptotic markers (B) are differentially expressed in PCa cell lines after treatment with BI 2536 and BI 6727.

Numbers above Western blots indicate densitometric quantification of protein expression levels relative to untreated controls, normalized to the housekeeper vinculin.

concentrations of BI 6727, while there was an absence of any detectable protein when treated with BI 2536. Cyclin B1, another mitotic marker, increases late in G2-phase, and is degraded by cell-division cycle protein 20 (CDC20) when cells progress through the spindle assembly checkpoint (SAC), marking the progression from metaphase to anaphase.^{25, 31} DU145 and LNCaP cells had increased levels of cyclin B1 after BI treatment, suggesting cells are arrested early in mitosis. On the other hand, cyclin B1 protein levels remained unchanged compared to untreated controls in PC3 cells after treatment with BI 2536. Treatment of PC3 cells with high nanomolar concentrations of BI 6727 yielded an increase in cyclin B1 expression. This suggested that upon treatment with BI compounds, PC3 cells do undergo mitotic arrest, albeit at higher doses of BI compounds than DU145 and LNCaP cells. We next assessed the levels of CDC20, a protein that plays a key role in the anaphase promoting complex.³¹ Activation of the SAC leads to proteolysis of CDC20, causing a (pro)metaphase arrest. Treatment of DU145 and LNCaP cells with BI compounds led to a decrease in CDC20 protein levels, demonstrating activation of the SAC. However, PC3 cells did not show an appreciable change in CDC20 protein levels. This further adds to the notion that PC3 cells lack a strong SAC, which results in cells progressing through mitosis despite Plk1 inhibition, as was evident in the MTS assay results (Fig. 1A).

Prolonged mitotic arrest may trigger apoptosis.^{32, 33} To assess whether BI treatment leads to induction of apoptosis, we assessed poly (ADP-ribose) polymerase (PARP) cleavage as a surrogate marker for apoptosis (Fig. 5B). Cleaved PARP was increased in all three PCa cell

lines with increasing dosage of both BI compounds. After treatment with BI 6727, DU145, and LNCaP cells demonstrated an increase in cleaved PARP at 10 nM, while cleaved PARP was increased in PC3 cells at concentrations of 50 nM and higher only. Thus, both BI 2536 and BI 6727 have the ability to trigger apoptosis in all PCa cell lines, albeit to variable degrees. Since PC3 cells do undergo apoptosis, we wondered whether these cells are arrested in a cell cycle phase other than mitosis. We used p21 as a marker for G1 arrest and were surprised to find a significant dose-dependent induction of p21 in PC3 cells on treatment with both compounds (Fig. 6A). Analysis of mRNA levels of p21 revealed that PC3 cells demonstrated a marked increase in p21 transcripts after treatment with both compounds, while p21 was induced in DU145 cells only at higher nanomolar concentrations of BI 6727 (Fig. 6B). Since both PC3 and DU145 cells are deficient in p53, induction of p21 on Plk1 inhibition is through a p53-independent mechanism. Induction of p21 in PC3 cells suggests a G1 arrest; however, p21 is also known to cause G2 arrest in p53-deficient cells.^{34, 35} To distinguish between these possibilities, we conducted a fluorescence-activated cell sorting (FACS) analysis of PCa cells after treatment with BI 2536 and BI 6727 (Fig. 6C). Cells were stained for phosphorylated histone H3 to differentiate between cells in G2 phase and in mitosis. Our data indicated that all PCa cell lines exhibited mitotic arrest after BI treatment as indicated by the phosphorylated H3-positive fraction. However, the degree of arrest differed among cell lines. DU145 cells were most sensitive to BI 6727, followed by LNCaP and PC3 cells. LNCaP cells were slightly more sensitive to BI 2536, with 98% of the cells accumulated in mitosis at the highest concentration, while in DU145 and PC3 cells, only 67 and 45% of the cells were in mitosis, respectively. Although the baseline percentage of PC3 cells in G2 phase was low compared to other PCa cell lines, an increase in cells in G2 phase was observed on treatment with either BI compound. Thus, at higher concentrations, the BI compounds caused DU145 and LNCaP cells to accumulate in mitosis and PC3 cells to accumulate both in the G2 and mitotic phase of the cell cycle arrest, eventually triggering apoptosis. Moreover, LNCaP cells exhibited an increase in polyploid cells after Plk1 inhibition.

Combinations of Plk1 inhibitors BI 6727 or BI 2536 with HDAC inhibitors VPA or SAHA have synergistic effects in vitro.

On the basis of previous findings that HDAC inhibitors downregulate Plk1 expression, we argued that combining HDAC inhibitors with Plk1 inhibitors would increase cell death, as the combination would maximize inhibition of the Plk1 pathway. Besides, we and others have shown that HDAC inhibitors can inhibit growth and proliferation of cancer cells through multiple pathways, such as cell cycle arrest, apoptosis, angiogenesis inhibition, and senescence.^{15, 36, 37} To ascertain whether downregulation of Plk1 by HDAC inhibitors at the transcript level was translated to protein expression levels, we determined protein expression levels of Plk1 by Western blots after treatment of DU145 and PC3 cells with HDAC inhibitors VPA and SAHA. As expected, Plk1 expression was decreased after treatment with

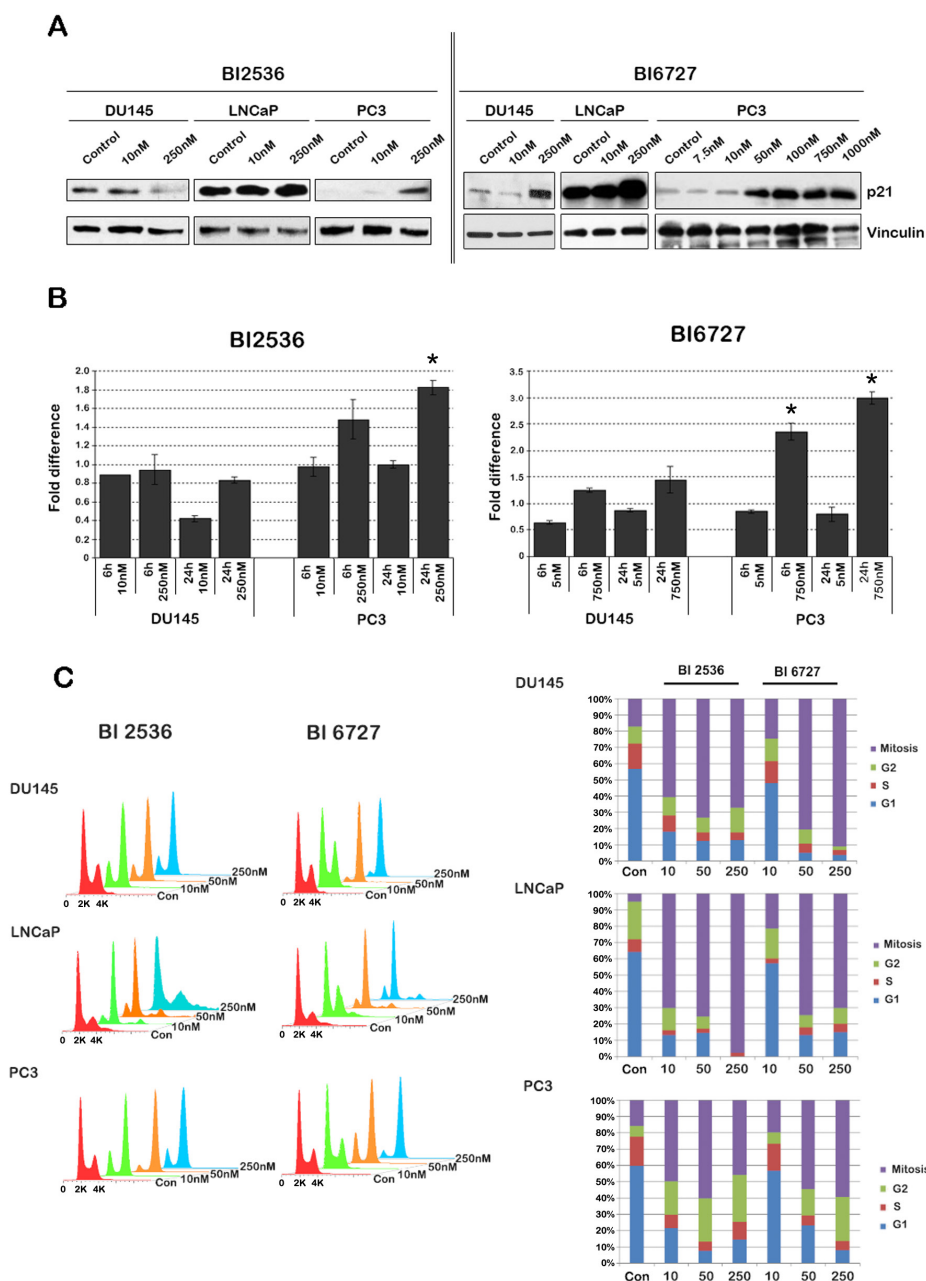


Figure 6. DU145 and LNCaP cells are arrested exclusively in mitosis after BI 2536 and BI 6727 treatment, while PC3 cells accumulate both in G2- and M-phase. A) Western blot analysis of p21 after treatment of PCa cells with BI 2536 or BI 6727 for 24 h. B) Real time RT-PCRs performed after 6 and 24 h of BI treatment of DU145 and PC3 cells to measure p21 mRNA levels. *, $p < 0.01$; concentrations in nM. C) Flow cytometry performed after treatment of PCa cells with BI 2536 and BI 6727 for 24 h to measure the percentage of cells at a respective cell cycle stage.

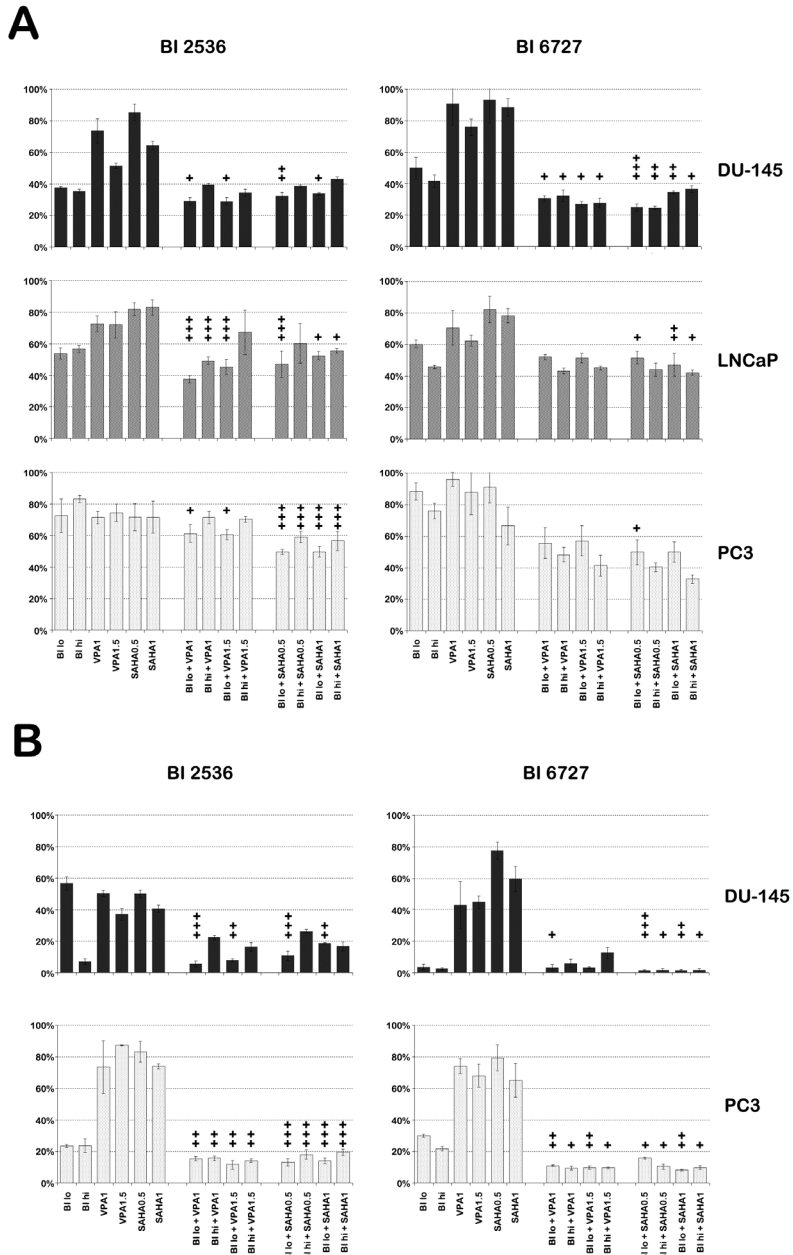


Figure 7. Combination treatment of Plk1 inhibitors BI 2536 (left) and BI 6727 (right) with HDAC-inhibitors VPA and SAHA (vorinostat) in PCa cell lines results in synergy, as indicated by results from MTS (A) and clonogenic (B) assays. +, synergism; ++, strong synergism; +++, very strong synergism, as determined by Calcsyn (Biosoft). For DU145 cells: BI 2536 Lo, 10 nM; BI 2536 Hi, 100 nM (MTS) or 250 nM (clonogenics); BI 6727 Lo, 10nM; BI 6727 Hi, 25nM. For LNCaP cells: BI Lo, 50 nM; BI Hi, 250nM. For PC3 cells: BI 2536 Lo, 50 nM; BI 2536 Hi, 250 nM; BI 6727 Lo, 300 nM; BI 6727 Hi, 500 nM. VPA in mM, SAHA in μ M.

the HDAC inhibitors at concentrations that correspond with serum plasma levels established in cancer patients treated with VPA (3 mM) or SAHA (6.17 μ M) at the maximum tolerated dose (MTD) or below in phase I clinical trials (data not shown).^{38, 39} Next, we treated PCa cells with combinations of Plk1 inhibitors BI 2536 or BI 6727 and HDAC inhibitors VPA or SAHA. Proliferation and survival were assessed by MTS and clonogenic assays, respectively (Fig. 7). For the MTS assays (Fig. 7A), two concentrations were chosen per inhibitor based on the IC₅₀ values for the respective cell line. Concentrations used in clonogenic assays were derived from results of the MTS assays (Fig. 7B). All concentrations used were at or below the plasma serum levels at the MTD in patients as determined in clinical studies.^{13, 23, 38, 39} Clonogenic results suggested that combining the BI compounds with HDAC inhibitors had a synergistic effect in DU-145 and PC3 cells (Fig. 7B and Table 1). There were three important features evident from these studies. First, combinations of HDAC inhibitors with BI 2536 had a greater synergistic effect on all PCa cell lines than combinations with BI 6727. The lower synergistic effect in combinations with BI 6727 was most likely due to this compound being highly effective as a single agent at very low concentrations: i.e., BI 6727 at a concentration of 10 nM resulted in 5% clonogenic survival in DU145 cells compared to untreated DU145 cells (Fig. 7B). Second, combining BI compounds with SAHA led to more synergy than combinations with VPA. This is well illustrated in MTS assays performed after treatment of PCa cells with BI 6727: while combinations of BI 6727 with SAHA did exhibit a synergistic effect, in general, combinations of BI 6727 with VPA failed to exhibit clear synergy, as synergism was limited to DU145 cells. Third, and most important, increasing treatment concentrations overall diminished synergistic effects; at times, it even seemed to result in antagonistic effects. A clear example is evident in clonogenic assays performed with DU145 cells treated with BI 2536 and/or VPA: at low concentrations of the single agents, survival of DU145 cells was inhibited by 40–50%, while the combination of the two compounds inhibited survival of DU145 cells highly effectively (>90%); on the other hand, at higher nanomolar concentrations, cells were effectively inhibited by BI 2536 alone (<10% clonogenic survival), while the combination of BI 2536 with VPA led to 15–30% clonogenic survival (Fig. 7B). To further validate that combination of low-dose HDAC inhibitor and Plk1 inhibitors does lead to an enhanced decrease in clonogenic potential, we performed the clonogenic experiments with a range of Plk1 inhibitor concentrations with low doses of HDAC inhibitors (data not shown). We did find a significant increase in cell death in both DU145 and PC3 cells at lower concentrations of Plk1 inhibitors when combined with HDAC inhibitors.

In summary, both BI 2536 and BI 6727 were successful in inhibiting PCa growth and survival *in vitro*. Combining these Plk1 inhibitors with HDAC inhibitors VPA or SAHA resulted in a synergistic effect depending on the type of inhibitor, the concentration of the inhibitor and the PCa cell line.

Discussion

Insights gained from various studies indicate that cancer is fueled by multiple pathways, which are either mutated or amplified, to gain growth and invasive potential. The oncogene addiction hypothesis, put forward by Weinstein, states that cancer cells are addicted to such molecular pathways, which, if targeted, could reverse or inhibit the cancer phenotype.⁴⁰ This has been the rationale for several successful targeted therapies such as tamoxifen and trastuzumab for breast cancer, and imatinib for gastrointestinal cancers and leukemia.⁴¹⁻⁴³ A protein upregulated in several cancers that could serve as a target in PCa is Plk1. Multiple small molecules have been generated to inhibit Plk1 and are currently in clinical trials.² Our study investigates two such compounds, BI 2536 and BI 6727. Multiple phase II clinical trials have been conducted with BI 2536.⁹⁻¹² All trials concluded that BI 2536 was well tolerated.⁹ A newer, potentially improved compound is BI 6727.^{2, 7, 8} Phase I dose escalation studies in patients with advanced solid tumors reported partial responses in patients with sarcoma, head and neck cancer, urothelial cancer, ovarian cancer and melanoma, the limiting side effect being of hematological origin, similar to BI 2536.^{13, 44} Currently, a phase II clinical trial for this compound is being performed in patients with metastatic urothelial cancer.⁴⁵ Preliminary results of this study are promising, the drug being well tolerated in patients, 14% of the patients having a partial response and 24% of the patients having stable disease. In the current study, we assessed the effect of BI 2536 and BI 6727 in PCa cells at the molecular level. We further assessed the *in vitro* potency of both compounds as chemotherapeutics in the treatment of PCa, both as a single agent and in combination with HDAC inhibitors. Our results reveal that both BI 2536 and BI 6727 are potent inhibitors of PCa cell growth, BI 6727 being the more effective of the two. However, we did observe a difference in response to the BI compounds across PCa cell lines. We argued that this differential response could be on account of inherent molecular differences between cell lines. Our data indicate that changes in Plk1 activity as a result of treatment with the BI compounds differ among the cell lines: while Plk1 activity was effectively decreased in DU145 and LNCaP cells upon treatment, the activity in PC3 cells was relatively refractory to BI treatment. As our study does not include mutation analysis of Plk1 in PCa cell lines, the possibility of PC3 cells harboring a Plk1 mutant which render the cells less sensitive to the BI compounds cannot be ruled out. Alternatively, differences in response may arise from differential reliance on Plk1 among PCa cells, which may reflect the heterogeneity in molecular pathways that drive PCa. Another difference may be the presence of a strong SAC in DU145 and LNCaP cells compared to PC3 cells, which may account why PC3 cells are not effectively arrested in mitosis after drug treatment. Nonetheless, we found that the BI compounds can arrest PC3 cells in G2 and M phase, decrease their clonogenic potential, and induce apoptosis, albeit at higher nanomolar concentrations. Arrest of PC3 cells in G2-phase by the Plk1 inhibitors may suggest that Plk1 has functions beyond mitosis.⁴ Plk1 inhibitors, such as BI 6727, have a relatively low toxicity profile in humans, but more than

Table 1. Combination indices of different combinations of Plk1 inhibitors with HDAC-inhibitors in DU145, LNCaP and PC3 prostate cancer cell lines.

Inhibitor combination	Combination index				
	MTS assays			Clonogenic assays	
	DU145	LNCaP	PC3	DU145	PC3
BI2536					
BI lo + VPA1	0.447	0.000	0.554	0.152	0.294
BI hi + VPA1	53.814	0.019	1.164	4.963	0.299
BI lo + VPA1.5	0.670	0.012	0.426	0.290	0.170
BI hi + VPA1.5	1.103	318.620	0.714	3.381	0.189
BI lo + SAHA0.5	0.232	0.031	0.079	0.090	0.013
BI hi + SAHA0.5	25.581	6.777	0.042	5.939	0.021
BI lo + SAHA1	0.488	0.451	0.078	0.279	0.029
BI hi + SAHA1	1855.938	0.494	0.032	3.115	0.048
BI 6727					
BI lo + VPA1	0.444	0.813	0.737	0.690	0.246
BI hi + VPA1	0.686	1.034	0.847	10.378	0.327
BI lo + VPA1.5	0.538	0.981	0.951	0.835	0.215
BI hi + VPA1.5	0.656	1.399	0.901	96.972	0.346
BI lo + SAHA0.5	0.062	0.395	0.684	0.079	0.432
BI hi + SAHA0.5	0.132	0.835	0.731	0.451	0.398
BI lo + SAHA1	0.216	0.253	1.055	0.129	0.218
BI hi + SAHA1	0.616	0.681	0.894	0.398	0.400

For DU145 cells: BI 2536 Lo, 10 nM; BI 2536 Hi, 100 nM (MTS) or 250 nM (clonogenics); BI 6727 Lo, 10nM; BI 6727 Hi, 25nM. For LNCaP cells: BI Lo, 50 nM; BI Hi, 250nM. For PC3 cells: BI 2536 Lo, 50 nM; BI 2536 Hi, 250 nM; BI 6727 Lo, 300 nM; BI 6727 Hi, 500 nM. VPA in mM, SAHA in μ M

half of the patients with metastatic urothelial cancer treated with BI 6727 had progressive disease after six weeks of treatment.⁴⁵ Better treatment outcomes for PCa patients could be accomplished by combining BI compounds with other chemotherapeutics, to accomplish an additive or synergistic effect while keeping toxicities manageable. To our knowledge, only one phase II clinical trial assessing combinational therapy with a BI compound is currently being performed, in which BI 6727 is combined with cisplatin and carboplatin in patients with advanced solid tumors.⁴⁴ In this study, preliminary data show a partial response in 8% and stable disease in 50% of the patients. In our study we combined BI 2536 and BI 6727 with HDAC inhibitors. Taking lead from our previous AFA study and those of others which indicated that HDAC inhibitors downregulate mitotic checkpoint genes, Plk1 being one of them,^{17, 46} we reasoned that Plk1 would be inhibited from a transcriptional angle by the HDAC inhibitors and by direct enzyme inhibition by the Plk1 inhibitors, leading to enhanced cell death. As hypothesized, combining BI compounds with HDAC inhibitors had synergistic effects both in MTS and clonogenic assays. Synergism was more pronounced in the combination of BI 2536 with HDAC inhibitors and when both types of inhibitors were added in lower concentrations. Intriguingly, higher concentrations of BI compounds and HDAC inhibitors had the same effect as a single agent or were even found to be seemingly

antagonistic. Therefore, our data underscores the importance of correct dosing of both compounds to achieve the desired effect.

In summary, this study tested the Plk1 inhibitors BI 2536 and BI 6727 in PCa cells, and elucidated the molecular mechanism by which the BI compounds effectively inhibit PCa cells. Furthermore, this study clearly indicates that the combination of two cell-cycle arresting chemotherapeutical agents, Plk1 inhibitors with HDAC inhibitors, has a synergistic effect in inhibiting PCa cells. However, to fully validate this claim, we feel *in vivo* studies are warranted before further evaluation in clinical trials for PCa.

Acknowledgments

The authors wish to thank Boehringer Ingelheim for the Plk1 inhibitors, dr. Kyung S. Lee for providing the GST-PBIPtide-A6 vector, Eugene Kim for his help with the Western blots and prof. J.W.R. Nortier and prof. E. van der Wall for their support and discussion.

References

1. de Cárcer G, Manning G, Malumbres M. From Plk1 to Plk5: Functional evolution of polo-like kinases. *Cell Cycle* 2011;10(14):2255-2262.
2. Chopra P, Sethi G, Dastidar SG, Ray A. Polo-like kinase inhibitors: an emerging opportunity for cancer therapeutics. *Expert Opin Investig Drugs* 2010;19(1):27-43.
3. el-Bahassi M. Polo-like kinases and DNA damage checkpoint: beyond the traditional mitotic functions. *Exp Biol Med (Maywood)* 2011;236(6):648-657.
4. Takaki T, Trenz K, Costanzo V, Petronczki M. Polo-like kinase 1 reaches beyond mitosis--cytokinesis, DNA damage response, and development. *Curr Opin Cell Biol* 2008;20(6):650-660.
5. Schmit TL, Ledesma MC, Ahmad N. Modulating polo-like kinase 1 as a means for cancer chemoprevention. *Pharm Res* 2010;27(6):989-998.
6. Deeraksa A, Pan J, Sha Y et al. Plk1 is upregulated in androgen-insensitive prostate cancer cells and its inhibition leads to necroptosis. *Oncogene* 2012.
7. Schoffski P. Polo-like kinase (PLK) inhibitors in preclinical and early clinical development in oncology. *Oncologist* 2009;14(6):559-570.
8. Rudolph D, Steegmaier M, Hoffmann M et al. BI 6727, a Polo-like kinase inhibitor with improved pharmacokinetic profile and broad antitumor activity. *Clin Cancer Res* 2009;15(9):3094-3102.
9. Pandha HS, Protheroe A, Wylie J et al. An open label phase II trial of BI 2536, a novel Plk1 inhibitor, in patients with metastatic hormone refractory prostate cancer (HRPC). [abstract]. *J Clin Oncol* 2008;26:14547.
10. Gandhi L, Chu QS, Stephenson J et al. An open label phase II trial of the Plk1 inhibitor BI 2536, in patients with sensitive relapse small cell lung cancer (SCLC) [abstract]. *J Clin Oncol* 2009;27:15s:8108.
11. Sebastian M, Reck M, Waller CF et al. The efficacy and safety of BI 2536, a novel Plk-1 inhibitor, in patients with stage IIIB/IV non-small cell lung cancer who had relapsed after, or failed, chemotherapy: results from an open-label, randomized phase II clinical trial. *J Thorac Oncol* 2010;5(7):1060-1067.
12. Schoffski P, Blay JY, de Greve J et al. Multicentric parallel phase II trial of the polo-like kinase 1 inhibitor BI 2536 in patients with advanced head and neck cancer, breast cancer, ovarian cancer, soft tissue sarcoma and melanoma. The first protocol of the European Organization for Research and Treatment of Cancer (EORTC) Network Of Core Institutes (NOCI). *Eur J Cancer* 2010;46(12):2206-2215.
13. Schoffski P, Awada A, Dumez H et al. A phase I, dose-escalation study of the novel Polo-like kinase inhibitor volasertib (BI 6727) in patients with advanced solid tumours. *Eur J Cancer* 2012;48(2):179-186.
14. Rokhlin OW, Glover RB, Guseva NV, Taghiyev AF, Kohlgraf KG, Cohen MB. Mechanisms of cell death induced by histone deacetylase inhibitors in androgen receptor-positive prostate cancer cells. *Mol Cancer Res* 2006;4(2):113-123.
15. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* 2007;26(37):5541-5552.
16. Kortenhorst MS, Zahurak M, Shabbeer S et al. A multiple-loop, double-cube microarray design applied to prostate cancer cell lines with variable sensitivity to histone deacetylase

- inhibitors. *Clin Cancer Res* 2008;14(21):6886-6894.
17. Kachhap SK, Rosmus N, Collis SJ et al. Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. *PLoS One* 2010;5(6):e11208.
 18. Yasunaga Y, Nakamura K, Ewing CM, Isaacs WB, Hukku B, Rhim JS. A novel human cell culture model for the study of familial prostate cancer. *Cancer Res* 2001;61(16):5969-5973.
 19. Park JE, Li L, Park J et al. Direct quantification of polo-like kinase 1 activity in cells and tissues using a highly sensitive and specific ELISA assay. *Proc Natl Acad Sci U S A* 2009;106(6):1725-1730.
 20. Yun SM, Jung KH, Lee H et al. Synergistic anticancer activity of HS-173, a novel PI3K inhibitor in combination with Sorafenib against pancreatic cancer cells. *Cancer Lett* 2013;331(2):250-261.
 21. Cordoba EV, Arnaiz E, De La Mata FJ et al. Synergistic activity of carboxilane dendrimers in combination with maraviroc against HIV in vitro. *AIDS* 2013.
 22. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984;22:27-55.
 23. Mross K, Frost A, Steinbild S et al. Phase I dose escalation and pharmacokinetic study of BI 2536, a novel Polo-like kinase 1 inhibitor, in patients with advanced solid tumors. *J Clin Oncol* 2008;26(34):5511-5517.
 24. Lee KS, Oh DY, Kang YH, Park JE. Self-regulated mechanism of Plk1 localization to kinetochores: lessons from the Plk1-PBIP1 interaction. *Cell Div* 2008;3:4.
 25. Strebhardt K. Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy. *Nat Rev Drug Discov* 2010;9(8):643-660.
 26. Lens SM, Voest EE, Medema RH. Shared and separate functions of polo-like kinases and aurora kinases in cancer. *Nat Rev Cancer* 2010;10(12):825-841.
 27. Chan EH, Santamaria A, Sillje HH, Nigg EA. Plk1 regulates mitotic Aurora A function through betaTrCP-dependent degradation of hBora. *Chromosoma* 2008;117(5):457-469.
 28. Hanisch A, Wehner A, Nigg EA, Sillje HH. Different Plk1 functions show distinct dependencies on Polo-Box domain-mediated targeting. *Mol Biol Cell* 2006;17(1):448-459.
 29. Crane R, Gadea B, Littlepage L, Wu H, Ruderman JV. Aurora A, meiosis and mitosis. *Biol Cell* 2004;96(3):215-229.
 30. De Luca M, Lavia P, Guarguaglini G. A functional interplay between Aurora-A, Plk1 and TPX2 at spindle poles: Plk1 controls centrosomal localization of Aurora-A and TPX2 spindle association. *Cell Cycle* 2006;5(3):296-303.
 31. Bolanos-Garcia VM. Assessment of the mitotic spindle assembly checkpoint (SAC) as the target of anticancer therapies. *Curr Cancer Drug Targets* 2009;9(2):131-141.
 32. Weaver BA, Cleveland DW. Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer Cell* 2005;8(1):7-12.
 33. Valsasina B, Beria I, Alli C et al. NMS-P937, an orally available, specific small-molecule polo-like kinase 1 inhibitor with antitumor activity in solid and hematologic malignancies. *Mol Cancer Ther* 2012;11(4):1006-1016.
 34. Cazzalini O, Scovassi AI, Savio M, Stivala LA, Prosperi E. Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. *Mutat Res* 2010;704(1-3):12-20.
 35. Cayrol C, Knibiehler M, Ducommun B. p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. *Oncogene* 1998;16(3):311-320.
 36. Richon VM, Sandhoff TW, Rifkind RA, Marks PA. Histone deacetylase inhibitor selectively

- induces p21WAF1 expression and gene-associated histone acetylation. *Proc Natl Acad Sci U S A* 2000;97(18):10014-10019.
37. Shabbeer S, Kortenhorst MS, Kachhap S, Galloway N, Rodriguez R, Carducci MA. Multiple Molecular pathways explain the anti-proliferative effect of valproic acid on prostate cancer cells in vitro and in vivo. *Prostate* 2007;67(10):1099-1110.
 38. Atmaca A, Al-Batran SE, Maurer A et al. Valproic acid (VPA) in patients with refractory advanced cancer: a dose escalating phase I clinical trial. *Br J Cancer* 2007;97(2):177-182.
 39. Rubin EH, Agrawal NG, Friedman EJ et al. A study to determine the effects of food and multiple dosing on the pharmacokinetics of vorinostat given orally to patients with advanced cancer. *Clin Cancer Res* 2006;12(23):7039-7045.
 40. Weinstein IB, Joe AK. Mechanisms of disease: Oncogene addiction--a rationale for molecular targeting in cancer therapy. *Nat Clin Pract Oncol* 2006;3(8):448-457.
 41. Riggs BL, Hartmann LC. Selective estrogen-receptor modulators -- mechanisms of action and application to clinical practice. *N Engl J Med* 2003;348(7):618-629.
 42. Deininger MW, Goldman JM, Lydon N, Melo JV. The tyrosine kinase inhibitor CGP57148B selectively inhibits the growth of BCR-ABL-positive cells. *Blood* 1997;90(9):3691-3698.
 43. Baselga J, Tripathy D, Mendelsohn J et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol* 1996;14(3):737-744.
 44. Deleporte A, Dumez H, Awada A et al. Phase I trial of volasertib (BI 6727), a polo-like kinase 1 (Plk1) inhibitor, in combination with cisplatin or carboplatin in patients with advanced solid tumors [abstract]. *J Clin Oncol* 2011;29:3031.
 45. Stadler WM, Vaughn DJ, Sonpavde G et al. Clinical outcome of single agent volasertib (BI 6727) as second-line treatment of patients (pts) with advanced or metastatic urothelial cancer (UC) [abstract]. *J Clin Oncol* 2011;29:4567.
 46. Prystowsky MB, Adomako A, Smith RV et al. The histone deacetylase inhibitor LBH589 inhibits expression of mitotic genes causing G2/M arrest and cell death in head and neck squamous cell carcinoma cell lines. *J Pathol* 2009;218(4):467-477.

Chapter 8

Combining the pan-aurora kinase inhibitor AMG 900 with histone deacetylase inhibitors enhances antitumor activity in prostate cancer

Michel D. Wissing[†], Channing J. Paller[†], Janet Mendonca, Anup Sharma, Eugene Kim, Hea-Soo Kim, Madeleine S.Q. Kortenhorst, Stephanie Gerber, Marc Rosen, Faraz Shaikh, Marianna L. Zahurak, Michelle A. Rudek, Hans Hammers, Charles M. Rudin, Michael A. Carducci, Sushant K. Kachhap

[†]These authors contributed equally to this work.

Cancer Med. 2014 Jul 3. [Epub ahead of print]

Abstract

Histone deacetylase inhibitors (HDACIs) are being tested in clinical trials for the treatment of solid tumors. While most studies have focused on the re-expression of silenced tumor suppressor genes, a number of genes/pathways are downregulated by HDACIs. This provides opportunities for combination therapy: agents that further disable these pathways through inhibition of residual gene function are speculated to enhance cell death in combination with HDACIs. A previous study from our group indicated that mitotic checkpoint kinases such as PLK1 and Aurora A are downregulated by HDACIs. We used *in vitro* and *in vivo* xenograft models of prostate cancer (PCa) to test whether combination of HDACIs with the pan-aurora kinase inhibitor AMG 900 can synergistically or additively kill PCa cells. AMG 900 and HDACIs synergistically decreased cell proliferation activity and clonogenic survival in DU-145, LNCaP, and PC3 PCa cell lines compared to single agent treatment. Cellular senescence, polyploidy and apoptosis was significantly increased in all cell lines after combination treatment. *In vivo* xenograft studies indicated decreased tumor growth and decreased aurora B kinase activity in mice treated with low dose AMG 900 and vorinostat compared to either agent alone. Pharmacodynamics was assessed by scoring for phosphorylated histone H3 through immunofluorescence. Our results indicate that combination treatment with low doses of AMG 900 and HDACIs could be a promising therapy for future clinical trials against PCa.

Introduction

In recent years, the introduction of various novel therapies for prostate cancer (PCa), such as taxanes, has significantly extended survival of patients.^{1,2} Nevertheless, PCa remains the second deadliest cancer in the Western world.³ Therefore, research is needed to further improve clinical outcomes by identifying therapies with improved antitumor efficacy and/or reduced toxicities.

In the search for new molecular targets for PCa treatment, aurora kinases are a promising candidate.⁴ Three paralogous genes (aurora A, B, and C) comprise the aurora family of serine/threonine protein kinases in mammalian cells. Aurora A and B are essential regulators of mitosis, while aurora C primarily plays a role in meiosis. Aurora A is an oncogene upregulated in several tumor types.⁵ Its phosphorylation is required for cell cycle progression, centrosome maturation, and spindle assembly.^{6,7} Aurora B, also overexpressed in tumor cells, is most active during the G2/M-phase, and its phosphorylation is essential for the final steps of cytokinesis.^{6,8,9} Active aurora B phosphorylates histone H3 on serine 10, a molecular event vital for chromosome condensation and mitotic progression.^{6,8} Inhibition of aurora A and B inactivates the spindle assembly checkpoint, resulting in endoreduplication, polyploidy and eventually, apoptosis.^{6,10-12} The orally bioavailable pan-aurora kinase inhibitor AMG 900 aborts cytokinesis by inhibition of autophosphorylation of aurora kinases.¹¹ It is effective in multidrug resistant models, possibly through circumvention of the drug efflux effector P-glycoprotein.¹¹

AMG 900 may yield enhanced antitumor activity in the presence of additional cancer therapeutics.^{11,13} Previous studies by our group have demonstrated that several genes involved in mitotic checkpoints, including polo-like kinase 1 (Plk1) and aurora kinases, are downregulated by HDACIs.^{14,15} Recently we have demonstrated that combination of HDACIs with a Plk1 inhibitor synergistically induced apoptosis, decreased cell proliferation, and decreased clonogenic survival of PCa cells.¹⁶ Based on our success with Plk1 inhibitors, we hypothesized that addition of HDACIs could potentiate apoptosis in PCa cells that are treated with aurora kinase inhibitors. Further, HDACIs exhibit promising antitumor effects in PCa *in vitro* and *in vivo*,^{17,18} and have successfully been used in concert with other chemotherapeutics.^{19,20} Therefore, HDACIs could serve as a rational choice for complementing the apoptotic effects of AMG 900. Hence, we combined AMG 900 with the HDACIs VPA and vorinostat in PCa cells in our current study. We found that combination of HDACIs with AMG 900 has a synergistic antitumor effect, the HDACIs activating an apoptotic mechanism in aurora kinase-inhibited PCa cells.

Materials and methods

In vitro

Cell culture and treatment

PCa cell lines (DU-145, LNCaP, PC3) were obtained from ATCC. Cells were grown in RPMI-1640 (Invitrogen) with 10% fetal bovine serum (FBS) (Gemini) and maintained in a 37°C humidified incubator supplemented with 5% CO₂. VPA (Sigma-Aldrich) was prepared in RPMI at a 1M stock on the day of treatment of the cells. Vorinostat (AtonPharma) and AMG 900 (Amgen) were maintained in 10 mM DMSO stock solutions at -20°C and diluted in RPMI upon use. Compounds were administered concomitantly in combination studies.

Cell viability and synergy

MTS assays were performed with CellTiter 96™ Aqueous Non-Radioactive Cell Proliferation Assay reagent (Promega) according to the manufacturer's instructions. In brief, PCa cells were plated in 96-well plates, allowed to adhere overnight and treated with the selected compounds for 72 h. Subsequently, MTS reagent was added. Absorption at 490 nm was measured after approximately 2 h using a colorimetric plate reader (Molecular Devices).

To compare the antitumor effect of single agent treatments with combination treatment, synergy was determined using CalcuSyn software (Biosoft). CalcuSyn calculates a combination index (CI) at different levels of growth, using the formula for mutually non-exclusive mechanisms: $(D1/Dx1) + (D2/Dx2) + (D1 \cdot D2 / (Dx1 \cdot Dx2))$, where D1 and D2 are the doses of drug 1 and drug 2 in combination required to produce x percentage effect, and Dx1 and Dx2 are the doses of drug 1 and drug 2 alone required to produce the same effect. Synergy levels (no synergy (CI>0.9), moderate synergy (0.7<CI≤0.9, +), synergy (0.3<CI≤0.7, ++), strong synergy (0.1<CI≤0.3, +++), very strong synergy (CI≤0.1, ++++)) were determined from CI ranges, using the Chou-Talalay method following the manufacturer's instructions.^{21,}

22

Cell survival

Clonogenic assays were performed to assess long-term cell survival. PCa cells were plated in complete RPMI media. Upon reaching 50-60% confluency, drugs were added at the appropriate concentration and dishes were incubated for 48 h. Then, cells (1.25x10³ for DU-145 and PC3 cells, 2x10³ for LNCaP cells) were replated and grown in triplicate in 60 mm dishes containing fresh, complete RPMI media. After 10-14 days (depending on the doubling time of cell line), crystal violet stain (Sigma) was used to stain colonies and colonies were counted. All dishes from one cell line were stained at the same time point. The average number of colonies in DMSO-treated controls was considered 100% clonogenic survival in each separate cell line and in each separate experiment. Student's t-tests were performed to assess whether clonogenic survival of a cell line differed significantly between doses of a single agent; synergy was determined with CalcuSyn when comparing single agent

treatment with combination treatment.^{21, 22}

Cellular senescence

PCa cells were plated in 6-well plates (25-50x10³ cells per well) and allowed to adhere overnight. Compounds were added to the complete RPMI media for 48 h, after which senescent cells were stained using the senescence β -galactosidase staining kit (Cell Signaling Technology) according to the manufacturer's instructions. In brief, cells were washed in phosphate buffered saline (PBS) and fixed in Fixative Solution (2% formaldehyde and 0.2% glutaraldehyde in 1X PBS). After fixing, cells were washed in PBS and incubated with Staining Solution (containing 40 mM citric acid/sodium phosphate (pH 6.0), 150 mM NaCl, 2 mM MgCl₂, 5 nM potassium ferrocyanide, and 1 mg/ml X-gal in 5% dimethylformamide) in a 37°C incubator for 24 h. Cells were washed in PBS and visualized under an Olympus IX70 inverted microscope using an Uplan FL 10x phase contrast lens. Multiple images (>5) were taken from each well. Senescent (blue) and total cells were counted in five fields of approximately 30 cells/field for each treatment. Student's t-tests were performed to determine whether cellular senescence significantly differed between combination treatment and single agent treatment.

Fluorescence microscopy

Cells were drugged at 50-70% confluency for 48 h and fixed with neutral buffered formalin for 10 minutes, followed by permeabilization with 0.125% Triton X-100 for 5 minutes. Cells were blocked with 10% bovine serum albumin (BSA) overnight. Cells were probed with a primary antibody against phosphorylated aurora kinase A/B/C (antibody #2914, Cell Signaling Technology) at a 1:100 dilution followed by an Alexa Fluor-555 (Invitrogen) conjugated secondary antibody at a 1:400 dilution in blocking buffer. Cells were washed in PBS and further incubated with Alexa Fluor-488 conjugated phosphorylated histone H3 antibody (antibody #9713 at a 1:100 dilution, Cell Signaling Technology). Cells were counterstained with Hoechst 33258 and mounted on slides. Confocal images were taken with the Zeiss LSM 510 meta-confocal microscope (Carl Zeiss) using a 63x objective.

Flow cytometry

Cells were plated in 100 mm dishes and drugged at 50-70% confluency. Both floating and attached cells were collected 48 h after treatment, washed in PBS and fixed with 4% freshly-made paraformaldehyde. Cells were then permeabilized with 90% cold methanol. Permeabilized cells were stained with Alexa Fluor-488 conjugated phosphorylated histone H3 antibody (1: 100 dilution, Cell Signaling Technology). Nuclei were stained with propidium iodide (Sigma) in PBS containing 1% BSA. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences). Cell cycle analysis was performed using BD FACSDiva software and FlowJo.

Immunoblotting

Western blotting was performed as described previously.¹⁴ Loading volumes equal to 20 µg of total protein were used. Primary antibodies were diluted 1:1000 in blocking solution with the exceptions of vinculin (Millipore) and phosphorylated aurora A/B/C (antibody #2914, Cell Signaling Technology), which were diluted 1:4000 and 1:500, respectively. Conjugated secondary antibodies were diluted 1:4000 in blocking solution. Blocking solution (5% milk in TBST (100 mM Tris-HCl pH 7.4, 0.1% Tween 20, 150 mM NaCl in H₂O)) was used to dilute antibodies against p21 (BD Biosciences), cyclin B1 (Cell Signaling Technology), vinculin, and cleaved PARP (Cell Signaling Technology), as well as all secondary antibodies. 5% BSA was used for phosphorylated aurora A/B/C and phosphorylated and total histone H3 (Cell Signaling Technology). Blots were developed with ECL (GE) or Femto (Pierce Biotechnology) and scanned into a computer at a resolution of 300 dots per inch (dpi). Densitometric analyses were performed using ImageJ (Research Services Branch, National Institute of Mental Health); density of bands of the protein of interest was normalized to the density of bands of the housekeeper (actin, vinculin or total aurora A), and protein expression of treated cells was compared to the control.

In vivo

Animals

The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Johns Hopkins University (JHU). All IACUC guidelines and United States Department of Agriculture regulations were followed. The mice used in this study were 8-week old JHU Oncology NOD/SCID_s (JHU bred colony) and were housed under aseptic conditions on a 12 h light-dark cycle with food and water provided *ad lib*. Each cage contained ≤ 5 mice, which were differentiated by treatment groups.

Two million DU-145 PCa cells, suspended in complete RPMI media, were embedded in a 1:2 solution of Matrigel (BD Biosciences) and injected subcutaneously into the right flank of the mice. The tumor inoculation success rate was approximately 90%. Following a 3 week growth incubation period, tumor volume was estimated with digital calipers, using the standard formula: $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. Before treatment initiation, mice were stratified by tumor size and assigned into homogenous groups (8-9 per group). Once average tumor volume was above 200 mm³, treatment was initiated in all mice. Treatments were administered 4 consecutive days per week for a total duration of 4 weeks. Vorinostat (50 mg/kg) was administered once daily via intraperitoneal injections on mornings of days 1-4 of the dosing cycle. AMG 900 was administered through gavage on days 1 and 2 of each dosing cycle. Mice were treated with vehicle (2% hydroxypropyl methyl cellulose (HPMC) and 1% Tween 80 in deionized water (pH 2.2) with methane sulfonic acid (MSA)), or AMG 900 at a concentration of 3.75 mg/kg or 7.5 mg/kg (provided weekly by AMGEN in glycerin). Tumor size and mice bodyweights were measured on the day preceding each dosing cycle

(day 0/7) and the final day of dosing (day 4) each week. Tumors were harvested when the volume reached 1000 mm³, which occurred 26 to 35 days after start of the treatment. For histological assessment of the tumors, mice were perfused with 2% paraformaldehyde through a cardiac catheter, and then tumors were excised, infiltrated in sucrose, embedded in O.C.T. compound (Sakura Finetek) and stored at -80°C. Tissue sections were prepared for hematoxylin-eosin staining by fixing the tissue in formalin and embedding in paraffin after tumor excision.

Tumor growth analysis

In vivo data from the DU-145 xenograft model were analyzed with a random intercept hierarchical linear model. The primary statistical outcome was tumor volume. To adjust for the initial volume, tumor volumes on days 3 through 26 for each mouse were divided by the volume on day 0 and then the log of these values was taken for analysis. The intercept in this longitudinal model was specified such that it represented the log ratio of the final tumor volume to the initial volume (time was coded using negative numbers and 0 for the final day). The model had the formula: $\gamma_{it} = \beta_0 + \zeta_{0i} + \beta_1 \text{time}_{it} + \beta_2 \text{group}_i + \beta_3 (\text{group}_i * \text{time}_{it}) + \varepsilon_{it}$ where γ_{it} denotes the log ratio tumor volume for mouse i at time t , β_0 is the intercept representing the log ratio of the tumor volume on day 26 to the initial volume for the control group, β_1 is the linear effect of time for the control group, β_2 is the group effect on day 26, and β_3 is the group effect in terms of the linear effect of time. The mouse specific effect ζ_{0i} represented the deviation of each mouse from the group intercept. This corrected for the correlation between measurements taken on the same mouse. Since the mice in this experiment were considered a representative sample from a larger population, the effect was considered random and it was assumed that the population distribution from which they were sampled had a normal distribution.

Tissue Immunostaining

Sections (20 μ m) were cut from frozen tissues and mounted on microscope slides (Fisher Scientific). Sections were blocked in blocking buffer (5% goat serum, 0.5% BSA, 0.1% Triton X-100 and 0.01% sodium azide in PBS) and probed overnight with the primary antibody phosphorylated histone H3 in a 1:100 dilution (antibody #9713, Cell Signaling Technology). Sections were washed in PBS containing 0.1% Triton X-100 and probed overnight with Alexa Fluor-546 conjugated anti-rabbit antibody in a 1:500 dilution (Invitrogen). Subsequently, sections were washed in PBS and fixed with 10% formalin, and nuclei were stained with DAPI present in the mountant (Invitrogen). Coverslips were mounted on the slides and sections were imaged with a Nikon Eclipse Ti microscope at 20x (Nikon). The percentage of fluorescent cells was assessed in at least three fields of view per treatment group by dividing the density of red (phosphorylated histone H3 positive cells) by the density of blue (DAPI staining all nuclei) color with ImageJ. Student's t-tests were performed to assess

for statistically significant differences in the percentage of cells that stained positively for phosphorylated histone H3 and phosphorylated aurora A/B/C.

Results

AMG 900 and HDACIs inhibit aurora kinase expression and clonogenic survival of PCa cells.

After treatment of DU-145, PC3 and LNCaP PCa cells with AMG 900 for 12 h, the relative decrease in protein expression of phosphorylated aurora A/B/C was compared to protein levels of total aurora A. In all three cell lines, protein expression of phosphorylated aurora decreased in a dose-dependent manner at concentrations above 1 nM (Fig. 1A). Next, long-term clonogenic assays were performed to determine cell survival of PCa cells after AMG 900 treatment at 1 and 5 nM. At 5 nM, AMG 900 effectively inhibited clonogenic survival in all PCa cell lines (>70%) (Fig. 1B). These results mirrored those of Payton et al., who reported that the IC_{50} of AMG 900 in proliferation assays was around 5 nM for DU-145 and PC3 cells.¹¹ Based on aforementioned results, we selected AMG 900 at 1 nM (low dose, less effective) and 5 nM (high dose, effective) for drug combination studies. VPA and vorinostat were administered at concentrations of 1 mM and 1.5 mM, and 0.5 μ M and 1 μ M, respectively, as at these concentrations the compounds downregulated phosphorylated aurora A/B/C protein expression levels in DU-145 and PC3 cells (data not shown), while previous studies

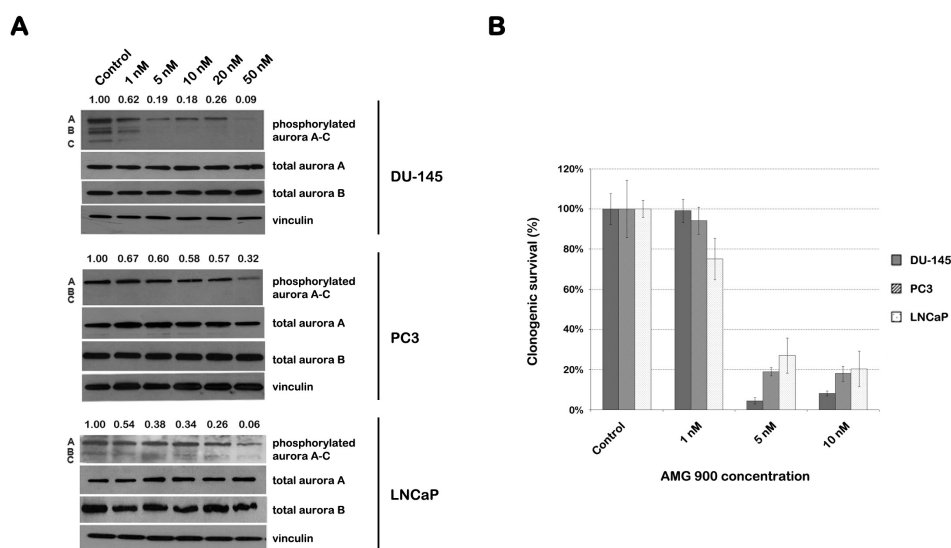


Figure 1. AMG 900 effectively targets PCa cells at concentrations above 1 nM. A) Western blot quantifying the protein levels of phosphorylated aurora A (48 kDa), B (40 kDa) and C (35 kDa) after treatment of PCa cells with AMG 900 (concentrations indicated above each lane) for 48 h. Bands were normalized to total levels of aurora A. B) Bar graph representing relative clonogenic survival after AMG 900 treatment of PCa cell lines. Colonies were counted in triplicate.

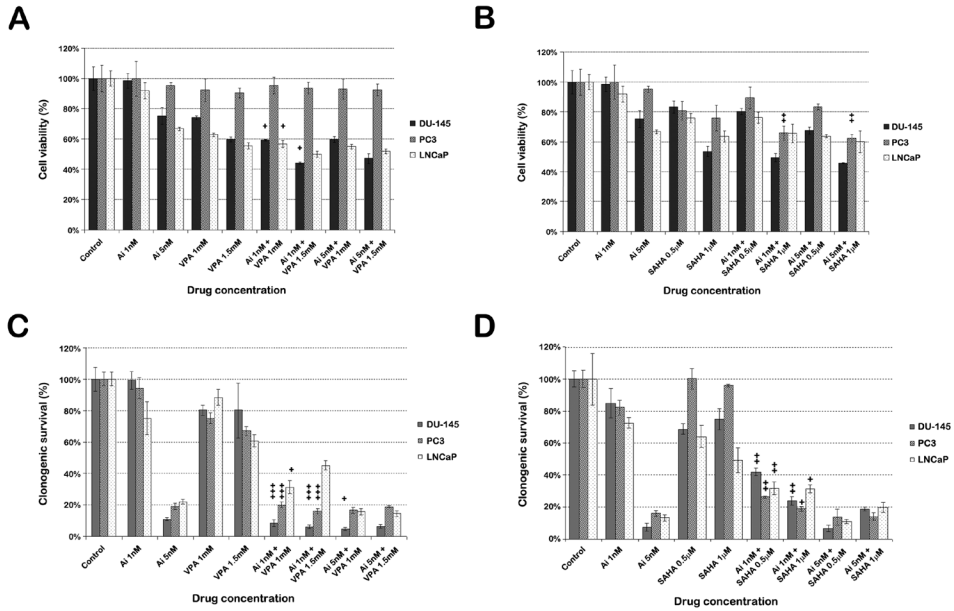


Figure 2. Combinations of AMG 900 with HDACIs VPA and vorinostat decrease the proliferation activity and long-term clonogenic survival of PCa cells compared to single agent use. A-B) Proliferation activity of PCa cells after treatment with AMG 900 and VPA (A) or vorinostat (B), as measured by MTS assays. C-D) Quantification of colonies to assess clonogenic survival of PCa cells after treatment with AMG 900 and VPA (C) or vorinostat (D). Ai, AMG 900; VPA, valproic acid; SAHA, suberanilohydroxamic acid (vorinostat); +, moderate synergy; ++, synergy; +++, strong synergy.

suggested acceptable toxicities at these concentrations.^{16, 18, 23}

Combinations of low dose AMG 900 with HDACIs decrease proliferation activity and clonogenic survival of PCa cells.

We employed MTS and clonogenic assays to assess the effect of combinations of AMG 900 with VPA and vorinostat on the proliferation activity and long-term survival of PCa cells compared to a single agent. In both assays, treatment of PCa cells with 1 nM AMG 900 did not result in antitumor activity, similar to previous results (compare Fig. 1 to Fig. 2). In MTS assays, low dose VPA combined with low dose AMG 900 showed enhanced inhibition of cell proliferation compared to high dose AMG 900 used as a single agent in both DU-145 and LNCaP cells (Fig. 2A). Moderate synergistic effects were observed in DU-145 cells treated with combinations of AMG 900 (1 nM) and VPA (1 mM and 1.5 mM) (CI=0.796 and CI=0.777, respectively), and in LNCaP cells treated with combinations of AMG 900 (1 nM) and VPA (1 mM) (CI=0.848). In PC3 cells the proliferation, as evaluated by MTS assays, was decreased by 10% at the most after treatment with AMG 900 and/or VPA. Combinations of vorinostat and AMG 900 enhanced the inhibition of cell proliferation in all three cell lines compared to treatment with single agents, except when combining a low dose of vorinostat (0.5 μM) with

a low dose of AMG 900 (1 nM) (Fig. 2B). Synergistic effects, as defined by CalcuSyn, were observed in PC3 cells treated with combinations of 1 μ M vorinostat and AMG 900 (1 nM and 5 nM) ($CI=0.375$ and $CI=0.558$, respectively).^{21, 22}

Cell death through aurora kinase inhibition may involve aborted cytokinesis progressing to apoptosis, which may not be registered as a change in a short-term proliferation assay. Therefore, we assessed the long-term effect of combination treatment on clonogenic survival in PCa cell lines. Treatment of PCa cells with 1 nM AMG 900 did not result in decreased clonogenic survival; however, combinations of low dose AMG 900 with either VPA or vorinostat resulted in a remarkably decreased clonogenic survival compared to single agent treatment (Fig. 2C-D). Subsequent analyses performed with CalcuSyn demonstrated that administration of AMG 900 (1 nM) combined with VPA (1 mM and 1.5 mM) resulted in a strongly synergistic decrease in survival compared to the single compounds in DU-145 and PC3 cells ($0.171 < CI < 0.260$) (Fig. 2C).^{21, 22} In LNCaP cells a moderately synergistic decrease in clonogenic survival was seen in combination treatments of low dose AMG 900 and low dose VPA ($CI=0.765$). Treatment of cells with combinations of AMG 900 (1 nM) and vorinostat (0.5 μ M and 1 μ M) resulted in a decrease in clonogenic survival that was synergistic or moderately synergistic in all three PCa cell lines ($0.340 < CI < 0.809$) (Fig. 2D). The higher dose of AMG 900 as a single agent severely inhibited clonogenic survival in all three cell lines. As a result, virtually no synergy could be measured in combination treatments involving high dose AMG 900.

Combinations of AMG 900 with VPA or vorinostat increase cellular senescence of PCa cells.

During MTS and clonogenic assays, we observed PCa cell phenotypes resembling a senescent morphology after treatment with AMG 900 with or without HDACIs. Treated cells displayed a flattened morphology compared to untreated controls, and some treated DU-145 and PC3 cells showed cytoplasmic vacuoles and granularity. We performed a Western blot analysis for p21 as a surrogate marker of cellular senescence in PCa cells treated with (combinations of) AMG 900 and HDACIs.²⁴ Protein levels of p21 were not assessed in combinations with high dose AMG 900, as during the course of the treatment, cells treated with this combination constituted mainly apoptotic cells confounding analysis. In line with previous studies,^{18, 25} protein expression of p21 was significantly increased after HDACI treatment compared to untreated controls; to a lesser extent AMG 900 treatment also resulted in an increase in p21 protein levels (Fig. 3A). Combination treatment further increased p21 levels, suggesting increased cellular senescence. To ascertain whether cellular senescence is indeed increased after combination treatment compared to single agent use in PCa cells, we stained DU-145, PC3 and LNCaP cells for senescence associated (SA) β -galactosidase enzyme activity (Fig. 3B). Consistent with our previously observed morphological transformations, DU-145 and PC3 cells treated with combinations of AMG 900 and HDACIs had increased SA

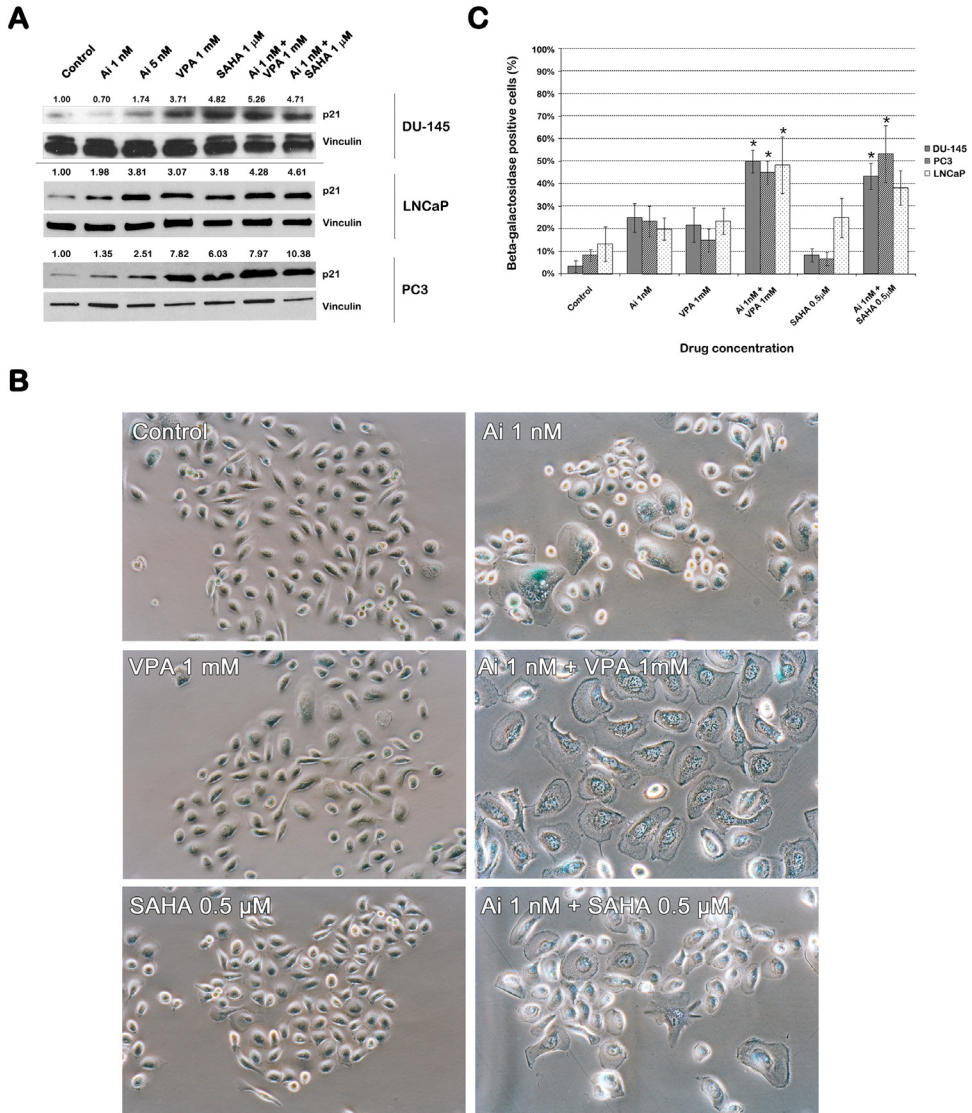


Figure 3. AMG 900 combined with HDACIs VPA or vorinostat increases cellular senescence in PCa cell lines compared to single agent use. A) Western blot for p21, a marker of cellular senescence or a G1/G2 phase cell cycle arrest, after treating DU-145, LNCaP and PC3 cells as indicated above each lane. Bands were normalized to the housekeeper vinculin. B) Representative images of PC3 cells after performing an SA β -galactosidase assay. Blue cells are SA β -galactosidase positive cells, indicating cellular senescence. C) Quantification of SA β -galactosidase positive staining after treatment of PCa cells with AMG 900 and/or HDACIs. Ai, AMG 900; VPA, valproic acid; SAHA, suberanilohydroxamic acid (vorinostat); *, combination treatments with a significantly increased percentage of senescent cells (p < 0.05).

β -galactosidase staining. LNCaP cells had a baseline level of blue SA β -galactosidase staining in untreated controls; combination treatment of LNCaP cells with AMG 900 and HDACIs resulted in increased SA β -galactosidase staining. Quantification of β -galactosidase positive cells confirmed that the percentage of senescent cells was significantly increased in PCa cells treated with combinations of AMG 900 and HDACIs (35-60% SA β -galactosidase positive cells) compared to cells treated with single agents alone ($\leq 25\%$ SA β -galactosidase positive cells) ($p \leq 0.05$), except for combination treatment of LNCaP cells with AMG 900 (1 nM) and vorinostat (0.5 μ M) compared to cells treated with vorinostat alone ($p = 0.116$) (Fig. 3C).

Combination treatment with AMG 900 and HDACIs increases PCa cells with multipolar spindles and polyploidy as compared to single agent treatment.

Since aurora kinases localize to distinct subcellular structures in mitotic cells, we probed DU-145 and PC3 cells with an antibody against phosphorylated aurora kinase A/B/C to investigate whether treatment results in differences in localization of these enzymes (Fig. 4). We co-stained the cells for phosphorylated histone H3 as histone H3 is phosphorylated at serine 10 by aurora B during mitosis and can thus be used as a marker for aurora B activity.²⁶ After treatment with AMG 900, alone or in combination with HDACIs, localization of aurora was limited to the spindle poles. It was further observed that PC3 cells treated with low dose AMG 900 and HDACIs exhibited multipolar spindles, similar to 5 nM of AMG 900, suggesting endoreduplication and polyploidy. Both cell lines showed a near complete loss in aurora staining after a combination treatment with 5 nM of AMG 900 and HDACIs. Of note, addition of AMG 900 also caused a decrease in histone H3 phosphorylation in a dose-dependent manner in both PCa cell lines, with DU-145 demonstrating a greater decrease compared to PC3 cells. These results demonstrate that AMG 900 inhibits aurora kinases in PCa cells, potentially causing an increase in multipolar polyploid cells.

To further quantify the effects of combining HDACIs and AMG 900 in PCa cells, we performed cell cycle analysis after treatment (Figs. 5A-B). Cells were stained with phosphorylated H3 as a marker of aurora kinase activity. It is also a marker for cells in mitosis. DU-145 cells exhibited the greatest dose-dependent decrease in phosphorylated histone H3 positive cells upon AMG 900 treatment (Fig. 5A). In both cell lines combination treatment resulted in an additional decrease in phosphorylated histone H3 positive cells, most evidently after combination therapy with the HDACI vorinostat. As expected, flow cytometry showed AMG 900 alone induced polyploidy in a dose-dependent manner with DU-145 cells exhibiting increased polyploidy as compared to LNCaP and PC3 (Fig. 5B). Combinations of vorinostat and low dose AMG 900, again to a greater extent than VPA and low dose AMG 900, led to a much greater increase in polyploidy as compared to single agents across PCa cell lines investigated.

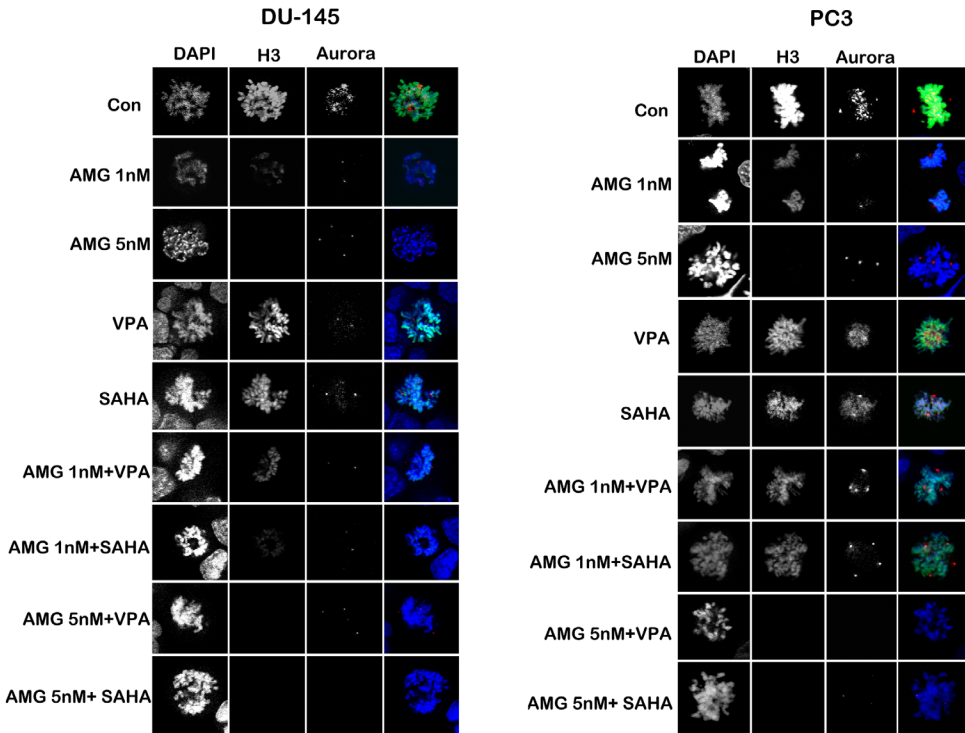


Figure 4. Confocal analysis of DU-145 and PC3 PCa cells treated with AMG 900 and HDACIs VPA and vorinostat stained for phosphorylated histone H3 (green) and phosphorylated aurora kinase A/B/C (red). Ai, AMG 900; VPA, valproic acid; SAHA, suberanilohydroxamic acid (vorinostat).

All above data pointed to enhanced antitumor effects after treatment with a combination of low concentration of AMG 900 with HDACIs. To investigate whether combinations led to increased apoptosis, we probed PCa cells for cleaved PARP, a marker for apoptosis. As expected, combination treatments with low dose AMG 900 resulted in an increase in PARP cleavage (Fig. 5C). DU-145 cells exhibited a greater increase in PARP cleavage as compared to PC3 cells. These data demonstrate that AMG 900 can effectively inhibit aurora kinase activity in PCa cells, although the degree of inhibition may differ between cell types. Addition of an HDACI further potentiates apoptosis.

Low dose AMG 900 in combination with vorinostat inhibits histone H3 phosphorylation and suppresses growth of DU-145 xenografts.

The effect of combination treatment with AMG 900 and HDACIs on *in vivo* tumor growth inhibition was evaluated in mice bearing DU-145 xenografts. Vorinostat was selected as HDACI in these experiments, as it is already approved for the treatment of cancers in humans and the treatment administration does not require continuous administration as does VPA in mice.¹⁸ Mice were treated with vehicle alone, with AMG 900 at 3.75 (low dose) or 7.5

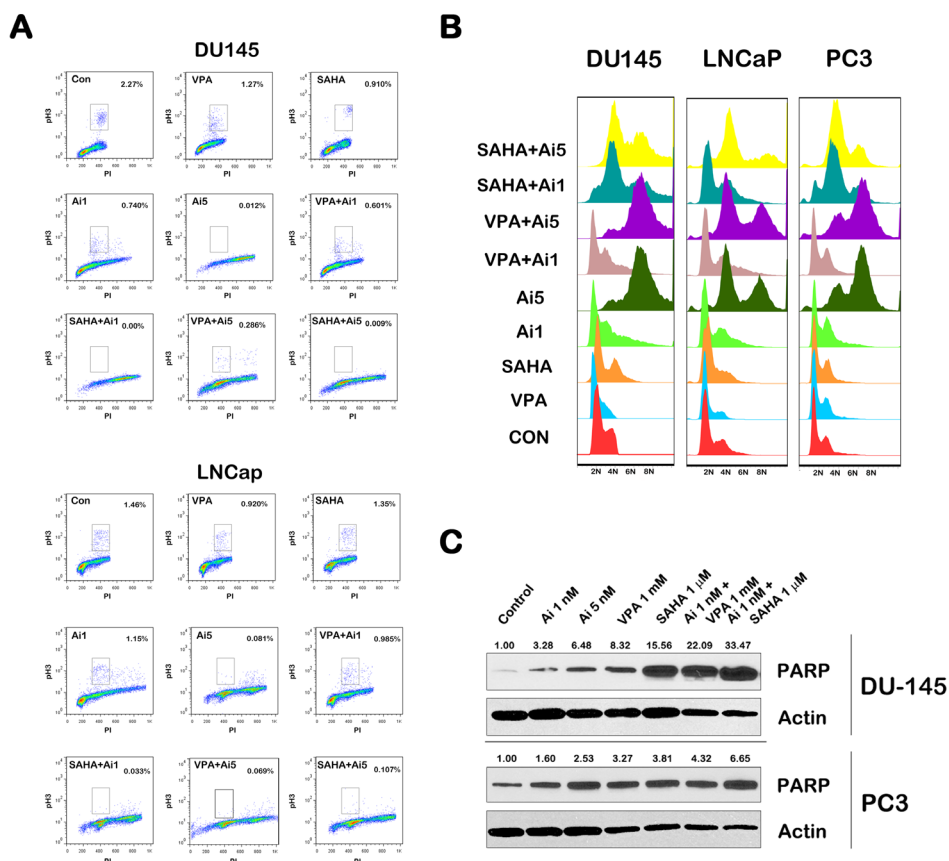


Figure 5. AMG 900 combined with HDACIs VPA or vorinostat (SAHA) decreases phosphorylated histone H3, and increases polyploidy and apoptosis in PCa cells. A-B) Flow cytometry analysis of PCa cell lines treated with AMG 900 and/or HDACIs. Cells were sorted by phosphorylated histone H3 expression (A) and by the number of sets of chromosomes (B). C) Western blot analysis for cleaved PARP in PCa cells treated with AMG 900 and/or HDACIs. Bands were normalized to the housekeeper actin. Ai, AMG 900; VPA, valproic acid; SAHA, suberanilohydroxamic acid (vorinostat).

mg/kg (high dose), and/or with vorinostat at 50 mg/kg, corresponding to doses that had been used previously with limited toxicity.^{11, 27} The mean tumor growth during treatment is depicted in Figure 6A. We used the random intercept model to assess the overall effects for time, group, and the time by group interaction, which were all significant (interaction p-value <0.0001). Specifically, the tumor growth rate in mice after single agent treatment with high dose AMG 900 and combination treatment with either low- or high dose AMG 900 and vorinostat was significantly reduced compared to the tumor growth rate in control mice (p-values 0.020, 0.014 and 0.036, respectively). In the group treated with a combination of low dose AMG 900 and vorinostat the average tumor growth rate was also lower than the tumor growth rate in groups treated with low dose AMG 900 alone or vorinostat alone (p=0.003 and p=0.008, respectively). Tumor growth rates in mice treated with low dose

AMG 900 and vorinostat combination treatment were similar to tumor growth rates in mice treated with high dose AMG 900 ($p=0.833$) and in mice treated with high dose AMG 900 and vorinostat ($p=0.721$).

These data are in concordance with our *in vitro* findings that the combination of low dose AMG 900 and HDACIs enhances inhibition of tumor cell growth. As the groups receiving combination treatment did not exhibit a significant difference in overall bodyweight compared to vehicle-treated controls and did not show signs of severe toxicity (no diarrhea or lethargy), our data suggest that combination treatment resulted in few toxicities in mice with stable weight.

Tumors from sacrificed mice were stained for phosphorylated histone H3 to assess aurora B inhibition (Fig. 6B). Vehicle and vorinostat treated tumors displayed about 4% phosphorylated histone H3 positive staining ($p=0.713$), while treatment with low dose AMG 900 decreased the percentage of phosphorylated histone H3 positive cells to about 2% ($p=0.004$) (Fig. 6C). Both combination treatment with low dose AMG 900 and vorinostat, and treatment with high dose AMG 900 alone or in combination with vorinostat resulted in 0.5% of the DU-145 cells being stained positively for phosphorylated histone H3, indicating significantly inhibited aurora B kinase activity in these tumors compared to vehicle treated tumors ($p<0.001$ and $p=0.001$, respectively). Inhibition of aurora B kinase activity did not differ between the combination treatment with low dose AMG 900 and vorinostat and high dose AMG 900 alone ($p=0.806$).

Discussion

Tumor cell resistance and dose-limiting toxicities frequently result in administration of molecularly targeted agents below the efficacy threshold in patients. The efficacy of treatment could be increased by rationally combining antitumor therapies. Previously, we applied Analysis of Functional Annotation (AFA) to analyze data from a microarray experiment with VPA- or vorinostat-treated PCa cells that demonstrated HDACI-induced changes in gene expression in these cells.²³ These data highlighted multiple pathways that were up- or downregulated by vorinostat and VPA in PCa cells.¹⁵ One can envisage pathways downregulated by HDACIs as opportunities for combining treatment modalities that are ineffective in the pathways' presence. Several genes involved in the mitotic spindle checkpoint were downregulated by HDACI treatment, such as aurora kinase inhibitors, pointing to combinations of HDACIs with mitotic spindle checkpoint inhibitors as a promising anticancer strategy.¹⁴ Combination therapy of aurora kinase inhibitors with HDACIs had promising results in preclinical experiments with blood cancers;^{28,29} such combinations have not been assessed in solid tumors. For this purpose, we successfully combined AMG 900, a pan-aurora kinase inhibitor, with HDACIs VPA and vorinostat in PCa cells in this study. AMG

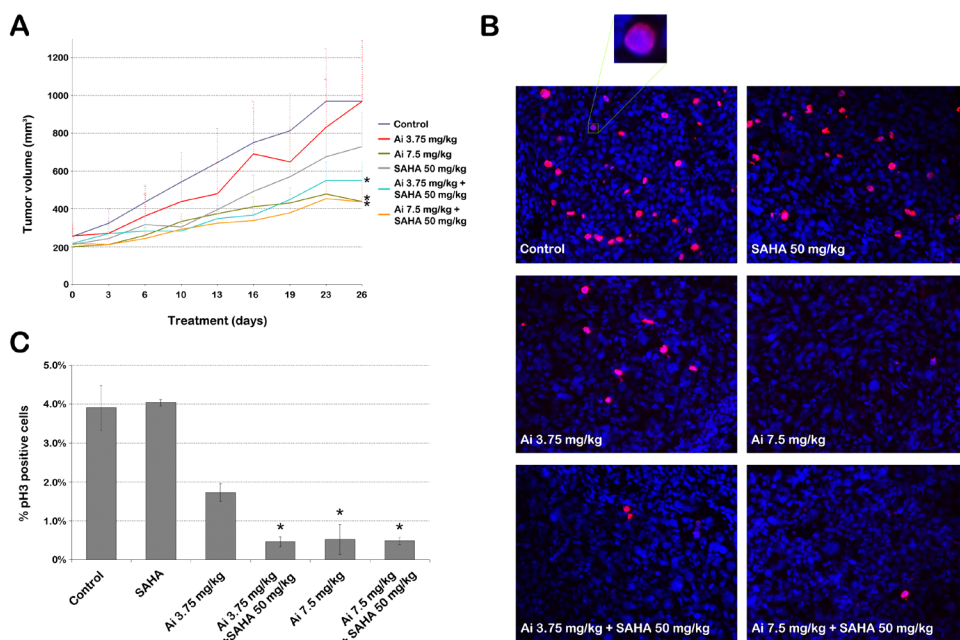


Figure 6. Low dose AMG 900 combined with vorinostat enhances growth suppression of DU-145 xenografts and inhibits histone H3 phosphorylation *in vivo*, similar to treatment with high dose AMG 900. A) Tumor growth curves of NOD/SCID mice bearing established DU-145 tumors treated with vehicle, AMG 900 and/or SAHA for a maximum of four weeks. B) Representative images of tumor sections stained for phosphorylated histone H3 (red) and DAPI (blue). C) Bar graph displaying the percentage of phosphorylated histone H3 positive cells per treatment group. Tumor volumes are represented as mean $\pm$ SEM (n=9). *, groups with a significantly decreased tumor growth rate (A) or less phosphorylated histone H3 (C) compared to the control group ($p \leq 0.05$). pH3, phosphorylated histone H3; Ai, AMG 900; SAHA, suberanilohydroxamic acid (vorinostat).

900 is currently being tested in phase I clinical trials in patients with advanced solid tumors and in patients with acute leukemias;³⁰ vorinostat is FDA-approved with a primary indication for cutaneous T-cell lymphoma.

Our data indicate that combining aurora kinase inhibitors with HDACIs yields additive and even synergistic effects in inhibiting growth of both androgen-dependent (LNCaP) and androgen-independent (DU-145, PC3) PCa cell lines. We propose that multiple factors contribute to the observed synergy: (1) HDACIs target cells in different stages of the cell cycle (interphase) than AMG 900 (mitosis).^{11, 31} It is conceivable that cells which escape targeting by AMG 900 and progress through mitosis would be inhibited by HDACIs. (2) HDACIs may complement the action of the aurora kinase inhibitor by directly upregulating expression of genes that trigger the apoptotic/senescence pathway, such as p21, p27, and Bcl-2.^{28, 32} (3) HDACIs also target aurora kinases but from a transcriptional angle instead of by direct catalytic inhibition; therefore, the aurora kinase pathway is targeted more effectively with

combination therapy.³³⁻³⁵ Furthermore (4), our data indicate that combinations of HDACIs and AMG 900 could trigger senescence. Senescence is known to be induced by activation of p21/Cip1 via p53.²⁴ However, AMG 900 induces p21/Cip1 proteins in LNCaP, which has a wild type p53, and PC3 and DU-145 cells, which are p53 null and mutant, respectively. This indicates that p21 is induced independently of p53. In line with this observation, HDACIs are known to induce p21 and senescence independently of the p53-p21 pathway.^{32, 36, 37} Intriguingly, the antitumor effect of AMG 900, enhanced with HDACIs, was more pronounced in clonogenic assays than in MTS assays. In line with previous studies with aurora kinase inhibitors, we hypothesized endoreduplication to be the mechanism of action.^{10, 11, 38} Though this process inexorably leads to cell death, it may, prior to that point, leave mitochondrial activity unaffected. Our cell cycle and microscopic imaging analysis results demonstrated an increase in polyploidy and senescence after AMG 900 treatment, supporting this theory.

Consistent with our *in vitro* observations, tumor growth rates were lower in mice with a DU-145 xenograft model that were treated with a combination of low dose AMG 900 and vorinostat, than in comparable mice treated with each individual agent at low doses. Growth rates in mice given combination treatment were equivalent, however, to those found in mice treated with high dose AMG 900 alone. Prior research noted that AMG 900 blocks phosphorylated histone H3 in a dose-dependent manner in multiple human tumor xenograft models.¹¹ Pharmacodynamic analysis in our study demonstrated that low dose AMG 900 in combination with low dose vorinostat blocks phosphorylated histone H3 with efficacy similar to that of high dose AMG 900.

In conclusion, combination treatment of low dose AMG 900 with HDACIs could prove to be a viable mode of therapy in solid tumors such as PCa. Clinically, a regimen combining lower concentrations of HDACIs and AMG 900 could thereby yield 1) increased efficacy through synergistic or additive mechanisms, and/or 2) decreased toxicity. As VPA, vorinostat and AMG 900 differ in dose-limiting toxicities, the combination is not expected to severely increase the odds of a dose-limiting toxicity.^{30, 39-41} Therefore, this combination of targeted therapies could be a candidate for clinical trials as forward-seeking translational research aimed at improving clinical outcomes in cancer patients.

Acknowledgments

The authors wish to thank AMGEN for providing the pan-aurora kinase inhibitor AMG 900 for both the *in vitro* and *in vivo* assays, dr. Michael McCaffery and Marcus Sanchez for their help analyzing the β -galactosidase staining images, and Georges Habineza and Whei Moriarty for their help and discussion.

References

1. Tannock IF, de Wit R, Berry WR et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351(15):1502-1512.
2. de Bono JS, Oudard S, Ozguroglu M et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* 2010;376(9747):1147-1154.
3. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013;63(1):11-30.
4. Hilton JF, Shapiro GI. Aurora kinase inhibition as an anticancer strategy. *J Clin Oncol* 2014;32(1):57-59.
5. Das K, Lorena PD, Ng LK et al. Aurora-A expression, hormone receptor status and clinical outcome in hormone related cancers. *Pathology* 2010;42(6):540-546.
6. Carmena M, Earnshaw WC. The cellular geography of aurora kinases. *Nat Rev Mol Cell Biol* 2003;4(11):842-854.
7. Sardon T, Peset I, Petrova B, Vernos I. Dissecting the role of Aurora A during spindle assembly. *EMBO J* 2008;27(19):2567-2579.
8. Perez-Cadahia B, Drohic B, Davie JR. H3 phosphorylation: dual role in mitosis and interphase. *Biochem Cell Biol* 2009;87(5):695-709.
9. Chieffi P, Cozzolino L, Kisslinger A et al. Aurora B expression directly correlates with prostate cancer malignancy and influence prostate cell proliferation. *Prostate* 2006;66(3):326-333.
10. Girdler F, Gascoigne KE, Evers PA et al. Validating Aurora B as an anti-cancer drug target. *J Cell Sci* 2006;119(Pt 17):3664-3675.
11. Payton M, Bush TL, Chung G et al. Preclinical evaluation of AMG 900, a novel potent and highly selective pan-aurora kinase inhibitor with activity in taxane-resistant tumor cell lines. *Cancer Res* 2010;70(23):9846-9854.
12. Yang H, Burke T, Dempsey J et al. Mitotic requirement for aurora A kinase is bypassed in the absence of aurora B kinase. *FEBS Lett* 2005;579(16):3385-3391.
13. Lee EC, Frolov A, Li R, Ayala G, Greenberg NM. Targeting Aurora kinases for the treatment of prostate cancer. *Cancer Res* 2006;66(10):4996-5002.
14. Kachhap SK, Rosmus N, Collis SJ et al. Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. *PLoS One* 2010;5(6):e11208.
15. Kortenhorst MS, Wissing MD, Rodriguez R et al. Analysis of the genomic response of human prostate cancer cells to histone deacetylase inhibitors. *Epigenetics* 2013;8(9):907-920.
16. Wissing MD, Mendonca J, Kortenhorst MS et al. Targeting prostate cancer cell lines with polo-like kinase 1 inhibitors as a single agent and in combination with histone deacetylase inhibitors. *FASEB J* 2013;27(10):4279-4293.
17. Abbas A, Gupta S. The role of histone deacetylases in prostate cancer. *Epigenetics* 2008;3(6):300-309.
18. Shabbeer S, Kortenhorst MS, Kachhap S, Galloway N, Rodriguez R, Carducci MA. Multiple Molecular pathways explain the anti-proliferative effect of valproic acid on prostate cancer cells in vitro and in vivo. *Prostate* 2007;67(10):1099-1110.
19. Hwang JJ, Kim YS, Kim MJ, Kim DE, Jeong IG, Kim CS. Histone deacetylase inhibitor potentiates anticancer effect of docetaxel via modulation of Bcl-2 family proteins and tubulin in hormone refractory prostate cancer cells. *J Urol* 2010;184(6):2557-2564.

20. Bots M, Johnstone RW. Rational combinations using HDAC inhibitors. *Clin Cancer Res* 2009;15(12):3970-3977.
21. Chou TC, Talalay P. Analysis of combined drug effects: a new look at a very old problem. *Trends Pharmacol Sci* 1983;4:450-454.
22. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res* 2010;70(2):440-446.
23. Kortenhorst MS, Zahurak M, Shabbeer S et al. A multiple-loop, double-cube microarray design applied to prostate cancer cell lines with variable sensitivity to histone deacetylase inhibitors. *Clin Cancer Res* 2008;14(21):6886-6894.
24. Roninson IB. Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. *Cancer Lett* 2002;179(1):1-14.
25. Xu WS, Perez G, Ngo L, Gui CY, Marks PA. Induction of polyploidy by histone deacetylase inhibitor: a pathway for antitumor effects. *Cancer Res* 2005;65(17):7832-7839.
26. Wei Y, Mizzen CA, Cook RG, Gorovsky MA, Allis CD. Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in *Tetrahymena*. *Proc Natl Acad Sci U S A* 1998;95(13):7480-7484.
27. Butler LM, Agus DB, Scher HI et al. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. *Cancer Res* 2000;60(18):5165-5170.
28. Kretzner L, Scuto A, Dino PM et al. Combining histone deacetylase inhibitor vorinostat with aurora kinase inhibitors enhances lymphoma cell killing with repression of c-Myc, hTERT, and microRNA levels. *Cancer Res* 2011;71(11):3912-3920.
29. Fiskus W, Wang Y, Joshi R et al. Cotreatment with vorinostat enhances activity of MK-0457 (VX-680) against acute and chronic myelogenous leukemia cells. *Clin Cancer Res* 2008;14(19):6106-6115.
30. Carducci MA, Shaheen MF, Paller CJ et al. First-in-human study of AMG 900, an oral pan-Aurora kinase inhibitor, in adult patients (pts) with advanced solid tumors. *J Clin Oncol* 30, 2012 (suppl; abstr 3009).
31. Richon VM, Sandhoff TW, Rifkind RA, Marks PA. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. *Proc Natl Acad Sci U S A* 2000;97(18):10014-10019.
32. DiBernardo G, Squillaro T, Dell'Aversana C et al. Histone deacetylase inhibitors promote apoptosis and senescence in human mesenchymal stem cells. *Stem Cells Dev* 2009;18(4):573-581.
33. Gully CP, Velazquez-Torres G, Shin JH et al. Aurora B kinase phosphorylates and instigates degradation of p53. *Proc Natl Acad Sci U S A* 2012;109(24):E1513-E1522.
34. Lorenzo C, Liao Q, Hardwicke MA, Ducommun B. Pharmacological inhibition of aurora-A but not aurora-B impairs interphase microtubule dynamics. *Cell Cycle* 2009;8(11):1733-1737.
35. Fadri-Moskwik M, Weiderhold KN, Deeraksa A et al. Aurora B is regulated by acetylation/deacetylation during mitosis in prostate cancer cells. *FASEB J* 2012;26(10):4057-4067.
36. Blagosklonny MV, Robey R, Sackett DL et al. Histone deacetylase inhibitors all induce p21 but differentially cause tubulin acetylation, mitotic arrest, and cytotoxicity. *Mol Cancer Ther* 2002;1(11):937-941.
37. Gediya LK, Belosay A, Khandelwal A, Purushottamachar P, Njar VC. Improved synthesis of histone deacetylase inhibitors (HDIs) (MS-275 and CI-994) and inhibitory effects of HDIs

- alone or in combination with RAMBAs or retinoids on growth of human LNCaP prostate cancer cells and tumor xenografts. *Bioorg Med Chem* 2008;16(6):3352-3360.
38. Weaver BA, Cleveland DW. Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer Cell* 2005;8(1):7-12.
 39. Atmaca A, Al-Batran SE, Maurer A et al. Valproic acid (VPA) in patients with refractory advanced cancer: a dose escalating phase I clinical trial. *Br J Cancer* 2007;97(2):177-182.
 40. Bruserud O, Stapnes C, Ersvaer E, Gjertsen BT, Ryningen A. Histone deacetylase inhibitors in cancer treatment: a review of the clinical toxicity and the modulation of gene expression in cancer cell. *Curr Pharm Biotechnol* 2007;8(6):388-400.
 41. Bradley D, Rathkopf D, Dunn R et al. Vorinostat in advanced prostate cancer patients progressing on prior chemotherapy (National Cancer Institute Trial 6862): trial results and interleukin-6 analysis: a study by the Department of Defense Prostate Cancer Clinical Trial Consortium and University of Chicago Phase 2 Consortium. *Cancer* 2009;115(23):5541-5549.

Chapter 9

Nuclear Eg5 (kinesin spindle protein) expression predicts docetaxel response and prostate cancer aggressiveness

Michel D. Wissing[†], Ellen S. de Morrée[†], Vincent O. Dezentjé, Jeroen T. Buijs, Ronald R. de Krijger, Vincent T.H.B.M. Smit, Wytse M. van Weerden, Hans Gelderblom, Gabri van der Pluijm

[†]These authors contributed equally to this work.

Oncotarget, in press

Abstract

Novel biomarkers predicting prostate cancer (PCa) aggressiveness and PCa docetaxel therapy response are needed. In this study, the correlation between nuclear Eg5-expression, PCa docetaxel response and PCa aggressiveness was assessed. Immunohistochemical staining for nuclear Eg5 was performed on 117 archival specimens from 110 PCa patients treated with docetaxel between 2004 and 2012. Samples were histologically categorized as positive/negative.

Median follow-up time from diagnosis was 11.6 years. Nuclear Eg5-expression was significantly related to docetaxel response ($p=0.036$) in tissues acquired within three years before docetaxel initiation. Nuclear Eg5-expression was not related to Gleason-score ($p=0.994$). Survival of patients after docetaxel initiation did not differ based on nuclear Eg5-expression ($p=0.540$). Analyzing samples taken before hormonal therapy, overall survival and time to docetaxel use were significantly decreased in patients with nuclear Eg5-expressing tumors ($p<0.01$). Eg5-positive nuclei were found more frequently in T4-staged tumors ($p=0.04$), Gleason 8-10 tumors ($p=0.08$), and in metastasized tumors ($p<0.01$). Multivariate analyses indicated that nuclear Eg5-expression may be an independent parameter for tumor aggressiveness. Limitations of a retrospective analysis apply.

In conclusion, nuclear Eg5-expression may be a predictive biomarker for docetaxel response in metastatic castrate-resistant PCa patients and a prognostic biomarker for hormone-naïve PCa patients. Prospective validation studies are needed to validate nuclear Eg5 as a biomarker.

Introduction

Metastatic castrate-resistant prostate cancer (mCRPC) is the second deadliest cancer in men in the Western world.¹ Primary first-line therapy for most mCRPC patients consists of the taxane docetaxel with prednisone,^{2,3} although various other mCRPC therapies have recently been introduced.⁴⁻⁸ About 48% of patients initially respond to docetaxel therapy;² eventually all patients progress during or after docetaxel therapy, usually within a few months after their last cycle. As docetaxel inhibits depolarization of microtubules regardless of cell type,⁹ toxicities may be severe, such as polyneuropathy and bone marrow suppression.² To prevent or restrict unnecessary docetaxel use, and to determine the optimal treatment sequence for individual mCRPC patients,¹⁰ biomarkers predicting docetaxel response need to be identified and implemented in clinical practice.¹¹

We hypothesized that nuclear Eg5 (Kindle Spindle Protein/KSP/KIF11/kinesin-5) may be such a marker. Eg5 separates spindle poles of a mitotic cell by crosslinking two antiparallel microtubules and moving to the plus-ends of both microtubules.¹² Due to its essential function in mitosis, multiple Eg5-inhibitors have been developed for anticancer therapy, such as ispinesib.¹³ Two studies with ispinesib focused particularly on mCRPC patients, with ambiguous results. In a phase I study, six out of fourteen mCRPC patients had stable disease (SD) for ≥ 18 weeks and one patient had a prostate-specific antigen (PSA)-decrease of $>50\%$ when ispinesib was combined with docetaxel in mCRPC patients.¹⁴ In a phase II study in which ispinesib was administered as monotherapy, no responses were reported.¹⁵ Twenty out of 21 patients had been treated with docetaxel prior to ispinesib. Immunohistochemistry analysis on archival tumor tissue from sixteen patients indicated that only one tumor stained positive for Eg5. It was concluded that ispinesib is not effective in primary prostate cancer (PCa) due to its low mitotic index, resulting in low Eg5 expression. However, considering their similar mechanism of action, an alternative explanation could be that cross-resistance occurs between docetaxel and Eg5-inhibitors.

Recent studies indicate that Eg5 may also play a role in intracellular transport in the cytoplasm, suggesting that Eg5-inhibitors may target Eg5 expressing non-mitotic cells too.^{16, 17} Xing et al. analyzed archival specimens from 80 patients with clinically localized PCa; half stained positive for Eg5, while benign prostate cells did not express Eg5.¹⁸ Considering the low mitotic index of PCa cells regardless of disease stage,¹⁹ these data suggest that Eg5 may indeed be expressed in non-mitotic PCa cells too.²⁰

Combining aforementioned findings, initial Eg5 expression of PCa may have been decreased once tumors have become docetaxel resistant.^{14, 15, 18} This led to our hypothesis that Eg5 may be a predictive marker for docetaxel response. Based on recent findings that patients with high Gleason-scores respond better to taxane-based therapy,²¹ we further hypothesized that Eg5 may be a prognostic marker for tumor aggressiveness and clinical outcome.

Materials and methods

Collection of patient material and data

Formalin fixed and paraffin embedded (FFPE) human PCa samples (biopsies, transurethral resections of prostate (TUR-P), or radical prostatectomies), stored at room temperature, were collected from pathology archives of Leiden University Medical Center, Reinier de Graaf Gasthuis and Erasmus Medical Center Rotterdam. mCRPC patients who had pathological material available taken before docetaxel therapy were included. The study was carried out in accordance with the Dutch code of conduct for the secondary use of human tissues; informed consent was therefore not required when enough material remained to serve the patient's and family's needs.²² Additional patient information was collected anonymously in a database. Approval was obtained from the Medical Ethics Committee of Leiden University Medical Center (P12.219).

Immunohistochemistry

Samples (3µm sections) were stained for Eg5 using a polyclonal anti-Eg5 antibody (1:1500, HPA006916, Sigma-Aldrich) on an automated immunohistochemistry stainer (Ventana Benchmark Ultra) (Fig. 1). This stainer utilized the ultraView Universal DAB Detection Kit (760-500, Ventana) for visualization of antibodies. The kit consisted of various enzyme labeled secondary antibodies that bind to primary antibodies; the complex was visualized with hydrogen peroxidase substrate and a 3,3'-diaminobenzidine (DAB) tetrahydrochloride (DAB) chromogen. For antigen retrieval, ULTRA CC1, an EDTA-Tris pH 8.4 solution, was used (950-224, Ventana). Representative images were taken at 20x10 under an Olympus BX41 microscope (Olympus Optical Co., Ltd.) from each slide using a colorview IIIu camera (Olympus), and analyzed with Cell[^]B imaging software (version 2.4108-181207). If an image was representative for the whole slide, only one picture was taken; otherwise, three representative views were imaged per slide.

Data analysis

Images were examined and scored blindly and independently by two researchers (MDW, ESdM). A clear contrast between nuclear and cytoplasmic Eg5 staining was evident (Fig. 1). Recent studies have indicated that intracellular functions of Eg5 may differ based on its subcellular localization;¹⁷ not all functions may be related to docetaxel response. Therefore, samples were scored for positive or negative staining of nuclei, cytoplasm or any cellular compartment (nucleus and/or cytoplasm).

Samples were considered positive when in one high-power field of view (20x10) at least four cancer cells were positive, regardless of intensity. This cut-off value ensured that random mitotic cells, infrequently found in the negative control too, were excluded. For analysis, average scores from both observers were calculated. If >50% of all scores per sample were positive for Eg5, the sample was considered Eg5-positive; otherwise it was considered Eg5-

negative.

Clinical endpoints

Clinical endpoints used in this study include survival from docetaxel initiation, overall survival (OS), time to symptomatic mCRPC and best therapy response.

Time to symptomatic mCRPC was defined as time between PCa diagnosis and docetaxel initiation. OS was calculated as time between diagnosis and patient death. If patients had not died or were lost to follow-up, survival was censored at the day the patient was last known to be alive before July 20th, 2013. Tumor aggressiveness was based on OS, time to symptomatic mCRPC, Gleason-score, and TNM-classification. Determination of best disease response (progressive disease, partial response) followed PCa working group guidelines as described previously, and could indicate PSA response and/or response as viewed on imaging such as computer tomography.^{23, 24}

Statistical analyses

Microsoft Excel 2003 was used for basic statistical analyses; student's t-tests were conducted for comparisons. SPSS (version 20) was used for the Kaplan-Meier analyses of survival and time to symptomatic mCRPC; log-rank tests were used to compare these parameters between groups. Multivariate analyses were performed using the Cox-regression model. P-values ≤ 0.050 were considered statistically significant.

Results

Patient and tissue characteristics

In total, 117 samples were collected from 110 mCRPC patients. These patients had been diagnosed with PCa between 1994 and 2011 and treated with docetaxel between July 15th, 2004 and December 24th, 2012. Median time to follow-up from date of PCa diagnosis was 11.6 years (interquartile range 8.7-14.2 years). Clinicopathological parameters are listed in Table 1. Median age of patients when diagnosed with PCa was 64 years. Median Gleason-score of tumors was 8. About two-thirds of patients had ≥ 2 measured metastatic localizations when docetaxel was initiated. Of note, tumor imaging methods such as CT-scans were not performed in all patients, underestimating the number of metastatic lesions. All patients had been medically and/or surgically castrated. In general, patients had been heavily pretreated: patients had received up to five therapies before docetaxel therapy.

For immunohistochemistry, tonsil and healthy prostate tissue served as positive and negative controls, respectively (Figs. 1A-B). Obtained PCa tissue consisted primarily of biopsies (70.0%) (Table 1). In the tumor samples, a clear distinction was observed between samples with nuclear Eg5 staining (5.1%), cytoplasmic Eg5 staining (19.7%), and samples staining

Table 1. Characteristics of mCRPC patients (n=110), their disease and treatment, and of the obtained tissue (n=117).

Patient Age		
At time of prostate cancer diagnosis [median (range)]	64	(43-84)
At time of tissue sampling [median (range)]	65	(43-86)
At time of start docetaxel [median (range)]	69	(46-87)
Disease characteristics (diagnostic imaging)		
Gleason-score [median (range)]		
All patients	8	(4-10)
Hormone-naïve patients	8	(4-10)
Number of metastatic lesions [number of patients (%)]		
1	37	(33.6%)
2	49	(44.5%)
≥3	24	(21.8%)
Confirmed localization of metastases [number of patients (%)]		
Lymph node	71	(64.5%)
Bone	106	(96.4%)
Liver	10	(9.1%)
Lung/pleura	16	(14.5%)
brain	1	(0.9%)
Treatment characteristics		
Pretreatment [number of patients (%)]		
Androgen-deprivation therapy	109	(99.1%)
Radical prostatectomy	15	(13.6%)
TUR-P	25	(22.7%)
Surgical castration	4	(3.6%)
Lymph node dissection	34	(30.9%)
Radiotherapy prostate	34	(30.9%)
Radiotherapy metastases	40	(36.4%)
Other	4	(3.6%)
Docetaxel treatment		
# courses [median (range)]	1	(1-3)
# cycles [median (range)]	6	(1-20)
Best response [number of patients (%)]:		
progressive disease	22	(20.0%)
stable disease	38	(34.5%)
partial response	49	(44.5%)
Docetaxel rechallenge [number of patients (%)]	7	(6.4%)
Posttreatment [number of patients (%)]		
Cabazitaxel	91	(82.7%)
Abiraterone	16	(14.5%)
Enzalutamide	30	(27.3%)
Radiotherapy	6	(5.5%)
Strontium-89	47	(42.7%)
Samarium-153	24	(21.8%)
Mitoxantrone	4	(3.6%)
Other	15	(13.6%)
Other	8	(7.3%)
Obtained pathological material		
Type of material [number of samples (%)]		
Biopsy	82	(70.0%)
TUR-P	24	(20.5%)
Radical prostatectomy	11	(9.4%)
Disease stage [number of samples (%)]		
hormone-naïve	87	(74.4%)
pre-docetaxel	112	(95.7%)
within three years of start docetaxel	61	(52.1%)
mCRPC post-docetaxel	5	(4.3%)

Survival		
OS in years [median (IQR)]	4.8	(2.6-9.3)
Lost-to-follow-up [number of patients (%)]	18	(16.4%)

IQR, interquartile range; mCRPC, metastatic castrate-resistant prostate cancer; OS, overall survival; TUR-P, transurethral resection of the prostate

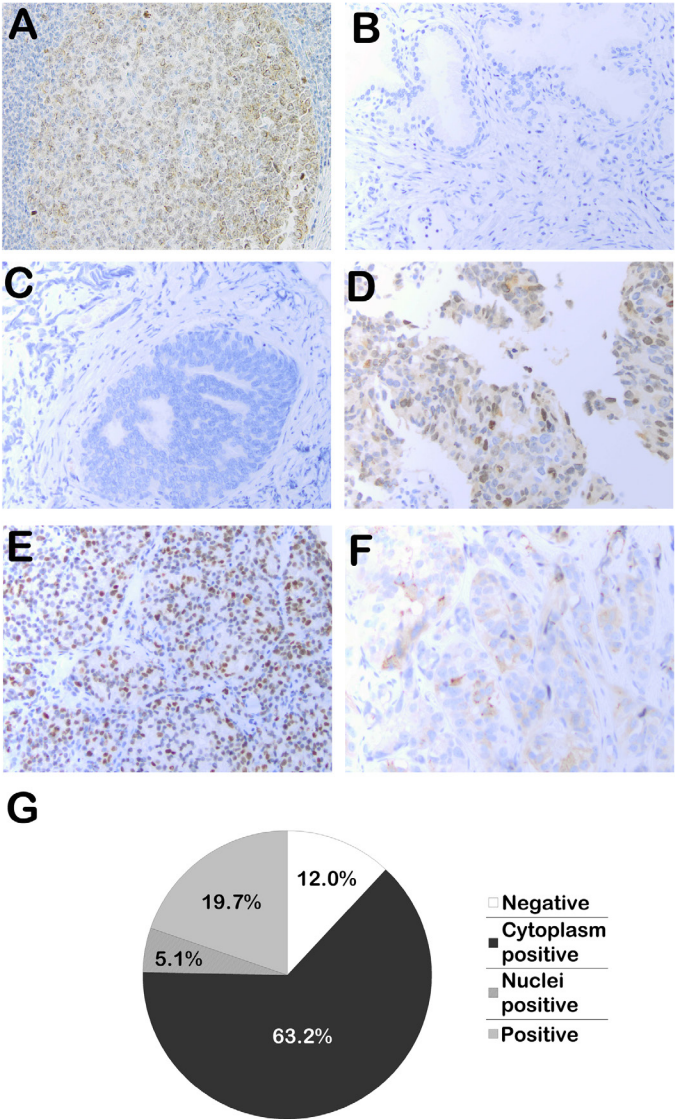


Figure 1. Immunohistochemical analysis of Eg5 expression in human clinical samples. A) Positive control: lymphatic tissue in a tonsil. B) Negative control: healthy prostate tissue. C) Prostate cancer (PCa) sample staining negative for Eg5. D) PCa sample with Eg5 expression in both the nuclei and cytoplasm. E) PCa sample with nuclear Eg5 expression. F) PCa sample with cytoplasmic Eg5 expression. G) Percentages indicate the frequencies samples with this subcellular staining pattern were found in our sample set (n=117).

positive for Eg5 in both compartments (63.2%), irrespective of the samples' age (Figs. 1C-G). Samples were scored for nuclear or cytoplasm staining separately. Interobserver agreement of scoring was 98.1%.

Immunohistochemical Eg5 expression and docetaxel response

Eg5 expression varied in tumors from some patients who had multiple biopsies taken before docetaxel therapy. This variability always reflected a disappearance of Eg5 expression over time. It is unknown whether these changes occurred as the tumor evolved spontaneously or due to other therapies, such as androgen-deprivation therapy. Therefore, correlation between Eg5 expression and docetaxel response was evaluated for all patients ($n=110$) as well as for patients with samples taken within three years before docetaxel start ($n=61$). A clear trend was observed between nuclear Eg5 expression and a better response to docetaxel therapy (Fig. 2A). This correlation was significant in patients from whom tissue was taken within three years before docetaxel initiation: 71.9% of these patients with nuclear Eg5 expression had a PR versus 36.4% of patients without nuclear Eg5 expression ($p=0.036$). Conversely, cytoplasmic or any Eg5 expression did not predict docetaxel response (data not shown).

As a previous report identified Gleason-scores as a predictive marker for docetaxel response, it was tested whether a correlation existed between Gleason-score and docetaxel response in our set of patient samples (data not shown). Gleason-score was not related to docetaxel response, neither in all patients ($p=0.343$) nor in patients with tissue available in the three

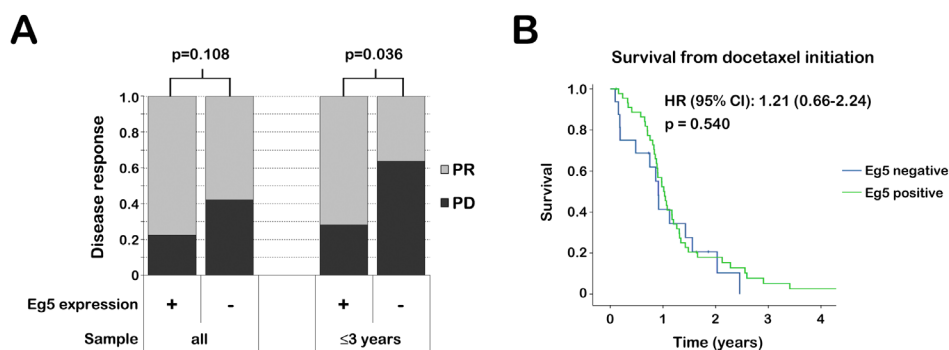


Figure 2. Nuclear Eg5 expression and docetaxel response in mCRPC patients. A) Best disease response to docetaxel therapy in mCRPC patients, grouped by nuclear Eg5 expression of their tumor. Patients with stable disease (SD) were excluded from this analysis. The most recent PCa tissue before docetaxel therapy was analyzed from all patients (left) or only from patients who had tissue available within three years before docetaxel therapy (right). B) Overall survival (OS) after docetaxel initiation. Patients were excluded when they only had PCa tissue available acquired more than three years before docetaxel therapy. Selected mCRPC patients were grouped based on nuclear Eg5 expression of their tumor. PD, progressive disease; PR, partial response.

years before docetaxel initiation ($p=0.884$). Furthermore, Gleason-score and nuclear Eg5 expression were not related in this latter subpopulation ($p=0.994$), suggesting that nuclear Eg5 expression was an independent marker of docetaxel response.

We further explored the correlation between docetaxel response and Eg5 expression by investigating patients who had a PCa sample taken before and after docetaxel treatment. Only five patients matched these criteria. While cytoplasmic Eg5 expression did not alter in these patients, three out of four tumors with positive Eg5 nuclei before docetaxel therapy did not have nuclear Eg5 expression after docetaxel treatment. These three patients had progressive disease upon discontinuation of docetaxel. On the other hand, the patient whose tumor expressed nuclear Eg5 pre- and post-docetaxel discontinued docetaxel therapy due to unacceptable toxicities. Despite the small patient number, these results suggested that loss of nuclear Eg5 expression may be related to docetaxel resistance.

Intriguingly, although patients with nuclear Eg5 expression had a better response to docetaxel (Fig. 2A), no difference in OS, calculated from the start of docetaxel therapy to death, was evident between tumors based on nuclear Eg5 expression ($p=0.540$) (Fig. 2B).

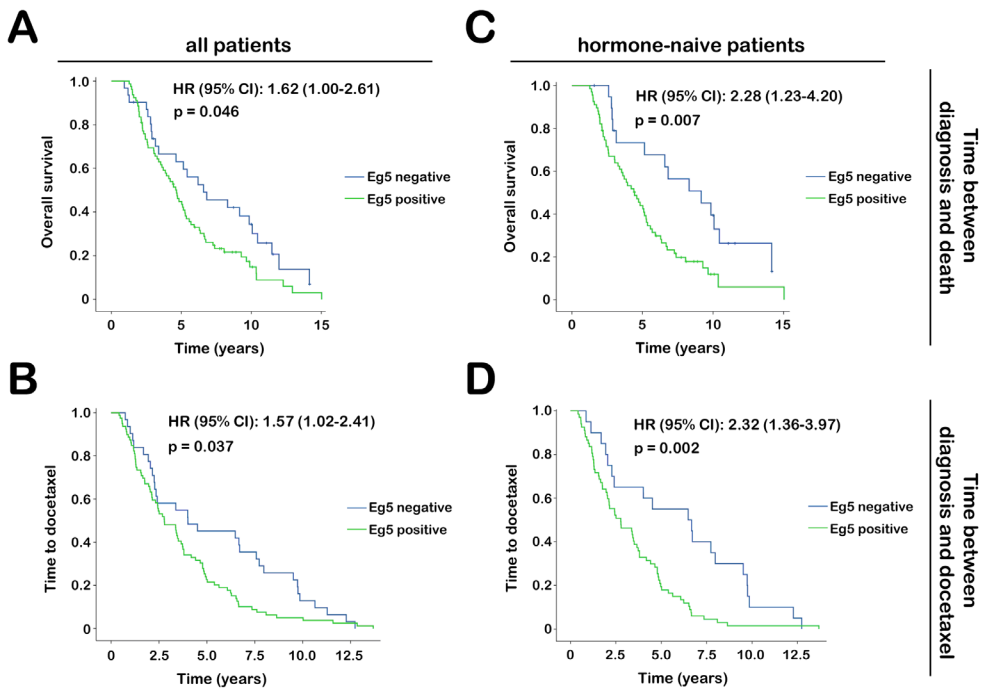


Figure 3. Tumor aggressiveness in mCRPC patients based on Eg5 expression. Patients were selected of whom PCa tissue acquired within three years (left) or three months (right) of diagnosis was available. Patients were divided in groups based on nuclear Eg5 expression. Median OS (top) and time to symptomatic mCRPC (bottom) were compared between patients groups.

Immunohistochemical nuclear Eg5 expression and tumor aggressiveness

We evaluated whether tumors with nuclear Eg5 expression behaved more aggressively. Analyzing samples from all 110 patients, patients with tumors with nuclear Eg5 expression had a significantly decreased OS (median 6.6 versus 4.7 years, $p=0.046$) (Fig. 3A). Time from diagnosis to symptomatic mCRPC was also decreased (median 4.0 versus 2.8 years, $p=0.037$) (Fig. 3B). When selecting samples from hormone-naïve patients ($n=87$), differences in OS and time to symptomatic mCRPC were even more pronounced ($p=0.010$ and $p=0.006$, respectively) (Figs. 3C-D). In this subset of patients, nuclear Eg5 expression was related to Gleason-score ($p=0.014$) and TNM-classification (tumor stage, $p=0.052$; any metastases, $p=0.007$; distant metastases, $p=0.021$); no correlation existed between nuclear Eg5 expression and age (Fig. 4).

Multivariate analyses were performed to test whether the correlation between nuclear Eg5 expression and tumor aggressiveness (OS and time to symptomatic mCRPC) remained evident when correcting for potential confounding variables, such as Gleason-score (Table 2). When including all patients, addition of most covariates resulted in no statistically significant correlation between nuclear Eg5 expression and OS or time to symptomatic mCRPC. This included correction for age, while this variable was neither related to nuclear Eg5 expression nor to prognosis, suggesting the study was underpowered for such analyses. However, a trend towards positive nuclear Eg5 expression and aggressive tumors was evident. When assessing hormone-naïve patients, a clearly positive trend existed between nuclear Eg5 expression and tumor aggressiveness regardless of the covariate added (hazard

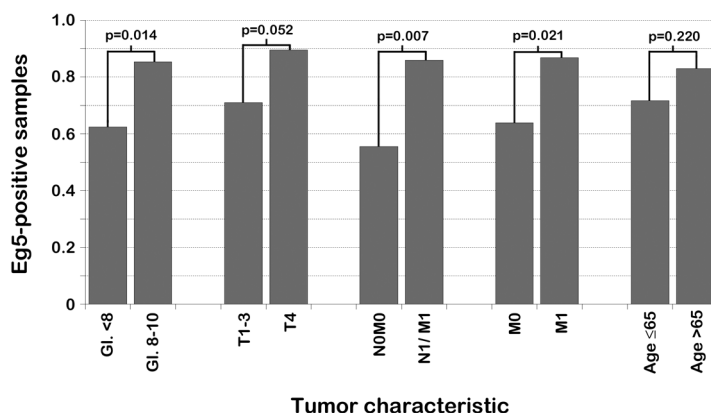


Figure 4. Correlation between PCa characteristics and nuclear Eg5 expression in hormone-naïve PCa patients. Tumors were divided in groups based on Gleason-score and TNM-classification upon diagnosis; the percentage of tumors with Eg5 expressing nuclei was compared.

GI, Gleason; T, tumor stage according to TNM-classification; N, lymph node metastases (0, no metastases; 1, metastases); M, distant metastases (0, no metastases; 1, metastases), N1/M1, any metastases (lymph node and/or distant metastases).

ratio >1.75), suggesting a potential independent prognostic value for nuclear Eg5 expression. The correlation between nuclear Eg5 expression and time to symptomatic mCRPC was significant in all subgroup analyses, except when metastases (N1 and/or M1) were added as a covariate ($p=0.063$).

Discussion

Research has been ongoing to identify prognostic biomarkers and biomarkers predictive for therapy response in PCa that have improved accuracy compared to established biomarkers such as serum PSA levels and Gleason-score, with some success.¹¹ Urokinase plasminogen activator and its inhibitor PAI-1, and Ki-67 have been identified as potential prognostic biomarkers of PCa.^{25, 26} Cytoplasmic localization of the androgen-receptor and increased blood serum levels of Macrophage Inhibitory Cytokine 1 (MIC-1) have been identified as potential markers for PCa docetaxel response.^{27, 28} PCa tumors expressing class III beta-tubulin were relatively insensitive to PCa therapy: class III beta-tubulin expression resulted in faster recurrence after radical prostatectomies, a decreased docetaxel response and decreased survival.²⁹ Unfortunately, none of these markers are available yet for use in clinical practice.¹¹ Additional studies, such as the one we present here, are needed to identify a biomarker to be used in clinical practice.

In the current study, we found that nuclear Eg5 expression in PCa was associated with improved antitumor efficacy of docetaxel, independently of patient's Gleason-score. Furthermore, we identified nuclear Eg5 as a prognostic marker in hormone-naïve PCa patients: patients whose tumor expressed nuclear Eg5 had a decreased median OS and progressed more rapidly to mCRPC. Similar findings were reported in non-small lung cancer patients: patients with Eg5 expressing tumors had a better response to chemotherapy, but a lower OS.³⁰ Similarly, Eg5 expression was related to worse clinical outcome in renal cell carcinoma patients.³¹

Once docetaxel was initiated, survival of mCRPC patients was similar irrespective of nuclear Eg5 expression. This may indicate that nuclear Eg5 expressing tumors initially respond well to docetaxel, resulting in decreased patient mortality. However, once these Eg5 expressing tumors progress, these tumors behave more aggressively, increasing patient death. This trend could indeed be derived from the survival curve (Fig. 2B) and might explain why survival of patients with nuclear Eg5 expression is not increased after docetaxel treatment despite responding better to docetaxel therapy. Alternatively, other factors may have resulted in the similar survival curve, such as unequal patient and treatment characteristics between groups other than Eg5 expression.

Table 2. Multivariate analysis using the Cox-regression model exploring potential confounders for the correlation between nuclear Eg5 expression and tumor aggressiveness.

Covariate	All patients		Hormone-naïve patients	
	OS [HR (95% CI)]	TTD [HR (95% CI)]	OS [HR (95% CI)]	TTD [HR (95% CI)]
Age	1.56 (0.95-2.54)	1.56 (1.00-2.42)	2.13 (1.13-4.02)	2.29 (1.31-3.98)
Gleason	1.48 (0.91-2.40)	1.45 (0.94-2.23)	1.76 (0.92-3.36)	1.78 (1.00-3.18)
Gleason <7 and ≥7	1.58 (0.95-2.63)	1.50 (0.95-2.38)	2.61 (1.35-5.07)	2.67 (1.50-4.75)
Gleason <8 and ≥8	1.48 (0.91-2.40)	1.46 (0.95-2.26)	1.84 (0.79-3.49)	1.93 (1.09-3.41)
T stage	1.75 (1.03-2.99)	1.59 (0.99-2.53)	2.56 (1.32-4.97)	2.64 (1.47-4.75)
Any metastases	1.52 (0.81-2.85)	1.28 (0.74-2.22)	2.25 (0.99-5.13)	1.97 (0.96-4.02)
Distant metastases	1.40 (0.79-2.49)	1.25 (0.75-2.08)	1.84 (0.87-3.87)	2.33 (1.14-4.79)
Number of metastases	1.64 (1.01-2.65)	1.59 (1.04-2.44)	2.61 (1.35-5.07)	2.67 (1.50-4.75)

CI, confidence interval; HR, hazard ratio; OS, overall survival; T stage, T stage according to TNM classification; TTD, time to docetaxel therapy.

Nuclear Eg5 expression could provide a useful tool for clinical practice. Interobserver agreement between researchers was very high (98.1%), as no subjective degrees of positive staining (mild/moderate/strong) were used. Positive/negative scoring requires little interpretation from the pathologist. Determination of Eg5 expression at the time of diagnosis would be non-invasive, as tissue material has already been acquired. Additional tissue sampling once the mCRPC stage has been reached, could aid the physician in deciding when to initiate docetaxel therapy. Patients whose tumor expresses nuclear Eg5 may benefit from early docetaxel treatment; patients with Eg5-negative tumors may be recommended to initiate other therapies first, as docetaxel response is more limited.

In the current study, a retrospective design was chosen, resulting in several limitations. FFPE PCa samples were collected from pathology archives; these samples were taken for diagnostic purposes (biopsies) and consisted of residual materials from surgical procedures such as TUR-P or radical prostatectomies. Therefore, the sample set we created was heterogeneous in origin. However, contrary to other tumors such as breast cancer, only limited tissue material is available from PCa patients during their disease, as many patients have a prostatectomy early in their disease and primarily suffer from bone metastases, which are not easily accessible. Furthermore, additional tissue sampling is often not needed, as it currently would not influence further therapy decisions. Therefore, although our initial patient number was relatively large, only limited tissue material was available from patients shortly before docetaxel initiation. Hence, all samples taken within three years of docetaxel initiation were collected for analysis. This led to a heterogeneous cohort of samples (both biopsies and residual surgical material), representing various stages of PCa disease. Furthermore, patients may have received various treatments between tissue sampling and docetaxel initiation. E.g., antiandrogen treatment significantly changed gene expression profiles of prostate cancer.³² It is unknown whether such treatment specifically

affects nuclear Eg5 expression. To overcome these challenges, a prospective study will be needed in which tissue will be collected shortly before docetaxel initiation to confirm the correlation between nuclear Eg5 expression and docetaxel response. However, such a study will need to overcome ethical and practical challenges.

In addition, our patient population was underpowered for multivariate analyses in hormone-naïve patients. Further prospective studies are warranted to validate whether nuclear Eg5 expression may serve as an independent prognostic biomarker.

Previous studies found that PCa patients with aggressive tumors respond well to docetaxel, but also respond better to cabazitaxel, suggesting that aggressive tumors respond well to taxanes in general.^{24, 33} Therefore, additional studies are needed to assess whether Eg5 predicts response to cabazitaxel too. Finally, our study results suggest that loss of Eg5 expression may be related to docetaxel resistance. Although ispinesib had limited antitumor efficacy after docetaxel, our study and previous phase I findings suggest that ispinesib may be effective when administered before or concomitantly with docetaxel, when up to 70% of tumors express nuclear Eg5.¹⁴ However, combination therapy with docetaxel would need direct comparison to docetaxel monotherapy. Eg5-inhibitors may provide further clinical benefit when selecting mCRPC patients based on nuclear Eg5 expression (personalized medicine).

In conclusion, nuclear Eg5 expressing PCa is aggressive, but responds well to docetaxel. Loss of nuclear Eg5 expression may be associated with docetaxel resistance. Determining nuclear Eg5 expression in PCa samples may aid to improve timing to initiate docetaxel therapy in individual PCa patients. Additional prospective studies are needed to confirm the predictive and prognostic value of nuclear Eg5.

Acknowledgments

The authors wish to thank Natalja ter Haar and Michelle Osse for their help with the immunohistochemistry experiments. The authors further wish to thank Carolien Haazer and Edith van Druten for their help collecting the patient samples from the Reinier de Graaf Gasthuis.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013;63(1):11-30.
2. Tannock IF, de Wit R, Berry WR et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351(15):1502-1512.
3. Petrylak DP, Tangen CM, Hussain MH et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* 2004;351(15):1513-1520.
4. de Bono JS, Oudard S, Ozguroglu M et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* 2010;376(9747):1147-1154.
5. de Bono JS, Logothetis CJ, Molina A et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011;364(21):1995-2005.
6. Ryan CJ, Smith MR, de Bono JS et al. Abiraterone in metastatic prostate cancer without previous chemotherapy. *N Engl J Med* 2013;368(2):138-148.
7. Scher HI, Fizazi K, Saad F et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med* 2012;367(13):1187-1197.
8. Parker C, Nilsson S, Heinrich D et al. Alpha emitter radium-223 and survival in metastatic prostate cancer. *N Engl J Med* 2013;369(3):213-223.
9. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. *Nat Rev Cancer* 2004;4(4):253-265.
10. van Soest RJ, van Royen ME, de Morree ES et al. Cross-resistance between taxanes and new hormonal agents abiraterone and enzalutamide may affect drug sequence choices in metastatic castration-resistant prostate cancer. *Eur J Cancer* 2013;49(18):3821-3830.
11. Armstrong AJ, Eisenberger MA, Halabi S et al. Biomarkers in the management and treatment of men with metastatic castration-resistant prostate cancer. *Eur Urol* 2012;61(3):549-559.
12. Kapitein LC, Peterman EJ, Kwok BH, Kim JH, Kapoor TM, Schmidt CF. The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. *Nature* 2005;435(7038):114-118.
13. Lad L, Luo L, Carson JD et al. Mechanism of inhibition of human KSP by ispinesib. *Biochemistry* 2008;47(11):3576-3585.
14. Blagden SP, Molife LR, Seebaran A et al. A phase I trial of ispinesib, a kinesin spindle protein inhibitor, with docetaxel in patients with advanced solid tumours. *Br J Cancer* 2008;98(5):894-899.
15. Beer TM, Goldman B, Synold TW et al. Southwest Oncology Group phase II study of ispinesib in androgen-independent prostate cancer previously treated with taxanes. *Clin Genitourin Cancer* 2008;6(2):103-109.
16. Wissing MD, van Diest PJ, van der Wall E, Gelderblom H. Antimitotic agents for the treatment of patients with metastatic castrate-resistant prostate cancer. *Expert Opin Investig Drugs* 2013;22(5):635-661.
17. Wakana Y, Villeneuve J, van Galen J, Cruz-Garcia D, Tagaya M, Malhotra V. Kinesin-5/Eg5 is important for transport of CARTS from the trans-Golgi network to the cell surface. *J Cell Biol* 2013;202(2):241-250.
18. Xing ND, Ding ST, Saito R et al. A potent chemotherapeutic strategy in prostate cancer: S-(methoxytrityl)-L-cysteine, a novel Eg5 inhibitor. *Asian J Androl* 2011;13(2):236-241.
19. Berges RR, Vukanovic J, Epstein JI et al. Implication of cell kinetic changes during the

- progression of human prostatic cancer. *Clin Cancer Res* 1995;1(5):473-480.
20. Wissing MD, Carducci MA, Gelderblom H, van Diest PJ. Tales of how great drugs were brought down by a flawed rationale--letter. *Clin Cancer Res* 2013;19(5):1303.
 21. van Soest RJ, de Morree ES, Shen L, Tannock IF, Eisenberger MA, de Wit R. Initial Biopsy Gleason Score as a Predictive Marker for Survival Benefit in Patients with Castration-resistant Prostate Cancer Treated with Docetaxel: Data from the TAX327 Study. *Eur Urol* 2013.
 22. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. *For. BMJ* 2002;325(7365):648-651.
 23. Scher HI, Halabi S, Tannock I et al. Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. *J Clin Oncol* 2008;26(7):1148-1159.
 24. Wissing MD, van Oort IM, Gerritsen WR et al. Cabazitaxel in patients with metastatic castration-resistant prostate cancer: results of a compassionate use program in the Netherlands. *Clin Genitourin Cancer* 2013;11(3):238-250.
 25. Gupta A, Lotan Y, Ashfaq R et al. Predictive value of the differential expression of the urokinase plasminogen activation axis in radical prostatectomy patients. *Eur Urol* 2009;55(5):1124-1133.
 26. Rubio J, Ramos D, Lopez-Guerrero JA et al. Immunohistochemical expression of Ki-67 antigen, cox-2 and Bax/Bcl-2 in prostate cancer; prognostic value in biopsies and radical prostatectomy specimens. *Eur Urol* 2005;48(5):745-751.
 27. Darshan MS, Loftus MS, Thadani-Mulero M et al. Taxane-induced blockade to nuclear accumulation of the androgen receptor predicts clinical responses in metastatic prostate cancer. *Cancer Res* 2011;71(18):6019-6029.
 28. Zhao L, Lee BY, Brown DA et al. Identification of candidate biomarkers of therapeutic response to docetaxel by proteomic profiling. *Cancer Res* 2009;69(19):7696-7703.
 29. Ploussard G, Terry S, Maille P et al. Class III beta-tubulin expression predicts prostate tumor aggressiveness and patient response to docetaxel-based chemotherapy. *Cancer Res* 2010;70(22):9253-9264.
 30. Saijo T, Ishii G, Ochiai A et al. Eg5 expression is closely correlated with the response of advanced non-small cell lung cancer to antimitotic agents combined with platinum chemotherapy. *Lung Cancer* 2006;54(2):217-225.
 31. Sun D, Lu J, Ding K et al. The expression of Eg5 predicts a poor outcome for patients with renal cell carcinoma. *Med Oncol* 2013;30(1):476.
 32. Holzbeierlein J, Lal P, LaTulippe E et al. Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance. *Am J Pathol* 2004;164(1):217-227.
 33. Oudard S, de Bono JS, Özgüroglu M et al. Impact of cabazitaxel (Cbz) + prednisone (P; CbzP) on overall survival (OS) at 2 yrs and in patients (pts) with aggressive disease: post-hoc analyses of TROPIC trial. [abstract]. *Ann Oncol* 2012;23 (Suppl 9; abstr 1857).

Chapter 10

Radium-223 chloride: extending life in prostate cancer patients by treating bone metastases

Michel D. Wissing, Fijs W.B. van Leeuwen, Gabri van der Pluijm, Hans Gelderblom

Clin Cancer Res. 2013 Nov 1;19(21):5822-7

Abstract

The treatment scope for patients with metastatic castrate-resistant prostate cancer (mCRPC) is rapidly expanding. On May 15th, 2013, the US Food and Drug Administration (FDA) approved radium-223 chloride ($^{223}\text{RaCl}_2$) for the treatment of mCRPC patients whose metastases are limited to the bones. Radium-223 is an α -emitting alkaline earth metal ion, which, similar to calcium ions, accumulates in the bone. In a phase III study (ALSYMPCA), mCRPC patients with bone metastases received best standard-of-care treatment with placebo or $^{223}\text{RaCl}_2$. At a prespecified interim analysis, the primary endpoint of median overall survival was significantly extended by 3.6 months in patients treated with radium-223 compared with placebo ($p < 0.001$). The radioisotope was well tolerated and gave limited bone marrow suppression. $^{223}\text{RaCl}_2$ is the first bone-targeting antitumor therapy that received FDA approval based on a significantly extended median overall survival. Further studies are required to optimize its dosing and to confirm its efficacy and safety in cancer patients.

Introduction

Prostate cancer is the most prevalent and second deadliest cancer in men in the United States and Europe.¹ Most morbidity and virtually all mortality from prostate cancer occur once the tumor has become metastatic and castrate-resistant (mCRPC).² Therefore, research aimed at the development of novel therapies for prostate cancer has primarily focused on this patient group. Although a decade ago no therapy existed with a proven significant benefit on the median overall survival of mCRPC patients, patients now have the options to be treated with multiple life-extending therapies. These therapies consist of agents that selectively target the androgen pathway (abiraterone acetate, enzalutamide) and taxanes (docetaxel, cabazitaxel), which target microtubules.³⁻⁸ Furthermore, the immunotherapy sipuleucel-T has been approved for its use in asymptomatic or minimally symptomatic mCRPC patients.^{9,10}

Prostate cancer primarily metastasizes to the bone.¹¹ Bone metastases may lead to severe morbidity, such as bone marrow failure, pathological fractures, and spinal cord compression, reducing quality of life and potentially resulting in death.^{12,13} Hence, specific treatment of bone metastases may significantly lower the burden of prostate cancer disease.^{14,15} Multiple agents have been approved by the United States Food and Drug Administration (US FDA) for the palliative treatment of bone metastases in mCRPC patients, such as external beam radiotherapy and the β -emitting radiopharmaceuticals strontium-89 (1.5-2.2 MBq/kg) and samarium-153 (37 MBq/kg) (Table 1). Such therapies result in symptomatic relief in more than half of patients.^{16,17} However, the duration of response is limited; the effect of these treatments on overall survival has not been studied. Moreover, as surrounding tissue, including the bone marrow, is damaged as well, significant adverse events such as bone marrow failure may occur in treated patients.

Table 1. Characteristics of radiopharmaceutical agents regularly used in clinic for the treatment of prostate cancer related bone metastases.

Therapeutic isotope	Rhenium-186	Samarium-153	Strontium-89	Radium-223
Half-life (days)	3.8	1.9	50.5	11.4
Administered agent	¹⁸⁶ Re-HEDP	¹⁵³ Sm-EDTMP	⁸⁹ SrCl ₂	²²³ RaCl ₂
Binding inducing factor	Bisphosphonate ligand	Lexidronam ligand	Ca ²⁺ similarity of Sr ²⁺	Ca ²⁺ similarity of Ra ²⁺
Therapeutic irradiation	1 β -particle	1 β -particle	1 β -particle	4 α -particles, 2 β -particles
Stable decay product	¹⁸⁶ Os	¹⁵³ Eu	⁸⁹ Y	²⁰⁷ Pb
Standard dose	1295 MBq	37 MBq/kg	1.5-2.2 MBq/kg	50 kBq/kg
FDA approval based on	not approved	relief of bone pain	relief of bone pain	improved median overall survival
Targeted prostate cancer population	patients with painful skeletal metastases	patients with confirmed osteoblastic metastatic bone lesions	patients with painful skeletal metastases	CRPC patients with symptomatic bone metastases and no known visceral metastatic disease

On May 15th, 2013, the FDA approved radium-223 chloride ($^{223}\text{RaCl}_2$; Xofigo; previously named alpharadin) for the treatment of bone metastases in mCRPC patients based on interim results from a phase III randomized clinical trial, the ALSYMPCA study (Alpharadin in the treatment of patients with symptomatic bone metastases in castration-resistant prostate cancer). This marks the first FDA-approved radionuclide that has been shown to extend overall survival in mCRPC patients in a phase III study. In this review, we will discuss the (pre)clinical development of $^{223}\text{RaCl}_2$, focusing primarily on the most recent results from the ALSYMPCA study. Subsequently, we discuss the FDA approval and future implications this approval may have in clinical practice.

Radium-223 chloride

$^{223}\text{RaCl}_2$ is a water-soluble radium salt. In ionic form, radium accumulates in bones at areas with increased bone turnover because of chemical similarity to calcium ions; both are alkaline earth metals.^{18, 19} Radium-223 is an α -emitting radioisotope that decays via seven daughter nuclides before it stabilizes as lead-207 (Fig. 1). During the decay of each radium-223 isotope, four α -particles and two electrons (β -particles) are emitted (Table 1). Both α - and β -irradiation can induce local therapy by inducing damage in the surrounding tissue. Because of the size and high energy of α -particles, these particles are highly effective in inducing double-strand breaks in DNA within 100 μm . The half-life of radium-223 is 11.4 days; the half-lives of its daughter nuclides range from seconds to minutes. These daughter nuclides do not have a chemical similarity to calcium ions. Therefore, the half-lives of radon-219 (4.0 s), bismuth-211 (2.1 min) and thallium-207 (4.8 min) seem to be long enough to allow diffusion from the primary accumulation site.

Early preclinical and clinical studies

In a preclinical study, nude rats with MT-1 human breast cancer xenografts were treated with pamidronate, a bisphosphonate used against skeletal complications of cancer, with or without radium-223.¹⁸ Although all rats treated with pamidronate only had to be sacrificed within 21 days, 40% of rats treated with pamidronate and radium-223 at 10 or 30 kBq survived beyond 50 days. Compared with β -emitting particles such as strontium-89, rodents treated with radium-223 showed no signs of bone marrow suppression or other toxicities.²⁰ In a phase I study, $^{223}\text{RaCl}_2$ was administered to 15 prostate and 10 breast cancer patients with bone metastases.²¹ Patients received a single i.v. injection of radium-223 with activities up to 250 kBq/kg. More than 50% of patients reported pain relief, while toxicity was low. Grade 3 leukopenia occurred in three patients; the maximum-tolerated dose (MTD) was not established. Radium-223 accumulated in the skeleton, particularly in sites with metastases. In the blood, radioactivity levels diminished quickly: to 6% after 1 hour and to <1% 24 hours

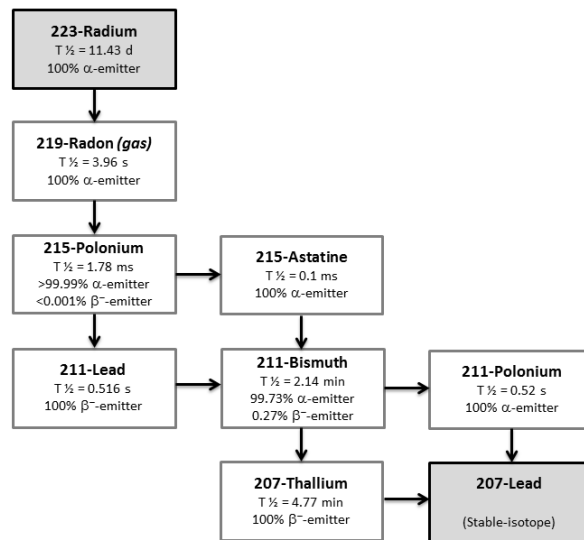


Figure 1. Radioactive decay of radium-223. Radium-223 decays via seven daughter nuclides to lead-207, resulting in the emission of both α-particles (vertical step in diagram) and β-particles (horizontal step in diagram). T½, half-life.

after injection. Recently, similar findings were presented from another phase I study.²² In this study, ten mCRPC patients received radium-223 up to 200 kBq/kg, among which six patients received a second dose of 50 kBq/kg. The MTD was again not established, and radium-223 was rapidly cleared from the blood, primarily into the small bowel.

A subsequent phase II study compared treatment of 50 kBq/kg radium-223 with placebo treatment in 64 CRPC patients with painful bone metastases.^{23, 24} Median time to skeletal-related event (SRE) was 14 weeks in the radium-223-treated group versus 11 weeks in patients treated with placebo ($p = 0.257$).²³ Low toxicity of radium-223 was confirmed in this phase II study. None of the 33 $^{223}\text{RaCl}_2$ -treated patients discontinued treatment because of adverse events. Grades 3 and 4 hematological adverse events occurred in 3 (9.1%) and 0 (0%) radium-223-treated patients and in 1 (3.3%) and 1 (3.3%) placebo-treated patients, respectively. Severe non-hematological adverse events occurred in 3 (9.1%) $^{223}\text{RaCl}_2$ -treated patients and in 5 (16.7%) placebo-treated patients. In a follow-up report 24 months after the first injection of study medication, no long-term treatment-related toxicity was noted.²⁴ Median overall survival was 65 weeks in the $^{223}\text{RaCl}_2$ -treated group, and 46 weeks in the placebo-treated group ($p = 0.056$).

ALSYMPCA

The ALSYMPCA phase III study was initiated in 2008, comparing the efficacy and safety of

$^{223}\text{RaCl}_2$ with that of placebo (a saline injection) in patients with symptomatic CRPC with bone metastases.²⁵ Patients needed to have at least two bone metastases, diagnosed by bone scintigraphy. Patients were eligible if they had previously received docetaxel, if they were unfit for docetaxel, if they declined therapy with docetaxel, or if docetaxel was not available. Patients were excluded if they had visceral metastases. Patients received an i.v. injection (50 kBq/kg) once every 4 weeks for a maximum of 6 cycles.

In total, 921 patients were included at 135 study locations worldwide, primarily in North America, Australia and Europe.²⁶ Six hundred fourteen patients received radium-223, of whom 352 had received docetaxel before $^{223}\text{RaCl}_2$ treatment (57.3%). In the placebo group, a similar percentage had received prior docetaxel (56.7% (174 patients)). Other baseline characteristics, such as age, disease stage, and baseline opioid use, were similar between the two treatment groups as well.²⁵

The primary endpoint of the ALSYMPCA study was overall survival. In general, the median overall survival in patients treated with radium-223 was extended by 3.6 months compared with placebo-treated patients ($p < 0.001$). For patients who had received docetaxel before participation in the ALSYMPCA trial, median overall survival was 14.4 months in $^{223}\text{RaCl}_2$ -treated patients versus 11.3 months in placebo-treated patients (hazard ratio (HR) = 0.71; 95% confidence interval (CI) 0.56-0.89); for patients who had not received prior docetaxel, the median overall survival durations were 16.1 months and 11.5 months, respectively (HR = 0.74; 95% CI 0.56-0.99).²⁵

The key secondary endpoint in the ALSYMPCA trial was time to symptomatic skeletal event (SSE), defined as the time to first use of external-beam radiotherapy to relieve skeletal symptoms, new symptomatic pathologic bone fractures, spinal cord compression, or tumor-related orthopedic surgery. Imaging to assess for skeletal events was only performed when clinically indicated to avoid registration of asymptomatic fractures. Time to SSE was 15.6 months in $^{223}\text{RaCl}_2$ -treated patients versus 9.8 months in the placebo-treated group ($p < 0.001$; HR = 0.66; 95% CI 0.52-0.83).²⁵ Time to initial opioid use and time to external beam radiotherapy were both increased in patients treated with radium-223 (HR = 0.670 and HR = 0.621, respectively).²⁷ Sixteen and 24 weeks after initiation of radium-223 treatment, patients had significantly less pain compared with baseline ($p < 0.001$ and $p = 0.001$, respectively). Further analysis revealed that an increase in the levels of total alkaline phosphatase was associated with an increased risk for death in the patient population studied in the ALSYMPCA trial ($p < 0.0001$).²⁸ In patients treated with radium-223, a $\geq 30\%$ reduction in total alkaline phosphatase levels compared with the baseline was seen in 47% of patients versus 3% of placebo-treated patients.²⁵

In general, radium-223 was well tolerated by patients, with grade 3 or 4 adverse events occurring more frequently in the placebo-treated group (62%) than in patients treated with radium-223 (56%). The only reported non-hematologic grade ≥ 3 adverse events that occurred more frequently in the $^{223}\text{RaCl}_2$ -treated patient group were anorexia (2% versus 1%) and a

decreased appetite (2 patients versus 0 patients). Comparing grade ≥ 3 hematologic adverse events in the $^{223}\text{RaCl}_2$ -treated group with the placebo group, anemia occurred in 13% and 13%, neutropenia in 3% and 1%, and thrombocytopenia in 6% and 2%, respectively.²⁵ Analysis of these hematologic adverse events revealed that a baseline total alkaline phosphatase of ≥ 220 U/L strongly predicted anemia.²⁹ Apart from the use of radium-223 instead of placebo, other baseline predictors for neutropenia and thrombocytopenia were prior docetaxel use and more than six bone metastases. However, prior external beam radiotherapy to the bone was associated with a decrease in anemia and neutropenia.

FDA approval

Interim results from the ALSYMPCA phase III clinical trial led to approval of radium-223 by the FDA for the treatment of mCRPC patients who have symptomatic bone metastases and no visceral metastases. It was recommended to be administered at 50 kBq/kg every 4 weeks with a maximum of 6 doses, which is equal to the treatment regimen in the ALSYMPCA trial. This approval makes radium-223 the first agent available for CRPC patients that significantly increases overall survival by exclusively treating bone metastases.

Despite its decision to approve radium-223, the FDA required four additional studies, besides final analysis of ALSYMPCA study results.³⁰ Non-compliance with this decision, or negative results could result in the FDA revoking the approval.

As mentioned previously, no MTD for radium-223 has been established.^{21, 22} Results from phase I studies suggested concentrations higher than 50 kBq/kg could be administered to patients with relatively few changes in the toxicity profile of the agent. Two recent phase II studies confirm that treatment up to 100 kBq/kg has similar toxicities compared with radium-223 at 50 kBq/kg, while the efficacy of radium-223 is increased.^{31, 32} Therefore, the FDA required a randomized phase II study to further assess the efficacy and safety of radium-223 in CRPC patients with bone metastases at concentrations higher than 50 kBq/kg. If these results suggest a beneficial risk-benefit profile for higher doses, an additional phase III study will be required to confirm the optimal activity level.

The FDA required the company to perform three studies to further assess the safety of radium-223: an observational study in 1200 CRPC patients with bone metastases, evaluating the long-term safety of radium-223 administered at the recommended dose; a randomized clinical trial in CRPC patients with bone metastases and no visceral metastases to further assess the safety of radium-223, particularly for enhanced assessment of the effect of radium-223 on healthy bone marrow and secondary malignancies, such as acute myeloid lymphoma (AML) and myelodysplastic syndrome (MDS);³³ finally, an assessment of the short- and long-term safety of a radium-223 rechallenge, for which the company was required to perform a study in which CRPC patients with bone metastases were re-treated with radium-223.

Discussion

With the FDA approval of $^{223}\text{RaCl}_2$ for CRPC patients with symptomatic bone metastases regardless of prior chemotherapy, the treatment scope for mCRPC patients has further expanded. By excluding patients with visceral metastases, the most rational step for oncologists would be to use radium-223 primarily as a first-line therapy in mCRPC patients. That said, a subpopulation may also be eligible for radium-223 treatment as second-line therapy or later.

Considering the results of radium-223 treatment in the robustly designed and well-conducted ALSYMPCA trial, it is expected that the approval and consequently clinical use of radium-223 will expand beyond the United States in the near future. In addition, radium-223 may improve the quality of life and survival for patients with other tumor types who suffer from bone metastases. However, such expansions require additional clinical investigations. Finally, the FDA approval of radium-223 as the first metastasis-targeting agent based on an improved median overall survival confirms that selectively treating metastases may be an effective strategy in patients with advanced solid tumors for whom palliative treatment is the only option, strengthening the development of such agents.

A major limitation of the ALSYMPCA phase III study is that the group of patients selected for this study may not correspond to the patient population in clinical practice that will receive radium-223 treatment. Patients with visceral metastases were excluded from participation in the trial. Considering that β -emitting radionuclides, such as samarium-153, have been shown to induce pain relief in patients with advanced prostate cancer, further research is required to address whether radium-223 provides clinical benefit for patients with visceral metastases.^{16, 17} Based on the approval of radium-223, physicians may decide to administer this agent to this group of patients as well, although its efficacy has not been proven. Furthermore, patients were excluded from the ALSYMPCA study when docetaxel was available, patients were fit for (and willing to receive) docetaxel treatment and had not received docetaxel before. Nevertheless, the FDA does not exclude this group of patients for radium-223 treatment in its approval letter. Until the required post-marketing studies have been conducted that will indicate whether mCRPC patients eligible for docetaxel treatment will also benefit from radium-223, its clinical benefit in this patient group remains uncertain. Currently, no long-term follow-up is known for patients in the ALSYMPCA trial, limiting the toxicity profile of the drug. Although the high number of therapeutic emissions in the decay process of radium-223 may yield a strong therapeutic effect, the daughter nuclides, which do not have affinity to the bone, may diffuse throughout the body and cause damage in healthy tissue. Although no bone marrow suppression was seen in the short term, it will be important to follow patients over time to ensure that there are no long-term harmful effects from radium-223 treatment. Most of the FDA's post-marketing requirements therefore focus on drug safety, particularly on the long-term effects of radium-223 treatment and safety of radium-223 rechallenge at a later stage.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013;63(1):11-30
2. American Cancer Society [Internet]. Dublin, OH; c2013 [updated 2013 May 15; cited 2013 Aug 1]. Survival rates for prostate cancer. Available from: <http://www.cancer.org/cancer/prostatecancer/detailedguide/prostate-cancer-survival-rates>.
3. Tannock IF, de Wit R, Berry WR et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351(15):1502-1512.
4. de Bono JS, Oudard S, Ozguroglu M et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* 2010;376(9747):1147-1154.
5. de Bono JS, Logothetis CJ, Molina A et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011;364(21):1995-2005.
6. Fizazi K, Scher HI, Molina A et al. Abiraterone acetate for treatment of metastatic castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301 randomised, double-blind, placebo-controlled phase 3 study. *Lancet Oncol* 2012;13(10):983-992.
7. Scher HI, Fizazi K, Saad F et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med* 2012;367(13):1187-1197.
8. Ryan CJ, Smith MR, de Bono JS et al. Abiraterone in metastatic prostate cancer without previous chemotherapy. *N Engl J Med* 2013;368(2):138-148.
9. Small EJ, Schellhammer PF, Higano CS et al. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. *J Clin Oncol* 2006;24(19):3089-3094.
10. Kantoff PW, Higano CS, Shore ND et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010;363(5):411-422.
11. Lange PH, Vessella RL. Mechanisms, hypotheses and questions regarding prostate cancer micrometastases to bone. *Cancer Metastasis Rev* 1998;17(4):331-336.
12. Coleman RE. Clinical features of metastatic bone disease and risk of skeletal morbidity. *Clin Cancer Res* 2006;12(20 Pt 2):6243s-6249s.
13. Nieder C, Haukland E, Pawinski A, Dalhaug A. Anaemia and thrombocytopenia in patients with prostate cancer and bone metastases. *BMC Cancer* 2010;10:284.
14. Goyal J, Antonarakis ES. Bone-targeting radiopharmaceuticals for the treatment of prostate cancer with bone metastases. *Cancer Lett* 2012;323(2):135-146.
15. Saylor PJ, Armstrong AJ, Fizazi K et al. New and emerging therapies for bone metastases in genitourinary cancers. *Eur Urol* 2013;63(2):309-320.
16. Sartor O, Reid RH, Hoskin PJ et al. Samarium-153-Lexidronam complex for treatment of painful bone metastases in hormone-refractory prostate cancer. *Urology* 2004;63(5):940-945.
17. Serafini AN. Samarium Sm-153 leixidronam for the palliation of bone pain associated with metastases. *Cancer* 2000;88(12 Suppl):2934-2939.
18. Henriksen G, Bristol K, Bruland OS, Fodstad O, Larsen RH. Significant antitumor effect from bone-seeking, alpha-particle-emitting (223)Ra demonstrated in an experimental skeletal metastases model. *Cancer Res* 2002;62(11):3120-3125.
19. van Leeuwen FW, Verboom W, Reinhoudt DN. Selective extraction of naturally occurring

- radioactive Ra²⁺. *Chem Soc Rev* 2005;34(9):753-761.
20. Henriksen G, Fisher DR, Roeske JC, Bruland OS, Larsen RH. Targeting of osseous sites with alpha-emitting ²²³Ra: comparison with the beta-emitter ⁸⁹Sr in mice. *J Nucl Med* 2003;44(2):252-259.
 21. Nilsson S, Larsen RH, Fossa SD et al. First clinical experience with alpha-emitting radium-223 in the treatment of skeletal metastases. *Clin Cancer Res* 2005;11(12):4451-4459.
 22. Carrasquillo JA, O'Donoghue JA, Pandit-Taskar N et al. Phase I pharmacokinetic and biodistribution study with escalating doses of (²²³)Ra-dichloride in men with castration-resistant metastatic prostate cancer. *Eur J Nucl Med Mol Imaging* 2013;40(9):1384-1393.
 23. Nilsson S, Franzen L, Parker C et al. Bone-targeted radium-223 in symptomatic, hormone-refractory prostate cancer: a randomised, multicentre, placebo-controlled phase II study. *Lancet Oncol* 2007;8(7):587-594.
 24. Nilsson S, Franzen L, Parker C et al. Two-year survival follow-up of the randomized, double-blind, placebo-controlled phase II study of radium-223 chloride in patients with castration-resistant prostate cancer and bone metastases. *Clin Genitourin Cancer* 2013;11(1):20-26.
 25. Parker C, Nilsson S, Heinrich D et al. Alpha emitter radium-223 and survival in metastatic prostate cancer. *N Engl J Med* 2013;369(3):213-223.
 26. clinicaltrials.gov [Internet]. Bethesda, MD: National Institutes of Health, c1993-2013 [updated 2013 July 30; cited 2013 Aug 1]. A Phase III Study of Radium-223 Dichloride in Patients With Symptomatic Hormone Refractory Prostate Cancer With Skeletal Metastases (ALSYMPCA). Available from <http://clinicaltrials.gov/ct2/show/study/NCT00699751>.
 27. Nilsson S, Sartor AO, Bruland OS, Fang F, Aksnes AK, Parker C. Pain analyses from the phase III randomized ALSYMPCA study with radium-223 dichloride (Ra-223) in castration-resistant prostate cancer (CRPC) patients with bone metastases. *J Clin Oncol* 2013;31(suppl):Abstract 5038.
 28. Sartor AO, Amariglio R, Wilhelm S et al. Correlation between baseline variables and survival in the radium-223 dichloride (Ra-223) phase III ALSYMPCA trial with attention to total ALP changes. *J Clin Oncol* 2013;31(suppl):Abstract 5080.
 29. Parker C, Garcia-Vargas JE, O'Bryan-Tear CG, Fang F, Vogelzang NJ. Hematologic safety of Ra-223 dichloride (Ra-223) in castration-resistant prostate cancer (CRPC) patients with bone metastases from the phase III ALSYMPCA trial. *J Clin Oncol* 2013;31(suppl):Abstract 5060.
 30. United States Food and Drug Administration [Internet]. Silver Spring, MD; c2013 [updated 2013 May 14; cited 2013 Aug 1]. Summary review of Xofigo, application number: 203971Orig1s000. Available from http://www.accessdata.fda.gov/drugsatfda_docs/nda/2013/203971Orig1s000SumR.pdf.
 31. Parker CC, Pascoe S, Chodacki A et al. A randomized, double-blind, dose-finding, multicenter, phase 2 study of radium chloride (Ra 223) in patients with bone metastases and castration-resistant prostate cancer. *Eur Urol* 2013;63(2):189-197.
 32. Nilsson S, Strang P, Aksnes AK et al. A randomized, dose-response, multicenter phase II study of radium-223 chloride for the palliation of painful bone metastases in patients with castration-resistant prostate cancer. *Eur J Cancer* 2012;48(5):678-686.
 33. Leone G, Fianchi L, Voso MT. Therapy-related myeloid neoplasms. *Curr Opin Oncol* 2011;23(6):672-680.

Chapter 11

NDRG1 in prostate cancer biology and treatment

Chapter 11a

NDRG1 (N-myc downstream regulated gene 1) **[gene card]**

Michel D. Wissing, Nadine S. Rosmus, Michael A. Carducci, Sushant K. Kachhap

Atlas Genet Cytogenet Oncol Haematol. 2010; 14(8):776-780

Identity

Other names: CAP43; CMT4D; DRG-1; DRG1; GC4; HMSNL; NDR1; NMSL; PROXY1; RIT42; RTP; Rit42; TARG1; TDD5

HGNC (Hugo): NDRG1

Location: 8q24.22

DNA/RNA

NDRG1 consists of 60,085 basepairs, starting at basepair 134,318,596 and ending at basepair 134,378,680 from the p-terminus. It is a member of the NDRG family, consisting of NDRG1, NDRG2, NDRG3 and NDRG4 (of which three isoforms exist: NDRG-4B, NDRG-4Bvar and NDRG-4H), which are part of the alpha/beta hydrolase superfamily. The DNA of NDRG1 contains 16 exons (Fig. 1). It encodes a 3.0 kb mRNA with a coding region of 1.185 kb.

Protein

NDRG1 is a 43 kDa protein, composed of 394 amino acids, with an iso-electric point of 5.7 (Fig. 2). NDRG1 has an alpha/beta hydrolase-fold motif, however, the presence of hydrolytic catalytic activity is still questionable. NDRG1 has more than seven phosphorylation sites, among others a phosphopantetheine attachment site, protein kinase C, casein kinase II, tyrosine kinase, protein kinase A and calmodulin kinase II. NDRG1 is phosphorylated by protein kinase A and calmodulin kinase II, and is a physiological substrate of SGK1 and GSK-3 β kinase, a kinase involved in cancer growth and progression. NDRG1 is relatively ubiquitously expressed in normal human cells, and especially highly expressed in prostate, brain, kidney, placenta, ovarian, testicular and intestinal cells. NDRG1 is mostly found in epithelial cells. In cells, NDRG1 is primarily a cytoplasmic protein. 47.8% of the NDRG1 is expressed in the cytosol, 26.1% in the nucleus (such as in prostate epithelial cells), and 8.7% in the mitochondria (such as in proximal tubule cells in the kidney). NDRG1 is also found in the adherens junctions. Additionally, in intestinal and lactating breast epithelia NDRG1 is located in the plasma membrane. NDRG1 can also be found in vacuoles, the peroxisome, early and recycling endosomes, and the cytoskeleton.

NDRG1 amino acid sequence is 53% homologous to NDRG2, 62% to NDRG3, 62% to NDRG4, and 94% homologous to the mouse analog, Ndr-1 (also known as TDD5). NDRG1 homologs have been found in *Helianthus*, *Caenorhabditis*, *Xenopus* and *Drosophila*.

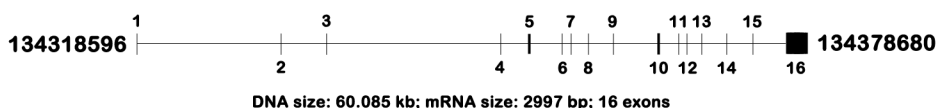


Figure 1. NDRG1 DNA

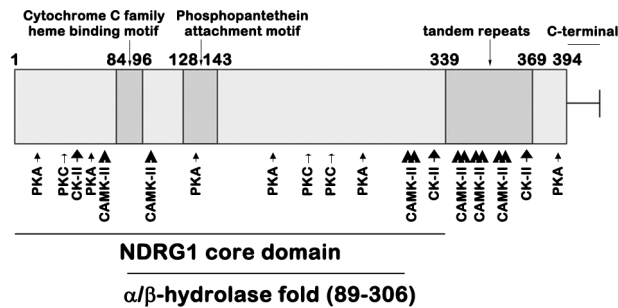


Figure 2. NDRG1 protein.

Function

NDRG1 is reported to be a metastasis suppressor gene which is downregulated in prostate, colon and breast cancers. It has been found to be a Rab4a effector protein that recruits to the recycling endosomes in the trans-Golgi network by binding to the lipid phosphatidylinositol 4-phosphate (PI4P), where it plays a role in the recycling of E-cadherin. NDRG1 also interacts with HSP70. NDRG1 co-localizes with APO A-I and A-II, and may be involved in lipid transport. The function of NDRG1 may be controlled at least in part by phosphorylation. It has also been identified as a stress response gene, upregulated by homocysteine and hypoxia. HIF-1-dependent and independent mechanisms have been implicated in NDRG1 induction. It is also controlled by AP-1 transcription factors. When exposed to stress, for example hypoxia, NDRG1 may play a cytoprotective role in normal healthy cells. There is evidence that NDRG1 is involved in the induction of differentiation. NDRG1 is downregulated under conditions of cell growth. NDRG1 expression peaks in the G1 and G2/M phases, and is lowest in the S phase. NDRG1 is also a microtubule-associated protein, which may play an important role in maintaining spindle structure during cell division. In Schwann cells, NDRG1 is essential for myelin sheath maintenance. Hence, NDRG1 is a multifunctional protein with roles that may be tissue- and/or cell-type specific.

Certain transcription factors such as myc, ERG1, HIF-1 α bind to the NDRG1 promoter region and regulate its expression.

NDRG1 is upregulated during colon epithelial cell differentiation. It is regulated by hormones such as androgens and estradiol. Small molecules such as Nhydroxy- N'-phenol-octane-1,8-dioic acid diamide, calcium ionophores like BAPTA, metal ions such as Nickel and Cobalt, iron chelators and differentiating agents, such as retinoic acid, induce NDRG1 expression. Additionally, NDRG1 is induced during cellular DNA damage and endoplasmic reticulum stress.

Implications in solid cancers

NDRG1 has implications in prostate, breast, colon, renal, bladder, pancreatic and hepatic

cancer. Downregulation of NDRG1 in cancer worsens the prognosis of cancer. There is an inverse relationship in the levels of NDRG1 expression and the Gleason grade of the tumor in prostate cancer. A high PTEN (a tumor suppressor which positively regulates NDRG1) and NDRG1 expression improves survival rates in patients with breast and prostate cancer. In patients with colorectal cancer, the 2 year survival rate for patients with high NDRG1-expression was 82.4%, while for patients with a low NDRG1-expression it was only 69.6%. In pancreatic cancer patients, the median survival time for patients with high NDRG1-expression was 24.7 months, while the median survival time for patients with low NDRG1-expression was only 10.9 months. High expression of NDRG1 in colon tumors was found to correlate with increased resistance to irinotecan.

An inverse relationship exists between NDRG1 and the oncogenes N-myc and c-myc, suggesting that members of the myc family suppress expression of NDRG1. Experimental evidence exist that both N-myc and c-myc downregulate NDRG1 gene expression by directly binding to NDRG1 promoter.

NDRG1 is downregulated in colon, breast, prostate and pancreatic neoplasms, by c-myc and N-myc transcription factors. In cancer cells, NDRG1 expression is consistent through all phases in the cell cycle, instead of the biphasic expression in normal cells. PTEN expression is positively related to NDRG1 expression. NDRG1 is induced in cancer cells by histone deacetylase inhibitors and DNA methyl transferase inhibitors indicating that NDRG1 is regulated by chromatin modulation and DNA methylation.

Although NDRG1 has been reported to be downregulated in a variety of cancers, it has been shown to be upregulated in hepatic, pancreatic and kidney cancers. Induction of NDRG1 in these tumors is speculated to be in response to tumor stress or hypoxia, and NDRG1 is proposed as a marker for tumor hypoxia. However, in pancreatic cancer, cellular differentiation and not hypoxia was demonstrated to be the determining factor for NDRG1 expression. In renal cancer, induction of NDRG1 in the tumor tissue was restricted to infiltrating macrophages and not cancer cells.

NDRG1 is suggested to be an early target for p53. Loss of p53 expression in cancer is suggested to reduce NDRG1 expression. However, p53 knockout mice show expression of NDRG1, suggesting that there are other mechanisms regulating NDRG1 levels.

NDRG1 expression plays a role *in vitro* in primary tumor growth in prostate, breast, and bladder cancer: a higher expression of NDRG1 lowers the proliferation rates of these cancers. In pancreatic and bladder cancer cells, this reduction was proven *in vivo*: in pancreatic cells it was suggested that the reduced proliferation was caused by NDRG1 by modulating tumor stroma and angiogenesis. NDRG1 can recruit onto the recycling endosome in the trans-Golgi network by binding to phosphatidylinositol 4-phosphate. There, NDRG1 may be involved in the transport of various cargo back to the cells' surface. At the molecular level, NDRG1 may stabilize the E-cadherin molecule by recycling it back to the cells' surface, thereby preventing tumor invasion.

Implications in hereditary motor and sensory neuropathy-Lom (HMSNL) / Charcot-Marie-Tooth disease (CMT 4D)

HMSNL/CMT 4D is caused by the Gypsy founder mutation in NDRG1, homozygote R148X, also called homozygote C564t. In patients with CMT disease, apart from the R148X mutation, another disease-causing mutation was identified, namely IVS8-1G>A (g.2290787G>A), which results in skipping of exon 9. The homozygote phenotype of this mutation was very closely related to the phenotype of HMSNL/CMT 4D patients. HMSNL/CMT 4D is a hereditary autosomal recessive disease, resulting in demyelination of peripheral nerves. It is the most common form of demyelinating CMT disease in the Roma population. The disease results in severe disability in adulthood. It begins consistently in the first decade of life with a gait disorder, followed by upper limb weakness in the second decade and, in most subjects, by deafness setting in in the third decade of life. Sensory loss affecting all modalities is present; sensory loss and motor involvement predominates distally in the limbs. Skeletal deformity, particularly foot deformities, are frequent.

Implications in atherosclerosis

Patients with HMSNL were found to have a high total cholesterol: HDL-C ratio. Atherosclerosis is an important factor for the development of cardiovascular diseases, like myocardial infarction and angina pectoris. NDRG1 contributes to HDL-C (high-density lipoproteincholesterol) levels most likely by its phosphopantetheine-binding domain interacting with the high-density lipoproteins apolipoprotein A-I and AII.

References

- Kokame K, Kato H, Miyata T. Homocysteine-responder genes in vascular endothelial cells identified by differential display analysis. GRP78/BiP and novel genes. *J Biol Chem* 1996 Nov 22;271(47):29659-29665
- Krauter-Canham R, Bronner R, Evrard JL, Hahne G, Friedt W, Steinmetz A. A transmitting tissue- and pollen-expressed protein from sunflower with sequence similarity to the human RTP protein. *Plant Sci* 1997; 129: 191-202.
- van Belzen N, Dinjens WN, Diesveld MP et al. A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms. *Lab Invest* 1997 Jul;77(1):85-92
- Kalaydjieva L, Nikolova A, Turnev I et al. Hereditary motor and sensory neuropathy--Lom, a novel demyelinating neuropathy associated with deafness in gypsies. Clinical, electrophysiological and nerve biopsy findings. *Brain* 1998 Mar;121 (Pt 3):399-408
- Kurdistani SK, Arizti P, Reimer CL, Sugrue MM, Aaronson SA, Lee SW. Inhibition of tumor cell growth by RTP/rit42 and its responsiveness to p53 and DNA damage. *Cancer Res* 1998 Oct 1;58(19):4439-4444
- Zhou D, Salnikow K, Costa M. Cap43, a novel gene specifically induced by Ni2+ compounds. *Cancer Res* 1998 May 15;58(10):2182-2189
- Piquemal D, Joulia D, Balaguer P, Basset A, Marti J, Combes T. Differential expression of the RTP/

- Drg1/Ndr1 gene product in proliferating and growth arrested cells. *Biochim Biophys Acta* 1999 Jul 8;1450(3):364-373
- Salnikow K, An WG, Melillo G, Blagosklonny MV, Costa M. Nickel-induced transformation shifts the balance between HIF-1 and p53 transcription factors. *Carcinogenesis* 1999 Sep;20(9):1819-1823
- Shimono A, Okuda T, Kondoh H. N-myc-dependent repression of ndr1, a gene identified by direct subtraction of whole mouse embryo cDNAs between wild type and N-myc mutant. *Mech Dev* 1999 May;83(1-2):39-52
- Ulrix W, Swinnen JV, Heyns W, Verhoeven G. The differentiation-related gene 1, Drg1, is markedly upregulated by androgens in LNCaP prostatic adenocarcinoma cells. *FEBS Lett* 1999 Jul 16;455(1-2):23-26
- Agarwala KL, Kokame K, Kato H, Miyata T. Phosphorylation of RTP, an ER stress-responsive cytoplasmic protein. *Biochem Biophys Res Commun* 2000 Jun 16;272(3):641-647
- Guan RJ, Ford HL, Fu Y, Li Y, Shaw LM, Pardee AB. Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer. *Cancer Res* 2000 Feb 1;60(3):749-755
- Kalaydjieva L, Gresham D, Gooding R et al. N-myc downstreamregulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom. *Am J Hum Genet* 2000 Jul;67(1):47-58
- Salnikow K, Costa M, Figg WD, Blagosklonny MV. Hyperinducibility of hypoxia-responsive genes without p53/p21- dependent checkpoint in aggressive prostate cancer. *Cancer Res* 2000 Oct 15;60(20):5630-5634
- Salnikow K, Su W, Blagosklonny MV, Costa M. Carcinogenic metals induce hypoxia-inducible factor-stimulated transcription by reactive oxygen species-independent mechanism. *Cancer Res* 2000 Jul 1;60(13):3375-3378
- Gómez-Casero E, Navarro M, Rodríguez-Puebla ML et al. Regulation of the differentiation-related gene Drg-1 during mouse skin carcinogenesis. *Mol Carcinog* 2001 Oct;32(2):100-109
- Unoki M, Nakamura Y. Growth-suppressive effects of BPOZ and EGR2, two genes involved in the PTEN signaling pathway. *Oncogene* 2001 Jul 27;20(33):4457-4465
- Zoroddu MA, Kowalik-Jankowska T, Kozlowski H, Salnikow K, Costa M. Ni(II) and Cu(II) binding with a 14-aminoacid sequence of Cap43 protein, TRSRSHSTSEGRSR. *J Inorg Biochem* 2001 Mar;84(1-2):47-54
- Lachat P, Shaw P, Gebhard S, van Belzen N, Chaubert P, Bosman FT. Expression of NDRG1, a differentiation-related gene, in human tissues. *Histochem Cell Biol* 2002 Nov;118(5):399-408
- Qu X, Zhai Y, Wei H et al. Characterization and expression of three novel differentiationrelated genes belong to the human NDRG gene family. *Mol Cell Biochem* 2002 Jan;229(1-2):35-44
- Salnikow K, Kluz T, Costa M et al. The regulation of hypoxic genes by calcium involves c-Jun/AP-1, which cooperates with hypoxiainducible factor 1 in response to hypoxia. *Mol Cell Biol* 2002 Mar;22(6):1734-1741
- Bandyopadhyay S, Pai SK, Gross SC et al. The Drg-1 gene suppresses tumor metastasis in prostate cancer. *Cancer Res* 2003 Apr 15;63(8):1731-1736
- Hunter M, Bernard R, Freitas E et al. Mutation screening of the N-myc downstream-regulated gene 1 (NDRG1) in patients with Charcot-Marie-Tooth Disease. *Hum Mutat* 2003 Aug;22(2):129-135
- Li J, Kretzner L. The growth-inhibitory Ndr1 gene is a Myc negative target in human neuroblastomas and other cell types with overexpressed N- or c-myc. *Mol Cell Biochem* 2003 Aug;250(1-2):91-105
- Masuda K, Ono M, Okamoto M et al. Downregulation of Cap43 gene by von Hippel-Lindau tumor suppressor protein in human renal cancer cells. *Int J Cancer* 2003 Jul 20;105(6):803-810
- Taketomi Y, Sugiki T, Saito T et al. Identification of NDRG1 as an early inducible gene during in vitro maturation of cultured mast cells. *Biochem Biophys Res Commun* 2003 Jun 27;306(2):339-346
- Bandyopadhyay S, Pai SK, Hirota S et al. Role of the putative tumor metastasis suppressor gene Drg-1

- in breast cancer progression. *Oncogene* 2004 Jul 22;23(33):5675-5681
- Bandyopadhyay S, Pai SK, Hirota S et al. PTEN upregulates the tumor metastasis suppressor gene Drg-1 in prostate and breast cancer. *Cancer Res* 2004 Nov 1;64(21):7655-7660
- Kim KT, Ongusaha PP, Hong YK et al. Function of Drg1/Rit42 in p53-dependent mitotic spindle checkpoint. *J Biol Chem* 2004 Sep 10;279(37):38597-38602
- Okuda T, Higashi Y, Kokame K, Tanaka C, Kondoh H, Miyata T. Ndr1-deficient mice exhibit a progressive demyelinating disorder of peripheral nerves. *Mol Cell Biol* 2004 May;24(9):3949-3956
- Stein S, Thomas EK, Herzog B et al. NDRG1 is necessary for p53-dependent apoptosis. *J Biol Chem* 2004 Nov 19;279(47):48930-48940
- Sugiki T, Murakami M, Taketomi Y, Kikuchi-Yanoshita R, Kudo I. N-myc downregulated gene 1 is a phosphorylated protein in mast cells. *Biol Pharm Bull* 2004 May;27(5):624-627
- Cui DX, Zhang L, Yan XJ et al. A microarray-based gastric carcinoma prewarning system. *World J Gastroenterol* 2005 Mar 7;11(9):1273-1282
- Hunter M, Angelicheva D, Tournev I et al. NDRG1 interacts with APO AI and A-II and is a functional candidate for the HDL-C QTL on 8q24. *Biochem Biophys Res Commun* 2005 Jul 15;332(4):982-992
- Shah MA, Kemeny N, Hummer A et al. Drg1 expression in 131 colorectal liver metastases: correlation with clinical variables and patient outcomes. *Clin Cancer Res* 2005 May 1;11(9):3296-3302
- Chen B et al. N-myc down-regulated gene 1 modulates the response of term human trophoblasts to hypoxic injury. *J Biol Chem* 2006 Feb 3;281(5):2764-2772
- Kovacevic Z, Richardson DR. The metastasis suppressor, Ndr1-1: a new ally in the fight against cancer. *Carcinogenesis* 2006 Dec;27(12):2355-2366
- Maruyama Y, Ono M, Kawahara A et al. Tumor growth suppression in pancreatic cancer by a putative metastasis suppressor gene Cap43/NDRG1/Drg-1 through modulation of angiogenesis. *Cancer Res* 2006 Jun 15;66(12):6233-6242
- Kachhap SK, Faith D, Qian DZ et al. The N-Myc down regulated Gene1 (NDRG1) Is a Rab4a effector involved in vesicular recycling of E-cadherin. *PLoS One* 2007 Sep 5;2(9):e844
- Ellen TP, Ke Q, Zhang P, Costa M. NDRG1, a growth and cancer related gene: regulation of gene expression and function in normal and disease states. *Carcinogenesis* 2008 Jan;29(1):2-8

Chapter 11b

Identification of cetrimonium bromide and irinotecan as compounds with synthetic lethality against NDRG1 deficient prostate cancer cells

Michel D. Wissing, Janet Mendonca, Eunice Kim, Eugene Kim, Joong S. Shim, Nadine S. Kaelber, Huub Kant, Hans Hammers, Therese Commes, Paul J. van Diest, Jun O. Liu, Sushant K. Kachhap

Cancer Biol Ther. 2013 May 1;14(5):401-10

Abstract

The N-myc downstream regulated gene 1 (NDRG1) has been identified as a metastasis-suppressor gene in prostate cancer (PCa). Compounds targeting PCa cells deficient in NDRG1 could potentially decrease invasion/metastasis of PCa. A cell based screening strategy was employed to identify small molecules that selectively target NDRG1 deficient PCa cells. DU-145 PCa cells rendered deficient in NDRG1 expression by a lentiviral shRNA-mediated knockdown strategy were used in the primary screen. Compounds filtered from the primary screen were further validated through proliferation and clonogenic survival assays in parental and NDRG1 knockdown PCa cells. Screening of 3360 compounds revealed irinotecan and cetrimonium bromide (CTAB) as compounds that exhibited synthetic lethality against NDRG1 deficient PCa cells. A three-dimensional (3-D) invasion assay was utilized to test the ability of CTAB to inhibit invasion of DU-145 cells. CTAB was found to remarkably decrease invasion of DU-145 cells in collagen matrix. Our results suggest that CTAB and irinotecan could be further explored for their potential clinical benefit in patients with NDRG1 deficient PCa.

Introduction

The majority of morbidity and mortality in prostate cancer (PCa) patients is caused by metastases.¹ The ability of cancer cells to metastasize is a multistep process that involves intravasation of cells from their primary site into blood vessels and extravasation into target organs.² Inhibiting any step of the metastatic process is hypothesized to negatively impact the spread of cancer, thereby providing clinical benefit for cancer patients. Failure of cancer therapies can to a large extent be attributed to a failure to halt or contain metastasis, particularly in PCa. To improve the clinical outcome for PCa patients, it is crucial to target key molecular mechanisms/pathways involved in the metastatic process of PCa cells.

In the past two decades, multiple genes have been identified that suppress the formation and growth of cancer metastases without affecting primary growth of the tumor, such as KAI1, CD44, NM23, PEBP1, RECK, MAP2K4 and the N-myc downstream regulated gene 1 (NDRG1).^{3,4} NDRG1 is downregulated in highly metastatic PCa cells.⁵ Our group has identified NDRG1 as a Rab4a GTPase effector protein involved in the vesicular recycling of the adhesion molecule E-Cadherin, thereby preventing its degradation and possibly preventing metastasis of cancer cells.⁶ More recently, Liu et al. reported that NDRG1 negatively modulates Wnt- β -signaling during metastatic progression via interaction with the Wnt receptor LRP6.⁷ Therefore, drugs that selectively target tumor cells deficient in NDRG1 could potentially decrease PCa invasion by modulating the E-Cadherin and the Wnt- β -signaling pathway. However, compounds that selectively target NDRG1-deficient prostate cancer cells are yet to be identified.

In the current study, we aimed to identify compounds that selectively target NDRG1 deficient PCa cells. For this purpose, we raised isogenic DU-145, LNCaP and PC3 cell lines that differed in their NDRG1 expression by stably knocking down (KD) NDRG1 expression using a lentiviral shRNA vector. By performing a drug library screen, we aimed to identify compounds that would be less toxic to cells by themselves, but prove to be synthetically lethal in (PCa) cells that had lower NDRG1 expression. Parental and NDRG1 KD DU-145 cells were utilized for the primary screen; all three PCa cell lines were used for validation of compounds identified from the primary screen. The screen was performed using the Johns Hopkins drug library (JHDL), a small-molecule library with 3,360 compounds consisting mostly of FDA-approved drugs and other bioactive molecules.⁸

In this study, the topoisomerase I inhibitor irinotecan and the cationic surfactant cetrimonium bromide (CTAB) were identified as compounds that are synthetically lethal *in vitro* in NDRG1 deficient PCa cell lines. These compounds warrant further investigation as potential chemotherapeutic agents that could target NDRG1 deficient invasive PCa cells.

Materials and methods

Cell lines and treatment

PC3, DU-145 and LNCaP PCa cell lines were obtained from ATCC. Human prostate fibroblasts were obtained from a prostate biopsy on a 62-year old PCa patient with a Gleason score of 4 and kindly provided by dr. J. Isaacs. All cell lines were grown and maintained in RPMI-1640 media (Invitrogen) supplemented with 10% fetal bovine serum (complete RPMI media). Cells were maintained in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

JHDL compounds were stored at -20°C in 200 µM stock solutions in DMSO; compounds were dissolved in H₂O when they were not soluble in DMSO. Irinotecan (I1406, Sigma-Aldrich) and CTAB (H9151, Sigma-Aldrich) were stored as 10 mM stock solutions in DMSO at -20°C. For experiments the compounds were diluted in complete RPMI media to obtain the desired final concentration.

Knockdown experiment

Stable shRNA KD for NDRG1 in PCa cell lines were generated using MISSION lentiviral systems (Sigma) according to the manufacturer's recommendations. Control cells were generated using non-mammalian shRNA constructs. Briefly, 1.6×10^4 cells were plated in a 96-well plate and incubated overnight. Cells were transduced with lentiviral particles at a Multiplicity of Infection (MOI) of 1, 2 and 3 with hexadimethrine bromide at a final concentration of 8 µg/ml. Transduced clones were selected with puromycin. KDs were assessed by Western blotting. Clones that demonstrated maximum KD with the lowest MOI were selected.

For topoisomerase I KD, siRNA for topoisomerase I (Santacruz Biotech) was transfected in DU-145 cells using Lipofectamine 2000 reagent (Invitrogen). KD was assessed 48 h after transfection by Western blotting.

Drug library screen

Parental DU-145 cells and DU-145 NDRG1 KD cells were seeded in 100 µl complete RPMI media in 96-well plates (1.5×10^3 cells/well). The cells were incubated overnight to allow for attachment, and subsequently treated with compounds from the JHDL at a final concentration of 10 µM. Cells treated with 0.05% DMSO were used as negative controls. Plates were incubated for 48 h, after which cell viability was measured using CellTiter 96™ AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's specifications. Absorption at 490 nm was determined using a SoftMax Pro plate reader (Molecular Devices). Viability of treated cells was compared to viability of DMSO-treated control cells (relative cell viability). Then, relative cell viability of DU-145 NDRG1 KD cells was compared to relative cell viability of DU-145 cells ('DU-145 NDRG1 KD/DU-145'-ratio).

IC₅₀ assessment

To assess IC₅₀ values of selected compounds from the library screen in various PCa cell lines,

MTS assays were performed in a similar way as in the aforementioned screen. In brief, cells were plated (DU-145 cells at 1.5×10^3 cells/well, LNCaP cells at 2×10^3 cells/well and PC3 cells at 1×10^3 cells/well; all cells were dissolved in 100 μ l complete RPMI media), allowed to adhere overnight and treated with the small molecules for a duration of 48 h. MTS reagent (Promega) was added for 2-3 h, after which a colorimetric reading was performed using the SoftMax Pro plate reader (Molecular Devices). Viability of treated cells was compared to viability of DMSO-treated control cells (relative cell viability). Independent two-sample t-tests were performed to compare the relative cell viability in parental cells to the relative cell viability in NDRG1 KD cells. Variances were assumed to be equal in parental and NDRG1 KD cells of the same cell line. When comparing DU-145 cells to human prostate fibroblasts, the variance was assumed to be unequal.

Clonogenic assay

Clonogenic assays were performed to assess long-term survival after treatment with small molecules. Briefly, cells were plated in 60 mm dishes and treated for 48 h. Following treatment, 1×10^3 cells from each dish were plated in triplicate in 60 mm dishes and incubated for 10-12 days. Colonies were fixed and stained with a crystal violet solution (Sigma); dishes were scanned with a computer scanner (Microtek) and counted manually. Student's t-tests were performed to assess p-values.

Immunoblotting and densitometry

Immunoblotting and densitometry were performed as described previously.⁹ Cleaved PARP primary antibody (9541, Cell Signaling Technology) and topoisomerase I primary antibody (TG2012-2, TopoGEN) were diluted 1:1000, NDRG1 primary antibody¹⁰ 1:4000, Vinculin primary antibody (05-286, Millipore (Upstate)) 1:10,000; all antibodies were dissolved in blocking buffer (5% milk in TBST (100 mM Tris-HCl pH 7.4, 0.1% Tween 20, 150 mM NaCl in H₂O)). Secondary antibodies were dissolved in blocking buffer (5% milk in TBST) at 1:4000 dilution.

3-D invasion assay

Trypsinized DU-145 and DU-145 NDRG1 KD cells were suspended in complete RPMI media containing 0.5% methylcellulose. Ten microliters of suspended cells were placed on the inner side of the lid of a sterile bacterial petri plate and cultured as a hanging drop over a humidified plate in a CO₂ incubator for 16 h. The generated spheroids were used for 3-D invasion assays by embedding them in collagen matrix (BD Biosciences), which was prepared according to the manufacturer's instructions. CTAB was administered and the spheroids were imaged daily using a Nikon Eclipse Ti microscope (Nikon) under phase contrast. For calculation of the invasion index the total area over which the spheroid had dispersed (including invading and non-invading cells) and the area of non-invading cells (at the center

of the spheroid) were measured using ImageJ. Values were expressed as an average of at least 3 invasion index calculations using the formula $\text{invasion index} = 1 - (\text{non-invading area} / \text{total area})$. Comparisons were done using the student's t-test.

Results

Library screen for compounds targeting DU-145 cells based on NDRG1 expression

To identify novel chemotherapeutic agents that potentially decrease invasion of PCa cells, a synthetic lethal screen was devised based on NDRG1 expression in DU-145 cells. The goal was to identify PCa targeting compounds that selectively inhibit NDRG1 deficient PCa cells, thereby decreasing invasiveness. For this purpose, we created isogenic PCa cell lines that differed in NDRG1 expression. NDRG1 shRNA lentiviral constructs successfully generated a stable KD of NDRG1 expression in DU-145 cells (DU-145 NDRG1 KD), as judged by the 99% decrease in NDRG1 protein levels in these cells (Fig. 1A). As high-throughput screening assays are prone to generating a high degree of false positives,¹¹ our screen was robustly designed in a three tier fashion to weed out false positives. The primary screen involved treatment of isogenic parental DU-145 and DU-145 NDRG1 KD cells with compounds from the JHDL at a concentration of 10 μM for 48 h. Our objective was to identify compounds that selectively inhibit DU-145 NDRG1 KD cells and parental DU-145 cells. Therefore, hits were defined as compounds that selectively inhibited DU-145 NDRG1 KD cells or parental DU-145 cells by changing the 'DU-145 NDRG1 KD/DU-145'-proliferation ratio to < 0.7 or > 1.5 , respectively. Furthermore, as we were only interested in PCa targeting compounds, only compounds that inhibited cell proliferation of PCa cells by 10% or more were selected. Of the 3,360 compounds in the primary screen, nineteen compounds were selected (Fig. 1B). Ten of these compounds selectively inhibited NDRG1 deficient DU-145 cells.

The nineteen compounds identified as hits in the primary screen were put through a secondary screening process wherein MTS assays were performed in triplicates (Fig. 1C). As a result of this approach, four compounds which inhibited NDRG1 deficient cells $\geq 30\%$ more effectively in their proliferation ('DU-145 NDRG1 KD/DU-145'-ratio < 0.7) were identified, namely, stearyltrimethylammonium chloride (STAC), cupric chloride (CuCl_2), neocuproine (NCP) and cetyltrimethyl ammonium chloride (CTAC) (Fig. 1D). Furthermore, irinotecan hydrochloride trihydrate was included in our list of hits as well, as it was just below our threshold with a 'DU-145 NDRG1 KD/DU-145'-ratio of 0.71, and as previous studies reported a positive correlation between NDRG1 expression and irinotecan resistance in patients with colorectal tumors.^{12,13} None of the compounds tested resulted in a 'DU-145 NDRG1 KD/DU-145'-proliferation ratio > 1.5 .

CuCl_2 and NCP were excluded from further research. CuCl_2 was excluded as the difference in proliferation activity in cells after treatment with this inorganic salt was not significant and could not be repeated in additional experiments (data not shown). NCP was excluded

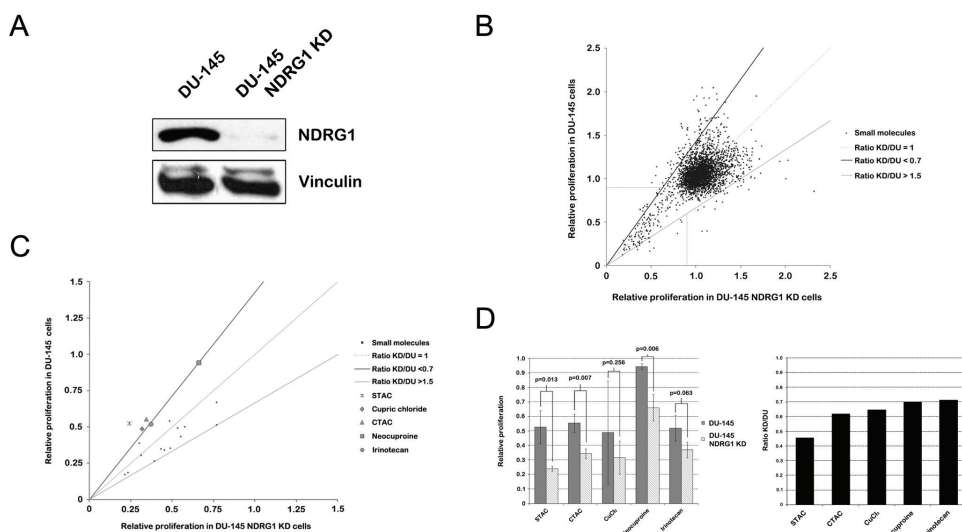


Figure 1. Screening of the JHDL identifies stearyltrimethylammonium chloride (STAC), cetrimonium chloride (CTAC), cupric chloride (CuCl_2), neocuproine (NCP) and irinotecan as compounds that selectively inhibit NDRG1 deficient DU-145 cells. A) Western blot for NDRG1 performed with DU-145 cell lysates after stable knockdown of NDRG1 by shRNA. Control cells had been transduced with non-mammalian shRNA constructs. B) Primary screen with compounds of the JHDL at 10 μM . Compounds were selected when inhibiting cell proliferation by at least 10% (horizontal and vertical line), and having a 'DU-145 NDRG1 KD/DU-145'-ratio of <0.7 (area above dark diagonal line) or >1.5 (area under light diagonal line). C) Secondary screen with the selected compounds from the primary screen to discard false positives. MTS assays were performed in triplicate; compounds that changed the 'DU-145 NDRG1 KD/DU-145'-ratio to <0.7 and irinotecan were selected. D) Bar graphs representing proliferation activity (left) and the 'DU-145 NDRG1 KD/DU-145'-ratio (right) of selected compounds from the secondary DU-145 screen. Ratio KD/OU, 'DU-145 NDRG1 KD/DU-145'-ratio.

as this compound would most likely not provide any clinical benefit in metastasis-prone PCa patients, the small molecule acting on healthy cells, such as astrocytes, as well.¹⁴ The other hits from the secondary screen (STAC, CTAC and irinotecan) were selected for the tertiary screen, in which series of MTS and clonogenic assays were performed at varying concentrations in multiple PCa cell lines to conclusively rule out false positives and to ascertain that the inhibitory effect of the compounds was not merely restricted to one PCa cell line. The results of this tertiary screen will be presented next.

Irinotecan selectively targets NDRG1 deficient PCa cells.

Irinotecan (also known as Camptothecin-11 or CPT-11) is a DNA topoisomerase I inhibitor that is mainly used for the treatment of colorectal carcinomas. Due to the known positive correlation between irinotecan resistance and NDRG1 expression in colon cancer, the compound could be used to validate our screening results.^{12, 13, 15} Furthermore, irinotecan was considered an interesting compound to explore for its use in PCa therapy, as the

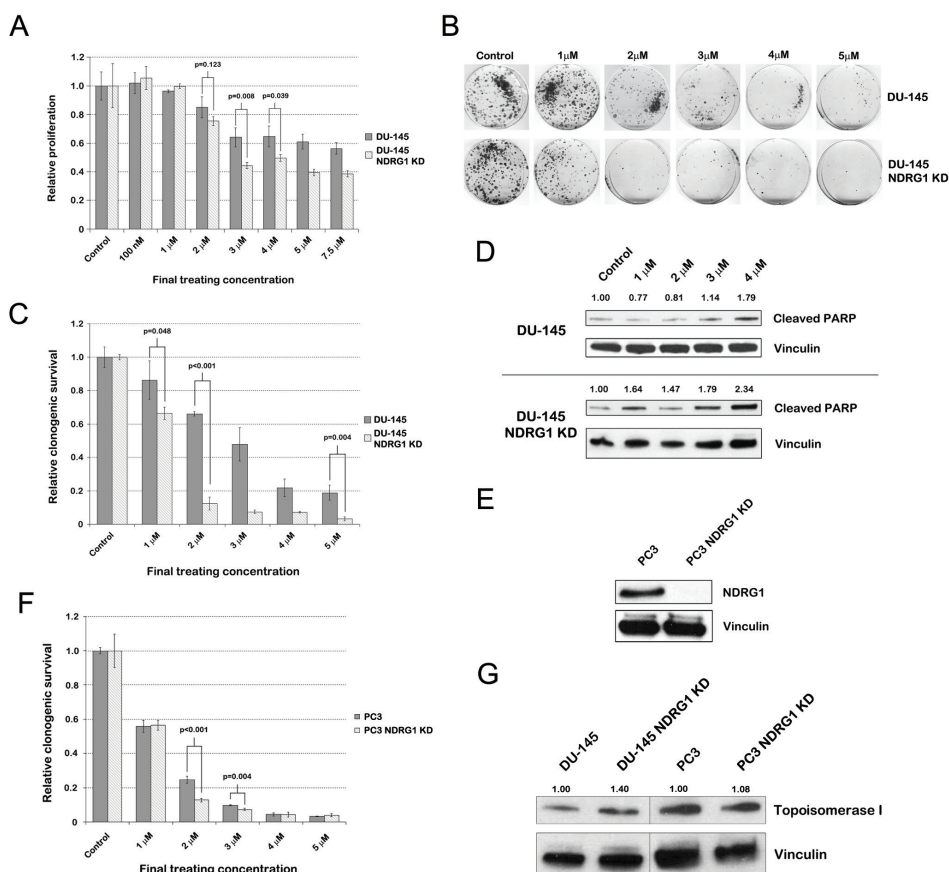


Figure 2. Irinotecan selectively targets NDRG1 deficient DU-145 and PC3 cells by induction of apoptosis. A) MTS assays performed after 48 h treatment of parental DU-145 and DU-145 NDRG1 KD cells with irinotecan. Student's t-tests were performed to assess p-values. B) Images displaying clonogenic survival of parental DU-145 and DU-145 NDRG1 KD cells after irinotecan treatment. C) Bar graph quantifying the percentage of clonogenic survival in DU-145 cells. D) Protein expression levels of cleaved PARP, an apoptotic marker, were assessed after treatment of DU-145 cells with irinotecan. Densitometry was performed with ImageJ. E) Western blots confirmed successful knockdown (KD) of NDRG1 protein expression in PC3 cells after transduction of the cells with shRNA that stably knocks down NDRG1 expression. F) Bar graph quantifying the percentage of clonogenic survival in PC3 cells after irinotecan treatment. G) Western blots for topoisomerase I, performed with cell lysates from untreated DU-145 and PC3 cells. Densitometry was performed.

compound is already FDA approved for treatment against other cancer types.

We assessed the sensitivity of parental DU-145 and DU-145 NDRG1 KD cells to irinotecan by performing MTS assays with varying concentrations of the compound. After 48 h of treatment, the IC_{50} of parental DU-145 cells was $>7.5 \mu M$, while the IC_{50} of NDRG1 KD cells was $2.5 \mu M$ (Fig. 2A). Furthermore, at concentrations between 3 and $7.5 \mu M$, DU-145 cells

demonstrated a statistically significant difference in sensitivity to irinotecan based on the NDRG1 status of the DU-145 cells, DU-145 NDRG1 KD cells having up to 30% decreased cell viability compared to parental DU-145 cells ($p < 0.04$). These results were in line with the results from the secondary screen (compare Fig. 2A to Fig. 1D). Long-term clonogenic survival demonstrated that DU-145 NDRG1 KD cells have about 50% less clonogenic survival after irinotecan treatment as compared to parental DU-145 cells ($p < 0.05$) (Figs. 2B-C). Furthermore, Western blots for cleaved PARP confirmed that apoptosis was increased after 48 h of irinotecan treatment in NDRG1 deficient DU-145 cells (Fig. 2D). To exclude that the differential effect caused by irinotecan was not a cell-line dependent response, we performed clonogenic assays in another PCa cell line as well, namely PC3 cells. Stable KD of NDRG1 in PC3 cells significantly reduced NDRG1 protein expression levels (Fig. 2E). PC3 NDRG1 KD cells were significantly more sensitive to irinotecan at final treating concentrations between 2 and 3 μM ($p < 0.01$) (Fig. 2F). Furthermore, PC3 cells were more sensitive to irinotecan compared to DU-145 cells in general, as irinotecan at a concentration of 4 μM effectively inhibited PC3 cells regardless of their NDRG1 expression, decreasing clonogenic survival to <5% compared to untreated controls. Since an increase in topoisomerase I expression is reported to be one of six general mechanisms for cellular resistance against irinotecan,¹⁶ we analyzed topoisomerase I expression through Western blotting (Fig. 2G). We did find a slight increase in topoisomerase I expression in NDRG1 KD DU-145 and PC3 cells. To rule out that sensitivity to irinotecan in NDRG1 deficient cells is due to off target effects, we performed a transient knockdown of topoisomerase I in DU-145 and DU-145 NDRG1 KD cells and evaluated proliferation through MTS assays. In concordance with the irinotecan data, NDRG1 deficient cells showed a decrease in proliferation after topoisomerase I knockdown (data not shown).

CTAB selectively targets NDRG1 deficient PCa cells.

Two other hits in our secondary screen of the JHDL were STAC and CTAC, two cationic surfactants with similar characteristics. Of these two compounds, CTAC was more selective for NDRG1 deficient DU-145 cells than STAC (Fig. 1D). CTAC was more selective for NDRG1 deficient LNCaP cells compared to parental LNCaP cells as well (data not shown). The two compounds drew our interest, as similar cationic surfactants such as cetrimonium bromide (CTAB) have shown to have an antitumor effect.¹⁷⁻¹⁹ As CTAC was more selective for NDRG1 deficient PCa cells than STAC, and as CTAC and CTAB, which only differ in the halide groups, have a similar efficacy as antitumor agents, we continued our study with CTAB.¹⁷ This compound was also preferred as efficacy and safety in mammals have been studied more extensively with CTAB.

We performed MTS assays with varying concentrations of CTAB in parental DU-145 and DU-145 NDRG1 KD cells (Fig. 3A). CTAB appeared more potent in inhibiting PCa cells than CTAC, which might be caused by the difference in the halide group (compare Fig. 3A with Fig.

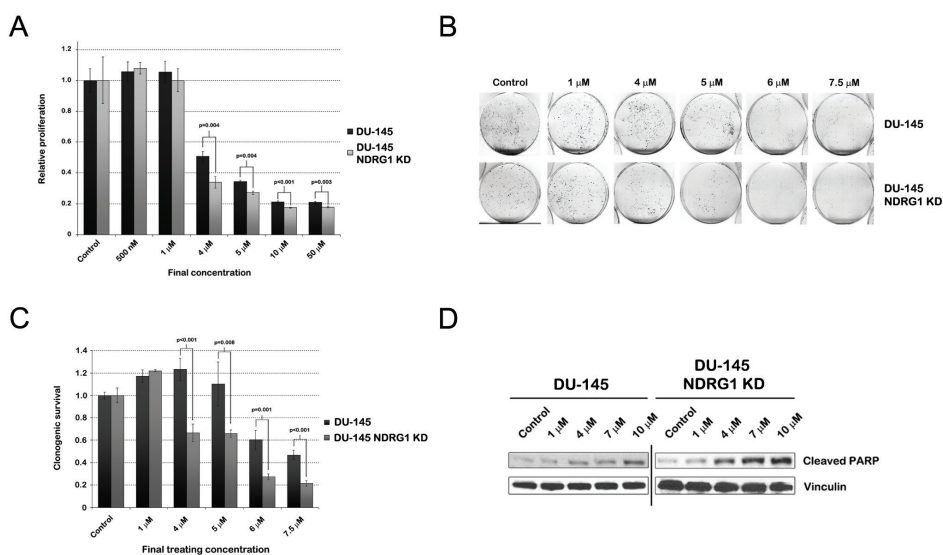


Figure 3. CTAB selectively targets DU-145 NDRG1 KD cells by induction of apoptosis. A) MTS assays performed after treatment of parental DU-145 and DU-145 NDRG1 KD cells with CTAB. Student's t-tests were performed to assess p-values. B) Images displaying clonogenic survival of parental DU-145 and DU-145 NDRG1 KD cells after CTAB treatment. C) Bar graph quantifying the percentage of clonogenic survival in DU-145 cells after CTAB treatment. D) Protein expression levels of cleaved PARP, an apoptotic marker, were assessed after treatment of DU-145 cells with CTAB for 48 h.

1D). Although the IC_{50} in parental DU-145 and DU-145 NDRG1 KD cells differed by 0.5 μ M only (4 and 3.5 μ M, respectively, data not shown), multiple independently performed MTS assays indicated a significant decrease in cell viability of DU-145 NDRG1 KD cells compared to parental DU-145 cells at concentrations of 4 μ M and above, DU-145 NDRG1 KD cells being up to 15% more effectively inhibited ($p \leq 0.004$). Next we assessed differences in long-term survival by performing clonogenic assays. At final concentrations between 4 and 7.5 μ M, NDRG1 deficient DU-145 cells clearly exhibited less clonogenic survival than parental DU-145 cells (Figs. 3B-C). Due to a lack of viable cells after 48 h treatment with 10 μ M CTAB, no clonogenics could be performed at this concentration. As the difference between parental DU-145 and DU-145 NDRG1 KD cells was more pronounced in the long-term clonogenic survival assay than in the short-term MTS assay, we hypothesized that CTAB causes a differential induction of apoptosis in the cell lines. To test this hypothesis, we performed a Western blot for cleaved PARP, a surrogate marker for apoptosis. Cells were treated with CTAB for 48 h and lysates were probed for cleaved PARP. As expected, NDRG1 deficient DU-145 cells had increased levels of cleaved PARP compared to parental DU-145 cells (Fig. 3D). In line with previous results, this difference in cleaved PARP expression was most pronounced at concentrations of 4 and 7 μ M.

To rule out that the inhibitory effect of CTAB observed in cancer cells is caused by general toxicity, we treated human prostate fibroblast cells with CTAB at concentrations at which it is effective in inhibiting PCa cells, and performed MTS assays (Fig. 4A). In human prostate fibroblasts, the IC_{50} for CTAB was about 30 μ M, while in parental DU-145 cells the IC_{50} was about 4 μ M ($p < 0.05$ at concentrations of $\geq 3 \mu$ M). This result encouraged us to determine the efficacy of CTAB in other PCa cell lines as well. In concordance with earlier observations, cell proliferation in NDRG1 deficient LNCaP cells was more effectively inhibited compared to parental LNCaP cells at concentrations above 5 μ M, as determined by MTS assays ($p < 0.02$) (Fig. 4B). Again CTAB was more potent than CTAC in the JHDL screening. Since LNCaP cells have weak adhesive properties compared to other PCa cell lines, technical limitations prevented confirming this result by clonogenic assays. Therefore, PC3 cells were used to confirm and validate the effect of CTAB in clonogenic assays. This assay indicated that CTAB selectively inhibited NDRG1 deficient PC3 cells at final concentrations between 2.5 and 6 μ M ($p \leq 0.001$) (Figs. 4C-D).

Our results indicate that NDRG1 deficient PCa cells are more sensitive to CTAB than NDRG1

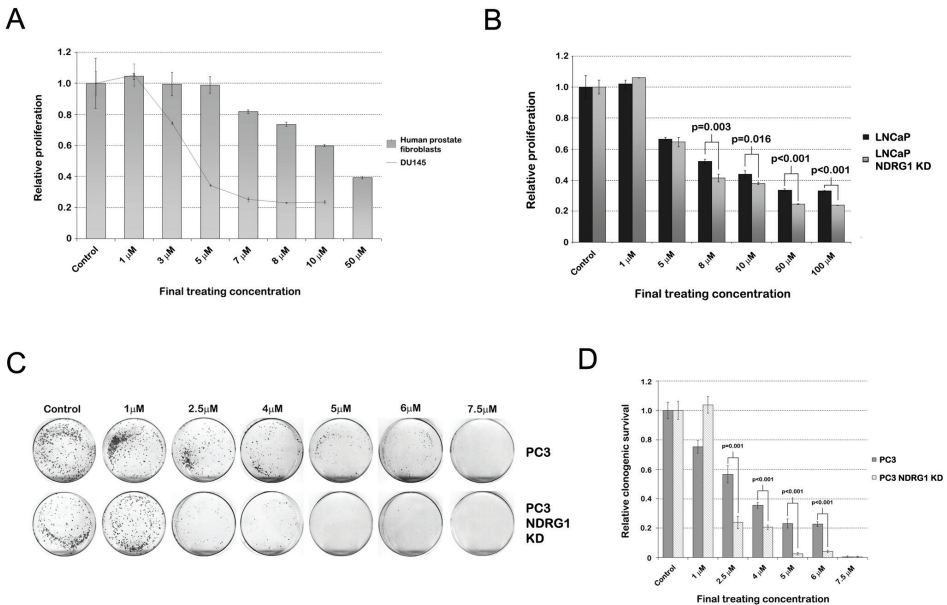


Figure 4. While non-cancerous human prostate fibroblasts are relatively unaffected by CTAB, NDRG1 KD cells are selectively targeted in multiple PCa cell lines. A) MTS assays were performed with human prostate fibroblasts and DU-145 cells after CTAB treatment. B) MTS assays performed after treatment of parental LNCaP and LNCaP NDRG1 KD cells with CTAB. Student's t-tests were performed to assess p-values. C) Images displaying clonogenic survival of parental PC3 and PC3 NDRG1 KD cells after CTAB treatment. D) Bar graph quantifying the percentage of clonogenic survival in PC3 cells after CTAB treatment. NDRG1 deficient PC3 cells had significantly lower survival when treated with CTAB at concentrations between 2.5 and 6 μ M ($p \leq 0.001$).

expressing cells at concentrations which were well tolerated in xenografts.¹⁷ To test whether CTAB also decreases invasion of PCa at these concentrations, we conducted a 3-D invasion assay in collagen type I matrix (Fig. 5). A 3-D invasion assay is superior to the Boyden chamber invasion assay, as the cells exhibit properties and behavior which are physiologically closer to *in vivo* settings. Untreated spheroids, consisting of parental DU-145 cells, increased in size over the course of 48 h. However, barely any invasive structures were observed (Fig. 5A). On the other hand, untreated spheroids consisting of DU-145 NDRG1 KD cells exhibited highly invasive behavior (Fig. 5B).²⁰ Treatment of spheroids with 5 μ M CTAB resulted in a decrease in growth of spheroids in both cell lines, indicating that CTAB was effectively targeting both DU-145 cell lines. Invasion of the DU-145 NDRG1 KD spheroids into the collagen matrix was markedly reduced after CTAB treatment, the invasion index of untreated controls being 0.57, while the invasion index of CTAB treated cells was 0.26 (Fig. 5C). Thus, apart from selectively targeting NDRG1 deficient DU-145 cells, CTAB significantly reduced invasion in NDRG1 deficient DU-145 cells as well ($p = 0.01$).

Discussion

In the constant search for improved therapy options for cancer patients, there is a growing need for novel chemotherapeutic agents that target metastatic tumors. Chemotherapeutics that target PCa invasion is limited, a major limitation for the development of such chemotherapeutics being the lack of a good model for invasive PCa. We reasoned that engineering PCa cell lines through overexpression of metastasis related oncogenes or KD/knockout of metastasis suppressor genes could be useful in identifying compounds that selectively inhibit PCa invasion. For this purpose, the PCa metastasis suppressor gene NDRG1 was knocked down to generate a cell based screen in this study. By performing a synthetically lethal screen, comparing sensitivity for compounds between wildtype PCa cells and the engineered cells, compounds that selectively target metastasis-prone PCa cells were identified. This type of screens has gained prominence due to its success in identifying cytotoxic agents that target cells with mutations in a particular gene.^{21,22} The JHDL used in our study has previously been used to identify new antitumor agents, such as mycophenolic acid, nitroxoline, itraconazole and ketoconazole as potential inhibitors of angiogenesis;^{8, 23, 24} ebisen oxide as an inhibitor of α -methylacyl coenzyme A racemase (AMACR) in PCa, a metabolic enzyme that stimulates PCa cell growth;²⁵ glafenine as an inhibitor of the ATP-binding cassette transporter ABCG2, an enzyme associated with multidrug resistance;²⁶ and digoxin and other cardiac glycosides as inhibitors of HIF-1 α synthesis, thereby blocking tumor growth.^{21,27} As most small molecules in the JHDL are FDA-approved, pharmacokinetic and safety profiles are already known, and the identified compounds can progress rapidly from preclinical development to phase II clinical trials.²⁸ Such a repurposing strategy saves time between preclinical research and implementation of the drug in clinic, and reduces

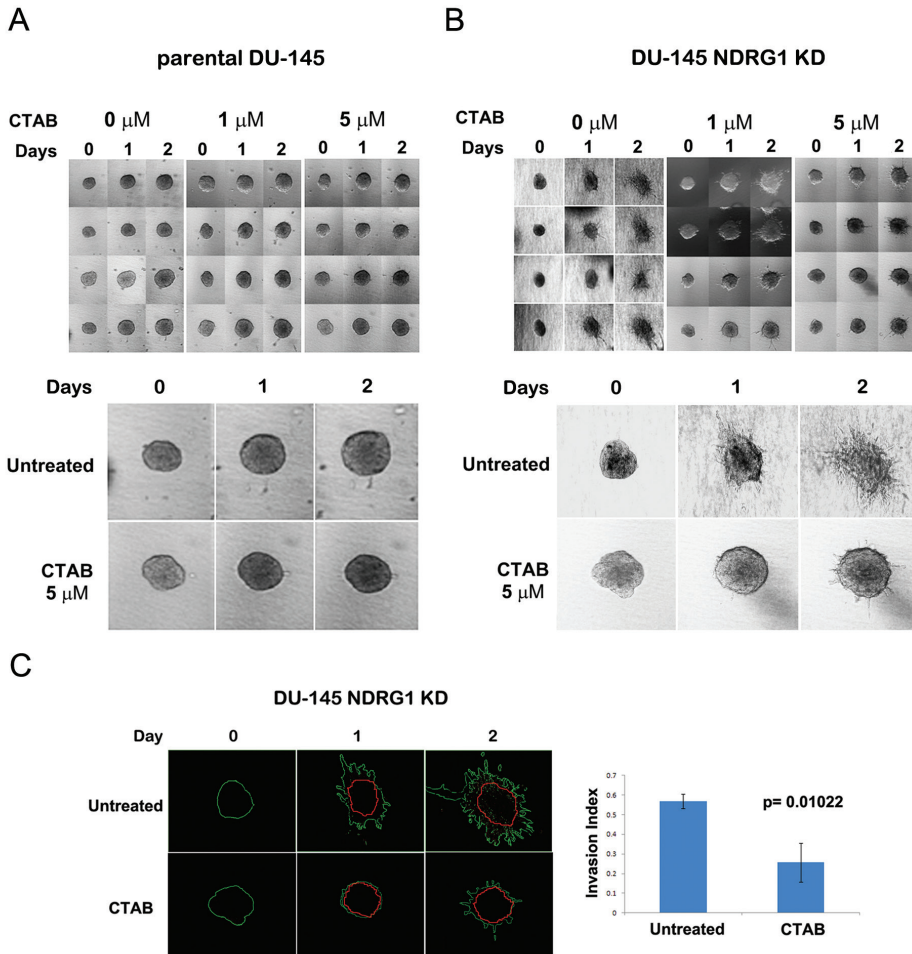


Figure 5. CTAB treatment results in decreased invasion of DU-145 NDRG1 KD cells. A-B) 3-D invasion assays performed with parental DU-145 (A) and DU-145 NDRG1 KD (B) cells treated with CTAB. C) Calculation of the invasion index. The red area contains non-invading cells and the green area contains both invading and non-invading cells (left). The graph depicts the invasion index on day 2 of CTAB treatment for DU-145 NDRG1 KD cells (right).

costs.^{28, 29}

Although these chemical libraries have demonstrated their success in identifying novel compounds for cancer treatment,²¹ they do have certain limitations which have to be taken into account during screening. Some limitations are inherent to the nature of high-throughput screens, while others include practical limitations related to the nature of the compounds present in the library. A few limitations worth mentioning include: 1) screening libraries often go through many freezing/thawing cycles, while the structure and activity of

drugs may alter over time; 2) screening libraries are incomplete and often not up-to-date with the most recent drug developments; 3) concentrations chosen for the screen may be outside the effective range of the particular compound, creating false negative results; 4) screening results contain false positive results due to experimental limitations (i.e. to limit the use of resources the primary screen is performed only once); and 5) cutoff values to select for hits are chosen arbitrarily. However, robustness of the primary screen in this study was underscored by the identification of known antitumor agents such as cardiac glycosides and the type II topoisomerase inhibitor mitoxantrone as inhibitors of DU-145 proliferation (data not shown).²¹ The strength of our screen was further enhanced as compounds were validated rigorously through a three tier approach, weeding out false positives.

Due to aforementioned limitations of high-throughput screens, it is important to interpret the data in the light of known information about the drugs. This is well illustrated in our screening results with irinotecan. Although compounds were selected in our secondary screen when having a 'DU-145 NDRG1 KD/DU-145'-ratio of ≤ 0.70 , hence we selected compounds that selectively target NDRG1 KD cells 30% more effectively than parental DU-145 cells, irinotecan was taken ahead for further investigation as well, despite its 'DU-145 NDRG1 KD/DU-145'-ratio of 0.71. The rationale for selection of this compound is based on reports which indicate a negative correlation between NDRG1 expression and sensitivity of cells to irinotecan;^{12, 13, 15} our data confirm this inverse relationship. Our data further suggest that NDRG1 KD upregulates topoisomerase I expression, thereby increasing the sensitivity of NDRG1 KD PCa cells to irinotecan. Therefore, irinotecan may prove to be of clinical use in patients with advanced NDRG1 deficient PCa.³⁰ Irinotecan is FDA approved for its use as a chemotherapeutic agent in metastatic colorectal cancer. The compound has also been studied in PCa: its effect in PCa was demonstrated for the first time in 1996, when it was being tested successfully in PC3 cells *in vitro* and in the Dunning R3327 AT6.3 rat model *in vivo*.³¹ Subsequently, a phase II clinical trial was performed in hormone-refractory PCa patients.³² In this study, the best response established was stable disease in eight out of fifteen patients (53%), which was defined as a PSA decrease of $<50\%$, or a PSA increase of $<25\%$ for at least four weeks. As NDRG1 expression of the tumors was not assessed in the clinical trial, it is tempting to speculate that patients with stable disease might have had PCa with low NDRG1 protein expression.³³ However, it is also possible that most patients selected for this study had higher NDRG1 expression levels, as the ECOG performance status of all PCa patients but one was 0/1, irinotecan being given as a first line therapy to patients with hormone-refractory PCa, while patients with NDRG1 deficient PCa generally have a more aggressive disease.

The other compounds we identified that selectively target NDRG1 deficient cells and could potentially be used in the clinic, are CTAB/CTAC and STAC. CTAB is a cationic micellar surfactant, part of the group of quaternary ammonium compounds. It is used as a topical

antiseptic and part of a group of molecules that (potentially) plays a role in cancer treatment in diverse ways. A cell-based phenotype-driven high-throughput screen recently identified CTAB as a potential compound in the treatment of head and neck cancer (HNC), inducing caspase activated apoptosis by inhibition of H^+ -ATP synthase activity and depolarization of the mitochondrial membrane potential, thereby decreasing ATP levels in the cell.¹⁷ In *in vivo* experiments CTAB inhibited tumor formation and delayed tumor growth. At the concentrations used in this study, mice had no evidence of toxicity or lethality, similar to our *in vitro* findings in human prostate fibroblasts. Furthermore, cetrimide (of which CTAB is one component) is used as an effective cytotoxic lavage solution for use during surgery of breast carcinomas,³⁴ and other quaternary ammonium compounds, such as benzethonium chloride, have shown to exhibit antitumor activity as well.³⁵

CTAB was identified in our screen as a compound that selectively inhibits proliferation of PCa cells in general, and NDRG1 deficient PCa cells in particular. While screenings involving simple endpoint proliferation assays often yield the development of highly selective and potent compounds, such assays provide limited information on how potential therapeutics influence complex multifaceted biological events such as tumor invasion. To investigate whether CTAB affects the invasive capacity of NDRG1 deficient cells, we performed a 3-D spheroid invasion assay. Our data demonstrate that NDRG1 deficient PCa cells exhibit collective migration on a 3-D collagen matrix, which gets significantly inhibited after CTAB treatment. These results, combined with the antitumor activity of CTAB in HNC as indicated by Ito et al.¹⁷ urge further exploration of CTAB for its use in cancer therapy.

In summary, this study identifies cetrimonium bromide (CTAB) and irinotecan as clinically established compounds that selectively inhibit NDRG1 deficient PCa cells. This study gives leads for future studies about the function of NDRG1 and pathways in which this protein is involved, our results suggesting that NDRG1 might have a functional relationship with topoisomerase I. Future *in vivo* and clinical studies need to be performed to assess whether administration of CTAB or irinotecan to patients with NDRG1 deficient PCa is clinically beneficial. If these studies confirm the preclinical data presented here, CTAB or irinotecan could potentially be used in personalized medicine in combination with conventional or new PCa treatment methods, to prevent invasion of tumor cells in patients with NDRG1 deficient advanced PCa.

Acknowledgments

The authors wish to thank profs. E. van der Wall, H. Gelderblom and J.W.R. Nortier for their support and discussion, and dr. J.T. Isaacs and S. Chen for their provision of the human prostate fibroblast cell line.

References

1. seer.cancer.gov [Internet]. Table 23.8 Cancer of the Prostate (Invasive): 5-Year Relative and Period Survival (Percent) by Race, Diagnosis Year, Stage and Age. Available at: http://seer.cancer.gov/csr/1975_2007/results_merged/sect_23_prostate.pdf. Accessed: August 20, 2012.
2. Suva LJ, Washam C, Nicholas RW, Griffin RJ. Bone metastasis: mechanisms and therapeutic opportunities. *Nat Rev Endocrinol* 2011;7(4):208-218.
3. Karayi MK, Markham AF. Molecular biology of prostate cancer. *Prostate Cancer Prostatic Dis* 2004;7(1):6-20.
4. Shoushtari AN, Szmulewitz RZ, Rinker-Schaeffer CW. Metastasis-suppressor genes in clinical practice: lost in translation? *Nat Rev Clin Oncol* 2011;8(6):333-342.
5. Bandyopadhyay S, Pai SK, Gross SC et al. The Drg-1 gene suppresses tumor metastasis in prostate cancer. *Cancer Res* 2003;63(8):1731-1736.
6. Kachhap SK, Faith D, Qian DZ et al. The N-Myc down regulated Gene1 (NDRG1) Is a Rab4a effector involved in vesicular recycling of E-cadherin. *PLoS One* 2007;2(9):e844.
7. Liu W, Xing F, Iizumi-Gairani M et al. N-myc downstream regulated gene 1 modulates Wnt-beta-catenin signalling and pleiotropically suppresses metastasis. *EMBO Mol Med* 2012;4(2):93-108.
8. Chong CR, Qian DZ, Pan F et al. Identification of type 1 inosine monophosphate dehydrogenase as an antiangiogenic drug target. *J Med Chem* 2006;49(9):2677-2680.
9. Kachhap SK, Rosmus N, Collis SJ et al. Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. *PLoS One* 2010;5(6):e11208.
10. Piquemal D, Joulia D, Balaguer P, Basset A, Marti J, Commes T. Differential expression of the RTP/Drg1/Ndr1 gene product in proliferating and growth arrested cells. *Biochim Biophys Acta* 1999;1450(3):364-373.
11. Sink R, Gobec S, Pecar S, Zega A. False positives in the early stages of drug discovery. *Curr Med Chem* 2010;17(34):4231-4255.
12. Motwani M, Sirotnak FM, She Y, Commes T, Schwartz GK. Drg1, a novel target for modulating sensitivity to CPT-11 in colon cancer cells. *Cancer Res* 2002;62(14):3950-3955.
13. Shah MA, Kemeny N, Hummer A et al. Drg1 expression in 131 colorectal liver metastases: correlation with clinical variables and patient outcomes. *Clin Cancer Res* 2005;11(9):3296-3302.
14. Chen SH, Lin JK, Liu SH, Liang YC, Lin-Shiau SY. Apoptosis of cultured astrocytes induced by the copper and neocuproine complex through oxidative stress and JNK activation. *Toxicol Sci* 2008;102(1):138-149.
15. Zheng Y, Wang LS, Xia L et al. NDRG1 is down-regulated in the early apoptotic event induced by camptothecin analogs: the potential role in proteolytic activation of PKC delta and apoptosis. *Proteomics* 2009;9(8):2064-2075.
16. Xu Y, Villalona-Calero MA. Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. *Ann Oncol* 2002;13(12):1841-1851.
17. Ito E, Yip KW, Katz D et al. Potential use of cetrimonium bromide as an apoptosis-promoting anticancer agent for head and neck cancer. *Mol Pharmacol* 2009;76(5):969-983.
18. Boca SC, Astilean S. Detoxification of gold nanorods by conjugation with thiolated

- poly(ethylene glycol) and their assessment as SERS-active carriers of Raman tags. *Nanotechnology* 2010;21(23):235601.
19. Hauck TS, Ghazani AA, Chan WC. Assessing the effect of surface chemistry on gold nanorod uptake, toxicity, and gene expression in mammalian cells. *Small* 2008;4(1):153-159.
 20. Friedl P, Hegerfeldt Y, Tusch M. Collective cell migration in morphogenesis and cancer. *Int J Dev Biol* 2004;48(5-6):441-449.
 21. Zhang H, Qian DZ, Tan YS et al. Digoxin and other cardiac glycosides inhibit HIF-1 α synthesis and block tumor growth. *Proc Natl Acad Sci U S A* 2008;105(50):19579-19586.
 22. Guo W, Wu S, Liu J, Fang B. Identification of a small molecule with synthetic lethality for K-ras and protein kinase C ι . *Cancer Res* 2008;68(18):7403-7408.
 23. Chong CR, Xu J, Lu J, Bhat S, Sullivan Jr. DJ, Liu JO. Inhibition of angiogenesis by the antifungal drug itraconazole. *ACS Chem Biol* 2007;2(4):263-270.
 24. Shim JS, Matsui Y, Bhat S et al. Effect of nitroxoline on angiogenesis and growth of human bladder cancer. *J Natl Cancer Inst* 2010;102(24):1855-1873.
 25. Wilson BA, Wang H, Nacev BA et al. High-throughput screen identifies novel inhibitors of cancer biomarker alpha-methylacyl coenzyme A racemase (AMACR/P504S). *Mol Cancer Ther* 2011;10(5):825-838.
 26. Zhang Y, Byun Y, Ren YR, Liu JO, Latterra J, Pomper MG. Identification of inhibitors of ABCG2 by a bioluminescence imaging-based high-throughput assay. *Cancer Res* 2009;69(14):5867-5875.
 27. clinicaltrials.gov [Internet]. Bethesda (MD): US National Institutes of Health (NIH); 2010 [updated 2011 Nov 29; cited 2012 Dec 6]. Available from: <http://clinicaltrials.gov/ct2/show/NCT01162135>
 28. Chong CR, Sullivan Jr. DJ. New uses for old drugs. *Nature* 2007;448(7154):645-646.
 29. DiMasi JA, Hansen RW, Grabowski HG. The price of innovation: new estimates of drug development costs. *J Health Econ* 2003;22(2):151-185.
 30. Song Y, Oda Y, Hori M et al. N-myc downstream regulated gene-1/Cap43 may play an important role in malignant progression of prostate cancer, in its close association with E-cadherin. *Hum Pathol* 2010;41(2):214-222.
 31. Lievano G, Mirochnik Y, Rubenstein M, Shaw M, Guinan P. Antitumor effect of CPT-11, a new derivative of camptothecin, against human prostate cancer (PC-3) in vitro and prostate rat tumor (AT-3) in vivo. *Methods Find Exp Clin Pharmacol* 1996;18(10):659-662.
 32. Reese DM, Tchekmedyian S, Chapman Y, Prager D, Rosen PJ. A phase II trial of irinotecan in hormone-refractory prostate cancer. *Invest New Drugs* 1998;16(4):353-359.
 33. Caruso RP, Levinson B, Melamed J et al. Altered N-myc downstream-regulated gene 1 protein expression in African-American compared with caucasian prostate cancer patients. *Clin Cancer Res* 2004;10(1 Pt 1):222-227.
 34. Park KG, Chetty U, Scott W, Miller W. The activity of locally applied cytotoxics to breast cancer cells in vitro. *Ann R Coll Surg Engl* 1991;73(2):96-99.
 35. Yip KW, Mao X, Au PY et al. Benzethonium chloride: a novel anticancer agent identified by using a cell-based small-molecule screen. *Clin Cancer Res* 2006;12(18):5557-5569.

Chapter 12

Small molecule screening of prostate cancer cells identifies tilorone dihydrochloride to selectively inhibit cells based on cyclin-dependent kinase 5 expression

Michel D. Wissing, Tikva Dadon, Eunice Kim, Klaus B. Piontek, Joong S. Shim, Nadine S. Kaelber, Jun O. Liu, Sushant K. Kachhap, Barry D. Nelkin

Oncology Rep. 2014 Jul;32(1):419-24

Abstract

Background: Cyclin-dependent kinase 5 (CDK5) is a potential target for prostate cancer treatment, the enzyme being essential for prostate tumor growth and metastases formation. In the current study, we identified agents that target prostate cancer cells based on CDK5 expression.

Methods: CDK5 activity was suppressed by transfection of PC3 prostate cancer cells with a dominant-negative construct (PC3 CDK5dn). PC3 CDK5dn and PC3 control cells were screened for compounds that selectively target cells based on CDK5 expression, utilizing the Johns Hopkins Drug Library. MTS proliferation, clonogenic and 3-D growth assays were performed to validate selected hits.

Results: Screening of 3360 compounds identified rutilantin, ethacridine lactate and cetalkonium chloride as compounds that selectively target PC3 control cells and a tilorone analog as selective inhibitor of PC3 CDK5dn cells. A PubMed literature study indicated that tilorone may have clinical use in patients. Validation experiments confirmed that tilorone treatment resulted in decreased PC3 cell growth and invasion; PC3 cells with inactive CDK5 were inhibited more effectively.

Conclusions: Tilorone selectively targets PC3 cells based on CDK5 expression. Future studies are needed to unravel the mechanism of action of tilorone in CDK5 deficient prostate cancer cells and to test combination therapies with tilorone and a CDK5-inhibitor for potential use in clinical practice.

Introduction

Although novel therapies have recently been introduced into clinical practice for the treatment of advanced prostate cancer, prostate cancer has remained the second deadliest cancer in men in the United States in 2014.¹ New therapeutic targets and strategies are urgently needed to further improve the clinical outcome of patients with prostate cancer.

One promising potential therapeutic target is cyclin-dependent kinase 5 (CDK5). CDK5 is a serine/threonine kinase structurally similar to other CDKs.² CDK5 does not appear to have a major role in cell cycle regulation.^{3,4} It has been well characterized for its dominant role in the development of the central nervous system, including roles in neuronal migration, differentiation and adhesion.^{5,6} We and others subsequently showed that CDK5 plays an important role in cancer development and metastasis.⁷⁻¹² In prostate cancer cells, we demonstrated that CDK5 was critical for cytoskeletal integrity, cell migration and invasion, and, *in vivo*, for metastasis.⁷ In pancreatic cancer, CDK5 is intrinsic to KRAS signaling through the centrally important Ral signal transduction pathway, thus providing a potential “druggable” target for mutant KRAS tumors.⁸ Together, these studies indicated that inhibition of CDK5, alone or in combination with other agents, may provide an effective therapeutic strategy for these and other cancer types.

In the current study we set out to identify agents that would be particularly effective in combination with CDK5 inhibition in prostate cancer cells. Therefore, we performed a screen of the Johns Hopkins Drug Library (JHDL). The JHDL is a collection of 3360 pharmaceutical compounds that have successfully completed safety testing in humans for a variety of applications.^{13,14} This library has been used successfully for repurposing of compounds for cancer therapy, including identification of digoxin as an HIF-1 α inhibitor,¹⁵ and itraconazole as an angiogenesis inhibitor.¹⁶ We have previously employed the JHDL to identify cetrimeronium bromide and irinotecan as compounds with increased antitumor activity against prostate cancer cells expressing low levels of the metastasis suppressor gene N-myc downregulated gene 1 (NDRG1).¹⁷ Here, we performed a similar JHDL screening with prostate cancer cells which differ in CDK5 activity. Tilorone was identified as a compound with *in vitro* synthetically lethality in CDK5 deficient prostate cancer cells.

Materials and methods

Cell culture

PC3 prostate cancer cell lines were obtained from ATCC. These cells are derived from a bone metastasis from a 62-year old prostate cancer patient. Human prostate fibroblasts, kindly provided by dr. J. Isaacs, were obtained from a prostate biopsy on a 62-year old prostate cancer patient with a Gleason score of 4. Both cell lines were grown and maintained in

RPMI-1640 (Invitrogen) media supplemented with 10% fetal bovine serum. Cells were cultured in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

Creation of PC3 CDK5dn cell line

Loss of CDK5 function was accomplished in PC3 cells by transfection of a dominant-negative construct containing a D144N mutation, kindly provided by dr. L.H. Tsai (Harvard Medical School).¹⁸ The protocol used has been described previously.⁷ In brief, the construct was subcloned in a bidirectional Tet vector, pBI-EGFP (BD Biosciences), which had a zeocin resistance gene added for selection (kindly provided by dr. K. Schuebel, Johns Hopkins University School of Medicine). pBI-EGFP empty vector or pBI-EGFP CDK5dn vector were transfected into PC3 cells which contained a Tet-Off promoter construct, pTTa (BD Biosciences).

Western blotting

Western blotting was performed as described previously.¹⁹ Ten micrograms of protein were loaded on the gel. Primary antibodies were dissolved in blocking buffer (5% milk in TBST (100 mM Tris-HCl pH 7.4, 0.1% Tween 20, 150 mM NaCl in H₂O)). A 1:1000 dilution was used for anti-CDK5 (Sigma-Aldrich); anti-vinculin (Millipore (Upstate)) was diluted 1:4000. Secondary antibodies were diluted at a 1:4000 dilution. Normalization of band intensity was done with the housekeeper protein vinculin. Developed blots were scanned using a Microtek scanner.

Wound healing assay

Wound healing assays were performed with confluent PC3 control (containing the empty pBI-EGFP vector) or PC3 CDK5dn cells. A rubber-tipped scraper was used to scrape off an area of cells. Light microscopic images were taken immediately and 24 h after scraping.

Small-molecule library screening

The JHDL library has been described previously.^{13, 14, 17} Storage and screening of JHDL compounds was done as described previously.¹⁷ Briefly, PC3 control and CDK5dn cells were seeded in 96-well plates (1x10³ cells per well) and allowed to adhere overnight. Then, 5 µl of drugs, stored as stock solutions of 200 µM in DMSO/H₂O, was added to complete RPMI media, so that cells were treated at a final concentration of 10 µM. After 48 h of treatment, 20 µl MTS reagent from the CellTiter 96™ AQ_{ueous} Non-Radioactive Cell Proliferation Assay (a reagent containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS); Promega) was added to each well for a duration of 2-4 h at 37°C. Plates were analyzed using a SoftMax Pro plate reader (Molecular Devices). Proliferation of treated cells was compared with proliferation of DMSO-treated PC3 control or CDK5dn cells (proliferation index). Proliferation indices of PC3 CDK5dn cells were compared to proliferation indices of PC3 control cells. A PubMed study

was performed to assess the clinical use of potential hits.

MTS assays

MTS assays were performed to measure the antiproliferative effect of tilorone treatment. Tilorone dihydrochloride (Sigma-Aldrich) was stored as a 10 mM stock solution in DMSO at -20°C. One thousand PC3 cells were plated in 96-well plates containing 100 µl complete RPMI media. At circa 50% confluence, tilorone dihydrochloride was administered. For experiments the compound was diluted in complete RPMI media to obtain the desired final concentration. After treatment for 72 h (tilorone monotherapy), MTS reagent was added, and absorption at 490 nm was determined using a SoftMax Pro plate reader (Molecular Devices). Proliferation indices were calculated; untreated PC3 control or CDK5dn cells were used as a control. Student's t-tests were performed to assess p-values.

Clonogenic assays

Clonogenic assays were performed to assess long-term survival after tilorone treatment. Prostate cancer cells were plated in 60 mm dishes and allowed to adhere. At 50-60% confluency, cells were treated with tilorone for 72 h. Subsequently, 1×10^3 cells from each dish were plated in triplicate in 60 mm dishes and incubated in complete RPMI media for 12 days. Colonies were fixed and stained with a solution containing 90% methanol and 10% crystalviolet solution (2.3% crystal violet, 0.1% ammonium oxalate and 20% ethylalcohol; Sigma). Colonies were scanned with a computer scanner (Microtek) and counted manually. Student's t-tests were performed to evaluate whether differences between cell lines were statistically significant.

3-D growth assay

3-D growth assays were performed utilizing the same protocol described previously.¹⁷ In short, spheroids were generated by culturing PC3 cells for 16 h as a hanging drop over a humidified plate in a CO₂ incubator in complete RPMI media containing 0.5% methylcellulose. Spheroids were embedded in collagen matrix (BD Biosciences), treated with tilorone, and imaged using a Nikon Eclipse Ti microscope (Nikon) on the day of treatment and six days after treatment start. Spheroid and total (spheroid plus sprouts) areas were measured with ImageJ. Fold increases were calculated by dividing the spheroid/total area at day 6 by the spheroid/total area on day 0 for each individual spheroid. For each cell line and time point, fold increases of four spheroids were averaged. Statistical analyses were performed using student's t-tests.

Results

Suppression of CDK5 activity

PC3 prostate cancer cells were chosen for the JHDL compound screen due to their highly metastatic potential and androgen independence, thereby resembling aggressive metastatic castrate-resistant prostate cancer. CDK5 activity was inhibited by transfection and selection of a dominant-negative mutation (CDK5 144N). These PC3 CDK5dn cells had a higher protein level of total CDK5 as compared to PC3 control cells (PC3 cells transfected with an empty vector) (Fig. 1A). A wound healing assay confirmed that CDK5 was functionally inactive in these cells: unlike the PC3 control cells, PC3 CDK5dn cells did not have the ability to invade the scraped surface area (Fig. 1B).

Library screen for compounds targeting PC3 cells based on CDK5 activity

A high-throughput screening assay was performed to select compounds that target PC3 cells based on CDK5 activity. PC3 control and CDK5dn cells were treated with all compounds of the JHDL at 10 μ M for 48 h. To identify hits that selectively target PC3 cells based on CDK5 expression, we selected all compounds in which the proliferation index ratio (CDK5dn/control) was below 0.5 or above 1.5 (Fig. 2A). Furthermore, hits had to inhibit cell proliferation of PC3 cells by at least 10%, as we were specifically interested in compounds that inhibited cell growth (horizontal and vertical line in graph). We also selected all compounds that inhibited cell proliferation in PC3 cells by more than 70% (bottom left corner of graph), as we were interested in identifying potential highly effective antitumor agents. In total, 41 hits were selected for further evaluation.

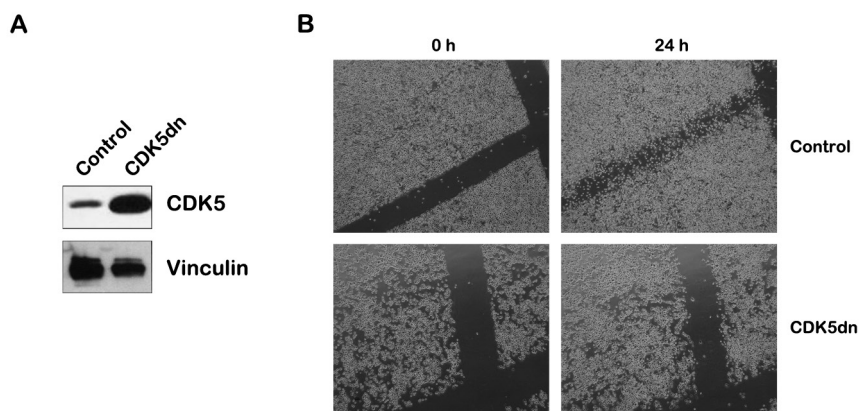


Figure 1. Suppression of CDK5 activity by transfection of a dominant-negative construct. A) PC3 cells were transfected with a pBI-EGFP vector with or without a CDK5 dominant-negative construct; clones in which transfection was successful were selected. A Western blot for CDK5 was performed with these PC3 CDK5dn and control cell lysates. B) Wound healing assay with PC3 CDK5dn and control cells to confirm CDK5 inactivity in PC3 CDK5dn cells.

A secondary screen was performed in which selected hits from the primary screen were added at 10 μ M for 48 h to PC3 control and CDK5dn cells in triplicate, to weed out false positive results (Fig. 2B). Cutoff values were slightly less strict than in the primary screen: compounds were considered a hit when the ratio of proliferation indices (CDK5dn/control) was below 0.7 or above 1.4. This resulted in the identification of three compounds that selectively target CDK5-expressing PC3 cells: rutilantin, ethacridine lactate and cetalkonium chloride (Fig. 2C). These compounds have not been used as antitumor agents, and their potential clinical use as intravenous antitumor agents seems limited.²⁰⁻²³ Another compound, tilorone analog R9536-DA, was highly effective in inhibiting both isogenic PC3 cell lines (>70% inhibition), but inhibited proliferation of PC3 CDK5dn cells somewhat more effectively (ratio CDK5dn/control: 0.687). Tilorone and its analogs have antiviral activity, acting at least in part as interferon inducers,²⁴⁻²⁶ and have been shown preclinically and clinically to have antitumor activity too.^{27, 28}

Tilorone selectively targets PC3 cells with low CDK5 activity

We continued our experiments with freshly dissolved tilorone dihydrochloride. After 72 h of tilorone treatment at various concentrations, its IC_{50} was established in MTS assays at 8-12 μ M in PC3 CDK5dn cells and 15 μ M in PC3 control cells (Fig. 3A). At 8 μ M, proliferation activity was decreased by 24% and 47% in PC3 control and CDK5dn cells, respectively ($p=0.001$). To assess toxicity of tilorone in normal prostate cells, MTS assays were performed with tilorone treatment of human prostate fibroblasts (Fig. 3B). Sensitivity of these cells to tilorone was similar to that of PC3 control cells.

The inhibitory effect of tilorone in PC3 cells was further assessed by performing clonogenic assays (Fig. 3C). PC3 CDK5dn cells were also significantly more sensitive than PC3 control cells to tilorone in this assay. Treatment with 10 μ M tilorone resulted in clonogenic survival of 40% in PC3 CDK5dn cells, and 72% in PC3 control cells ($p=0.002$).

A spheroid growth assay was performed to assess 3-D tumor growth and invasion of PC3 cells upon tilorone treatment (Fig. 4). Both PC3 control and PC3 CDK5dn cells had comparable increases in spheroid size over six days. However, total size (the size of spheroids plus sprouts) had a higher fold increase in PC3 control cells, confirming that untreated PC3 CDK5dn cells had a decreased invasive potential as compared to PC3 control cells. When tilorone was administered at 5 μ M, PC3 control spheroids had a similar growth and invasive pattern as untreated PC3 control cells ($p=0.59$) (Fig. 4B left graph). However, when tilorone was administered at the same concentration to PC3 CDK5dn cells, a significant decrease in both spheroid size and total size was observed ($p<0.01$), suggesting that tilorone successfully inhibits spheroid growth and invasion of PC3 CDK5dn cells when administered at 5 μ M (Fig. 4B right graph). At 10 μ M, both isogenic cell lines had a decreased invasive potential.

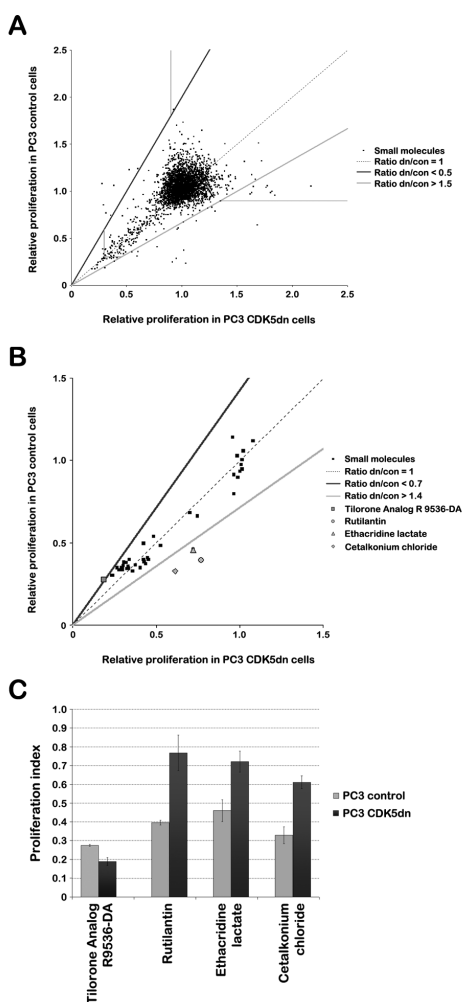


Figure 2. Screening of the JHDL identifies four compounds targeting PC3 cells selectively based on CDK5 activity. A) Primary screen with 3360 compounds of the JHDL, by performing MTS assays after 48 h of treatment at 10 μ M. Compounds were selected when the proliferation index ratio CDK5dn/control was <0.5 or >1.5. Compounds were excluded as a hit when not resulting in a decreased cell proliferation of 10% in one cell line compared to DMSO-treated controls. Compounds inhibiting cell proliferation by >70% were selected too. B) Secondary screen by performing MTS assays in triplicate with PC3 cells treated with 41 hits from the primary screen. Compounds were selected when the proliferation index ratio CDK5dn/control was below 0.7 or above 1.4. C) The four compounds selected in the secondary screen.

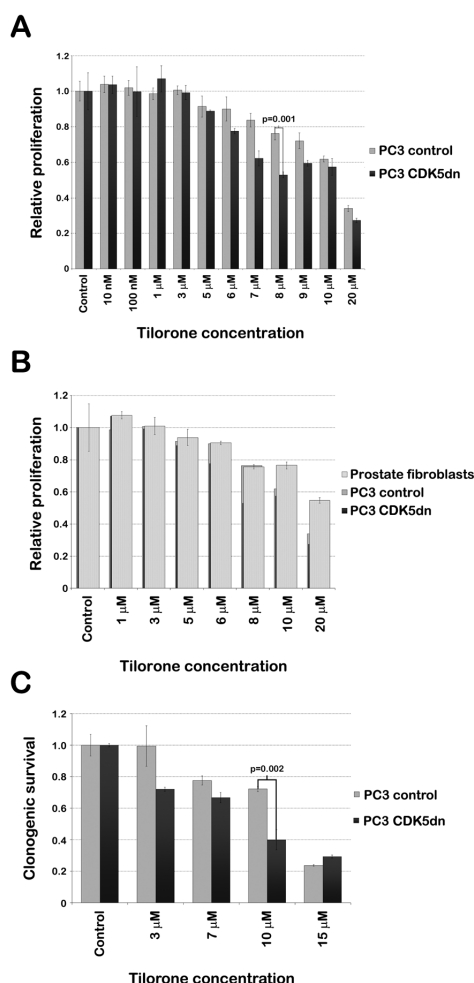


Figure 3. Validation of tilorone dihydrochloride as a compound that selectively targets PC3 cells based on CDK5 activity. A) MTS assays performed after treating PC3 CDK5dn and control cells for 72 h with tilorone. B) MTS assays performed after 72 h treatment of healthy human prostate fibroblasts with tilorone to determine toxicity of tilorone in healthy cells. C) Clonogenic survival assays performed after 72 h treatment of PC3 cells with tilorone, further validating the screening results.

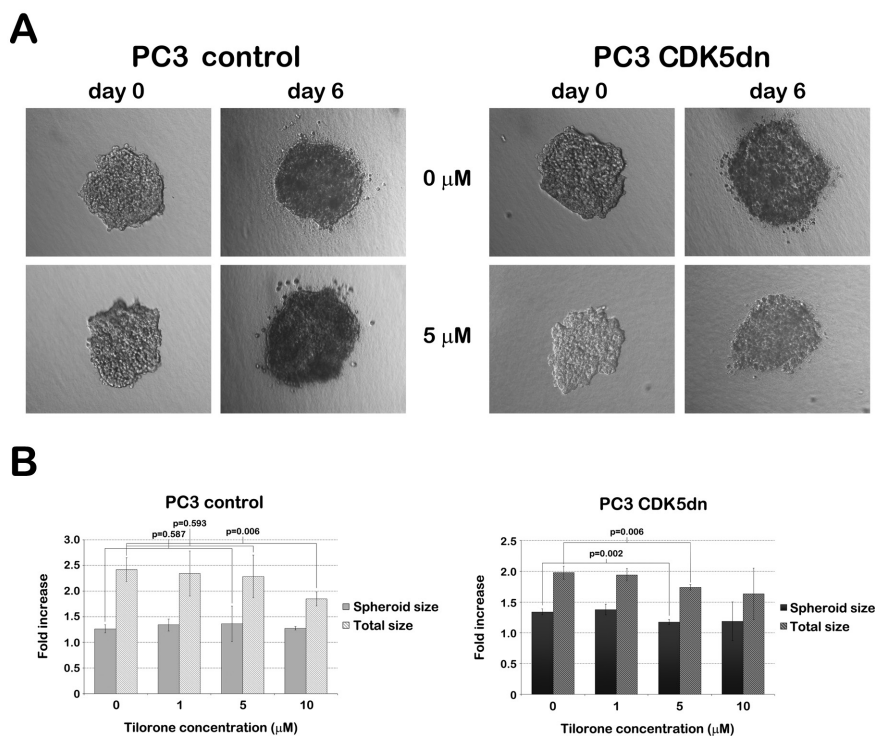


Figure 4. 3-D hanging drop growth assay performed to assess 3D spheroid growth and invasive potential of PC3 cells upon tilorone treatment. A) Representative images of PC3 control (left) and CDK5dn (right) cells at day 0 and day 6, either untreated or treated with 5 μM tilorone. B) Fold increase in spheroid and total size after six days of tilorone treatment at various concentrations.

Discussion

The JHDL, a library of well characterized pharmaceutical compounds, was developed to facilitate drug repurposing studies.²⁹ The extensive *in vivo* toxicity and pharmacokinetic profiles of compounds in the library allow rapid subsequent development of these compounds. Several compounds from the JHDL have been advanced to clinical trials for cancer and other therapeutic applications.^{13, 14, 16, 17, 30-32}

In the current study we screened the JHDL for compounds that differentially inhibit cancer cell growth in the presence of CDK5 inhibition; tilorone and a tilorone analog were identified as agents that selectively target CDK5-deficient PC3 prostate cancer cells. Tilorone (Amixin IC) is employed clinically in some countries as an orally active antiviral agent.²⁵ Tilorone has been tested in humans for the treatment of cerebral gliomas, laryngeal papillomatosis and breast cancer.^{28, 33, 34} Although antitumor efficacy was reported, interest in tilorone for cancer therapy has subsided. Recently, Zhou et al. reported new tilorone analogs with improved

anticancer activity.³⁵ These analogs may be promising to examine, especially in combination with CDK5 inhibition.

In addition to the possibility that tilorone may be promising in combination with CDK5 inhibition, the identification of tilorone as an agent that selectively targets cells with inactive CDK5 suggests potential classes of drugs to potentiate the efficacy of CDK5 inhibition. Tilorone has been characterized as an interferon inducer.²⁴ This suggests that interferon itself, or an alternative interferon inducer such as a TLR agonist, may be useful in combination with a CDK5 inhibitor. Nevertheless, other mechanisms may be involved. For example, tilorone is a DNA intercalating agent as well,²⁴ and one may envision that it may modulate chromatin structure and gene expression. Other functions of tilorone, including signaling pathway and transcription factor interactions,^{36, 37} may also be involved. Further studies are needed to unravel the exact mechanism of action by which tilorone selectively targets CDK5-negative prostate cancer cells.

Acknowledgments

The authors wish to thank profs. P.J. van Diest and E. van der Wall for their support and discussion, and profs. M.A. Carducci and J.T. Isaacs for their provision of lab materials.

References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;64(1):9-29.
2. Tarricone C, Dhavan R, Peng J, Areces LB, Tsai LH, Musacchio A. Structure and regulation of the CDK5-p25(ncK5a) complex. *Mol Cell* 2001;8(3):657-669.
3. Rosales JL, Lee KY. Extraneuronal roles of cyclin-dependent kinase 5. *Bioessays* 2006;28(10):1023-1034.
4. Lalioti V, Pulido D, Sandoval IV. Cdk5, the multifunctional surveyor. *Cell Cycle* 2010;9(2):284-311.
5. Dhavan R, Tsai LH. A decade of CDK5. *Nat Rev Mol Cell Biol* 2001;2(10):749-759.
6. Cicero S, Herrup K. Cyclin-dependent kinase 5 is essential for neuronal cell cycle arrest and differentiation. *J Neurosci* 2005;25(42):9658-9668.
7. Strock CJ, Park JI, Nakakura EK et al. Cyclin-dependent kinase 5 activity controls cell motility and metastatic potential of prostate cancer cells. *Cancer Res* 2006;66(15):7509-7515.
8. Feldmann G, Mishra A, Hong SM et al. Inhibiting the cyclin-dependent kinase CDK5 blocks pancreatic cancer formation and progression through the suppression of Ras-Ral signaling. *Cancer Res* 2010;70(11):4460-4469.
9. Hsu FN, Chen MC, Chiang MC et al. Regulation of androgen receptor and prostate cancer growth by cyclin-dependent kinase 5. *J Biol Chem* 2011;286(38):33141-33149.
10. Demelash A, Rudrabhatla P, Pant HC et al. Achaete-scute homologue-1 (ASH1) stimulates migration of lung cancer cells through Cdk5/p35 pathway. *Mol Biol Cell* 2012;23(15):2856-2866.
11. Hsu FN, Chen MC, Lin KC et al. Cyclin-dependent kinase 5 modulates STAT3 and androgen receptor activation through phosphorylation of Ser727 on STAT3 in prostate cancer cells. *Am J Physiol Endocrinol Metab* 2013;305(8):E975-E986.
12. Pozo K, Castro-Rivera E, Tan C et al. The role of cdk5 in neuroendocrine thyroid cancer. *Cancer Cell* 2013;24(4):499-511.
13. Chong CR, Qian DZ, Pan F et al. Identification of type 1 inosine monophosphate dehydrogenase as an antiangiogenic drug target. *J Med Chem* 2006;49(9):2677-2680.
14. Chong CR, Xu J, Lu J, Bhat S, Sullivan Jr. DJ, Liu JO. Inhibition of angiogenesis by the antifungal drug itraconazole. *ACS Chem Biol* 2007;2(4):263-270.
15. Rudin CM, Liu W, Desai A et al. Pharmacogenomic and pharmacokinetic determinants of erlotinib toxicity. *J Clin Oncol* 2008;26(7):1119-1127.
16. Zhang H, Qian DZ, Tan YS et al. Digoxin and other cardiac glycosides inhibit HIF-1 α synthesis and block tumor growth. *Proc Natl Acad Sci U S A* 2008;105(50):19579-19586.
17. Wissing MD, Mendonca J, Kim E et al. Identification of cetrimeron bromide and irinotecan as compounds with synthetic lethality against NDRG1 deficient prostate cancer cells. *Cancer Biol Ther* 2013;14(5):401-410.
18. Nikolic M, Dudek H, Kwon YT, Ramos YF, Tsai LH. The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. *Genes Dev* 1996;10(7):816-825.
19. Kachhap SK, Rosmus N, Collis SJ et al. Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. *PLoS One* 2010;5(6):e11208.
20. O'Meara S, Al-Kurdi D, Ologun Y, Ovington LG. Antibiotics and antiseptics for venous leg ulcers. *Cochrane Database Syst Rev* 2010;(1):CD003557.

21. Hou SP, Fang AH, Chen QF, Huang YM, Chen OJ, Cheng LN. Termination of second-trimester pregnancy by mifepristone combined with misoprostol versus intra-amniotic injection of ethacridine lactate (Rivanol(R)): a systematic review of Chinese trials. *Contraception* 2011;84(3):214-223.
22. Daull P, Lallemand F, Garrigue JS. Benefits of cetalkonium chloride cationic oil-in-water nanoemulsions for topical ophthalmic drug delivery. *J Pharm Pharmacol* 2013.
23. Hume V, Westwood JC, Appleyard G. The anti-viral action of Rutilantin A. *J Gen Microbiol* 1965;38:143-151.
24. Krueger RE, Mayer GD. Tilorone hydrochloride: an orally active antiviral agent. *Science* 1970;169(3951):1213-1214.
25. Mayer GD, Krueger RF. Tilorone hydrochloride: mode of action. *Science* 1970;169(951):1214-1215.
26. Tazulakhova EB, Parshina OV, Guseva TS, Ershov FI. Russian experience in screening, analysis, and clinical application of novel interferon inducers. *J Interferon Cytokine Res* 2001;21(2):65-73.
27. Adamson RH. Antitumor activity of tilorone hydrochloride against some rodent tumors: preliminary report. *J Natl Cancer Inst* 1971;46(2):431-434.
28. Cummings FJ, Gelman R, Skeel RT et al. Phase II trials of Baker's antifol, bleomycin, CCNU, streptozotocin, tilorone, and 5-fluorodeoxyuridine plus arabinosyl cytosine in metastatic breast cancer. *Cancer* 1981;48(3):681-685.
29. Chong CR, Sullivan Jr. DJ. New uses for old drugs. *Nature* 2007;448(7154):645-646.
30. Shim JS, Matsui Y, Bhat S et al. Effect of nitroxoline on angiogenesis and growth of human bladder cancer. *J Natl Cancer Inst* 2010;102(24):1855-1873.
31. Shim JS, Rao R, Beebe K et al. Selective inhibition of HER2-positive breast cancer cells by the HIV protease inhibitor nelfinavir. *J Natl Cancer Inst* 2012;104(20):1576-1590.
32. Yang HC, Xing S, Shan L et al. Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. *J Clin Invest* 2009;119(11):3473-3486.
33. Lisianyi MI, Skitiak SA. Use of amiksin in complex therapy of cerebral gliomas. *Lik Sprava* 2002;(8):121-123.
34. Karimova FS, Ivanchenko GF, Grigorian SS. The treatment of laryngeal papillomatosis with interferon inducers. *Vestn Otorinolaringol* 2000;(5):54-57.
35. Zhou D, Tuo W, Hu H et al. Synthesis and activity evaluation of tilorone analogs as potential anticancer agents. *Eur J Med Chem* 2013;64:432-441.
36. Ratan RR, Siddiq A, Aminova L et al. Small molecule activation of adaptive gene expression: tilorone or its analogs are novel potent activators of hypoxia inducible factor-1 that provide prophylaxis against stroke and spinal cord injury. *Ann N Y Acad Sci* 2008;1147:383-394.
37. Schrimpf MR, Sippy KB, Briggs CA et al. SAR of alpha7 nicotinic receptor agonists derived from tilorone: exploration of a novel nicotinic pharmacophore. *Bioorg Med Chem Lett* 2012;22(4):1633-1638.

Chapter 13

Summarizing discussion - Future perspectives on treatment of patients with metastatic castrate-resistant prostate cancer

Prostate cancer has the highest incidence of all cancers in men in the Netherlands.¹ Its incidence rises with age. Virtually all mortality caused by prostate cancer occurs after the tumor has become metastatic and castrate-resistant (mCRPC). At this stage, prostate cancer treatment is strictly palliative. However, only 10-20% of all prostate cancer patients reach this disease stage within five years after diagnosis.² Therefore, a majority of patients dies with prostate cancer, but not because of the disease. Nevertheless, due to its high prevalence in the population, prostate cancer is still the second deadliest cancer in men, being only surpassed by lung cancer.^{3,4} In this thesis, multiple approaches to improve mCRPC outcome were studied, focusing on novel chemotherapies for mCRPC patients.

Until 2004, mCRPC patients were treated with mitoxantrone, which improves quality of life but has not shown to extend overall survival.⁵ That year docetaxel was introduced as first-line therapy for mCRPC patients. This taxane extended median overall survival by 2.4 months compared to mitoxantrone treatment in the TAX-327 trial.⁶ Docetaxel targets cells by stabilizing microtubules. Its mechanism of action results in a variety of antitumor effects in the cell, such as mitotic arrest, inhibition of transnuclear localization of the androgen receptor, and p53 induced apoptosis.^{7,8} However, docetaxel is not specific for cancer cells, and as microtubules play essential roles in healthy cells too, docetaxel treatment may result in severe adverse events, such as polyneuropathy and bone marrow suppression. Furthermore, only about half of mCRPC patients respond to docetaxel treatment, and eventually all tumors become docetaxel resistant.⁶ Therefore, additional research aims to further improve survival and quality of life of mCRPC patients.

Over the past few years, various novel second-line mCRPC therapies have been introduced into the clinic, such as cabazitaxel (a second-generation taxane),⁹ abiraterone acetate (an inhibitor of the enzyme cytochrome P450 c17 (CYP17)),^{10,11} and enzalutamide (an androgen receptor antagonist)¹². These agents have been studied or are being studied as first-line therapy as well; however, currently known results from studies such as COU-AA-302, a phase III study assessing abiraterone in asymptomatic or minimally symptomatic mCRPC patients, have not resulted in a change in policy regarding first-line mCRPC therapy in the Netherlands, one reason being that the control-arm received prednisone, which is not standard of care in the Netherlands.¹³

As multiple second-line mCRPC therapies have received approval, it has become unclear what therapy sequence is preferred after docetaxel therapy. In **Chapter 4**, we studied therapy outcome in mCRPC patients receiving cabazitaxel and abiraterone after docetaxel therapy. Differences in survival were minimal and non-conclusive, also as patient characteristics were not completely equal between groups due to the retrospective nature of the study. Cabazitaxel had a lower antitumor efficacy in mCRPC patients who had previously been

treated with abiraterone than in patients who received cabazitaxel before abiraterone; nevertheless, some patients still had clinical benefit by treatment with cabazitaxel. In patients treated with abiraterone after cabazitaxel, abiraterone was only slightly less effective than in patients receiving abiraterone prior to cabazitaxel. Future studies are needed to evaluate the cause of this decreased antitumor efficacy in more advanced mCRPC patients. As a final note, the CAST-study confirmed that toxicity caused by cabazitaxel, some life-threatening, remains a considerable issue with cabazitaxel therapy.

In the CAST-study, abiraterone and particularly cabazitaxel had a decreased antitumor efficacy when administered in the third-line compared to second-line therapy. Similar results were reported in patients receiving enzalutamide after docetaxel and abiraterone, abiraterone after docetaxel and enzalutamide, and docetaxel after abiraterone.¹⁴⁻¹⁸ These findings may simply be a consequence of the patients having more advanced disease. Alternatively, cross-resistance between various mCRPC therapies may occur, e.g. as all therapies interact in some way with the androgen receptor pathway. Early preclinical studies indicated cross-resistance between taxanes and abiraterone.¹⁹ However, this theory currently lacks a thorough scientific basis: aforementioned results need to be confirmed and mechanisms for cross-resistance would need to be identified. Thirdly, these therapies may only target a subgroup of prostate cancer cells, while other critical cancer cells are not inhibited by the therapy. E.g., stem cell-like prostate cancer cells may not be targeted by these therapies, resulting in proliferation and differentiation of these cells and acquired resistance.²⁰ It has also been suggested that epithelial-to-mesenchymal transition (EMT) may result in docetaxel resistance through reduced expression of miR-200C and miR-205, while such mesenchymal cells are prone to metastasize.²¹

Apart from aforementioned considerations regarding treatment sequence, the optimal time to initiate and end treatment needs more investigation. Another limitation of the COU-AA-302 study is that it is unclear whether treatment at this early stage has an advantage over treating patients once they have become symptomatic. As all therapies are palliative and have adverse events, it would be best to postpone treatment as long as possible, unless clinical outcome would be worse. Until this has been studied more extensively, it seems rational to avoid treatment in non-symptomatic patients.

Treatment is currently discontinued once patients start progressing on their tumor. Furthermore, taxane treatment is generally discontinued after ten cycles. However, it is unknown whether this is the best moment to discontinue treatment. Increasing treatment duration may invoke an extra delay for therapies with improved antitumor efficacy, particularly now that multiple therapy options are available for mCRPC patients. On the other hand, even when patients have minimal progressive disease, e.g. slowly increasing PSA levels while no progression is observed during radiographic assessments, patients may

still be benefitting from this therapy.

Currently, mCRPC patients are being viewed as a homogenous population when making therapy decisions, while cancer is a heterogeneous disease. As discussed in **chapter 2**, race may be one factor in tumor heterogeneity in prostate cancer patients. Since prostate cancer prevalence and mortality differs across races, the importance of recruiting a patient population in clinical trials whose racial demographics adequately represent the average patient population in clinic is stressed in this chapter. Furthermore, markers that identify mCRPC patient populations that will respond better to therapy need to be identified. This has become more crucial now that multiple therapies are available: by treating patients with ineffective therapies, other, potentially effective therapies are being delayed, while toxicity of the ineffective therapy may be severe. Results from the Dutch compassionate use program with cabazitaxel (**chapter 3**) and the CAST-study (**chapter 4**) suggest that patients who respond well to docetaxel, i.e. completed at least 10 cycles of docetaxel, have a better response to cabazitaxel therapy than patients who had receive less than 10 cycles of docetaxel. Others have found that Gleason-score predicts docetaxel response.²² In **chapter 9**, we report that nuclear Eg5-expression may be a biomarker predicting docetaxel response as well. Additional research is needed to identify predictive markers for therapy response, as well as markers that are able to follow therapy response more accurately during treatment. Such markers would prevent overtreatment and may aid in determining what therapy to initiate in an individual patient.

Nuclear Eg5-expression may not only be a marker for docetaxel response, but our results indicated that nuclear Eg5-expression may predict tumor aggressiveness too. While Gleason-score is now most frequently used to assess tumor aggressiveness, this assessment is limited: patients with a Gleason 6 or higher may have highly indolent disease, while others with a Gleason 6 progress rapidly into highly aggressive mCRPC. Results from multivariate analyses in our study suggested that nuclear Eg5-expression is a biomarker of tumor aggressiveness, independent of Gleason-score. If our results can be confirmed in an independent larger study population, determination of Eg5-expression in biopsies taken from patients may aid in determining whether a patient can be treated more conservatively or needs aggressive treatment, such as treatment with taxanes at an earlier disease stage.

Due to the success of taxanes, researchers hypothesized that inhibitors of proteins that play essential roles during mitosis, commonly overexpressed in cancer, may be effective while causing fewer side effects. This rationale led to the development of amongst others Eg5-, polo-like kinase 1 (Plk1-) and aurora kinase-inhibitors (**Chapter 5 section A**). Early clinical studies with these inhibitors had disappointing results, amongst others as toxicities such as bone marrow suppression prevented treatment with effective dosages. For some

researchers, this casts doubt whether such therapies may be effective at all.²³ In short, it was argued that mitotic inhibitors may have been unsuccessful as tumor cells in patients divide at a slower pace than tumor cells *in vitro* and *in vivo*. Due to the differences between preclinical models and tumors in patients, it was expected that mitotic inhibitors will have limited antitumor efficacy in humans. In **Chapter 5 section B**, we explain that such statements are too simplified. We argue that while the proteins have originally been identified due to their role in mitosis, they seem to play a more extensive role in tumor progression, such as regulation of the androgen receptor pathway.²⁴⁻²⁶ Considering the wide overexpression of these proteins in (prostate) cancer compared to healthy tissue,²⁷⁻³⁰ we think their roles as oncogene need to be investigated in more detail, and their clinical potential more thoroughly explored. The development of mitotic inhibitors is currently in full swing for a wide variety of cancers, among others prostate cancer.

Improvement of the antitumor effect of mitotic inhibitors in prostate cancer patients can be achieved by selecting patients based on the expression of the targeted protein in the tumor. For example, Eg5 and Plk1 are overexpressed in about half of prostate cancer patients; therefore, *a priori* selection of these patients would prevent overtreatment in about half of patients.^{27, 28} Since all mCRPC patients have had prostate biopsies taken and/or underwent prostate surgery, tumor tissue is readily available, hence this assessment would be non-invasive. Increased specificity of antimitotic agents may decrease toxicities. Intracellular levels in the tumor may be improved by developing agents that have low affinity to pumps such as P-glycoprotein, which transports docetaxel and other agents out of the tumor cell, limiting their efficacy.³¹ Combination therapy may enhance antitumor efficacy.

Similar to breast cancer, colorectal cancer and other cancer types, it is expected that in the future treatment of mCRPC will not consist of monotherapy but a combination of antitumor agents, due to the heterogeneity of the disease and as (stem cell-like) cancer cells have the ability to bypass pathways in order to survive. To speed up the clinical development of such combination therapies and to prevent unnecessary phase II studies with combination therapies that will not have an enhanced antitumor effect, we developed and tested Analysis of Functional Annotation (AFA) as an objective strategy to create a rationale for combination therapies (**Chapter 6**). Based on our AFA results with histone deacetylase inhibitors (HDACIs), we hypothesized that combinations of mitotic inhibitors with HDACIs would have an enhanced antitumor effect. In **Chapter 7** and **Chapter 8** we show that combination therapies of HDACIs with Plk1- and aurora kinase-inhibitors, respectively, indeed have synergistic antitumor effects in preclinical studies. This combination therapy could potentially be tested in humans, and may reduce bone marrow suppression and other adverse events, as lower concentrations can be used to accomplish similar antitumor effects.

Research is ongoing to identify additional targets for prostate cancer therapy. As most morbidity and mortality from prostate cancer is caused by metastases, patients may benefit from therapies that exclusively target metastases. Recently it has been reported that radium-223 extends the median overall survival of mCRPC patients by solely targeting bone metastases, underlining the importance of metastases-targeting agents (**Chapter 10**).³² Two targets to prevent or treat metastases are NDRG1, a metastasis-suppressor gene in prostate cancer (**Chapter 11 section a**), and CDK5, an enzyme necessary for prostate cancer metastases.^{33, 34} In **Chapter 11 section b**, we identify irinotecan and cetrimonium bromide as agents that selectively target NDRG1-deficient prostate cancer cells; tilorone selectively targets CDK5-deficient prostate cancer cells (**Chapter 12**). Furthermore, other targeted therapies may improve antitumor efficacy too, such as therapies that prevent epithelial-mesenchymal transition (EMT) or stimulate mesenchymal-epithelial transition (MET), therapies that target the microenvironment of the tumor, and therapies that prevent/target angiogenesis so that the tumor is deprived of its necessary oxygen and nutrients to grow and survive. Therapies that specifically target stem cell-like prostate cancer cells may be crucial, as these cells are resistant to most of the currently available therapies, while recent research suggested that such cells (the castration-resistant cells expressing the NK3 homeobox 1 (Nkx3-1) (CARNs)) may initiate mCRPC after castration.^{35, 36} Future research will need to identify and establish the clinical benefit of therapy aimed at these specific molecular/cellular targets.

Over the past years, significant progress has been made regarding the treatment of mCRPC patients. Multiple novel therapies have been introduced into clinical practice: cabazitaxel, abiraterone, enzalutamide and radium-223. Other therapies are at advanced stages of clinical development, such as ipilimumab, tasquinimod, custirsen and cabozantinib. Additional molecular targets and its targeted therapies are slowly but steadily identified in preclinical studies. Although the development of new therapies with increased efficacy and/or decreased toxicity remains of high importance, due to this progress it has become crucial that studies optimize the use of existing therapies, so that maximum clinical benefit can be accomplished in mCRPC patients with existing therapies. Therefore, research assessing the optimal treatment strategy with available therapies and the development of improved biomarkers, both for therapy response and tumor aggressiveness, has become of high importance too. Such research may further improve survival and the quality of life of mCRPC patients.

References

1. <http://cijfersoverkanker.nl/p=5124a6e31b232>[Internet]. Incidence rate of cancers in men in the Netherlands. [in Dutch]. Nederlandse Kankerregistratie (NKR), integraal kankercentrum Nederland (IKNL). Utrecht, NL. 2013.
2. Kirby M, Hirst C, Crawford ED. Characterising the castration-resistant prostate cancer population: a systematic review. *Int J Clin Pract* 2011;65(11):1180-1192.
3. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;64(1):9-29.
4. http://cijfersoverkanker.nl/selecties/Dataset_1/img5102c95ec2307[Internet]. Mortality rate of cancers in men in the Netherlands. [in Dutch]. Nederlandse Kankerregistratie (NKR), integraal kankercentrum Nederland (IKNL). Utrecht, NL. 2013.
5. Tannock IF, Osoba D, Stockler MR et al. Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points. *J Clin Oncol* 1996;14(6):1756-1764.
6. Tannock IF, de Wit R, Berry WR et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351(15):1502-1512.
7. Darshan MS, Loftus MS, Thadani-Mulero M et al. Taxane-induced blockade to nuclear accumulation of the androgen receptor predicts clinical responses in metastatic prostate cancer. *Cancer Res* 2011;71(18):6019-6029.
8. Giannakakou P, Nakano M, Nicolaou KC et al. Enhanced microtubule-dependent trafficking and p53 nuclear accumulation by suppression of microtubule dynamics. *Proc Natl Acad Sci U S A* 2002;99(16):10855-10860.
9. de Bono JS, Oudard S, Ozguroglu M et al. Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* 2010;376(9747):1147-1154.
10. de Bono JS, Logothetis CJ, Molina A et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011;364(21):1995-2005.
11. Fizazi K, Scher HI, Molina A et al. Abiraterone acetate for treatment of metastatic castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301 randomised, double-blind, placebo-controlled phase 3 study. *Lancet Oncol* 2012;13(10):983-992.
12. Scher HI, Fizazi K, Saad F et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med* 2012;367(13):1187-1197.
13. Ryan CJ, Smith MR, de Bono JS et al. Abiraterone in metastatic prostate cancer without previous chemotherapy. *N Engl J Med* 2013;368(2):138-148.
14. Mezynski J, Pezaro C, Bianchini D et al. Antitumour activity of docetaxel following treatment with the CYP17A1 inhibitor abiraterone: clinical evidence for cross-resistance? *Ann Oncol* 2012.
15. Schrader AJ, Boegemann M, Ohlmann CH et al. Enzalutamide in Castration-resistant Prostate Cancer Patients Progressing After Docetaxel and Abiraterone. *Eur Urol* 2013.
16. Bianchini D, Lorente D, Rodriguez-Vida A et al. Antitumour activity of enzalutamide (MDV3100) in patients with metastatic castration-resistant prostate cancer (CRPC) pre-treated with docetaxel and abiraterone. *Eur J Cancer* 2013.
17. Lortot Y, Bianchini D, Ileana E et al. Antitumour activity of abiraterone acetate against metastatic castration-resistant prostate cancer progressing after docetaxel and enzalutamide (MDV3100). *Ann Oncol* 2013.

18. Noonan KL, North S, Bitting RL, Armstrong AJ, Ellard SL, Chi KN. Clinical activity of abiraterone acetate in patients with metastatic castration-resistant prostate cancer progressing after enzalutamide. *Ann Oncol* 2013;24(7):1802-1807.
19. van Soest RJ, de Morree ES, Teubel W et al. Pre-clinical evidence for cross-resistance between taxanes and abiraterone in castration-resistant prostate cancer (mCRPC). [abstract]. European Cancer Congress 2013. 2013 Sep 27 - Oct 1; Amsterdam, the Netherlands. Abstract 2929.
20. McCubrey JA, Chappell WH, Abrams SL et al. Targeting the cancer initiating cell: the Achilles' heel of cancer. *Adv Enzyme Regul* 2011;51(1):152-162.
21. Puhr M, Hoefer J, Schafer G et al. Epithelial-to-mesenchymal transition leads to docetaxel resistance in prostate cancer and is mediated by reduced expression of miR-200c and miR-205. *Am J Pathol* 2012;181(6):2188-2201.
22. van Soest RJ, de Morree ES, Shen L, Tannock IF, Eisenberger MA, de Wit R. Initial Biopsy Gleason Score as a Predictive Marker for Survival Benefit in Patients with Castration-resistant Prostate Cancer Treated with Docetaxel: Data from the TAX327 Study. *Eur Urol* 2013.
23. Komlodi-Pasztor E, Sackett DL, Fojo AT. Inhibitors targeting mitosis: tales of how great drugs against a promising target were brought down by a flawed rationale. *Clin Cancer Res* 2012;18(1):51-63.
24. Hou X, Li Z, Huang W et al. Plk1-dependent microtubule dynamics promotes androgen receptor signaling in prostate cancer. *Prostate* 2013;73(12):1352-1363.
25. Lorenzo C, Liao Q, Hardwicke MA, Ducommun B. Pharmacological inhibition of aurora-A but not aurora-B impairs interphase microtubule dynamics. *Cell Cycle* 2009;8(11):1733-1737.
26. Liu XS, Song B, Liu X. The substrates of Plk1, beyond the functions in mitosis. *Protein Cell* 2010;1(11):999-1010.
27. Xing ND, Ding ST, Saito R et al. A potent chemotherapeutic strategy in prostate cancer: S-(methoxytrityl)-L-cysteine, a novel Eg5 inhibitor. *Asian J Androl* 2011;13(2):236-241.
28. Weichert W, Schmidt M, Gekeler V et al. Polo-like kinase 1 is overexpressed in prostate cancer and linked to higher tumor grades. *Prostate* 2004;60(3):240-245.
29. Buschhorn HM, Klein RR, Chambers SM et al. Aurora-A over-expression in high-grade PIN lesions and prostate cancer. *Prostate* 2005;64(4):341-346.
30. Chieffi P, Cozzolino L, Kisslinger A et al. Aurora B expression directly correlates with prostate cancer malignancy and influence prostate cell proliferation. *Prostate* 2006;66(3):326-333.
31. Payton M, Bush TL, Chung G et al. Preclinical evaluation of AMG 900, a novel potent and highly selective pan-aurora kinase inhibitor with activity in taxane-resistant tumor cell lines. *Cancer Res* 2010;70(23):9846-9854.
32. Parker C, Nilsson S, Heinrich D et al. Alpha emitter radium-223 and survival in metastatic prostate cancer. *N Engl J Med* 2013;369(3):213-223.
33. Feldmann G, Mishra A, Hong SM et al. Inhibiting the cyclin-dependent kinase CDK5 blocks pancreatic cancer formation and progression through the suppression of Ras-Ral signaling. *Cancer Res* 2010;70(11):4460-4469.
34. Strock CJ, Park JI, Nakakura EK et al. Cyclin-dependent kinase 5 activity controls cell motility and metastatic potential of prostate cancer cells. *Cancer Res* 2006;66(15):7509-7515.
35. Wang X, Kruihof-de Julio M, Economides KD et al. A luminal epithelial stem cell that is a cell of origin for prostate cancer. *Nature* 2009;461(7263):495-500.
36. Germann M, Wetterwald A, Guzman-Ramirez N et al. Stem-like cells with luminal progenitor phenotype survive castration in human prostate cancer. *Stem Cells* 2012;30(6):1076-1086.

Appendices

A. Nederlandse samenvatting

B. Publicaties

C. Over de auteur

D. Dankwoord

Nederlandse samenvatting

Prostaatkanker is de meest voorkomende kanker in Nederlandse mannen. De incidentie van prostaatkanker stijgt met de leeftijd. Prostaatkanker leidt vrijwel alleen tot sterfte wanneer de tumor castratie-resistent en gemetastaseerd (mCRPC) is. Dit palliatieve stadium wordt door slechts 10-20% van alle prostaatkankerpatiënten binnen vijf jaar bereikt. De meerderheid van prostaatkankerpatiënten overlijdt dan ook aan andere aandoeningen dan de tumor. Desondanks is prostaatkanker na longkanker de dodelijkste kanker onder Nederlandse mannen. Gezien dit hoge sterftecijfer is het van belang om therapeutische opties voor mCRPC patiënten te verbeteren. In het afgelopen decennium zijn deze opties reeds aanzienlijk verbeterd. Was voorheen alleen symptoomverlichting mogelijk om de kwaliteit van leven te verbeteren, sinds 2004 zijn er systemische therapieën (o.a. docetaxel, cabazitaxel, abiraterone acetaat, enzalutamide) beschikbaar gekomen die de mediane overleving van patiënten met enkele maanden verlengen. Echter, omdat niet alle patiënten een respons op deze systemische therapieën hebben en uiteindelijk alle patiënten tumorprogressie hebben ondanks behandeling, is het noodzakelijk verder onderzoek te doen naar therapieën voor mCRPC patiënten. In dit proefschrift wordt vanuit verschillende invalshoeken dit vraagstuk nader onderzocht.

Prostaatkanker is een heterogene ziekte. Dit komt onder andere tot uiting in de demografische spreiding van de ziekte: terwijl Aziaten en Indianen over het algemeen minder prostaatkanker krijgen dan blanken, hebben mensen met een donkere huidskleur juist relatief vaak prostaatkanker. Daarnaast leidt prostaatkanker in de Verenigde Staten vaker tot sterfte in de Afro-Amerikaanse en Indiaanse bevolkingsgroepen. Het is daarom goed voorstelbaar dat de werking van een therapie bij blanke Europeanen/Amerikanen anders is dan bij andere bevolkingsgroepen. In **hoofdstuk 2** wordt onderzoek gedaan naar de deelname van minderheden in klinische studies die bij de United States Food and Drug Administration ingediend zijn voor de markttoelating van nieuwe therapieën. Hierbij wordt geconcludeerd dat in deze studies minderheden over het algemeen ondervetegenwoordigd waren. Daarnaast is de deelname van minderheden niet toegenomen over de laatste twee decennia. Dit wordt mogelijk veroorzaakt door toenemende rekrutering van patiënten in landen waar deze minderheden in minder grote aantallen aanwezig zijn (zoals landen in het voormalige Oostblok), maar ook lijken pogingen tot verbeterde deelname van minderheden in Westerse landen niet succesvol. Door verbeterde deelname van minderheden in klinische studies, zal het begrip van de werkzaamheid van therapieën in minderheden verbeteren, zodat ook deze patiënten de optimale therapieën krijgen.

In Nederland worden mCRPC patiënten momenteel in eerste instantie met het taxaan

docetaxel behandeld. Deze chemotherapie grijpt aan op microtubuli in cellen. Hiermee voorkomt docetaxel onder andere dat cellen succesvol kunnen delen, maar grijpt het ook in op andere tumor-gerelateerde processen, zoals regulatie van het tumorsuppressorgen p53 en de androgeen receptor. Echter, microtubuli spelen ook een belangrijke rol in gezonde cellen, taxanen maken hier geen onderscheid tussen. Daarom resulteert behandeling met docetaxel in soms aanzienlijke bijwerkingen, zoals polyneuropathie en beenmergsuppressie. Daarnaast reageert slechts $\pm 50\%$ van de patiënten op docetaxel, en ontwikkelen uiteindelijk alle patiënten resistentie tegen behandeling. Sinds enkele jaren hebben patiënten die behandeld zijn met docetaxel keuze uit verschillende andere therapieën, waaronder een ander taxaan, cabazitaxel, of hormoontherapie, abiraterone acetaat.

In **hoofdstuk 3** worden de resultaten van het Nederlandse compassionate use programma samengevat, waarin mCRPC patiënten met cabazitaxel behandeld zijn. Dit programma werd in Nederland gestart naar aanleiding van de resultaten van de TROPIC-studie, een fase III studie waarin cabazitaxel behandeling werd vergeleken met mitoxantrone in patiënten die progressie hadden tijdens of na docetaxel behandeling. Een compassionate use programma geeft patiënten, voor wie geen reguliere (levensverlengende) behandeling mogelijk is, de mogelijkheid om een nieuwe behandeling te krijgen die nog niet door de verzekeraars vergoed wordt, maar waarvan wel verwacht wordt dat dit in de nabije toekomst zal gebeuren. Inclusie in dit programma vindt in een beperkt aantal centra plaats, in het geval van cabazitaxel in vijf ziekenhuizen; de veiligheid van de behandeling moet geregistreerd en gerapporteerd worden. Uit de gegevens van het compassionate use programma blijkt dat cabazitaxel beter getolereerd werd in de klinische setting dan verwacht zou worden op basis van de resultaten van de TROPIC-studie. Verder suggereren onze resultaten dat patiënten die goed reageren op docetaxel over het algemeen ook een betere respons op cabazitaxel hebben. In juni 2011, een jaar na de start van dit programma, werd besloten dat cabazitaxel door Nederlandse verzekeraars vergoed zal worden.

Enkele maanden na deze beslissing werd op basis van de resultaten van de COU-AA-301 studie besloten om abiraterone te vergoeden voor mCRPC patiënten met progressie tijdens of na docetaxel. Dit betekent dat momenteel cabazitaxel en abiraterone dezelfde indicatie hebben. Echter, welke volgorde het beste aangehouden kan worden, is niet bekend. Om deze reden werd de CAST-studie opgezet, waarvan de resultaten beschreven zijn in **hoofdstuk 4**. In deze retrospectieve studie zijn in het totaal 326 Nederlandse patiënten gevolgd die met cabazitaxel en/of abiraterone behandeld zijn. Een eerdere studie suggereerde dat docetaxel na abiraterone een verminderd antitumor effect heeft. Ook in de CAST-studie is de respons op cabazitaxel na abiraterone kleiner dan wanneer cabazitaxel voor abiraterone wordt gegeven, hoewel sommige patiënten nog steeds goed reageren op cabazitaxel na abiraterone. Deze verminderde werking wordt in mindere mate ook bij abiraterone voor en

na cabazitaxel gezien. Verder blijkt uit de CAST-studie dat ondanks de toenemende ervaring met cabazitaxel, bijwerkingen van deze therapie ernstig kunnen zijn; bij een paar procent leiden de bijwerkingen zelfs tot het overlijden van de patiënt. Deze bevindingen kunnen artsen een handvat bieden wanneer gekozen moet worden tussen cabazitaxel en abiraterone ter behandeling van mCRPC patiënten na docetaxel. Toekomstige studies moeten uitwijzen of resistentie van de tumor tegen abiraterone ook tot resistentie tegen cabazitaxel leidt, of dat andere oorzaken tot de verminderde werking van cabazitaxel in de derde lijn leiden.

Het succes van de taxanen in mCRPC patiënten heeft geleid tot een zoektocht naar behandelingen die eiwitten remmen die noodzakelijk zijn voor succesvolle celdelingen, in de hoop een behandeling te vinden die meer selectief prostaatkankercellen remt. Zoals beschreven in **hoofdstuk 5 sectie a** heeft dit onder andere geleid tot de ontwikkeling van kinesin spindle protein (Eg5-), polo-like kinase 1 (Plk1-) en aurora kinase (AK-)remmers. De eerste klinische studies met dit soort medicijnen hadden niet het gewenste resultaat, o.a. omdat beenmergsuppressie door de therapie verhinderde dat hogere en effectievere concentraties gebruikt konden worden. Dit leidde tot twijfel bij sommige onderzoekers of kankerpatiënten überhaupt baat zullen hebben bij dit soort therapieën. In **hoofdstuk 5 sectie b** wordt uiteengelegd waarom deze twijfel voorbarig is, en waarom te verwachten is dat in de toekomst deze behandelingen mogelijk wel in de kliniek toegepast zullen worden. Momenteel is de ontwikkeling van Plk1- en AK-remmers als kankerbehandeling in volle gang in preklinische en klinische studies.

Op dit moment worden mCRPC patiënten alleen met systemische monotherapieën behandeld. Echter, het is de verwachting dat het remmen van verscheidene oncologische processen door combinatietherapie meer effect heeft dan behandeling met monotherapie. Daarom is het onderzoek naar combinatietherapieën groeiende. In **hoofdstuk 6** wordt Analysis of Functional Annotation (AFA) beschreven als een methode om op rationele wijze nieuwe combinatietherapieën te ontwikkelen. Voor deze analyse is gebruik gemaakt van prostaatkankercellen behandeld met histon deacetylase remmers (HDACIs). HDACIs moduleren transcriptie van genen door remming van histon deacetylases, enzymen die het DNA strak rond histonen wikkelen waardoor dit DNA niet afgelezen wordt. Hierdoor beïnvloeden HDACIs de expressie van vele (oncologische) processen, en leiden ze onder andere tot herexpressie van genen die tumorgroei onderdrukken. Hoewel HDACIs al worden toegepast voor de behandeling van lymfomen, viel het antitumor effect in solide tumoren tot nu toe tegen. Echter, gezien de talrijke cellulaire veranderingen die HDACIs teweeg brengen en relatief weinig bijwerkingen van HDACIs, vormt deze chemotherapie een uitstekende kandidaat voor combinatietherapie. Uit onze AFA blijkt dat HDACIs mogelijk gebruikt kunnen worden vóór behandeling met immunotherapie (zoals sipuleucel T of ipilimumab, beide in ontwikkeling voor de behandeling van mCRPC in Nederland). Daarnaast suggereert deze

analyse dat HDACIs in combinatie met remmers van mitotische eiwitten mogelijk tot een verbeterd antitumor effect leiden.

In **hoofdstuk 7** en **hoofdstuk 8** wordt geconcludeerd dat de combinatie van HDACIs met respectievelijk Plk1-remmers en AK-remmers inderdaad leidt tot een synergistisch antitumor effect *in vitro* en *in vivo*. Echter, cellijnen reageren verschillend op deze combinatiebehandeling, zowel qua gevoeligheid als qua intracellulaire veranderingen. Verder onderzoek zal uitgevoerd moeten worden om vast te stellen of combinatietherapie ook een positief antitumor effect in de kliniek heeft, en welke eigenschappen van tumoren en patiënten tot een beter resultaat van deze combinatietherapie leiden.

Zoals eerder genoemd, vormen prostaatkankerpatiënten een heterogene populatie. Deze heterogeniteit komt tot uiting in demografische verschillen, maar ook in de agressiviteit van de tumor en de reactie van de tumor op chemotherapie: terwijl in sommige patiënten langdurige regressie wordt bereikt na chemotherapie, zijn tumoren in andere patiënten compleet ongevoelig voor chemotherapie. Echter, omdat momenteel niet bekend is welke patiënten wel en niet zullen reageren op therapie, worden patiënten momenteel als een homogene populatie behandeld. Op zoek naar een marker voor therapie-respons zodat wel onderscheid tussen patiënten valt te maken, wordt in **hoofdstuk 9** de hypothese getest dat Eg5 mogelijk een marker is voor de gevoeligheid van prostaatkankercellen voor docetaxel. Eg5 is een enzym dat aangrijpt op de microtubuli. Uit onze studie blijkt dat tumoren met nucleaire Eg5-expressie beter reageren op docetaxel. Daarnaast blijkt Eg5 een marker voor tumor-agressiviteit: patiënten met tumoren die Eg5 tot expressie brengen in de nucleus, hadden bij diagnose aggressievere tumoren. Verder grootschalig onderzoek moet uitwijzen of Eg5 inderdaad als biomarker in de kliniek gebruikt kan worden, waarbij patiënten met tumoren met nucleaire Eg5 expressie mogelijk gebaat zijn bij eerdere behandeling met docetaxel.

De meeste morbiditeit en sterfte van prostaatkanker wordt veroorzaakt door metastasen. Patiënten zouden daarom baat kunnen hebben bij het selectief behandelen en/of voorkomen van metastasen. Deze theorie is recentelijk bevestigd door radium-223 chloride: deze radionuclide behandelt alleen botmetastasen, maar verlengt de overleving van mCRPC patiënten met enkele maanden (**hoofdstuk 10**). Sinds februari 2014 is deze behandeling beschikbaar voor Nederlandse mCRPC patiënten. Echter, meer onderzoek is nodig naar de lange termijngevolgen van radium-223, alsmede naar de plaats van deze behandeling in het reeds bestaande therapiespectrum. Daarnaast zijn studies nodig om de effectiviteit van radium-223 in andere tumoren waar botmetastasen een groot deel van de morbiditeit en mortaliteit veroorzaken, zoals borstkanker, te onderzoeken.

Eerder onderzoek heeft aangetoond dat het gen NDRG1 een metastase suppressor gen in

o.a. prostaatkanker is (**hoofdstuk 11 sectie a**), terwijl expressie van CDK5 juist noodzakelijk is voor de metastasering van prostaatkanker. In **hoofdstuk 11 sectie b** en **hoofdstuk 12** zijn tumor-cellijnen gecreëerd die volledig identiek zijn, op de expressie van respectievelijk NDRG1 en CDK5 na. Gebruikmakend van een bibliotheek met medicijnen die voor uiteenlopende aandoeningen gebruikt worden, de zogenaamde Johns Hopkins Drug Library, is onderzoek gedaan naar medicijnen die selectief tumorcellen remmen gebaseerd op de expressie van deze genen. Uit deze studies blijken dat irinotecan en cetrimonium bromide selectief prostaatkankercellen remmen die weinig NDRG1 tot expressie brengen. Tilorone remt selectief prostaatkankercellen die weinig CDK5 tot expressie brengen.

Ten slotte worden in **hoofdstuk 13** de eerdere hoofdstukken samengevat en in een toekomstig perspectief geplaatst. Geconcludeerd wordt dat met de introductie van cabazitaxel, abiraterone, enzalutamide en radium-223 chloride de afgelopen jaren veel vooruitgang is geboekt qua behandelopties voor mCRPC patiënten; de verwachting is dat in de nabije toekomst meer behandelingen voor mCRPC patiënten op de markt zullen komen. Deze snelle ontwikkelingen leiden tot een verschuiving van prioriteiten in mCRPC onderzoek: waar voorheen in onderzoek voornamelijk gefocust werd op de ontwikkeling van nieuwe, verbeterde therapieën, een prioriteit die overigens blijft bestaan, wordt het steeds belangrijker om therapieën beter af te stemmen op de individuele eigenschappen van de patiënt. Dit kan bereikt worden door betere biomarkers die zowel vóór als tijdens behandelingen voorspellen bij welke therapieën mCRPC patiënten het meeste baat zullen hebben. Een verbeterd begrip van mechanismen waardoor prostaatkankercellen resistent worden tegen bepaalde therapieën, kan ertoe leiden dat behandelingen waarvoor de tumor resistent is (geworden), niet (meer) gegeven worden. De behandelvolgorde is mogelijk van invloed op het te behalen klinische voordeel, afhankelijk van de individuele eigenschappen van de tumor en patiënt. Combinatietherapieën zijn mogelijk effectiever dan monotherapieën. Ten slotte moet verder onderzocht worden wat het optimale moment is om van behandeling te wisselen en wanneer de bijwerkingen van de behandeling niet meer opwegen tegen de te behalen gezondheidswinst. Hoewel mCRPC nog niet te genezen valt, kunnen verbeterde therapieën de kwaliteit van leven van patiënten aanzienlijk verbeteren en mogelijk de ziekte dusdanig uitstellen dat prostaatkanker in plaats van een dodelijke ziekte steeds meer een chronische ziekte wordt.

Publicaties

Wissing M, Rosmus N, Carducci M, Kachhap S. NDRG1 (N-myc downstream regulated gene 1). Atlas Genet Cytogenet Oncol Haematol. 2010; 14(8):776-780.

Kachhap SK, Rosmus N, Collis SJ, Kortenhorst MS, **Wissing MD**, Hedayati M, Shabbeer S, Mendonca J, Deangelis J, Marchionni L, Lin J, Höti N, Nortier JW, Deweese TL, Hammers H, Carducci MA. Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. PLoS One. 2010 Jun 18;5(6):e11208. PMID: 20585447

Isaacs JT, Antony L, Dalrymple SL, Brennen WN, Gerber S, Hammers H, **Wissing M**, Kachhap S, Luo J, Xing L, Bjork P, Olsson A, Björk A, Leanderson T. Tasquinimod is an allosteric modulator of HDAC4 survival signaling within the compromised cancer microenvironment. Cancer Res. 2013 Feb 15;73(4):1386-99. PMID: 23149916

Wissing MD, Mendonca J, Kim E, Kim E, Shim JS, Kaelber NS, Kant H, Hammers H, Commes T, van Diest PJ, Liu JO, Kachhap SK. Identification of cetrimonium bromide and irinotecan as compounds with synthetic lethality against NDRG1 deficient prostate cancer cells. Cancer Biol Ther. 2013 May 1;14(5):401-10. PMID: 23377825

Wissing MD, Carducci MA, Gelderblom H, van Diest PJ. Tales of how great drugs were brought down by a flawed rationale--Letter. Clin Cancer Res. 2013 Mar 1;19(5):1303. PMID: 23393074

Wissing MD, van Diest PJ, Van der Wall E, Gelderblom H. Antimitotic agents for the treatment of patients with metastatic castrate-resistant prostate cancer. Expert Opin Investig Drugs. 2013 May;22(5):635-61. PMID: 23586879

Wissing MD, Van Oort IM, Gerritsen WR, Van den Eertwegh AJM, Coenen JLLM, Bergman AM, Gelderblom H. Cabazitaxel in patients with metastatic castration-resistant prostate cancer: results of a compassionate use program in the Netherlands. Clin Genitourin Cancer. 2013 Sep;11(3):238-250.e1. PMID: 23659772

Wissing MD, Kortenhorst MS, Rodríguez R, Kachhap SK, Jans JJ, Van der Groep P, Verheul HM, Gupta A, Aiyetan PO, van der Wall E, Carducci MA, van Diest PJ, Marchionni L. Analysis of the genomic response of human prostate cancer cells to histone deacetylase inhibitors. Epigenetics. 2013 Sep 1;8(9):907-20. PMID: 23880963

Wissing MD, Mendonca J, Kortenhorst MS, Kaelber NS, Gonzalez M, Kim E, Hammers H, van Diest PJ, Carducci MA, Kachhap SK. Targeting prostate cancer cell lines with polo-like kinase 1 inhibitors as a single agent and in combination with histone deacetylase inhibitors. *FASEB J*. 2013 Oct;27(10):4279-93. PMID: 23884428

Wissing MD, van Leeuwen FW, van der Pluijm G, Gelderblom H. Radium-223 chloride: extending life in prostate cancer patients by treating bone metastases. *Clin Cancer Res*. 2013 Nov 1;19(21):5822-7. PMID: 24052017

Wissing MD, Dadon T, Kim E, Piontek KB, Shim JS, Kaelber NS, Liu JO, Kachhap SK, Nelkin BD. Small-molecule screening of PC3 prostate cancer cells identifies tilorone dihydrochloride to selectively inhibit cell growth based on cyclin-dependent kinase 5 expression. *Oncol Rep*. 2014 Jul;32(1):419-24. PMID: 24841903

Wissing MD, Kluetz PG, Ning YM, Bull J, Merenda C, Murgo AJ, Pazdur R. Under-representation of racial minorities in prostate cancer studies submitted to the US Food and Drug Administration to support potential marketing approval, 1993-2013. *Cancer*. 2014 Jun 25. [Epub ahead of print]. PMID: 24965506

Wissing MD, Paller CJ, Mendonca J, Sharma A, Kim E, Kim HS, Kortenhorst MS, Gerber S, Rosen M, Shaikh F, Zahurak ML, Rudek MA, Hammers H, Rudin CM, Carducci MA, Kachhap SK. Combining the pan-aurora kinase inhibitor AMG 900 with histone deacetylase inhibitors enhances antitumor activity in prostate cancer. *Cancer Med*. 2014 Jul 3. [Epub ahead of print]. PMID: 24989836

Wissing MD, De Morrée ES, Dezentjé VO, Buijs JT, De Krijger RR, Smit VTHBM, Van Weerden WM, Gelderblom H, Van der Pluijm G. Nuclear Eg5 (kinesin spindle protein) expression predicts docetaxel response and prostate cancer aggressiveness. *Oncotarget*. In press.

Over de auteur

Michel Daniël Wissing is geboren te Tilburg op 27 juli 1988. Nadat hij in 2005 zijn VWO diploma summa cum laude behaalde aan het Hofstad Lyceum in Den Haag, begon Wissing met de studie geneeskunde aan de Rijksuniversiteit Leiden. In juni 2006 behaalde hij zijn propedeuse cum laude, waarna hij een semester studeerde aan het Karolinska Institutet te Stockholm, Zweden.

Na in 2007 geselecteerd te zijn voor het Excellente Studententrajec van het Leids Universitair Medisch Centrum, begon Wissing zijn onderzoekscarrière tijdens zijn geneeskundestudie bij de afdeling neuroradiologie (supervisor: dr. J. van der Grond). Hier deed hij onderzoek naar de invloed van glucose-inname op het brein middels functionele MRI. In mei 2009 reisde Wissing voor zijn wetenschapsstage naar het Johns Hopkins Medical Institutions te Baltimore, Verenigde Staten, waar hij in het laboratorium van prof. dr. M.A. Carducci en dr. S.K. Kachhap onderzoek verrichtte naar nieuwe chemotherapeutica ter behandeling van prostaatkanker (Nederlandse supervisor: prof. dr. J.W.R. Nortier). Wegens veelbelovende resultaten werd besloten het onderzoek uit te breiden tot een promotieonderzoek.

In maart 2011 behaalde Wissing zijn doctoraalexamen voor geneeskunde. Vanaf juni 2011 was hij in dienst bij het Leids Universitair Medisch Centrum in het kader van het MD/PhD programma, waarbij hij zijn onderzoekswerkzaamheden verspreidde over het Leids Universitair Medisch Centrum (supervisors: prof. dr. A.J. Gelderblom en dr. G. van der Pluijm), Johns Hopkins Medical Institutions (supervisors: dr. S.K. Kachhap, prof. dr. B.D. Nelkin en dr. L.M. Marchionni), Universitair Medisch Centrum Utrecht (supervisor: prof. dr. P.J. van Diest) en de United States Food and Drug Administration (supervisor: A. Murgo). In de laatste twee jaar breidde Wissing zijn promotieonderzoek uit naar klinische studies, waarvoor hij samenwerkte met onder andere het Erasmus Medisch Centrum in Rotterdam en het Reinier De Graaf ziekenhuis in Delft. Voor zijn onderzoek heeft Wissing in 2012 de Pieter de Mulder Award mogen ontvangen.

Per december 2013 continueert Wissing zijn opleiding geneeskunde bij de Rijksuniversiteit Leiden en is hij gestart met de co-schappen.

Dankwoord

Bij het tot stand komen van dit proefschrift waren veel mensen betrokken, omdat het uitgevoerde onderzoek in vier instituten is uitgevoerd, en de klinische studies in samenwerking met 13 Nederlandse ziekenhuizen zijn uitgevoerd. Ik wil iedereen die een bijdrage heeft geleverd aan de studies in dit proefschrift bij dezen hartelijk bedanken voor zijn/haar inspanning. Een aantal wil ik hiervoor in het bijzonder bedanken.

Als eerste zou ik graag mijn promotoren, prof. dr. A.J. Gelderblom en prof. dr. P.J. van Diest hartelijk willen bedanken. Ik vond het erg prettig hoe jullie mij de ruimte gaven om ideeën te bedenken en uit te voeren, maar snel ingrepen als ik hier in vast liep. Hans, dankzij jouw hands-on aanpak verliep mijn overgang van Hopkins naar het LUMC vloeiend, en heb ik met plezier de laatste jaren van mijn promotietraject in het LUMC afgemaakt. Paul, toen jij akkoord ging mijn Nederlandse begeleider te zijn, had je ongetwijfeld niet gedacht dat het zo'n klus zou worden, maar zoals je al zei, het is uiteindelijk zeker de moeite waard geweest. Dank voor je inzet en enthousiasme.

Dit onderzoek was nooit van start gegaan zonder dr. M.S.Q. Kortenhorst. Mady, bedankt voor het mogelijk maken van wat begon als een studiereis naar Hopkins, en erg leuk dat we over de jaren contact bleven houden.

My colleagues from Johns Hopkins I would like to acknowledge in English, in particular dr. S.K. Kachhap, dr. L.M. Marchionni and C.J. Paller, MD. Sushant, the skills I learnt while working in your lab form a robust basis for my future in cancer research, thank you for sharing your knowledge. Luigi, your knowledge about bio-informatics and your ability to translate it to understandable English were invaluable, it was a pleasure working with you. Channing, your ability to rapidly solve problems was inspiring; thank you for the productive collaborations. I would further like to thank prof. dr. B.D. Nelkin, dr. H. Hammers and all other PIs and colleagues who helped me with my research and made me feel at home in Baltimore, in particular Georges N'dikuyeze, Stephanie Gerber, Nadine Kaelber, Robert Delaney and all the Baltimorean Dutchies.

I would also like to thank the colleagues at CDER at the United States Food and Drug Administration for the inspiring internship. Here I would especially like to thank dr. A.J. Murgo. Without your involvement, this internship would not have been possible, and your endless patience answering all my questions was highly appreciated and motivating. But also all other colleagues, particularly Richard Pazdur, Paul Kluetz, Max Ning, Gideon Blumenthal, Edvardas Kaminskas, Dianne Spillman, Beverly Gallauresi, Emily McFadden and Jura Viesulas, I would like to thank for making my internship an utterly interesting experience.

In Leiden zou ik graag prof. dr. P.C.M. Pelger en dr. G. van der Pluijm van de afdeling urologie bedanken. Gabri, jouw kennis, collegialiteit, en enthousiasme maakten onze samenwerking zeer prettig, productief en inspirerend. Bij de afdeling oncologie wil ik graag prof. dr. J.W.R.

Nortier bedanken voor de begeleiding tijdens de eerste twee jaren van mijn onderzoek. Ook ben ik in het LUMC de afdeling pathologie dank schuldig voor hun hulp en begeleiding, voornamelijk prof. dr. V.T.H.B.M. Smit, E.M. Osse en N.T. ter Haar. Verder wil ik prof. dr. E. van der Wall en prof. dr. J.J.M. van der Hoeven bedanken voor hun begeleiding en beoordeling van mijn proefschrift.

Tenslotte wil ik alle collega's bij de afdelingen urologie en oncologie bedanken voor de fijne samenwerking, belangstelling, ondersteuning, en gezellige tijd tijdens de laatste twee jaar van mijn onderzoek.

Het is onmogelijk om alle collega's bij andere Nederlandse ziekenhuizen individueel te bedanken die hebben meegeholpen met de studies in dit proefschrift, maar, jullie enthousiasme en inzet om samen te werken aan deze projecten heb ik zeer gewaardeerd. In het bijzonder zou ik prof. dr. R. De Wit en dr. A.M. Bergman willen bedanken voor hun input, en Ellen de Morree voor de intensieve samenwerking tijdens het Eg5 project.

Geen proefschrift komt tot stand zonder steun van naasten, die ik in het bijzonder ontving van mijn ouders, broer, en partner.

Gemma, René, Stefan, het geduld waarmee jullie mijn verhalen aanhoorden was oneindig. Ondanks de lange tijd in het buitenland, stond de deur bij jullie altijd open. Heel erg bedankt voor jullie steun. En Stefan, wat fijn dat je als paranimf naast me staat tijdens mijn promotie. Ook ben ik verheugd dat Frans Steenwinkel, als dispuutsgeenoot, huisgeenoot, mede-Baltimoron en uiteindelijk buurjongen, mijn andere paranimf zal zijn. Ik heb altijd genoten van onze vele avondlange discussies op verschillende continenten.

Cally, we leerden elkaar kennen toen ik net begon aan wat een vijf jaar durend avontuur zou worden. Jouw aanwezigheid, begrip, hulp en steun tijdens mijn promoveren zijn van onschatbare waarde geweest. De beslissing om mee naar Nederland te verhuizen terwijl ik mijn promotie en studie afmaak, was niet alleen een grote stap voor jou, maar ook voor onze relatie. Bedankt!

