Cover Page

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Author: Slingerland, Marije
Title: Exploring novel formulations and new classes of anticancer drugs in solid tumors
Issue Date: 2014-09-11

Exploring novel formulations and new classes of anticancer drugs in solid tumors

Marije Slingerland

Publication of this thesis was sponsored by:
AZL Onderzoeks- en Ontwikkelingskrediet (OOK) Apotheek
Stichting Doelfonds Klinische Oncologie Bontius

Image cover Palau/Shutterstock.com
Layout Renate Siebes, Proefschrift.nu
Printed by Ipskamp Drukkers B.V.
ISBN 978-94-92026-00-2

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Exploring novel formulations and new classes of anticancer drugs in solid tumors

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 donderdag 11 september 2014
klokke 13.45 uur

door

Marije Slingerland

geboren te Leiden
in 1980

PROMOTIECOMMISSIE

Promotores	prof. dr. A.J. Gelderblom
	prof. dr. H.-J. Guchelaar
Overige leden	prof. dr. P.H. van der Graaf
	prof. dr. ir. J.J.M. van der Hoeven
	prof. dr. A.H.J. Mathijssen (Erasmus Universiteit Rotterdam)

Voor mijn kinderen

CONTENTS

1

Introduction, aim and outline of this thesis

Cancer, the leading cause of death in many developed countries, is responsible for almost one third of all deaths worldwide. Every year almost 0.5% of the world population is diagnosed with cancer.[1] It is expected that cancer is set to become a major cause of morbidity and mortality in the next few decades in every region of the world, irrespective of level of resource. Recently Bray *et al*. predicted an increase in the incidence of all cancer cases from 12.7 million new cases in 2008 to 22.2 million by 2030.[2]

Nowadays many different treatment options for cancer are known: local therapies including surgery and radiotherapy and systemic therapy including chemotherapy, hormonal therapy, immunotherapy and targeted therapy. Unfortunately, many current anticancer drugs have non-ideal pharmaceutical and pharmacological properties, which can lead to adverse consequences, including suboptimal therapeutic activity, dose-limiting side effects and poor patient quality of life. Novel formulations of anticancer drugs are necessary to overcome these problems. The general aim and scope of this thesis is to explore several novel formulations and new classes of anticancer drugs in solid tumors.

NOVEL FORMULATIONS

The first part of this thesis focuses on two novel formulations, namely liposomal drug formulations and camptothecin glycoconjugate BAY 56-3722 (formerly BAY 38-3441).

Liposomal drug formulations

In **Chapter 2**, as a prelude to the next chapters, the liposomal anticancer drugs that are available in the clinic are reviewed. Liposomes are simple, self-assembling systems that consist of a bilayer membrane surrounding an aqueous interior compartment. They are generally formed from naturally occurring phospholipids and cholesterol.[3] Considerable flexibility is possible in the design of liposomes with regard to, for example, their composition, size and drug release characteristics. Liposomal nanoparticles are designed to be multifunctional, with different components providing control over such properties as elimination half lives, permeability, biodistribution and targeting specificity.[4] At present, several liposomal anticancer drugs are available in the clinic or are in advanced stages of clinical development. Approved drugs include pegylated liposomal doxorubicin (Doxil®/Caelyx®), nonpegylated liposomal doxorubicin (Myocet®), liposomal daunorubicin (DaunoXome®) and liposomal cytarabine (DepoCyte®). Although almost all studies show that liposomal formulations of anticancer drugs are less toxic than the non-encapsulated

formulations, some liposome-specific adverse effects such as various skin reactions, and also hypersensitivity reactions, were reported.

In **Chapter 3**, a dose-escalating phase I study of LiPlaCis, a liposomal formulated platinum compound, in patients with advanced solid tumors is reported. In **Chapter 4** we describe a randomized two-period crossover, clinical bioequivalence study comparing the pharmacokinetics and safety of liposome-entrapped paclitaxel easy-to-use (LEP-ETU) formulation versus paclitaxel in Cremophor® EL (Taxol®) in patients with advanced cancer.

BAY 56-3722

In **Chapter 5**, we report the fate of BAY 56-3722 (formerly BAY 38-3441), a camptothecin glycoconjugate and the unique situation of a clinical hold after enrollment of 25 patients during a phase II study. This phase II study evaluates the antitumor activity of BAY 56-3722 in patients with recurrent or metastatic inoperable colorectal cancer (CRC) resistant to irinotecan.

NEW CLASSES OF ANTICANCER DRUGS

Besides novel formulations, also new classes of anticancer drugs for solid tumors such as histone deacetylase (HDAC) inhibitors and cardiac glycosides could be useful in the treatment of cancer. The second part of this thesis focuses on HDAC inhibitors and cardiac glycosides.

HDAC inhibitors

The histone deacetylase inhibitors are a group of targeted agents which are characterized as class I-specific or as pan-deacetylase (pan-DAC) inhibitors, which show activity against both classes I and II HDACs. A lot of research was focused on the treatment of hematological malignancies, but in the last decade also clinical trials with HDAC inhibitors in solid tumors were conducted. In **Chapter 6**, as a prelude to the next chapter, the clinical trials in solid tumors of HDAC inhibitors are reviewed. We demonstrate that despite promising results in the treatment of hematological malignancies, HDAC inhibitors have generally not been effective in clinical trials involving solid tumors.

Chapter 7 describes a phase I, open-label, multicenter study to evaluate the pharmacokinetics and safety of oral panobinostat in patients with advanced solid tumors and various degrees of hepatic function.

Cardiac glycosides

Cardiac glycosides have a long history in the treatment of cardiac disease. However, several preclinical studies and also two phase I studies have shown that cardenolides may also have anticancer effects. The mechanisms of the anticancer effects of cardenolides may include intracellular decrease of K^+ and increase of Na^+ and Ca^{2+}; intracellular acidification; inhibition of IL-8 production and the TNF-α/NF-κB pathway; inhibition of DNA topoisomerase II and activation of the Src kinase pathway. In **Chapter 8** we give an overview of these possible mechanisms and discuss their early development in cancer therapeutics. In **Chapter 9** we summarize the preclinical data and the preliminary results of a prematurely stopped clinical phase I trial with UNBS1450, a semisynthetic cardenolide glycoside derivative. This drug is considered a promising anticancer agent targeting overexpressed sodium pump α subunits in malignant tumors.

An English and Dutch summary of this thesis, including future perspectives, is presented in **Chapter 10**.

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PART I

Novel formulations

2

Marije Slingerland
Henk-Jan Guchelaar
Hans Gelderblom

Liposomal drug formulations in cancer therapy: 15 years along the road

Drug Discov Today 2012;17:160-6.

SUMMARY

Liposomes as pharmaceutical drug carriers were developed to increase antitumor efficacy and decrease drug toxicity. Doxorubicin HCl liposomal injection was the first liposomal encapsulated anticancer drug to receive clinical approval. To date, virtually all traditional anticancer drugs have been encapsulated in liposomes.

The majority of clinical studies only support the concept of a decreased toxicity and better tolerability of the liposomal anticancer drug. Although liposomal anticancer drugs have grown to maturity in several indications and are now in widespread further development programmes using their theoretical advantages to fulfill the high expectations, further studies are warranted – including the development of novel liposomal formulations.

INTRODUCTION

Many current anticancer drugs have non-ideal pharmaceutical and pharmacological properties such as low aqueous solubility, irritant properties, lack of stability, rapid metabolism, unfavorable pharmacokinetics and non-selective drug distribution, which can lead to a number of adverse consequences, including lack of or suboptimal therapeutic activity, dose-limiting side effects and poor patient quality of life.[1] From the drug delivery perspective, this might not only result in low bioavailability of the anticancer drug at the site of action (i.e. inside the cancer cells) but also in high organ toxicity that limits the maximal tolerable dose. Nanoscale drug delivery systems, defined as drug delivery systems having particle diameters of approximately 100 nm or less, are attracting considerable attention as a means of overcoming some of the limitations of conventional anticancer drug therapy. Liposomes and other lipid-based drug delivery systems are the archetypal nanoscale drug delivery systems. The first product, liposomal amphotericin B (Ambisome®), which is indicated for fungal infections, received clinical approval in 1990. Liposomes are simple, self-assembling systems that consist of a bilayer membrane surrounding an aqueous interior compartment. They are generally formed from naturally occurring phospholipids and cholesterol, rendering them readily biodegradable (Figure 2.1).[2] Considerable flexibility is possible in the design of liposomes with regard to, for example, their composition, size and drug release characteristics. Liposomal nanoparticles are designed to be multifunctional, with different components providing control over such properties as elimination half lives, permeability, biodistribution and targeting specificity.[1]

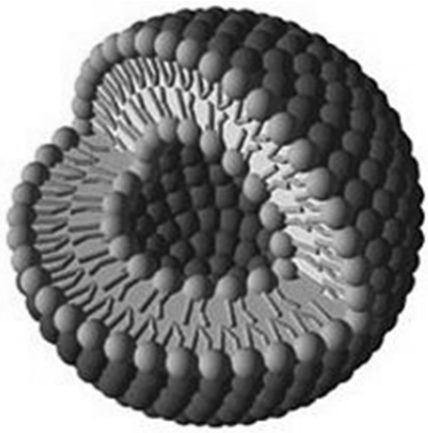

Figure 2.1 Liposome.

Doxorubicin HCl liposomal injection (Caelyx® in Europe, Doxil® in the USA), which received marketing approval in 1995, was the first nanoscale delivery system to receive clinical approval in cancer therapy for acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma.[3] Currently, virtually all traditional anticancer drugs have been encapsulated in liposomes using different technologies and many of them have entered clinical trials as cancer-imaging agents and/or anticancer therapeutics, indicating that this is a rapidly developing field that justifies review.

Here, we focus on the liposomal anticancer drugs that are available in the clinic, including discussion on the specific adverse effects of liposomes. We start with a short description of the principles of liposomal delivery.

Principles of liposomal drug delivery

Theoretically, liposomes have a couple of advantages over non-capsulated drugs,[4] first of which is their improved pharmacokinetics and drug release. In 2010, in a meta-analysis, Sidone *et al*. demonstrated that the pharmacokinetic (PK) variability of liposomal agents is 2.7 fold or 16.7 fold greater than non-liposomal agents, measured by ratio of the coefficient of variation (CV) to AUC, AUC CV%, and ratio of AUC_{max} to AUC_{min}, respectively.[5] A second advantage of liposomal drugs is their enhanced cellular penetration, for which exist different mechanisms, such as fusion of the liposomal membrane with the cellular plasma membrane.[4]

A third advantage is the possibility of selectively targeting anticancer drugs to the tumor, preventing the side effects of drugs related to effects in healthy tissues and enhancing the uptake of the drug by the targeted cells.[4] The fourth theoretical advantage of liposomal drugs is the ability to include several active ingredients in one complex liposomal drug delivery system. Clinical evidence supports the hypothesis of Goldie and Coldman: that treating cancers with all the available effective agents simultaneously provides the greatest chance of eliciting a cure.[6] Combination chemotherapy carried out with synergistic drugs is considered as a basis for improving its effectiveness. The ultimate goal of research is to prepare a product that encompasses traditional cytotoxic agents and new molecularly targeted modalities with optimum therapeutic effects and acceptable toxicity for healthy tissues, although this is difficult to achieve.[6]

Clinical use of liposomal drugs

At present, several liposomal anticancer drugs are available in the clinic (Table 2.1) or are in advanced stages of clinical development (Table 2.2). Approved drugs include pegylated liposomal doxorubicin (Doxil®/Caelyx®), nonpegylated liposomal doxorubicin (Myocet®), liposomal daunorubicin (DaunoXome®) and liposomal cytarabine (DepoCyte®).

We searched the literature (Pubmed) on this topic using a combination of the medical subject heading (MeSH) terms 'antineoplastic agents', 'daunorubicin', 'cytarabine', 'cisplatin' and 'clinical trials phase III', as well as search terms 'pegylated liposomal doxorubicin', 'chemotherapy', 'anticancer', 'antineoplastic', 'liposomal', 'liposomic', 'liposomes' and 'liposome', on 3 September 2011.

Liposomal formulations of anthracyclines are being used today for the treatment of AIDS-associated Kaposi's sarcoma, ovarian cancer and breast cancer.

AIDS-associated Kaposi's sarcoma

In the 1990s there were already positive reports of liposomal formulations of anthracyclines with high response rates in the treatment of AIDS-related Kaposi's sarcoma. In 1996, Gill *et al*. convincingly showed that a nonpegylated liposomal formulation of daunorubicin 40 mg/m^2 given every two weeks had considerably less toxicity than the doxorubicin, bleomycin and vincristine regimen without compromising efficacy. The overall response rate was 25% versus 28%.[7] In 1998 Stewart *et al*. reported on a multicentre phase III study that compared pegylated liposomal doxorubicin with the combination of bleomycin and vincristine and showed that the liposomal product is an effective treatment for AIDS-related Kaposi's sarcoma with a higher overall response rate (58.7% versus 23.3%, $P < 0.001$) than the bleomycin and vincristine combination. They reported that it was well tolerated but more myelosuppressive.[8] In 1998 Northfelt *et al*. reported on a phase III study that compared pegylated liposomal doxorubicin 20 mg/m^2 given every two weeks with doxorubicin, bleomycin and vincristine, during which patients that received pegylated liposomal doxorubicin experienced less toxicity and a higher overall response rate (45.9% versus 24.8%, $P < 0.001$).[9] In 2010 Cianfrocca *et al*. demonstrated in a phase III study that treatment with either paclitaxel or pegylated liposomal doxorubicin appears to produce significant improvements in pain and swelling in patients with advanced, symptomatic, AIDS-associated Kaposi's sarcoma treated in the highly active antiretroviral therapy (HAART) era. Comparing the paclitaxel and pegylated liposomal doxorubicin results revealed similar overall response rates (56% versus 46%, $P = 0.49$).[10]

Table 2.1 Overview of approved liposomal anticancer drugs

Available liposomal anticancer drug	Indication (for exact indication see text)	Phase III study	Refs
Nonpegylated liposomal doxorubicin	AIDS-related Kaposi's sarcoma	Stewart *et al*. 1998	8
		Northfelt *et al*. 1998	9
		Cianfrocca *et al*. 2010	10
	Metastatic ovarian cancer	Gordon *et al*. 2001	11
		Pignata *et al*. 2009	15
		Markman *et al*. 2010	16
		Pujade-Lauraine *et al*. 2010	17
	Metastatic breast cancer	Keller *et al*. 2004	18
		Chan *et al*. 2004	20
		Sparano *et al*. 2009	21
		Alba *et al*. 2010	22
	Multiple myeloma	Rifkin *et al*. 2006	24
		Orlowski *et al*. 2007	26
		Sonneveld *et al*. 2008	25
Liposomal daunorubicin	AIDS-related Kaposi's sarcoma	Gill *et al*. 1996	7
	Acute myeloid leukemia	Latagliata *et al*. 2008	27
Liposomal cytarabine	Lymphomas or leukemia with meningeal spread	Glantz *et al*. 1999	28

Ovarian carcinoma

In 2001, a phase III study in patients with epithelial ovarian carcinoma that had recurred after, or was not responsive to, first-line platinum-based chemotherapy was published by Gordon *et al*. to compare the efficacy and safety of pegylated liposomal doxorubicin and topotecan. They concluded that the comparable efficacy (overall response rates: 19.7% versus 17.0%, $P = 0.390$), favorable safety profile and convenient dosing support the role of pegylated liposomal doxorubicin as a valuable treatment option in this patient population.[11] Based on phase II results[12-14] and efficacy data from this phase III study, Caelyx® received FDA approval in June 1999 for the treatment of metastatic carcinoma of the ovary in patients with disease that is refractory to paclitaxel- and platinum-based chemotherapy regimens. Since the approval, much research has been done on liposomal doxorubicin. In 2009, based on the efficacy of pegylated liposomal doxorubicin in relapsed ovarian cancer, Pignata *et al*. demonstrated in a phase III study that pegylated liposomal doxorubicin plus carboplatin also has activity as a first-line treatment for advanced ovarian cancer (overall response rate of 68%, which exceeded the minimum required for study continuation).[15] In 2010 Markman *et al*. demonstrated in their phase III study that carboplatin plus pegylated

Table 2.2 Some liposomal chemotherapeutic anticancer drugs at various stages of development

Drug	Encapsulated chemotherapeutic agent	Development stage	Refs
ThermoDox®	Doxorubicin	Phase II	45
JNS002	Doxorubicin	Phase II	41
Liposomal annamycin	Annamycin	Phase II	46
LEM	Mitoxantrone	Preclinical	47
SPI-77	Cisplatin	Phase II	48-51
Lipoplatin	Cisplatin	Phase III	52
LiPlaCis	Cisplatin	Phase I	53
L-NDDP/aroplatin	Cisplatin analogue	Phase II	54, 55
MBP426	Oxaliplatin	Phase I	56
NL CPT-11	Nanoliposomal camptothecin	Trial	http://www.clinicaltrials.gov/
L9NC	9-nitro-20(S)-camptothecin	Trial	http://www.clinicaltrials.gov/
IHL-305	Irinotecan	Phase I	57
LE-SN38	SN38 (active metabolite of irinotecan)	Trial	http://www.clinicaltrials.gov/
PEP02	Irinotecan	Phase I	58
OSI211	Lurtotecan	Phase II	59, 60
TLI	Topotecan	Trial	http://www.clinicaltrials.gov/
PNU-93914	Paclitaxel	Trial	http://www.clinicaltrials.gov/
LEP-ETU	Paclitaxel	Trial	http://www.clinicaltrials.gov/
Marqibo®	Vincristine	Phase II	61
VLI	Vinorelbine	Trial	http://www.clinicaltrials.gov/
CPX-1	Fixed combination of irinotecan and floxuridine	Phase I	62
CPX-351	Fixed combination of cytarabine and daunorubicin	Phase I	63

liposomal doxorubicin in recurrent ovarian cancer had a favorable impact on progression-free survival (12 versus 8 months, $P = 0.02$), although the effect on overall survival was not statistically significant (median: 31 versus 18 months, $P = 0.2$).[16] In 2010 Pujade-Lauraine *et al*. published a randomized, multicentre, phase III noninferiority trial that demonstrated superiority in progression-free survival (11.3 versus 9.4 months, $P = 0.005$), and a better therapeutic index of pegylated liposomal doxorubicin with carboplatin over standard carboplatin and paclitaxel.[17]

Breast cancer

Also, in patients with metastatic breast cancer liposomal doxorubicin seemed to be effective. In 2004, Keller *et al*. published a randomized phase III trial to compare the efficacy of

pegylated liposomal doxorubicin with that of a common salvage regimen in patients with taxane-refractory advanced breast cancer. Patients in the control group received either vinorelbine or mitomycin C plus vinblastine, regimens previously shown to have moderate efficacy (median overall survival: 10.4 months versus 9.0 months, $P = 0.57$). They concluded that pegylated liposomal doxorubicin has efficacy comparable to that of common salvage regimens in patients with taxane-refractory metastatic breast cancer, thereby representing a useful therapeutic option.[18] The same year, O'Brien *et al*. published a phase III trial to demonstrate that efficacy of pegylated liposomal doxorubicin is comparable to doxorubicin (progression-free survival 6.9 versus 7.8 months, hazard ratio (HR) = 1.00), with significantly reduced cardiotoxicity (HR = 3.16, $P < 0.001$), myelosuppression, vomiting and alopecia in first-line treatment of women with metastatic breast cancer.[19] Also in 2004 Chan *et al*. showed that liposomal doxorubicin is an acceptable alternative to epirubicin as a first-line treatment for patients with metastatic breast cancer (overall response rates: 46% and 39%, $P = 0.42$).[20] In 2009 Sparano *et al*. demonstrated that pegylated liposomal doxorubicin was more effective than docetaxel alone in women with metastatic breast cancer who experienced relapse at least 1 year after prior adjuvant anthracycline therapy (median time to progression: 7.0-9.8 months, $P = 0.000001$; and the overall response rate from 26% to 35%, $P = 0.0085$), although overall survival was similar among the two groups (HR = 1.02, 95% CI, 0.86-1.22). This was without an increase in cardiac toxicity, although mucocutaneous toxicity was more common.[21] In 2010 Alba *et al*. demonstrated in their phase III study that maintenance chemotherapy with pegylated liposomal doxorubicin is well tolerated and offers improved time to progression of 3.3 months (8.4 versus 5.1 months, $P = 0.0002$) in patients with metastatic breast cancer following first-line chemotherapy.[22]

Hematological malignancies

For a few years liposomal anthracyclines have also been tested in the treatment of hematological malignancies.

In 2003 Dimopoulos *et al*. reported a multicentre trial that indicated that vincristine, doxorubicin and dexamethasone bolus and vincristine, liposomal doxorubicin and dexamethasone can be administered to outpatients and can provide an equal opportunity of rapid response in many patients with multiple myeloma (overall response of 61.4% and 61.3%).[23] In 2006 Rifkin *et al*. published a phase III trial to show that pegylated liposomal doxorubicin, vincristine and dexamethasone provide similar efficacy (objective response rates: 44% versus 41% progression-free survival, $P = 0.69$; and overall survival, $P = 0.67$)

with significant reduction in toxicity with doxorubicin, vincristine and dexamethasone in patients with newly diagnosed multiple myeloma. Notwithstanding these promising results, the authors concluded that the optimal management of patients with newly diagnosed myeloma still requires further study.[24] Sonneveld *et al*. showed in 2008 that pegylated liposomal doxorubicin plus bortezomib significantly prolonged time to progression compared with bortezomib alone (270 days versus 205 days) in patients with recurrent or refractory multiple myeloma who received prior thalidomide/lenalidomide therapy.[25] A year earlier, in the same phase III study, Orlowski *et al*. showed that pegylated liposomal doxorubicin plus bortezomib compared with bortezomib alone improved time to progression (6.5 months versus 9.3 months) in relapsed or refractory multiple myeloma.[26]

Recently, Latagliata *et al*. explored the efficacy of liposomal daunorubicin versus daunorubicin in acute myeloid leukemia patients aged older than sixty years. Liposomal daunorubicin seemed to improve overall survival and disease-free survival in the long-term follow-up, because of a reduction on late relapses (59% versus 78% at 24 months, $P = 0.064$). The authors concluded that liposomal daunorubicin could have a possible beneficial role in acute myeloid leukemia treatment although further testing would be useful.[27]

Liposomal cytarabine is approved for the treatment of lymphomas with meningeal spread and is the only liposomal drug administered for intrathecal administration. Although liposomal cytarabine is increasingly used for the treatment (and prophylaxis) of central nervous system involvement in patients with leukemia or lymphoma, many of the recently presented clinical trials on liposomal cytarabine were retrospective in nature or used this drug on a compassionate use basis. So far, one randomized phase III study has shown significantly better response rates in patients with lymphomatous meningitis who received liposomal cytarabine compared with cytarabine. The authors of this randomized trial concluded that liposomal cytarabine injected once every two weeks produced a high response rate (71% versus 15%, $P = 0.006$) and a better quality of life as measured by Karnofsky score ($P = 0.041$) relative to that upon treatment with free cytarabine injected twice a week.[28]

Epithelial malignancies

Liposomal cisplatin was developed for the treatment of epithelial malignancies. Initial safety and response results of a randomized phase III study with liposomal cisplatin in the treatment of advanced squamous cell carcinoma of the head and neck showed that liposomal cisplatin seems to reduce the renal and hematological toxicity, as compared

with conventional cisplatin, to a clinically relevant extent. This reduction of side effects will influence the chance to preserve the dose-density of chemotherapy and, thereby, the efficacy of treatment. The efficacy results showed 38.8% objective partial remission in the cisplatin arm of the trial versus 19% in the lipoplatin arm. However, 64% of the patients achieved stable disease while being treated with lipoplatin/5-fluorouracil (5-FU), versus 50% in the cisplatin/5-FU arm.[29] In 2010, Stathopoulos *et al*. showed in a phase III study that liposomal cisplatin in combination with paclitaxel was much less toxic than the cisplatin in combination with paclitaxel, whereas time to tumor progression (6.5 versus 6 months) and survival (9 versus 10 months) were similar in chemotherapy-naive patients with inoperable non-small cell lung cancer.[30]

The majority of clinical studies we have described are only supporting the concept of a decreased toxicity and better tolerability of the liposomal anticancer drug, there is a lack of available information regarding the greater clinical antitumor activity. None of the studies showed a better overall survival for the liposomal drug when directly compared to the non-liposomal variant. One of the reasons for this could be the inefficient drug release from the liposomes, as described by Seynhaeve *et al*. in 2007, showing that intact Doxil® liposomes could be visualized within living tumor cells.[31]

Because no direct comparative data are available on the efficacy of the drugs further studies with novel liposome encapsulated anticancer drugs are warranted to provide conclusive evidence for increased efficacy.

Also, no direct comparative data are available on the tumor distribution of drugs by administering the same doses given as a free drug or incapsulated in liposomes. As far as the distribution is concerned, one should perform studies giving the free drug and the liposomal formulation at the same time – labeling the drug in different ways and thus having interpretable results on the difference of distribution according to the method of administration.

Liposome-specific adverse effects

Although almost all studies show that liposomal formulations of anticancer drugs are less toxic than the non-encapsulated formulations, some liposome-specific adverse effects such as various skin reactions, and also hypersensitivity reactions, were reported.

Skin reactions

In 2000, Lotem *et al*. reported a study to show skin toxic effects of polyethylene-glycol-coated liposomal doxorubicin. In 60 patients four patterns of skin eruptions were seen: (i) hand-foot syndrome; (ii) diffuse follicular rash; (iii) intertrigo-like eruptions; and (iv) new formation of melanotic macules. The most common effect was the hand-foot syndrome, which was more pronounced, frequent and disabling with short dose intervals. This side effect is not a side effect of doxorubicin itself. Compared with doxorubicin, liposomal doxorubicin has a long elimination half-life and is highly stable, thus providing a slow release pool of drug to tumor and other tissues. It preferably localizes in the skin and deposits a substantial fraction of the administered drug locally. Inflamed skin is especially susceptible to liposome localization. The palms, soles and areas of repeated friction or trauma apparently achieve increased concentrations of liposomal doxorubicin as a result of the rich capillary network at their thickened papillary dermis and increased blood flow.[32]

Hypersensitivity reactions

Chan *et al*. described an episode of hypersensitivity reaction associated with the infusion of liposomal doxorubicin in an ovarian cancer patient during her first cycle of chemotherapy.[33] Hypersensitivity or infusion reactions with (pegylated) liposomes are well known and yet poorly understood. This type of hypersensitivity reaction is an acute transient malaise that develops in patients within minutes of vesicle infusion and is typically observed only during the first cycle of exposure. The hemodynamic, respiratory, cutaneous and subjective manifestations include hypotension or hypertension, dyspnea, flushing, rash and feeling of choking. Up to 30.8% of the patients experienced any type of hypersensitivity reaction.[30, 34-42] Although in practice severe hypersensitivity reactions to liposomal formulations are very uncommon. Unlike most chemotherapy, induced hypersensitivity reactions are IgE-mediated and the mechanism of liposomal reaction is described as a type I hypersensitivity reaction related to complement activation.[43] Slowing of the rate, or stopping the infusion, along with standard measures of anaphylaxis prevention and treatment (e.g. antihistamines, corticosteroids, epinephrine, bronchodilators or supportive therapy with fluids) usually seem to be sufficiently effective. However, considering that cardiopulmonary distress is a major physiological consequence that can lead to cardiac anaphylaxis, the prediction and prevention of this reaction seems to be crucial in patients with cardiovascular abnormalities. Liposome reactions in such patients can be life threatening, despite all treatment and preventive measures.[44]

Concluding remarks

In recent years, liposomes as pharmaceutical drug carriers have received considerable and increasing attention. Several phase II and III studies have shown increased antitumor efficacy and decreased toxicity and also several liposomal anticancer drugs are already available in the clinic for Kaposi's sarcoma, ovarian cancer and breast cancer. Further studies with liposome-encapsulated anticancer drugs, including the development of novel liposomal formulations, are warranted to provide evidence for increased efficacy and tolerability as compared with their non-liposomal counterparts. Fifteen years down the road we can conclude that liposomal anticancer drugs have grown to maturity in several indications and are in broad further development using their theoretical advantages to fulfill the high expectations.

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Maja de Jonge*
Marije Slingerland*
Walter Loos
Erik Wiemer
Herman Burger
Ron Mathijssen
Judith Kroep
Margret den Hollander
Diane van der Biessen
Mei-Ho Lam
Jaap Verweij
Hans Gelderblom

* Authors contributed equally

Early cessation of the clinical development of LiPlaCis, a liposomal cisplatin formulation

Eur J Cancer 2010;46:3016-21.

ABSTRACT

Purpose

To evaluate the safety and tolerability of LiPlaCis, a liposomal formulated platinum compound, in patients with solid tumors and to determine the maximum tolerated dose (MTD) of intravenous (i.v.) LiPlaCis and to assess plasma and urine pharmacokinetics and plasma biomarkers.

Patients and methods

Patients with solid tumors without standard therapeutic options were enrolled to receive LiPlaCis administered as a 1 h infusion without additional hydration every 3 weeks until RECIST progression or unacceptable toxicity. Cohorts of 3-6 patients were treated at each dose level until MTD was reached.

Results

Eighteen patients were enrolled and 64 cycles were delivered. At the first dose level 3 patients experienced an infusion reaction. Despite prophylactic premedication and prolongation of the infusion to 2 h in further patients, three other patients had mild acute infusion reactions. Toxicity at the fifth dose level of 120 mg consisted of grade 2 renal toxicity, reversible after hydration in 2 patients and grade 4 thrombocytopenia in one of these patients. Peak plasma concentrations and AUC were dose proportional. The interpatient variability in the clearance of total LiPlaCis-derived platinum was 41%. Platinum was excreted via the urine mainly during the first 24 h after administration. Investigated plasma biomarkers $sPLA_2$ and SC5b-9 were related to, but not predictive for, acute infusion reactions.

Conclusion

The observed safety profile suggests no benefit over standard cisplatin formulations and LiPlaCis will require reformulation to enable further development.

INTRODUCTION

Cisplatin-based anticancer therapies are widely used in the treatment of solid tumors. Dose-limiting toxicities include renal-tubular dysfunction, peripheral-neuropathy and ototoxicity, the first of which is due to rapid renal clearance of cisplatin and can be largely prevented by extensive pre- and post-hydration surrounding cisplatin administration.[1-3]

Widening cisplatin's therapeutic window by making the drug more tumor selective seems attractive. Liposomal drug delivery could serve this purpose, but was previously limited by the fast clearance from the blood. Addition of polyethylene glycol to the surface of liposomes resolved this problem and leads to preferential trapping and accumulation of liposomes in the leaky tumor vasculature resulting in enhanced drug exposure at the tumor site.[4]

However, in particular true for hydrophilic drugs like cisplatin, which cannot readily pass the liposomal lipid membrane, liposomal degradation and subsequent drug release into the tumor is an essential prerequisite for effect. The absence of drug release from the liposomes and the resulting absence of DNA-adduct formation, explained the lack of activity of SPI-077, a liposomal cisplatin formulation.[5,6]

LiPlaCis is a novel liposomal formulation of cisplatin. The LiPlaCis liposomes (i.e. LiPlasomes) are designed to be degraded by secretory phospholipase A_2 (PLA_2), a relatively tumor selective enzyme and thereby release the encapsulated cisplatin.[7,8] The use of enzymes, such as PLA_2, for triggered-drug release provides a novel tumor selective drug delivery approach. Preclinical proof of principle has been demonstrated *in vitro* and *in vivo*.[9,10]

The aim of this study was to define the maximum tolerated dose (MTD), the recommended phase II dose, pharmacokinetics and pharmacodynamics, as well as the preliminarily antitumor effects of a three-weekly schedule of LiPlaCis in patients with solid tumors.

PATIENTS AND METHODS

Drug formulation

This study was an open-label, dose-escalating phase I study of LiPlaCis in patients with advanced solid tumors. LiPlaCis was supplied by LiPlasome Pharma A/S as a concentrate for infusion in vials containing 2 mL (1 mg/mL) each as a white opalescent dispersion. The product must be stored at T = -80°C in order to ensure stabilization of the liposomes. The liposomes of LiPlaCis are composed of 1,2-disteaeoyl-*sn*-glycero-3-phosphocholine,1,2-

distearoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)] (sodium salt) and 1,2-disteaeoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt). In addition, sodium chloride, sucrose and disodium hydrogen phosphate are added to stabilize the liposomes. After thawing in a water bath at room temperature the content of the vials was added to a polyvinylchloride bag with saline to a total volume of 500 mL. The solution was kept at room temperature protected from light until administration which had to take place within 8 h after preparation.

Study design

LiPlaCis was administered intravenously in 1 h once every 3 weeks as long as there was no evidence of progressive disease (PD) or unacceptable toxicity. Escalation followed in a 3 + 3 design with increase of 20-100% from the previous dose level based on toxicity and pharmacokinetics. The MTD was defined as the dose with two or more patients with dose-limiting toxicity (DLT) in a cohort of 3 or 6 patients. Toxicity was evaluated using the CTC version 3.0.[11] DLT was defined as CTC grade 4 neutropenia, grade 4 thrombocytopenia, or grade 3 thrombocytopenia complicated with bleeding, persistent grade 2 neurotoxicity, persistent serum creatinine $> 2 \times$ upper limit of normal (ULN), drug-related non-hematological grade 3-4 toxicity or a delay in re-treatment with LiPlaCis of more than 2 weeks. The recommended dose for phase II (RD) was defined as the immediate-dose level below MTD.

If 2 patients at any dose level experienced an infusion reaction of at least grade 2, reduction in infusion rate and premedication would be introduced. No prophylactic anti-emetics were administered. Once 2 patients experienced nausea or vomiting grade 2 or more, prophylactic use of anti-emetics would be introduced for both the patients in question and the remaining patients. Hydration was not used routinely, however if nephrotoxicity was observed in a patient, both pre- and post-hydration would be introduced for the remaining cycles of this patient. In case of nephrotoxicity in multiple patients, a routine pre- and post-hydration schedule was to be implemented. Patients with measurable disease were assessed for antitumor activity by RECIST every 3 cycles and patients without measurable disease were assessed clinically.[12] Each subject, receiving at least 1 cycle was assigned a best response. The analysis of safety was based on the subjects who received at least one dose of LiPlaCis.

Eligibility criteria

Eligibility included a histological- or cytological-documented locally advanced or metastatic solid tumor refractory to standard therapy or for which no effective therapy existed and ECOG performance status 0-2. Required laboratory values included: absolute neutrophil count > 1.5×10^9/L, platelet count > 100×10^9/L, hemoglobin > 9 g/dL, total bilirubin < $1.5 \times$ ULN, alkaline phosphatase < $2.5 \times$ ULN, creatinine and blood urea within normal limits, unless creatinine clearance was < 60 mL/min calculated according to Cockcroft–Gault formula, aspartate aminotransferase and alanine aminotransferase < $2.5 \times$ ULN, or < $5 \times$ ULN in case of liver metastases. The study was approved by the institutional ethical committee and patients gave written informed consent prior to treatment.

Pharmacokinetics and pharmacodynamics

Serial blood samples for plasma total LiPlaCis-derived platinum (i.e. cisplatin-derived liposomal- associated plus non-liposomal associated platinum) concentration measurements as well as for secretory phospholipase A_2 ($sPLA_2$) and the complement activation marker SC5b-9 were collected over a 5-d period following the start of the infusion in cycle 1. Blood-samples were collected in standard blood collection lithium-heparin tubes prior to LiPlaCis infusion, halfway the infusion, 5 min before the end of infusion and 30 min, 1, 3, 6, 8, 24, 47, 71 and 95 h after the end of infusion. In case the infusion was stopped due to an acute infusion reaction, additional blood samples were collected at the point of interruption and restart of the infusion. Samples were centrifuged within 10 min after collection at 2800-3000 g for 10 min at 4°C. The plasma supernatant was stored at T < -70°C upon analysis.

Urine samples for the analysis of LiPlaCis-derived platinum concentrations were collected as voided in standard polypropylene containers prior to start of the infusion and during the following period after start of infusion: 0-6, 6-12, 12-24, 24-48, 48-72 and 72-96 h. The total volume was recorded and 3-mL aliquot of each portion stored at T < -70°C upon analysis.

Total LiPlaCis-derived platinum was determined by a validated, atomic absorption spectrophotometer method for cisplatin-derived platinum, with a lower limit of quantitation established at 0.200 µg/mL, essentially reported previously.[13] Pharmacokinetic parameter estimates of platinum were derived from weighted ($1/y$) two-compartmental model analysis using WinNonlin version 5.2.1 (Pharsight Corp., Mountain View, CA; model 10). Urine concentrations of total LiPlaCis-derived platinum were determined likewise. The lower limit of quantitation was validated at 1.00 µg/mL platinum in urine.

Plasma concentrations of $sPLA_2$ were determined by an enzyme immunoassay (EIA) based on the double-antibody 'sandwich' technique specific for type IIa $sPLA_2$ ($sPLA_2$ human Type IIA EIA Kit, Cayman, Ann Arbor, Michigan, United States of America (USA)). Plasma samples were also analyzed for SC5b-9, the terminal complement complex (TCC, SC5b-9) generated by the assembly of C5 through C9 as a consequence of activation of the complement system, using a SC5b-9 Plus EIA Kit (Quidel, San Diego CA, USA). ELISA measurements were performed according to the manufacturer's protocols.

Statistical analysis

Statistical analysis was performed using SPSS version 15.0. Potential differences in PK parameters between subgroups of patients were evaluated with ANOVA and *T*-test, whilst correlations were tested with linear regression analysis.

RESULTS

Patients, doses and toxicity

From May 2008 to November 2009 18 patients were enrolled into this study. Baseline patient demographics and disease characteristics are outlined in Table 3.1.

Overall, a total of 64 cycles of LiPlaCis were administered with a median of 3 cycles per patient (range 1-15). Three patients were treated at the first dose level of 10 mg with one infusion reaction grade 2. Therefore three additional patients were enrolled at dose level 1. In one of them a grade 2 infusion reaction occurred within a few minutes of starting the first infusion, but without significant systemic reactions. After administration of clemastine and dexamethasone, the patients received the remainder of the infusion successfully. Due to the fact that 2 patients experienced a > grade 2 reaction requiring treatment, reduction of infusion rate to 50% and routine premedication with a combination of clemastine 2 mg i.v. and dexamethasone 10 mg i.v., were introduced. Three patients were treated at dose levels 2-5 (20, 40, 80 and 120 mg), without first cycle DLTs. At each dose level 2, 4 and 5, 1 patient had a grade 2 infusion reaction. The patient at dose level 4 experienced a recurrent-infusion reaction (grade 3) in the second cycle despite additional premedication and was taken off study. Grade 1-2 nephrotoxicity, reversible after hydration, was observed in 1 patient at the second dose level and 2 patients at the fifth dose level. At dose level 5, 1 patient had a DLT in the second cycle consisting of grade 4 thrombocytopenia, grade 2

Table 3.1 Baseline demographics and patient characteristics

Baseline characteristics	Patients (*n* (%))	
Gender		
Male	10	(55)
Female	8	(45)
Age, years		
Median (range)	58	(39-75)
ECOG performance status		
0	4	(22)
1	14	(78)
Tumor type		
Breast	2	(11)
Melanoma	2	(11)
Esophagus	2	(11)
Prostate	2	(11)
Parotic carcinoma	2	(11)
Oro-/hypopharyngeal cancer	2	(11)
Urothelial carcinoma	2	(11)
Other*	4	(22)

* = Adenoid cystic carcinoma, cancer of unknown primary, non-small cell lung cancer, sarcoma

renal toxicity and schistocytes based on hemolytic uremic syndrome/thrombotic thrombocytopenic purpura (HUS/TTP) (Table 3.2). A second patient treated at this dose also developed a grade 2 renal toxicity in the first treatment cycle. Given the frequent infusion reactions and the high incidence of renal toxicity implicating no apparent practical benefit over standard formulated cisplatin it was concluded that without reformulation further development was precluded.

Response per RECIST was assessed in 12 patients, because 6 patients stopped treatment before their first planned disease evaluation after cycle 3 (2 patients on their own request, 2 because of recurrent infusion reactions and 2 because of DLT). Three from the 12 evaluable patients had stable disease (SD) at 9 weeks whereas the remaining 9 patients showed PD.

Pharmacokinetics

All 18 patients were evaluable for plasma-pharmacokinetic analysis. The observed plasma concentration, time data could be best fitted by a two-compartmental model in 17 patients,

whilst a one-compartmental model best fitted the data of 1 patient treated at the first dose-level of 10 mg, for which platinum could be quantitated only up to 8 h after end of infusion (i.e. platinum concentrations below 0.200 µg/mL). A summary of the pharmacokinetic parameters is presented in Table 3.3. Peak plasma concentrations were observed at or shortly after the end of infusion, irrespective of infuse duration. Peak plasma concentrations and

Table 3.2 Number of patients with treatment-related adverse events in all cycles

Adverse event		10 mg (*n* = 6)		20 mg (*n* = 3)		40 mg (*n* = 3)		80 mg (*n* = 3)		120 mg (*n* = 3)	
	Grade	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4
Leucopenia		1		1		1		1		–	
Neutropenia		–		–		–		–		–	
Thrombocytopenia		2		–		–		–		–	1
Anemia		3		3		1		2		2	
Nausea		3		2		1		2		2	
Vomiting		2		–		–		–		1	
Diarrhea		–		1		–		–		–	
Mucositis		–		–		–		1		–	
Nephrotoxicity		1		3		–		–		2	
Neurotoxicity		2		2		1		1		2	
Fatigue		4		3		2		1		2	
AST/ALT		4		1		1		1		2	
Infusion reaction		3		1		–		1[a]	1[a]	1	

[a] Same patient

Table 3.3 Mean ± SD plasma pharmacokinetics[a] of total LiPlaCis-derived platinum during course 1

Dose (mg)	Number of patients	C_{max}[b] (µg/mL)	$T_{1/2}\alpha$ (h)	$T_{1/2}\beta$ (h)	AUC (µg·h/mL)	CL (mL/h)	Vss (L)
10	6	1.62 ± 0.46	3.26 ± 0.56[c]	80 ± 21[c]	93.1 ± 31.8[c]	77.5 ± 29.6[c]	7.85 ± 0.81[c]
20	3	2.95 ± 0.32	3.44 ± 0.35	113 ± 46	258 ± 110	56.2 ± 21.1	7.91 ± 0.12
40	3	5.50 ± 1.18	5.50 ± 1.36	141 ± 59	559 ± 259	52.9 ± 20.7	9.21 ± 0.46
80	3	11.3 ± 0.23	3.98 ± 0.42	116 ± 35	888 ± 248	61.9 ± 18.5	9.31 ± 1.15
120	3	18.8 ± 7.50	5.04 ± 1.81	132 ± 90	2711 ± 2643	49.9 ± 34.1	6.43 ± 1.73
All	18	-	4.13 ± 1.26[d]	112 ± 51[d]	-	61.8 ± 25.3[d]	8.11 ± 1.34[d]

[a] Two-compartmental, except for 1 patient in 10 mg cohort which could be best fitted to a one-compartmental model

[b] Visually observed, in most cases (12 of 18) 0.5 or 1 h after end of infusion

[c] *n* = 5 (excluding patient fitted to one-compartmental model)

[d] *n* = 17 (excluding patient fitted to one-compartmental model)

AUC were dose-proportional. The interpatient variability in the clearance of total LiPlaCis-derived platinum was 41% and increased to 46% after correction for patient's individual body surface area. In addition, clearance was independent of gender ($P = 0.95$; *T*-test) and dose ($P = 0.59$; ANOVA) and was not related to age ($P = 0.94$; linear regression analysis). In Figure 3.1, the average total platinum concentration corrected for dose versus time curve is presented.

Platinum was below the lower limit of quantitation in most urine samples, especially at the lower dose-levels and after 24 h. Urinary excretion, however, seemed independent of the dose and if quantifiable, during the first 24 h after administration on average approximately 20% of the dose was excreted via the urine.

Pharmacodynamics

$sPLA_2$ levels could be analyzed in 17 patients and SC5b-9 levels in 14 patients. Plasma levels of $sPLA_2$ and SC5b-9 were readily detected in both baseline (pre-treatment) samples as well as on-therapy samples. Although the absolute $sPLA_2$ baseline plasma concentrations showed a high variability ranging from 1.5 to 13.7 ng/mL with a median of 6.3 ng/mL, relative $sPLA_2$ plasma levels were not affected upon administration of the liposomal- encapsulated cisplatin (LiPlaCis). The spectrum of SC5b-9 baseline levels displayed an even higher variation (range: 18-723 ng/mL; median: 233 ng/mL). All patients with a clinically-manifested acute infusion reaction showed an immediate increase in SC5b-9 levels after start of treatment

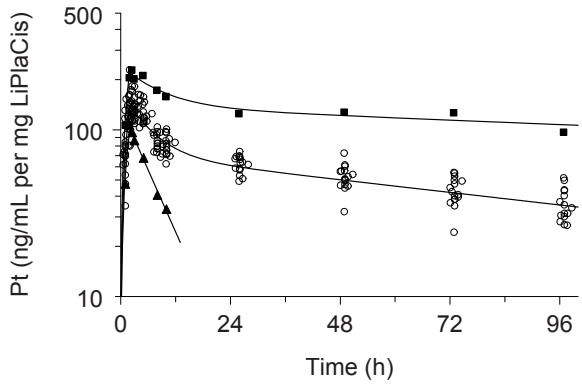

Figure 3.1 Average dose normalized concentration-time curve of LiPlaCis-derived platinum in plasma fitted to a two-compartmental model. The patients with the relative fast clearance (fitted to a one-compartmental model), treated at the first dose-level of 10 mg and the patient with the relative slow clearance, treated at the dose-level of 120 mg, are presented with the closed symbols.

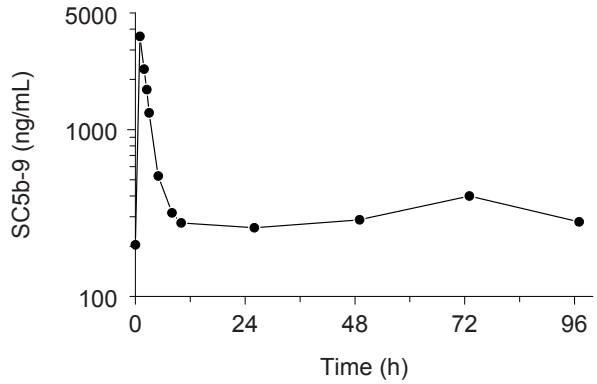

Figure 3.2 Plasma levels of the terminal complement complex (SC5b-9) in a typical representative patient with LiPlaCis infusion reaction. SC5b-9 concentrations were determined by ELISA in plasma samples before infusion with LiPlaCis (t = 0), halfway during the infusion (t = 60 min), 5 min before the end of infusion (t = 115 min) and at regular time points (30 min, 1, 3, 6, 8, 24, 47, 71 and 95 h) after end of infusion. Evidently, a clear increase in SC5b-9 levels was noted during and directly after LiPlaCis administration, indicative of the acute infusion reaction of this patient to LiPlaCis.

that returned to (pre-treatment) baseline within 24 h (Figure 3.2). However, the baseline $sPLA_2$ plasma levels in the patients with an acute infusion reaction (n = 5) did not differ from those observed in the other 12 patients (mean ± SD: 5.9 ± 1.5 ng/mL versus 7.5 ± 4.3 ng/mL; P = 0.42; T-test). Furthermore, no correlation was found between the baseline $sPLA_2$ levels and the plasma PK parameter $T_{1/2}\alpha$ (Pearson's coefficient correlation: r = 0.044; P = 0.87), indicating secretory PLA_2 levels are not associated with the plasma half-life of the LiPlasomes.

DISCUSSION

This study was designed to evaluate the tolerability, pharmacokinetics and pharmacodynamics of LiPlaCis, a novel liposomal formulation of cisplatin. LiPlaCis has a different toxicity profile compared to cisplatin; it seems to be less emetogenic at the dose levels studied. However, many patients experienced an acute infusion reaction related to the liposomal formulation requiring premedication with corticosteroids, which provides a disadvantage. Even more importantly, just like cisplatin, LiPlaCis induced renal toxicity and did not have the desired

kidney-sparing effect. The severity seemed to increase with the dose administered. Already at the dose level of 20 mg, grade 1 nephrotoxicity was observed in 3 patients 2 of which had confounding factors (pneumonia and nausea). The third patient had pre-existing grade 1 renal impairment. At the subsequent dose levels another 2 patients developed grade 1-2 renal toxicity. At the 120 mg dose level 2 patients developed grade 2 nephrotoxicity. In 1 patient this was due to HUS-TTP, which has been ascribed in the past to cisplatin-based chemotherapy, but is a rare side-effect.[14]

Lipoplatin, another liposomal-cisplatin formulation recently entered phase III studies. During the phase I study renal toxicity was not observed. The main toxicities of Lipoplatin constituted neutropenia, anemia and nausea and vomiting, all limited to grade 1-2.[15] In a subsequent phase Ib study combining Lipoplatin with 5-fluorouracil and radiotherapy 18% (2/11) of the patients developed grade 1 renal toxicity to which dehydration caused by gastrointestinal discomfort might have contributed.[16] This still contrasts the 33% (6/18) of the patients in our present study with LiPlaCis who developed renal toxicity.

Another major drawback of LiPlaCis was the frequent observation of non-dose related grade 2 infusion reactions despite premedication. Also after administration of Lipoplatin infusion reactions were observed albeit at an incidence of only 8.3%.[16] For pegylated-liposomal formulation of doxorubicin the percentage of acute infusion reaction is up to 9%.[17] This reaction that is typical for liposomal formulations, occurred at a rather (too) high incidence (7/18 patients, 39%) in our study. The infusion reactions were accompanied with complement activations, illustrated by an immediate increase in plasma SC5b-9 level (Figure 3.2).

Pharmacokinetic profiles of total LiPlaCis-derived platinum could be best fitted to a two-compartmental model. The initial half-life ($T_{1/2}\alpha$) most likely reflects the half-life of the intact circulating liposome, whilst the secondary half-life ($T_{1/2}\beta$) is considered to primarily reflect the half-life of extra-liposomal plasma-protein bound platinum. Although the LiPlasomes were constructed to be specifically degraded by $sPLA_2$ and plasma $sPLA_2$ concentrations were highly variable between patients, no correlation between the baseline levels of $sPLA_2$ and the initial half-life of LiPlaCis-derived platinum was observed. Potentially, other factors contributed to the degradation of the LiPlasomes. Total plasma-platinum clearance following LiPlaCis was slower compared to total-platinum clearance following the administration of free cisplatin and Lipoplatin, however was slightly faster compared to SPI-077 derived platinum.[15,18,19] Urinary excretion was slightly lower compared to free cisplatin and half of the excretion as observed after the administration of Lipoplatin.[15,20]

Although the toxicity pattern of LiPlaCis differed from cisplatin toxicity, renal damage was not prevented by the formulation. Acute infusion reactions required addition of extensive premedication that in turn could not completely prevent a high incidence of acute infusion reactions. Reformulation of LiPlaCis seems to be warranted prior to further development.

ACKNOWLEDGEMENT

The study was financially supported by LiPlasome Pharma A/S.

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Marije Slingerland
Henk-Jan Guchelaar
Hilde Rosing
Max Scheulen
Laurence van Warmerdam
Jos Beijnen
Hans Gelderblom

Bioequivalence of liposome-entrapped paclitaxel easy-to-use (LEP-ETU) formulation and paclitaxel in polyethoxylated castor oil: a randomized, two-period crossover study in patients with advanced cancer

Clin Ther 2013;35:1946-54.

ABSTRACT

Background

Preclinical studies comparing paclitaxel formulated with polyethoxylated castor oil with the sonicated formulation of liposome-entrapped paclitaxel (LEP) have demonstrated that LEP was associated with reduced toxicity while maintaining similar efficacy. Preliminary studies on the pharmacokinetics in patients support earlier preclinical data, which suggested that the LEP easy-to-use (LEP-ETU) formulation and paclitaxel formulated with castor oil may have comparable pharmacokinetic properties.

Objectives

Our objectives were: 1) to determine bioequivalence of paclitaxel pharmaceutically formulated as LEP-ETU (test) and paclitaxel formulated with castor oil (reference); and 2) to assess the tolerability of LEP-ETU following intravenous administration.

Methods

Patients with advanced cancer were studied in a randomized, two-period crossover bioequivalence study. Patients received paclitaxel 175 mg/m^2 administered as an intravenous infusion over 180 minutes, either as a single-treatment cycle of the test formulation followed by a single-treatment cycle of the reference formulation, or vice versa.

Results

Thirty-two of 58 patients were evaluable and were included in the analysis for bioequivalence. Mean total paclitaxel C_{max} values for the test and reference formulations were 4955.0 and 5108.8 ng/mL, respectively. Corresponding $AUC_{0-\infty}$ values were 15853.8 and 18550.8 ng·h/mL, respectively. Treatment ratios of the geometric means were 97% (90% CI, 91%-103%) for C_{max} and 84% (90% CI, 80%-90%) for $AUC_{0-\infty}$. These results met the required 80% to 125% bioequivalence criteria. The most frequently reported adverse events after LEP-ETU administration were fatigue, alopecia, and myalgia.

Conclusion

At the studied dose regimen, LEP-ETU showed bioequivalence with paclitaxel formulated with polyethoxylated castor oil.

INTRODUCTION

Paclitaxel is an antimicrotubule agent that prevents cell division by promoting the assembly and stabilization of microtubules and is active in a broad spectrum of malignancies.[1] The most commonly used 3-weekly regimen is 175 mg/m^2 over 3 hours given by intravenous infusion.

Because paclitaxel is extremely insoluble in water as well as in other vehicles commonly used in parenteral dosage formulations, the current injectable formulation consists of paclitaxel solubilized in 50:50 (vol/vol) polyethoxylated castor oil and dehydrated alcohol (USP) and must be diluted to a concentration of 0.3 to 1.2 mg/mL before use. Despite the dilution, the amount of polyethoxylated castor oil necessary to deliver the required doses of paclitaxel is significantly higher than that administered with any other marketed pharmaceutical injectable drug and may cause serious or fatal hypersensitivity episodes in humans.[2] In the initial clinical experience with paclitaxel, the incidence of severe infusion-related hypersensitivity reactions was approximately 20%.[3,4] Premedication with a corticosteroid, diphenhydramine, and H_2 antagonist has decreased the frequency of severe infusion-related hypersensitivity in 2% to 4% of patients, permitting the manageable administration of the drug.[5] Nevertheless, infusion-related hypersensitivity reactions remain a significant problem. In addition, polyethoxylated castor oil contributes to the nonlinear pharmacokinetic behavior of paclitaxel at higher doses.[2,6] Other major clinical toxicities associated with the use of paclitaxel are myelosuppression, peripheral neuropathy, myalgia/arthralgia, cardiovascular events, alopecia, and gastrointestinal toxicity.[7] Neutropenia is dose dependent and has dose-limiting toxicity. The acute toxicities not only limit dose intensification but also can necessitate dose reduction in individual patients, potentially decreasing the effectiveness of the treatment.

Approaches such as liposomal drug formulation have been pursued to further improve drug delivery, to increase the stability of the drug product, and to improve the safety profile.[8-15] One promising approach has been the use of electrically charged lipids to achieve an electrostatic attraction between the charged lipid and oppositely charged drug to create a stable liposome drug formulation. The use of synthetic electrostatic cardiolipin has enabled the liposome encapsulation of a variety of chemotherapeutic agents, including liposome-encapsulated doxorubicin (LED); liposome-encapsulated mitoxantrone (LEM); liposome-encapsulated SN-38 (LE-SN38), the active metabolite of irinotecan; liposome-encapsulated c-raf antisense oligonucleotide (LErafAON), initially as a sonicated formulation and now as an easy-to-use formulation (LErafAON-ETU); and paclitaxel, initially as a sonicated formulation

as liposome-encapsulated paclitaxel (LEP) and now as an easy-to-use formulation (LEP-ETU).[16-21] Liposome products LED, LEM, LE-SN38, LErafAON, LErafAON-ETU, and LEP have all been evaluated in preclinical studies and in phase I clinical trials.[16-21]

The LEP-ETU formulation is sterile, stable, and easy to use. The mean particle size of the liposomes is about 150 nm before and after lyophilization, and the drug-entrapment efficiency is > 90%. Stability data indicated that the lyophilized LEP-ETU was physically and stable for at least 12 months at 2°C to 8°C and chemically stable for at least 12 months at 25°C. Moreover, the formulation can be diluted to ~0.25 mg/mL without drug precipitation or change in particle size. *In vitro* drug-release study in phosphate-buffered saline (PBS; pH 7.4) showed that < 6% of the entrapped paclitaxel was released after 120 hours, indicating that the drug in an entrapped formulation is highly stable at physiologic temperatures.[22] The liposome-entrapped formulation of paclitaxel was developed aiming at an improved drug safety profile. This approach enables the elimination of the solvent polyethoxylated castor oil and the formulation of paclitaxel with a mixture of well-characterized, negatively charged, synthetic phospholipids and cholesterol. The LEP-ETU formulation allows for the possible administration of paclitaxel to patients without the need for premedication with corticosteroids because the well-characterized, synthetic phospholipids and cholesterol appear to be better tolerated than polyethoxylated castor oil. Moreover, an improved safety profile may enhance efficacy by facilitating the administration of higher cumulative doses. In addition, the entrapment of paclitaxel in liposomes should at least maintain or possibly improve the antitumor properties of paclitaxel while offering the advantage of a shorter infusion time. Indeed, preclinical studies comparing paclitaxel in castor oil with the previous, sonicated formulation, LEP, demonstrated that LEP was associated with reduced toxicity while maintaining efficacy compared with injectable paclitaxel.[23]

Analysis of the pharmacokinetics in patients treated in the extended dosing cohort supports earlier preclinical data, which suggested that paclitaxel in LEP-ETU and paclitaxel in castor oil have comparable pharmacokinetic properties.[24]

Determination of the bioequivalence of this new paclitaxel drug formulation with that of conventional paclitaxel formulated with polyethoxylated castor oil is warranted and was the aim of the current randomized, two-period crossover study.

PATIENTS AND METHODS

Study design and patients

This multicenter, randomized two-period crossover, clinical bioequivalence study was initiated in October 2004 and compared the pharmacokinetics of LEP-ETU and paclitaxel in castor oil in patients with advanced cancer. The inclusion criteria were: 1) age ≥18 years; 2) histologic diagnosis of advanced non-hematologic malignancy for which there is no curative therapy and for which treatment with single-agent paclitaxel was appropriate in the opinion of the physician; 3) Eastern Cooperative Oncology Group performance status of 0/1; 4) life expectancy of ≥12 weeks; 5) recovered from acute toxicities of prior treatment; and 6) adequate hematologic, kidney, and liver function. The study was approved by the ethics committees and institutional review boards of the collaborating institutions (Cancer Institute of New Jersey; University Clinic, Essen, Germany; General Hospital, St. Georg, Germany; Academic Medical Centre, Amsterdam, The Netherlands; Catharina Hospital, Eindhoven, The Netherlands; and Leiden University Medical Centre, Leiden, The Netherlands) and all patients signed informed consent before any study-related procedure.

Treatment

The injectable formulation of paclitaxel, as solubilized in 50:50 (vol/vol) polyethoxylated castor oil (trademark: Cremophor® EL) and dehydrated alcohol, (trademark: Taxol®) was used as the reference formulation. The LEP-ETU (developed by NeoPharm) used was developed as described by Zhang *et al*. in 2004 (test formulation).[22] The liposomes were prepared under Good Manufacturing Practice conditions. LEP-ETU was supplied as a lyophilized cake containing 30 mg paclitaxel. It was prepared for administration by reconstitution in 12.5 mL sterile water for injection to yield 2 mg/mL paclitaxel and diluted in 0.9% normal saline. The chemical composition of LEP-ETU is summarized in Table 4.1.

Each patient was randomized to receive a dose of 175 mg/m^2 paclitaxel as test formulation in study cycle 1, followed by the same dose of the reference formulation in study cycle 2, or vice versa. The washout period was 3 weeks. The test and reference formulations of paclitaxel (each at a concentration of 0.5 mg/mL) were administered by intravenous infusion over 180 minutes. Patients were premedicated with a fixed regimen of antihistamines (H_1- and H_2-antagonists) and dexamethasone prior to each dose of study medication to prevent infusion-related hypersensitivity reactions and to facilitate direct comparison of both paclitaxel treatments during both cycles. Patients were carefully monitored, particularly during the infusion.

Table 4.1 Chemical composition of LEP-ETU

Component	LEP-ETU
Paclitaxel	2.0 mg/mL
1,2-dioleoyl-sn-glycero-3-phosphocholine (DPOC)	54 mg/mL
Cholesterol (CH)	1.5 mg/mL
Tertramyristoyl cardiolipin (TMCA)	4.9 mg/mL
D-alpha tocopheryl acid succinate	0.3 mg/mL
Sucrose	200 mg/mL
Sodium chloride	9.0 mg/mL
Dehydrated ethanol	Removed during evaporation and lyophilization processes
Sterile water for injection	12.5 mL
Total volume of reconstituted product	15.0 mL
Total lipid	60 mg/mL
Lipid-to-drug molar ratio (DPOC: CH: TMCA)	90:5:5
Total lipid-to-drug molar ratio	33:1
Drug entrapment efficiency	≥ 85%

Safety assessments

National Cancer Institute Common Toxicity Criteria for Adverse Events version 3.0 was used to describe and grade all toxicities and adverse events (AEs). The relationship of AEs to study drug was documented by the Investigator as unrelated or unlikely, possibly, probably, or definitely related.

Pharmacokinetic evaluations

Blood samples (8 mL) for pharmacokinetic evaluation were collected in lithium heparinized collection tubes at each cycle, at each of the following time points: 0 (prior to start of infusion), 60, 120, and 165 minutes after the start of infusion; 30 seconds prior the end of infusion; and 15, 30, 45, 60, 120, 240, 360, 480 minutes and 22 to 26, 44 to 52, and 68 to 76 hours after infusion end. Plasma samples were stored at -20°C until analysis.

An HPLC-MS/MS method has been validated for the determination of paclitaxel concentration in human heparinized plasma. Total levels (free, protein bound, and liposomal levels) of the analytes were quantified. The analytes were quantified using $^{13}C_6$-paclitaxel as internal standard. The plasma sample clean-up procedure was performed by liquid-liquid extraction using *tert*-butylmethylether. After mixing and centrifuging, the aqueous layer was frozen instantly in a dry ice-ethanol mixture, and the organic solvent was decanted

into a clean tube. After evaporation of the solvent, the residue was reconstituted, and 25-µL aliquots were injected onto the analytical column. The analytical column was a Zorbax Extend-C18 column (Agilent Technologies, Inc, Santa Clara, California; 150 × 2.1 mm internal diameter, 5-µm particle size). A mixture of 10-mM ammonium hydroxide-methanol (30:70 vol/vol) was used as eluent. With an eluent flow of 0.2 mL/min, the run time was ~9 minutes. Positively charged ions were created at atmospheric pressure and were transferred to an API 3000 triple quadrupole mass spectrometer (Sciex, Thornhill, Canada). The transitions for paclitaxel were selected from *m/z* 854 → 509 and for the internal standard from *m/z* 860 → 515. The validated concentration ranges were from 0.25 to 1000 ng/mL for paclitaxel.

Pharmacokinetic analysis

The primary analysis was conducted on AUC and C_{max} values of paclitaxel in plasma following the administration of study drug. The analysis followed the approach for establishing average bioequivalence as presented in the US Food and Drug Administration guidance Statistical Approaches to Establishing Bioequivalence.[25] In this guidance, it is recommended that standard *in vivo* bioequivalence study designs be based on the administration of either single or multiple doses of the test drug and reference drug products to subjects on separate occasions, with random assignment to the two possible sequences of drug product administration. The guidance further recommends that statistical analysis for pharmacokinetic measures, such as AUC and C_{max}, be based on the two one-sided tests procedure to determine whether the average values for the pharmacokinetic measures determined after administration of the test and reference products were comparable. This approach is termed "average bioequivalence" and involves the calculation of a 90% CI for the ratio of the averages (population geometric means) of the measures for the test and reference drug products. To establish bioequivalence, the calculated CI should fall within a bioequivalence limit, usually 80% to 125% for the ratio of the product averages. In addition to this general approach, the guidance provides specific recommendations for: 1) logarithmic transformation of pharmacokinetic data; 2) methods to evaluate sequence effects; and 3) methods to evaluate outlier data.

The pharmacokinetic parameters of paclitaxel were determined as follows. $AUC_{0-\infty}$ was calculated as AUC_{0-lqc} + lqc/(-β), were lqc is the last quantifiable concentration and β is the slope from the linear regression of the natural logarithmic concentration versus time during the terminal phase. C_{max} was the peak observed plasma concentration. T_{max} was the time to C_{max}. The $t_{½}$ value was calculated as ln(2)/-β, for paclitaxel. The λ_z value was the first-order rate constant associated with the terminal portion of the curve.

If the paclitaxel concentration in the sample taken immediately prior to the end of infusion was less than that in the sample taken 15 minutes before the end of infusion, then the latter value was used in the calculations of AUC.

Actual sampling times, rather than scheduled sampling times, were used in all computations involving sampling times, except for predose. However, for ease of presentation, scheduled sampling times are presented in data listings and graphic presentations.

Plasma concentration values below quantifiable limits were treated as zero in computation of mean concentration values and individual patient-computed parameters.

Statistical analysis

From preliminary data on the pharmacokinetic properties of paclitaxel in plasma after the administration of LEP-ETU at 175 mg/m^2 over 90 minutes in advanced cancer patients, it was determined that the between-subject %CV of $AUC_{0-\infty}$ was ~0.4. It was assumed that the within-subject %CV was ~75% of the between-subject coefficient of variation. This would lead to a %CV of $0.4 \times 0.75/\sqrt{2} = 0.213$ for the 2×2 crossover design. Sample size calculation indicated that, accounting for early withdrawals, up to 54 patients would need to be treated to yield 32 evaluable patients. The sample size was determined using nQuery software (Statistical Solutions, Boston Massachusetts). A sample of 16 evaluable patients in each sequence, for a total of 32 evaluable patients in this study, should have 80% power to reject the null hypothesis that the two treatments are not bioequivalent using the 80% to 125% bioequivalence criteria for the ratio of the means in nontransformed scale at a 0.05 level of significance. Because C_{max} has shown a smaller %CV in preliminary data, it was determined that this sample size would be sufficient to show bioequivalence of both $AUC_{0-\infty}$ and C_{max}.

RESULTS

Fifty-eight patients were enrolled into this study. The characteristics of the patients are summarized in Table 4.2. Thirty-eight patients completed both cycles of treatment per protocol. Six patients discontinued due to early disease progression, 9 patients were discontinued from the study by the sponsor, 3 patients voluntarily withdrew, 1 patient discontinued due to a protocol violation (nonevaluable pharmacokinetic parameters), and 1 patient died due to disease progression.

Table 4.2 Baseline characteristics of the patients in this study of the pharmacokinetic properties and tolerability of LEP-ETU

Characteristic		*N*	%
Age group	< 45	12	21
	45-54	15	26
	55-64	18	31
	> 64	13	22
Gender	Female	38	66
	Male	20	34
Race	Asian	2	3
	Black/African American	4	7
	White	51	88
	Other	1	2
Ethnicity	Hispanic/Latino	2	3
	Non-Hispanic or -Latino	56	97
Primary tumor	Breast	10	17
	Colon	7	12
	Esophagus	5	9
	Sarcoma	5	9
	Bladder	4	7
	Lung	4	7
	Ovarian	4	7
	Other	19	33

Means of $AUC_{0-\infty}$ for the test and reference formulations were 15853.8 and 18550.8 ng·h/mL, respectively, and means of C_{max} were 4955.0 and 5108.8 ng/mL. The relative bioavailability of the test compared with the reference formulation was 84% with respect to $AUC_{0-\infty}$, while the C_{max} ratio was 97%–both meeting the 80% to 125% bioequivalence range per the US Food and Drug Administration guidance.[25]

Figure 4.1 shows mean total plasma paclitaxel concentration-time profiles for the test and reference formulations at a single dose of 175 mg/m^2 over 180 minutes.

Paclitaxel is stable in human plasma for at least 10 months. All study samples were analyzed within the time period of 6.5 months.

The nature and incidence of AEs related to the administration of LEP-ETU that were reported during the study are presented in Table 4.3. Fifteen patients in the study experienced a total of 23 AEs. Nine of these AEs were considered as either possibly, probably, or definitely related to administration of the relevant drug (test or reference). All of these events occurred during

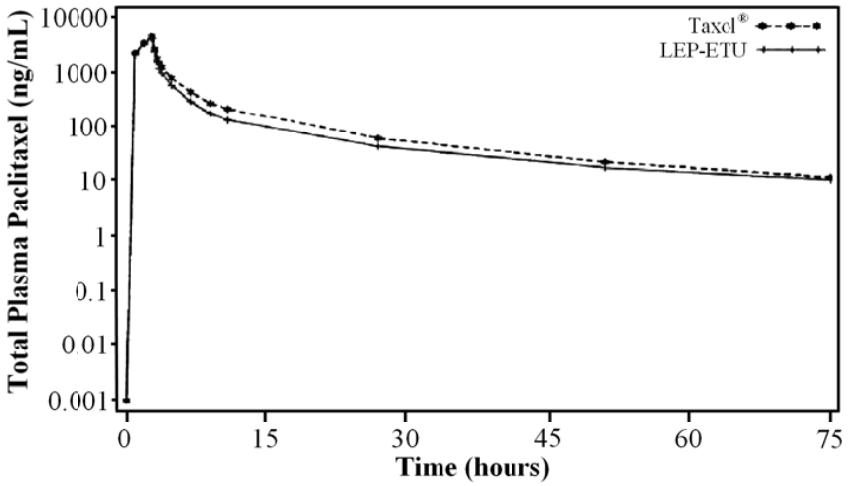

Figure 4.1 Mean plasma paclitaxel (= total levels) concentrations over time ($N = 32$). Test drug, liposome-entrapped paclitaxel easy-to-use (LEP-ETU); reference drug, injectable formulation consists of paclitaxel solubilized in 50:50 (vol/vol) polyethoxylated castor oil and dehydrated alcohol. Both formulations were infused intravenously at a dosage of 175 mg/m^2 paclitaxel over 180 minutes.

Table 4.3 Adverse events considered possibly, probably, or definitely related to study drug, by maximum severity, after 1 cycle of treatment*

Category/Adverse Event/Grade	Test/Reference (*N* = 30)	Reference/Test (*N* = 28)
Infection		
Febrile neutropenia, grade 3	0	1
Blood/bone marrow		
Leukopenia		
grade 3	1	1
grade 4	0	1
Neutropenia		
grade 3	2	2
grade 4	3	2
Gastrointestinal		
Dehydration, grade 3	1	0
Vomiting, grade 3	0	1
Neurology		
Dizziness, grade 3	1	0

* Test drug, liposome-entrapped paclitaxel easy-to-use (LEP-ETU); reference drug, injectable formulation consists of paclitaxel solubilized in 50:50 (vol/vol) polyethoxylated castor oil and dehydrated alcohol. Both formulations were infused intravenously at a dosage of 175 mg/m^2 paclitaxel over 180 minutes.

cycle 1 and thus their causality could be attributed to the study drug administered during that cycle. Four of the patients (who experienced a total of 7 AEs: dizziness, bone pain, vomiting, fatigue, pyrexia, hypertension, and angina pectoris) received the test formulation in cycle 1, and 2 of the patients (who experienced 2 separate AEs: febrile neutropenia and pyrexia) received the reference formulation in cycle 1. One patient prematurely discontinued from treatment due to a serious AE (a disease-related pulmonary embolism) after receiving the test formulation in cycle 1.

In this study, 11 of the 58 patients (19%) experienced neutropenia, all in cycle 1. Five patients experienced grade 4 neutropenia (3 patients after receiving the test formulation, and 2 patients after receiving the reference formulation), and 5 patients experienced grade 3 neutropenia (3 patients after receiving the test formulation, and 2 patients after receiving the reference formulation). One additional patient experienced grade 3 febrile neutropenia (after receiving the reference formulation), as well as grade 2 neutropenia (after receiving the reference formulation). None of these patients were discontinued from the study due to these events. No thrombocytopenia was observed. A single patient (2%) in this study had disease-related grade 3 anemia, which occurred during cycle 1 while the patient was receiving the reference formulation. Grade 2 anemia was reported as an AE in 5 patients (9%) (3 patients after receiving the test formulation, and 2 patients after receiving the reference formulation). In addition, 1 case of grade 2 iron-deficiency anemia (after receiving the reference formulation) was reported (2%).

DISCUSSION

In this bioequivalence and phase I tolerability study, LEP-ETU was bioequivalent to the reference formulation of paclitaxel. The most frequently reported AEs with LEP-ETU were fatigue, alopecia, and myalgia.

The rationale for developing a liposomal formulation of paclitaxel was to attempt to improve the safety profile of paclitaxel by eliminating the drug-formulation component polyethoxylated castor oil, which has been associated with toxicities, while maintaining or enhancing efficacy.

The previous sonicated formulation, LEP, was evaluated in a phase I clinical trial in patients with advanced malignancies.[21]

The product utilized in this study was the easy-to-use liposomal formulation of LEP, LEP-ETU. Several preclinical studies of this formulation have been conducted. In a mouse model, LEP

was shown to have had equal or superior efficacy in inhibiting tumor growth compared with paclitaxel in several tumor types.[22,26-28]

In 2008, Fetterly *et al*. reported on a phase I study of LEP-ETU.[24] A maximum tolerated dose of 325 mg/m^2 was established following the occurrence of dose-limiting toxicities in 2 separate patients (neutropenia and ataxia (sensory to neuropathy) in 1 patient each) at the 375-mg/m^2 dose level. Analysis of the pharmacokinetic data from patients treated in the extended-dosing cohort supports earlier preclinical data, which suggested that LEP-ETU and paclitaxel formulated with castor oil have comparable pharmacokinetic properties. The investigators concluded that LEP-ETU could be administered safely at higher doses than conventional paclitaxel. Modeling and simulation studies predict that LEP-ETU 325 mg/m^2 q3w will provide an acceptable rate of neutropenic events relative to those observed with conventional paclitaxel 175 mg/m^2 q3w.[24] A 275-mg/m^2 dose may offer an improved therapeutic index.[24] In addition, another clinical study of this product has been performed.[29]

In January 2005, the FDA approved albumin-bound paclitaxel for injectable suspension (trademark: Abraxane®) for the treatment of breast cancer after failure of combination chemotherapy for metastatic disease or relapse within 6 months of adjuvant chemotherapy. Our study was initiated in October 2004, just before, so we could not compare LEP-ETU with albumin-bound paclitaxel. The difference between LEP-ETU and albumin-bound paclitaxel is that LEP-ETU is a conventional (nonstabilized) nanosome, and albumin-bound paclitaxel is a nanoparticle.[30] It is unclear whether LEP-ETU has pharmacologic or cytotoxic advantages over albumin-bound paclitaxel.

To face the clinical problems of paclitaxel, 2 main strategies have been employed: 1) improving the properties of paclitaxel by a different and innovative drug formulation; and 2) adopting the classic route of medical chemistry, to obtain novel molecules with a better therapeutic index and the ability to (partly) overcome drug resistance.[31] Besides albumin-bound paclitaxel, quite a lot of different new drug formulations have been developed since 2004, with hopeful results. Also solid pharmaceutical formulations of paclitaxel were developed. Clinical studies with these novel formulations are currently ongoing.[32] However LEP-ETU is, together with paclitaxel combined with neutral and positive lipids (trademark: EndoTAG®), the only liposomal paclitaxel that has reached phase II clinical trials.[33]

Study imitations

We recognize that this study had some shortcomings. First of all, the number of patients who dropped out of the study was concerningly high. This high dropout rate was most likely due

to a poor patient selection. Second, because of some difficulties with the sponsor, it took almost 7 years to get to a publication. Nevertheless, we feel obligated to the participants in the study and to science in general to have submitted this study for publication.

Conclusions

The current randomized, two-period crossover, clinical bioequivalence study was designed to directly compare the pharmacokinetics of paclitaxel following intravenous administration of LEP-ETU (test) and paclitaxel solubilized in polyethoxylated castor oil and dehydrated alcohol (reference). Analysis of AUC and C_{max} pharmacokinetic parameters from this study has established that the test and reference formulations are bioequivalent and, based on the results of this bioequivalence study, next-phase clinical studies are planned to further develop this new liposomal paclitaxel formulation.

ACKNOWLEDGMENTS

We are thankful to the LEP-ETU PK BE study team for their contribution to the study.

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5

Marije Slingerland
Hans Gelderblom

The fate of camptothecin glycoconjugate: report of a clinical hold during a phase II study of BAY 56-3722 (formerly BAY 38-3441), in patients with recurrent or metastatic colorectal cancer resistant/refractory to irinotecan

Invest New Drugs 2012;30:1208-10.

SUMMARY

Introduction

BAY 56-3722 (formerly BAY 38-3441) is a glycoconjugated camptothecin, which was considered an attractive drug to assess in colorectal cancer (CRC).

Patients and methods

Phase II study design evaluating the antitumor activity of BAY 56-3722 i.v. 320 mg/m^2 daily for 3 days every 3 weeks in patients with recurrent or metastatic inoperable CRC resistant to irinotecan.

Results

Twenty-four patients received the study treatment. Triggered by adverse events in two other studies with this compound the study was put on a clinical hold while the safety data were reviewed for the entire program. After the review Bayer decided to withdraw BAY 56-3722 from all clinical investigations.

Discussion

We felt it was our obligation to share this interrupted phase II study for two reasons: to report the fate of camptothecin glycoconjugate and to report the unique situation of a clinical hold during a phase II study.

INTRODUCTION

Since more than a decade the topoisomerase I inhibitor irinotecan has been one of the most important drugs in the treatment of metastatic CRC although its single agent activity in second line is only 20% and its toxicity is considerable.[1] Especially in the pre-cetuximab/panitumab and bevacizumab era new camptothecin analogues with improved activity and less toxicity were therefore warranted. BAY 56-3722 (formerly BAY 38-3441) is a camptothecin glycoconjugate that generates camptothecin upon cleavage. BAY 56-3722 consists of a carbohydrate (fucose) moiety attached to the camptothecin toxophore by a peptide spacer. The camptothecin delivered from BAY 56-3722 acts by binding to and stabilizing the topoisomerase I DNA complex, leading to an accumulation of double-stranded DNA breaks upon replication, ultimately causing cell death. The lactone form is associated with its antitumor activity, whereas the carboxylate form is inactive.[2,3]

BAY 56-3722 was considered an attractive drug to assess in CRC. First, there were *in vitro* data suggesting the utility of BAY 56-3722 in a variety of CRC lines. Secondly, the two main body tissues with highest levels of radioactivity after administration of BAY 56-3722 were liver (3.0%) and the large intestine (3.6%). This could provide a potential advantage for BAY 56-3722 over other chemotherapy agents in patients with metastatic tumors in the liver. BAY 56-3722 was evaluated *in vivo* in a panel of human tumor xenografts in nude mice.[4] In most of these experiments, BAY 56-3722 was tested in comparison with doses of topotecan and not with irinotecan, which would have been more appropriate. BAY 56-3722 was more efficacious at maximum tolerated dose than topotecan and exhibited less gastrointestinal toxicity and myelosuppression. In patients BAY 56-3722 has been studied on three schedules, once every 21 days, daily for 3 days every 21 days and daily for 5 days every 21 days.[3,5,6] In the phase I study where a daily $\times$ 5 schedule is explored, there appears to be a fourfold increase in the camptothecin AUC comparing day 1 to day 5 suggesting that this schedule might be the most likely schedule to have antitumor activity.[5]

The present phase II study was designed in the beginning of this century to study the antitumor activity, safety and tolerability of BAY 56-3722 using a daily schedule for 3 days every 3 weeks.

PATIENTS AND METHODS

The study was conducted at 13 centers in Canada, the USA and the Netherlands. Patients received BAY 56-3722 i.v. over 30 min daily for 3 days every 3 weeks until objective evidence of tumor progression, unacceptable toxicity, consent withdrawn or until the investigator deemed that continuation of treatment adds no more benefit for the patient.

Tumor response measurements were made according to WHO criteria at baseline and every 6 weeks for the entire duration of treatment.[7]

The study was planned to enroll a maximum of 140 evaluable patients. A three stage enrolment procedure would be used (null hypothesis: underlying response rate is less than or equal to 10%; alternative hypothesis: true response rate is more than or equal to 20%; one-sided alpha of 0.025; power of 90%). A futility analysis was planned when 20 evaluable patients were treated and followed for tumor response for a maximum of six cycles. If none of these patients responded (no PR or CR) to therapy termination of the study was warranted. If at least one patient responded (5%), an additional 60 patients were planned to be enrolled. The second futility analysis would count the number of responders out of the 80 patients at the end of maximum six cycles: if the number of responders would be less than 10% the likelihood of success would be sufficiently low to warrant discontinuation of the study. If the number of responders would be more than 20% the regimen would be considered active and the study might be closed in preparation for phase III. Nevertheless, if 9-15 responders were obtained, additional 60 patients would be enrolled and response rate would be evaluated at the end of cycle 6 to determine if the drug was active enough to start phase III.

Adverse events were graded by the National Cancer Institute (NCI) Common Toxicity Criteria (CTC) version 2.0.[8]

Informed consent and protocol were reviewed and approved by the appropriate local ethics or review boards before study initiation.

Patients were considered eligible if they had histologically confirmed recurrent or metastatic colorectal cancer with documented progression during or within 6 months after treatment with irinotecan. Required were adequate bone marrow, renal and liver functions and signed informed consent.

RESULTS

Twenty-five patients were enrolled in this study. Twenty-four patients received at least one dose of study treatment and were therefore included in the safety evaluation. One patient did not qualify to receive study medication due to a protocol inclusion criteria violation.

Of the 24 patients in the safety population, 18 (75%) discontinued study treatment because of disease progression, 4 (17%) because of consent withdrawn, and 2 (8%) because of study termination by the sponsor. Of the four patients that withdrew consent, one withdrew it after only one dose of study drug, another one after cycle 1, a third patient due to opting for treatment with capecitabine, and the last patient due to clinical deterioration.

The futility analysis that was planned for this study after the first 20 eligible patients were enrolled could not be completed due to an initial clinical hold as well as later discontinuation of the BAY 56-3722 development program.

This study was put on a clinical hold while the safety data were reviewed for the entire BAY 56-3722 development program. This review was triggered by events in two other studies in the program. Once this review was completed, the clinical hold was removed (after 5 weeks). At the time of the clinical hold, only two patients were taken off study because of lack of the essential IRB approval to go through. At the time when the clinical hold was removed, patients had to undergo a new tumor assessment and show no disease progression in order to continue study drug treatment. Only one patient qualified; that patient received two additional cycles of treatment.

At least one treatment-emergent event was reported by 23 of the 24 patients (96%). One patient with non-insulin dependent diabetes and coagulant use experienced one episode each of grade 4 rectal bleeding and hypoglycemia. Grade 3 non-hematological adverse events were experienced by eight patients. Three patients experienced a total of four adverse events that were considered serious. Two of these events, grade 2 creatinine elevation and grade 3 renal/genitourinary-other (bilateral hydronephrosis), were considered possibly drug-related. All four serious adverse events resolved. No patients developed grade 4 hematological or biochemical toxicities. Three patients had grade 3 toxicities.

DISCUSSION

Development in systemic therapy options for CRC is moving fast. This study was conducted in the pre-cetuximab/panitumab and bevacizumab era. BAY 56-3722, selected for this phase II study, was a promising drug in diseases that were resistant to other topisomerase I inhibitors because of the enhanced stability of the active lactone moiety of the drug with enhanced preclinical antitumor activity and a favorable toxicity profile. Based on three phase I studies further phase II studies in several tumor types were undertaken with the preferred BAY 56-3722 regimen. None of these studies have been published and we felt that this was an omission. Therefore we decided to share our results and the fate of this drug in the current publication. This study was put on a clinical hold while the safety data were reviewed for the entire program, because of excessive toxicity in three patients with hepatocellular carcinoma in two studies in the program, this study not being one of them. Since, after review, this toxicity appeared to be disease related, patients were allowed to continue treatment after 4 weeks provided that there was no disease progression in our study. During the clinical hold for toxicity reasons Bayer undertook a voluntary action to withdraw camptothecin glycoconjugate (BAY 56-3722, formerly BAY 38-3441) from further clinical development due to observed safety issues, lack of therapeutic benefit, and poor enrolment in other studies. Due to this decision we were not able to draw conclusions whether this drug is active or not in colorectal cancer. Prematurely stopped studies as a result of a decision of the sponsor not to further develop a study drug (based on results in other studies) are extremely rare and the (temporary) withdrawal of the drug during the study puts the patient and the treating physician/local study team in a difficult position. The clinical hold was undertaken for safety reasons in the first place which is easier to accept than for economic reasons. We felt it was our obligation to share this interrupted phase II study for two reasons: to report the fate of camptothecin glycoconjugate and to report the unique situation of a clinical hold during a phase II study.

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PART II

New classes of anticancer drugs

6

Marije Slingerland
Henk-Jan Guchelaar
Hans Gelderblom

Histone deacetylase inhibitors: an overview of the clinical studies in solid tumors

Anticancer Drugs 2014;25:140-9.

ABSTRACT

The histone deacetylase inhibitors (HDACi) are a group of small molecules that target histone deacetylases (HDACs) by inhibiting their activity. HDACi have a long history of use in neurology and psychiatry as anti-epileptics and mood stabilizers. More recently, they have been investigated as possible treatments for cancer. HDACi have undergone rapid clinical development in recent years, on the basis of their preclinical *in vitro* and *in vivo* antitumor activity in hematological malignancies and solid tumors. Many HDACi have entered phase I-III clinical trials. Among the HDACi, vorinostat and romidepsin are currently the most extensively studied. In 2006 and 2009, respectively, they received approval by the United States Food and Drug Administration for treatment of cutaneous T-cell lymphoma and romidepsin for the treatment of peripheral T-cell lymphoma. Other HDACi, such as panobinostat and valproic acid, also demonstrated activity as therapeutic anticancer agents. In this article we give an overview of the clinical studies of HDACi in solid tumors. We start with a short description of the working mechanism of HDACi in general.

HISTONE DEACETYLASE INHIBITORS

In addition to genetic mutations, epigenetic changes play an important role in the onset and progression of cancer.[1] Epigenetic changes are defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence. Changes to the patterns of epigenetic alterations are common in cancer, and epigenetic dysregulation may be a preliminary transforming event often observed in early-stage tumors and benign neoplasms.[2,3] DNA and histones are the main compounds of nucleosomes, which are the structural units of chromatin that are important for wrapping eukaryotic DNA. Gene expression is affected by changes in the structural configuration of chromatin to a relatively open or more closed form, which alters the accessibility of DNA for transcription.[4] Transcription factor binding to DNA is mainly regulated through changes in chromatin conformation. This in turn is governed by chemical modifications such as the acetylation and deacetylation of lysine residues in the amino tails of the histones. The opposing activities of histone acetyltrans-ferases (HATs) and histone deacetylases (HDACs) tightly regulate gene expression through chromatin modification (Figure 6.1). HATs, by acetylating histones, produce an open chromatin structure, resulting in greater accessibility of regulatory proteins to DNA. HDACs, by contrast, catalyze acyl group removal, leading to a closed chromosomal configuration and transcriptional repression. Histone proteins were traditionally considered to be the primary focus for HDAC and HAT activities. However,

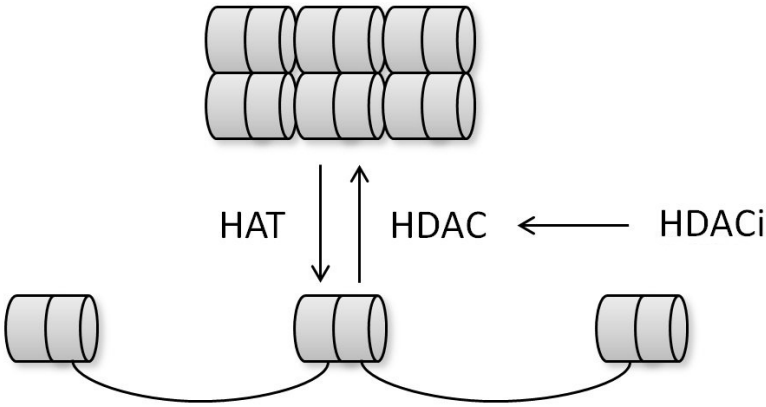


Figure 6.1 Histone acetyltransferase (HAT), histone deacetylase (HDAC), and histone deacetylase inhibitors (HDACi). The opposing activities of HATs and HDACs: HATs, by acetylating histones, produce an open chromatin structure; HDACs catalyze acyl group removal, leading to a closed chromosomal configuration.

acetylation also plays a crucial role in contexts other than histone and DNA-dependent processes. A considerable number of nonhistone proteins that play an important role in cell cycle proliferation and apoptosis are also being regulated by HAT and HDAC, for example transcription factors such as p53, E2F1, and NF-κB, which play important roles in tumor onset and antitumor response, as well as proteins that, instead of regulating gene expression, regulate the cellular cytoskeleton (α-tubulin), DNA repair (Ku70), and protein stabilization (Hsp90).[5] Hsp90, a nonhistone HDAC substrate, plays a major role in the proper wrapping and stability of several oncoproteins. HDAC activity also controls cell protein turnover through the aggresome pathway. Interference of this pathway results in the accumulation of misfolded protein aggregates, finally leading to apoptosis in tumor cells through autophagy.[6] These observations have revealed that the antitumor activity of histone deacetylase inhibitor (HDACi) includes effects on nonhistone proteins, implicated in many oncogenic pathways, in combination with epigenetic changes.

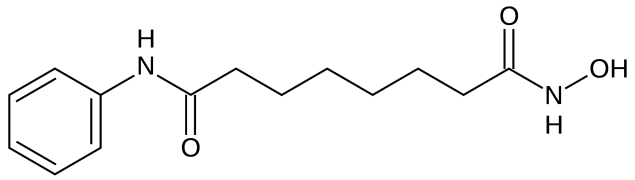
Already in 2001, Lin *et al*. stated that deregulation of HDAC activity in association with chromosomal translocated proteins is closely implicated in blocking differentiation and tumor suppressor genes, resulting in stimulating leukemogenesis.[7] The use of HDACi to reverse aberrant epigenetic changes in cancer cells, because of this important link, has emerged as a potential strategy for the treatment of solid tumors and hematological malignancies. The additional activity of deacetylases on nonhistone proteins provides HDACi with the opportunity to reverse and prevent the effects of aberrant deacetylation through epigenetic modifications and via effects on nonhistone protein targets, which are important in oncogenesis.[8,9]

CLINICAL STUDIES OF HISTONE DEACETYLASE INHIBITORS IN SOLID TUMORS

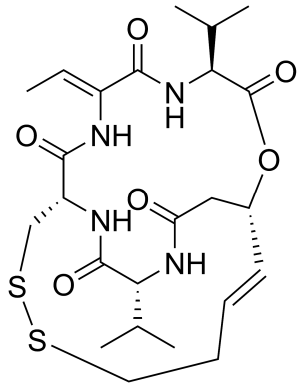
Vorinostat

Vorinostat (suberoylanilide hydroxamic acid; Zolinza®) inhibits HDAC by binding to a zinc ion in the catalytic domain of the enzyme (Figure 6.2).[10] Vorinostat demonstrated activity in murine xenograft models and it was additive or synergistic when combined with chemotherapy drugs in induction of differentiation and apoptosis of various cancer cell lines.[11] In 2006, the US Food and Drug Administration (FDA) granted regular approval to vorinostat for the treatment of cutaneous T-cell lymphoma (CTCL) in patients with progressive, persistent, or recurrent disease on or following two systemic therapies.[12]

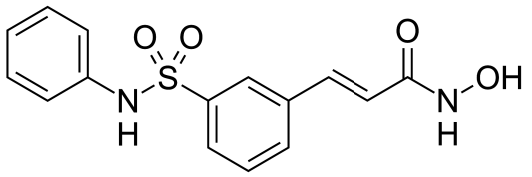
Vorinostat



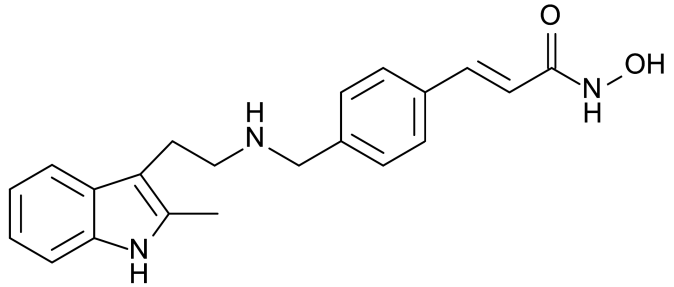
Romidepsin



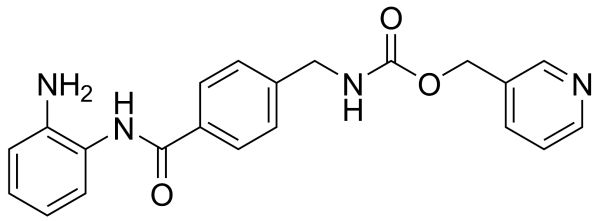
Belinostat



Panobinostat



Entinostat



Valproic acid

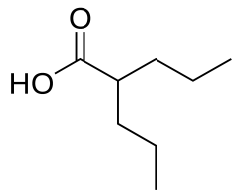


Figure 6.2 Structures of histone deacetylase inhibitors.

The pivotal study supporting approval was a single-arm open-label phase II trial.[13] An additional single-center study enrolled 33 patients with baseline and demographic features similar to the pivotal trial.[14] Despite the demonstrated effect in CTCL and other hematological tumors, unfortunately no such success has been demonstrated in solid tumors, although the phase I trials seemed rather encouraging. In two phase I studies with, respectively, intravenously and orally administered vorinostat Kelly *et al*. concluded that daily intravenous vorinostat was well tolerated, inhibited the biological target *in vivo*, and had antitumor activity in solid tumors. Oral vorinostat had linear pharmacokinetics (PK) and good bioavailability, inhibited HDAC activity in peripheral-blood mononuclear cells, could be safely administered chronically, and had a broad range of antitumor activity.[15,16] In 2007, Ramalingam *et al*. demonstrated in a phase I study that both schedules of vorinostat (400 mg orally daily 14 days or 300 mg twice daily 7 days) were tolerated well in combination with carboplatin (area under the concentration versus time curve = 6 mg/ml·min) and paclitaxel (200 mg/m^2) and that encouraging anticancer activity was noted in patients with previously untreated non-small cell lung cancer (NSCLC).[17] On the basis of these results, Vansteenkiste *et al*. conducted an early phase II trial of oral vorinostat in relapsed or refractory breast cancer, colorectal cancer, and NSCLC.[18] Sixteen patients (median age, 62 years; median 5.5 prior therapies) were enrolled. Six patients received 400 mg twice daily, six received 300 mg twice daily, and four received 200 mg twice daily (14 days/3 weeks). Dose-limiting toxicities (DLTs) at the 400 or 300 mg twice daily level were anorexia, asthenia, nausea, thrombocytopenia, vomiting, and weight loss. No DLTs were observed at the 200 mg twice daily level. Disease stabilization was observed in eight (50%) patients, but there were no confirmed responses. The median time to progression was only 33.5 days. Eleven patients (69%) discontinued because of clinical adverse events (AEs). The most common drug-related AEs were anorexia (81%), fatigue (62%), nausea (62%), diarrhea (56%), vomiting (56%), thrombocytopenia (50%), and weight loss (50%). Drug-related AEs of at least grade 3 included thrombocytopenia (50%), anemia (12%), asthenia (12%), and nausea (12%). They concluded that vorinostat on a daily oral schedule for 14 days/3 weeks was tolerable at 200 mg twice daily only, but that no responses were observed in this study because most patients had limited drug exposure, which did not allow a reliable efficacy analysis. In 2009, Woyach *et al*. could also not demonstrate a therapeutic effect of vorinostat in patients with metastatic radioiodine-refractory thyroid carcinoma.[19] Also in a phase II trial by Luu *et al*. in 2008 in metastatic breast cancer patients, vorinostat did not show adequate single-agent activity.[20] Other phase II trials with vorinostat in patients with recurrent head and/or metastatic head and neck cancer, respectively, by Blumenschein *et al*., recurrent platinum-refractory ovarian or primary peritoneal carcinoma by Modesitt *et*

al., relapsed NSCLC by Traynor *et al*., and recurrent glioblastoma multiforme by Galanis *et al*. all showed limited to no activity.[21-24] Study results with vorinostat in combination with, respectively, 5-fluorouracil/leucovorin in refractory colorectal cancer and bortezomib in recurrent glioblastoma were also disappointing.[25,26] However, in 2009 Ramalingam *et al*. published a phase II randomized, double-blind, placebo-controlled study evaluating the efficacy of vorinostat in combination with carboplatin and paclitaxel in patients with advanced-stage NSCLC.[27] Ninety-four patients initiated protocol therapy. The confirmed response rate was 34% with vorinostat versus 12.5% with placebo ($P = 0.02$). There was a trend toward improvement in median progression-free survival (PFS) (6.0 versus 4.1 months; $P = 0.48$) and overall survival (OS) (13.0 versus 9.7 months; $P = 0.17$) in the vorinostat arm. Grade 4 platelet toxicity was more common with vorinostat (18 versus 3%; $P < 0.05$). Nausea, emesis, fatigue, dehydration, and hyponatremia were also more frequent with vorinostat. In 2011, Munster *et al*. published their phase II trial of vorinostat combined with tamoxifen for the treatment of patients with hormone therapy-resistant breast cancer, which showed that this combination was well tolerated and exhibits encouraging activity in reversing hormone resistance.[28] Forty-three patients (median age 56 years (31-71)) were treated. Twenty-five patients (58%) received prior adjuvant tamoxifen, 29 (67%) failed one prior chemotherapy regimen, 42 (98%) progressed after one, and 23 (54%) after two aromatase inhibitors. The objective response rate by Response Evaluation Criteria In Solid Tumors (RECIST) criteria was 19% and the clinical benefit rate (response or stable disease (SD) > 24 weeks) was 40%. The median response duration was 10.3 months (confidence interval (CI): 8.1-12.4).

Romidepsin

Romidepsin (depsipeptide; Istodax®) acts as a prodrug with the disulfide bond undergoing reduction within the cell to release a zinc-binding thiol (Figure 6.2).[29-31] The thiol reversibly interacts with a zinc atom in the binding pocket of zinc-dependent HDAC to lock its activity. Romidepsin was licensed by the US FDA in 2009 for CTCL on the basis of two phase II trials conducted in a total of 167 patients suffering from relapsed, refractory, or advanced CTCL.[32,33] In 2011, romidepsin was also approved by the US FDA for peripheral T-cell lymphoma (PTCL) on the basis of the results from two studies: a phase II multicenter international open-label single-arm study in patients with PTCL who had failed at least one prior systemic therapy, which was presented at the 2010 American Society of Hematology annual meeting; and a single-arm clinical study in patients with PTCL who had failed prior therapy.[34,35] A series of phase I and phase II trials of romidepsin were conducted in patients with solid tumors, all with disappointing results. In 2002 Sandor *et al*. conducted a

phase I trial in patients with refractory neoplasms.[36] DLT was observed, and the maximum tolerated dose (MTD) exceeded 24.9 mg/m^2. The DLTs included grade 3 fatigue (three patients), grade 3 nausea and vomiting (one patient), grade 4 thrombocytopenia (two patients), and grade 4 cardiac arrhythmia (one patient, atrial fibrillation). The MTD was defined at the seventh dose level (17.8 mg/m^2). Reversible ST/T changes and mild reversible dysrhythmias were observed on the post-treatment electrocardiogram (ECG). There were no clinically significant changes in left ventricular ejection fraction. One patient with renal cell carcinoma (RCC) achieved a partial response (PR). Because of the refractory nature of metastatic human RCC and to follow up on this anecdotal response observed in the phase I studies, a single-arm, phase II, multi-institutional study was conducted to assess the antitumor activity of romidepsin in metastatic RCC.[37] The 29 evaluable patients, who were accrued so that 25 patients who received at least three doses of romidepsin could be observed, were heavily pretreated with a median of two previous systemic therapies and a 2-year median duration of metastatic disease. Twenty-four patients (83%) had clear-cell histology. The most common serious toxicities were fatigue, nausea, vomiting, and anemia. Two patients developed a prolonged QT_c interval, one patient each developed grade 3 atrial fibrillation and tachycardia, and there was one sudden death. Two patients experienced an objective response (one complete response (CR)) for an overall response rate (ORR) of 7% (95% CI: 0.8-23%). Schrump *et al*. could also not observe any objective responses in their phase II trial of romidepsin in lung cancer patients.[38] In this trial 19 patients were evaluable for toxicity assessment; 18 were evaluable for treatment response. Myelosuppression was dose-limiting in one individual. No significant cardiac toxicities were observed. In colorectal cancer patients romidepsin also seemed not to be effective. Whitehead *et al*. included 28 patients with previously treated colorectal cancer with advanced disease in a phase II trial of romidepsin, two of whom were ineligible.[39] One eligible patient refused all treatment and was not analyzed. For the 25 remaining patients, performance status was 0 in 16 patients and 1 in nine patients. Ten patients had received one prior chemotherapy regimen and 15 two prior regimens. Out of the 25 eligible and analyzable patients accrued in the first stage of the protocol, no objective responses were observed and the study was permanently closed. Four patients had SD as the best response. Twenty-five patients were assessed for toxicity. No grade 4 or greater toxicities were seen. Fourteen of the 25 patients experienced grade 3 toxicities, the most common of which were fatigue and anorexia. Molife *et al*. found minimal antitumor activity in chemotherapy-naive patients with castration-resistant prostate cancer in their phase II trial with romidepsin.[40] Thirty-five patients were enrolled in this study. Two patients achieved a confirmed radiological PR (RECIST) lasting for at least 6 months, along with a confirmed prostate-specific antigen decline of at least 50%. Eleven

patients experienced toxicity necessitating early discontinuation. The commonest AEs were nausea (30 patients; 85.7%), fatigue (28 patients; 80.0%), vomiting (23 patients; 65.7%), and anorexia (20 patients; 57.1%). There was no significant cardiac toxicity. In 2010 Otterson *et al*. published the results of their phase II trial of romidepsin in chemosensitive recurrent small cell lung cancer (SCLC).[41] Sixteen patients (10 male, six female) were accrued to the first stage of this study. Most (11 patients, 69%) presented with extensive-stage SCLC, and all had received prior chemotherapy, with 11 having received prior radiation. Eastern Cooperative Oncology Group performance status was excellent with 0 in six patients (38%) and 1 in 10 patients. No objective responses were seen, and SD was the best response seen in three patients (19%). Toxicity was modest with three patients suffering grade 3 toxicity (lymphopenia, insomnia, nausea, vomiting, and hyponatremia) and one patient suffering grade 4 thrombocytopenia. Median PFS was 1.8 months, and median OS was 6 months. They concluded that romidepsin given on a weekly schedule in patients with chemosensitive, recurrent SCLC was inactive. Iwamoto *et al*. found in their phase I/II trial that romidepsin was also ineffective for patients with recurrent glioblastomas.[42] Two dose cohorts were studied in the phase I component of the trial (13.3 and 17.7 mg/m^2/day). Patients in the phase II component were treated with intravenous romidepsin at a dosage of 13.3 mg/m^2/day on days 1, 8, and 15 of each 28-day cycle. Eight patients were treated in the phase I component. A similar romidepsin PK profile was demonstrated between patients receiving enzyme-inducing anti-epileptic drugs and those not receiving enzyme-inducing anti-epileptic drugs. Thirty-five patients with glioblastoma were accrued to the phase II component. There was no objective radiographic response. The median PFS was 8 weeks and only one patient had a PFS time of at least 6 months (PFS6 = 3%). At publication, 34 patients (97%) had died, with a median survival duration of 34 weeks. In 2012 Jones *et al*. published the results of their phase I trial that was conducted to determine the MTD for two schedules of romidepsin plus gemcitabine in patients with advanced solid tumors in which gemcitabine had previously demonstrated clinical activity.[43] The recommended phase II dose was 12 mg/m^2 romidepsin plus 800 mg/m^2 gemcitabine on days 1 and 15 every 28 days. They concluded that the results suggested additive hematologic toxicities of romidepsin plus gemcitabine, but the level of antitumor activity observed warranted more formal trials of this combination to further assess safety and efficacy. Also in 2012, Sherman *et al*. published their single-institution Simon two-stage phase II clinical study to evaluate the clinical activity of romidepsin and radioactive iodine (RAI) re-uptake in RAI-refractory thyroid carcinoma.[44] They observed preliminary signs of *in vivo* reversal of RAI resistance after treatment with romidepsin. However, no major responses were observed and accrual was poor after a grade 5 AE. Haigentz *et al*. conducted a phase II

trial in patients with advanced squamous cell carcinoma of the head and neck.[45] Objective responses were not observed, although two heavily pretreated patients had brief clinical disease stabilization. Observed toxicities were expected, including frequent severe fatigue.

Belinostat

Belinostat (PXD101) is a hydroxamic acid HDACi with anti-proliferative and HDAC inhibitory activities *in vitro* (Figure 6.2).[46] Belinostat has growth inhibitory and pro-apoptotic activities in a variety of human tumor cell lines at nanomolar concentrations. *In vivo*, belinostat inhibits growth in human tumor xenografts without apparent toxicity to the host mice.[46] Growth inhibition *in vitro* and *in vivo* is associated with a marked increase in the level of acetylation of histone proteins.[46]

In 2008, Steele *et al*. conducted a phase I study to determine the safety, DLT, MTD dose, and PK and pharmacodynamic profiles of belinostat in patients with advanced refractory solid tumors. Forty-six patients received belinostat at one of six dose levels (150-1200 mg/m^2/day). DLTs were grade 3 fatigue (one patient at 600 mg/m^2; one patient at 1200 mg/m^2), grade 3 diarrhea combined with fatigue (one patient at 1200 g/m^2), grade 3 atrial fibrillation (one patient at 1200 mg/m^2; one patient at 1000 mg/m^2), and grade 2 nausea/ vomiting leading to inability to complete a full 5-day cycle (two patients at 1000 mg/m^2). The MTD was 1000 mg/m^2/day. SD was observed in a total of 18 (39%) patients, including 15 treated for at least four cycles. Of the 24 patients treated at the MTD (1000 mg/m^2/day), 50% achieved SD.[47] Lassen *et al*. showed in their phase I trial that the combination of belinostat and carboplatin and/or paclitaxel in patients with solid tumors was well tolerated, with no evidence of PK interaction. The MTD of belinostat was 1000 mg/m^2/day for days 1-5, with paclitaxel 175 mg/m^2 and carboplatin area under the curve (AUC) 5 administered on day 3. Grade 3/4 AEs were (*n*; %): leucopenia (5; 22%), neutropenia (7; 30%), thrombocytopenia (3; 13%) anemia (1; 4%), peripheral sensory neuropathy (2; 9%), fatigue (1; 4%), vomiting (1; 4%), and myalgia (1; 4%). The PK of belinostat, paclitaxel, and carboplatin were unaltered by the concurrent administration. There were two PRs (one rectal cancer and one pancreatic cancer). A third patient (mixed mullerian tumor of ovarian origin) showed a complete cancer antigen-125 response. In addition, six patients showed an SD lasting for at least 6 months.[48] In 2009, Ramalingam *et al*. concluded in a phase II study that belinostat was not active as monotherapy against recurrent malignant pleural mesothelioma.[49] Other phase II trials could only demonstrate limited activity.[50-52] However, in 2012 Dizon *et al*. demonstrated that belinostat, carboplatin, and paclitaxel combined (BelCaP) was reasonably well tolerated and

demonstrated clinical benefit in heavily pretreated patients with epithelial ovarian cancer. Thirty-five women were treated. The median age was 60 years (range, 39-80 years), and patients had received a median of three prior regimens (range, 1-4). Fifty-four percent had received more than two prior platinum-based combinations; 16 patients (46%) had primary platinum-resistant disease, whereas 19 patients (54%) recurred within 6 months of their most recent platinum treatment. The median number of cycles of BelCaP administered was 6 (range, 1-23). Three patients had a CR, and 12 had a PR, for an ORR of 43% (95% CI: 26-61%). When stratified by primary platinum status, the ORR was 44% among resistant patients and 63% among sensitive patients. The most common drug-related AEs related to BelCaP were nausea (83%), fatigue (74%), vomiting (63%), alopecia (57%), and diarrhea (37%). With a median follow-up of 4 months (range, 0-23.3 months), 6-month PFS is 48% (95% CI: 31-66%). Median OS was not reached during study follow-up.[53]

Panobinostat

Panobinostat (LBH589) is a hydroxamic acid and acts as a non-selective HDACi (Figure 6.2). In 2010 the first phase I trial was published by Rathkopf *et al*. In this phase I trial 16 patients with castration-resistant prostate cancer were included. In arm I, oral panobinostat (20 mg) was administered on days 1, 3, and 5 for 2 consecutive weeks followed by a 1-week break. In arm II, oral panobinostat (15 mg) was administered on the same schedule in combination with docetaxel 75 mg/m^2 every 21 days. DLTs were grade 3 dyspnea (arm I) and grade 3 neutropenia greater than 7 days (arm II). In arm I, all patients developed progressive disease despite accumulation of acetylated histones in peripheral-blood mononuclear cells. In arm II, five of eight patients (63%) had at least a 50% decline in prostate-specific antigen, including one patient whose disease had previously progressed on docetaxel.[54] In 2011 Jones *et al*. showed in their phase I trial that the combination of panobinostat and gemcitabine was limited by myelosuppression. The recommended doses for further study were intermittent oral panobinostat administered at a dose of 10 mg three times weekly for 2 weeks in combination with gemcitabine 800 mg/m^2 administered intravenously on days 1 and 8 every 21 days.[55] Fukutomi *et al*. concluded in 2012, in their phase I trial, that panobinostat administered orally once daily on Monday, Wednesday, and Friday of each week was well tolerated at doses up to 20 mg in Japanese patients. Dose escalation did not proceed after exploration of the 20 mg dose due to emerging global clinical data at that time.[56] Morita *et al*. reported a phase I study to evaluate intravenous panobinostat given on days 1 and 8 of a 21-day cycle in Japanese patients with solid tumors. They concluded that the MTD was 20 mg/m^2.[57] Drappatz *et al*. concluded in their phase I study of panobinostat

in combination with bevacizumab for recurrent high-grade glioma that the recommended doses for further study are oral panobinostat 30 mg three times per week, every other week, in combination with bevacizumab 10 mg/kg every other week.[58] However, in 2012 Strickler *et al*. concluded in their phase I trial that adding everolimus to panobinostat and bevacizumab did not have an acceptable safety and tolerability profile.[59] DLTs in cohort 1 included grade 2 esophagitis and grade 3 oral mucositis; DLTs in cohort 1 were grade 2 ventricular arrhythmia and grade 2 intolerable skin rash. Common AEs were diarrhea (50%), headache (33%), mucositis/stomatitis (25%), hyperlipidemia (25%), and thrombocytopenia (25%). In a phase I trial Jones *et al*. investigated panobinostat in combination with paclitaxel and carboplatin in patients with solid tumors. They concluded that the recommended phase II dose is panobinostat 10 mg orally three times weekly in combination with paclitaxel 175 mg/m^2 and carboplatin AUC 5 administered intravenously on day 1 of every 21-day cycle.[60] Unfortunately, the phase II results of panobinostat were very disappointing. Hainsworth *et al*. concluded that panobinostat had no activity in patients with refractory renal carcinoma and Wang *et al*. could not support the treatment of advanced pancreatic cancer with bortezomib in combination with panobinostat in their clinical study.[61-62]

Entinostat

Entinostat (MS-275) is a benzamide derivative with potent HDAC inhibitory and antitumor activity in preclinical models (Figure 6.2). Several phase I trials have been performed since 2005. Ryan *et al*. conducted a phase I study that demonstrated that the entinostat oral formulation on the daily schedule (once daily 28 every 6 weeks (daily), starting dose 2 mg/m^2) was intolerable at the dose and schedule explored. The q14-day schedule was reasonably well tolerated. DLTs were nausea, vomiting, anorexia, and fatigue.[63] In 2007, Kummar *et al*. showed that entinostat was well tolerated at a dose of 6 mg/m^2 administered weekly with food for 4 weeks every 6 weeks. No grade 4 toxicities were observed. Grade 3 toxicities were reversible and consisted of hypophosphatemia, hyponatremia, and hypoalbuminemia.[64] Gore *et al*. showed that entinostat was well tolerated at doses up to 6 mg/m^2 every other week or 4 mg/m^2 weekly for 3 weeks followed by 1 week of rest and resulted in biologically relevant plasma concentrations and antitumor activity. Twice-weekly dosing was not tolerable due to asthenia, and further evaluation of this schedule was halted. The recommended dose for further disease-focused studies is 4 mg/m^2 given weekly for 3 weeks every 28 days or 2-6 mg/m^2 given once every other week.[65] Another phase I trial showed that the combination of entinostat and 13-cis retinoic acid was reasonably well tolerated. The recommended phase II doses are entinostat 4 mg/m^2 once weekly and 13-cis retinoic acid

1 mg/kg/day. Grade 3 toxicity included hyponatremia, neutropenia, and anemia. Fatigue grade 1 and 2 was a common side effect.[66] Unfortunately, the limited phase II results were rather disappointing: no objective responses in pretreated metastatic melanoma and no improvement in the outcomes of patients with advanced NSCLC treated with erlotinib combined with entinostat when compared with erlotinib monotherapy.[67,68] However, in 2011 Juergens *et al*. published their phase I/II trial of combined epigenetic therapy with azacitidine, inhibitors of DNA methylation, and entinostat in extensively pretreated patients with recurrent metastatic NSCLC. This therapy was well tolerated and objective responses were observed, including a CR and a PR in a patient who remains alive and without disease progression approximately 2 years after completing protocol therapy. Median survival in the entire cohort was 6.4 months (95% CI: 3.8-9.2), comparing favorably with existing therapeutic options. Demethylation of a set of four epigenetically silenced genes known to be associated with lung cancer was detectable in serial blood samples in these patients and was associated with improved PFS ($P = 0.034$) and OS ($P = 0.035$). Four of 19 patients had major objective responses to subsequent anticancer therapies given immediately after epigenetic therapy.[69]

Valproic acid

Valproic acid (divalproex sodium; Depakote®) relieves repression of transcription factors that recruit HDACs and activates transcription from diverse promoters (Figure 6.2). Valproic acid causes hyperacetylation of the N-terminal tails of histones H3 and H4 *in vitro* and *in vivo* and it inhibits HDAC activity, most probably by binding to the catalytic center and thereby blocking substrate access.[70,71] In 2005, Chavez-Blanco *et al*. published their phase I study titled 'Histone acetylation and histone deacetylase activity of magnesium valproate in tumor and peripheral blood of patients with cervical cancer'. Twelve newly diagnosed patients with cervical cancer were treated with magnesium valproate after a baseline tumor biopsy and blood sampling at the following dose levels (four patients each): 20, 30, or 40 mg/kg for 5 days through the oral route. On day 6, tumor and blood sampling were repeated and the study protocol ended. Tumor acetylation of H3 and H4 histones and HDAC activity were evaluated by western blot and colorimetric HDAC assay, respectively. Plasma levels of valproic acid were determined on day 6 once the steady state was reached. Toxicity of treatment was evaluated at the end of the study period. All patients completed the study medication. Mean daily dose for all patients was 1890 mg. Corresponding means for the doses 20, 30, and 40 mg/kg were 1245, 2000, and 2425 mg, respectively. Depressed level of consciousness grade 2 was registered in nine patients. Ten patients were evaluated for

H3 and H4 acetylation and HDAC activity. After treatment, we observed hyperacetylation of H3 and H4 in the tumors of nine and seven patients, respectively, whereas six patients demonstrated hyperacetylation of both histones. Plasma levels of valproic acid ranged from 73.6 to 170.49 mg/ml. Tumor deacetylase activity decreased in eight patients (80%), whereas two had either no change or a mild increase. There was a statistically significant difference between pretreatment and post-treatment values of HDAC activity (mean, 0.36 versus 0.21, two-tailed *T*-test $P < 0.0264$). There was no correlation between H3 and H4 tumor hyperacetylation with plasma levels of valproic acid. It was concluded that magnesium valproate at a dose between 20 and 40 mg/kg inhibits deacetylase activity and hyperacetylates histones in tumor tissues.[72] Arce *et al*. demonstrated in their proof-of-principle study that treatment with hydralazine and magnesium valproate exerts its proposed molecular effects of DNA demethylation, HDAC inhibition, and gene reactivation in primary tumors of patients with breast cancer. Importantly, this doxorubicin-associated and cyclophosphamide-associated treatment was safe and well tolerated, and appeared to increase the efficacy of chemotherapy.[73] Several phase I studies of valproic acid alone or in combination with another agent were performed: valproic acid followed by the topoisomerase II inhibitor epirubicin in advanced solid tumors, alone in patients with refractory advanced cancer, in combination with 5-azacytidine in patients with advanced cancers, in combination with all-trans-retinoic acid intravenously in patients with advanced solid tumor malignancies, and in combination with 5-aza-20-deoxycytidine (decitabine) in patients with advanced-stage NSCLC.[74-78] Some phase II trials were also performed. Candelaria *et al*. conducted a phase II study in 17 patients who were evaluable for toxicity and 15 for response. Primary sites included were cervix (three), breast (three), lung (one), testis (one), and ovarian (seven) carcinomas. A clinical benefit was observed in 12 (80%) patients: four PR and eight SD. The most significant toxicity was hematologic. Reductions in global DNA methylation, HDAC activity, and promoter demethylation were observed.[79] The combination of valproic acid and chemoimmunotherapy did not produce results overtly superior to standard therapy in patients with advanced melanoma.[80] In combination with karenitecin, a topoisomerase I inhibitor, valproic acid was associated with disease stabilization in 47% of patients with metastatic poor prognosis melanoma.[81] Scherpereel *et al*. demonstrated that valproic acid plus doxorubicin appeared to be an effective chemotherapy regimen in good performance score (80-100) patients with refractory or recurrent mesothelioma, for which no standard therapy was available.[82] The pilot phase II study by Mohammed *et al*. showed that valproic acid may have a role in treating low-grade neuroendocrine carcinoma.[83] However, in 2011 Coronel *et al*. published their randomized phase III, placebo-controlled study of hydralazine and valproate (HV) added to cisplatin-

topotecan in advanced cervical cancer. This study represents the first randomized clinical trial to demonstrate a significant advantage in PFS for epigenetic therapy over one of the current standard combination chemotherapies in cervical cancer. Patients received hydralazine at 182 mg for rapid or 83 mg for slow acetylators, and valproate at 30 mg/kg, beginning a week before chemotherapy and continuing until disease progression. Response, toxicity, and PFS were evaluated, and 36 patients (17 cisplatin topotecan (CT) plus HV and 19 CT plus placebo (PLA)) were included. The median number of cycles was 6. There were four PRs to CT + HV and one in CT + PLA. There was SD in five (29%) and six (32%) patients, respectively, whereas eight (47%) and 12 (63%) showed progression (P = 0.27). At a median follow-up time of 7 months (1-22), the median PFS is 6 months for CT + PLA and 10 months for CT + HV (P = 0.0384, two tailed).[84]

Mocetinostat, chidamide, SB939, and LAQ824

Some other HDACi were only studied in single phase I studies, for example mocetinostat (MGCD0103), chidamide (CS055/HBI-8000), SB939, and LAQ824.[85-89] The recommended phase II dose of mocetinostat was 45 mg/m^2/day. DLTs consisting of fatigue, nausea, vomiting, anorexia, and dehydration were observed in three (27%) of 11 and two (67%) of three patients treated at the 45 and 56 mg/m^2/day dose levels, respectively.[85] With chidamide no DLTs were identified in the two times per week for 4 consecutive weeks every 6-week cohorts up to 50 mg. DLTs were grade 3 diarrhea and vomiting in two patients in the three times per week for 4 consecutive weeks every 6-week cohort at 50 mg, respectively.[86] In a phase I study by Yong *et al*. the MTD of SB939 was 80 mg/day. DLTs were fatigue, hypokalemia, troponin T elevation, and QT_c prolongation.[87] Razak *et al*. demonstrated that the recommended phase II dose of SB939 was 60 mg given for 5 consecutive days every 2 weeks. The most frequent non-hematologic AEs of at least possible attribution to SB939 were fatigue, nausea, vomiting, anorexia, and diarrhea.[88] DLTs of LAQ824 were transaminitis, fatigue, atrial fibrillation, raised serum creatinine, and hyperbilirubinemia. On the basis of these data in the phase I trial, De Bono *et al*. concluded that future efficacy trials with LAQ824 should evaluate doses ranging from 24 to 72 mg/m^2.[89]

DISCUSSION

Despite promising results in the treatment of CTCL, HDACi have generally not been effective in clinical trials involving solid tumors. Many clinical trials have assessed the efficacy of vorinostat against different solid tumors, including refractory breast, colorectal, non-small cell lung, and thyroid cancers. Disappointingly, almost none of the patients in these trials showed PR or CR to treatment, but the prevalence of drug-induced side effects was very high.[18,19] Romidepsin has also been evaluated as a monotherapy against solid tumors. Similarly to vorinostat, romidepsin has also been ineffective against solid tumors and also induced serious side effects. Before its approval by the FDA, there were six cases of unexpected death in patients treated with romidepsin, one attributed to pulmonary embolus and the other five cases attributed to sudden cardiac arrest.[90,91]

The same disappointing results were found with studies of belinostat, panobinostat, and entinostat in solid tumors. Valproic acid is the only HDACi that completed a phase III trial in solid tumors, which demonstrated a significant advantage in PFS over one of the current standard combination chemotherapies in cervical cancer; however, the results were preliminary and should be taken as such. Current published studies indicate that so far HDACi have serious limitations, including ineffectively low concentrations in solid tumors and cardiac toxicity, including T-wave flattening, ST segment depression, and QT interval prolongation, which is hindering their progress in the clinic.[92] Although it is not completely understood why HDACi seem more effective in hematological malignancies than in solid tumors, it is suggested that in hematological malignancies, such as CTCL and multiple myeloma, the short PK half-life of HDACi compounds may not preclude their effectiveness, compared with less permeable solid tumors, in which their instability is a problem.[57] It is also possible that HDACi are not selective enough for solid tumors, which means that they are not target specific and are not delivered selectively. An interesting question is whether HDAC expression in a given tumor might predict the therapeutic response to HDACi. As in other targeted therapies, it is probable that treatment response is greater in those patients who strongly express HDACs in their cancer cells. Translational studies including this topic should be attached to clinical trials on HDACi to find adequate biomarkers for the future. The hope of up-and-coming cancer treatments of all kinds is to deliver high potency at the site of action, while eliminating the toxicities that result from off-target effects. Gryder *et al*. recently suggested that designing and developing HDACi with extremely high potency and selectivity for a unique molecular entity and not others and directing the medicine to the location of interest would help to overcome the problems

Table 6.1 Open clinical trials (with histone deacetylase inhibitors in solid tumors) recruiting patients

Title	Phase	ClinicalTrials.gov Identifier
Safety and tolerability study of RAD001 and LBH589 in all solid tumors with enrichment for EBV driven tumors	1	NCT01341834
Belinostat for solid tumors and lymphomas in patients with varying degrees of hepatic dysfunction	1	NCT01273155
Azacitidine and MS-275 in treating patients with recurrent advanced non-small cell lung cancer	1/2	NCT00387465
High-dose vorinostat and fractionated stereotactic body radiation therapy in treating patients with recurrent glioma	1	NCT01378481
A phase I study of belinostat in combination with cisplatin and etoposide in adults with small cell lung carcinoma and other advanced cancers	1	NCT00926640
Vorinostat in children	1/2	NCT01422499
High-dose or low-dose vorinostat in combination with carboplatin or paclitaxel in treating patients with advanced solid tumors	1	NCT01281176
Clinical study of vorinostat in combination with etoposide in pediatric patients < 21 years at diagnosis with refractory solid tumors	1/2	NCT01294670
Vorinostat and lapatinib in advanced solid tumors and advanced breast cancer to evaluate response and biomarkers	2	NCT01118975
Adjuvant valproate for high grade sarcomas	1	NCT01010958
Sorafenib and LBH589 in hepatocellular carcinoma (HCC)	1	NCT00823290
Study to evaluate panobinostat (DACi) pharmacokinetics and safety in solid tumors and varying renal function	1	NCT00997399

of HDACi in solid tumors.[92] While searching for 'HDAC inhibitor solid tumor' we found only 12 open clinical trials on http://www.clinicaltrials.gov recruiting patients (Table 6.1). But to fulfill the high expectations in solid tumors and to overcome the existing problems, a great deal of research is still necessary.

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Marije Slingerland
Dagmar Hess
Sally Clive
Sunil Sharma
Per Sandström
Niklas Loman
Maria Grazia Porro
Song Mu
Edward Waldron
Sue-zette Valera
Hans Gelderblom

A phase I, open-label, multicenter study to evaluate the pharmacokinetics and safety of oral panobinostat in patients with advanced solid tumors and various degrees of hepatic function

Submitted.

ABSTRACT

Purpose

To evaluate the pharmacokinetics and safety of oral panobinostat in patients with advanced solid tumors and varying degrees of hepatic function.

Patients and methods

Patients with advanced solid malignancies, acceptable bone marrow and renal function, and normal (control group) or impaired hepatic function, per NCI-ODWG criteria, were eligible. All patients received a single oral dose of 30 mg panobinostat for pharmacokinetic studies lasting 1 week (core phase). Subsequently, patients received three times weekly panobinostat for as long as the patient had benefit (extension phase safety assessment). Core phase serial blood samples were collected predose and over 96 hours postdose and assayed for panobinostat.

Results

Twenty-five patients were enrolled with a median age of 58 years, (range 45-76). Fifteen patients had hepatic dysfunction (8 mild, 6 moderate and 1 severe). Approximate reduction in plasma panobinostat clearance was 30% and 51%, with concomitant 43% and 105% increase in exposure, for patients with mild and moderate hepatic dysfunction respectively. Median peak plasma concentrations were 1.4 and 1.8 fold higher in the mild and moderate groups as compared to those in the normal group. Hepatic impairment did not alter panobinostat absorption with T_{max} unchanged at 2 hours. The safety data were consistent with known safety profile of panobinostat in patients with advanced cancers and normal liver function.

Conclusion

Despite increased plasma exposure, patients with mild or moderate hepatic dysfunction could be safely treated with the same dose of panobinostat as patients with normal hepatic function.

INTRODUCTION

Panobinostat is a potent pan-deacetylase inhibitor (pan-DACi) with low nanomolar activity against all class I, II, and IV histone deacetylase enzymes.[1,2] This activity is exerted by direct inhibition of histone deacetylases, modulating both histone and nonhistone proteins that regulate various cell signaling pathways.[3-7] Panobinostat has shown preclinical and clinical activity as a single agent and in combination with other chemotherapeutic agents in multiple tumor types.[8-13] The common toxicities associated with panobinostat include fatigue, thrombocytopenia, nausea, vomiting, and diarrhea. The disposition, metabolism, and excretion of panobinostat were studied in advanced cancer patients via trace radiolabeled ^{14}C material. These studies indicate that both liver and kidney are involved in the metabolism and elimination of the parent compound.[14] Panobinostat and its numerous inactive metabolites are excreted almost equally in bile/feces (44-77% of the dose) and urine (29-51% of the dose) of patients. The elimination is primarily in the form of metabolites with unchanged panobinostat in urine accounting for less than 2.5% and in feces for less than 3.5% of the dose.[14] To date, the safety and pharmacokinetics (PK) of panobinostat have been characterized in cancer patients with adequate hepatic function and no data are available in patients with hepatic dysfunction. Hepatic dysfunction, either as result of metastatic invasion, or as a pre-existing medical condition, is frequently observed in cancer patients necessitating dose adjustments to avoid toxicity.

Therefore, we conducted a phase I open-label multicenter study to evaluate the pharmacokinetics (PK) and safety of oral panobinostat in cancer patients with varying degrees of hepatic impairment.

PATIENTS AND METHODS

Study design

Eligible patients were stratified by the degree of hepatic dysfunction. The National Cancer Institute (NCI), Organ Dysfunction Working Group (ODWG) criteria[15] for classifying hepatic dysfunction as normal, mild, moderate and severe, based on serum bilirubin and AST (aspartate transaminase) levels are given in Table 7.1. The sample size was based on FDA guidance for the industry with planned 22-28 evaluable patients dosed in the PK study.[16]

There were two parts to the study. Part 1 (core phase) evaluated the PK of panobinostat in each hepatic function group after a single, 30 mg oral dose with food. Blood sampling was

Table 7.1 Definition of hepatic function groups and planned dose levels scheme in the study part 2 (extension phase)

	NCI-ODWG hepatic function/impairment group			
	Normal	**Mild**	**Moderate**	**Severe**
Bilirubin level	≤ ULN	≤ ULN > 1.0-1.5 ULN	> 1.5-3 ULN	> 3 ULN
AST level	≤ ULN	AST > ULN Any AST	Any AST	Any AST
Dose level	**Panobinostat dosing schedule**			
Starting dose	30 mg TIW QW	30 mg TIW QW	30 mg TIW QW	30 mg TIW QOW
Dose level -1	30 mg TIW QOW	30 mg TIW QOW	30 mg TIW QOW	20 mg TIW QOW
Dose Level -2	20 mg TIW QOW	20 mg TIWQOW	20 mg TIW QOW	

AST: aspartate aminotransferase; ULN: upper limit of normal; TIW: three times a week; QW: weekly; QOW: every other week.

carried out predose and over 96 hours postdose. Part 2 (extension phase) was initiated 7 days after start of core phase to characterize the safety profile of panobinostat. Panobinostat 30 mg/day was administered three times a week weekly, or every other week, depending on the patient's degree of hepatic dysfunction. In patients with severe liver dysfunction, a lower starting dose of 20 mg panobinostat three times a week every other week was also considered. Treatment cycles were repeated every 28 days (Table 7.1).

Treatment was continued until disease progression, unacceptable toxicity or withdrawal of informed consent. Initially, patients with normal hepatic function and mild or moderate hepatic dysfunction were enrolled in the study. A decision to enroll patients with severe hepatic impairment was made following review of the preliminary safety data of all patients who completed the core phase and cycle 1 of the extension phase, of which at least 3 patients were from the moderate hepatic dysfunction group.

Eligibility criteria

Patients with normal or abnormal liver function (including those with liver metastases and presence of biliary shunts), an Eastern Cooperative Oncology Group (ECOG) performance status < 2, and age > 18 years, were considered eligible if they had a documented diagnosis of advanced solid tumor for which no standard systemic therapy exists. Exclusion criteria were prior DACis, valproic acid treatment, any concomitant anticancer therapy, use of medication that affects renal or hepatic function, active central nervous system disease or

brain metastasis, evidence of another malignancy not in remission or any other concurrent severe or uncontrolled medical condition.

Pharmacokinetic assessments

Serial whole blood samples of 3 mL for PK analysis were collected in the core phase on day 1 at predose and 0.5 (30 min), 1, 2, 4, 7, and 24 (day 2), 48 (day 3), 72 (day 4), and 96 (day 5) hours postdose. In addition, one 6 mL whole blood sample was collected at predose on day 1 for protein binding analysis. Plasma was assayed for panobinostat concentration using a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method.[14,17] Percent protein binding at baseline was assessed *ex vivo* by radiolabeling each plasma sample using ^{14}C panobinostat. Percent protein binding of panobinostat was quantified by spiking predose patient plasma samples with panobinostat to achieve 30 and 100 ng/mL concentration levels. These concentrations represent the typical and highest C_{max} achievable in humans after oral administration of panobinostat.

Statistical assessments

PK parameters were estimated using non-compartmental analysis. PK parameters including peak plasma concentration C_{max}, time to reach peak plasma concentration T_{max}, area under curve $AUC_{0-\infty}$ and AUC_{last}, last observable concentration C_{last}, time to last concentration T_{last}, elimination half-life $T_{1/2}$, total body clearance CL/F and apparent volume of distribution Vz/F were derived based on analysis of plasma panobinostat concentration data. A linear mixed model analysis was performed to account for differences in age and body surface area (BSA).

Adverse events were graded according to NCI-CTCAE, version 3.0[18] and recorded throughout the study until 28 days after the last dose of panobinostat.

Tumor assessments were performed at baseline and followed up during the course of the study according to RECIST criteria, version 1.0.[19] With efficacy being an exploratory study endpoint, the best overall response at the end of treatment was based on the investigator's evaluation. No formal analysis of tumor measurements was conducted for this study.

Study ethics

The study protocol was approved by the institutional review board at each participating institution with all patients providing written informed consent.

RESULTS

Patient disposition and baseline characteristics

A total of 25 patients were enrolled in the study and received oral panobinostat (10 patients with normal hepatic function, 8 and 6 patients with mild and moderate hepatic impairment respectively). One patient with severe hepatic impairment was subsequently enrolled and received the single PK dose of panobinostat of 30 mg and completed the PK assessments during the core phase before withdrawing due to increased bilirubin levels; this patient was included in the PK and safety population. One patient in the mild hepatic impairment group was excluded from the PK population due to vomiting within 4 hours of the single PK dose of panobinostat. Patient disposition and baseline characteristics, overall and by hepatic function group, are summarized in Table 7.2. Overall, the median age was 58 years (range, 45-76) with 56% of patients being male. 28%, 68%, and 4% of patients had an ECOG performance status of 0, 1, and 2, respectively. The most common malignancy was colon cancer, which was seen in 24% of patients.

Patient exposure

All patients took the dose of 30 mg panobinostat during the PK core phase. All patients started the extension phase with the dose regimen of 30 mg three times a week, weekly. None received the lowest dose level of 20 mg/day three times a week, every other week. Patients received a median of 1 cycle of treatment (range, 0.1-3.7) including medians (ranges) of 1.2 (0.1-3.5), 0.9 (0.1-2.2) and 1.6 (0.5-3.7) in patients with normal hepatic function, mild and moderate hepatic impairment, respectively. Three patients received ≥ 2 cycles. In the majority of patients, the exposure to study treatment was less than 2 months. The mean duration of exposure in the extension phase was 1.35 months in all patients. Overall 76% of patients received up to 2 months of treatment. Most patients required dose reduction to 30 mg three times a week, every other week within the first 2 weeks of treatment. Patients received a median of 7.8 mg/day of panobinostat (range, 0.0-12.9), representing 60% of the median planned dose of 12.9 mg/day. The mean relative dose intensity (DI) was 0.63 in all patients with slightly higher values in patients with mild liver impairment (0.73).

The main reason for treatment discontinuation was disease progression in 18 (72%) patients, including 9 (90%), 5 (62.5%), and 4 (66.7%) patients in the normal function, mild and moderate hepatic impairment groups, respectively. In addition, 3 (12%) patients refused further participation and 4 (16%) discontinued because of adverse events.

Table 7.2 Patient disposition and baseline characteristics overall and by hepatic function group

	Hepatic function/impairment group				
Panobinostat dose Core PK phase, *n* (%)	**All (*N* = 25)**	**Normal (*n* = 10)**	**Mild (*n* = 8)**	**Moderate (*n* = 6)**	**Severe (*n* = 1)**
30 mg single dose	25 (100)	10 (100)	8 (100)	6 (100)	1 (100)
Extension phase, *n* (%)					
30 mg TIW QW	24 (96)	10 (100)	8 (100)	6 (100)	
30 mg TIW QOW	1 (4)				1 (100)
Evaluable for PK, *n* (%)	24 (96)	10 (100)	7 (87.5)	6 (100)	1 (100)
Evaluable for safety, *n* (%)	25 (100)	10 (100)	8 (100)	6 (100)	1 (100)
Median age, y (range)	58 (45-76)	52 (45-76)	54 (46-67)	65 (59-74)	58 (58-58)
Male, *n* (%)	14 (56)	4 (40)	4 (50)	5 (83)	1 (100)
Female, *n* (%)	11 (44)	6 (60)	4 (50)	1 (16.7)	
Caucasian, *n* (%)	25 (100)	10 (100)	8 (100)	6 (100)	1 (100)
Cancer type, *n* (%)					
Colon	6 (24)	1 (10)	1 (12.5)	3 (50)	1 (100)
Prostate	3 (12)	1 (10)	1 (12.5)	1 (16.7)	0
Rectum	3 (12)	0	2 (25)	1 (16.7)	0
Lung	2 (8)	1 (10)	1 (12.5)	0	0
Uterus	2 (8)	2 (20)	0	0	0
Other[a]	9 (45)	5 (50)	3 (37.5)	1 (16.7)	0
ECOG PS, *n* (%)					
0	7 (28)	5 (50)	0	2 (33.3)	0
1	17 (68)	5 (50)	7 (87.5)	4 (66.7)	1 (100)
2	1 (4)	0	1 (12.5)	0	0

[a] Including: 1 mesothelioma, 1 gastric, 1 peritoneum, 1 melanoma, 1 fallopian tubes (normal group); 1 gall bladder, 1 ovarian, 1 endometrium (mild group); 1 liver (moderate group).

Pharmacokinetics

PK samples and data were available for 24 patients across the hepatic function groups. PK parameters from non-compartmental analysis grouped by hepatic function are listed in Table 7.3. Mean plasma concentration profiles for panobinostat are presented in Figure 7.1. The absorption of panobinostat was not affected by hepatic function as median T_{max} was similar across all groups. The median $AUC_{0-\infty}$ in the mild and moderate hepatic function group was approximately 35% and 84% higher than the normal group. Individual estimates of the $AUC_{0-\infty}$ between mild and normal group largely overlapped. Geometric mean of $AUC_{0-\infty}$ in the normal, mild and moderate group were 150.3, 214.8, and 308.0 ng·h/mL, respectively. This represents a 43% increase in the mild and 105% increase in the moderate groups as

Table 7.3 Summary of panobinostat plasma PK profile by hepatic function group

	Hepatic function/impairment group			
Panobinostat PK parameter (unit)	**Normal (*n* = 10)**	**Mild (*n* = 7)**	**Moderate (*n* = 6)**	**Severe (*n* = 1)**
T_{max} (h)	2.0 (0.5-7.0)	2.0 (0.5-4.0)	2.0 (1.0-4.0)	2.0 (2.0-2.0)
C_{max} (ng/mL)	18.5 (81.18)	29.1 (57.3)	33.9 (50.9)	31.2 (NE)
AUC_{0-48} (ng·h/mL)	125.0 (70.3)	183.9 (54.2)	249.9 (43.2)	235.4 (NE)
$AUC_{0-\infty}$ (ng·h/mL)	150.3 (72.3)	214.8 (56.3)	308.0 (44.2)	272.3 (NE)
AUC_{last} (ng·h/mL)	140.5 (73.3)	204.3 (56.2)	284.9 (42.6)	263.9 (NE)
CL/F (mL/h)	199647 (72.3)	139658 (56.3)	97399 (44.2)	110187 (NE)
Vz/F (mL)	8295077 (54.7)	5826678 (48.1)	4863991 (35.1)	3156940 (NE)
$T_{1/2}$ (h)	28.8 (27.3)	26.3 (27.6)	34.6 (31.5)	19.9 (NE)
C_{last} (ng/mL)	0.24 (0.13- 0.42)	0.27 (0.11- 0.46)	0.52 (0.17- 0.61)	0.29 (NE)
T_{last} (h)	96.0 (47.9- 96.3)	96.0 (72.0- 96.6)	96.0 (95.8- 96.0)	96.0 (96.0- 96.0)

Values are geometric mean (% CV), except for C_{last}, T_{max}, and T_{last} (median; range); NE: not evaluable.

compared with the normal group. The percent coefficient of variance (CV) associated with the geometric mean was large, ranging between 44-72%, reflecting the large PK variability of panobinostat. After adjusting for baseline age and BSA in a linear mixed model analysis the adjusted geometric means $AUC_{0-\infty}$ were similar to the unadjusted geometric means in the normal and mild group and slightly lower in the moderate group at 151.6, 214.6 and 291.8 ng·h/ml, respectively. This represents a 42% increase in the mild and a 92% increase in the moderate groups when compared with the normal group. Median peak plasma concentration C_{max} was 1.4 (mild) and 1.8 (moderate) fold higher as compared to those in the normal group. The terminal half-life estimated across normal, mild and moderate groups were similar, between 26 to 35 hours. This is consistent with the terminal half-life derived from the final parameter estimates of the population PK analysis in patients with normal hepatic function. Using Child Pugh's classification,[20] mild and moderate liver impairment patients had median $AUC_{0-\infty}$ approximately 60% above the normal group. Percent panobinostat bound to plasma protein were similar at panobinostat concentrations of 30 and 100 ng/mL. At clinically relevant peak plasma concentration of 30 ng/mL, percent protein binding in the mild impairment group was similar to those in the normal group at 83%, and decreased to between 77 to 74% in the moderate and severe groups. Protein binding adjusted free $AUC_{0-\infty}$ for the normal, mild and moderate groups were 24.7, 36.3 and 70.4 ng·h/mL, respectively. PK parameters of the severe patient did not differ from those of the moderate group.

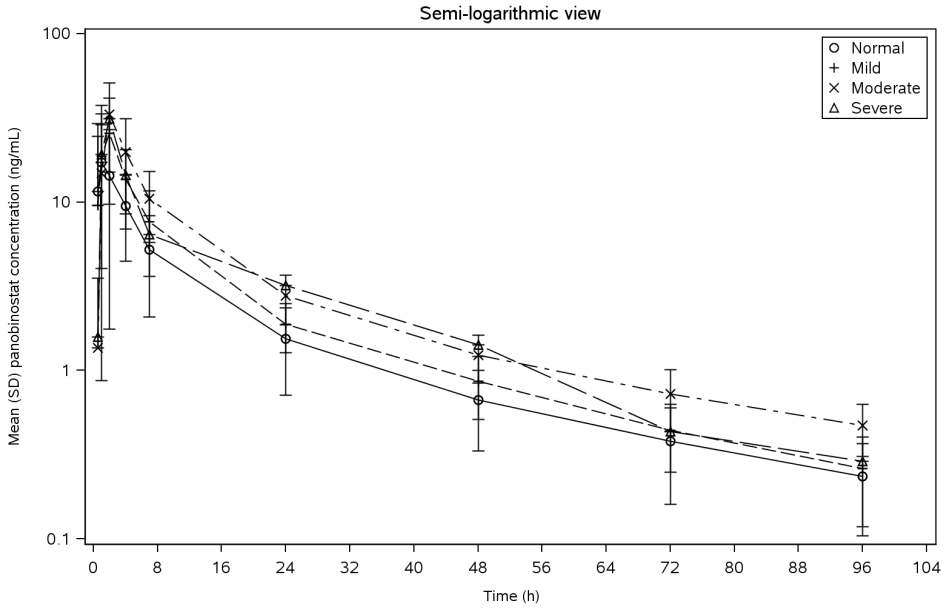

Figure 7.1 Arithmetic mean (SD) panobinostat plasma concentration-time profiles following a single 30 mg dose, by hepatic function group.

No patient received concomitant CYP3A4 inhibitors or inducers during the study core PK phase, thus the data in this study were not affected by such medications.

Safety

All patients treated with panobinostat experienced at least one adverse event (AE), and AEs of grade ≥ 3 were recorded for 92% of patients. The safety profile of panobinostat and the most common drug-related AEs (all grades, and grade ≥ 3) are summarized in Table 7.4 stratified by hepatic function group. Rates of grade ≥ 3 drug-related AEs were 70% in patients with normal liver function, 62.5% and 83.3% in patients with mild and moderate liver impairment, respectively. Fatigue, nausea, thrombocytopenia and diarrhea were the most common drug-related AEs of grade ≥ 3. Serious adverse events (SAEs) were reported in 36% of patients, mostly patients with normal liver function. The most common drug-related SAEs were diarrhea, nausea, vomiting, and fatigue (2 patients each). Overall, 4 patients (16%) had at least one AE leading to study drug discontinuation, fatigue being the most frequent. One unexpected SAE (grade 3 vasculitis) occurred during this study in a patient with moderate hepatic impairment. There were no clinically significant changes

Table 7.4 Safety profile of panobinostat overall and by hepatic function group, including most common drug-related adverse events of any grade (reported in ≥ 30% of patients) and of grade ≥ 3 severity (reported in ≥ 10% of patients)

Adverse event, *n* (%)	Hepatic function/impairment group				
	All ***N*** **= 25**	**Normal** ***n*** **= 10**	**Mild** ***n*** **= 8**	**Moderate** ***n*** **= 6**	**Severe** ***n*** **= 1**
Any adverse event	22 (88)	9 (90)	7 (87.5)	6 (100)	0
Nausea	17 (68)	7 (70)	6 (75)	4 (66.7)	0
Fatigue	15 (60)	7 (70)	4 (50)	4 (66.7)	0
Vomiting	14 (56)	7 (70)	4 (50)	3 (50)	0
Decreased appetite	13 (52)	5 (50)	4 (50)	4 (66.7)	0
Thrombocytopenia	12 (48)	5 (50)	1 (12.5)	6 (100)	0
Diarrhea	10 (40)	6 (60)	4 (50)	0	0
Any grade ≥ 3 adverse event	17 (68)	7 (70)	5 (62.5)	5 (83.3)	0
Fatigue	7 (28)	4 (40)	2 (25)	1 (16.7)	0
Nausea	4 (16)	3 (30)	1 (12.5)	0	0
Thrombocytopenia	4 (16)	3 (30)	0	1 (16.7)	0
Diarrhea	3 (12)	2 (20)	1 (12.5)	0	0
Any serious adverse event	9 (36)	6 (60)	2 (25)	1 (16.7)	0
Discontinuation due to adverse event	4 (16)	1 (10)	1 (12.5)	1 (16.7)	1 (100)
On-study deaths	5 (20)	2 (20)	1 (12.5)	2 (33.3)	0

in hematology or biochemistry parameters. The safety data from this study was consistent with the known safety profile of single agent oral panobinostat in patients with advanced cancers and adequate liver function.

A total of 6 patients died with 5 deaths occurring while on study treatment or within 28 days of the last dose of panobinostat, but were not treatment related. Most of the deaths were due to progression of underlying malignancy and one death was recorded as pulmonary edema in presence of disease progression.

Efficacy

No complete or partial responses were observed for the 24 patients in the extension phase. Stable disease was the best overall response in 4 patients (16%), including one in the normal group with lung cancer, one in the mild group with endometrial cancer and two in the moderate group with prostate and liver cancer. Early progressive disease (PD) was noted in 14 patients (56%).

DISCUSSION

The primary objective of this study was to assess the effect of various degrees of impairment in hepatic function on the pharmacokinetics and safety of panobinostat. The FDA guidance[16] for industry, recommends a PK study in patients with impaired hepatic function if hepatic metabolism and/or excretion accounts for a substantial portion (>20 percent of the absorbed drug) of the elimination of a parent drug or active metabolite. This is essential for dosage recommendations in clinical practice.

This study used a design whereby all enrolled patients (N = 25) received a single initial fixed panobinostat dose. This optimizes PK comparisons across all hepatic function groups. Hepatic dysfunction, per NCI-ODWG criteria based on bilirubin level, are similar to other studies of anticancer agents in patients with hepatic dysfunction.[21-24] This study showed that systemic exposure of panobinostat was increased with increasing hepatic impairment. Imbalances in patient demographics, such as age and BSA, may have contributed to the observed differences in the panobinostat plasma exposure among patients with normal, mild, moderate and severe hepatic functions. After adjusting for age and BSA in a linear mixed model analysis the adjusted geometric means of the hepatic impairment groups were not substantially affected. Due to the large PK variability of panobinostat, adjusted geometric means of normal, mild and moderate groups were associated with wide confidence intervals. The impact of change in adjusted geometric means, seen between hepatic function groups was deemed not clinically significant based on the covariate relationship identified in the population PK analysis.

Panobinostat is extensively metabolized primarily through non-CYP-mediated pathways. CYP pathways contribute < 50% to the overall metabolism of panobinostat.[14] Conversely, the clinical impact of CYP pathways inhibition, has been shown to be minor, as co-administration of panobinostat with a strong CYP3A4 inhibitor, ketoconazole increased panobinostat exposure to < twofold.[25]

Protein binding in the mild and normal group of 83% was within the range of historical values, but was lower in the moderate and severe hepatic impaired patients (74 to 77%). The extent of increase in free $AUC_{0-\infty}$ in mild and moderate groups were somewhat similar to those not adjusted for protein binding, reflecting the limited role of protein binding on the free drug exposure for a moderately bound drug like panobinostat.

Clinical safety profile of panobinostat was qualitatively and quantitatively consistent with known safety data in patients with advanced malignancies and adequate hepatic function

treated in previous single agent oral studies.[3-5,8-11] The dose of 30 mg given three times a week on a weekly schedule was moderately tolerated by all patients regardless of their liver function. This is expressed by the low relative DI (0.63) seen in the majority of patients regardless of their liver function. A limitation of the study is the short duration of exposure to drug (median of 1.35 months), due to disease progression. A rapid decline in patient condition often occurs in patients with advanced solid cancers and hepatic dysfunction due to lack of effective therapy. Nevertheless this study demonstrated the impact of hepatic impairment on the systemic exposure of panobinostat.

The clinical relevance of liver-function-related PK changes in regard to safety could not be adequately established as increased exposures of panobinostat did not lead to corresponding increases in main toxicities, thrombocytopenia and QT_c prolongation. In regards to thrombocytopenia, PK/PD modeling analyses have shown a dose-schedule dependent relationship between oral panobinostat treatment and platelet response.[26] Since platelet kinetics are largely dependent on the baseline platelet count, tumor group and panobinostat dose and schedule, systemic exposure alone is not sufficient to predict overall risk of thrombocytopenia. Schedule and/or dose reduction have been successfully implemented to manage thrombocytopenia risk when patients experience decreased platelet counts during panobinostat treatment.

QT_c prolongation has drawn attention during a phase I study with continuous intravenous administration of panobinostat;[27] however in the current study as well as in the other studies using single agent panobinostat, this does not seem to be a major issue.[3-5,9-11,28] The lack of QT_{cF} signal evidenced by intensive ECG monitoring throughout the study is consistent with historical data indicating a < 1% incidence of grade 3 QT_c prolongation across the clinical oral dose range of 20-40 mg. In patients with normal or impaired liver function, the only observed QT_c abnormalities were few increases in QT_{cF} < 60 msec.

In summary, the results of this PK study in cancer patients with varying degrees of hepatic impairment have shown that the systemic exposure of panobinostat increases with the severity of organ impairment. The extent of increase is less than twofold in the presence of moderate liver impairment. The safety findings suggest that the increasing degree of hepatic impairment did not appear to substantially increase toxicity in the hepatic dysfunction groups and that the rates of grade ≥ 3 adverse events and serious adverse events in patients with hepatic impairment are within the range of the rates in patients with normal hepatic function. Therefore an exposure-response relationship for safety could not be established in patients with mild to moderate liver dysfunction. Therapeutic management of these

patients should aim at assuring that effective doses are delivered with careful monitoring and treatment modifications, based on patient's safety and tolerability. Conversely the lack of data for severe hepatic impairment would suggest great caution in administering panobinostat to this vulnerable patient population. This study has been complemented by a parallel trial in cancer patients with varying degrees of renal impairment recently completed.

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8

Marije Slingerland
Claudia Cerella
Henk-Jan Guchelaar
Marc Diederich
Hans Gelderblom

Cardiac glycosides in cancer therapy: from preclinical investigations towards clinical trials

Invest New Drugs 2013;31:1087-94.

SUMMARY

Cardiac glycosides have a long history in the treatment of cardiac disease. However, several preclinical studies as well as two phase I studies have shown that cardenolides may also possess anticancer effects. The mechanisms of these anticancer effects may include intracellular decrease of K^+ and increase of Na^+ and Ca^{2+}; intracellular acidification; inhibition of IL-8 production and of the TNF-α/NF-κB pathway; inhibition of DNA topoisomerase II and activation of the Src kinase pathway. To date three cardiac glycosides have been developed for treatment of cancer and were tested in a phase I clinical trial to determine dose-limiting toxicities and maximum tolerated dose. Future studies of this novel class of anticancer drugs are warranted to determine their possible role in cancer treatment.

INTRODUCTION

Cardiac glycosides have been used in the treatment of cardiac disease for more than 200 years and were already known to the ancient Egyptians over 3000 years ago.[1] Cardiac glycosides contain a common molecular structure comprised of a steroid nucleus, an unsaturated lactone ring at the C-17 position, and one or more glycosidic residues at the C-3 position.[2,3] Chemically, cardiac glycosides can be divided into two groups: cardenolides and bufadienolides. Cardenolides contain a lactone ring of five members and bufadienolides are characterized by a 6-membered unsaturated lactone ring.

Common cardenolides include digoxin, digitoxin, digitoxigenin, lantoside C and ouabain (Figure 8.1). From a therapeutic point of view, the most important cardiac glycosides are digoxin and digitoxin as they are both used for the treatment of cardiac congestion and some types of cardiac arrhythmias, such as atrial fibrillation.

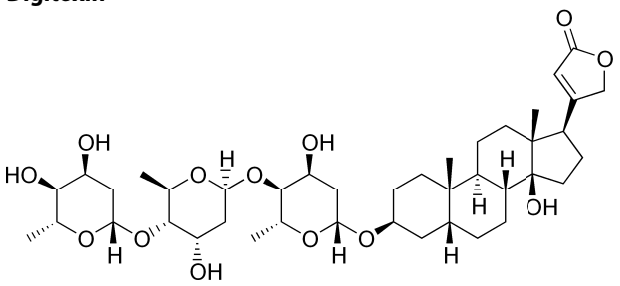
A variety of reports suggested that cardiac glycosides may have anticancer properties. In the 1960s clear inhibition of malignant cells of cardiac glycosides *in vitro* was reported. Almost two decades later, observation of the altered morphology of breast cancer cells from women on digitalis by Stenkvist *et al*. showed more benign characteristics than cancer cells from control patients not on digitalis.[4,5] Stenkvist *et al*. also showed that 5 years after the mastectomy, the recurrence among patients not taking digitalis was 9.6 times that in patients taking digitalis.[6]

In this manuscript, we will give an overview of the possible mechanisms involved in the anticancer activity of cardiac glycosides and discuss their early development in cancer therapeutics.

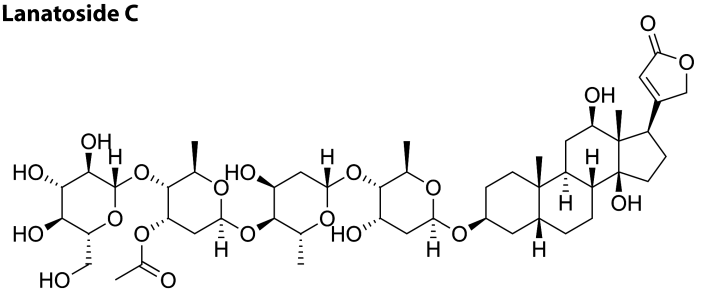
POSSIBLE CYTOTOXIC MECHANISMS OF ACTION

It is well known that cardiac glycosides, such as digitoxin, inhibit the activity of the Na^+/K^+-ATPase (also known as the Na^+ pump or Na^+/K^+ pump). This pump is a transmembrane enzyme that acts as an electrogenic ion transporter in the plasma membrane of all mammalian cells. Each cycle of the Na^+/K^+-ATPase activity extrudes three Na^+ from the cell, moves two K^+ into the cell and utilizes one ATP. The primary role of the Na^+/K^+-ATPase is therefore, to maintain high intracellular K^+ and low intracellular Na^+. This pump also has an important role in regulating cell volume, cytoplasmic pH and Ca^{2+} levels through the Na^+/H^+ and Na^+/Ca^{2+} exchangers, respectively, and in driving a variety secondary transport processes

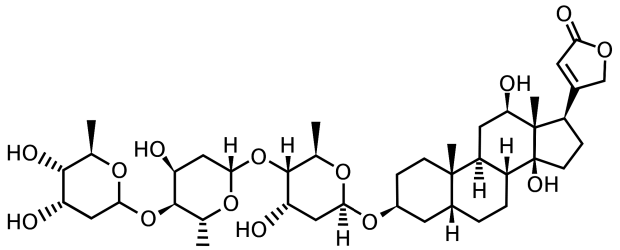
Digitoxin



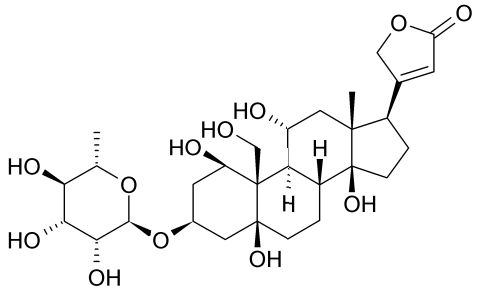
Lanatoside C



Digoxin



Ouabain



Digitoxigenin

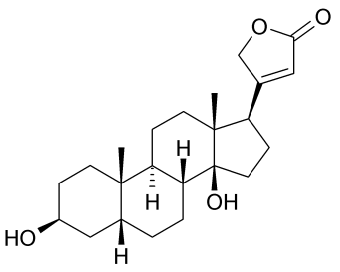


Figure 8.1 Chemical structures of common cardenolides.

such as Na^+ dependent glucose and amino acid transport.[7,8] Inhibiting Na^+/K^+-ATPase by cardiac glycosides leads to higher levels of intracellular Ca^{2+}, which leads to a decrease in heart rate and an increase in contractility of the heart. However, the decrease in intracellular K^+ and increase in intracellular Na^+ and Ca^{2+} following inhibition of the Na^+/K^+-ATPase may also induce apoptosis.[9-14] Inhibition of the Na^+/K^+-ATPase by digitoxin and subsequent increase in intracellular Ca^{2+} led to the induction of apoptosis of prostate cancer cells.[15,16]

Besides inducing apoptosis by intracellular decrease of K^+ and of Na^+ and intracellular Ca^{2+}, cytotoxic mechanisms of action include intracellular acidification; inhibition of IL-8 production and the TNF-α/NF-κB pathway; inhibition of DNA topoisomerase II and activation of the Src kinase pathway (Figure 8.2). Whether the Na^+/K^+-TPase is the primary target of cardiac glycosides or not is actually a matter of intense debate.[17]

Intracellular decrease of K^+ and increase of Na^+ and Ca^{2+}

Inducing apoptosis by excessive K^+ efflux and intracellular K^+ depletion are early key steps in apoptosis.[9] Physiological concentration of intracellular K^+ acts as a repressor of apoptotic effectors. Loss of cellular K^+, a common event in apoptosis of many cell types, may trigger the apoptotic cascade including caspase cleavage, cytochrome c release, and

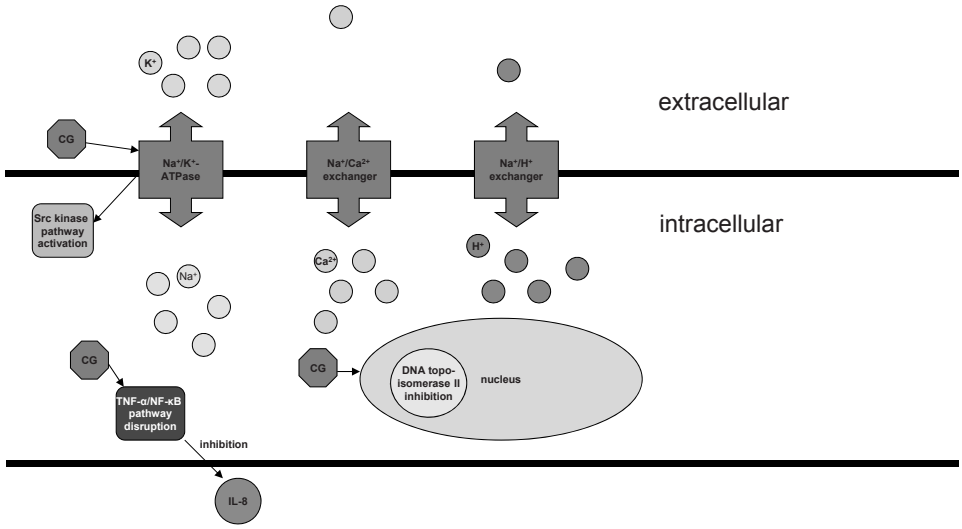


Figure 8.2 Proposed mode of action of cardiac glycosides. Cardiac glycosides (CG) induce apoptosis by intracellular decrease of K^+ and of Na^+ and intracellular Ca^{2+}. Other cytotoxic mechanisms of action include intracellular acidification; inhibition of IL-8 production and the TNF-α/NF-κB pathway; inhibition of DNA topoisomerase II and activation of the Src kinase pathway.

endonuclease activation. Pro-apoptotic disruption of K^+ homeostasis can be mediated by over-activated K^+ channels or ionotropic glutamate receptor channels, and most likely, accompanied by reduced K^+ uptake due to dysfunction of Na^+/K^+-ATPase. Studies indicate that also mitochondrial K^+ channels and K^+ homeostasis play important roles in apoptosis.[9-11]

During apoptosis, there is compelling evidence indicating an early increase in intracellular Na^+ followed by a decrease in both intracellular K^+ and Na^+ suggesting a regulatory role for these cations during both the initial signaling, and the execution phase of apoptosis. Studies have shown that the Na^+/K^+-ATPase is involved in controlling perturbations of Na^+ and K^+ homeostasis during apoptosis.[14]

Also cellular Ca^{2+} overload, or perturbation of intracellular Ca^{2+} compartmentalization, can cause cytotoxicity and trigger either apoptotic or necrotic cell death.[15]

Intracellular acidification

Published data suggests that intracellular alkalinisation can produce malignant transformation.[18-25] It is also suggested that alkalinisation may be required for the development and maintenance of the transformed phenotype cancer cells and may be implicated in key cancer related processes.[18-25] In contrast, it has been observed that intracellular acidification can induce apoptosis in cancer cells and play an important role in the induction of apoptosis by different stimuli.[24,26-32] For example, Rich *et al*. demonstrated that apoptosis of leukemic cells accompanies reduction of intracellular pH after targeted inhibition of the Na^+/H^+ exchanger.[24] Moreover stress-activated protein kinase pathway activation and mitochondrial-derived hydrogen peroxide acts as an effector mechanism leading to induction of apoptosis by intracellular acidification.[26,27]

These observations indicate that induction of intracellular acidification possesses anticancer effects. Interestingly, cardiac glycosides induce intracellular acidification in cancer cells as the inhibition of the Na^+/K^+-ATPase may increase intracellular concentrations of Na^+, reduce the activity of the Na^+/H^+ exchanger and trigger intracellular acidification.

Inhibition of IL-8 production and the TNF-α/NF-κB pathway

Inhibition of IL-8 production and the TNF-α/NF-κB pathway is another mechanism of cardiac glycosides to produce anticancer effects. As production of IL-8 has been associated with important processes involved in tumor progression such as apoptosis resistance, angiogenesis or metastasis, inhibition of its expression is therefore thought to produce

anticancer effects.[33-35] Juncker *et al*. demonstrated that the hemi-synthetic cardenolide UNBS1450 leads to inhibition of IL-8 synthesis via NF-κB pathway disruption leading to apoptotic cell death.[36] Srivastava *et al*. showed similar results for digitoxin[37] whereas Yang *et al*. demonstrated that cardiac glycosides were potent blockers of the TNF-α/NF-κB pathway, which results in apoptosis, as NF-κB induces the expression of genes that are inhibitors of apoptosis.[38]

Inhibition of DNA topoisomerase II

Recently published data suggest that digitoxin may inhibit topoisomerase II. Because of their central role in DNA replication, transcription and repair processes, topoisomerase II inhibitors are a category of drugs commonly used in the treatment of malignancies by inducing apoptosis.[39,40] López-Lázaro *et al*. demonstrated that a renal adenocarcinoma cancer cell line was hypersensitive to digitoxin and died by apoptosis. *In vitro* experiments showed that digitoxin induced levels of DNA-topoisomerase II cleavable complexes comparable to etoposide, a topoisomerase II poison widely used in cancer chemotherapy. Cells exposed to digitoxin for 30 min showed low but statistically significant levels of DNA-topoisomerase II cleavable complexes; however these complexes disappeared after 24 h exposure.[39] The same research group also showed that digitoxin, at concentrations commonly found in the plasma of cardiac patients, significantly reduced etoposide and idarubicin-induced topoisomerase II cleavable complexes in leukemia cells.[40] Also other cardiac glycosides, such as ouabain, digoxin, proscillaridin and bufalin, have shown to inhibit topoisomerase II.[41,42] Bielawski *et al*. demonstrated that digoxin, ouabain and proscillaridin A exerted significant inhibitory effects on the proliferation of breast cancer cells. Of the two cardiac glycosides, proscillaridin A was more effective at inhibiting the proliferation of breast cancer cells than digoxin or ouabain.[41] Hashimoto *et al*. showed that bufalin caused a marked decrease in the steady-state level of topo II alpha mRNA in human leukemia cells, which led to a decrease in the amount and activity of the enzyme and to the induction of apoptosis.[42]

Activation of the Src kinase pathway

Multiple studies have established that the binding of cardiac glycosides to Na^+/K^+-ATPase not only inhibits the ATPase activity but also stimulates protein tyrosine kinases such as Src. This process is the consequence of an additional function played by Na^+/K^+-ATPase besides its control of ionic cellular homeostasis, which is already the trigger of complex intracellular signalization pathway forming a signalosome. Accordingly, pools of non-

pumping Na^+/K^+-ATPase are localized in plasma membrane caveolae, where it clusters with other plasma membrane proteins and receptors, including growth factor receptors (i.e., the epidermal growth factor receptor EGFR).[43] Binding of Na^+/K^+-ATPase by cardiac glycosides may in turn unleash several kinase-dependent cascades, which are implicated in cell proliferation. Activated Src in turn transactivates EGFR, resulting in the assembly and activation of multiple signaling cascades controlled by the extracellular signal-regulated kinase (ERK)1/2 and phospholipase C-γ/protein kinase C pathways.[44] Liang *et al*. suggested that cells contain a pool of Src-interacting Na^+/K^+-ATPase that not only regulate Src activity but also serve as receptors for ouabain to activate protein kinases.[44] One year before, in 2005, Kometiani *et al*. showed in breast cancer cell lines that ouabain-induced cell growth inhibition may be mediated by activation/transactivation of Src/EGFR by Na^+/K^+-ATPase, which leads to activation of ERK1/2, increase in the levels of the cell cycle inhibitor $P21^{Cip1}$ and subsequent growth arrest.[45] Kometiana *et al*. also demonstrated that digoxin and digitoxin concentrations close to or at therapeutic plasma levels had effects both on proliferation and ERK1/2 similar to those of ouabain, supporting the proposed potential value of digitalis drugs for the treatment of breast cancer.[45] The existence of signalosomes where Na^+/K^+-ATPase plays a non-ionic activity has highlighted an endogenous activity of cardiac glycosides. Ouabain is endogenously produced[46] and circulating in the plasma, it acts in a paracrine/endocrine fashion and its levels are considered critical to determine several physio-pathological responses.[47-49] Interestingly, these endogenous biological effects correlate with a complex signaling cascade involving kinases.[50] The discovery of these non-canonical functions has very recently suggested a role for Na^+/K^+-ATPase as hormone receptor.[51] Altogether, these findings suggest in a very next future important hints in the elucidation of anticancer effects ascribed to cardiac glycosides and help in the explanation of preventive effects observed in patients under treatment with digitalis especially towards forms of hormonal cancer.

IMPACT OF CARDIAC GLYCOSIDES ON CANCER CELLS

Cardiac glycosides exert anti-proliferative and cytocidal effects on different cancer cell models.[17,52] Their ability to impair cancer cell viability represents a main hallmark of their anticancer activities. Nevertheless, multiple types of cell death are triggered by cardenolides and bufadienolides. The induction of apoptosis has been frequently reported. Both extrinsic and intrinsic apoptosis pathways were triggered. Moreover, the sensitization to other therapeutic agents has been also described. In a consistent number of reports, cardiac

glycosides led to the accumulation of cells essentially in the S phase[53,54] and G2/M[55-58] phase. This event has been correlated to the elicitation of intracellular reactive oxygen species.[55,57] Besides, in adherent cancer cell models, cardiac glycosides have been shown able to activate an autophagic cell death.[17] This dual cytocidal ability underlines the promising use of cardiac glycosides especially for the treatment of those forms of cancer that are resistant to agents inducing apoptosis. Nevertheless, the mechanisms determining the kind of cell death accomplished upon treatment with cardiac glycosides remain still unclear and debated. One possibility is that sustained autophagy may be commonly activated as a first response by the cells followed by a switch to apoptosis in cancer cells prone to activate programmed forms of cell death. In contrast, autophagic cell death may be undertaken as a kind of final backup cell death modality whenever apoptosis cannot take place. This hypothesis implies that cardiac glycosides may induce stress conditions that potentially lead to alterations of metabolic activities. Finally, very recently clinically used cardiac glycosides, as digoxin and digitoxin, have been shown to induce immunogenic cell death.[59] Interestingly, among the parameters determining immunogenic cell death is the autophagy-dependent secretion of ATP.[60]

OBSERVATIONAL STUDIES

In the last decades observational studies have shown that digitalis may have an anticancer effect. In 1979, Stenkvist *et al*. reported that breast cancer cells from patients while taking digitalis for chronic heart disease were smaller and more uniform in morphology than breast cancer cells not exposed to cardiac glycosides.[5] Also the tumor mass was smaller at diagnosis in patients taking digitalis compared to patients not taking digitalis. The risk of recurrence was 9.6 times higher in the group of patients who were not taking digitalis.[6] Later, Goldin *et al*. conducted a retrospective trial of 127 cancer patients. They found only one cancer death (of a total of 21 deaths) within patients taking digitalis, suggesting that the use of cardiac glycosides may also prevent the development of cancer.[61]

Two large case control studies could nevertheless not show a significant protective benefit.[62,63] The authors of the large case-control study in Norway concluded that elevated morbidity and mortality in the digitoxin population disturbed efforts to isolate eventual anticancer effects of digitoxin.[62]

However, in 2008, Ahern *et al*. suggested in their case control study that digoxin treatment moderately increases the risk of invasive breast cancer among postmenopausal women instead of reducing it.[64]

PRECLINICAL STUDIES IN CANCER

The unusual species-dependent sensitivity to growth inhibition of cardiac glycosides across a broad spectrum of tumor cells is the reason for the paucity of animal data.

In the past decade there has been a substantial increase in the number of *in vitro* and *in vivo* studies regarding the effects of cardiac glycosides on the growth of human malignant tumor cells. In 1967 Shiratori already reported about the growth inhibitory effect of cardiac glycosides on neoplastic cells[65] and many research reports followed.

CARDIAC GLYCOSIDES IN PHASE I CLINICAL TRIAL

To date, there are three cardiac glycosides or derivatives that have been developed for treatment of cancer and were assessed in a phase I clinical trial. The initial product was Anvirzel™, an aqueous extract of Nerium oleander, the second was PBI-02504, a super critical CO_2 extract of Nerium oleander and the third UNBS1450, a semisynthetic cardenolide derivate of 2″-oxovoruscharin extracted from Calotropis procera, a desert shrub.[36,52]

In 2000, Manna *et al*. demonstrated that oleandrin inhibits the activation of NF-κB and AP-1 and their associated kinases.[66] Smith *et al*. showed that Anvirzel™, like oleandrin, inhibits fibroblast growth factor (FGF)-2 export *in vitro* from prostate cancer cells in a concentration- and time-dependent fashion and may, therefore, contribute to the antitumor activity of this treatment for cancer.[67]

Based on these preclinical data, a phase I study started and Mekhail *et al*. reported in 2006 the results of this study of Anvirzel™.[68] The study reported a phase I trial to determine the maximum tolerated dose (MTD) and safety of Anvirzel™ in 18 patients with advanced, refractory solid tumors. Patients were randomized to receive this agent by intramuscular injection at doses of 0.1, 0.2 and 0.4 ml/m^2/day with subsequent patients receiving 0.8 or 1.2 ml/m^2/day sequentially. Eighteen patients were enrolled and completed at least one treatment cycle of 3 weeks. Most patients developed mild injection site pain (78%). Other toxicities included fatigue, nausea, and dyspnea. Traditional dose-limiting toxicity was not seen, but the MTD was defined by injection volume as 0.8 ml/m^2/day. No objective antitumor responses were seen. They concluded that Anvirzel™ can be safely administered at doses up to 1.2 ml/m^2/day, with the amount administered intramuscularly limited by volume. The recommended phase II dose level is 0.8 ml/m^2/day.

PBI-05204 has recently completed testing for safety in Phase I clinical trial.[69] The publication of conclusions is in process and the initial findings were presented at the annual meeting of the American Society of Clinical Oncology (ASCO) in June 2011. PBI-05204 (Oleandrin), inhibits the α-3 subunit Na^+/K^+-ATPase pump. Relative expression of the α-3 subunit in tumor cells correlates with proliferation. Oleandrin inhibits FGF-2 export, activation of NF-κB, phosphorylation of Akt, p70S6K and decreases mTOR activity. In this first-in-human study, the authors sought to determine the MTD/recommended phase II dose and to define the pharmacokinetics (PK) and pharmacodynamics (PD) of PBI-05204 in advanced cancer patients. Forty-six patients were dosed at 8 dose levels (DL) of PBI-05204 (0.6 to 10.2 mg/day). Two dose-limiting toxicities occurred at DL 8 (grade 3 proteinuria, fatigue) thus the MTD was DL 7. Most common adverse events (AEs) were fatigue (56.1%), abdominal pain (41.5%), constipation (41.5%), nausea (41.5%), and diarrhea (39.0%). Cardiac disorders were reported in 10 patients (24.4%), all grade 1, except for one patient with grade 2 supraventricular tachycardias (SVT). Of the 45 evaluable patients, 7 showed a stable disease for > 4 months, with bladder, colorectal, fallopian tube, breast, appendical and pancreatic carcinoma (2 patients). They concluded that PBI-05204 is well tolerated up to 10.2 mg/day with very little AEs or cardiotoxicity.

UNBS1450, has also been tested in an open-label, dose escalation study to evaluate the safety, tolerability and pharmacokinetics of this single agent, administered once every 3 weeks in separate cohorts of patients with advanced solid tumors or lymphoma. Chemical modifications of 2″-oxovoruscharin (a novel cardenolide extracted from Calotropis procera) has led to the identification of UNBS1450.[70] The activity of the compound in preclinical cancer models, independent of cell type, has been tested *in vitro* on 57 human cancer models from 11 distinct histological types.[70] In aggressive and metastatic orthotopic NSCLC,[71,72] refractory prostate cancer[73] and glioma[74] models, UNBS1450 was more potent than tested reference compounds, including paclitaxel, irinotecan, oxaliplatin, mitoxantrone and temozolomide.[71-75] UNBS1450 was the most potent inhibitor of all three isozymes (α3β1, α2β1 and α1β1) with a potency ~6 to > 200 times greater than that ouabain (another cardenolide) and digoxin[74] The general mechanism of action associated with UNBS1450-mediated anticancer effects relates to the compound's propensity in disorganizing the actin cytoskeleton and thus non ATPase-mediated effects.[73-75] UNBS1450 can thus be considered both anti-proliferative (cytotoxic) and anti-migratory.[75,76] given that the actin cytoskeleton is essential to cytokinesis and to cancer cell migration.[77] In sharp contrast to digitalis-like cardenolides, UNBS1450 does not induce intracellular Ca^{2+} or Na^+ increase at concentrations at which it induces potent antitumor effects.[74,75] UNBS1450 induces both apoptotic and

non-apoptotic cell death processes depending on the cellular environment. Non-apoptotic cell death mechanisms such as lysosome membrane permeabilisation[71] and autophagy[74] were observed in solid tumors and thus may overcome major apoptosis resistance pathways responsible in part for the failure of therapeutics in certain cancers. Canonical intrinsic apoptosis was demonstrated by Juncker *et al*. in leukemia and lymphoma cellular models with an early degradation of anti-apoptotic Mcl-1, Bak and Bax activation leading to cytochrome C release, caspase-9, -7 and -3.[36] Experimental data involving NF-κB inhibition/deactivation evidenced it as an important new approach to the treatment of various malignancies was shown by the same authors.[36] UNBS1450 deactivates the cytoprotective NF-κB pathways at several points, in sharp contrast to specifically designed NF-κB inhibitors acting at one precise point.[72] In leukemia cells, UNBS1450 inhibits degradation of the IκB inhibitor of p50/p65 NF-κB heterodimers thus preventing transcription factor translocation in the nucleus. Using genomic and proteomic approaches, it was possible to evidence UNBS1450-mediated down-regulation of c-MYC gene, MYC oncoprotein-related genes, and genes with nucleolar functions.[15] UNBS1450-induced marked down-regulation of c-MYC expression in a number of human cancer cell lines lead to nucleolar disorganization resulting in impairment of cancer cell survival.[15] Unfortunately the phase I study was closed in 2011 by the sponsor because of bankruptcy before reaching the MTD after including 23 patients. Preliminary data will be published elsewhere.

CONCLUSION

Cardiac glycosides have a long history in the treatment of cardiac diseases, but several preclinical studies have shown that cardenolides have also anticancer effects. Two cardiac glycosides, Anvirzel™ and PBI-02504, completed testing for safety in a phase I clinical trial. Another phase I trial with UNBS1450 was closed early. Several mechanisms seem to participate in these anticancer effects. Additional in-depth preclinical research is required to find out the possible role for cardiac glycosides as primary anticancer agents as well as bona fide biological markers. As the pharmacological and safety profile of compounds like digitoxin is well known future clinical investigations should be accelerated.[78]

ACKNOWLEDGMENTS

Claudia Cerella is supported by a Waxweiler grant for cancer prevention research from the Action Lions Vaincre le Cancer. Research at Laboratory of Molecular and Cellular Biology of Cancer is financially supported by the Fondation de Recherche Cancer et Sang, the Recherches Scientifiques Luxembourg association, the Een Häerz fir kriibskrank Kanner association, the Action Lions Vaincre le Cancer association, the European Union and the Télévie Luxembourg. Marc Diederich is supported by the National Research Foundation by the Ministry of Science and Technology of Korea for Tumor Microenvironment Global Core Research Center grant, by the Seoul National University (SNU) Research grant and by the Research Settlement Fund for the new faculty of the SNU.

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9

Marije Slingerland
Florian Muller
Jan Ouwerkerk
Claudia Cerella
Marc Diederich
Hans Gelderblom

Preclinical evaluation and preliminary report of an incomplete phase I pharmacokinetic trial using UNBS1450, a sodium pump antagonist, in patients with advanced solid tumors

ABSTRACT

Introduction

UNBS1450, a semisynthetic cardenolide glycoside derivative is considered a promising anticancer agent targeting overexpressed sodium pump α subunits in malignant tumors. This paper summarizes preclinical data and the preliminary results of a non-completed clinical phase I trial with the compound.

Preclinical data

Experiments on a human hematopoietic cancer cell line, already previously used as cell model to investigate the effects of UNBS1450 were performed in order to evaluate the minimum exposure time to UNBS1450 required to trigger the commitment phase of apoptosis. With this purpose, two strategies were pursued. First, the histiocytic lymphoma U937 cells were incubated for different times with an apoptogenic concentration of UNBS1450 (20 nM) followed by recovery. Second, U937 cells were incubated with 10nM UNBS1450 for 1 h, a concentration/time mimicking the conditions during patients' treatment. In this instance, the experiment was also performed in presence of different percentages of fetal calve serum (FCS) (0.1-10%) used for cell culture cultivation to monitor any influence of serum to sequester the compound and therefore alter its action. The effect of UNBS1450 on viability (induction of apoptosis) and cell proliferation was then assessed following these conditions during the recovery phase.

Patients and methods used in the clinical trial

A phase I trial to evaluate the safety, tolerability and pharmacokinetics of single agent, UNBS1450 administered once every three weeks in separate cohorts of patients with advanced solid tumors not amenable to established forms of therapy was conducted.

Results of the clinical trial

The study was closed by the sponsor because of bankruptcy before reaching the maximum tolerated dose (MTD) after including 23 patients. The half-life of UNBS1450 was very short being around 0.1 h within the tested dose range. There appeared an approximately linear relationship with dose for both mean maximum plasma drug concentration (C_{max}) and area under the curve 0-t where t is last time at which drug was quantifiable (AUC_{last}) values over the dose range 90-615 µg/patient. There were no Response Evaluation Criteria In Solid Tumors (RECIST) responses in any of the patients.

Conclusion

The primary endpoint of the clinical phase I was not reached due to early termination of the study for non-scientific reasons. The available preclinical work could not guide us in adapted scheduling of the patients. More research is necessary to establish the optimal dose and schedule of UNBS1450 for future phase I/II studies.

INTRODUCTION

The sodium pump, sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase), has been suggested as an interesting oncology target. It serves as a versatile signal transducer and it plays a key role in cell adhesion. Several malignancies are characterized by an overexpression of its α subunits.[1]

Numerous studies investigated the changes in the transmembrane transport of cations during the course of malignant cell transformation, due to increases in Na^+/K^+-ATPase activity, specifically through the upregulation of the Na^+/K^+-ATPase α subunits.[1] This was confirmed in a large proportion of clinical non-small cell lung carcinoma (NSCLC) samples[2] while more than 50% of glioblastoma samples expressed 10 times more α1 messenger ribonucleic acid (mRNA) compared to samples from normal brains.[3] Those studies also pointed to a difference in the density of the enzyme, as well as isozyme expression, at the plasma cell membrane of tumor cells.[3]

Cardiotonic steroids (CSs), and notably cardenolides, are the natural ligands and inhibitors of the Na^+/K^+-ATPase, thus supporting the possibility of their potential development as anticancer agents targeting overexpressed Na^+/K^+-ATPase α subunits.[4,5] While CSs have been widely used for the treatment of heart failure, early epidemiological evaluations have indicated lower mortality rates in cancer patients who were on digitalis, a cardenolide, at the time of first diagnosis.[6-9] To date, the development of CSs as anticancer agents has been impaired by a presumed narrow therapeutic margin resulting from the theoretical risk to induce cardiovascular side effects.[1,10]

Chemical modifications of 2"-oxovoruscharin (a novel cardenolide extracted from Calotropis procera) based on an understanding of the structure activity relationship within the series, has led to the identification of UNBS1450.[11] The activity of the compound in preclinical cancer models, independent of cell type, has been tested *in vitro* on 57 human cancer models from 11 distinct histological types.[11] In aggressive and metastatic orthotopic NSCLC,[4,5] refractory prostate cancer[12] and glioma[3] models, UNBS1450 was more potent than tested reference compounds, including paclitaxel, irinotecan, oxaliplatin, mitoxantrone and temozolomide.[3-5,12,13]

UNBS1450 was the most potent inhibitor of all three isozymes (α3β1, α2β1 and α1β1) with a potency ~6 to > 200 times greater than that ouabain (another cardenolide) and digoxin.[3] The general mechanism of action associated with UNBS1450-mediated anticancer effects relates to the compound's propensity in disorganizing the actin cytoskeleton.[3,12,13]

UNBS1450 can thus be considered both anti-proliferative (cytotoxic) and anti-migratory[13,14] given that the actin cytoskeleton is essential to cytokinesis and to cancer cell migration.[15] In sharp contrast to digitalis-like cardenolides, UNBS1450 does not induce intracellular Ca^{2+} or Na^{+} increase at concentrations at which it induces potent antitumor effects.[3,13] UNBS1450 induces non-apoptotic cell death processes (such as lysosome membrane permeabilization[4] and autophagy[3]) and thus may overcome major apoptosis resistance pathways responsible in part for the failure of therapeutics in certain cancers. Experimental data involving NF-κB inhibition/deactivation evidenced it as an important new approach to the treatment of various malignancies.[15] UNBS1450 at 10 nM (its mean anti-proliferative IC50 concentration) deactivates the cytoprotective NF-κB pathways at several points, in sharp contrast to specifically designed NF-κB inhibitors acting at one precise point.[5] Furthermore, the anticancer activity of UNBS1450 is not affected by chemotherapy resistance expressed in cancer cells. UNBS1450 kills chemoresistant cells harboring the multidrug resistance phenotype (PgP overexpression) and/or apoptosis-resistant cancer cells with the same efficacy as it does for chemosensitive cancer cells.

Using genomic and proteomic approaches, it was possible to evidence UNBS1450-mediated down-regulation of c-MYC gene, MYC oncoprotein-related genes, and genes with nucleolar functions.[12]

UNBS1450-induced marked down-regulation of c-MYC expression in a number of human cancer cell lines lead to nucleolar disorganization resulting in impairment of cancer cell survival.[12] The present data suggest that c-MYC could be used as a marker of UNBS1450-mediated antitumor activity. An exploratory cardiovascular study in dogs comparing the effects of intravenously administered digoxin and UNBS1450 showed similar effects on the cardiovascular system. There was no evidence of an increased toxicity or increased pro-arrhythmic effects of UNBS1450 compared to digoxin. The structural uniqueness of UNBS1450 taken with its ability to i) disorganize the actin cytoskeleton, ii) disorganize nucleolar structure and functions, iii) kill chemoresistant and/or apoptosis-resistant cancer cells, and iv) deactivate constitutively activated cytoprotective signaling pathways and to induce lysosomal membrane permeabilization and/or autophagy-related cell death thus overcoming major pathways responsible for the failure of cancer chemotherapy, support its development as an anticancer agent targeting overexpressed sodium pump α subunits.

On the basis of the above mentioned presumed antitumor properties of UNBS1450 we started a classical phase I study with the drug.

MATERIAL AND METHODS

Cell culture

U937 cells (histiocytic lymphoma) were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% (v/v) FCS (Lonza, Verviers, Belgium) and 1% (v/v) antibiotic-antimycotic (BioWhittaker, Verviers, Belgium) at 37°C and 5% of CO_2. Experiments were performed in culture medium containing 10% of FCS, unless otherwise indicated. UNBS1450 was a kind gift of Unibioscreen (Brussels, Belgium).

Washout experiments

Cells were seeded at a density of 3.0×10^5 cells/ml and incubated with 20 nM UNBS1450 for different times (0, 1, 2, 4, 6, 8, 10, and 12 h). Then cells were washed and resuspended in the same volume of fresh medium for recovery. As a positive control, cells were cultured in the presence of UNBS1450 throughout the experiment. Apoptosis was analyzed 24-48 h after the start of the treatment (time 0= T_0).

Determination of apoptosis

a) Analysis of nuclear fragmentation. Percentage of apoptotic cells was quantified as the fraction of apoptotic nuclei (different stages of nuclear fragmentation) assessed by fluorescence microscopy (Leica-DM IRB microscope, Lecuit, Luxembourg) upon staining with the DNA-specific dye Hoechst 33342 (Sigma, Bornem, Belgium). The fraction of cells with nuclear apoptotic morphology was counted (at least 300 cells in at least three independent fields).[16,17]

b) Mitochondrial membrane potential analysis. At the indicated time points, U937 cells were loaded with 50 nM MitoTracker Red (MTR; Molecular Probes)[16,18] at 37°C for 20 min, and immediately analyzed by flow cytometry using a BD FACScalibur (BD Biosciences, San José, CA, USA), tuned at 488 nm, standard band pass filters FL3 (630 nm). Data were recorded for further analysis with Cell Quest software (http://www.bdbiosciences.com/features/products/display_product.php?keyID=92). The mean fluorescence value was determined by counting at least 10000 cells. Data were further analyzed using FlowJo 8.8.7 software (Tree Star Inc).

c) Induction of apoptosis was molecularly confirmed by Western blot analysis of caspase-3 cleavage (see also section below).

Western Blot analysis

Cells were washed with cold phosphate buffered saline (PBS), and cells extracts were prepared using M-PER® Mammalian Protein Extraction Reagent (Pierce, Erembodegem, Belgium) completed with a protease inhibitor cocktail (Roche, Luxembourg), 1 µM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 5 mM sodium fluoride (Sigma, Bornem, Belgium). Cells were incubated 15 min at 4°C in lysis buffer and centrifuged at 14000 g, 15 min, 4°C. Twenty µg of proteins were separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%), transferred onto polyvinylidene difluoride membranes. Following a 1 h incubation period in 5% non-fat milk in PBS-Tween, membranes were probed with primary antibodies to Mcl-1 and caspase-9 (Cell Signaling Technology, Leiden, The Netherlands; 1:1000 in PBS-T/BSA 5%) and caspase-3 (Santa Cruz, Biotechnology, Boechout, Belgium; 1:1000 in PBS-T/Milk 5%). Protein bands were visualized via chemiluminescence using the ECL+ Western Blotting Detection System Kit® (GE 36 Healthcare, Roosendaal, The Netherlands), following incubation with secondary antibodies horseradish peroxidase (HRP)-conjugated (Mcl-1 and caspase-9: 1:4000, anti-rabbit; caspase-3: 1:4000, anti-mouse; Santa Cruz). Equal loading of samples was controlled using β-actin (Sigma, 1:10000, in PBS-T/Milk 5%; secondary antibody: 1:10000, anti-mouse, in PBS-T/Milk 5%, Santa Cruz).

PATIENTS AND METHODS USED IN THE CLINICAL TRIAL

Study design

This was an open-label, dose escalation study to evaluate the safety, tolerability and pharmacokinetics of single agent, UNBS1450 administered once every three weeks in separate cohorts of patients with advanced solid tumors not amenable to established forms of therapy with curative intent.

The primary objective of this study was to determine the MTD and to establish the recommended phase II dose of UNBS1450 when administered intravenously once every three weeks. The secondary objectives of this study were to describe the safety profile of UNBS1450, including the dose-limiting toxicity (DLT); to assess the pharmacokinetic (PK) profile of UNBS1450; to study preliminary evidence of pharmacodynamic (PD) relationships with UNBS1450 systemic exposure; to perform UNBS1450-related explorative immunomonitoring in peripheral blood; to assess the preliminary antitumor activity of UNBS1450; to study the expression of the Na^+/K^+-ATPase pump α subunits in the study

population for potential correlation with clinical responses; to evaluate c-MYC as a potential surrogate marker for antitumor efficacy of UNBS1450 in sequential tumor biopsies.

Dose escalation followed a classical 3 + 3 design.[19] Up to 6 patients were enrolled in this study per dosing cohort. Any patient who received at least 1 dose of UNBS1450 was evaluable for safety. However patients were required to complete at least the initial 21 days of treatment period/observation to be evaluable for determination of MTD, unless they were withdrawn due to a DLT prior to day 22. Patients continued to receive UNBS1450 for as long as the investigator felt it was appropriate but would be discontinued from study drug in case of clinically and/or radiographically documented disease progression; the occurrence of unacceptable toxicity; failure to recover from hematological and/or non-hematological toxicity despite a dosing delay of up to 14 days; medical or ethical reason, including noncompliance and pregnancy (following discussion between the investigator and sponsor); and/or patient's request or investigator's recommendation. Discontinuation of treatment for non-medical reasons (e.g. bankruptcy) was not mentioned in the protocol or contract.

Eligibility criteria

The inclusion and exclusion criteria were histologically or cytologically confirmed malignancy that was advanced and/or metastatic and refractory to established forms of therapy or for which no effective therapy exists with curative intent; age 18 years or older; an Eastern Cooperative Oncology Group Performance status (ECOG PS) ≤ 2; left ventricular ejection fraction (LVEF) (by echocardiography) > 55% and no uncontrolled ischemic heart disease; a predicted life expectancy of at least 3 months and adequate hematopoietic, hepatic, cardiac, renal, and thyroid function; no sign of arrhythmias or conduction abnormalities; normal electrolytes; not taking any of the following medication: digoxin, digitoxin or molsidomin, and agents with similar mode of action.

Study procedures

Patients received UNBS1450 intravenously once every 3 weeks via central or peripheral intravenous line. The dose was administered over a 1 h infusion, but the actual infusion time could be adapted depending on clinical signs.

Eleven PK samples were collected on day 1 during cycles 1 and 3, between 0 and 5 h after start of infusion (for a 1 h infusion time: prior to dosing, and 20, 40, 60 (just before end of

infusion), 65, 70, 75, 80, 90, 105, and 4 h post start of infusion). Blood samples were taken prior to drug administration on day 1 of cycles 2 and 4.

As this was the first UNBS1450 exposure to humans, the most suitable PD parameters were used during the trial, based on findings made while treating and observing the patients. If possible, the pharmacodynamic biomarker c-MYC and other upstream or downstream markers of the pathway would be assessed in tumor samples and any changes related to PK and clinical outcome.

Blood counts and clinical chemistry (including serum Na^+, K^+, Ca^{2+}, Mg^{2+}, liver function tests, bilirubin, creatinine, blood urea nitrogen (BUN), alkaline phosphatase, total protein, albumin, blood glucose), CBC (complete blood count), and thyroid stimulating hormone (TSH) were obtained at baseline, predose on day 1 (if > 7 days after baseline), and once weekly. Creatine kinase (including MB isoenzyme analysis) and troponin I were also assessed at baseline, predose on day 1, on day 2, and at least weekly on every cycle. Urinalysis were obtained at baseline and repeated predose on day 1 of each cycle. Electrocardiograms (ECGs) were performed at baseline, during infusion, up to 2-4 h post dose on day 1 of each and on day 2 at 24 h post dose, prior to dosing on day 22, and at last study visit. Echocardiography was performed at baseline, on day 22, at end of dosing, and 15 days after last cycle. Echocardiography was assessed by a cardiologist. Physical exams were performed every 3 weeks.

UNBS1450 were supplied in injectable, ready for use, clear glass vials. Each vial contained 10.5 ml (including overfill) with 10 µg UNBS1450/ml of saline solution. The amount of UNBS1450 present per vial was 100 µg. Dosing of UNBS1450 was reduced and/or interrupted for any hematological and non-hematological toxicities related to UNBS1450. Treatment for all patients was repeated provided they reached pre-specified hematological and non-hematological recovery levels (e.g., absolute neutrophil count (ANC) 1.5×10^9/L; platelet count 100×10^9/L; non-hematological toxicity ≤ common toxicity criteria (CTC) grade 1 or ≤ 1 grade worse than baseline severity, etc.). If adequate recovery of these levels was not achieved at time of next dose, dosing was postponed until they were reached. Intra-patient dose escalation was not permitted. Dosing was interrupted for any patient with heart rate-corrected QT (QT_c) ≥ 470 msec during any ECG. Provided there was no significant cardiac toxicity, dosing might resume at the next lower dose level when QT_c has decreased to ≤ 440 msec. Any patient with persistent QT_c > 470 msec for more than 1 day (confirmed by a follow-up 10-sec ECG on the next day) was withdrawn. Dosing was stopped if there was any development of clinically significant cardiac arrhythmia or an absolute decrease

of ≥ 10% in the LVEF from baseline. Once a patient's dose was reduced for a drug-related toxicity, the dose was not re-escalated.

Criteria for evaluation

Safety was assessed via physical examination, vital signs, clinical laboratory tests (CBC, clinical chemistry, urinalysis), ECG, echocardiography, and adverse events.

Response assessments (physical examination, CT scan, etc.) were performed every 2 cycles and evaluated according to RECIST version 1.0.[20] Plasma concentration versus time profiles of UNBS1450 was obtained from the analysis of plasma samples. PK parameters were calculated for each subject. Parameters included AUC_{last}, area under the curve extrapolated from 0-∞ ($AUC_{0-\infty}$), C_{max}, %AUC extrapolated, half-life alpha ($T_{1/2}$ alpha) and $T_{1/2}$ beta, clearance (Cl), volume of distribution (V_z) and time of maximum plasma drug concentration (T_{max}). A standard 3 + 3 dose phase I dose escalation scheme was used. Pharmacokinetic parameter estimates were summarized by dose cohort using descriptive statistics: N, mean, median, minimum, maximum. In addition, geometric means with 90% confidence intervals were calculated for $AUC_{0-\infty}$, AUC_{0-t}, C_{max}, drug concentration at 4 h post initiation of drug infusion (C_{4h}).

RESULTS OF THE CLINICAL TRIAL

From October 2008 to October 2010 23 patients were enrolled into seven cohorts in this study in two investigational sites in Belgium and the Netherlands. Patients in cohorts 1 to 7 received single doses of 90, 140, 210, 265, 350, 465 and 615 µg/patient of UNBS1450 as a 60 min i.v. infusion respectively. Two patients, included in the 23 patients, had to be replaced after cycle 1 drug administration owing to non drug-related adverse events. Additionally, two patients in each of cohorts 2, 3 and 5 and one patient from cohorts 4, 6 and 7 have completed 3 cycles of compound administration (a cycle = one administration every 3 weeks).

In October 2010 the study was closed by the sponsor because of financial reasons. Because of this, MTD was not reached. Not enough data for a response evaluation were available because of the sudden end of the trial.

PK results

Given the very short half-life of UNBS1450 determined in the first three cohorts, sampling time points were revised a first time from cohort 4 in order to get more usable data at the early times post administration, and for a second time for cohort 7 where only the last time point during the infusion was changed (from 60 to 55 min). Accordingly, blood samples from cohort 7 were taken predose, at 20, 40 and 55 min during the infusion, and then at 5, 10, 15, 20, 30, 45 and 180 min post end of infusion while for cohorts 1-3, they were taken predose, at 20 and 40 min during the infusion, immediately before the end of the infusion (60 min) and then at 5, 15, 45, 90, 180, 300 and 560 min post infusion. Corresponding plasma samples were analyzed using a validated Liquid Chromatography - Mass Spectrometry and Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) method with a lower limit of quantification (LLOQ) of 0.1 ng/mL at the CRO Notox. As a result of the change in the sampling time points, it was believed more robust PK parameters would have been determined since cohort 4. However, certain calculated PK parameters notably clearance and volume of distribution should still be considered preliminary estimates given the compound's short half-life and inability to follow drug concentrations in plasma generally beyond 0.75 h post end of infusion, despite an appreciably sensitive quantitative method. Furthermore, given the compound's short half-life, C_{max} and T_{max} values were likely to be very sensitive to even small errors around sampling times. PK parameters were been determined using a non-compartmental analysis model (Table 9.1).

As explained previously, in this clinical study for the overwhelming majority of patients across all dose groups, T_{max} has been surprisingly observed earlier than the end of infusion. It had been postulated that this could be due to problems of drug delivery attributable to the infusion pump potentially exacerbated by the extremely short half-life of the compound. Consequently, a change in PK sampling time points was proposed for cohort 7, namely the 60 min post start of infusion sampling time being changed to 55 min, in order to avoid possible sample collection after the infusion had been completed. Unfortunately, this change did not bring the expected outcome, as certainly in two individuals T_{max} was again observed before the end of infusion at 40 min.

There appeared an approximately linear relationship with dose for both mean C_{max} and AUC_{last} values over the dose range 90-615 µg/patient. However, at 465 µg/patient, mean C_{max} and AUC_{last} values were lower than might have been expected and only marginally increased over corresponding values determined in cohort 5 dosed at 350 µg/patient (Figure 9.1). Mean C_{max} and AUC_{last} values from cohort 7 dosed at 615 µg/patient however

Table 9.1 Summary of PK data obtained after 1 cycle

Pharmacokinetics of UNBS1450 (mean ± SD, t_{max}: median (range))	Cycle 1						
	A single dose of 90 µg UNBS1450	**A single dose of 140 µg UNBS1450**	**A single dose of 210 µg UNBS1450**	**A single dose of 265 µg UNBS1450**	**A single dose of 350 µg UNBS1450**	**A single dose of 465 µg UNBS1450**	**A single dose of 615 µg UNBS1450**
n	3	3	3	4[e]	3	4	3
C_{4h} (ng/mL)	0 ± -	0 ± -	0 ± -	0 ± -	0 ± -	0 ± -	0 ± -
C_{max} (ng/mL)	0.752 ± 0.07305	1.089 ± 0.2972	1.733 ± 0.9001	2.544 ± 0.295	4.211 ± 1.316	4.299 ± 0.9800	8.078 ± 0.6890
T_{max} (h)	0.67 (0.33-0.67)	0.67 (0.67-0.67)	0.33 (0.33-0.67)	0.67 (0.33-0.67)	0.67 (0.67-1.00)	0.67 (0.33-1.00)	0.67 (0.67-1.08)
AUC_{last} (ng·h/mL)	0.6492 ± 0.07965	0.8366 ± 0.2462	1.448 ± 0.6409	2.133 ± 0.1093	3.627 ± 1.003	3.886 ± 1.217	5.396 ± 1.133
$AUC_{0-\infty}$ (ng·h/mL)	NA	NA	NA	NA	NA	NA	NA
λ_{alpha} (1/h)	6.751-7.118[c]	4.883[d] ± 2.435[d]	8.317-9.130[c]	10.21 ± 4.470	7.890[d] ± 3.999[d]	7.561 ± 2.547	5.812 ± 3.166
$t_{1/2alpha}$ (h)	0.09738-0.1027[c]	0.1640[d] ± 0.06730[d]	0.07592-0.08334[c]	0.0846 ± 0.05313	0.1019[d] ± 0.04218[d]	0.09994 ± 0.03287	0.1408 ± 0.05979
λ_{beta} (1/h)	NA	NA	NA	1.978[d] ± 0.3999[d]	NA	NA	NA
$t_{1/2beta}$ (h)	NA	NA	NA	0.3616[d] ± 0.08244[d]	NA	NA	NA
CL (L/h[a])	125.1-127.9[c]	167.3[d]-47.65[d]	96.17-169.2[c]	123.5 ± 7.001	100.6 ± 26.65	128.2 ± 40.16	116.6 ± 25.61
V_z (L[a])	17.57-18.94[c]	42.20[d] ± 27.16[d]	10.53-20.35[c]	14.75 ± 8.532	15.50 ± 8.265	18.77 ± 9.597	24.32 ± 13.89
CL (L/h[b])	NA	NA	NA	117.5[d] ± 3.822[d]	NA	NA	NA
V_z (L[b])	NA	NA	NA	60.97[d] ± 11.73[d]	NA	NA	NA

C^{4h} drug concentration at 4 h post initiation of drug infusion; C_{max} maximum plasma drug concentration; T_{max} time of maximum plasma drug concentration; AUC_{last} area under the curve 0-t where t is last time at which drug was quantifiable; $AUC_{0-\infty}$ area under the curve extrapolated from 0-∞; $t_{1/2}$ half-life; CL total drug clearance; V_z Elimination phase volume of distibution; NA not assessable; 0 = NQ = not quantifiable (< 0.100 ng/mL); [a] calculation based on λ_{alpha}; [b] calculation based on λ_{beta}; [c] *n* = 2, individual values reported; [d] accurate determination not possible; [e] *n* = 3 for λ_{beta}, $t_{1/2beta}$, CL_{beta} and $V_{z\,beta}$

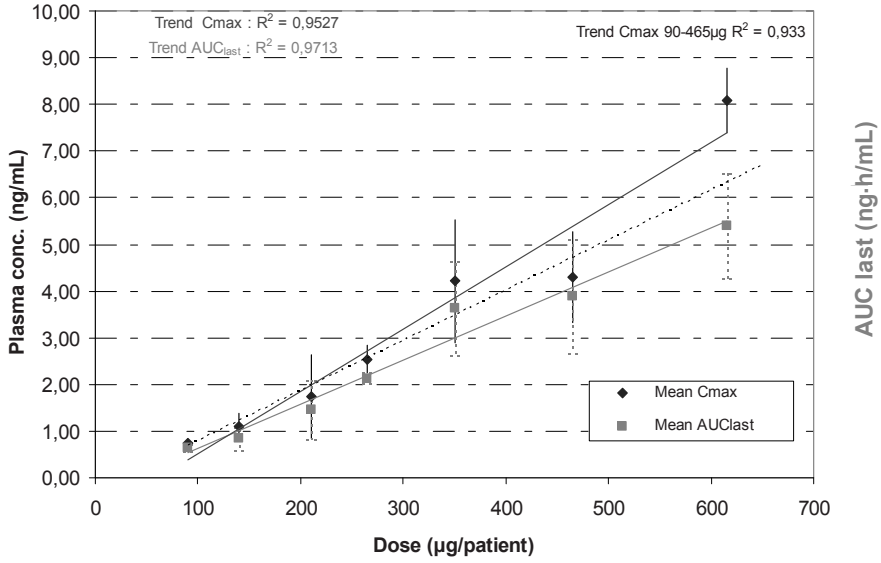

Figure 9.1 Mean C_{max} and AUC_{last} values versus dose.

appear to indicate that any previous suggestion that systemic exposure at 465 µg/patient may have plateaued and showed non-linearity was not the case, and was likely due to the limited data forcing the comparison of small size dose groups of different individuals (*n* = 3 or 4) who have received doses not corrected for body surface area.

However, when dose normalized individual C_{max} and AUC_{last} values were compared, there appeared to be a slight trend for a disproportional increase in both these parameters with increasing dose over the range 90-615 µg/patient (Figures 9.2 and 9.3).

In vitro evaluation of the minimum exposure time to UNBS1450 required to trigger the commitment phase of apoptosis

To determine the time required for UNBS1450 to trigger the commitment phase of apoptosis, we exposed U937 cells to 20 nM UNBS1450 (a concentration we previously reported as apoptogenic).[21] Cells were incubated for different times, then, the treatment was washed out and cells were resuspended in fresh medium for recovery. Apoptosis was estimated respectively at 24 and 48 h (as described in Material and methods). Figure 9.4 (panels A-B) shows that a treatment time > 8 h is required to trigger apoptosis, with a more relevant accumulation of apoptotic cells starting from 10-12 h of continuous treatment. The Western

blot analysis of caspase-9 and -3 cleavage further confirmed the results got by estimating apoptosis by two different consolidated approaches, as the analysis of nuclear morphology and fragmentation and the loss of mitochondrial membrane potential (Figure 9.4C). The difference with the positive control was not due to a delay in apoptosis: the same analysis

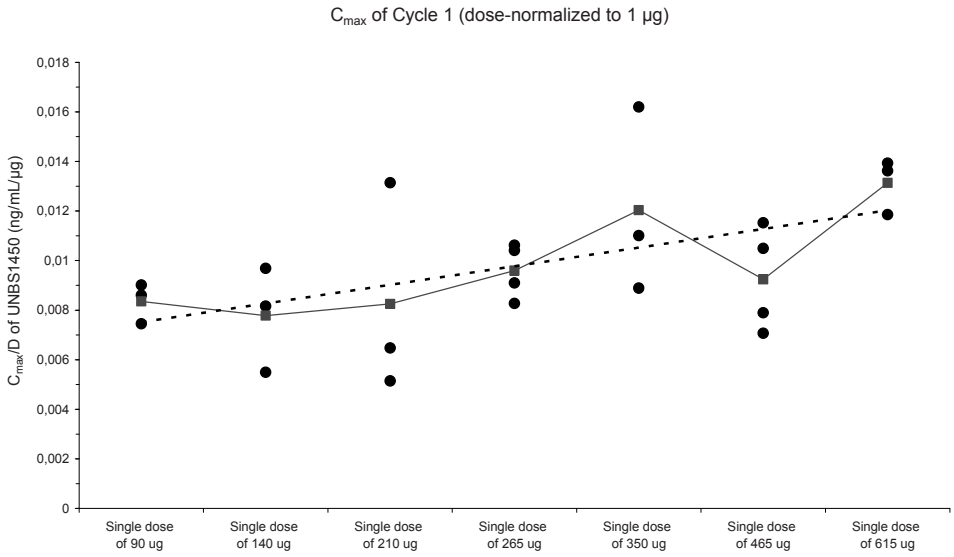

Figure 9.2 Dose normalized C_{max} versus dose group.

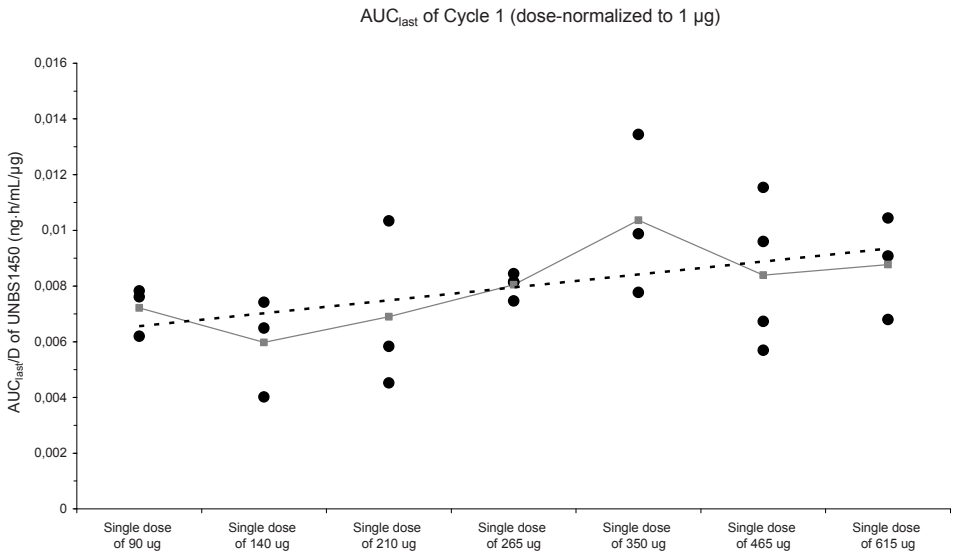

Figure 9.3 Dose normalized AUC_{last} versus dose group.

performed after 48 h did not show any accumulation of apoptotic cells in samples exposed to washout experiments.

We have identified Mcl-1 protein as the earliest Bcl-2 protein targeted by UNBS1450 in U937 cells.[21] The findings so far refer to a continuous treatment of the cells with 20 nM

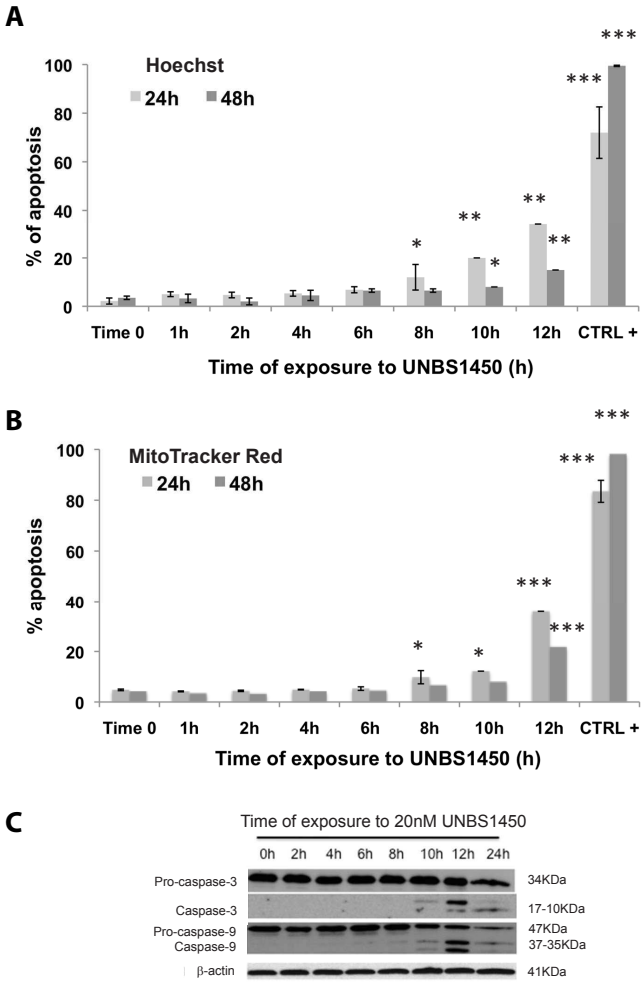

Figure 9.4 Washout experiments on U937 cells. U937 cells were treated at T_0 with 20 nM UNBS1450. Then at the indicated times, the compound was removed and cells were resuspended in fresh medium. At T_0 + 24 h and T_0 + 48 h, the impact on cell viability of the different times of exposures to UNBS1450 was evaluated by considering **(A)** the nuclear fragmentation as assessed by Hoechst staining and fluorescence microscope observation; **(B)** the mitochondrial membrane potential as analyzed by MTR staining and FACS analysis. **(C)** The induction of apoptosis was further confirmed by caspase-3 cleavage. The results are the mean of three independent experiments or representative of three experiments. Significant difference compared to untreated cells: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

UNBS1450.[21] Next, we explored Mcl-1 protein status during washout experiments with UNBS1450 in U937 cells. The Western blot analysis reported in Figure 9.5 shows Mcl-1 protein levels as estimated after 24 h from the start of treatment with 20 nM UNBS1450 (T_0; see also Materials and methods). Mcl-1 appeared down-regulated with the complete disappearance of the protein at times > 12 h.

Since the treatment with UNBS1450 was performed with cell culture medium containing 10% FCS, a percentage which may sequester and, therefore, limit the cytotoxic activity of UNBS1450, we cultured U937 cells in medium with different concentrations of FCS (0.1-10%) in the presence of UNBS1450. Then, U937 cells were cultured again in fresh medium with 10% FCS during the recovery phase. Concentration and time of exposure to UNBS1450 (10 nM; 1 h) were chosen to mimic the concentration used in patients treatment and the turnover of the compound into the body as emerging from the clinical trials. After 24 h and 48 h, the analysis of apoptosis excluded any relevant impact of UNBS1450 on U937 cell viability (data not shown).

Next, we wanted to investigate whether in the same conditions, a cytostatic effect might take place. When the challenge with UNBS1450 was performed in a medium containing 0.1% FCS, we witnessed a significant reduction of the cell concentration in UNBS1450 treated versus untreated cells at 24 h as well as after 48 h of recovery (Figure 9.6A-B). To ascertain whether this reduction effectively corresponded to a reduced cell growth at both time points of recovery, we calculated the 24 h/0 h and 48 h/24 h (of recovery) cell proliferation ratio, which is directly proportional to the doubling time of the cells. The analysis revealed that the cytostatic effect was limited to the early 24 h of recovery, whereas at longer times the rate of cell proliferation was completely restored.

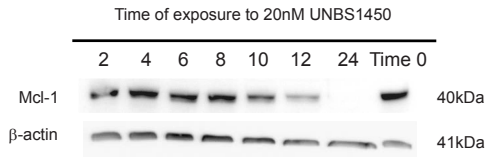

Figure 9.5 During washout experiments the decrease of Mcl-1 protein fits with the commitment to apoptosis. U937 cells were treated at T_0 with 20 nM UNBS1450. Then at the indicated time points, the compound was removed and cells resuspended in fresh medium. At T_0 + 24 h, the impact on Mcl-1 of the different exposures times to UNBS1450 was evaluated by Western blot analysis. The results are representative of three independent experiments with comparable results.

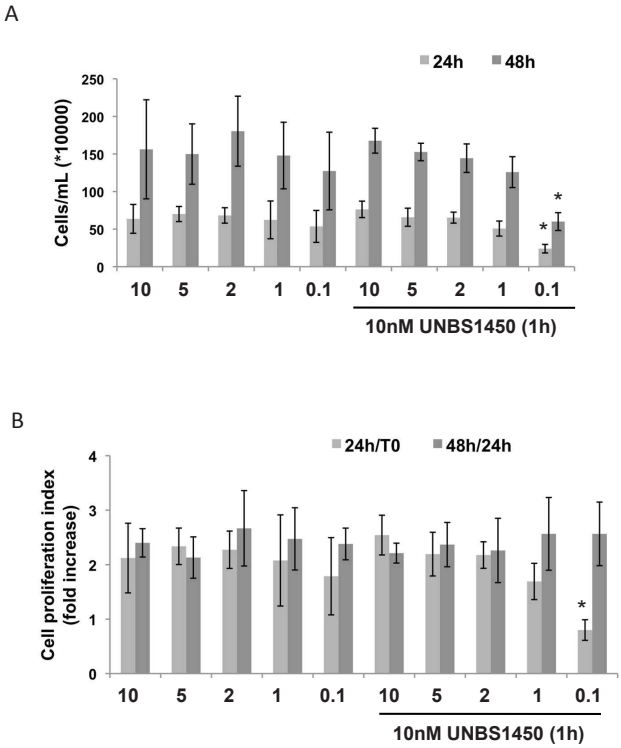

Figure 9.6 Analysis of the impact of FCS on the cytostatic effects of UNBS1450. U937 cells were treated for 1 h with 10 nM UNBS1450 in a medium containing the percentage of FCS indicated in the panels. Then at the indicated times, the compound was removed and cells were resuspended in 10% FCS fresh medium (T_0) for recovery. **(A)** At T_0 + 24 h (24 h) and T_0 + 48 h (48 h) the cell concentration was estimated by Trypan Blue exclusion assay as described in Material and methods. **(B)** Cell proliferation index between T_0 + 24 h and T_0 (24 h/T_0; light grey bars); T_0 + 48 h and T_0 + 24 h (48 h/24 h; dark grey bars). The results are the mean of three independent experiments +/- SD. Significant difference compared to untreated cells: * $P < 0.05$.

DISCUSSION

This study was designed to translate preclinical evidence into a clinical phase I study evaluating the safety, tolerability and pharmacokinetics of UNBS1450 administered once every three weeks in separate cohorts of patients with advanced solid tumors or lymphoma not amenable to established forms of therapy with curative intent. The selected route of administration was intravenous because the compound was poorly available when given orally. The intravenous route is also the safest for such a potentially cardiotoxic compound

as it enabled to perform close cardiac monitoring in each patient who would enter this phase I protocol.

Preclinical pharmacology studies using *in vitro* and animal models indicate that UNBS1450 is characterized by marked anticancer activity due to both anti-proliferative and anti-migratory (anti-metastatic) features resulting from the propensity of UNBS1450 in disorganizing the actin cytoskeleton, which leads to cell death through autophagy, rather than through apoptosis. *In vitro*, UNBS1450 kills apoptosis-resistant cancer cells, including multidrug resistant cancer cells. UNBS1450 belongs to the same chemical family as digoxin, a cardiotonic steroid used to treat congestive heart failure. Cardiotonic steroid receptors relate to the α subunits of the sodium pump (the Na^+/K^+-ATPase). The α-1 subunit of the sodium pump is overexpressed in 30-40% of a large set of solid cancers, including gliomas, melanomas, renal cell carcinomas, non-small cell lung cancers, and colon cancers. Overexpression of the sodium pump α-1 subunit is also suspected in breast, prostate, and head and neck cancers. The therapeutic ratio with respect to the safety profile/antitumor activity of digoxin is too weak to be used as a potential anticancer agent. On the contrary, UNBS1450 displays tenfold higher binding affinity for the α-1 subunit of the sodium pump than digoxin. With a toxicity profile similar to digoxin, UNBS1450 shows an antitumor activity at least 10 times more pronounced, designating it as a potential candidate for clinical application in oncology, especially where no effective curative therapy exists as it is the case for advanced and/or refractory prostate, breast, non-small cell lung, colon and renal cancers, and for melanomas and glioblastomas. This compound could also find potential use in the treatment of metastatic cancers, knowing that 90% of cancer patients die today from their metastases.

Because of financial reasons this phase I study was closed before reaching the MTD, so it is not possible to establish the recommended phase II dose of UNBS1450. It has to be emphasized again that with such a very short life compound, timing deviations around the sampling time can have a big impact on C_{max}/T_{max} values, while the limited drug concentration-time profile post end of infusion make calculation of accurate pharmacokinetic parameters difficult.

UNBS1450 requires treatment times > 8 h to significantly induce apoptosis in the U937 cancer cell model, when used at the apoptogenic concentration of 20 nM. Mcl-1, which we identified previously as an anti-apoptotic protein early affected by UNBS1450 resulted impacted within the same time required for committing cells to the death.[21] The low concentrations required to affect cancer cells (in the range of nanomolar concentrations) and the fact that UNBS1450 appears to be particularly active on Mcl-1, an intracellular

molecular target currently at the center of many investigations to find out new anticancer therapeutics prompts to explore in the future any further strategies based on the use of this cardiac glycoside in targeting Mcl-1 as potential suitable approach in clinics to fight many forms of cancer.

The percentage FCS does not exert any specific impact on UNBS1450 apoptogenic properties, when used for 1 h at 10 nM. UNBS1450 exerts a cytostatic effect on U937 cells during the first 24 h of recovery when cells are treated in 0.1% FCS medium.

These results may be the base to evaluate specific protocols of administration concerning the number of applications and the lag time between one administration and another, therefore about relevance of tempting different protocols of cycles of treatment alternated to recovery phase.

Moreover, although the absence of a direct impact on cell viability when challenging cells for 1 h with 10 nM of the compound, it would be worth to evaluate whether UNBS1450 may sensitize U937 cells to further cytocidal treatment in combination experiments with known chemotherapeutic agents. Alternatively, it would be considered to evaluate whether UNBS1450 may sensitize U937 cells to further cytostatic treatment in combination experiments with known cytostatic agents. Both assays may provide information about any potential chemoadjuvant activity of this compound, which remains to be determined.

Looking back, we realize that the preclinical work was not extensive enough to start with this phase I trial. Based on washout experiments we think the optimal dose may have been much higher and the optimal schedule more intensively. Because leukemia cells seem to be the most sensitive cells we suggest that a followup phase I study is done in patients with hematological malignancies.

ACKNOWLEDGMENTS

Florian Muller is supported by the Marian Aldred award from Think Pink Luxembourg. Claudia Cerella is supported by a Waxweiler grant for cancer prevention research from the Action Lions Vaincre le Cancer. Research at Laboratory of Molecular and Cellular Biology of Cancer is financially supported by the Fondation de Recherche Cancer et Sang, the Recherches Scientifiques Luxembourg association, the Een Häerz fir kriibskrank Kanner association, the Action Lions Vaincre le Cancer association, the European Union and the Télévie Luxembourg. Marc Diederich is supported by the National Research Foundation by the Ministry of Science and Technology of Korea for Tumor Microenvironment Global

Core Research Center grant, by the Seoul National University (SNU) Research grant and by the Research Settlement Fund for the new faculty of the SNU.

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10

Summary and future perspectives

In this chapter the reported studies and reviews presented in this thesis are summarized.

The first part of this thesis focuses on some novel formulations, especially camptothecin glycoconjugate BAY 56-3722 (formerly BAY 38-3441) and liposomal drug formulations. The second part of this thesis focuses on histone deacetylase (HDAC) inhibitors and cardiac glycosides, especially UNBS1450. **Chapter 1** gives a general introduction and describes the general aim of this thesis to explore some novel formulations and new classes of anticancer drugs in solid tumors. It also describes the outline of the thesis.

NOVEL FORMULATIONS

Liposomal drug formulations

In **Chapter 2**, a review of liposomal anticancer drugs is presented. The main advantages, 1) improved pharmacokinetics and drug release; 2) enhanced cellular penetration; 3) tumor targeting and 4) multi-ingredients systems, and an up-to-date overview of the current clinical development are discussed. Furthermore, some liposome-specific adverse effects such as various skin reactions, and also hypersensitivity reactions, are described. We concluded that further studies with liposome-encapsulated anticancer drugs, including the development of novel liposomal formulations, are warranted to provide evidence for increased efficacy and tolerability as compared with their non-liposomal counterparts.

A dose-escalating phase I study of LiPlaCis, a liposomal formulated platinum compound, in patients with advanced solid tumors is reported in **Chapter 3**. This phase I dose-escalating study was conducted to define the maximum tolerated dose (MTD), the recommended phase II dose, pharmacokinetics and pharmacodynamics, as well as the preliminarily antitumor effects of a three-weekly schedule of LiPlaCis in patients with solid tumors. Although the toxicity pattern of LiPlaCis differed from cisplatin toxicity, renal damage was not prevented by the formulation. Acute infusion reactions required addition of extensive premedication that in turn could not completely prevent a high incidence of acute infusion reactions. The observed safety profile suggested no benefit over standard cisplatin formulations and LiPlaCis reformulation is required to enable further development. Recently a new phase I dose-escalating study with LiPlaCis started to find the recommended phase II dose.

In **Chapter 4** a randomized, clinical bioequivalence study comparing the pharmacokinetics and safety of liposome-entrapped paclitaxel easy-to-use (LEP-ETU) formulation versus paclitaxel in Cremophor® EL (Taxol®) in patients with advanced cancer is reported.

Our objectives were to (1) to determine bioequivalence of paclitaxel pharmaceutically formulated as LEP-ETU and as Taxol® and (2) to assess the safety and tolerability of LEP-ETU following intravenous administration. Thirty two of the 58 patients were evaluable patients and were analyzed for bioequivalence. The number of patients that dropped out of the study was concerning high. This high drop-out rate was most likely due to a poor patient selection. Mean total paclitaxel C_{max} values for LEP-ETU and Taxol® were 4955.0 ng/mL and 5108.8 ng/mL, respectively. Mean total paclitaxel $AUC_{0-\infty}$ values for LEP-ETU and Taxol® were 15853.8 ng·h/mL and 18550.8 ng·h/mL, respectively. Ratios of the geometric means of LEP-ETU divided by Taxol® for C_{max} were 97% (90% CI, 91%-103%) and for $AUC_{0-\infty}$ were 84% (90% CI, 80%-90%). These results meet the required 80-125% bioequivalence criteria. The most frequently reported adverse events after LEP-ETU administration were fatigue, alopecia, and myalgia.

BAY 56-3722

Chapter 5 describes a phase II study of BAY 56-3722 (formerly BAY 38-3441), a camptothecin glycoconjugate, in patients with recurrent or metastatic inoperable colorectal cancer resistant to irinotecan. Patients received BAY 56-3722 i.v. 320 mg/m^2 daily for 3 days every 3 weeks. Twenty-four patients received the study treatment. Triggered by adverse events in two other studies with this compound the study was put on a clinical hold while the safety data were reviewed for the entire program. We felt it was our obligation to report the fate of BAY 56-3722 and the unique situation of a clinical hold during a phase II study.

NEW CLASSES OF ANTICANCER DRUGS

HDAC inhibitors

The HDAC inhibitors recently are being investigated as possible treatments for cancer. The HDAC inhibitors are a group of targeted agents which are characterized as class I-specific or as pan-deacetylase (pan-DAC) inhibitors, which show activity against both classes I and II HDACs. Two of them, vorinostat and romidepsin, are already approved by the US Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL). Romidepsin is also approved for the treatment of peripheral T-cell lymphoma (PTCL). Much research was focused on the treatment of hematological malignancies, but the last decade also clinical trials with HDAC inhibitors in solid tumors were conducted. Despite promising results in the treatment of hematological malignancies, HDAC inhibitors have generally not

been effective in clinical trials involving solid tumors. In **Chapter 6**, a review of the clinical trials in solid tumors of HDAC inhibitors is presented.

In **Chapter 7** a phase I study to evaluate the pharmacokinetics and safety of oral panobinostat in patients with advanced solid tumors and various degrees of hepatic function is reported. This study demonstrated the impact of hepatic impairment on the systemic exposure of panobinostat and showed that patients with mild or moderate hepatic function could be safely treated with the same dose of panobinostat as patients with normal hepatic dysfunction, despite somewhat higher pharmacokinetic values.

Cardiac glycosides

Besides novel formulations, also 'old drugs' for example cardiac glycosides, could be useful in the treatment of cancer. Cardiac glycosides have a long history in the treatment of cardiac disease. However, several preclinical studies as well as two phase I studies have shown that cardenolides may also possess anticancer effects. The mechanisms of these anticancer effects may include intracellular decrease of K^+ and increase of Na^+ and Ca^{2+}; intracellular acidification; inhibition of IL-8 production and of the TNF-α/NF-κB pathway; inhibition of DNA topoisomerase II and activation of the Src kinase pathway. In **Chapter 8** a review of cardiac glycosides in cancer therapy is presented. To date only three cardiac glycosides have been developed for treatment of cancer and were tested in a phase I clinical trial to determine dose-limiting toxicities and maximum tolerated dose.

Chapter 9 reports the preclinical data and the preliminary results of a non-completed clinical phase I trial with UNBS1450, a semisynthetic cardenolide glycoside derivative. The primary endpoint of the clinical phase I was not reached due to early termination of the study for non-scientific reasons. The available preclinical work could not guide us in adapted scheduling of the patients. To establish the optimal dose and schedule of UNBS1450 for future phase I/II studies more research is necessary.

CONCLUSION

Many current anticancer drugs have non-ideal pharmaceutical and pharmacological properties, which can lead to adverse consequences, including lack of or suboptimal therapeutic activity, dose-limiting side effects and poor patient quality of life. In this thesis we focused on some novel formulations, especially camptothecin glycoconjugate BAY 56-3722 (formerly BAY 38-3441) and liposomal drug formulations, hoping to overcome some of

these problems. We also focused on 'old drugs' for new indications, as an example HDAC inhibitors and cardiac glycosides.

Unfortunately, the outcomes of some of the presented studies were disappointing: a clinical hold during the phase II study of BAY 56-3722, no benefit of LiPlaCis over standard cisplatin formulations and a non-completed clinical phase I trial with UNBS1450.

It is known that many phase I and phase II trials do not result in new treatment options used in daily practice. It is also known that publishing negative trial results is seen as less attractive and is also more difficult than publishing positive trial results. But sharing these results is essential for improving the knowledge necessary for the development of future research by the scientific community. For example a new phase I dose-escalating study with LiPlaCis started because in our phase I study a recommended dose for a phase II study was never reached which is now the aim of this phase I dose-escalating study. In addition, based on the preclinical evaluation and preliminary report of the incomplete phase I pharmacokinetic trial using UNBS1450 we now know that based on washout experiments the optimal dose may have been much higher and the optimal schedule more intensively.

Beside the disappointing outcomes of some of the presented studies, we demonstrated that LEP-ETU and Taxol® met the required 80-125% bioequivalence criteria and we showed that patients with mild or moderate hepatic function could be safely treated with the same dose of panobinostat as patients with normal hepatic function.

The reviews of liposomal anticancer drugs, HDAC inhibitors and cardiac glycosides all showed that to fulfill the high expectations of all these formulations and new drugs and to overcome the existing problems much research is still necessary.

11

Samenvatting en toekomstperspectief

In dit hoofdstuk worden de beschreven studies en reviews van dit proefschrift samengevat en bediscussieerd. Het eerste deel van het proefschrift concentreert zich op nieuwe formuleringen, in het bijzonder camptothecine glycoconjugaat BAY 56-3722 (voorheen BAY 38-3441) en liposomale formuleringen. Het tweede deel van dit proefschrift richt zich op histone deacetylase (HDAC) remmers en cardiale glycosiden, in het bijzonder UNBS1450. **Hoofdstuk 1** geeft een algemene inleiding en beschrijft het doel van het proefschrift, het onderzoeken van enkele nieuwe formuleringen en nieuwe klassen van antikankermedicatie in solide tumoren. Het beschrijft eveneens de indeling van het proefschrift.

NIEUWE FORMULERINGEN

Liposomale antikankermedicatie

In **Hoofdstuk 2** wordt een review over liposomale antikankermedicatie gepresenteerd. De belangrijkste voordelen, 1) het verbeteren van de farmacokinetiek en het beschikbaar maken van het medicament; 2) het vergroten van de cellulaire penetratie; 3) het doelgericht benaderen van de tumor en 4) de mogelijkheid meerdere medicamenten tegelijkertijd toe te dienen, worden bediscussieerd, evenals een overzicht van de actuele klinische ontwikkelingen. Daarnaast beschrijven we enkele liposoomspecieke bijwerkingen zoals huidreacties en overgevoeligheidsreacties. We concludeerden dat er meer studies met liposomale antikankermedicatie, inclusief het ontwikkelen van nieuwe liposomale formuleringen, nodig zijn om te bewijzen dat deze middelen ten opzichte van niet-liposomale middelen effectiever zijn en minder bijwerkingen hebben.

In **Hoofdstuk 3** wordt een dosis-escalatie fase I studie met LiPlaCis, een liposomale formulering van cisplatin, bij patiënten met vergevorderde solide tumoren beschreven. Deze fase I dosis-escalatie studie werd uitgevoerd bij patiënten met solide tumoren om de maximum getolereerde dosis (MTD), de aanbevolen fase II dosis, de farmacokinetiek en farmacodynamiek en antitumoreffecten van driewekelijks LiPlaCis vast te stellen. Hoewel het toxiciteitsprofiel van LiPlaCis verschilt van dat van cisplatin, werd renale schade niet voorkomen met deze formulering. Ondanks toediening van uitgebreide premedicatie kon een hoge incidentie van acute infusiereacties niet geheel worden voorkomen. Het geobserveerde veiligheidsprofiel liet geen voordelen zien ten opzichte van standaard cisplatin en herformulering van LiPlaCis is noodzakelijk om verdere ontwikkeling mogelijk te maken. Recent is een nieuwe fase I dosis-escalatie studie met LiPlaCis gestart om de aanbevolen fase II dosering vast te stellen.

In **Hoofdstuk 4** wordt een gerandomiseerde, klinische bio-equivalentiestudie beschreven, die de farmacokinetiek en veiligheid van liposomaal paclitaxel (liposome-entrapped paclitaxel easy-to-use (LEP-ETU)) vergelijkt met die van paclitaxel in Cremophor® EL (Taxol®) bij patiënten met vergevorderde kanker. Ons doel was om (1) de bio-equivalentie vast te stellen van paclitaxel geformuleerd als LEP-ETU en Taxol® en (2) de veiligheid en tolereerbaarheid van LEP-ETU vast te stellen na intraveneuse toediening. Tweeëndertig van de 58 patiënten werden geëvalueerd en geanalyseerd ter beoordeling van bio-equivalentie. Het aantal patienten dat uitviel in de studie was zorgwekkend hoog. Meest waarschijnlijk was dit het gevolg van een slechte patiëntenselectie. De gemiddelde totale paclitaxel C_{max} waarden voor LEP-ETU en Taxol® waren resepectievelijk 4955.0 ng/mL en 5108.8 ng/mL. De gemiddelde totale paclitaxel $AUC_{0-\infty}$ waarden voor LEP-ETU en Taxol® waren respectievelijk 15853.8 ng·h/mL en 18550.8 ng·h/mL. De verhoudingen van het geometrische gemiddelde van LEP-ETU gedeeld door Taxol® voor C_{max} waren 97% (90% CI, 91%-103%) en voor $AUC_{0-\infty}$ 84% (90% CI, 80%-90%). Op basis van de vereiste 80-125% bio-equivalentiecriteria kon geconcludeerd worden dat beide formuleringen bio-equivalent zijn. De meest frequente bijwerkingen die werden gezien na toediening van LEP-ETU waren vermoeidheid, alopecia en myalgie.

BAY 56-3722

Hoofdstuk 5 beschrijft een fase II studie van BAY 56-3722 (voorheen BAY 38-3441), een camptothecine glycoconjugaat, bij patiënten met terugkerend of gemetastaseerd inoperabel colorectaal carcinoom refractair voor irinotecan. Patiënten werden elke 3 weken gedurende 3 dagen dagelijks behandeld met BAY 56-3722 i.v. 320 mg/m^2. Vierentwintig patiënten namen deel aan de studie. In verband met bijwerkingen in 2 andere studies met hetzelfde middel werd de studie voortijdig beëindigd. We voelden het als onze verplichting om deze studie te publiceren om het lot van BAY 56-3722 te beschrijven, evenals de unieke situatie van het voortijdig beëindigen van een fase II studie.

NIEUWE KLASSEN VAN ANTIKANKERMEDICATIE

HDAC-remmers

De HDAC-remmers zijn recent onderzocht als mogelijke middelen in de behandeling van kanker. De HDAC-remmers zijn een groep van doelgerichte middelen die gekarakteriseerd worden als klasse I specifieke remmers of als pan-deacetylase (pan-DAC) remmers, die zowel tegen klasse I als II HDACs actief zijn. Twee HDAC-remmers, te weten vorinostat en romi-

depsine, zijn reeds goedgekeurd door de US Food and Drug Administration (FDA) voor de behandeling van het cutane T-cel lymfoom (CTCL). Romidepsine is eveneens goedgekeurd voor de behandeling van het perifere T-cel lymfoom (PTCL). Veel onderzoek was gefocust op de behandeling van hematologische maligniteiten, maar het laatste decennium zijn er ook klinische onderzoeken met HDAC-remmers bij patiënten met solide tumoren uitgevoerd. Ondanks veelbelovende resultaten bij de behandeling van hematologische maligniteiten, zijn HDAC-remmers in het algemeen niet effectief gebleken in klinische studies bij solide tumoren. In **Hoofdstuk 6** geven we een overzicht van de klinische onderzoeken verricht bij patiënten met solide tumoren.

In **Hoofdstuk 7** worden de resultaten van een fase I studie beschreven naar de farmacokinetiek en veiligheid van oraal panobinostat bij patiënten met vergevorderde solide tumoren en verschillende mate van leverfunctiestoornissen. Deze studie laat het gevolg van een verminderde leverfunctie zien op de systemische expositie van panobinostat en toont dat patiënten met milde of middelmatige leverfunctiestoornissen veilig behandeld kunnen worden met dezelfde dosering panobinostat als patiënten met een normale leverfunctie, ondanks wat hogere farmacokinetiekwaarden.

Cardiale glycosiden

Naast nieuwe formuleringen, zouden ook 'oude medicamenten', zoals bijvoorbeeld cardiale glycosiden, bruikbaar kunnen zijn in de behandeling van kanker. Cardiale glycosiden hebben een lange geschiedenis in de behandeling van hartziekten. Daarnaast laten een aantal preklinische en ook 2 fase I onderzoeken zien dat cardiale glycosiden mogelijk ook antikankereigenschappen bezitten. De mechanismen van deze antikankereffecten zijn onder andere afname van intracellulair K^+ en toename van Na^+ en Ca^{2+}, intracellulaire verzuring, remming van IL-8 productie en van de TNF-α/NF-κB route, remming van DNA topoisomerase II en activatie van de Src kinase route. **Hoofdstuk 8** beschrijft een review over cardiale glycosiden in de behandeling van kanker. Vandaag de dag zijn er drie cardiale glycosiden die ontwikkeld zijn voor de behandeling van kanker en die getest zijn in fase I onderzoek om de dosis limiterende toxiciteit en MTD vast te stellen.

Hoofdstuk 9 beschrijft de preklinische data en voorlopige resultaten van een niet afgeronde fase I studie met UNBS1450, een synthetisch cardenolide-glycosidederivaat. Het primaire einddoel van deze klinische fase I studie werd niet bereikt omdat de studie vroegtijdig werd beëindigd om niet-wetenschappelijke redenen. Het beschikbare preklinische onderzoek gaf ons geen adequaat doseringsschema voor de patiënten. Om de

optimale dosering vast te stellen van UNBS1450 voor toekomstige fase I/ II studies is meer onderzoek nodig.

CONCLUSIE

Veel hedendaagse antikankermiddelen hebben geen ideale farmaceutische en farmacologische eigenschappen, hetgeen nadelige gevolgen kan hebben, zoals een suboptimale therapeutische activiteit, dosislimiterende bijwerkingen en een slechte kwaliteit van leven van patiënten. In dit proefschrift richtten we ons op nieuwe formuleringen, te weten camptothecine glycoconjugaat BAY 56-3722 (voorheen BAY 38-3441) en liposomale formuleringen in de hoop deze problemen te overwinnen. We richtten ons ook op 'oude medicamenten' voor nieuwe indicaties, zoals bijvoorbeeld HDAC-remmers en cardiale glycosiden.

Helaas waren de uitkomsten van sommige van de beschreven studies teleurstellend: een voortijdige beëindiging van een fase II studie met BAY 56-3722, geen voordeel van LiPlaCis ten opzichte van de standaard cisplatinformulering en een niet afgeronde fase I studie met UNBS1450.

Het is bekend dat veel fase I en fase II studies niet resulteren in nieuwe behandelingen voor de dagelijkse praktijk. Het is eveneens bekend dat het publiceren van negatieve studieresultaten als minder aantrekkelijk wordt beschouwd en moeilijker is dan het publiceren van positieve studieresultaten. Maar het is essentieel om deze resultaten te delen om de kennis voor de ontwikkeling van toekomstig onderzoek te verbeteren. Zo is recent een nieuwe fase I dosis-escalatie studie gestart met LiPlaCis omdat in onze fase I studie de aanbevolen dosis voor fase II onderzoek nooit bereikt is. Daarbij, gebaseerd op de preklinische evalutie en het voorlopige rapport van de incomplete fase I farmacokinetiekstudie met UNBS1450, weten we nu, op basis van de washout experimenten, dat de optimale dosering waarschijnlijk veel hoger is en het optimale doseringsschema intensiever.

Naast de teleurstellende uitkomsten van sommige van de beschreven studies lieten we ook zien dat LEP-ETU en Taxol® voldoen aan de 80-125% bio-equivalentiecriteria en lieten we zien dat patiënten met milde of middelmatige leverfunctiestoornissen veilig behandeld kunnen worden met dezelfde dosering panobinostat als patiënten met een normale leverfunctie.

De reviews van liposomale antikankermiddelen, HDAC-remmers en cardiale glycosiden laten zien dat er nog veel onderzoek nodig is om aan de hoge verwachtingen van deze nieuwe formuleringen en nieuwe medicamenten te voldoen en de bestaande problemen te overwinnen.

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* Authors contributed equally

Curriculum vitae

Marije Slingerland werd geboren op 26 september 1980 te Leiden. In 1998 behaalde zij haar gymnasiumdiploma aan het Ichthus College te Enschede. In 1999 behaalde zij cum laude het propedeutisch examen van de studie Biomedische Wetenschappen aan de Universiteit Leiden en startte zij met de studie Geneeskunde. Haar propedeutisch examen en haar doctoraalexamen behaalde zij in 2000, respectievelijk 2003, beide cum laude. In augustus 2005 behaalde zij haar artsexamen. Aansluitend startte zij met de opleiding tot internist in het Rijnland ziekenhuis te Leiderdorp om in 2008 haar opleiding voort te zetten in het Leids Universitair Medisch Centrum te Leiden (opleiders dr. F.H.M. Cluitmans, prof. dr. J.A. Romijn, prof. dr. J.H. Bolk, prof. dr. J.W.A. Smit, prof. dr. J.W.R. Nortier). In december 2011 volgde de registratie tot internist-oncoloog. Tijdens haar opleiding begon zij met haar promotieonderzoek onder leiding van prof. dr. A.J. Gelderblom en later ook prof. dr. H.-J. Guchelaar, hetgeen heeft geresulteerd in dit proefschrift. In aansluiting op de registratie was zij aanvankelijk tijdelijk als internist-oncoloog werkzaam in het Rijnland ziekenhuis te Leiderdorp tot juli 2012. Sindsdien is zij aangesteld als staflid bij de afdeling Klinische Oncologie in het Leids Universitair Medisch Centrum. Haar aandachtsgebieden zijn hoofd-halstumoren, tumoren van de bovenste tractus digestivus, melanomen en fase I studies.

De auteur van dit proefschrift woont samen met Martin Meijer en zij hebben twee zoons, Jens en Timo.

Dankwoord

Graag wil ik iedereen die direct of indirect heeft bijgedragen aan het tot stand komen van dit proefschrift hartelijk bedanken.