Chapter 11b

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

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Abstract

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

Introduction

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

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

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

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

Materials and methods

Cell lines and treatment

PC3, DU-145 and LNCaP PCa cell lines were obtained from ATCC. Human prostate fibroblasts were obtained from a prostate biopsy on a 62-year old PCa patient with a Gleason score of 4 and kindly provided by dr. J. Isaacs. All cell lines were grown and maintained in RPMI-1640 media (Invitrogen) supplemented with 10% fetal bovine serum (complete RPMI media). Cells were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

JHDL compounds were stored at -20°C in 200 µM stock solutions in DMSO; compounds were dissolved in H_2O when they were not soluble in DMSO. Irinotecan (I1406, Sigma-Aldrich) and CTAB (H9151, Sigma-Aldrich) were stored as 10 mM stock solutions in DMSO at -20°C. For experiments the compounds were diluted in complete RPMI media to obtain the desired final concentration.

Knockdown experiment

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

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

Drug library screen

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

IC_{50} assessment

To assess IC_{50} values of selected compounds from the library screen in various PCa cell lines,

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

Clonogenic assay

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

Immunoblotting and densitometry

Immunoblotting and densitometry were performed as described previously.[9] Cleaved PARP primary antibody (9541, Cell Signaling Technology) and topoisomerase I primary antibody (TG2012-2, TopoGEN) were diluted 1:1000, NDRG1 primary antibody[10] 1:4000, Vinculin primary antibody (05-286, Millipore (Upstate)) 1:10,000; all antibodies were dissolved in blocking buffer (5% milk in TBST (100 mM Tris-HCl pH 7.4, 0.1% Tween 20, 150 mM NaCl in H_2O)). Secondary antibodies were dissolved in blocking buffer (5% milk in TBST) at 1:4000 dilution.

3-D invasion assay

Trypsinized DU-145 and DU-145 NDRG1 KD cells were suspended in complete RPMI media containing 0.5% methylcellulose. Ten microliters of suspended cells were placed on the inner side of the lid of a sterile bacterial petri plate and cultured as a hanging drop over a humidified plate in a CO_2 incubator for 16 h. The generated spheroids were used for 3-D invasion assays by embedding them in collagen matrix (BD Biosciences), which was prepared according to the manufacturer's instructions. CTAB was administered and the spheroids were imaged daily using a Nikon Eclipse Ti microscope (Nikon) under phase contrast. For calculation of the invasion index the total area over which the spheroid had dispersed (including invading and non-invading cells) and the area of non-invading cells (at the center

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

Results

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

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

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

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

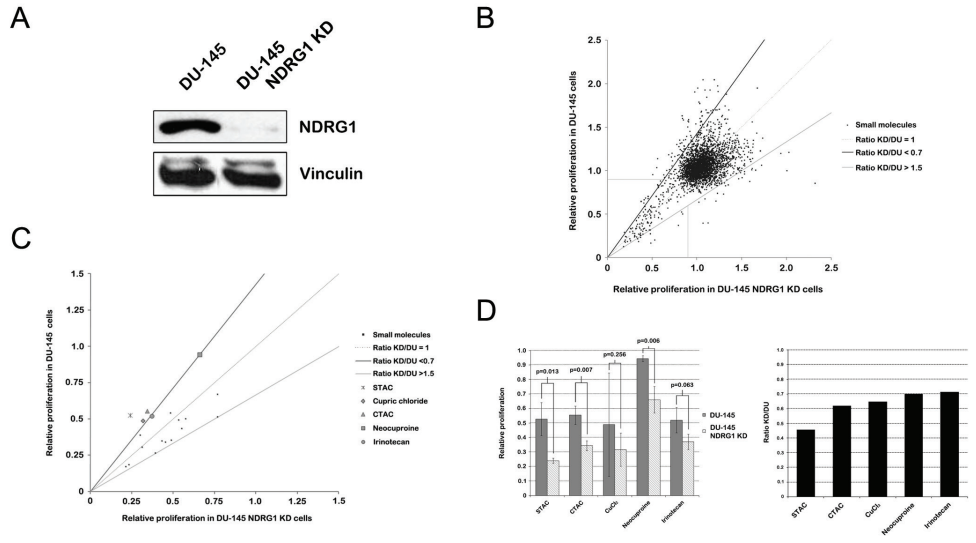

Figure 1. Screening of the JHDL identifies stearyltrimethylammonium chloride (STAC), cetrimonium chloride (CTAC), cupric chloride ($CuCl_2$), neocuproine (NCP) and irinotecan as compounds that selectively inhibit NDRG1 deficient DU-145 cells. A) Western blot for NDRG1 performed with DU-145 cell lysates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

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

Irinotecan selectively targets NDRG1 deficient PCa cells.

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

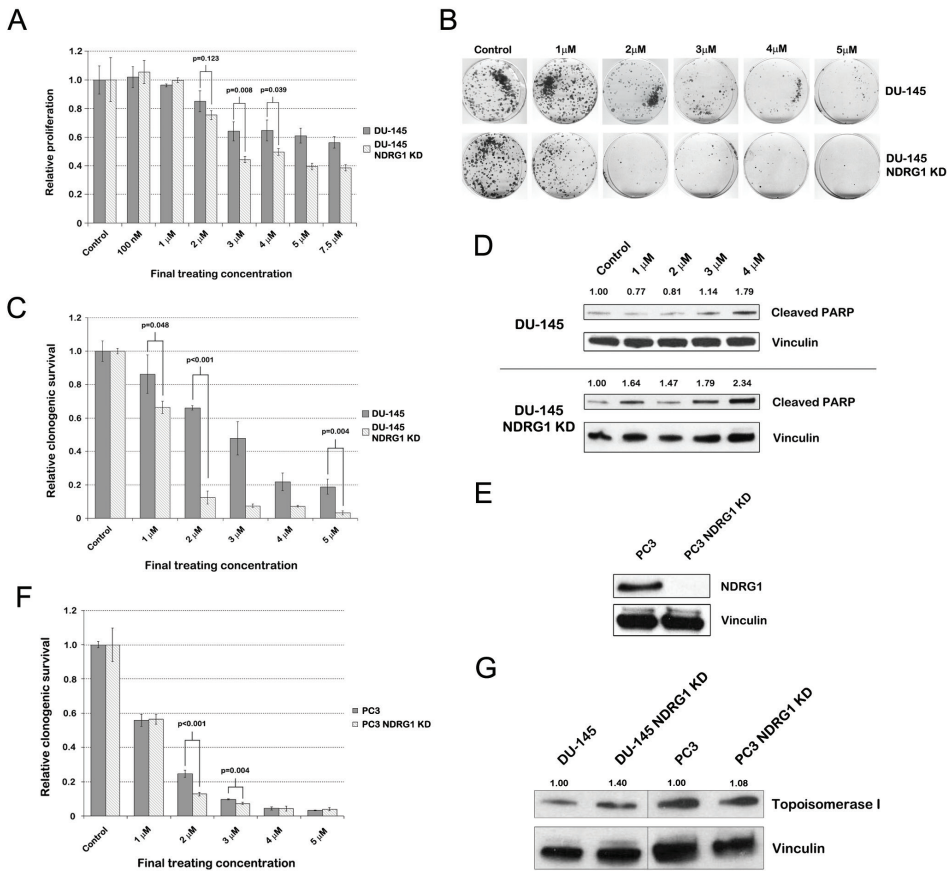

Figure 2. Irinotecan selectively targets NDRG1 deficient DU-145 and PC3 cells by induction of apoptosis. A) MTS assays performed after 48 h treatment of parental DU-145 and DU-145 NDRG1 KD cells with irinotecan. Student's t-tests were performed to assess p-values. B) Images displaying clonogenic survival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

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

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

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

CTAB selectively targets NDRG1 deficient PCa cells.

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

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

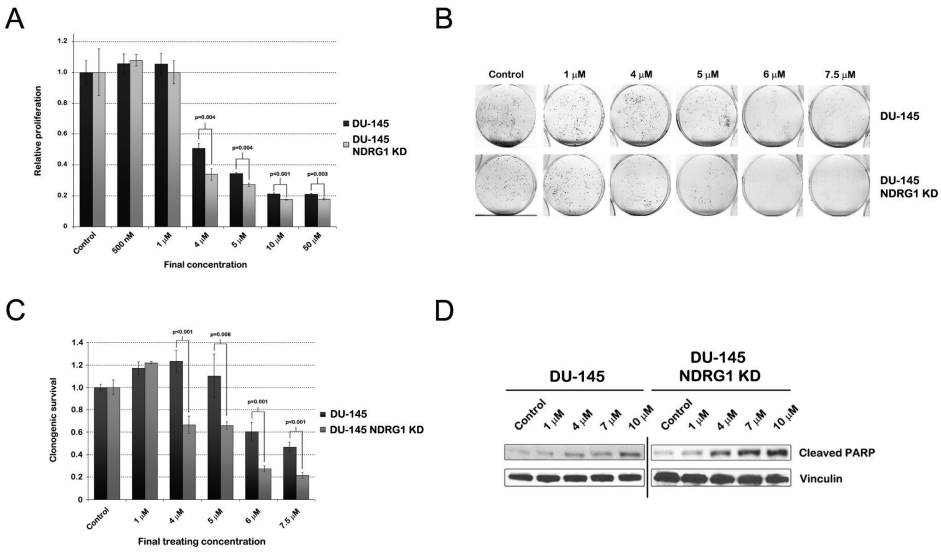

Figure 3. CTAB selectively targets DU-145 NDRG1 KD cells by induction of apoptosis. A) MTS assays performed after treatment of parental DU-145 and DU-145 NDRG1 KD cells with CTAB. Student's t-tests were performed to assess p-values. B) Images displaying clonogenic survival of parental DU-145 and DU-145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

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

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

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

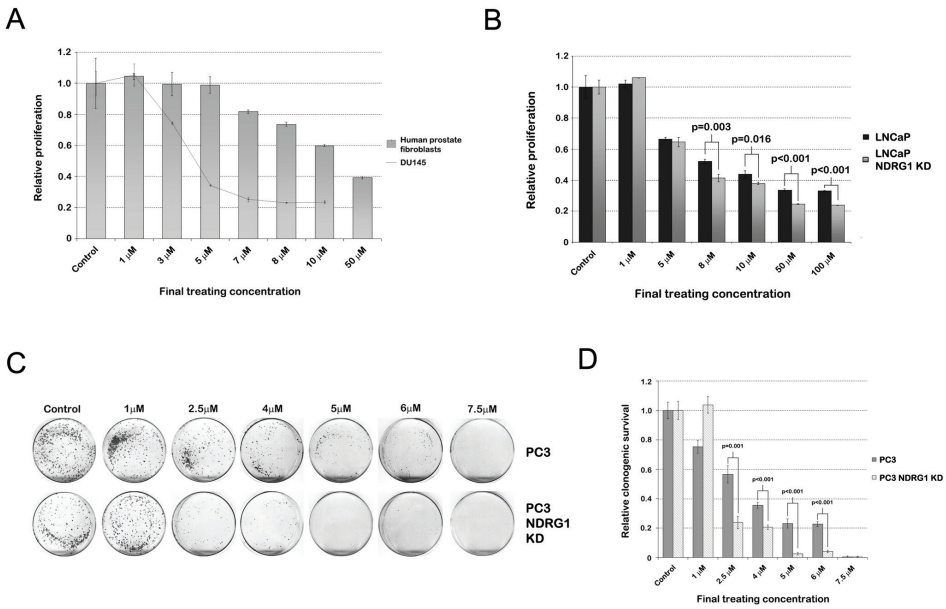

Figure 4. While non-cancerous human prostate fibroblasts are relatively unaffected by CTAB, NDRG1 KD cells are selectively targeted in multiple PCa cell lines. A) MTS assays were performed with human prostate fibroblasts and DU-145 cells after CTAB treatment. B) MTS assays performed after treatment of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

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

Discussion

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

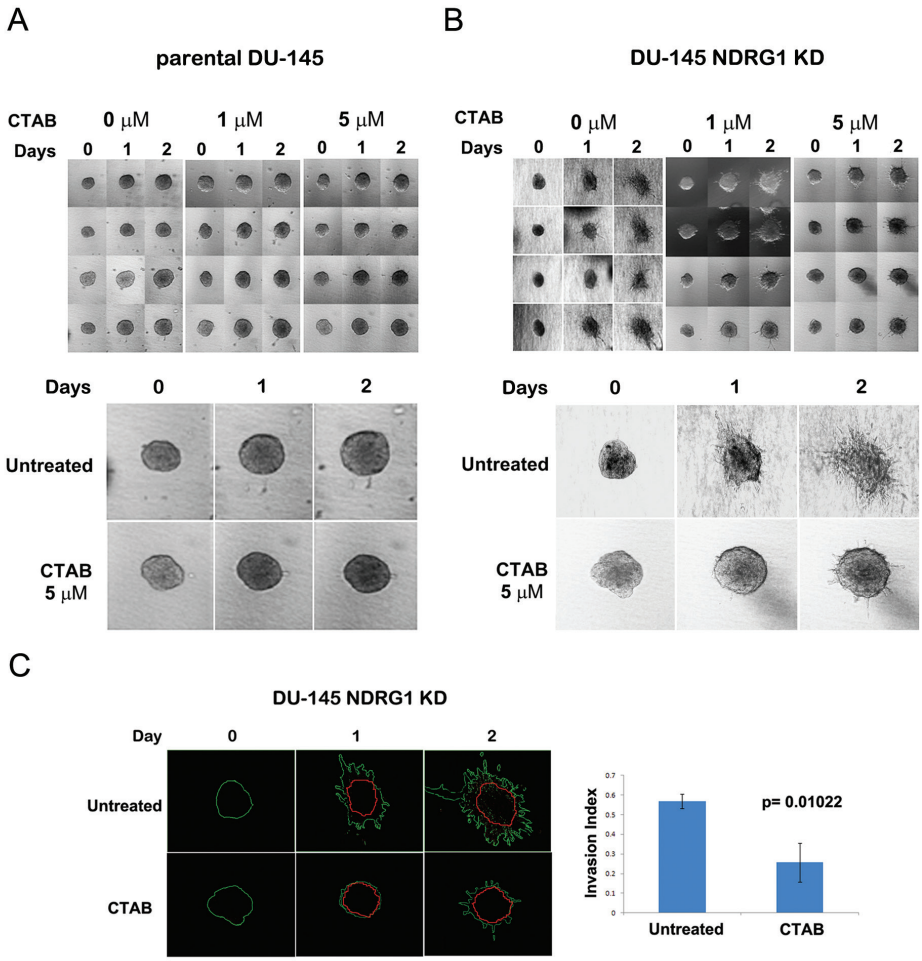

Figure 5. CTAB treatment results in decreased invasion of DU-145 NDRG1 KD cells. A-B) 3-D invasion assays performed with parental DU-145 (A) and DU-145 NDRG1 KD (B) cells treated with CTAB. C) Calculation of the invasion index. The red area contains non-invading cells and the green area contains both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

costs.[28, 29]

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

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

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

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

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

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

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

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