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Minnelide Inhibits Androgen Dependent, Castration Resistant Prostate Cancer Growth By Decreasing Expression of Androgen Receptor Full Length and Splice Variants

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Abstract

BACKGROUND.—With almost 30,000 deaths per year, prostate cancer is the second-leading cause of cancer-related death in men. Androgen Deprivation Therapy (ADT) has been the corner stone of prostate cancer treatment for decades. However, despite an initial response of prostate cancer to ADT, this eventually fails and the tumors recur, resulting in Castration Resistant Prostate Cancer (CRPC). Triptolide, a diterpene triepoxide, has been tested for its anti-tumor properties in a number of cancers for over a decade. Owing to its poor solubility in aqueous medium, its clinical application had been limited. To circumvent this problem, we have synthesized a water-soluble pro-drug of triptolide, Minnelide, that is currently being evaluated in a Phase 1 clinical trial against gastrointestinal tumors. In the current study, we assessed the therapeutic potential of Minnelide and its active compound triptolide against androgen dependent prostate cancer both in vitro as well as in vivo.

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COMPETING INTERESTS

The University of Minnesota has filed a patent for MinnelideTM (which has been licensed to Minneamrita Therapeutics LLC, Moline, IL). RC has ownership interest in a patent. AS has ownership interests (including patents) and is a consultant/advisory board member for Minneamrita Therapeutics LLC. SB is a consultant for Minneamrita Therapeutics. This relationship is managed by University of Minnesota according to its conflict of interest policy. SD is a consultant with Astellas/Medivation. The other authors disclosed no potential conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

METHODS.—Cell viability was measured by a MTT based assay after treating prostate cancer cells with multiple doses of triptolide. Apoptotic cell death was measured using a caspase 3/7 activity. Androgen Receptor (AR) promoter-binding activity was evaluated by using luciferase reporter assay. For evaluating the effect in vivo, 22Rv1 cells were implanted subcutaneously in animals, following which, treatment was started with 0.21mg/kg Minnelide.

RESULTS.—Our study showed that treatment with triptolide induced apoptotic cell death in CRPC cells. Triptolide treatment inhibited AR transcriptional activity and decreased the expression of AR and its splice variants both at the mRNA and the protein level. Our studies show that triptolide inhibits nuclear translocation of Sp1, resulting in its decreased transcriptional activity leading to downregulation of AR and its splice variants in prostate cancer cells. In vivo, Minnelide (0.21mg/kg) regressed subcutaneous tumors derived from CRPC 22RV1 at our study endpoint. Our animal studies further confirmed that Minnelide was more efficacious than the standard of care therapies, Docetaxel and Enzalutamide.

CONCLUSION.—Our study indicates that Minnelide is very effective as a therapeutic option against CRPC at a dose that is currently tolerated by patients in the ongoing clinical trials.

Keywords

CRPC; Minnelide; triptolide; splice variant; Androgen receptor; Sp1

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer related death among men in the United States with an estimated 220,800 newly diagnosed cases and 27,540 deaths in 2015 [1]. Androgen Deprivation Therapy (ADT) has been the first line of systemic therapy in prostate cancer for decades. However, despite an initial response to ADT in nearly all patients, this eventually fails and leads to disease progression. This stage of the disease, referred to as Castration Resistant Prostate Cancer (CRPC), is responsible for virtually all prostate cancer death [2,3].

AR is the central molecule driving the growth and progression of prostate cancer. Maintained inactive in the cytosol in association with chaperones, AR is activated in the presence of androgen. The gene structure of AR is comprised of an N-terminal transactivation domain (NTD, encoded by exon 1), the DNA binding domain (DBD, exons 2 and 3), a short hinge region (exon 4), and the C-terminal ligand-binding domain (LBD; exons 4–8) where the androgenic ligands testosterone and dihydrotestosterone (DHT) bind [4]. The AR LBD is also the binding site for anti-androgens such as Enzalutamide, which function as competitive inhibitors. Binding to androgen alters the AR conformation dissociating it from the complex, resulting in its activation and translocation to the nucleus. Once in the nucleus, nuclear AR binds to AREs located throughout the genome and regulates the transcription of a number of genes involved in prostate growth, maintenance, and differentiation.

Over the past decade, there has been tremendous progress in our understanding of mechanisms underlying CRPC development and progression. Notably, this progress has

resulted in new therapies that improve overall survival and quality of life for CRPC patients. For example, progress in understanding that adrenal and intra-tumor androgens provide an important source of androgen receptor (AR) activation in CRPC cells led to the development of Abiraterone acetate as a therapy to suppress CYP17A1-dependent androgen synthesis in these tissues [5]. Additionally, characterization of AR gene amplification as a mechanism of AR protein overexpression and inappropriate transcriptional regulation led to the development of the second-generation AR antagonist, Enzalutamide [6]. However, despite these advances, resistance is frequent and CRPC remains a fatal disease. This has sparked new investigations into the mechanisms of resistance to Abiraterone and Enzalutamide. Remarkably, there is compelling evidence that AR transcriptional activity can persist even during therapy with these new agents, via mutations in androgen metabolism enzymes [7], mutations in the AR ligand binding domain (LBD) [8], and synthesis of constitutively active AR splice variants lacking the AR LBD [9,10]. Collectively, these findings indicate that the androgen/AR signaling axis remains an attractive target for therapy of CRPC, and highlight an urgent need for development of new, durable treatment strategies. Therapeutic resistance and CRPC progression is frequently associated with rising PSA levels (an AR-regulated gene), indicating persistent AR transcriptional activity despite active AR-targeted therapy [11,12]. Therefore, it is important to develop new treatment strategies that can durably suppress AR transcriptional activity and thereby combat development and progression of CRPC.

Triptolide, a diterpene triepoxide isolated from a Chinese herb *Tripterygium wilfordii*, has been tested for its anti-tumor properties in a number of cancers for over a decade. Owing to its poor solubility in aqueous medium, its clinical applications have been limited. To elude this issue, our group at the University of Minnesota has synthesized a water soluble pro-drug of triptolide, named Minnelide [13]. Minnelide has shown great promise in preclinical studies conducted in a number of cancers such as pancreatic cancer [13,14], lung cancer [15], liver cancer [16], and osteosarcoma [17]. Currently, Minnelide is being evaluated in a Phase 1 clinical trial against gastrointestinal tumors. Though triptolide has previously been studied in context of prostate cancer, it has not been evaluated against CRPC [18–20].

In the current study, we evaluated the effect of triptolide on CRPC and Enzalutamide resistant PCa in vitro and evaluated the efficacy of the water-soluble prodrug of triptolide, Minnelide on tumors derived from these cells in vivo. Our study showed that treatment with triptolide caused CRPC cell death in vitro, along with reduced expression and transcriptional activity of full-length AR and AR-Vs. Consistent with this, treatment of CRPC tumor bearing mice with Minnelide, the water-soluble pro-drug of triptolide, resulted in significantly reduced tumor burden when compared with the untreated animals.

MATERIALS AND METHODS

Cell Culture and Treatment

The 22Rv1 (#CRL-2505), DU-145 (#HTB-81), and LNCaP (#CRL-1740) cell lines were obtained from ATCC and cultured according to ATCC protocol (in 2014). ATCC ensures authenticity of these human cell lines using short tandem repeat (STR) analyses. All experiments with these cells were performed within 4 months of resuscitation of frozen cell

stocks prepared within 3 passages of receipt from ATCC. The C4–2 cell line was obtained from Dr. Katherine Stemke Hale at MD Anderson Cancer Center (in 2014), where they are routinely tested for authenticity by short tandem repeats genotyping and their response to androgens for growth and AR activity. All these 3 PCa cell lines were grown and propagated in RPMI supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 ug/ml streptomycin. All cells were maintained at 37°C in a humidified air atmosphere with 5% CO₂. The VCaP and PC3 cells were obtained from Dr.Scott Dehm at the University of Minnesota (in 2014). The PC3 cells were grown and propagated in RPMI 1,640 media supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 ug/ml streptomycin. The VCaP cells were grown and propagated in DMEM media supplemented with 20% fetal bovine serum (FBS), 100 units/ml penicillin and 100 ug/ml streptomycin.

Treatment of Cells With Triptolide, Enzalutamide, Mithramycin

Triptolide (EMD Millipore Chemicals, MA), Mithramycin (Sigma Aldrich), and Enzalutamide (Selleckchem, Houston, TX) were dissolved in DMSO (Sigma–Aldrich, St. Louis, MO) and added to the cells at the indicated concentrations after allowing cancer cells to adhere for 48 hr in media containing 10% FBS (for LNCaP, DU-145 and PC3), or 10% charcoal stripped FBS (CSS, for 22RV1, VCaP and C4–2). Cells treated without drug in media containing 10% FBS (for LNCaP and PC3), or 10% CSS (for 22RV1, VCaP and C4–2) served as controls. Human Sp1 siRNA (Qiagen) were used to silence expression of Sp1 in prostate cancer cell line 22Rv1. Transfections were done using HiPerfect (Qiagen) according to manufacturer's instructions.

Determination of Cell Viability

PCa cells (LNCaP: 4×10³/well, C4–2: 4×10³/well, 22RV1: 10×10³/well, PC3: 3×10³/well) were seeded in 96-well plates and allowed to adhere for 48hr at 37°C. Dojindo Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc, MD) was used to measure cell viability after drug treatment at indicated concentrations for 24–96hr. All experiments were performed in quadruplicate and repeated three times independently.

Flow Cytometry Detection of Cleaved Poly ADP-Ribose Polymerase (PARP) and Active Caspase-3

PCa cells were seeded in 10-cm plates, allowed to adhere for 48 hr and treated with triptolide at indicated concentrations. Cells were harvested at specified time points and incubated in fixation/permeabilization solution (BD cytofix/cytoperm™, BD Biosciences, San Jose, CA) for 30 min on ice. These fixed cells were then stained with PE conjugated monoclonal antibody for cleaved PARP (Asp 214, BD Biosciences) and FITC conjugated active caspase-3 (Asp 175, BD Biosciences) in presence of FcR blocking reagent (Miltenyi Biotech) for 30 min. Cells were then washed and FACS analyses were performed on a BD FACSCanto II (BD Biosciences, San Jose, CA) using FACSDiva (BD Biosciences) and FlowJo (Tree Star, Inc., Ashland, OR) software. All experiments were repeated three times independently.

Quantitative Real-Time Polymerase Chain Reaction for AR Full Length, AR 1/2/2b, AR 1/2/3/2b, and AR 1/2/3/ce3, PSA, and NKX3.1

PCa cells (1×10^6) were plated into 10 cm plates and allowed to adhere for 48 hr at 37°C before treatment. Triptolide was then added at indicated concentrations and cells were collected after 24 hr of drug treatment. RNA was extracted from cell lines as well as xenograft tumor tissue using TRIzol (Life Technologies, Grand Island, NY) reagent according to the manufacturer's instructions. Total RNA (2 µg) was used to make cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. NKX 3.1, prostate specific antigen (PSA) and 18S primers were purchased from Qiagen (QuantiTect primer assay, Qiagen, Valencia, CA). To measure AR full-length and splice variants, we used the following primer sequences:

	Forward	Reverse
AR full length	5'-AAC AGA AGT ACC TGT GCG CC-3'	5'TTC AGA TTA CCA AGT TTC TTC AGC-3'
AR 1/2/2b	5'-TGG ATG GAT AGC TAC TCC GG-3'	5'-TTC TGT CAG TCC CAT TGG TG-3'
AR 1/2/3/2b	5'-AAC AGA AGT ACC TGT GCG CC-3'	5'-TTC TGT CAG TCC CAT TGG TG-3'
AR 1/2/3/CE3	5'-AAC AGA AGT ACC TGT GCG CC-3'	5'TTC AGA TTA CCA AGT TTC TTC AGC-3'

All experiments were done in duplicate and repeated three times independently.

Measurement of AR Full Length and AR Splice Variants by Western Blot

PCa cells (1×10^6) were seeded in 10 cm plates and allowed to adhere for 48 hr at 37°C before treatment. Triptolide was then added at the indicated concentrations, and cells were collected at 48-, and 72-hr time points. Cell lysates were prepared by removing the culture media, washing the cells with 1X PBS and then adding Rapid Immuno-Precipitation Assay (RIPA) Buffer (Boston Bioproducts Inc, Ashland, MA) supplemented with protease (20 µl per ml of lysis buffer) and phosphatase (10 µl per ml of lysis buffer) inhibitors at 4°C. Proteins from xenograft tumor tissues were collected after homogenizing them in RIPA buffer. Total protein was quantitated (Pierce BCA Protein Estimation Kit, ThermoScientific, Rockford, IL), separated on a SDS-PAGE and transferred to a nitrocellulose membrane.

Anti-AR NTD (EMD Millipore) or anti-AR-V7 or AR1/2/3/CE3 (Precision Antibody, Columbia, MD) antibody was used to probe against full-length AR or splice variant AR, respectively. Actin expression was used as an internal control (Cell Signaling, Beverly, MA). All experiments were repeated independently three times. Western blots were quantified using ImageJ software (National Institutes of Health [NIH], Bethesda, MD) after normalizing to actin expression to calculate relative AR expression levels.

Luciferase Reporter Assay

AR transcription activity and Sp1 transcription activity was measured using Signal Reporter Assay for ARE and Sp1 binding elements, respectively (Qiagen). LNCaP and 22RV1 cells

(2×10^5 cells per well of 24-well plate) were transfected with 4ul of AR reporter plasmid, luciferase negative control and luciferase positive control using Attractene transfection reagent (Qiagen, Valencia, CA). After 18 hr of transfection, cells were treated with various drugs in RPMI+10% FBS for LNCaP cells and RPMI+10% CSS for 22RV1 cells. The luciferase activity was determined 24 hr after drug treatment using a dual luciferase reporter assay system (Promega, Fitchburg, WI), the signal was normalized to *Renilla* luciferase and values were expressed as relative luciferase units. All experiments were performed in duplicate and repeated independently three times.

Immunofluorescence

Prostate cancer cells 22Rv1 were grown in chamber slides and treated with 25 nM triptolide for 24 hr, fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton $\times$ 100. Anti-Sp1 antibody (Cell Signaling) was used at a dilution of 1:200 for 1h at room temperature. After washing, cells were incubated with secondary anti-bodies: 1:1200 dilution of Alexa-488-conjugated donkey anti-rabbit IgG (Molecular Probes) for 1 h at 4°C. The slides were washed and mounted using Prolong Gold anti-fade agent containing DAPI (Molecular Probes). Immunofluorescence images were obtained on a Nikon Eclipse Ti confocal microscope (Nikon, Melville, NY) using a 100 $\times$ oil-immersion objective.

CRPC Xenograft Model

All procedures were conducted according to the guidelines of the University of Minnesota Institutional Animal Care and Use Committee. Briefly, athymic male nude mice (4–6 weeks old; Charles River Laboratories, Raleigh, NC) were castrated and injected 1-week later with 2.0×10^6 22RV1 cells suspended in PBS and matrigel (1:1) subcutaneously into the right flank. Tumor volume was monitored using the formula: $0.524 \times \text{length} \times \text{width}^2$. When tumors reached 250mm³, mice were randomized into two groups (nine mice in the Minnelide treatment arm and eight mice in the control arm). Mice in the treatment arm received a daily intra-peritoneal injection of Minnelide 0.21 mg/kg/day, whereas mice in the control arm received a daily intra-peritoneal injection of saline (vehicle). Tumor volume was measured weekly until the average tumor volume was about 2 cm³ in the control arm. Tumor weights and volumes were documented after necropsy in order to assess the tumor burden in animals.

Comparison With Standard of Care

To study how Minnelide compared with the standard of care, 22RV1 cells were implanted in 40 nude mice (as in previous section) and randomized them to four treatment groups (10 mice in each arm): Control, Minnelide (0.21 mg/kg/day, intraperitoneal) treatment, Docetaxel treatment (10 mg/kg/wk, intra-peritoneal) and Enzalutamide treatment (10 mg/kg/day, oral gavage). Tumor volume was measured and documented weekly. Experiment was ended when the average tumor volume was about 2 cm³ in the control arm.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling (TUNEL) Assay for Measurement of In Situ Apoptosis

Paraffin-embedded prostate tumor xenograft tissue sections from control and Minnelide treated mice were processed for TUNEL assay according to the manufacturer's instructions (Roche, Indianapolis, IN). Counterstaining for total cells was done with propidium iodide. Coverslips were applied and fixed with paramount. Images were taken on a confocal microscope (Nikon) using a magnification of 20× for obtaining an overview and 100× for quantifying the images. A total of three random high power (100×) field images were taken per tumor section slide. The images were quantified using ImageJ software (NIH, MD) and the mean was calculated for the ratio of total TUNEL positive cells to total propidium iodide positive cells.

Statistical Analysis

Values are expressed as the mean $\pm$ standard error of the mean (SEM). The significance of the difference between the control and each experimental arms was analyzed by the unpaired Student's *t*-test and *P*<0.05 was considered statistically significant

RESULTS

Triptolide Decreases Cell Viability in Androgen Dependent and CRPC Cell Lines

The effects of treatment with triptolide and Enzalutamide on androgen dependent (LNCaP), and CRPC (C4–2, 22RV1) cell lines were examined at indicated concentrations for 24, 48, 72, and 96 hr. Triptolide treatment decreased cell viability in a dose and time dependent manner in androgen dependent (LNCaP) and CRPC (C4–2 and 22RV1) cells (Fig. 1A–C). In both androgen dependent (LNCaP) and CRPC (22RV1 and C4–2) cell lines, cell viability decreased by 75% after treatment with 25 nM triptolide for 72 hr. In contrast, though LNCaP cells showed slight sensitivity to the second-generation anti-androgen Enzalutamide (Fig. 1D), C4–2 and 22RV1 cells displayed robust growth in androgen depleted media and were unaffected by it (Fig. 1E and F). We calculated the IC₅₀ for triptolide treatment on a number of prostate cancer cell lines including normal prostate epithelial cells. Androgen dependent cell lines had a much lower IC₅₀ compared to the non-malignant prostate cells (BPH-1), PREC cells or AR negative prostate cancer cells (Supplementary Table I, Supplementary Fig. 1).

Triptolide Induces Apoptosis in Androgen Dependent and CRPC Cell Lines

To study if triptolide induced apoptotic cell death in PCa, we assayed for markers of apoptosis (active caspase-3 and cleaved PARP) in cells treated with triptolide at varying concentrations. Both androgen dependent (LNCaP), and CRPC (22RV1, C4–2) cell lines showed a dose- and time-dependent increase in apoptotic cells as indicated by active caspase-3 (Fig. 2A–C) and cleaved PARP positive population (Fig. 2D–F). An increase in active caspase-3 and cleaved PARP positive cells was evident within 24 hr after triptolide treatment and continued to increase with time. These results indicate that the observed decrease in PCa cell viability after triptolide treatment was due to caspase-dependent activation of apoptosis. Treatment of AR-ve cell line PC3 and DU145 also showed an

increase in caspase activity (Supplementary Fig. 1C). Consistent with the IC₅₀ of AR-ve cells, apoptosis occurred at a much higher dose in these cells.

Triptolide Decreases AR Transcriptional Activity Leading to Decreased mRNA Expression of AR Downstream Target and NKX3.1

Since AR is the central molecule driving the growth and progression of prostate cancer cells, we evaluated the effect of triptolide on its transcriptional activity using AR-responsive promoter-reporter construct in LNCaP and 22RV1 cell lines. As expected, both Enzalutamide and triptolide decreased AR transcriptional activity in LNCaP cells (Fig. 3A). In CRPC 22RV1 cells, however, triptolide inhibited AR transcriptional activity, but Enzalutamide had only a minimal effect (Fig. 3B). This was in keeping with the observation that triptolide treatment also inhibited expression of downstream AR targets PSA and NKX3.1 in LNCaP, C4-2, and 22RV1 cell lines (Fig. 3C and D). Interestingly, treatment with Enzalutamide did not decrease the mRNA expression of downstream targets NKX3.1 and PSA in 22RV1 cells (Supplementary Fig. 2A). Further, treatment of 22RV1 cells with DHT did not have any effect on the expression of AR regulated genes NKX3.1 and KLK3 (Supplementary Fig. 2B).

To assess if the decrease in transcriptional activity of AR after triptolide treatment was due to deregulation of AR expression, we studied the effect of triptolide on AR protein and mRNA levels. Triptolide decreased the mRNA expression of full length AR in LNCaP, C4-2 and 22RV1 cell line (Fig. 4A-C). Additionally, triptolide also decreased the AR splice variants in the CRPC cell line 22RV1 (Fig. 4D-F). Consistent with this, the protein expression of AR in both androgen dependent (LNCaP) and CRPC (C4-2 and 22RV1) cell lines were also decreased following triptolide treatment (Supplementary Fig. 3A-C). Protein analysis further revealed that AR1/2/3/CE3 splice variant expressed in the 22RV1 cells was also downregulated following triptolide treatment (Supplementary Fig. 3C and D).

To further verify this phenomenon in another prostate cancer cell line that has the AR splice variants, we used prostate cancer cell line V-CaP derived from the vertebral bone metastasis and evaluated the effect of triptolide on the splice variants. As seen with the 22RV1 cells, the mRNA expression of full length AR and its splice variant in these cells also decreased in a dose dependent manner after treatment with triptolide (Supplementary Fig. 4).

Triptolide Decreased AR and its Downstream Elements by Inhibition of Sp1 Activity

Previous studies from our laboratory have demonstrated that triptolide induced cell death may be mediated via down regulation of the transcription factor Sp1 in pancreatic cancer cells [21]. This study further showed that in pancreatic cancer cells, triptolide prevented translocation of Sp1 to the nucleus, thereby affecting its transcriptional activity. To study if this was happening in prostate cancer cells as well, we evaluated the effect of triptolide treatment on Sp1 expression. Our results showed that triptolide indeed decreased both the protein and the mRNA expression of Sp1 in the prostate cancer cell line 22RV1 at a dose of 50 nM but not at the sub-lethal dose of 25 nM (Fig. 5A and B). However, the transcriptional activity of Sp1 was decreased significantly at 25 nM triptolide, indicating that even though the cells were viable at this dose, they had decreased Sp1 activity (Fig. 5C). To evaluate this

further, we analyzed the nuclear localization of Sp1 following treatment with 25 nM triptolide using immunofluorescence. Our data clearly indicate that treatment with 25 nM triptolide resulted in an accumulation of Sp1 in the cytosol (Fig. 5D and E), which was responsible for its decreased transcriptional activity.

One of the major transcription factors that regulate transcription of AR is Sp1. Thus, to see if triptolide mediated downregulation of Sp1 was indeed decreasing the transcription of AR and its splice variant, we treated 22Rv1 cells with varying doses of Sp1 inhibition Mithramycin. As seen with triptolide, mithramycin decreased the viability of 22Rv1 cells (Fig. 5F). Further, silencing Sp1 using siRNA downregulated the expression of full length AR as well as its splice variants (Fig. 5G). To confirm if indeed triptolide induced cell death in 22Rv1 cells was mediated by Sp1, we next overexpressed Sp1-pCMV in these cells. Our results showed that Sp1 rescued 22Rv1 cells from triptolide induced cell death (Fig. 5H) and the reversed the downregulation of expression of AR FL mRNA (Fig. 5I).

Minnelide Inhibits Growth of CRPC Tumors Xenografts

To validate our findings in an animal model, we implanted 22RV1 cells subcutaneously in the flank of athymic nude mice 7 days after surgical castration. Treatment with Minnelide was started once the tumors reached an average volume of 250 mm³. At our study endpoint, xenografted CRPC tumors treated with Minnelide were significantly smaller in volume compared to tumors in the control arm (364.33±71.69 mm³ vs. 1950±549.81 mm³, *P*=0.023, Fig. 6A and B). In addition, xenograft tumors in the Minnelide arm had significantly smaller tumor weight compared to tumors in the control arm (370.35±64.5 mg vs. 1662.88±537.96 mg, *P*=0.047, Fig. 6C).

To compare the efficacy of Minnelide with the standards of care in PCa, we next expanded this model to evaluate Docetaxel and Enzalutamide and compare them with Minnelide. Our results showed that while Enzalutamide was not able to inhibit the growth of 22RV1 tumors over 5 weeks of drug treatment (1842.5±415.75 mm³ Enzalutamide vs. 1973.6±274.01 mm³ control, *P*=0.798) and docetaxel was only marginally effective, mice treated with Minnelide had significantly smaller tumor volume (164.65±30.92 mm³ vs. 1973.6±274.01 mm³, *P*<0.001) than mice in the control arm after 5 weeks of drug treatment (Fig. 6D and E).

As seen in vitro, tumors treated with Minnelide also showed apoptotic death as visualized by significantly higher %TUNEL positive area compared to tumors in the control arm (52.12±5.90 vs. 2.68±0.71, *P*<0.001, Supplementary Fig. 4A and B).

Consistent with our in vitro observation, xenograft tumors treated with Minnelide had significantly decreased mRNA expression of full-length AR (0.70±0.04 vs. 1.04±0.12, *P*=0.026, Supplementary Fig. 4B) and AR1/2/3/CE3 (0.43±0.04 vs. 1.02±0.07, *P*<0.001, Fig. 6F) than tumors in the control arm. In addition, expression of AR regulated genes like NKX3.1 and KLK3 were also decreased in the Minnelide treated tumors (Supplementary Fig. 5A).

DISCUSSION

Androgen Receptor (AR) activity is intimately linked with prostate cancer. Prostate cancer cells, like normal prostate cells require androgen to grow and proliferate. Adrenal and intratumoral androgen are the major activator of AR. AR is a ligand-dependent transcription factor that translocates to the nucleus and regulates transcription of genes that have the Androgen Response Elements (ARE) in their promoter. The binding of the AR to its native ligands 5 α -dihydrotestosterone (DHT) and testosterone initiates male sexual development and differentiation. In prostate cancer, genes with ARE typically regulate survival and proliferation of tumor cells. AR expression is maintained throughout prostate cancer progression. Though prostate cancer is treated by hormone deprivation therapy, the efficacy of this type of treatment is transient, as patients relapse after developing a castration-resistant form of the disease that is usually due to increased levels of AR expression or mutations that cause the AR to be resistant to anti-androgens. There is increasing evidence of CRPC dependence on the androgen-receptor (AR) signaling pathway and underlying mechanisms.

One of the major factors that contribute to CRPC is the presence of splice variants (AR-Vs) in the AR gene that lack the ligand-binding domain responsible for recognizing the androgen. As a result, these deregulated splice variant ARs are unresponsive to hormone withdrawal therapy leading to constitutively active AR signaling and uncontrolled proliferation of tumor cells [10,22,23].

Among the splice variants, AR-V7, encoded by contiguous splicing of AR exons 1/2/3/CE3, is significantly upregulated during PCa progression and its expression level is correlated with the risk of tumor recurrence after radical prostatectomy [24–26]. Further, AR1/2/3/CE3 in circulating tumor cells from patients with castration-resistant prostate cancer has been associated with resistance to Enzalutamide and Abiraterone [9]. Our study showed that triptolide treatment decreased the expression of both the full length AR as well as the AR splice variants at both protein and RNA level (Figs. 4 and 5). This resulted in a decreased transcriptional activity of AR leading to less PSA and NKX3.1 expression (Fig. 3). Androgen-deprivation therapy (ADT) is the mainstay of therapy for patients with locally advanced, metastatic and biochemically recurrent disease after failure of localized treatments. In this context our observation above has immense significance in developing therapy against all forms of AR dependent prostate cancers.

Minnelide has been extensively used by our group in multiple animal models for a number of cancers. Earlier preclinical studies indicate there is no toxicity associated with this compound at the doses in which it is effective against prostate cancer [13]. Our study has also confirmed that in the mouse models for prostate cancer the dose of Minnelide administered is not associated with any prostate specific toxicity (Supplementary Fig. 5B–D). This is very encouraging as we hope to expand these studies to a clinical trial early next year. The mechanism of action of triptolide is still unclear. Studies using cell-free systems using a dose of 10 mM have indicated that triptolide inhibits the XPB subunit of RNA polymerase IIB and shuts down global transcription [27]. However, for most cancer cells, the LD₅₀ for triptolide has been found to be less than 100 nmol/L, at which XPB remains

unaffected [28]. Recent literature from our group has shown that one of the potential targets of triptolide may be the transcription factor Sp1 [21].

Interestingly, Sp1 has also been shown to regulate the transcription and activity of AR in prostate cancer cells [29–30]. In the current study, we observed that triptolide at a sub-lethal dose (25nM) decreased transcriptional activity of Sp1 (and prevented its translocation to the nucleus) as a sub-lethal dose, while having minimal effect on its expression (Fig. 5A–E). This was consistent with the observation that upon downregulation of Sp1 by Mithramycin or siRNA, expression of full length AR or its splice variants in CRPC cell line 22Rv1 was decreased. It is thus possible in CRPC cells, triptolide is inhibiting AR transcription via inhibition of Sp1. This also explains why triptolide is effective against both AR positive prostate cancer cells (LNCaP, 22Rv1, VCaP) as well as AR-ve cells (PC3, DU145), even though the AR negative cells respond at a much higher concentration of the drug.

CONCLUSION

Our results reflect that Minnelide is inducing cell death in prostate cancer cells via downregulation of AR and its variants. Minnelide is currently being evaluated in a Phase 1 clinical trial against gastrointestinal cancer. Interestingly, the doses at which the prostate tumors showed sensitivity to Minnelide are well within the dose range that is being tested. This is extremely promising as evaluation of this compound in all forms of AR dependent prostate cancer may form the foundation for a clinical trial against this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Triptolide treatment for 24–96 hr in androgen dependent LNCaP (A) and CRPC C4–2 (B) and 22RV1 (C) cells results in dose and time dependent decrease in cell viability. Enzalutamide treatment in androgen dependent LNCaP cells (D) showed slight decrease in cell viability. CRPC C4–2 (E) and 22RV1 (F) cells show robust growth despite presence of Enzalutamide. Data expressed as the mean $\pm$ SEM of three independent experiments.

Fig. 2.

Triptolide treatment for 24 and 48 hr in androgen dependent LNCaP (A) and CRPC C4-2 (B) and 22RV1 (C) cells results in dose and time dependent increase in active caspase-3. Androgen dependent LNCaP (D) and CRPC C4-2 (E) and 22RV1 (F) cells also showed increase in cleaved PARP indicating apoptosis. Data expressed as the mean $\pm$ SEM of three independent experiments.

Fig. 3.

Triptolide treatment for 18 hr results in decrease of AR promoter activity in androgen dependent LNCaP (A) and CRPC 22RV1 (B) cell lines. Data expressed as the mean SEM of three independent experiments. Triptolide treatment for 24 hr results in dose dependent decrease in AR downstream target genes NKX3.1 mRNA level in androgen dependent LNCaP, 22RV1 and C4-2 cells (C). Similarly, PSA expression was also decreased in androgen dependent LNCaP, CRPC 22RV1, and C4-2 (D) cell following treatment with triptolide. Data expressed as the mean±SEM of three independent experiments.

Fig. 4.

Triptolide treatment for 24 hr results in dose dependent decrease in AR full-length mRNA level in androgen dependent LNCaP(A) and CRPC C4-2 (B) and 22RV1 (C) cell. In addition, triptolide treatments for 24 hr in results in dose dependent decrease in AR splice variants *AR 1/2/2b* (D), *AR 1/2/3/2b* (E), and *AR 1/2/3/CE3* (F) at mRNA level in 22RV1 cells. Data expressed as the mean $\pm$ SEM of three independent experiments.

Fig. 5.

Treatment with triptolide for 24 hr decreased expression of Sp1 at the mRNA level (A) and protein level (B). Triptolide also decreased the Sp1 transcriptional activity when measured using dual luciferase reporter assay for Sp1 (C). Immunofluorescence showed that Sp1 (FITC) accumulated in the cytosol after treatment with 25 nM triptolide for 24 hr. DAPI was used to stain for nucleus (D). Quantitation of cytosolic/nuclear staining for Sp1 confirmed the immunofluorescence observation (E). Inhibition of Sp1 using mithramycin in 22Rv1 decreased their cell viability (F). Silencing Sp1 decreased the full length AR as well as

splice variants (G). Overexpression of Sp1 rescued 22Rv1 cells from triptolide induced cell death (H) as well as AR-FL mRNA expression (I).

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Fig. 6.

Tumor volumes of 22RV1 cells xenografted subcutaneously in athymic nude mice were measured weekly (saline and Minnelide) to monitor tumor progression (A). Minnelide decreased end-of-study tumor volume (B) and tumor weight (C). To compare with the standard of care, xenografted tumors were treated with Docetaxel, Enzalutamide and Minnelide, and the tumor progression was documented (D). Minnelide treatment showed significantly decreased end-of-study tumor weight (E). Expression of AR and its splice variants were reduced in the tumors after treatment with Minnelide (F).