

Combination Therapy of Antiandrogen and XIAP Inhibitor for Treating Advanced Prostate Cancer

Michael Danquah • Charles B. Duke III • Renukadevi Patil • Duane D. Miller • Ram I. Mahato

Received: 4 February 2012 / Accepted: 9 March 2012 / Published online: 27 March 2012
© Springer Science+Business Media, LLC 2012

ABSTRACT

Purpose Overexpression of the androgen receptor (AR) and anti-apoptotic genes including X-linked inhibitor of apoptosis protein (XIAP) provide tumors with a proliferative advantage. Therefore, our objective was to determine whether novel antiandrogen (CBDIV17) and XIAP inhibitor based combination therapy can treat advanced prostate cancer.

Methods CBDIV17 and embelin-6g were synthesized and their effect on cell proliferation, apoptosis, cell cycle and AR and XIAP gene silencing determined.

Results CBDIV17 was more potent than bicalutamide and inhibited proliferation of C4-2 and LNCaP cells, IC_{50} for CBDIV17 was $\sim 12 \mu M$ and $\sim 21 \mu M$ in LNCaP and C4-2 cells, respectively, whereas bicalutamide had IC_{50} of $\sim 46 \mu M$ in LNCaP cells and minimal effect in C4-2 cells. CBDIV17 induced apoptosis more effectively compared to bicalutamide and significantly inhibited DNA replication. Combination of CBDIV17 and embelin resulted in supra-additive antiproliferative and apoptotic effects. Embelin downregulated AR expression and decreased androgen-mediated AR phosphorylation at Ser⁸¹. These hydrophobic drugs were solubilized using micelles prepared with polyethylene glycol-b-poly (carbonate-co-lactide) (PEG-b-p(CB-co-LA)) copolymer. Combination therapy inhibited prostate tumor growth more effectively compared to control or monotherapy *in vivo*.

Conclusions Our results demonstrated that CBDIV17 in combination with embelin can potentially treat advanced prostate cancer.

Electronic supplementary material The online version of this article (doi:10.1007/s11095-012-0737-1) contains supplementary material, which is available to authorized users.

R. I. Mahato (✉)
University of Tennessee Health Science Center
Department of Pharmaceutical Sciences
19 South Manassas, CRB RM 224
Memphis 38103-3308, Tennessee, USA
e-mail: rmahato@uthsc.edu
URL: <http://www.uthsc.edu/pharmacy/rmahato>

M. Danquah
University of Tennessee Health Science Center
Department of Pharmaceutical Sciences
19 South Manassas, CRB RM 226
Memphis 38103-3308, Tennessee, USA

C. B. Duke III • R. Patil
University of Tennessee Health Science Center
Department of Pharmaceutical Sciences
327 Johnson Building
Memphis 38163, Tennessee, USA

D. D. Miller
University of Tennessee Health Science Center
Department of Pharmaceutical Sciences
435 Pharmacy Building
Memphis 38163, Tennessee, USA

KEY WORDS androgen receptor • antiandrogen • bicalutamide • embelin • polymeric micelles • prostate cancer • XIAP

INTRODUCTION

Prostate Cancer is the most frequently diagnosed neoplasm and the second leading cause of cancer mortality affecting men in the United States (1). Nearly all prostate carcinomas are initially androgen dependent (2). Hence, the disease is classified as hormone-dependent or hormone-refractory depending on the sensitivity of the androgen receptor (AR) to androgens. To date, androgen ablation therapy using antiandrogens remains the gold standard for treating hormone-dependent prostate cancer and is effective in approximately 70 % of patients (3). In spite of its initial efficacy, androgen ablation results in remission lasting two

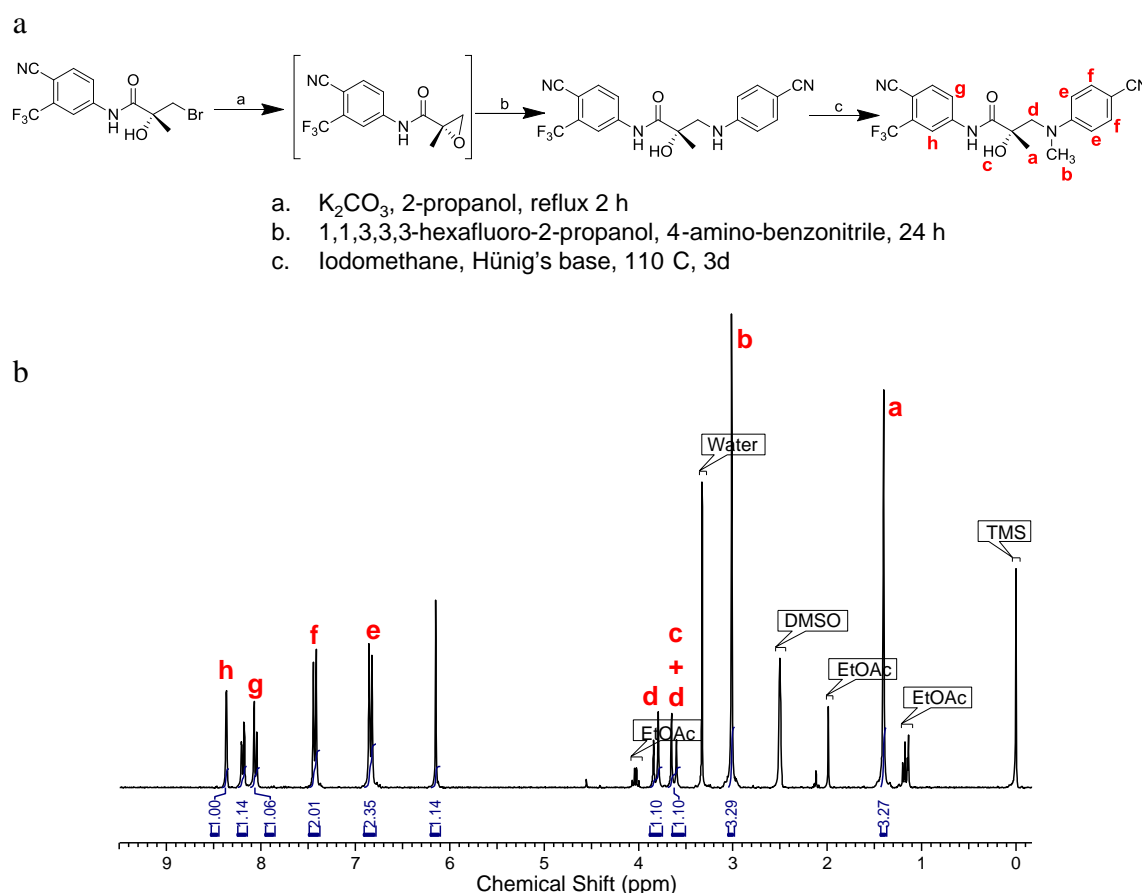


Fig. 1 Synthesis and characterization of (S)-N-(4-cyano-3-(trifluoromethyl)phenyl)-3-((4-cyanophenyl)(methyl)amino)-2-hydroxy-2-methylpropanamide (CBDIV17). **(a)** Synthesis scheme of CBDIV17. **(b)** ^1H NMR spectrum of CBDIV17.

to three years after which the disease recurs in its more aggressive hormone refractory form for which there is no cure (4,5). Consequently, there is an urgent need for novel therapeutic strategies for treating hormone refractory prostate cancer.

We have previously shown that combination therapy targeting AR and XIAP using bicalutamide and embelin is a promising approach to treat prostate cancer (6). However, first generation antiandrogens such as bicalutamide exhibit agonist characteristics upon prolonged treatment due to increased AR gene amplification (7,8). Therefore, we hypothesized new antiandrogens more effective than bicalutamide will be beneficial in treating advanced prostate cancer when combined with XIAP modulation. Hence, our group synthesized a novel antiandrogen ((S)-N-(4-cyano-3-(trifluoromethyl)phenyl)-3-((4-cyanophenyl)(methyl)amino)-2-hydroxy-2-methylpropanamide) (CBDIV17) using bicalutamide as a starting chemical scaffold due to its relatively high AR binding affinity and selectivity (Fig. 1a).

Systemic delivery of anticancer agents to solid tumors is still a challenge. Aside being toxic, current solubilizing agents such as Cremophor® EL and

DMSO contribute to erratic extravasation to tumors leading to sub-optimal therapeutic effects (9). Polymeric micelles are an elegant way to improve the solubility, stability and extravasation of drugs to tumors thereby enhancing therapeutic potential (6,9–12). They self-assemble from amphiphilic diblocks and are typically nanosized, spherical structures with a hydrophobic core which serves as a cargo space for hydrophobic drugs. We recently engineered and synthesized polyethylene glycol-b-poly (carbonate-co-lactide) (PEG-b-p(CB-co-LA)) copolymers with excellent drug loading and stability for optimal delivery of bicalutamide (10) and we will explore the use of this copolymer to formulate CBDIV17 and embelin in this work.

The focus of the present study is three fold. First to demonstrate the superiority of CBDIV17 in treating prostate cancer compared to bicalutamide. Second, to evaluate two XIAP inhibitors: embelin and 2,5-dihydroxy-3-(2-[4-(2-m-tolyl-ethyl)-phenyl]-ethyl)-[1,4] benzoquinone (embelin-6g) to determine the more appropriate compound to combine with CBDIV17. Third, to show that combination of CBDIV17 and chosen XIAP inhibitor is more potent than

control or monotherapy in inhibiting cell growth and inducing apoptosis *in vitro* as well as in regressing prostate cancer tumors in xenograft mouse model.

MATERIALS AND METHODS

Materials

SYBR Green real-time RT-PCR master mix and reverse transcription reagents were purchased from Applied Biosystems (Foster city, CA). Human total XIAP ELISA kit was purchased from R&D Systems (Minneapolis, MN). Primary and secondary antibodies were purchased from Abcam (Cambridge, MA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated and were used as received.

Synthesis of CBDIV17

CBDIV17 was synthesized by treating 3-Bromo-2-hydroxy-N-(4-isocyano-3-trifluoromethyl-phenyl)-2-methyl-propionamide (bromide N) with K_2CO_3 in 2-propanol at reflux for 2 h before filtering the solution. The reaction mixture was concentrated to a paste, dissolved in EtOAc, and washed with water before drying using Na_2SO_4 and concentrating to a resin. The epoxide intermediate was then treated with 4-aminobenzonitrile in 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol at 100 °C overnight. Flash chromatography on silica gel (EtOAc/Hexanes 20–100 %) afforded (S)-N-(4-cyano-3-(trifluoromethyl)phenyl)-3-((4-cyanophenylamino)-2-hydroxy-2-methylpropanamide), which was treated with MeI and Hünig's base in a pressure vessel for 3 days at 110 °C. Flash chromatography on silica gel (EtOAc/Hexanes 20–100 %) afforded CBDIV17.

In Vitro Cell Viability Assays

LNCaP and C4-2 cells (American Type Culture Collection) were incubated in RPMI 1640 media, 10 % fetal bovine serum and 1 % antibiotic-antimycotic at 37 °C in humidified environment of 5 % CO_2 and subcultured every 3–4 days to maintain exponential growth. Cells were seeded in 96-well plates at a density of 1×10^4 viable cells/well and incubated for 48 h. The cells were exposed to antiandrogen or XIAP inhibitor at various concentrations for specified time periods. At the end of treatment, 20 μ l of MTT (5 mg/ml) was added to each well, incubated for 3–4 h and analyzed at a test wavelength of 560 nm. Cell viability was expressed as a percentage of the intensity of controls. LNCaP cells are androgen dependent and non-bone metastatic prostate cancer cells with low tumorigenicity. In contrast, C4-2 cells are a subline

of LNCaP, but androgen independent and more tumorigenic compared to LNCaP cells. These cells lines were selected as *in vitro* models since they both express the androgen receptor and possess a threonine to alanine mutation of amino acid 877 (T877A) in the androgen receptor (13,14). Furthermore, LNCaP represents hormone dependent prostate cancer whereas C4-2 is a model for advanced prostate cancer.

Real-Time RT-PCR

RNA extraction and real-time RT-PCR was performed as previously described (6). Primers are as follows: AR: forward, 5'-AGC CAT TGA GCC AGG TGT AG-3'; reverse, 5'-CGT GTA AGT TGC GGA AGC C-3'. PSA: forward, 5'-GTG GGT CCC GGT TGT CT-3'; reverse, 5'-AGCCCAGCTCCCTGTCT-3'. XIAP: forward, 5'-TGT TTC AGC ATC AAC ACT GGC ACG-3'; reverse, 5'-GCA TGA CAA CTA AAG CAC CGG AC-3'. Cyclin D1: forward, 5'-GAT GCC AAC CTC CTC AAC GAC-3'; reverse, 5'-CTC CTC GCA CTT CTG TTC CTC-3'.

Western Blot Analysis

Cells were treated with embelin and embelin-6g for 96 h. Subsequently, cells were lysed using RIPA buffer (Sigma-Aldrich, St. Louis, MO) and protein concentration measured with bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). The lysate was boiled for 5 min, subjected to a 15 % SDS-PAGE and transferred to a PVDF membrane using iBlot™ system (Invitrogen, Carlsbad, CA). Membranes were blocked with 3 % BSA in 1x tris buffered saline (TBS) at room temperature for 1 h and then incubated with primary antibodies at 4 °C overnight, followed by incubation with secondary antibody conjugated with horseradish peroxidase (HRP) at room temperature for 1 h. The signal of target proteins was detected using Immuno-Star HRP chemiluminescent kit (BioRad, Hercules, CA) or Li-COR Odyssey® infrared imaging system (Li-COR, Lincoln, NE).

XIAP Detection Using ELISA

C4-2 cells were treated with embelin or embelin-6g for 48 h. Cells were lysed and XIAP concentration detected using Human XIAP ELISA kit as described in the manufacturer's protocol.

Apoptosis and Cell Cycle Analysis

Following treatment with 25 μ M antiandrogen for 72 h, cells were trypsinized and fixed in 70 % ice-cold ethanol and washed with PBS. Samples were resuspended in 500 μ l of

propidium iodide solution containing RNase A (BD Pharmingen, San Diego, CA) for 15 min at room temperature. Relative DNA content per cell was acquired by measuring DNA fluorescence using flow cytometry and subsequent analysis performed with Modfit program.

Confocal Microscopy

Confocal microscopy was used to elucidate the effect of XIAP inhibitor on DHT stimulated nuclear translocation of AR. Briefly, LNCaP and C4-2 cells were cultured in chamber slides in RPMI media and treated with XIAP inhibitor (5 μ M) or DMSO for 3 h and then incubated with DHT (1 nmol/L) for an additional 3 h. Cells were fixed in methanol at room temperature for 10 min, washed with PBS, and incubated with PBS containing 10 % (*w/v*) rabbit serum for 2 h at room temperature. Afterwards, the cells were treated with anti-AR antibody (1:100 in PBS buffer) 4 °C overnight. Cells were then washed with PBS and incubated with FITC-conjugated secondary antibody for 1 h at room temperature followed by counterstaining with DAPI. Slides were examined under a Zeiss LSM-710 confocal microscope at 63 \times objective lens magnification and data processed using a ZEN 2009 LE software.

Preparation and Characterization of Micelles

The film sonication method was used for loading drug into the core of PEG-b-p(CB-co-LA) micelles at a theoretical drug loading of 5 %. Briefly, 5 mg of drug and 95 mg of PEG-b-p(CB-co-LA) was dissolved in 5 ml methanol. The mixture was allowed to stir for 5 min and the solvent evaporated. The resulting film was hydrated and sonicated for 7 min using a Misonix ultrasonic liquid processor (Farmingdale, NY) with an output power of 25 W. The resultant formulation was then centrifuged at 5,000 rpm for 10 min to separate micelles from residual free drug. Subsequently, the supernatant was filtered using a 0.22 μ m nylon filter.

In Vivo Efficacy of CBDIV17 and Embelin-Loaded Micelles in Mouse Xenografts

All animal experiments were performed in a humane manner in accordance with NIH animal use guidelines and the protocol approved by the Animal Care and Use Committee (ACUC) at the University of Tennessee Health Science Center. Xenograft flank tumors were induced in 6 week old male athymic nu/nu mice (Jackson Laboratory, Bar Harbor, ME) by subcutaneous injection of 3 million C4-2 cells suspended in 1:1 media and matrigel. When tumors reached approximately 50 mm³, mice were randomized into four groups of 7 mice, minimizing

weight and tumor size differences. Each group was treated with intratumoral injection of blank micelles or drug-loaded micelles. Tumors were measured with a caliper prior to each injection, and their volumes calculated using the formula of ($\text{width}^2 \times \text{length}$)/2.

RESULTS

Synthesis and Characterization of CBDIV17

CBDIV17 was synthesized as shown in Fig. 1a and characterized by ESI and high-resolution mass spectrometry (HRMS), ¹H NMR (Fig. 1b) and ¹³C NMR. MS (ESI): calculated for C₂₀H₁₇F₃N₄O₂ 402.1, found 400.9 [M - H]. HRMS calculated for C₂₀H₁₇F₃N₄O₂ 403.13818 found 403.13696. ¹H NMR (300 MHz, *chloroform-d*) 10.42–10.51 (m, 1H), 8.33–8.41 (m, 1H), 8.14–8.24 (m, 1H), 8.06 (d, *J*=8.54 Hz, 1H), 7.43 (d, *J*=9.16 Hz, 2H), 6.84 (d, *J*=9.16 Hz, 2H), 6.15 (s, 1H), 3.81 (d, *J*=15.26 Hz, 1H), 3.62 (d, *J*=14.95 Hz, 1H), 3.01 (s, 3H), 1.37–1.43 (m, 3H) ppm (Fig. 1). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 175.4, 152.3, 142.9, 136.1, 132.7, 131.3 (q, *J*=31.5 Hz), 122.8, 122.4 (q, *J*=276.6 Hz), 120.2, 117.5 (q, *J*=5.1 Hz), 115.7, 112.0, 101.9, 95.9, 76.7, 59.3, 39.7, 39.4 (spt, DMSO), 23.7, 0.0 (s, TMS) ppm. HRMS and ¹³C NMR data are provided as [Supplementary Material](#).

CBDIV17 and Bicalutamide Inhibit LNCaP and C4-2 Cell Growth

We first assessed anticancer activity of CBDIV17 and bicalutamide in LNCaP and C4-2 cells. Both cells are AR positive and possess the T877A androgen receptor mutant. As shown in Fig. 2a and b, bicalutamide and CBDIV17 exhibited dose-dependent anticancer activities in both cells when treated with 0, 10, 25 and 50 μ M for 72 h. From our results, CBDIV17 was more potent than bicalutamide at each dose in both cell lines. IC₅₀ for CBDIV17 was 12 and 21 μ M in LNCaP and C4-2 cells, respectively. In contrast, bicalutamide had an IC₅₀ of 46 μ M in LNCaP with only a modest anticancer effect being observed at 50 μ M in C4-2 cells. Furthermore, a time course study using a drug concentration of 25 μ M for 24 and 72 h revealed the anticancer effect of bicalutamide and CBDIV17 to be time-dependent (Fig. 2c). Our data suggests CBDIV17 displays superior anticancer activity in LNCaP cells even after a short exposure time. Additionally, there is a dramatic increase in inhibition of prostate cancer cell growth for CBDIV17 compared to bicalutamide when cell exposure to drug is increased from 24 to 72 h.

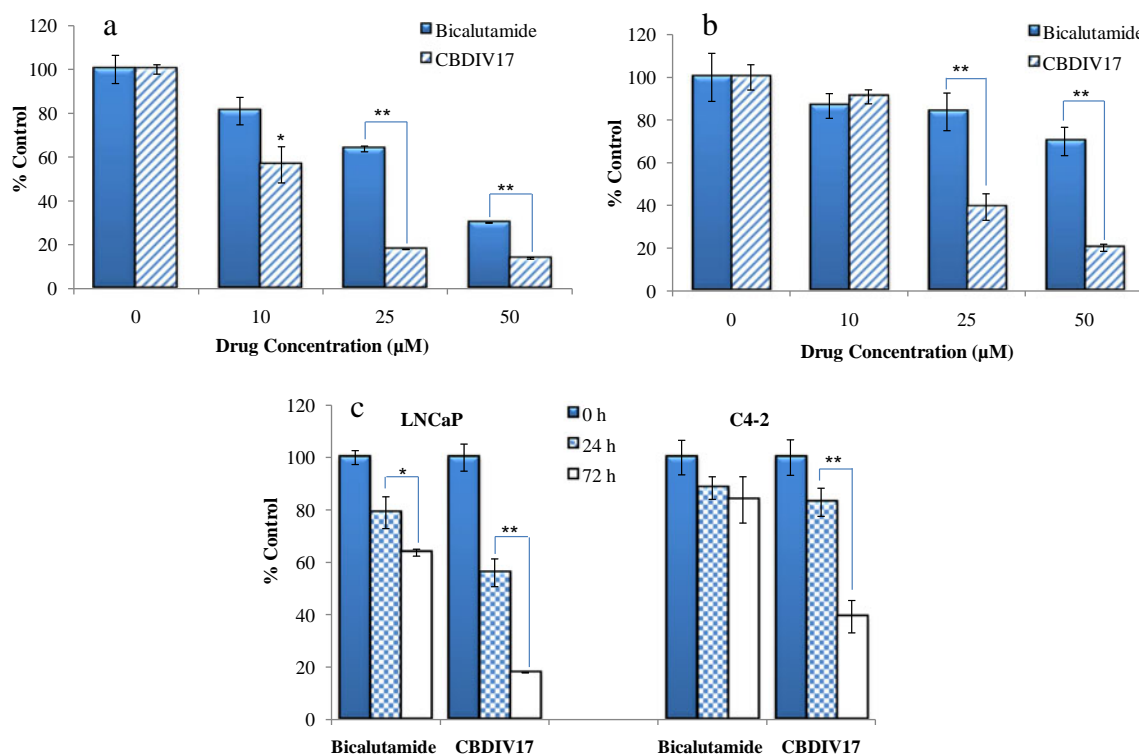
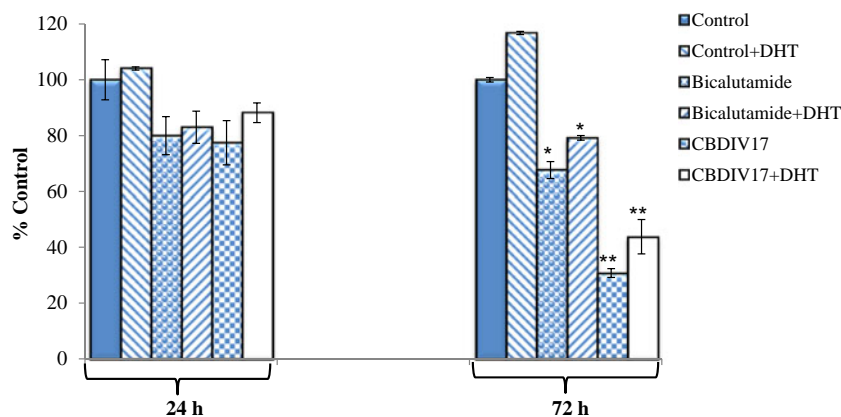


Fig. 2 Anticancer effect of antiandrogen on prostate cancer cells. **(a and b)** IC₅₀ of bicalutamide and CBDIV17 in LNCaP and C4-2 cells were calculated following treatment with 0, 10, 25 and 50 μM of drug for 72 h and cell viability determined by MTT assay. **(c)** Effect of antiandrogen with time was determined by exposing LNCaP and C4-2 cells to 25 μM of drug for 24 and 72 h. Cell viability was then determined by MTT assay. Results are represented as the mean ± SD of triplicates. **p* < 0.05; ***p* < 0.01 using Student's unpaired *t* test.

We next examined the influence of androgens on the anticancer activity of CBDIV17 since it is expected to function as a putative anti-androgen. LNCaP cells were cultured in androgen-depleted media with 5 % charcoal-stripped serum (CSS). Cells were treated for 24 or 72 h with or without 1 nM of the synthetic androgen 5α-Dihydrotestosterone (DHT) combined with DMSO (vehicle), CBDIV17 and bicalutamide (positive control). Our data suggests that treatment of cells with DHT stimulated cell growth (Fig. 3). However, even in the presence of DHT, cell growth was inhibited when cells were treated with bicalutamide or CBDIV17, with CBDIV17 being more potent than bicalutamide.

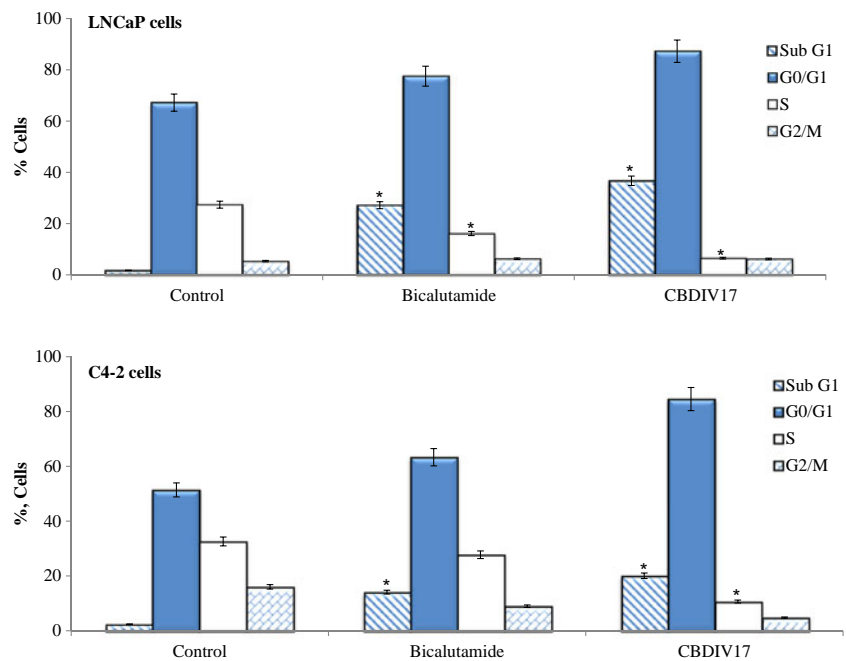
Fig. 3 Effect of bicalutamide and CBDIV17 on cell proliferation in the presence of androgen. LNCaP cells were cultured in androgen-depleted media with 5 % charcoal-stripped serum (CSS). Cells were treated for 24 or 72 h with or without 1 nM of the synthetic androgen 5α-Dihydrotestosterone (DHT) combined with DMSO, bicalutamide and CBDIV17. Results are represented as the mean ± SD of triplicates. **p* < 0.05; ***p* < 0.01 using Student's unpaired *t* test.



CBDIV17 and Bicalutamide Induce Apoptosis and Alter Cell Cycle of LNCaP and C4-2 Cells

The effect of bicalutamide and CBDIV17 on the cell cycle of LNCaP and C4-2 cells was determined following treatment with 25 μM of these drugs for 72 h. We observed a significant reduction in the percent of cells in S phase in both cell lines (Fig. 4). In LNCaP cells, 27 % of control cells were in the S phase, whereas 16 % of bicalutamide treated cells and 6.5 % of CBDIV17 treated cells were in the S phase. For C4-2 cells, 32.6 % of control cells were in the S phase whilst 27.7 % of bicalutamide treated cells and

Fig. 4 Effect of bicalutamide and CBDIV17 on apoptosis and cell cycle of LNCaP and C4-2 cells. Cells were treated with antiandrogen (25 μ M) for 72 h, stained with propidium iodide and analyzed on a flow cytometer. * p < 0.05 using Student's unpaired t-test.



10.7 % of CBDIV17 treated cells were in the S phase. Furthermore, drug treatment in LNCaP cells did not result in significant decrease in percent of cells in the G2/M phase. In contrast, percent of C4-2 cells in the G2/M phase decreased from 16 % in control cells to 8.9 % in bicalutamide treated cells and to 4.8 % in CBDIV17 treated cells.

Treatment with bicalutamide and CBDIV17 resulted in a significant increase in the percentage of cells in the G0/G1 phase in both cell lines. LNCaP cell population in the G0/G1 phase increased from 67 % in control cells to 76 % and 87 % for bicalutamide and CBDIV17 treated cells, respectively. For C4-2 cells, percentage of cells in G0/G1 phase increased from 51.4 % in control cells to 63.3 % and 84.5 % for bicalutamide and CBDIV17 treated cells, respectively. Finally, a significant increase in the sub-G1 phase (indicative of apoptosis) was observed upon administration of bicalutamide (13-fold) and CBDIV17 (18-fold) to LNCaP cells (Fig. 4). In C4-2 cells, treatment with bicalutamide resulted in a 7-fold increase in sub-G1 whereas CBDIV17 led to a 10-fold increase in percentage of cells in subG1 phase. These data demonstrate that CBDIV17 was more potent than bicalutamide in altering cell cycle leading to G1 arrest, reducing DNA synthesis and inducing cell death.

Embelin-6g is More Potent than Embelin in Repressing XIAP Expression

After demonstrating that CBDIV17 is more potent than bicalutamide, we next evaluated the mechanism and therapeutic potential of embelin and embelin-6g (Fig. 5a) to

determine which XIAP inhibitor should be used in conjunction with CBDIV17 for combination therapy. Since embelin-6g is reported to be a more potent XIAP inhibitor than embelin (15), we examined its effect on XIAP expression. Our observations indicated that embelin-6g repressed XIAP mRNA expression by 2-fold compared to embelin (Fig. 5b). We also assessed the effect of these drugs on XIAP protein using ELISA. Embelin-6g more potently inhibited XIAP expression compared to embelin with the effect being almost twice that of embelin (Fig. 5c).

Embelin and its Derivative Downregulate AR Expression and Repress AR Mediated Activity

In addition to elucidating the effect of embelin and embelin-6g on XIAP expression, we investigated the effect of these drugs on the androgen receptor signaling axis which is a key regulatory gene involved in prostate cancer. First, we examined the effect of embelin and embelin-6g on AR expression. 1×10^5 C4-2 cells were treated with drug (5 μ M) for 48 h and the amount of AR message was quantitatively determined by real-time RT-PCR. There was a significant decrease in AR mRNA levels (Fig. 6a) with both embelin and embelin-6g reducing AR levels by more than 50 % compared to control. Additionally, Western blot analysis revealed a decrease in AR protein amounts when C4-2 cells were treated with 5 μ M embelin or embelin-6g for 96 h (Fig. 6b).

Furthermore, we evaluated AR transcriptional activity by examining mRNA expression of prostate specific antigen (PSA). We observed 50 % decrease in PSA mRNA levels for both drugs (Fig. 6a). Additionally, we determined the

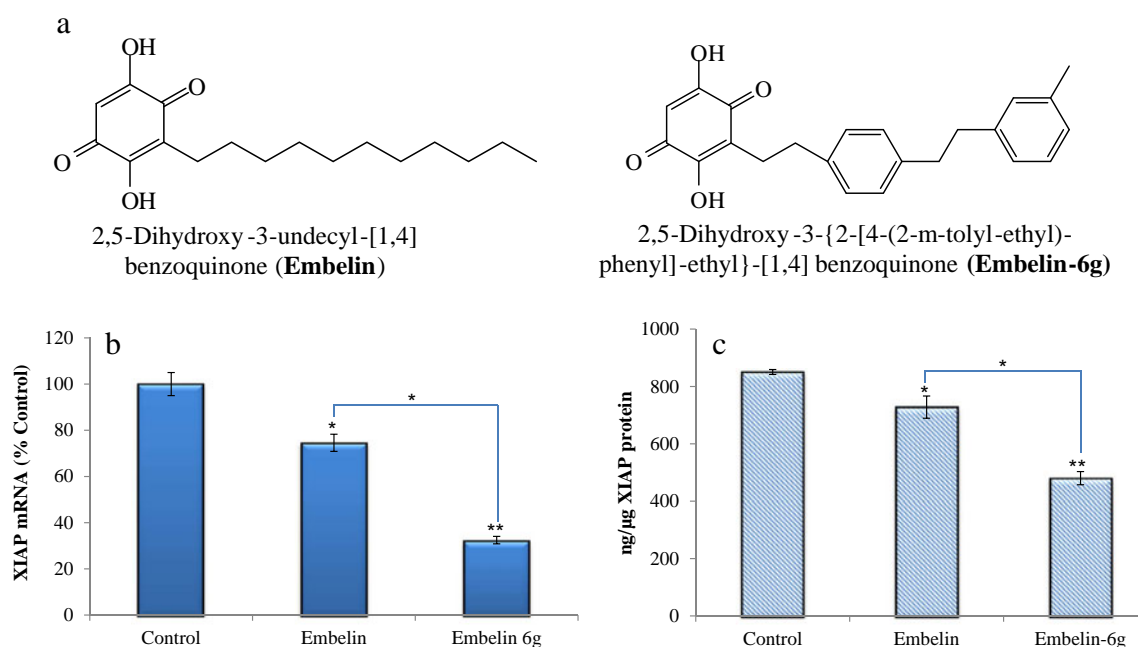


Fig. 5 Embelin and embelin-6g suppress XIAP expression. **(a)** Chemical structures of embelin and embelin-6g. **(b)** 1×10^5 C4-2 cells were treated with embelin or embelin-6g for 48 h, RNA extracted and XIAP expression at mRNA level determined using real-time RT-PCR. **(c)** 1×10^6 C4-2 cells were treated with embelin or embelin-6g for 96 h, protein isolated and XIAP expression at protein level determined using ELISA. * $p < 0.05$, ** $p < 0.01$ using Student's unpaired t-test.

effect of embelin and embelin-6g on intracellular PSA levels. Drug treatment resulted in a decrease in PSA protein expression compared to the control (Fig. 6b). These results demonstrate the ability of embelin to modulate AR levels and show direct correlation between AR protein amount and its subsequent ability to transactivate PSA.

Embelin Blocks DHT-Stimulated AR Nuclear Translocation and Phosphorylation

To determine whether embelin inhibits AR translocation in C4-2 cells we used confocal microscopy to determine the localization of AR in the nucleus. AR was diffusively distributed in the nucleus (Fig. 6c) but cytoplasmic AR translocation to the nucleus was significantly decreased upon incubation with embelin. In contrast, treatment with 1 nM DHT resulted in a majority of cytoplasmic AR translocating into the nucleus. Furthermore, since androgens stimulate phosphorylation of AR Ser81 especially in cells possessing T877A mutation, we examined the effect of embelin on AR phosphorylation (16). C4-2 cells were treated with various concentrations of embelin in the presence or absence of DHT. A concentration-dependent effect of AR Ser81 phosphorylation post embelin treatment was observed strongly suggesting embelin suppressed DHT-induced AR phosphorylation (Fig. 6d).

Embelin and its Derivative Inhibit Prostate Cancer Cell Growth

We next investigated the anticancer activity of embelin and embelin-6g by determining their IC_{50} values in LNCaP and C4-2 cells. Both drugs were found to be equally potent in inhibiting the proliferation of prostate cancer cells regardless of androgen status (Fig. 7a). IC_{50} was approximately 6.5 μ M.

To provide mechanistic insight into the effect of embelin and embelin-6g on cell proliferation, we examined whether these drugs modulate the expression of cyclin D1 which is a known marker of cell proliferation. Treatment of C4-2 cells with 5 μ M of embelin and embelin-6g for 48 h resulted in suppression of cyclin D1 expression by ~22 % (Fig. 7b). Western blot analysis also revealed inhibition of cyclin D1 when cells were treated with drug for 96 h (Fig. 7c).

Since we were interested in using a XIAP inhibitor which has fast acting activity for our combination study, we examined the anticancer activity of embelin and embelin-6g in LNCaP and C4-2 cells at 18 h post treatment. Our results (Fig. 7d) suggest embelin to be faster acting and more potent than embelin-6g in inhibiting cell growth especially in C4-2 cells. Therefore, we selected embelin as the XIAP inhibitor for further studies in our combination therapy.

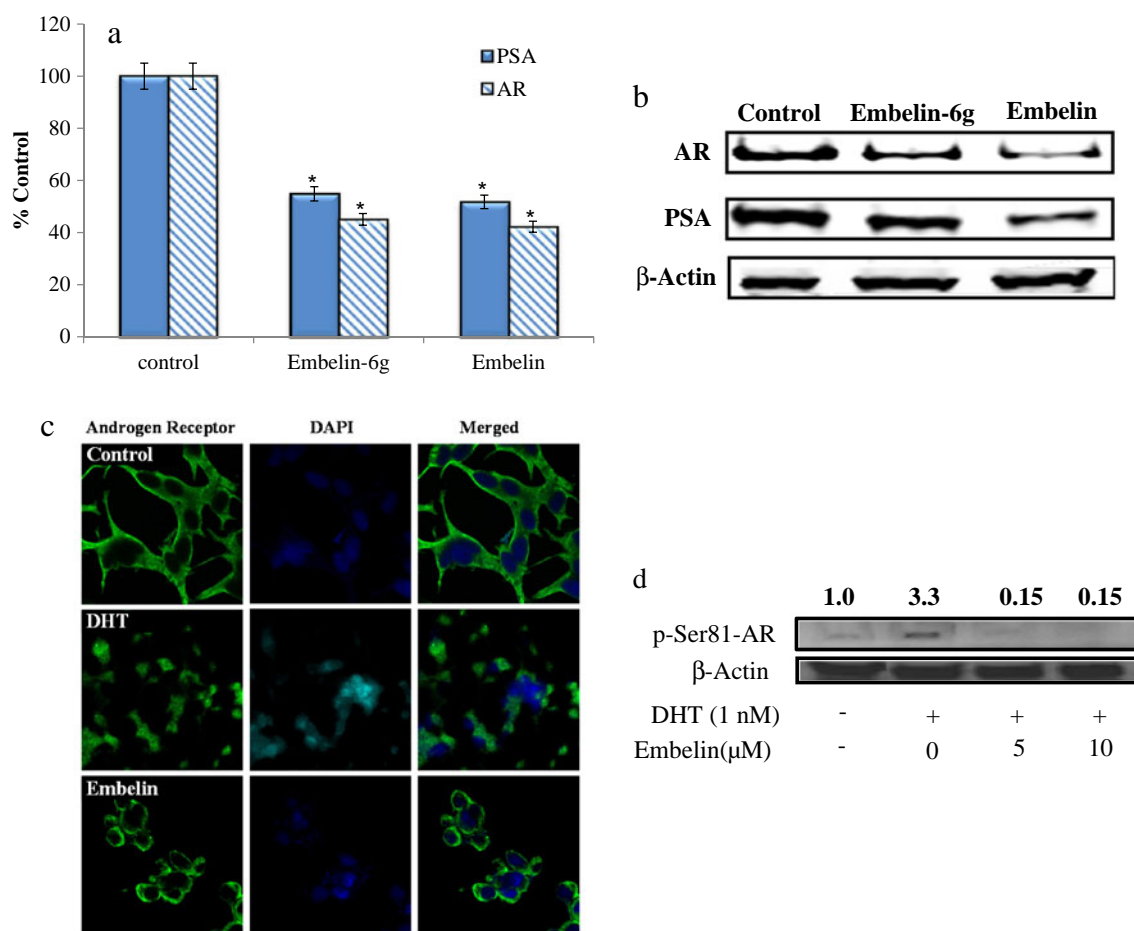


Fig. 6 Effect of embelin and embelin-6g on AR and PSA expression. **(a)** C4-2 cells were treated with embelin and embelin-6g ($5 \mu\text{M}$) for 48 h, RNA extracted and AR and PSA expression at mRNA level determined using real-time RT-PCR. **(b)** Cells were treated with embelin and embelin-6g ($5 \mu\text{M}$) for 96 h, protein isolated and AR and PSA expression at protein level determined using Western blot. **(c)** C4-2 cells were grown on chamber slides treated in the presence or absence of $5 \mu\text{M}$ embelin for 12 h prior to addition of 1 nM DHT. Cells were fixed, permeabilized and AR immunostained with rabbit anti-AR followed by FITC-conjugated secondary antibody and nucleus stained with DAPI. **(d)** Phosphorylated Ser81 AR protein levels in C4-2 cells were determined by Western blot analysis. Cells were grown in CSS and treated with Embelin (5 or $10 \mu\text{M}$), in the presence or absence of 1 nM DHT for 48 h.

Formulation of CBDIV17 and Embelin-Loaded PEG-b-p(CB-co-LA) Micelles and Their Effect on Prostate Cancer Cell Growth and Migration

Since CBDIV17 and embelin are extremely hydrophobic and we plan to test our combination therapy *in vivo*, we first formulated these drugs into Poly (ethylene glycol)-b-poly (carbonate-co-lactide) (PEG-b-p(CB-co-LA)) micelles. Micelle size ranged from ~ 83 to 90 nm (Table I) and the presence of drug did not significantly affect micelles size. Furthermore, (PEG-b-p(CB-co-LA)) micelles were monodisperse and had a PDI of approximately 0.11. Encapsulation efficiency for CBDIV17 was $91.2 \pm 2.3 \%$ at 5 % theoretical loading but dropped to $55.7 \pm 1.4 \%$ when theoretical loading increased to 10 %. In contrast, encapsulation efficiency for embelin was $38.4 \pm 1.0 \%$ at 5 % theoretical loading and $35.0 \pm 0.9 \%$ at 10 % theoretical loading. Therefore, a 5 % theoretical loading was used for formulating micelles for further studies.

Following micelle formulation and characterization, we investigated whether the combination of CBDIV17 and embelin was more potent than their monotherapy in treating prostate cancer. C4-2 cells were treated with embelin ($5 \mu\text{M}$) or CBDIV17 ($25 \mu\text{M}$) alone or in combination for 24 h. Combination therapy was found to be more effective than control or monotherapy (Fig. 8a). Both embelin and CBDIV17 resulted in approximately 20 % decrease in cell growth while combination therapy inhibited cell growth by about 70 %.

We also determined the effect of combining CBDIV17 and embelin on the migration potential of C4-2 cells using the scratch wound assay. We observed embelin to have no obvious effect on cell migration under our conditions whereas CBDIV17 had some modest effect. The group treated with the combination of CBDIV17 and embelin resulted in superior inhibition of cell migration compared to untreated control and each drug alone (Fig. 8b).

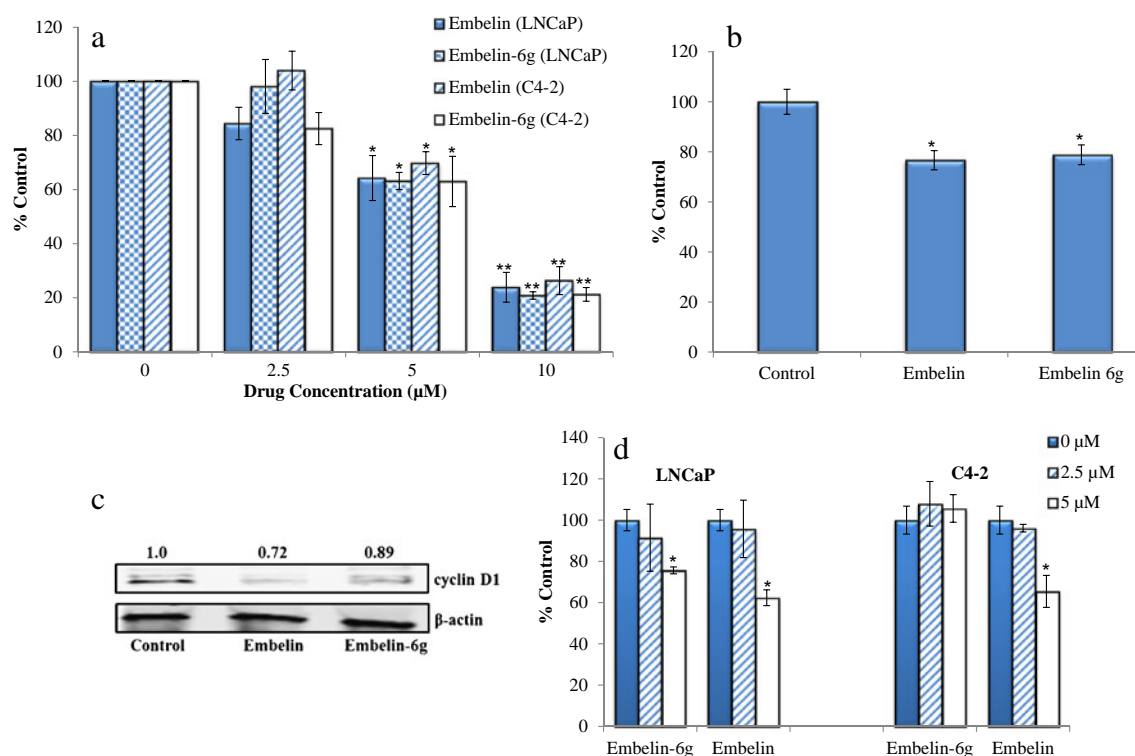


Fig. 7 Effect of embelin and embelin-6g on prostate cancer cell proliferation and cyclin D1, expression. **(a)** LNCaP and C4-2 cells were treated with XIAP inhibitor (0–10 μM) for 96 h and cell viability measured using MTT assay. **(b)** Cells were treated with XIAP inhibitors (5 μM) for 48 h, total RNA extracted and cyclin D1 expression at mRNA level determined using real-time RT-PCR. **(c)** 1×10^6 cells were treated with XIAP inhibitors for 96 h, protein isolated and cyclin D1 expression at protein level determined using Western blot. **(d)** LNCaP and C4-2 cells were treated with XIAP inhibitors (0, 2.5 and 5 μM) for 24 and cell viability measured using MTT assay. * $p < 0.05$, ** $p < 0.01$ using Student's unpaired t-test.

Effect of CBDIV17 and Embelin Combination on Apoptosis

Since CBDIV17 and embelin combination effectively inhibited cell proliferation *in vitro*, we further tested their effect on cell cycle and apoptosis in C4-2 cells. From Fig. 9a, there was a decrease in percentage of cells in the G2/M and S phases following treatment with CBDIV17. Treatment with 25 and 50 μM decreased percent of cells in the G2/M phase by 50 % while percentage of cells in the S phase

decreased by 30 %. Also, we found CBDIV17 showed dose-dependent increase in apoptosis (sub-G1). The percentage of cells in the sub-G1 phase was around 21 % at 25 μM and 38 % for 50 μM. In contrast, percentage of cells in the sub-G1 phase did not differ significantly from that of control for the concentrations of embelin tested. Notably, we observed combination of CBDIV17 (25 μM) and embelin (5 μM) induced apoptosis more potently compared to control or either treatment alone. Specifically, percentage of cells in the sub-G1 phase for combination therapy was 40-fold, 28-fold and 2-fold more, compared to control, embelin and CBDIV17, respectively. Additionally, combination of CBDIV17 (25 μM) and embelin (5 μM) resulted in percentage of cells in the sub-G1 phase comparable to that of 50 μM CBDIV17.

In Vivo Efficacy of CBD IV17 and Embelin-Loaded Micelles in Mouse Xenografts

Since CBDIV17 and embelin combination therapy effectively inhibited prostate cancer growth *in vitro*, we determined the effect of this combination approach in C4-2 xenograft tumor model. CBDIV17 and embelin were formulated using PEG-b-p(CB-co-LA) micelles and intratumoral injection of 10 mg/kg CBDIV17 and 10 mg/kg embelin-loaded

Table 1 Effect of Drug Loading on Poly (ethylene glycol)-b-poly (carbonate-co-lactide) (PEG-b-p(CB-co-LA)) Micelle Size and Encapsulation Efficiency

Theoretical loading (%)	Size (nm)	PDI	Encapsulation efficiency (%)
0	89.9 ± 0.7	0.10 ± 0.02	
CBD-IV 17			
5	86.4 ± 0.8	0.11 ± 0.02	91.2 ± 2.3
10	84.8 ± 0.2	0.12 ± 0.01	55.7 ± 1.4
Embelin			
5	85.6 ± 1.2	0.11 ± 0.01	38.4 ± 1.0
10	83.2 ± 0.5	0.10 ± 0.00	35.0 ± 0.9

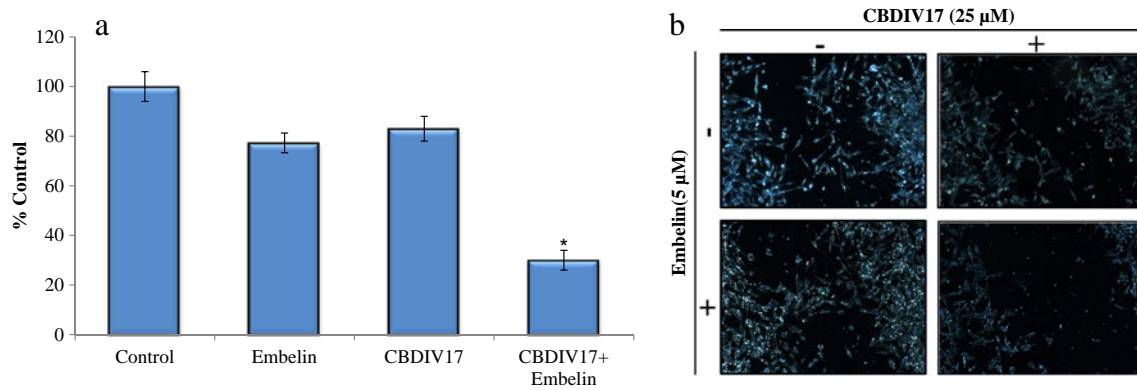


Fig. 8 Effect of CBDIV17 and embelin combination on prostate cancer cell growth and migration. **(a)** Anticancer effect of CBDIV17 and embelin, alone or in combination on C4-2 cells was determined following treatment of cells with 25 μ M of CBDIV17 and 5 μ M embelin for 24 h. and cell viability determined by MTT assay. * $p < 0.05$ using Student's unpaired t test. **(b)** C4-2 cells were grown to 70 % confluence in six well plates and three parallel wounds made using pipette tip. Cells were treated with CBDIV17 (25 μ M) or embelin (5 μ M) alone or in combination for 48 h after which cells were washed with ice cold 1 \times PBS and imaged under microscope.

micelles was administered on days 0, 3 and 7. CBDIV17 and embelin combination inhibited tumor growth more potently compared to the mice treated with empty micelles or monotherapy (Fig. 10a). On day 7 (last day of treatment), the tumor size of the combination group was 50 % of that in CBDIV17 group, 25 % of that in embelin group and 18 % of that in the control group (Fig. 10b). Furthermore, the body weight loss of mice was less than 5 % suggesting minimal toxicity of the treatment (Fig. 10c). Additionally, combination therapy increased tumor-doubling time compared to monotherapy and control. For instance, the time it took for tumor to double from 50 mm³ to 100 mm³ are as follows: the combination group took 14 days; CBDIV17 group took 7 days and less than 3 days for embelin and control group. Taken together, these results demonstrate that CBDIV17 and embelin combination therapy effectively inhibited tumor growth and can prolong survival.

DISCUSSION

Since prostate cancer is an androgen-dependent malignancy, current therapies including non-steroidal antiandrogens target the AR. However, first generation non-steroidal antiandrogens do not completely hinder AR activity and eventually become agonists in tumor cells partly due to elevated levels of AR. Furthermore, progression to hormone refractory prostate cancer is also characterized by resistance to apoptosis. Particularly, XIAP is overexpressed and inhibits caspases 9 and 3 thus contributing to a defect in the apoptotic machinery (17–20). Together, AR and XIAP overexpression may be a molecular reason leading to drug resistance and indicate that more potent antiandrogens, AR downregulating agents and XIAP inhibitors or their combination can effectively treat advanced prostate cancer. We have previously demonstrated that combination of

bicalutamide and embelin is more effective in treating prostate cancer compared to monotherapy (6). However, bicalutamide has limited efficacy in advanced prostate cancer (e.g., C4-2 cells) and assumes agonistic properties following prolonged treatment. Therefore, the primary objective of this study was to investigate whether a novel antiandrogen (CBDIV17) was more potent than bicalutamide and can effectively inhibit advanced prostate tumor growth when combined with XIAP inhibitor. We also further explored the molecular mechanism for our selected XIAP inhibitors.

To determine the comparative therapeutic benefits of CBDIV17 over bicalutamide, we evaluated the biological effects of both drugs in LNCaP and C4-2 cells by observing their effect on cell proliferation in the presence or absence of synthetic androgen and in inducing apoptosis. Our results show, CBDIV17 to be more potent than bicalutamide in inhibiting cell proliferation in a dose and time dependent manner (Fig. 2). Furthermore, inhibition of cell growth was only modestly reversed in the presence of DHT. This may possibly be due to better cytotoxic effects or its superior ability at the molecular level to retain antagonism and hence bypass antiandrogen resistance. Our observation suggests CBDIV17 analog to be more potent than bicalutamide regardless of AR sensitivity or presence of synthetic androgen and therefore has the potential for treating advanced prostate cancer.

Chen *et al.* synthesized new XIAP inhibitors with better binding affinities than embelin by modifying its hydrophobic tail. It was shown that embelin-6g has a two-fold better binding affinity to XIAP BIR3 compared to embelin and was potent in inhibiting proliferation of human breast and prostate cancer cell lines (15). Therefore, we were interested in how better XIAP binding affinity would translate in terms of potency in inhibiting cell growth and inducing apoptosis in prostate cancer cells. Although Chen *et al.* showed better binding affinity for embelin-6g they did not show its effect on XIAP gene expression. In our studies, we found embelin-6g

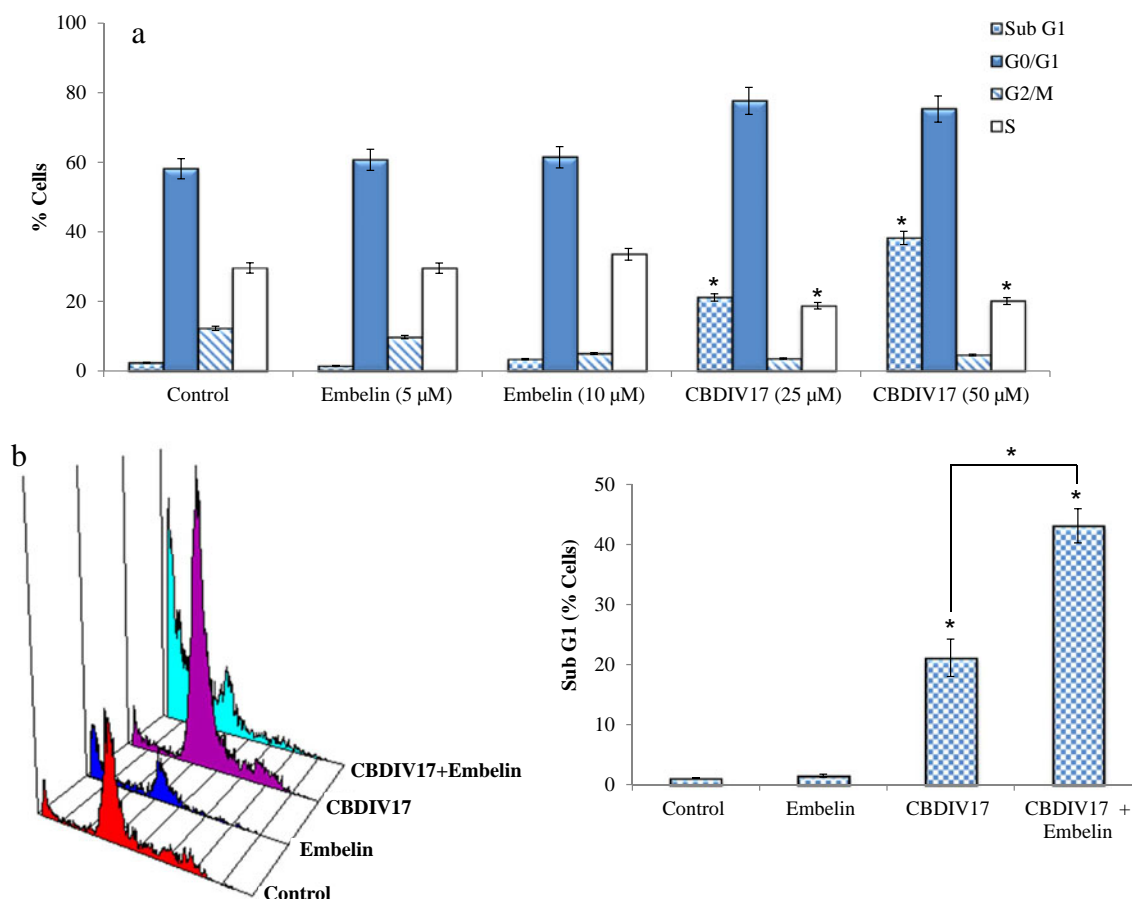


Fig. 9 Effect of embelin and CBDIV17 combination on cell cycle and apoptosis. **(a)** C4-2 cells were treated with vehicle, embelin (5 and 10 μ M) and CBDIV17 (25 and 50 μ M) for 72 h, stained with propidium iodide and analyzed on a flow cytometer. **(b)** Cells were treated with vehicle, embelin (5 μ M) and CBDIV17 (25 μ M) alone or in combination for 72 h, stained with propidium iodide and analyzed on a flow cytometer. *Left panel*, cell cycle distribution; *Right panel*, quantitative analysis of sub G1 phase. * $p < 0.05$ using Student's unpaired t test.

to be more potent than embelin in repressing XIAP gene expression. At both the mRNA and protein levels, embelin-6g appeared to be twice as effective as embelin (Fig. 5b and c). This result may logically extend from embelin-6g's two-fold better binding affinity compared to embelin. However, we observed embelin and embelin-6g to exhibit similar ability in inhibiting prostate cancer cell growth despite the difference in their effect on XIAP expression. In fact, our findings suggest embelin elicits a quicker therapeutic response compared to embelin-6g and was consequently more potent at short time intervals. Therefore, embelin was selected for combination studies with CBDIV17.

We also made an interesting finding regarding the ability of embelin and embelin-6g to disrupt AR signaling in prostate cancer cells. These XIAP inhibitors also appear to decrease AR levels in LNCaP and C4-2 cells. Similar results have also been reported in the literature where other drugs exhibit the ability to decrease AR expression (21–23). Examples of these AR down-regulating agents (ARDA) include quercetin, epicatechin, and curcumin. Purushottamachar *et al.* provided clear evidence concerning structural requirements common

to ARDAs (24). Typical features include one hydrophobic group, one aromatic group, and two hydrogen bond acceptors (24). Both embelin and embelin-6g satisfy these structural criteria and hence it may not be surprising that they are capable of depleting AR expression.

Although the precise mode of action of embelin and embelin-6g in down-regulating AR inhibition is not fully understood, it is likely that both drugs inhibit AR expression through a number of different mechanisms. Since both drugs decrease AR expression by more than 50 % on the mRNA level, it is possible that mRNA stability or inhibition of AR mRNA synthesis may contribute to the effect of these drugs on AR expression levels. It is also possible that embelin and embelin-6g hinder androgen-AR binding or may disrupt the Hsp90-AR complex. The likelihood of these mechanisms which have also been reported by Liu *et al.* (21) would be consistent with our results where we showed the ability of embelin to prevent AR nuclear translocation from the cytoplasm to the nucleus in C4-2 cells (Fig. 6c).

Combination therapy involving XIAP modulation is a promising approach for treating solid tumors including

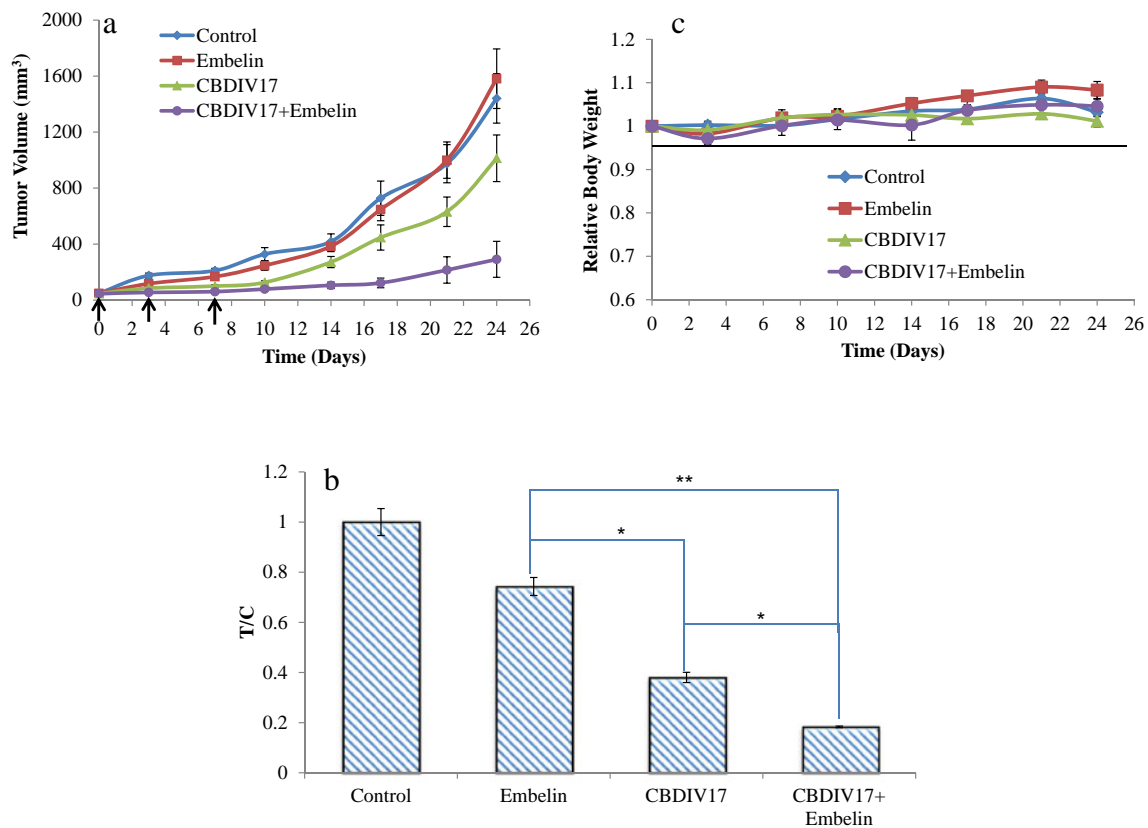


Fig. 10 Effect of CBDIV17 and embelin-loaded micelles on growth of tumors derived from C4-2 prostate cancer cells in nude mice. **(a)** Nude mice bearing 50 mm³ C4-2 tumors were given an intratumoral injection of 10 mg/kg CBDIV17 and 10 mg/kg embelin, alone or in combination (arrows indicate injection) and tumor size measured. Tumor growth regression was significantly higher for combination therapy group compared to control or monotherapy. Points are mean tumor size ($n=7$); bars, SE. **(b)** Tumor suppression (T/C) was computed as the ratio of mean tumor volume in treated groups compared with control when last injection was administered. **(c)** Relative body weight was expressed as the body weights at various times during treatment normalized with body weights at day 0. * $p < 0.05$, ** $p < 0.01$ using Student's unpaired t-test.

prostate cancer (6,25,26). We observed CBDIV17 and embelin combination to be more potent in inhibiting prostate cancer cell growth and in inducing apoptosis in advanced prostate cancer compared to control or monotherapy. This superior effect was found to be supra-additive and is consistent with our previous findings which showed synergism even for simultaneous administration of bicalutamide and embelin in C4-2 cells (6). The greater potency of CBDIV17 and embelin combination therapy may be attributed to the ability of embelin to lower the threshold required by XIAP to inhibit apoptosis thereby enhancing CBDIV17 activity several fold. From our findings, it is also possible that the ability of embelin to downregulate AR may play some role in the observed therapeutic effect of our combination therapy.

Since *in vivo* substantiation of our cellular observations is imperative for clinical translation, CBDIV17 and embelin were formulated into PEG-b-p(CB-co-LA) micelles to test our combination approach *in vivo* due to their extreme hydrophobicity and to avoid the detrimental side effects of solubilizing agents such as DMSO and Cremophor® EL (27). CBDIV17 had higher drug loading compared to

embelin. This may be due to the close structural similarity between CBDIV17 and bicalutamide for which PEG-b-p (CB-co-LA) was specifically designed to enhance drug loading (10). Consistent with our *in vitro* data, we found co-administration of embelin and CBDIV17 to be more potent in regressing prostate tumor growth compared to control or monotherapy (Fig. 10a and b). Tumor volume at the last injection and at the end of experiment for the combination group was approximately 20 % of control. A result demonstrating the ability of our combination therapy to suppress growth of advanced prostate cancer tumors. Also, CBDIV17 was effective in regressing tumor growth at the doses studied compared to embelin or control. However, tumor volume increased to 70 % of control at the end of the experiment compared to 40 % of control at the last injection. We found the embelin treated group to have tumor volume 75 % of the control group when the last injection was administered. Nonetheless, there was no difference between embelin treated group and control at the end of the study. Our results are in agreement with the findings of Dai *et al.* (28). However, they orally administered a 6-fold higher dose of embelin (60 mg/

kg) twice a week for three weeks and used PC-3 xenograft tumors which are AR negative.

In conclusion, we have demonstrated that CBDIV17 is more potent than bicalutamide in regressing prostate tumor growth and inducing apoptosis regardless of AR status. Additionally, we have shown that the combination of CBDIV17 and embelin is more potent in inhibiting cell growth and inducing apoptosis compared to control or monotherapy *in vitro* and *in vivo*. Our findings also reveal a novel mechanism regarding the ability of embelin and embelin-6g to alter AR signaling pathway. We are currently investigating the mode of action by which embelin down-regulates AR gene expression. We believe such mechanistic insight will lead to the design of highly potent analogs. The current study provides evidence that CBDIV17 and embelin combination is a promising therapeutic approach for treating hormone refractory prostate cancer.

ACKNOWLEDGMENTS & DISCLOSURES

This work is supported by an Idea Award (W81XWH-10-1-0969) from the Department of Defense Prostate Cancer Research Program. We would also like to thank Dr. Liguang Song for his help in obtaining HRMS data. Molecular weight measurement was performed at the Mass Spectrometry Center of the Department of Chemistry at the University of Tennessee at Knoxville using a JEOL (Peabody, MA) AccuTOF-D time-of-flight (TOF) mass spectrometer with a DART (direct analysis in real time) ionization source.

REFERENCES

- Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin*. 2010;60:277–300.
- Gittes RF. Carcinoma of the prostate. *N Engl J Med*. 1991;324:236–45.
- Kozlowski JM, Ellis WJ, Grayhack JT. Advanced prostatic carcinoma. Early *versus* late endocrine therapy. *Urol Clin North Am*. 1991;18:15–24.
- Bracarda S, de Cobelli O, Greco C, Prayer-Galetti T, Valdagni R, Gatta G, *et al*. Cancer of the prostate. *Crit Rev Oncol Hematol*. 2005;56:379–96.
- Goktasand S, Crawford ED. Optimal hormonal therapy for advanced prostatic carcinoma. *Semin Oncol*. 1999;26:162–73.
- Danquah M, Li F, Duke 3rd CB, Miller DD, Mahato RI. Micellar delivery of bicalutamide and embelin for treating prostate cancer. *Pharm Res*. 2009;26:2081–92.
- Scherand HI, Sawyers CL. Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *J Clin Oncol*. 2005;23:8253–61.
- Taplinand ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J Cell Biochem*. 2004;91:483–90.
- Danquah MK, Zhang XA, Mahato RI. Extravasation of polymeric nanomedicines across tumor vasculature. *Adv Drug Deliv Rev*. 2011;63:623–39.
- Danquah M, Fujiwara T, Mahato RI. Self-assembling methoxypoly (ethylene glycol)-b-poly(carbonate-co-L-lactide) block copolymers for drug delivery. *Biomaterials*. 2010;31:2358–70.
- Li F, Danquah M, Mahato RI. Synthesis and characterization of amphiphilic lipopolymers for micellar drug delivery. *Biomacromolecules*. 2010;11:2610–20.
- Li F, Danquah M, Singh S, Wu H, Mahato RI. Paclitaxel- and lapatinib-loaded lipopolymer micelles overcome multidrug resistance in prostate cancer. *Drug Deliv Transl Res*. 2011;1:240–8.
- Sun C, Shi Y, Xu LL, Nageswararao C, Davis LD, Segawa T, *et al*. Androgen receptor mutation (T877A) promotes prostate cancer cell growth and cell survival. *Oncogene*. 2006;25:3905–13.
- Wu Y, Chhipa RR, Zhang H, Ip C. The antiandrogenic effect of finasteride against a mutant androgen receptor. *Cancer Biol Ther*. 2011;11:902–9.
- Chen J, Nikolovska-Coleska Z, Wang G, Qiu S, Wang S. Design, synthesis, and characterization of new embelin derivatives as potent inhibitors of X-linked inhibitor of apoptosis protein. *Bioorg Med Chem Lett*. 2006;16:5805–8.
- Chang C, Saltzman A, Yeh S, Young W, Keller E, Lee HJ, *et al*. Androgen receptor: an overview. *Crit Rev Eukaryot Gene Expr*. 1995;5:97–125.
- Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, *et al*. A single BIR domain of XIAP sufficient for inhibiting caspases. *J Biol Chem*. 1998;273:7787–90.
- Fesik SW. Insights into programmed cell death through structural biology. *Cell*. 2000;103:273–82.
- Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, *et al*. Structural basis for the inhibition of caspase-3 by XIAP. *Cell*. 2001;104:791–800.
- Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES, *et al*. Structural basis of caspase-7 inhibition by XIAP. *Cell*. 2001;104:769–80.
- Liu S, Yuan Y, Okumura Y, Shinkai N, Yamauchi H. Camptothecin disrupts androgen receptor signaling and suppresses prostate cancer cell growth. *Biochem Biophys Res Commun*. 2010;394:297–302.
- Ren F, Zhang S, Mitchell SH, Butler R, Young CY. Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. *Oncogene*. 2000;19:1924–32.
- Chen L, Meng S, Wang H, Bali P, Bai W, Li B, *et al*. Chemical ablation of androgen receptor in prostate cancer cells by the histone deacetylase inhibitor LAQ824. *Mol Cancer Ther*. 2005;4:1311–9.
- Purushottamachar P, Khandelwal A, Chopra P, Maheshwari N, Gediya LK, Vasaitis TS, *et al*. First pharmacophore-based identification of androgen receptor down-regulating agents: discovery of potent anti-prostate cancer agents. *Bioorg Med Chem*. 2007;15:3413–21.
- Lecis D, Drago C, Manzoni L, Seneci P, Scolastico C, Mastrangelo E, *et al*. Novel SMAC-mimetics synergistically stimulate melanoma cell death in combination with TRAIL and Bortezomib. *Br J Cancer*. 2010;102:1707–16.
- Huerta S, Gao X, Livingston EH, Kapur P, Sun H, Anthony T. *In vitro* and *in vivo* radiosensitization of colorectal cancer HT-29 cells by the smac mimetic JP-1201. *Surgery*. 2010;148:346–53.
- Onetto N, Canetta R, Winograd B, Catane R, Dougan M, Grechko J, *et al*. Overview of Taxol safety. *J Natl Cancer Inst Monogr*. 1993;131–9.
- Dai Y, Desano J, Qu Y, Tang W, Meng Y, Lawrence TS, *et al*. Natural IAP inhibitor Embelin enhances therapeutic efficacy of ionizing radiation in prostate cancer. *Am J Cancer Res*. 2011;1:128–43.