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Research Paper

Micellar Delivery of Bicalutamide and Embelin for Treating Prostate Cancer

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Purpose. To examine the effect of bicalutamide and embelin on the growth of prostate cancer cells *in vitro* and *in vivo*
Methods. Cell viability was determined by MTT assay. Micelles were fabricated with polyethylene glycol-*b*-polylactic acid (PEG-PLA) copolymer and characterized in terms of particle size, micellar solubilization and drug loading, followed by evaluation in nude mice bearing LNCaP xenografts.
Results. Embelin induced caspase 3 and 9 activation in LNCaP and C4–2 cells by decreasing XIAP expression and was more potent than bicalutamide in killing prostate tumor cells irrespective of their androgen status. As analyzed by isobologram analysis the combination of bicalutamide and embelin was synergistic for C4–2 but additive and slightly antagonistic for LNCaP cells. Micellar formulation resulted in at least 60-fold increase in the aqueous solubility of bicalutamide and embelin. Tumor growth was effectively regressed upon treatment with bicalutamide, but the extent of tumor regression was significantly higher when bicalutamide was formulated in micelles. However, tumor response to bicalutamide stopped after prolonged treatment and began to grow. Sequential treatment with XIAP inhibitor embelin resulted in regression of these hormone refractory tumors.
Conclusion. Combined treatment with bicalutamide and embelin may be an effective strategy for treating hormone refractory prostate cancer.

KEY WORDS: androgen; bicalutamide; embelin; micelles; prostate cancer.

INTRODUCTION

Prostate cancer is the most pervasive malignancy diagnosed in men and remains the second leading cause of cancer-related mortality affecting men in the USA (1). The progression of prostate cancer has been found to be androgen-dependent with androgen playing a key role in the proliferation, differentiation, and survival of prostate cancer cells (2–5). Consequently, androgen ablation, especially the use of antiandrogens, has been used as a standard treatment for men with prostate cancer. Antiandrogens may be divided into two classes: steroidal and nonsteroidal. Steroidal antiandrogens, including cyproterone acetate, interfere with androgen receptor (AR) binding, blocks 5α-reductase, and have progesterone-like antigonadotropic activity. The clinical potency of cyproterone has been limited due to loss of erectile potency, influence on carbohydrate metabolism, and associated cardiovascular and hepatocellular toxicity (6,7).

Nonsteroidal antiandrogens have been developed to avoid the side effects associated with steroidal antiandrogen therapy. Three compounds, namely: flutamide (Eulexin™), nilutamide and bicalutamide (Casodex™), are available for clinical use. Among them, bicalutamide is the most widely used in androgen ablation therapy due to its relatively long half-life and tolerable side effects (8). The initial treatment of prostate cancer with bicalutamide yields response in up to 85% of patients. However, it is not curative, since prolonged treatment results in mutations in the AR, which converts bicalutamide from an antagonist into an agonist leading to drug resistance and the occurrence of hormone-refractory prostate cancer (HRPC).

Resistance to apoptosis is a common feature associated with the progression of prostate cancer from androgen-dependence to HRPC. This defect in the apoptotic machinery prevents neoplastic cells from being naturally eliminated due to the downregulation of proapoptotic and overexpression of antiapoptotic proteins, resulting in an imbalance between the rates of proliferation and apoptosis. Hence, regulators of apoptosis have emerged as attractive targets in the development of therapeutic strategies for treating prostate cancer. Among the key regulators of apoptosis is the inhibitor of apoptosis (IAP) family which suppress caspase activity endogenously preventing cell death (9–11). The most thoroughly characterized and most potent IAP protein is the X-chromosome-linked IAP (XIAP) (11). XIAP protein binds and inhibits the initiator caspase-9 and effector caspase-3 and caspase-7 through the binding of its BIR3 domain and the linker region between BIR1 and BIR2, respectively; consequently inhibiting both intrinsic and extrinsic apoptotic pathways (12–15). Recently, expression levels of XIAP in human

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prostate cancer cells was found to correlate with apoptotic resistance (16,17).

Embelin is a novel cell permeable small molecule inhibitor of XIAP, which was discovered by structure-based computational screening of a three-dimensional structure library of natural products derived from traditional Chinese medicine (18). Embelin binds to the XIAP BIR3 domain preventing its binding to and inhibition of caspase-9 and effector caspase-3 and caspase-7 (18). Also, embelin has been shown to possess antitumor and anti-inflammatory properties, as well as decrease testosterone levels (19,20). Recently, embelin was shown to block nuclear factor-κB (NF-κB) signaling pathway resulting in the suppression of NF-κB-regulated antiapoptotic and metastatic gene products, making it a potential effective suppressor of tumor cell survival, proliferation, angiogenesis, invasion, and inflammation (21).

Presently, there are limited treatment options for HRPC and a number of therapeutic strategies including combination therapy are the subject of intense research. Several studies have examined the effect of combining radiation, chemotherapeutic, and hormonal agents in treating prostate cancer. Traditionally, the exploration of combination therapy for treating hormone-refractory prostate cancer involves clinically combining well known cytotoxic chemotherapeutic agents (e.g., docetaxel and paclitaxel) and testing their potency in man. While these studies have shown some drug combinations to have synergistic effect in treating prostate cancer, the advantage may be marginal since their toxicity might exceed their benefit. A current approach in combination therapy requires a paradigm shift, in which the drug regimen incorporates chemotherapeutic agents which are selectively toxic to cancer cells. Therefore, one of our goals was to identify and explore a new combination of therapeutic agents for treating HRPC which is simultaneously potent and less toxic to humans; and can potentially be used for clinical treatment. Since embelin is a powerful inhibitor of XIAP and has minimal effects on normal human prostate epithelial cells (18), we investigated its cytotoxic effect as a single-agent and possible synergism in combination with bicalutamide in two androgen receptor containing human prostate cancer cell lines: LNCaP, which is androgen dependent and C4–2, which is androgen independent. The structures of bicalutamide and embelin are shown in Fig. 1A. We hypothesize that therapeutic combinations of bicalutamide and embelin will have a synergistic effect on growth inhibition and apoptosis of LNCaP and C4–2 cells as well as tumor regression in xenograft mouse models considering their mechanism of action (Fig. 1B).

Polymeric micelles are known to improve the solubility, stability, site specificity and hence therapeutic efficacy of hydrophobic drugs. These micelles self-assemble into nano-sized (20–60 nm), spherical structures with a hydrophobic core capable of solubilizing a considerable amount of highly water insoluble drugs. The stealth properties associated with poly(ethylene oxide) (PEO) hydrophilic corona of polymeric micelles prevents recognition by the reticuloendothelial system (RES) and therefore minimizes elimination of the micelles from the bloodstream (22). Thus, these so called 'stealth' properties of the PEO shell result in increased blood circulation times and prevention of plasma protein binding (23). In addition, the small size ensures preferential accumulation in tumor cells via the enhanced permeability and retention (EPR) effect (24,25).

In this study, polymeric micelles were fabricated using poly(ethylene glycol)-*b*-poly(lactic acid) [PEG-PLA] copolymer and used for enhancing the water solubility and bioavailability of bicalutamide and embelin to improve their efficacy. The enhanced drug water solubility and bioavailability translate into a reduction in the administered dose and possible toxicity in normal cells. In the current study, PLA was selected as the hydrophobic core-forming block since it is Food and Drug Administration (FDA) approved for clinical use and hence may facilitate the translation of our therapeutic agents for clinical applications. In addition, the *in vitro* synergistic or additive antiproliferative effects of combined bicalutamide and embelin, each possessing distinct cytotoxic mechanisms, was studied in both androgen dependent (LNCaP) and androgen independent (C4–2) human prostate cancer cells, while mechanistic studies on the induction of apoptotic cell death was also conducted. Finally, the ability of sequential exposure to bicalutamide-loaded micelles followed by embelin-loaded micelles to regress prostate cancer tumors in xenograft mice models was examined.

MATERIALS AND METHODS

Materials

PEG (5100)-b-PLA (4500) copolymer (Mn=9,600) was purchased from Polymer Source (Montreal, Canada). Embelin was purchased from Sigma–Aldrich (St. Louis, MO) while bicalutamide was synthesized as described by Mukherjee *et al.* (26) RPMI 1640 medium was obtained from Invitrogen (Carlsbad, CA) and human prostate cancer cells C4–2 and lymph node prostate adenocarcinoma (LNCaP) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Caspase-Glo™ 3/7, 8 and 9 assay and RNA extraction kits were purchased from Promega (Madison, WI). SYBR Green real time 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). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise stated and were used as received.

In vitro Cell Viability Assays

Human prostate cancer cells, LNCaP and C4–2 cells (American Type Culture Collection) were incubated in RPMI 1640 media supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 1% penicillin-streptomycin 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 5×10^3 viable cells/well and incubated for 48 h to permit cell attachment. The cells were exposed to bicalutamide or embelin at concentrations ranging from 1 to 100 μM for 24 h. At the end of treatment, 20 μl of MTT (5 mg/ml) was added to each well and incubated for 3–4 h. The plates were then centrifuged at 1,500×*g* for 2 min and the medium aspirated. The residual formazan crystals were solubilized

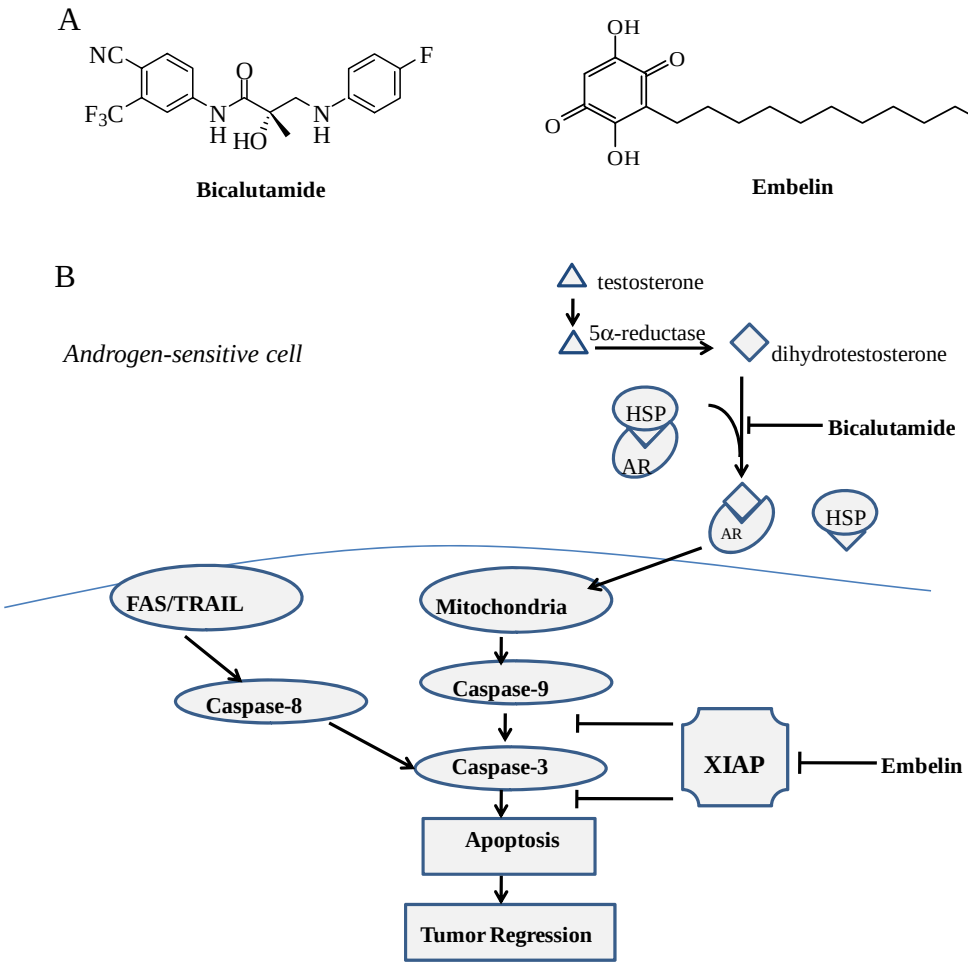

Fig. 1. Chemical structures of bicalutamide and embelin (**A**) and schematic diagram showing the combined effect of bicalutamide and embelin on apoptosis in androgen-sensitive cells and on tumor regression (**B**).

with 100 μl DMSO and the plate analyzed using a microplate reader recording absorbance values at a test wavelength of 560 nm. Cell viability for a given concentration was expressed as a percentage of the intensity of controls.

Caspase Detection

Caspase-Glo 3/7, 8 and 9 assay kits purchased from Promega (Madison, WI) were used to analyze caspase 3, 8 and 9 activities, respectively, as per the manufacturer's protocol. Briefly, 100 μl Caspase-Glo reagent was added to 100 μl of culture supernatants in 96-well plates and incubated at room temperature for 1 hour. The contents were then transferred into disposable culture tubes and luminescence was determined using a Berthold Detection Systems Sirius luminometer (Pforzheim, Germany).

XIAP Expression Analysis

The mRNA and protein level expression of XIAP in LNCaP and C4–2 cells after treatment with embelin for 48 h was assayed using quantitative real-time PCR and ELISA, respectively. Briefly, total RNA was isolated using RN easy mini isolation kit from Qiagen. RNA concentration was measured by UV spectrophotometry with a Biomate 3 spectrophotometer. One hundred and fifty nanograms of extracted RNA was converted into cDNA using Multi-Scribe reverse transcriptase and random hexamers (Applied Biosystems, Inc., Foster City, CA) by incubation at 25°C for 10 min, followed by reverse transcription at 48°C for 30 min and enzyme inactivation at 95°C for 5 min. To determine the level of XIAP expression, the following XIAP-specific primers were used: Forward: 5′-TGT TTC AGC ATC AAC ACT GGC ACG-3′; Reverse: 5′-TGC ATG ACA ACT AAA GCA CCG GAC-3′ (NM_001167). The PCR conditions included denaturation at 95°C for 10 min, followed by 40 cycles of amplification by sequential denaturation at 95°C for 15 s and primer annealing as well as strand extension for 1 min. XIAP gene expression was normalized to β-actin as internal control. To confirm the amplification specificity, the PCR products were subjected to melting curve analysis. The expressed XIAP levels were quantified and normalized to the total amount of cDNA used.

For XIAP protein expression, total protein was isolated by lysing cells. XIAP levels were measured as per the protocol of Human Total XIAP ELISA kit obtained from R&D Systems (Minneapolis, MN). Results were normalized by measuring the total protein using a BCA assay kit.

Isobologram Analysis

The dose-response interaction between bicalutamide and embelin at the point of IC_{50} was assessed to be synergistic, additive or antagonistic using the isobologram method of analysis of Steel and Peckham (27). Dose–response curves were plotted for the effects of bicalutamide and embelin on human prostate cancer LNCaP and C4–2 cell viability. From these curves, the combined drug IC_{50} values were determined for each curve. Specifically, LNCaP and C4–2 cells were treated with embelin (0, 5, 10, 25 and 50 μM) and bicalutamide (0, 10, 25 and 50 μM) either simultaneously or sequentially and cell viability assessed using MTT assay resulting in the above-mentioned dose-response curves. The combination index (CI) was calculated by the formula:

$$CI = \frac{d1}{D_{50}1} + \frac{d2}{D_{50}2} \quad (1)$$

where $D_{50}1$ is the dose of agent 1 (bicalutamide) required to produce 50 percentage effect alone, and $d1$ is the dose of agent 1 required to produce the same 50 percentage effect in combination with $d2$. Similarly, $D_{50}2$ is the dose of agent 2 (embelin) required to produce 50 percentage effect alone, and $d2$ is the dose of agent 2 required to produce the same 50 percentage effect in combination with d1. The CI values were interpreted as follows: <1.0, synergism; 1.0, additive effect; >1.0, antagonism. Each experiment was performed three times. The parameters $d1$ and $d2$ in Eq. 1 were obtained as follows: when a dose of bicalutamide ($d1$) was selected the incremental effect produced by adding embelin starting from 0 to 50 μM was assessed. The concentration of embelin that when combined with d1 resulted in the inhibition of 50% cell growth was designated $d2$.

Fabrication of Drug-Loaded Micelles

The film sonication method was used to load bicalutamide and embelin into the core of polyethylene glycol-polylactic acid (PEG-PLA) micelles. All experiments were performed using a theoretical loading of 5% unless otherwise stated. Briefly, 1 mg of bicalutamide or embelin and 19 mg of PEG-PLA 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 μm nylon filter. The micelle preparation was immediately lyophilized for 2 days and stored at 4°C to prolong shelf-life and avoid untimely release of the drug. The micelle yield was calculated using the equation below.

$$\text{Yield} = \frac{\text{weight of micelle}}{\text{weight of micelle} + \text{initial drug fed}} \times 100\% \quad (2)$$

Critical Micelle Concentration

Fluorescence spectroscopy was used to estimate the critical micelle concentration (CMC) of PEG-PLA copolymer using pyrene as a hydrophobic fluorescent probe. Nine samples of PEG-PLA dissolved in methanol with concentrations ranging from 1×10^{-8} to 1 g/L were prepared and allowed to equilibrate with a constant pyrene concentration of 6×10^{-7} M for 48 h at room temperature. The fluorescence spectra of pyrene were recorded with a Molecular Devices SpectraMax M2/M2^e spectrofluorometer (Sunnyvale, CA). An excitation wavelength of 390 nm was used and the emission spectra recorded from 320 to 450 nm with both bandwidths set at 2 nm. Peak height intensity ratio (I_3/I_1) of the third peak (I_3 at 338 nm) to the first peak (I_1 at 333 nm) was plotted against the logarithm of polymer concentration. The value of the CMC was obtained as the point of intersection of two tangents drawn to the curve at high and low concentrations, respectively.

Drug Loading Density and Encapsulation Efficiency

Drug loading was determined as follows: Lyophilized drug-loaded micelles were dissolved in methanol and the drug present in solution measured by ultraviolet spectroscopy. The weight of drug loaded in the micelles was calculated using a calibration curve. Background absorbance interference from PEG-PLA copolymer was accounted for by measuring the absorbance of blank PEG-PLA micelles under the same conditions. The micelle drug loading content and encapsulation efficiency were obtained by Eqs. 2 and 3.

$$\text{drug loading density} = \frac{\text{weight of drug in micelle}}{\text{weight of micelle}} \times 100\% \quad (3)$$

$$\text{drug encapsulation efficiency} = \frac{\text{weight of drug in micelle}}{\text{weight of drug originally fed}} \times 100\% \quad (4)$$

Size and Size Distribution

Mean particle size and size distribution of drug-loaded micelles were measured via dynamic light scattering using a Malvern instruments Zetasizer Nano Series (Worcestershire, UK). Samples were diluted to appropriate concentrations and analyzed at room temperature with a 90° detection angle. Mean micelle size was obtained as a Z-average which is an intensity mean. All measurements were repeated seven times and reported as the mean diameter ±SD for triplicate samples.

Solubility Studies of Drug-Loaded Micelles

The water solubility of free drug (bicalutamide or embelin) and drug-loaded micelles was determined by shaking an excess amount of free drug and 3 mg of drug-loaded micelles in 1 mL water, respectively. The suspension was then centrifuged at 8,000×g for 10 min and filtered on 0.2 μm cellulose membrane. The amount of bicalutamide in the saturated solution was evaluated by UV spectroscopy as described above.

In vivo Efficacy Assessment of Bicalutamide-Loaded Micelles in Xenografts

All animal experiments were performed in accordance with NIH animal use guidelines and the protocol approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. Xenograft flank tumors were induced in 8 week old male BALB/C nude mice purchased from The Jackson Laboratory (Bar Harbor, ME) by subcutaneous injection of three million LNCaP cells suspended in 1:1 media and matrigel. When tumors reached approximately 150 mm^3, mice were randomized into three groups of five mice, minimizing weight and tumor size differences. Each group was treated with intratumoral injection of saline, sonicated bicalutamide suspension or bicalutamide-loaded micelles (20 mg/kg) three times a week. Tumors were measured with a caliper prior to each injection, and their volumes calculated using the formula: $(\text{width}^2 \times \text{length})/2$.

RESULTS

Effect of Bicalutamide on Apoptosis

To determine whether prostate cancer cell death by bicalutamide is also associated with apoptosis, we determined the activation of initiator caspases 8 and 9, and effector caspase 3 at 48 h post-incubation of LNCaP cells with bicalutamide using caspase luminescence assay. Results were normalized by measuring the total protein using BCA protein assay kit. As shown in Fig. 2, relatively high levels of caspases 3 and 9 compared to caspase 8 were observed at the drug concentration of 50 and 100 μg/ml.

Effect of Embelin on Caspase Activation and XIAP Inhibiton

Resistance to apoptosis is a characteristic feature of prostate cancer and XIAP is known to inhibit the activation of the initiator caspase 9 and the effector caspase 3 which is the committed step in the apoptotic pathway. Hence, we determined the levels of caspase activation and XIAP expression levels in LNCaP and C4–2 cells after treatment with 5 μM embelin (XIAP inhibitor) for 48 h. As demonstrated in Fig. 3A, caspase activation was higher in the embelin treated cells. Specifically, caspase 8 had lower activation levels compared to caspases 3 and 9, with caspase 9 activity being the highest. However, the caspase 3 and 9 activation was found to be lower in C4–2 cells compared to LNCaP cells. To determine whether the differential levels of caspase activity in LNCaP and C4–2 cells correlate with XIAP expression, we measured baseline XIAP expression in both cells and the effect of embelin in inhibiting XIAP expression using ELISA and real time RT-PCR, respectively (Fig. 3B). The data show that C4–2 cells have higher baseline XIAP expression levels than LNCaP, and treatment with 5 μM embelin resulted in a decrease in XIAP expression in both cell lines. However, the decrease in XIAP expression upon treatment with embelin was more pronounced in LNCaP cells compared to C4–2 cells.

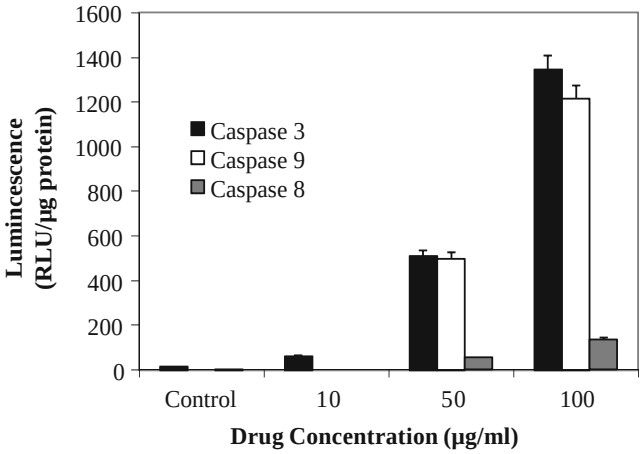

Fig. 2. Effect of bicalutamide on the activation of caspases in LNCaP cells. At 48 h postincubation with the drug at a dose of 0 (control), 10, 50 and 100 μg/ml, cells were lyzed to determine caspase 3, 8 and 9 activities in terms of relative light units (RLU) and total protein using a BCA protein assay kit. Results are presented as the mean ± SD of triplicates.

Effect of Bicalutamide and Embelin on Prostate Cancer Cell Growth

To determine whether embelin can be used for treating androgen dependent and hormone refractory prostate cancer, we determined the IC_{50} values of both bicalutamide and embelin in two androgen receptor containing human prostate cancer cells, androgen sensitive LNCaP and hormone refractory C4–2 prostate cancer cells. As shown in Fig. 4, embelin was more potent than bicalutamide in killing prostate tumor cells irrespective of their androgen status. LNCaP cells were more sensitive to bicalutamide compared to C4–2 cells, with a 50% inhibitory concentration (IC_{50}) of approximately 43 and 93 μM, respectively (Fig. 4A). Hence, C4–2 cells which are androgen independent and AR positive displayed a two-fold resistance to bicalutamide compared to LNCaP cells which are androgen dependent. In contrast, embelin exhibited superior antiproliferative activity in LNCaP and C4–2 cells, with IC_{50} of ~5 μM (Fig. 4B). However, both cell lines displayed similar sensitivity to embelin at the concentrations examined.

We used the isobologram method of Steel and Peckham (27) to assess whether the simultaneous or sequential combination of these two drugs could confer synergistic, additive, or antagonistic effects. Combination index (CI) was determined after treating 1×10^4 C4–2 cells with a combination of bicalutamide and embelin. Cell viability was determined by MTT assay and the resulting dose-response curves are shown in Fig. 5**.** CI was calculated by the formula: $CI=(d1/D_{50}1)+(d2/D_{50}2)$, where $D_{50}1$ is the dose of bicalutamide required to produce 50% effect alone, and $d1$ is the dose of bicalutamide required to produce the same 50% effect in combination with $d2$. $D_{50}2$ is similarly the dose of embelin required to produce 50% effect alone, and $d2$ is the dose of embelin required to produce the same 50% effect in combination with $d1$. The CI values are interpreted as follows: <1.0, synergism; 1.0, additive; and >1.0, antagonism. The data is reported in Tables I, II and III. These results suggest that the cytotoxic effect of bicalutamide and embelin combination was dependent on the treatment schedule and cell line. When the combination of these two drugs

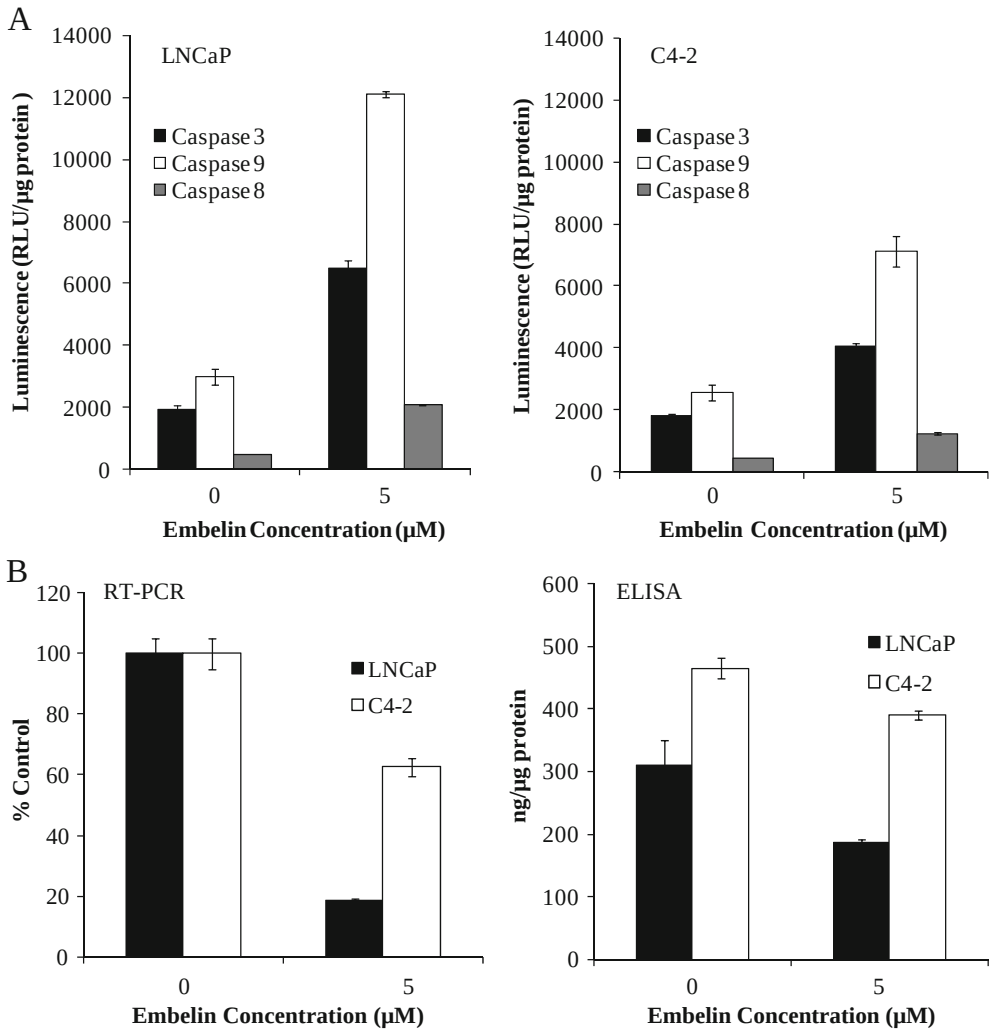

Fig. 3. Effect of embelin on caspase activation and XIAP inhibition in LNCaP and C4–2 prostate cancer cells. Embelin effectively activates caspases 3, 8 and 9 in LNCaP and C4–2 cells. Activation of caspases is higher in LNCaP cells which have comparatively lower XIAP expression. 1×10^6 LNCaP or C4–2 cells per well in six-well plates were treated with embelin for 48 h. Cells were lyzed to determine caspase 3, 8 and 9 activities in terms of relative light units (RLU) and total protein using a BCA protein assay kit. Results are presented as the mean ± SD of triplicates (**A**). XIAP expression in LNCaP and C4–2 cells using real time RT-PCR and ELISA (**B**).

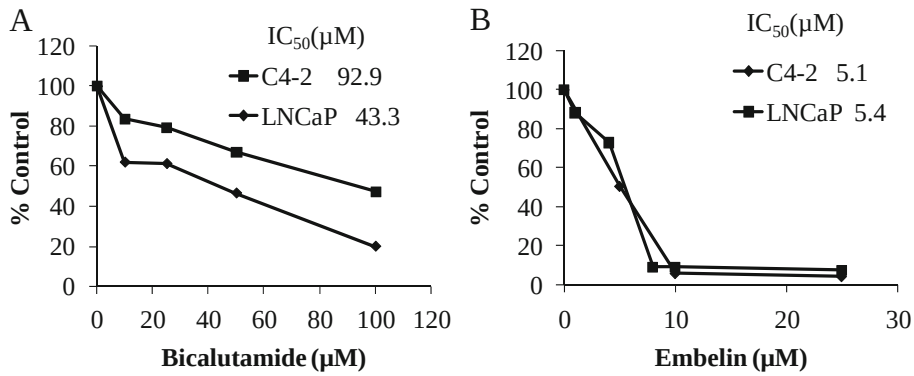

Fig. 4. Effect of drug treatment on cell viability in prostate cancer cells (C4–2 and LNCaP). **A** C4–2 and LNCaP cells treated with increasing concentrations of bicalutamide (0–100 µM) for 24 h. **B** C4–2 and LNCaP cells treated with increasing concentrations of embelin (0–25 µM) for 24 h. Results are expressed as the percentage of control.

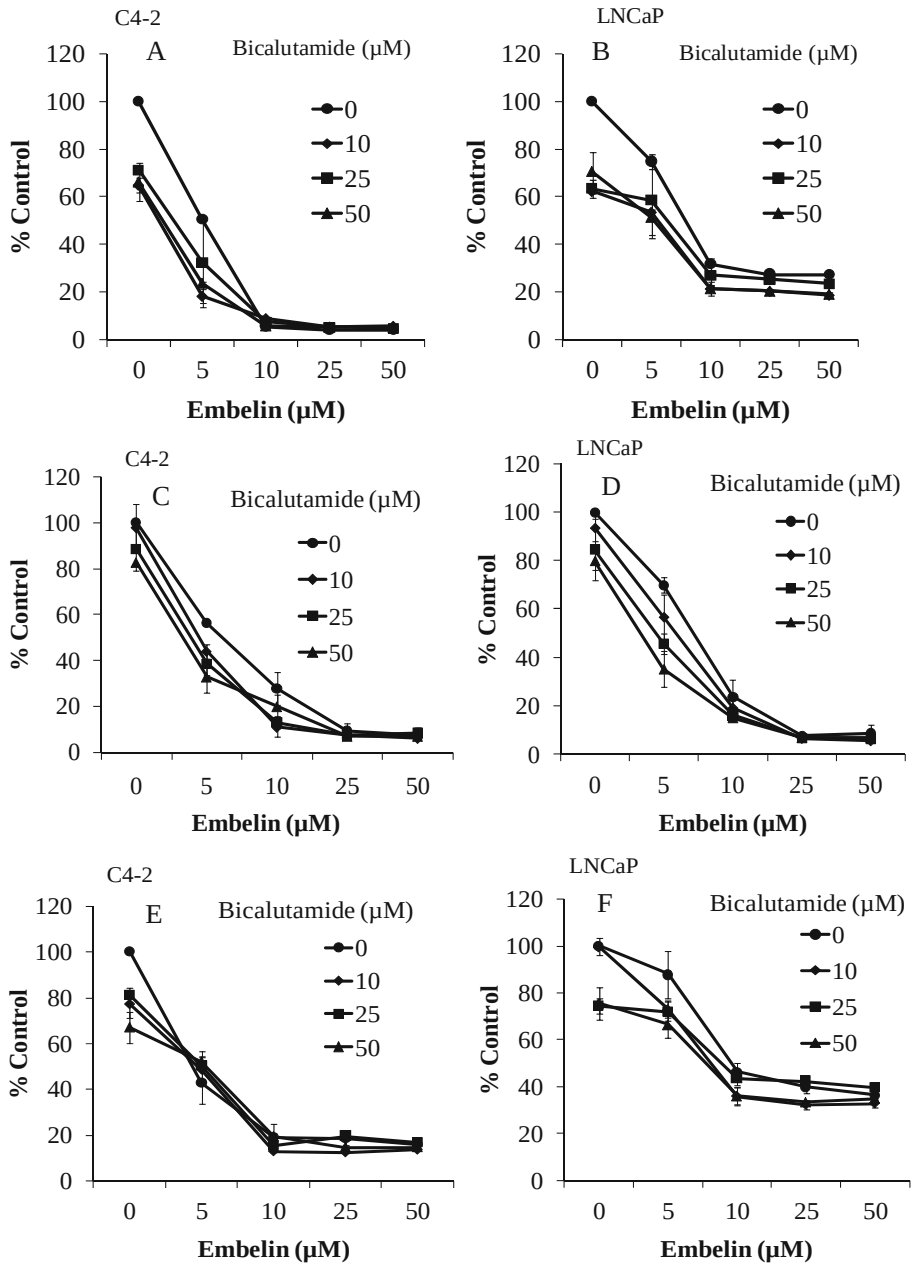

Fig. 5. Dose–response curves for bicalutamide and embelin combination in human prostate cancer cells. Simultaneous exposure (24 h) to bicalutamide and embelin in C4–2 and LNCaP cells, respectively (**A**) and (**B**). Sequential exposure (12 h incubation periods to bicalutamide followed by embelin in C4–2 and LNCaP cells, respectively (**C**) and (**D**). Sequential exposure (12 h incubation periods) to embelin followed by bicalutamide in C4–2 and LNCaP cells, respectively (**E**) and (**F**). The cell viability was measured using an MTT assay and plotted as a percentage of control.

were used, simultaneous exposure and sequential treatment of bicalutamide followed by embelin was synergistic in C4–2 cells, while additive and antagonistic effects were observed for LNCaP cells. Sequential treatment of embelin followed by bicalutamide was additive and antagonistic for both cell lines.

Characterization of Polymeric Micelles

Micelles were fabricated with polyethylene glycol-*b*-polylactic acid (PEG-PLA) di-block copolymer using the film sonication method. We determined the critical micelle concentration (CMC) using pyrene as a hydrophobic probe to observe micelle formation by monitoring polarity changes in the micellar microenvironment (28,29). Changes in the vibrational band intensity ratio (I_3/I_1) with polymer concentration was used to detect the onset of micelle formation, where I_3 at 338 nm and I_1 at 333 nm correspond to the (0,2) and (0,0) bands, respectively (30). The CMC obtained at the point of inflection of the I_3/I_1 *versus* the logarithm of polymer concentration curve was approximately 1 mg/L (data not shown). The particle size distribution of these micelles before

Table I. Synergistic Antiproliferative Activity of Bicalutamide and Embelin in Human Prostate Cancer Cells

Drug combination	Cell line	d1 (μM)	d2 (μM)	CI
Bicalutamide + Embelin	C4–2	10	1.59	0.42
	LNCaP	10	4.66	1.09
Bicalutamide → Embelin	C4–2	10	4.51	0.87
	LNCaP	10	5.90	1.05
Embelin → Bicalutamide	C4–2	10	5.65	1.22
	LNCaP	10	8.09	1.08

Combination Index (CI) of simultaneous and sequential treatment of bicalutamide and embelin in C4–2 and LNCaP cells. 5×10^3 cells were simultaneously treated with a combination of bicalutamide and embelin or sequentially [(bicalutamide followed by embelin) or (embelin followed by bicalutamide)]. Cell viability was determined by MTT assay. The combination index (CI) was calculated by the formula: $CI=(d1/D_{50}1)+(d2/D_{50}2)$, where $D_{50}1$ is the dose of bicalutamide required to produce 50% effect alone, and $d1$ is the dose of bicalutamide required to produce the same 50% effect in combination with $d2$. $D_{50}2$ is similarly the dose of embelin required to produce 50% effect alone, and $d2$ is the dose of embelin required to produce the same 50% effect in combination with d1. 10 μM bicalutamide was combined with 0, 5, 10, 25 and 50 μM embelin. The CI values are interpreted as follows: <1.0, synergism; 1.0, additive; and >1.0, antagonism. Each experiment was done in triplicate

Table III. Synergistic Antiproliferative Activity of Bicalutamide and Embelin in Human Prostate Cancer Cells

Drug combination	Cell line	d1 (μM)	d2 (μM)	CI
Bicalutamide + Embelin	C4–2	50	1.90	0.91
	LNCaP	50	4.27	1.94
Bicalutamide → Embelin	C4–2	50	3.26	1.09
	LNCaP	50	3.42	1.63
Embelin → Bicalutamide	C4–2	50	6.33	1.78
	LNCaP	50	7.71	1.96

Combination Index (CI) of simultaneous and sequential treatment of bicalutamide and embelin in C4–2 and LNCaP cells. 5×10^3 cells were simultaneously treated with a combination of bicalutamide and embelin or sequentially [(bicalutamide followed by embelin) or (embelin followed by bicalutamide)]. Cell viability was determined by MTT assay. The combination index (CI) was calculated by the formula: $CI=(d1/D_{50}1)+(d2/D_{50}2)$, where $D_{50}1$ is the dose of bicalutamide required to produce 50% effect alone, and $d1$ is the dose of bicalutamide required to produce the same 50% effect in combination with $d2$. $D_{50}2$ is similarly the dose of embelin required to produce 50% effect alone, and $d2$ is the dose of embelin required to produce the same 50% effect in combination with $d1$. 10 μM bicalutamide was combined with 0, 5, 10, 25 and 50 μM embelin. The CI values are interpreted as follows: <1.0, synergism; 1.0, additive; and >1.0, antagonism. Each experiment was done in triplicate

and after drug loading was measured by dynamic light scattering with mean particle size in the range of 30 to 50 nm.

Drug Encapsulation Efficiency and Micellar Solubility

The film sonication method was chosen for the fabrication of bicalutamide and embelin-loaded micelles since it yields micelles with higher drug loading (31). Preliminary experiments were performed to optimize formulation parameters such as sonication time, output power and the volume of water used for film hydration. This was done to obtain the highest drug loading possible. A sonication time of 7 min, output power of 25 W and hydration volume of 5 ml (*i.e.*, a micelle concentration of 4 mg/ml) proved optimal.

Table II. Synergistic Antiproliferative Activity of Bicalutamide and Embelin in Human Prostate Cancer Cells

Drug combination	Cell line	d1 (μM)	d2 (μM)	CI
Bicalutamide + Embelin	C4–2	25	2.74	0.81
	LNCaP	25	5.61	1.61
Bicalutamide → Embelin	C4–2	25	3.85	0.92
	LNCaP	25	4.47	1.20
Embelin → Bicalutamide	C4–2	25	5.80	1.41
	LNCaP	25	8.88	1.51

Combination Index (CI) of simultaneous and sequential treatment of bicalutamide and embelin in C4–2 and LNCaP cells. 5×10^3 cells were simultaneously treated with a combination of bicalutamide and embelin or sequentially [(bicalutamide followed by embelin) or (embelin followed by bicalutamide)]. Cell viability was determined by MTT assay. The combination index (CI) was calculated by the formula: $CI=(d1/D_{50}1)+(d2/D_{50}2)$, where $D_{50}1$ is the dose of bicalutamide required to produce 50% effect alone, and $d1$ is the dose of bicalutamide required to produce the same 50% effect in combination with $d2$. $D_{50}2$ is similarly the dose of embelin required to produce 50% effect alone, and $d2$ is the dose of embelin required to produce the same 50% effect in combination with $d1$; 10 μM bicalutamide was combined with 0, 5, 10, 25 and 50 μM embelin. The CI values are interpreted as follows: <1.0, synergism; 1.0, additive; and >1.0, antagonism. Each experiment was done in triplicate

To determine the maximum amount of drug that could be loaded into the micelles, the theoretical loading of bicalutamide and embelin into PEG-PLA micelles was systematically increased from 1% to 20% *w/w*. There was a linear relationship between the amount of embelin incorporated into the micelles with increasing theoretical loading. In contrast, the loading density for bicalutamide could not increase when the theoretical drug loading reached 10% or higher. At all theoretical loadings, the loading density of embelin was higher than that of bicalutamide especially at theoretical loading above 10% (Fig. 6A). From the experimental results, a theoretical loading of 5% *w/w* was chosen for all *in vitro* and *in vivo* experiments in this study since it was the optimum formulation in terms of drug loading, encapsulation efficiency, and yield.

The effect of micellar solubilization on the aqueous solubility of bicalutamide and embelin-loaded micelles was determined by shaking an excess amount of free drug or 3 mg of drug-loaded micelles in 1 ml water, respectively. Free bicalutamide and embelin exhibit very low water solubility of ~1 μg/ml, respectively. However, bicalutamide and embelin-loaded micelles resulted in a significant increase in drug solubility with increasing theoretical loading (Fig. 6B). At 10% *w/w* a 60-fold increase in water solubility was observed for a micelle concentration of 3 mg/ml. It is noteworthy that at each theoretical loading, the amount of drug brought into solution is essentially equivalent to the amount of drug successfully incorporated in the polymer.

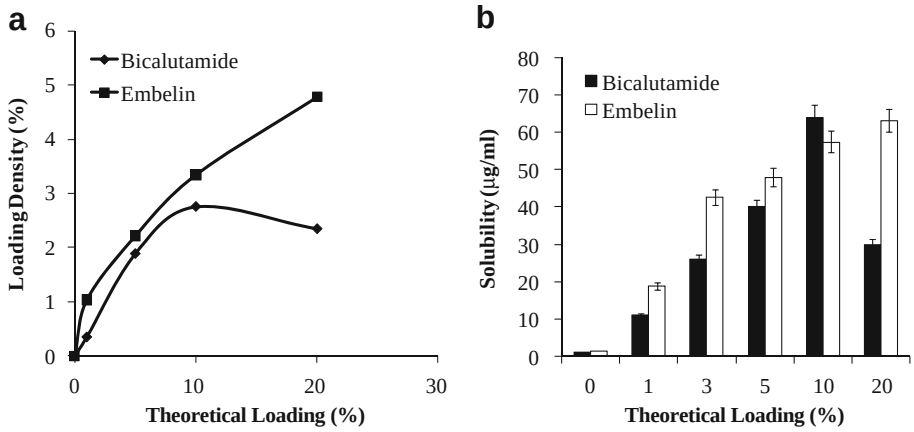

Fig. 6. Drug loading (**A**) and micellar solubilization (**B**) of bicalutamide and embelin. Water solubility of the drug was determined at 25°C. Both loading efficiency into PEG-PLA micelles and increase in aqueous solubility due to micellar solubization were higher for embelin than bicalutamide.

In vivo Efficacy Assessment of Drug-Loaded Micelles in Xenografts

Following *in vitro* characterization of bicalutamide and embelin loaded polymeric micelles in terms of particle size, micellar solubilization, drug loading and inhibitory effect on LNCaP cell proliferation, we decided to test bicalutamide-loaded micelles in mice bearing LNCaP xenografts. Xenograft flank tumors were induced in 8 week old male BALB/C nude mice by subcutaneous injection of three million LNCaP cells suspended in 1:1 media and matrigel. When tumors reached approximately 150 mm^3, mice were randomized into three groups of five mice. Each group was treated with intratumoral injection of saline, bicalutamide suspension and bicalutamide-loaded micelles at the dose of 20 mg/kg three times a week. Tumors were measured with a caliper prior to each injection, and their volumes calculated using the formula: ($width^2$ × length)/2. As shown in Fig. 7, tumor growth was effectively regressed upon treatment with bicalutamide up to 20 days post-treatment. Formulation into PEG-PLA polymeric micelles further regressed tumor growth and this effect was more significant in case of bicalulamide.

Unfortunately, these tumors became insensitive to bicalutamide at 20 days after treatment and began to grow. Since antiapoptotic proteins, including XIAP, is known to get upregulated in hormone refractory prostate cancer, we decided to treat these tumors subsequently with emblin, which is an effective XIAP inhibitor. As shown in Fig. 7, sequential treatment with embelin resulted in regression of hormone insensitive tumors.

DISCUSSION

Prostate cancer is the second leading cause of death in men in North America. Since androgens play an important role in progression of prostate cancer, androgen ablation and blockade of androgen action are the two most common modalities for treating prostate cancer. Although bicalutamide is one of the most widely used nonsteroidal antiandrogens for treating prostate cancer, it is highly hydrophobic and prolonged exposure leads to drug resistance, the occurrence of hormone refractory prostate cancer and an increased propensity for metastasis (32–35). Since hormone refractory tumors are resistant to apoptosis and overexpress XIAP, the objective of this study was to see whether small molecule XIAP inhibitor such as embelin can be used to treat bicalutamide irresponsive tumors.

Since the precise cellular mechanism by which bicalutamide induces apoptosis is still not well understood, we first measured the activity of caspases 3, 8 and 9, since caspase 9 upregulation represents mitochondrial pathway and caspase 8 upregulation represents extrinsic pathway (36). As shown in Fig. 2, there was dose dependent increase in all three caspases. However, the level was very high in the case of 3 and 9. Our results are in good agreement with the work of

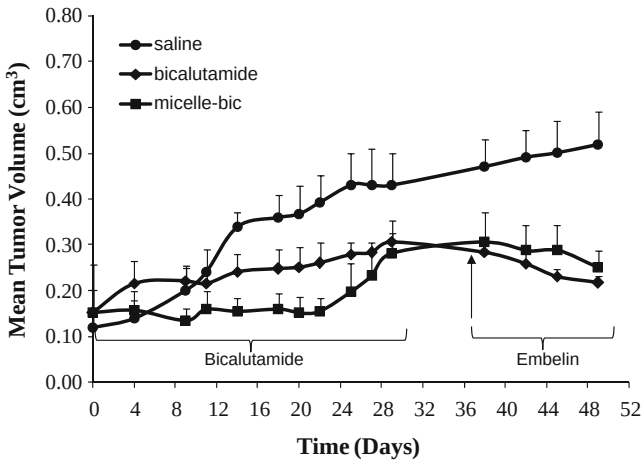

Fig. 7. Effect of bicalutamide and embelin-loaded micelles on growth of tumors derived from LNCaP prostate cancer cells in nude mice. Nude mice bearing 150-mm^3 LNCaP tumors were given an intratumoral injection of 20 mg/kg bicalutamide three times a week and tumor size was measured prior to each injection. Tumor growth regression was significantly higher for bicalutamide-loaded micelles compared to the free drug up to 20 days post-treatment. Since tumors became insensitive to bicalutamide at 20 days after treatment and began to grow, bicalutamide treatment was discontinued and embelin-loaded micelles were administered from day 28, which resulted in regression of hormone insensitive tumors. *Points* are mean tumor size (n=5); *bars*, SE.

Lee *et al.* (37), who also reported caspase 3 levels to be 5-fold higher than caspase 8 when LNCaP cells were treated with bicalutamide. Further, AR gene silencing has been reported to result in massive prostate tumor cell death through mitochondrial pathway regardless of their androgen sensitivity (38). These results suggest that bicalutamide mainly follows mitochondrial pathway.

Prolonged treatment with bicalutamide is known to convert androgen sensitive prostate cancer cells into apoptosis resistant hormone refractory cells due to overexpression of antiapoptotic genes including Bcl-2, Bcl-X_L and XIAP (39). Among them, XIAP is the most potent in inhibiting apoptosis via inhibition of effector caspases. XIAP inhibitors are known to inhibit caspases, promote mitochondrial permeability, Bcl-2 cleavage and Bak conformational change (40). Among various XIAP inhibitors, small molecule embelin is quite promising and known to inhibit caspase 9 and effector caspases (18). Therefore, we determined whether embelin can activate caspases in LNCaP and C4–2 cells (Fig. 3). We observed increased caspase 3 and 9 activity upon treatment with embelin in both cell lines. Our results are in good agreement with Nikolovska-Coleska *et al.* (18) who first showed the ability of embelin to induce caspase 3 and 9 activation in prostate cancer cells by inhibiting the activity of XIAP. While these authors studied the effect of embelin in LNCaP and other prostate cancer cells, they did not examine C4–2 cells and to the best of our knowledge no study using embelin has been done in C4–2 cells. In our study, we found caspase 3 and 9 activation to be lower in C4–2 cells compared to LNCaP cells which we attributed to higher levels of XIAP expression in C4–2 cells. ELISA and real time RT-PCR analysis on LNCaP and C4–2 cells revealed higher baseline XIAP expression in C4–2 cells and treatment with 5 μM embelin resulted in a decrease in XIAP expression in both cell lines. It is noteworthy that the decrease in XIAP expression upon treatment with embelin was more pronounced in LNCaP cells compared to C4–2 cells (Fig. 3). This outcome appears to correlate with the relative caspase 3 and 9 activation levels observed in LNCaP and C4–2 cells.

We examined the ability of bicalutamide and embelin for treating androgen dependent prostate cancer as well as hormone refractory advanced prostate cancer by observing their effect on cell proliferation. Embelin was more potent than bicalutamide in killing prostate tumor cells irrespective of their androgen status since it was effective in inhibiting the proliferation of androgen dependent LNCaP and hormone refractory C4–2 cells (Fig. 4). Isobologram analysis suggests that the simultaneous administration of bicalutamide and embelin and the sequential treatment of bicalutamide followed by embelin provide synergistic effect on the growth inhibition of hormone refractory C4–2 prostate cancer cells but additive and antagonistic effects in LNCaP cells **(**Fig. 5 and Tables I, II and III**)**. The efficacy of the above treatment schedules possibly stems from bicalutamide sensitizing the cells by androgen ablation to embelin mediated apoptosis. In contrast, the treatment schedule of embelin followed by bicalutamide was additive and antagonistic for both cell lines. However, since the definition of synergism is more stringent in the isobologram method of analysis compared to clinical synergism where drug combinations must simply produce a better tumor response than either drug alone, the observed antagonistic effects in our drug combination study may be equal or superior to that of bicalutamide or embelin alone (41).

Since bicalutamide and embelin are highly hydrophobic, their limited solubility in water can hamper their clinical use by systemic administration. Moreover, the historical use of DMSO to bring water insoluble drugs into true solution for clinical use is of great concern since DMSO is harmful to the liver and kidney and causes dose-dependent hemolysis. A potential approach to addressing these problems is to improve their aqueous solubility using polymeric micelles. Formulation of these drugs into PEG-PLA micelles significantly improved their aqueous solubility and drug loading density increased with increasing theoretical loading up to 20% for embelin and 10% for bicalutamide (Fig. 6). Embelin had higher drug loading compared to bicalutamide, possibly due to better hydrogen bonding between the carbonyl group of the PLA hydrophobic core-forming block and the hydroxyl groups of embelin. Similar observations has also been reported for quercetin which has three hydroxyl groups (42). Differences in their drug loading efficiency and micellar solubilization are also related to their different physicochemical properties as calculated using the Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (Ó 1994–2009 ACD/Labs): The log D (pH = 7), log P and pKa values for bicalutamide are 4.94, 9.44 and 11.49, respectively; and those of embelin are 1.20, 5.70 and 2.59, respectively. Micelle size was in the range of 30–50 nm, which is expected to allow enhanced capillary permeability and delivery to tumor and inflammatory sites. Furthermore, the small size of the micelles would facilitate the release of encapsulated drug due to the high surface area to volume ratio.

Following *in vitro* characterization, bicalutamide-loaded micelles were evaluated in mice bearing LNCaP xenografts. Tumor regression was more significant up to 20 days post-treatment with bicalutamide-loaded micelles compared to free bicalutamide. Unfortunately, tumors became insensitive to bicalutamide afterwards and began to grow. This finding is not surprising, since prostate cancer is known to become resistant to bicalutamide after prolonged treatment, leading to tumor cell proliferation. Subsequent treatment with emblin-loaded micelles resulted in regression of hormone insensitive tumors. This result clearly suggests that combination of bicalutamide with embelin has the potential to be used for treating advanced prostate tumor and metastasis. Sirotnak *et al.* have also shown synergistic effect of bicalultamide with ZD1839 (Iressa) which inhibits EGFR. However they used extremely high dose (150 mg/kg for ZD1839 and 100 mg/kg for bicalutamide) and did not measure tumor growth beyond 18 days. In our study there was significant tumor regression up to 20 days when we treated mice with bicalutamide-loaded micelles, but the tumors began to grow beyond 20 days and therefore we stopped treatment with bicalutamide on day 28 and treated mice with embelin a week later (43). Synergistic effect has also been shown in prostate cancer cell lines between bicalutamide and genistein which is an isoflavone (44).

A major goal of this study was to demonstrate proof of principle that the combination of an androgen receptor antagonist (bicalutamide) and XIAP inhibitor (embelin) was capable of regressing prostate cancer tumors. Since both bicalutamide and embelin are highly hydrophobic and the conventional use of DMSO to solubilize drugs is harmful to

the liver and kidney and causes dose-dependent hemolysis, we chose to use polymeric micelles. While the drug solubilization results are not dramatic, they were suitable for intratumoral injections for our xenograft models. For future studies involving systemic administration, we plan to optimize the selection of the hydrophobic block of our copolymer to increase the "cargo" space and enhance drug solubilization. Our laboratory is currently synthesizing polymers to address this issue and preliminary data suggests it may be able to enhance solubilization several fold compared to the poly lactic acid hydrophobic core used in this study. We are also conjugating targeting ligands to the polymeric micelles to enhance site-specific delivery after systemic administration. In conclusion, micellar delivery of antiandrogen and XIAP inhibitors has potential to treat advanced prostate cancer.

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