In vitro and In vivo Molecular Evidence for Better Therapeutic Efficacy of ABT-627 and Taxotere Combination in Prostate Cancer

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Abstract

Bone is the key metastatic site for prostate cancer. Endothelin 1 (ET-1) produced abundantly by prostate cancer cells binds to its receptor present on bone marrow stromal cells and favors osteoblastic response during bone metastases of prostate cancer. This suggests that interrupting ET-1 interaction with its endothelin A (ETA) receptor could be useful for inhibiting prostate cancer bone metastasis and, as such, may enhance the therapeutic activity of docetaxel (Taxotere), the most commonly used drug for the treatment of metastatic prostate cancer. Therefore, the goal of our study was to obtain preclinical data supporting our hypothesis that the combined use of ETA receptor antagonist (ABT-627; Atrasentan) with Taxotere will be superior in inducing apoptosis in vitro and inhibiting tumor growth in vivo in a SCID-hu model of experimental bone metastasis induced by C4-2b prostate cancer cells. In vitro studies were done on a panel of prostate cancer cell lines to understand the molecular basis of combination therapy, and we found that the combination was more effective in the inhibition of cell viability and induction of apoptosis in LNCaP and C4-2b cells (androgen receptor positive) but not in PC-3 cells. These results were correlated with inactivation of Akt/nuclear factor-kB and its target genes. For in vivo studies, the therapeutic regimen was initiated when the tumor began showing signs of growth and treatment was continued for 5 weeks. Tumor volume and serum prostate-specific antigen were used as terminal index to evaluate the therapeutic advantage of combination therapy relative to a single regimen and untreated control. At termination, we found a 90% reduction in tumor volume by combination treatment relative to the untreated control group. Most importantly, the antitumor activity was associated with the down-regulation of molecular markers in tumor tissues that were similar to those observed in vitro. [Cancer Res 2007;67(8):3818–26]

Introduction

Prostate cancer is the second leading cause of cancer-related deaths in men in the United States (1). Death is the result of metastatic hormone refractory disease in virtually the majority of patients. Prostate cancer metastasizes preferentially to bone, causing osteoblastic lesions with a progressive morbid course (2). Hence, the development of a novel and effective therapeutic strategy by which one could effectively inhibit hormone and/or chemotherapy refractory prostate cancer is urgently needed.

Normal healthy bone constantly undergoes remodeling through synchronized activities of osteoblasts, which synthesizes new bone, and osteoclasts, which resorbs old bone (3). Recent findings highlight the acquisition of osteoblastic phenotype with progression of prostate cancer, which is mediated by the interactions between prostate cancer cells and osteoblasts within the bone microenvironment. Some active bone factors, which are produced and secreted by prostate cancer cells and stimulate new bone formation and osteoblast proliferation, include fibroblast growth factor, bone morphogenetic proteins, platelet-derived growth factor, transforming growth factor β, insulin-like growth factor, pleiotrophin-osteoblast stimulating factor, and interleukins (interleukin-1 and interleukin-6; ref. 4). In addition, strong and emerging evidences suggest a critical role of the potent vasoconstrictor endothelin 1 (ET-1) in the pathogenesis and progression of prostate cancer–induced osteoblastic lesions (5, 6). Nelson et al. reported that ET-1 levels in plasma samples from men with hormone refractory prostate cancer are significantly higher compared with those from men with clinically localized disease and those without prostate cancer (7). Furthermore, the enzyme that degrades ET-1, neutral endopeptidase 24.11, is less prominent in prostate cancer, leading to the overproduction of ET-1 in men with prostate cancer (8). The action of ET-1 is mediated by G protein–coupled receptors–endothelin A (ET_{A}) and endothelin B (ET_{B}) receptor interaction (9, 10). ET_{A} is the predominant endothelin receptor in healthy prostatic epithelium and responsible for the clearance of circulating ET-1, thereby regulating ET-1 production (11). In prostate cancer, a significant reduction in the expression of ET_{B} has been reported (12). Thus, tumor cells producing ET-1 stimulates new bone formation in vitro and osteoblastic metastasis in vivo via the ET_{A} receptor (ref. 6). Based on this preexisting knowledge, we hypothesized that targeting ET_{A} receptor in combination with a conventionally used cytotoxic drug could influence the prostate cancer cell behavior within the bone microenvironment, resulting in a greater antitumor activity.

Several low molecular weight compounds antagonizing the ET_{A} receptor, thus interrupting cellular signaling affecting cell proliferation, have been evaluated in laboratory studies, as well as in clinical setting. The ET_{A} receptor antagonist ABT-627 (Atrasentan), which is p.o. bioavailable with selectivity (1800-fold), potentially binds to the ET_{A} receptor (K_{i} = 0.034 nmol/L), blocking the biological effects of ET-1, has been reported (13). In phase III of clinical trials, Atrasentan significantly delayed disease progression in men with prostate cancer bone metastases compared with
placebo-treated patients with similar characteristics (14). Laboratory studies using a combination treatment of ABT-627 with paclitaxel revealed additive antitumor, proapoptotic, and antiangiogenic effects in ovarian cancer cells (15). Another investigation conducted in nude mice showed that Atrasentan inhibited growth and neoangiogenesis in cervical cancer cell xenograft (16). Furthermore, novel molecular mechanistic pathways involved in metastasis-suppressing activity of Atrasentan during ovarian carcinoma progression have been recently published (17). However, to test this hypothesis in vivo, animal model of prostate cancer within the bone microenvironment having osteoblastic reactions are limited. We have previously found that C4-2b prostate cancer cells grown in a SCID-hu model of experimental bone metastases produced osteoblastic as well as osteolytic lesions. Therefore, we have used this model for the current in vivo study.

In this study, we have tested our hypothesis in vitro and especially in vivo using the SCID-hu model. Our data showed, for the first time, that ABT-627 treatment in combination with Taxotere caused greater antiproliferative and proapoptotic activity in vitro and antitumor activity in vivo. Our results were also correlated with mechanism-based evidence obtained from both in vitro and in vivo studies.

Materials and Methods

Cell culture and reagents. The human prostate carcinoma cell lines LNCaP (androgen receptor (AR) positive), C4-2b (AR positive), and PC-3 (AR negative) were maintained in exponential growth by twice weekly passage in RPMI 1410 (DME, Life Technologies, Inc., Gaithesburg, MD) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 10 mg/ml streptomycin in a humidified incubator containing 5% CO2 in air at 37°C.

The cell apoptosis ELISA assay kit and Ser73-phosphorylated Akt from Cell Signaling (Beverly, MA); anti-Bcl-2 and Bcl-xL antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Bax antibody from Trevigen, Inc. (Gaithersburg, MD); anti–poly[ADP-ribose] polymerase (PARP) antibody from Biomol Research (Plymouth, PA); antisurvivin antibody from R&D Systems (Minneapolis, MN); anti–β-actin antibody from Sigma Chemical Co. (St. Louis, MO). Taxotere (Aventis Pharmaceuticals, Bridgewater, NJ) was dissolved in 0.1 mol/L Na2CO3 to make a 10 mg/mL stock solution. Taxotere (Aventis Pharmaceuticals, Bridgewater, NJ) was dissolved in 0.1 mol/L Na2CO3 to make a 10 mg/mL stock solution.

Cell growth inhibition by cytotoxic agents. All three prostate cancer cell lines (LNCaP, C4-2b, and PC-3 cells) were seeded at a density of 3 × 10⁴ cells per well in 96-well microtiter culture plates. After overnight incubation, the medium was removed and replaced with a fresh medium containing different concentrations of ABT-627 (0–50 μmol/L) diluted from a 10-mmol/L stock. After 72 h of incubation with drug, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) was added to each well and incubated further for 2 h. Upon termination, the supernatant was aspirated and the MTT formazan formed by metabolically viable cells was dissolved in isopropanol (100 μL). The plates were mixed for 30 min on a gyrotary shaker, and the absorbance was measured at 595 nm on a plate reader (TECAN, Durham, NC).

Cell growth inhibition by cytotoxic agents. Cells were plated as described above and allowed to attach overnight. The culture medium was replaced with a fresh medium containing 25 μmol/L of ABT-627 and 1 mmol/L of the chemotherapeutic agent docetaxel (Taxotere) for an additional 72 h. For single agents, ABT-627 and Taxotere, cells were also exposed for 72 h. The effect of ABT-627 cotreatment on cell viability was examined by the MTT assay method as described above.

Quantification of apoptosis by ELISA. The cell apoptosis ELISA detection kit (Roche, Palo Alto, CA) was used to detect apoptosis after treatments with drugs individually and in combination according to manufacturer’s protocol. Briefly, LNCaP and C4-2b cells were treated with ABT-627 and/or Taxotere alone or in combination for 72 h. After treatment, the cytoplasmic histone/DNA fragments from these cells were extracted and bound to immobilized antihistone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by using an ULTRA multifunctional microplate reader (TECAN) at 405 nm.

Flow cytometric analyses for assessing the percentage of apoptotic cells. C4-2b cells were incubated in the presence or absence of 25 μmol/L of ABT-627, Taxotere (1.0 mmol/L), and combination with Taxotere and ABT-627 for 48 h. Free-floating cells were collected, and adherent cells were harvested with 0.25% trypsin and resuspended in RPMI 1640 media. Cells (1 × 10⁴) were centrifuged at 300 × g for 5 min and then washed once with PBS, fixed in 75% ethanol overnight at 4°C, and stained with 0.02 mg/mL of propidium iodide (PI) together with 0.1 mg/mL of RNase A. The DNA histogram from at least 10,000 PI-stained cells at an emission wavelength of 690 nm was measured using a Becton Dickinson fluorescence-activated cell sorting (FACS) caliber flow cytometer and analyzed with ModFit LT software (San Jose, CA). The percentage of apoptotic cells in the sub-G0-G1 was calculated.

Protein extraction and Western blot analysis. The prostate cancer cells C4-2b were plated and allowed to attach for 36 h. ABT-627 and/or Taxotere alone or in combination was directly added to the cell cultures at indicated concentrations and incubated for 72 h. Control cells were incubated in the medium without an equivalent concentration of solvent. After the treatment period, the cells were harvested in PBS by scraping them from culture dishes and collecting them by centrifugation. Cellular lysates were prepared by suspending the cells in 200 μL of lysis buffer (150 mmol/L NaCl, 1 mmol/L EGTA, 0.1% Triton X-100, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethyalsulfonyl fluoride (PMSF), 2 μg/mL leupeptin, and 2 μg/mL aprotinin). The cells were disrupted by sonication and extracted at 4°C for 30 min at maximal microfuge speed to remove debris. For Western blot analysis, each extract prepared as described equivalent to 50 μg total protein was separated on SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with specific antibodies. Detection of specific proteins was carried out with an enhanced chemiluminescence Western blotting kit according to manufacturer’s instructions (Pierce, Rockford, IL).

Assay of Akt kinase activity. Cells were incubated and treated as mentioned above. They were then washed twice with PBS and lysed in ice-cold lysis buffer (20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium PIPs, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 1 μg/mL leupeptin, and 1 mmol/L PMSF). The extracts were centrifuged to remove cellular debris, and the protein content of the supernatants was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce). Proteins (150 μg) were incubated with gentle rocking at 4°C overnight with immobilized Akt antibody cross-linked to agarose hydrazide beads. After the Akt was selectively immunoprecipitated from the cell lysates, the immunoprecipitated products were washed twice with lysis buffer and twice with kinase assay buffer (25 mmol/L Tris (pH 7.5), 10 mmol/L MgCl₂, 5 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 2 mmol/L DTT) and then resuspended in 40 μL of kinase assay buffer containing 200 μmol/L ATP and 1 μg GSX-3α/β fusion protein. The kinase assay reaction was allowed to proceed at 30°C for 30 min and stopped by the addition of Lameli SDS sample buffer. Reaction products were resolved by 10% SDS-PAGE, followed by Western blotting with antiphosphorylated GSX-3α/β antibody. For analysis of the total amount of Akt, 40 μg of protein from the lysate samples were resolved by 10% SDS-PAGE, followed by Western blotting with anti-Akt antibody.

Electrophoretic mobility shift assay. Nuclear protein extracts were prepared according to the method described by Chaturvedi et al. (18). Briefly, the cells were washed with cold PBS and suspended in 0.15 mL of lysis buffer (10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT; 1 mmol/L PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine). The cells were allowed to swell on ice for 20 min, and then 4.8 μL of 10% Nonidet P-40 was added. The
tubes were vigorously mixed on a vortex mixer for a few seconds and centrifuged for 120 s in a microfuge. The nuclear pellet was resuspended in 30 μL of ice-cold nuclear extraction buffer [20 mmol/L HEPES (pH 7.9), 0.4 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine] and incubated on ice with intermittent mixing. The tubes were then centrifuged for 5 min in a microfuge at 4°C, and the supernatant (nuclear protein extract) was collected in a cold Eppendorf tube and stored at −70°C for future use. The protein content was measured by BCA method.

Electrophoretic mobility shift assay (EMSA) was done by incubating 8 μg of nuclear protein extract with IRDye-700–labeled nuclear factor-κB (NF-κB) oligonucleotide. The incubation mixture included 2 μg of poly(dI-dC) in a binding buffer. The DNA-protein complex formed was separated from free oligonucleotide on 8.0% native polyacrylamide gel using buffer containing 50 mmol/L Tris, 200 mmol/L glycine (pH 8.5), and 1 mmol/L EDTA and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1 (Li-COR, Inc., Lincoln, Nebraska).

**Experimental animals.** Male homozygous CB-17 SCID/SCID mice (4 weeks old) were purchased from Taconic Farms (Germantown, NY). The mice were housed and maintained under sterile conditions in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH. The mice were used in accordance with Animal Care and Use Guidelines of Wayne State University under a protocol approved by the Institutional Animal Care and Use Committee. Mice received Lab Diet 5021 (Purina Mills, Inc., Richmond, IN).

Human bone and implantation of tumor cells. Human male fetal bone tissue was obtained by a third party nonprofit organization (Advanced Bioscience Resources, Alameda, CA), and written informed consent was obtained from the donor family, consistent with regulations issued by each state involved and the federal government. After 1 week of acclimatization, the mice were implanted with a single human fetal bone fragment as described previously (19). C4-2b cells were harvested from subconfluent cultures after a brief exposure to 0.25% trypsin and 0.2% EDTA. Trypsinization was stopped by adding a medium containing 10% FBS. The cells were washed once in serum-free medium and resuspended. Only suspensions consisting of a single cell with >90% viability was used for the injections. Cells (1 x 10⁶) in 20-μL serum-free RPMI medium were injected intrasosseously by insertion of a 27-gauge needle and Hamilton syringe through the mouse skin directly into the marrow surface of the previously implanted bone. In our previous experience with this model, we found a tumor take rate of ~90%.

**Experimental protocol.** As soon as the majority of the bone implants began to enlarge (now called a “bone tumor”) as determined by caliper measurements (30th day after cancer cell injection), mice were randomized into the following treatment groups (n = 7): (a) untreated control; (b) only ABT-627, 10 mg/kg body weight given i.p. everyday for 5 weeks since initiation of therapy; (c) Taxotere–one cycle, 5 mg/kg body weight given i.v. every 3rd day (total of four doses); and (d) ABT-627 plus Taxotere, following exactly the similar schedule as described for individual drugs. The volume of the bone tumor in each group was determined by twice weekly caliper measurements. The body weight of mice in each group was also measured. All mice were euthanized 1 day after the last dose of ABT-627 treatment (5 weeks) because large tumors were formed in the control mice, which required termination, and their final body weight and tumor volume were recorded. On autopsy, the tumor was neatly excised, freed of any extraneous adhering tissue, and subjected to ex vivo imaging on a Lo-Rad M-IV mammography unit using a magnified specimen technique. For routine H&E staining, one part of the tissue was fixed in formalin and embedded in paraffin; another part was rapidly frozen in liquid nitrogen, stored at −70°C, and subsequently used for preparation of nuclear protein extracts. H&E staining confirmed the presence of tumor.

**Serum prostate-specific antigen determination.** Commercially available ELISA kit (Anogen, Inc., Ontario, Canada) was used to determine serum concentration of prostate-specific antigen following manufacturer’s instruction.

**Tumor tissue nuclear protein extraction and EMSA.** Tissues were minced and incubated on ice for 30 min in 0.5 mL of ice-cold buffer A, composed of 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L DTT, 0.1% IGEPAL CA-630, and 0.5 mmol/L PMSF. The minced tissue was homogenized using a Dounce homogenizer (Kontes Co., Vineland, NJ) followed by centrifuging at 5000 × g at 4°C for 10 min. The crude nuclear pellet was suspended in 200 μL of buffer B [20 mmol/L HEPES (pH 7.9), 25% glycerol, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 0.5 mmol/L EDTA, 0.2 mmol/L EDTA, 0.5 mmol/L PMSF, and 4 μmol/L leupeptin] and incubated on ice for 30 min. The suspension was centrifuged at 16,000 × g at 4°C for 30 min. The supernatant (nuclear proteins) was collected and kept at −70°C until use. The protein concentration was determined using the BCA assay kit with bovine serum albumin as the standard (Pierce).

**Statistical analysis.** Data are represented as mean ± SD for the absolute values or percent of controls, as indicated in the vertical axis legend of Figs. 1–3, 5, and 6. The statistical significance of differential findings between experimental groups and control was determined by Student’s t test as implemented by Excel 2000 (Microsoft Corp., Redmond, WA). P values smaller than 0.05 were considered statistically significant.

**Results**

Effect of ABT-627 treatment on cell growth. To test the effect of ABT-627 treatment on androgen-dependent and androgen-independent prostate cancer cell proliferation, cells were treated...
with increasing concentrations of ABT-627 (0–50 μmol/L) for 72 h.

As shown in Fig. 1, cell viability was reduced in a dose-dependent manner only in LNCaP and C4-2b cells; PC-3 cells were relatively insensitive at equivalent concentration of the drug. As noted in LNCaP cells, the viability was reduced by 18%, 30%, and 60% with 10 μmol/L, 25 μmol/L, and 50 μmol/L of ABT-627 treatment for 72 h, respectively. Likewise, the treatment of C4-2b cells resulted in 15%, 32%, and 56% reduction in cell viability when exposed to identical concentrations of ABT-627 for a similar period. In contrast, PC-3 cells were comparatively resistant to the effect of ABT-627 and thus revealed only a marginal nonsignificant decrease in cell viability up to a concentration of 50 μmol/L of ABT-627 treatment. These results indicate that overall ABT-627 is an effective inhibitor of LNCaP and C4-2b prostate cancer cell growth.

**ABT-627 sensitizes prostate cancer cells to Taxotere-induced growth inhibition.** The effect of ABT-627 alone on the viability of three different prostate cancer cells was determined. Our data show a significant concentration-dependent inhibition of cell viability by ABT-627 only in two of the three investigated cell lines (Fig. 1). Subsequent studies were undertaken to examine whether the prostate cancer cells were more sensitive to the cytotoxic effect of a combined regimen of ABT-627 and Taxotere as determined by cell viability assay. For these studies, cells were treated with ABT-627 (25 μmol/L) alone or in combination with single dose of Taxotere (1 nmol/L) and viable cells were evaluated after 72 h by MTT assay (Fig. 2). We found that the combination treatment of cells with both ABT-627 and Taxotere for 72 h caused a significant 60% to 70% growth inhibition in LNCaP and C4-2b cells compared

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**Figure 2.** Effect of ABT-627 and Taxotere treatment on viability of prostate cancer cell lines LNCaP, C4-2b, and PC-3. Cells were either untreated or treated with either ABT-627 (25 μmol/L) alone, Taxotere (1 nmol/L) alone at designated concentration or treated simultaneously with ABT-627 and Taxotere for 72 h and then analyzed by MTT assay as described under Materials and Methods.

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**Figure 3.** A, sensitization of prostate tumor cells LNCaP and C4-2b to ABT-627–induced and Taxotere-induced apoptosis as determined by histone-DNA ELISA after 72 h treatment with ABT-627 (25 μmol/L) alone, Taxotere (1 nmol/L) alone, or the combination of ABT-627 and Taxotere incubated for 72 h. Increased apoptotic response was evident in combination treatment group relative to untreated control or single-agent treated group. B, cell cycle analysis using FACS. Exponentially growing C4-2b cells were either untreated or treated with Taxotere/ABT-627 for 72 h followed by FACS analysis for assessing apoptotic cells as measured by accumulation of cells in the sub-G0-G1 fraction.
with ~40% growth inhibition by single treatments (Fig. 2). However, once again PC-3 cells were found to be insensitive to this combined regimen, unlike LNCaP and C4-2b cells. These results suggest that the combination of ABT-627 and Taxotere elicits a significantly greater loss of viable prostate cancer cells relative to either agent alone. The inhibition of cell growth and viability as assessed by MTT could also be due to the induction of apoptosis induced by ABT-627 or chemotherapeutic agents. We therefore investigated whether ABT-627 could potentiate the effect of Taxotere by inducing apoptotic cell death compared with either agent alone.

**ABT-627 sensitizes C4-2b and LNCaP cells to apoptosis by Taxotere.** Two different approaches including histone-DNA ELISA and sub-G0-G1 fraction of PI-stained nuclei were used to investigate the degree of apoptosis. We observed apoptosis in prostate cancer cells treated with either ABT-627 (25 μmol/L) or Taxotere (1 mmol/L) alone. However, relative to single agent, combination treatment induced more apoptosis in LNCaP and C4-2b cell lines (Fig. 3A). These results are consistent with the inhibition of cell viability by MTT, suggesting that the loss of viable cells by ABT-627 and Taxotere is partly due to the induction of apoptotic cell death. These findings were complemented by FACS scan results wherein sub-G0-G1 fraction of PI-stained nuclei were increased by 2.88-fold relative to only Taxotere treatment (Fig. 3B).

**ABT-627 inhibits activation of NF-κB.** As documented previously, constitutive NF-κB found in nuclear extracts from prostate cancer cells enables survival by inhibiting apoptosis. To investigate whether the drug ABT-627 could abrogate constitutively expressed NF-κB, cells were treated with increasing concentrations of the drug (5, 10, and 25 μmol/L for 72 h) and subjected to gel shift assay (EMSA). As shown in Fig. 4A and B, ABT-627 resulted in a decreased NF-κB DNA binding activity in C4-2b cells in a dose-dependent and time-dependent manner. Interestingly, the combination of ABT-627 and Taxotere showed greater degree of down-regulation of the NF-κB DNA binding activity (Fig. 4C). These results show that ABT-627 not only down-regulates the NF-κB DNA binding activity but inhibits NF-κB even more in the presence of low concentration of Taxotere, which could be responsible for better cell killing by combination treatment.

**Cellular basis for ABT-627 and Taxotere augmenting signal for apoptosis.** To identify the mechanism of enhanced apoptotic response by ABT-627 and Taxotere combination treatment, we next assessed by Western immunoblotting PARP cleavage and modulation of the status of antiapoptotic proteins using C4-2b cells as representative cell type. Preliminary experiments were done to determine the optimal treatment schedule and dose of individual agents. Cells were treated with ABT-627 (25 μmol/L) and Taxotere for 72 h, and their whole-cell extract were subjected to Western immunoblottng. Our data showed that combination treatment substantially inhibited the levels of all markers tested that favors cell survival (Fig. 4D). Because PARP is a substrate for caspase activity and a reliable marker of apoptosis, we assessed the level of cleaved PARP. Individually, these agents showed low levels of PARP cleavage; however, the combination of ABT-627 and Taxotere resulted in the appearance of a stronger cleaved PARP band (Fig. 4D). Our results on the inactivation of NF-κB DNA binding
activity and subsequent decrease in the levels of Bcl-2, Bcl-xL, and survivin are consistent with transcriptional inactivation of Bcl-2, Bcl-xL, and survivin due to inactivation of NF-κB in combination treatment. Moreover, because inactivation of Akt phosphorylation also reportedly sensitizes cells to chemotherapeutic drugs, we examined the Akt activity by monitoring the phosphorylation of GSK-3α/β which is a substrate of Akt. We found that, although Taxotere and ABT-627 had an effect on down-regulation of phosphorylated Akt, the effect was much more pronounced in the combination group favoring apoptosis (Fig. 4D). These results confirms previous notion that ET-1 activates Akt activity through a PI-3 kinase-dependent mechanism and inhibition of ET-1 by ABT-627 results in the attenuation of PI-3 kinase–Akt survival pathway, causing inactivation of NF-κB and its target genes, leading to cell growth inhibition and induction of apoptotic cell death.

**ABT-627 augments in vivo therapeutic effect of Taxotere on tumor growth.** The above in vitro mechanistic results strongly support more efficient killing of prostate cancer cells by the novel combinatorial regimen comprising Taxotere and ABT-627. However, no in vivo preclinical studies has been done to date emulating the human situation commonly seen in patients presented with prostate cancer bone metastases accompanying osteoblastic lesions. Thus, we evaluated in vivo the efficacy of combinatorial regimen using SCID-hu model of experimental prostate cancer bone metastasis of C4-2b cells. The experimental scheme is summarized in Fig. 5A. We found that ABT-627 as well as Taxotere alone inhibited the C4-2b tumor growth within the bone environment to some extent (Fig. 5B). Interestingly, the combination of ABT-627 and Taxotere showed 90% inhibition in tumor growth relative to control, demonstrating enhanced inhibitory effect of ABT-627 and Taxotere combination in our model (Fig. 6B). To explore therapeutic benefit and unravel the molecular mechanism by which ABT-627 potentiated the antitumor and antimetastatic effect of Taxotere, we quantitated serum prostate-specific antigen and evaluated the antiapoptotic gene expression profiles by performing Western immunoblot and EMSA analysis using harvested tumor tissue extracts. We found a significant decrease in serum prostate-specific antigen (Fig. 6B), suggesting that ABT-627 alone, Taxotere alone, or their combinations did not induce any deleterious effects under present experimental conditions. These results clearly support the efficacy of combination of ABT-627 and Taxotere in inhibiting prostate tumor growth in a s.c. implanted fetal bone model of experimental metastases.

**In vivo NF-κB DNA binding activity and its downstream effector, survivin and Bcl-2.** We subsequently evaluated within two randomly selected tumor specimens per group to test whether ABT-627 and/or Taxotere drug combinations alter molecules that were observed during our in vitro studies. We measured the DNA binding activity of NF-κB and two of its downstream target genes, such as Bcl-2 and survivin, within tumor tissues. Our results clearly show that NF-κB and the expression of its downstream effector genes, Bcl-2 and survivin, was significantly down-regulated in specimens obtained from the combination group (Fig. 6C). These in vivo results were similar to our in vitro findings, suggesting that
the inactivation of NF-κB is, at least, one of the molecular events by which the drug combination potentiates antitumor activity in our experimental model. The down-regulation of the survivin and Bcl-2 protein expression also provides further evidence of loss of survival/proliferative signals within tumors treated with the drugs which corroborates our in vitro findings (Fig. 4D).

**Tumor histology.** All tumors harvested from the control and treated group of mice were subjected to histopathologic evaluation (Fig. 6D). In the control group of mice, the bone is seen severely destroyed by infiltrating tumor cells by osteolytic processes. Residual bone also showed active new bone formation (osteoblastic reaction) as shown by prominent osteoblasts lining and piling up on the surface of bone matrix. Osteoclasts are also focally seen. Significant histologic changes are seen in two of seven tumors in combination group. The neoplastic cells in these two tumors show marked cytoplasmic clearing and vacuolization. Tumor cells form smaller nests and associated with dense fibrosis. The bone fragments show much less prominent osteoblastic rim. The tumors in remaining animals are mainly composed of large irregular sheets or small nests of high-grade neoplastic cells with eosinophilic cytoplasm. The tumor cells are associated with variable hemorrhage, necrosis, cystic degeneration, and fibrosis. The bone fragments have a prominent osteoblastic rim. None of these changes showed obvious differences among these groups.

**Discussion**

Hormone-insensitive human prostate cancer with predilection to bone metastases exhibits chemoresistance toward commonly used conventional chemotherapeutic agents, suggesting that novel agents must be developed and tested for achieving better outcome in the treatment of hormone refractory and metastatic prostate cancer. Previous studies have shown that ET-1 produced abundantly by prostate cancer cells binds to its receptor present on bone marrow stromal cells and favors osteoblastic response during bone metastases of prostate cancer. These results suggest that the interruptions of ET-1 interaction with its receptor could be a useful strategy for inhibiting prostate cancer bone metastasis and, as such, may enhance the therapeutic activity of docetaxel (Taxotere), the most commonly used drug for the treatment of metastatic prostate cancer.
cancer. Docetaxel (Taxotere), a member of the taxane family, showed clinical activity in a wide spectrum of solid tumors, including breast, lung, ovarian, prostate cancers, etc. It binds to tubulin and deranges the equilibrium between microtubule assembly and disassembly during mitosis and, in turn, impairs mitosis and cell proliferation in tumors. Therefore, the goal of our study was to obtain preclinical data supporting our hypothesis that the combined use of ET-1 receptor antagonist, ABT-627 (Atrasentan), with Taxotere will be beneficial in inducing apoptosis in vitro and inhibiting tumor growth in vivo in a SCID-hu model of experimental bone metastasis induced by C4-2b prostate cancer cells.

In this report, we have presented evidence showing better therapeutic activity of Taxotere when combined with ABT-627 (Atrasentan) both in vitro and in vivo. Our results showed inhibition of tumor cell survival/proliferation and induction of apoptosis in vitro and in vivo, resulting in better antitumor activity of combination therapy in vivo. These results validate the potential advantage of combination therapy for advanced prostate cancer as shown in our experimental model. Our results are in concordance with recently published work by Akhavan et al. (20), demonstrating that endothelin receptor blockade enhances taxane effect in prostate cancer and that ABT-627 exhibited additive tumor effects when used in combination with taxane drugs both in vitro and in vivo using ETα overexpressing PPC-1 xenograft tumors and assessing the tumor growth rate.

Recent studies have shown that tumor cells produce ET-1, which has dual effect in stimulating osteoblast and tumor cell proliferation via ETA receptors. Thus, intervening ET-1 ligand–receptor interaction by the drug ABT-627 impedes ETα receptor signaling, suggesting that this drug is effective in preventing osteoblastic bone lesions and tumor growth arrest, invasion, and angiogenesis in vivo (6). Interestingly, the drug ABT-627 alone showed limited effect on tumor growth in our model system; however, antitumor activity was significantly increased by using one cycle of cytotoxic chemotherapeutic agent, Taxotere, commonly used for the treatment of advanced prostate cancer. Furthermore, our mouse model of experimental bone metastasis carrying human bone xenograft creates the bone marrow microenvironment that is closely similar to those seen in bone metastatic disease of prostate cancer. One remarkable finding was that the tumor cells induced new bone formation (osteoblastic lesions) within the implanted bone, and this feature is commonly seen in patients with advanced prostate cancer. In addition, we also found osteolytic processes in this model, which are also seen in bone metastatic disease in humans. Thus, we believe that this model of experimental bone metastases is a useful model for further understanding of the biological role and pathophysiologic role of tumor-bone interaction of prostate cancer metastasis, which is likely to assist in designing better therapies for bone metastatic disease of prostate cancer.

Our in vitro studies revealed that the drug ABT-627 has differential sensitivity toward human prostate cancer cells, as concluded from its ability to inhibit the growth of LNCaP and C4-2b cells (AR positive) but not the growth of PC3 cells that are AR negative. However, what role AR plays in determining the sensitivity of prostate cancer cells toward ABT-627 is not known and, as such, could be an interesting topic on its own right for future investigation. Consistent with these observations, we further evaluated, under in vitro conditions, the response of prostate cancer cells to combination treatment with cytotoxic chemotherapeutic agent, Taxotere; and again we found enhanced killing of LNCaP and C4-2b cells but not of PC-3 cells. Additional studies conducted using C4-2b cells complimented the cell viability results and revealed pronounced apoptosis using a sensitive DNA-histone ELISA and FACS-PI staining for analysis of sub-G0-G1 cells after 72 h coinubation with ABT-627 and Taxotere. Further, mechanistic studies using C4-2b cells correlated with cell growth inhibition and induction of apoptosis with pronounced effect observed in combination group compared with single agents. FACS scan analysis results revealed that the drug combination increased cell numbers in the sub-G0-G1 phase, suggesting apoptotic cell death.

A recent report published by Nelson et al. documented that ET-1 induced phosphorylation of Akt in prostate cancer cells (21). Studies published by our laboratory and others have shown that Akt directly activates NF-κB signaling (22). In other cell types, ET-1 reportedly signals through Erk1/2 to induce Akt phosphorylations (23). This clearly reveals that ET-1 has diverse cellular responses and, as such, multiple pathways converging to promote tumor cell proliferation and survival signaling. Thus, blocking endothelin axis with novel drugs, such as ABT-627, knocks down cell survival response to ETα signaling by inactivation of Akt/NF-κB signaling pathway. Our results using C4-2b cells showed more pronounced down-regulation of phosphorylated Akt in combination treatment compared with single agents.

To further test whether there is any possible link between endothelin axis and induction of apoptotic cell death using the proposed combinatorial regimen, we focused our attention on modulation of an important transcription factor, NF-κB, which is regulated by Akt. Consistent with existing reports in the literature documenting that ET-1 induces NF-κB activation, we evaluated NF-κB DNA binding activity using nuclear protein extracts of prostate cancer cells (C4-2b) treated with ETα receptor antagonist (ABT-627). Increasing concentration of ABT-627 reduced the basal constitutive level of NF-κB DNA binding activity, and these results are consistent with previously published reports establishing the link between NF-κB activation and endothelin axis (ETα receptor blockade). Several lines of evidence supports that the NF-κB pathway is an important target to prevent prostate cancer bone metastases, and published literatures suggest that NF-κB reciprocally regulates genes involved in metastases and apoptosis. We propose a hypothesis wherein ET-1 activates NF-κB through autocrine loop and that ET-1 antagonists abrogate NF-κB activation favoring proapoptotic environment, leading to greater killing when combined with chemotherapeutic agents. The C4-2b prostate cancer cells with high-basal level of constitutive NF-κB is likely to favor active transcription of prosurvival genes whose expression results in increased proliferation. In addition, numerous studies have shown that the production of angiogenic factors in inducing angiogenesis are regulated through NF-κB binding to promoter sequence of vascular endothelial growth factor, resulting in increased neovascularization in tumors. Thus, ABT-627–mediated down-regulation of NF-κB could result in a cascade of molecular events including up-regulation of effectors of apoptosis and reduction in the number of blood vessels within the tumor, ultimately resulting in the inhibition of cell survival, proliferation, and induction of cell death and collectively causing antitumor activity. As previously suggested by Nelson et al. (21) and others, we also believe that up-regulation of Akt activity and NF-κB accounts for the majority of the effects elicited by ET-1 signaling. Previous studies reported in the literature revealed elevated circulating plasma ET-1 in men with hormone refractory prostate cancer (13.2 ng/mL) compared with normal controls (5.1 pg/mL). It is
likely that ET-1 concentrations in circulation would be even lower than the local tissue concentrations, wherein prostate cancer cells are elicitng an autocrine mechanism of ET-1 signaling through the ET_A receptor, augmenting uncontrolled proliferation and, as such, supports our results.

Other studies targeting combination of ET_A receptor antagonist and chemotherapeutic drugs are beginning to emerge. Del Bufalo et al. (24) and Rosano et al. (15) have shown that the drug ABT-627 attenuated the action of ET-1 and sensitized the ovarian tumor cells to paclitaxel. Furthermore, in a nude mouse model carrying CaSki xenograft, the combined treatment of ABT-627 along with paclitaxel was very effective in inhibiting tumor growth (16). Consistent with these results, our findings reinforce the hypothesis that prolonged treatment with ET_A receptor antagonists along with antitumor chemotherapeutic agents should be more effective for the treatment of metastatic prostate cancer in the future.

In conclusion, the combination treatment significantly inhibited tumor cell growth, induced apoptosis, and inhibited the growth of C4-2b cells in the bone microenvironment in our SCID-hu model of human prostate cancer bone metastasis. These results suggest that the combination of ABT-627 and Taxotere could be a promising regimen for the treatment of prostate cancer and its metastases. Further testing of combinations of various drugs with ABT-627 and attenuation of survival signaling is likely to assist in designing mechanism-based anticancer therapies, particularly for metastatic prostate cancer for which there is currently no curative treatment.

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