

BKM1740, an Acyl-Tyrosine Bisphosphonate Amide Derivative, Inhibits the Bone Metastatic Growth of Human Prostate Cancer Cells by Inducing Apoptosis

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Abstract Purpose: Survivin overexpression has been associated with an unfavorable outcome in human PCa; however, its role in metastasis remains elusive. We aim to (a) evaluate the clinical implications of survivin expression in PCa bone metastasis; (b) determine *in vivo* efficacy of BKM1740, a small-molecule compound, against PCa skeletal growth and survival; and (c) investigate molecular mechanism by which BKM1740 augments apoptosis in bone metastatic PCa cells.

Experimental Design: Survivin expression was analyzed in PCa specimens and experimental models. Bone metastatic C4-2 and ARCaP_M cell lines were used to evaluate the *in vitro* effects of BKM1740 and molecular mechanism for the induction of apoptosis. C4-2 cells were grown intratibially in athymic nude mice to evaluate the *in vivo* efficacy of BKM1740. Tumor growth in mouse bone was assessed by serum prostate-specific antigen and radiography and confirmed by immunohistochemical analyses.

Results: Survivin expression is positively associated with clinical PCa bone metastasis. BKM1740 induced apoptosis in PCa cells by repressing survivin. Mice with established C4-2 tumors in tibia showed a marked decrease in serum prostate-specific antigen and much improved bone architecture radiographically after treatment with BKM1740. Immunohistochemical assays of mouse tumor samples confirmed that the *in vivo* effects were mediated by inhibition of survivin and induction of apoptosis.

Conclusions: Survivin expression is associated with PCa bone metastasis. BKM1740 treatment specifically inhibited survivin and induced apoptosis *in vitro* and was efficacious in retarding PCa skeletal growth in a mouse model. BKM1740 is a promising small-molecule compound that could be used to treat PCa bone metastasis.

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Bone metastasis and skeletal complications are the major contributing factors to human prostate cancer (PCa) morbidity and mortality (1). The gain of function of antiapoptotic factors and/or loss of function of proapoptotic proteins may allow PCa cells to evade apoptosis during dissemination and growth in bone tissue (2). As a member of the inhibitor of apoptosis family, survivin intersects multiple survival signals and is highly differentially expressed in cancer (3). In PCa, survivin overexpression has frequently been associated with an unfavorable outcome. Survivin expression is significantly elevated in tumors with a high Gleason score and lymph node metastasis (4, 5). Survivin also mediates resistance to antiandrogen therapy, chemotherapy, and irradiation (6–8). However, despite the well-defined function of survivin in antagonizing death signals during tumorigenesis, little is known about its role in tumor invasion and metastasis (9).

Currently, several strategies are being pursued to target survivin expression or interrupt its antiapoptotic function. Some have been in clinical trials for a variety of human cancers (see ref. 10 for review). Some small-molecule antagonists exert their antitumor function by indirectly targeting pathways implicated in survivin regulation (such as STA-21 inhibition

Translational Relevance

Bone metastasis is the leading cause of prostate cancer (PCa) death. Current therapy does not improve patient survival. It is critical to identify novel targets for developing efficacious treatments. This study showed that overexpression of survivin, a critical antiapoptotic protein, is associated with clinical PCa progression and bone metastasis. We developed BKM1740, a small-molecule acyl-tyrosine bisphosphonate amide derivative, and found that BKM1740 efficaciously retards PCa skeletal growth in athymic nude mice. Mechanistic study confirmed that the *in vivo* effects were mediated by specific inhibition of survivin and induction of apoptosis. This study validated the clinical implications of survivin expression in PCa bone metastasis and may have a significant effect on the development of efficacious and safe therapeutic regimens for metastatic PCa and other types of cancers.

of the signal transducer and activator of transcription 3 pathway or flavopiridol inhibition of cyclin-dependent kinase 1 activity) or by perturbing protein-protein interaction between survivin and its partners (such as shepherdin, a peptidyl antagonist that may disrupt the interaction between heat shock protein 90 and survivin). Some agents have been developed to directly suppress survivin expression, including an antisense molecule (LY218130B) and transcriptional repressors (YM155 and EM-1421). In established human PCa PC-3 xenografts, YM155, a small imidazolium-based compound, was shown to specifically suppress survivin transcription (11). Two phase I trials for YM155 have been completed in 41 patients with PCa, non-Hodgkin lymphoma, or colorectal cancer. The treatment was well tolerated and exhibited encouraging antitumor efficacy (12). Nonetheless, the portfolio of truly survivin-directed antagonists or suppressors available for clinical testing, particularly in metastatic cancer, is small (10).

Bradykinin-related compounds are emerging as promising antitumor agents (13–15). In experimental models for human PCa and lung cancer, a bradykinin antagonist peptide dimer, B-9870 (CU201), and its nonpeptide mimetic, BKM-570, suppress tumor growth and act synergistically with standard chemotherapy drugs such as cisplatin and Taxotere (16–18). Mechanistic study indicated that the bradykinin-related compounds induce caspase-dependent apoptosis in cancer cells while inhibiting angiogenesis and reducing tissue permeability mediated by matrix metalloproteinases (MMP) in tumors (15). Therefore, these compounds may be pluripotent anticancer agents. To explore novel drugs that may specifically target bone metastatic PCa cells, we developed a BKM-570 analogue conjugated with an aminobisphosphonate group. This compound, termed BKM1740, was found to be efficacious in retarding skeletal growth of human PCa cells through direct inhibition of survivin in a xenograft model.

Materials and Methods

Cell lines and culture conditions. Human PCa cell lines LNCaP, C4-2 (19), ARCaP_E, and ARCaP_M (20) were regularly maintained in

T-medium (Invitrogen) supplemented with 5% fetal bovine serum (Sigma), 100 IU/L penicillin G, and 100 µg/L streptomycin at 37°C under 5% CO₂.

Chemicals. BKM1740 was developed and synthesized by Gera in the Stewart laboratory according to previously described methods (18, 21, 22). For the *in vitro* and *in vivo* studies, the BKM1740 was dissolved in DMSO (Sigma) at 10 mg/mL stock solution and its purity was determined to be a minimum of 99% by high-performance liquid chromatography.

Cell proliferation assay. Cell proliferation was measured using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay; Promega]. Briefly, cells suspended in T-medium plus 5% fetal bovine serum were added to 96-well plates at 5,000 per well in sextuplicate. After 24 h of culture, BKM1740 or DMSO was added in various concentrations, and cells were cultured for the indicated time. Combined MTS/phenazine methosulfate solution (20 µL/well) was added to the cells and the absorbance at 490 nm was recorded after 1 h of incubation at 37°C using a microplate reader (Bio-Rad Laboratories). Cell viability was expressed as relative survival with controls recorded as 100%.

Reporter assay. Cells were seeded at a density of 1.5×10^5 per well in 12-well plates 24 h before transfection. pSurvivin-luc1430 (23) and pRL-TK (as internal control; Promega) were introduced using Lipofectamine 2000 (Invitrogen). A Dual-Luciferase Reporter Assay kit (Promega) was used to determine the firefly luciferase activity and *Renilla* luciferase activity. Data were presented as relative luciferase activity (firefly luciferase activity normalized to *Renilla* luciferase activity).

Western blot analysis. Total cell lysates were prepared using radio-immunoprecipitation assay buffer (Santa Cruz Biotechnology). Protein concentrations in the supernatants were measured with the bicinchoninic acid protein assay kit (Pierce Biotechnology). Total protein (50 µg) was loaded to each lane, resolved on a 4% to 12% NuPAGE Bis-Tris-buffered (pH 7.0) polyacrylamide gel (Invitrogen), and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was incubated with anti-survivin (Novus Biologicals), anti-caspase-3, anti-cleaved caspase-3, anti-caspase-8, anti-cleaved caspase-8, anti-caspase-9, anti-poly(ADP-ribose) polymerase (Cell Signaling), and anti-myeloid cell leukemia-1 (Mcl-1) and MMP-9 (Santa Cruz Biotechnology), with anti-β-actin (Sigma) or anti-EF1α (BD Transduction Laboratories) as loading controls. The reactive bands were visualized by an enhanced chemiluminescence assay kit (Amersham Pharmacia Biotech). Quantification of band intensity was measured by densitometry and analyzed with ImageJ (NIH). Relative protein expression was expressed as fold change compared with control (β-actin or EF1α).

Reverse transcription-PCR. Total RNA was prepared using a Qiagen RNeasy kit. One microgram was used as a template in a reaction using the SuperScript III One-Step reverse transcription-PCR (RT-PCR) kit (Invitrogen). The primer pairs specific for human survivin are 5'-ccaccgcatctcatca-3' (forward) and 5'-gcactttctcgcagttc-3' (reverse); for human Mcl-1, 5'-gaggaggaggaggcaggtt-3' (forward) and 5'-gtcccgtttgtccttaca-3' (reverse). The primers for vascular endothelial growth factor (VEGF) and glyceraldehyde-3-phosphate dehydrogenase were described previously (24). The thermal profile is 23 cycles for human survivin, Mcl-1, and VEGF amplification or 20 cycles for glyceraldehyde-3-phosphate dehydrogenase, with 94°C, 15 s; 55°C, 30 s; and 68°C, 60 s. Quantification of band intensity was measured by densitometry and analyzed with ImageJ. Relative mRNA abundance was expressed as fold change compared with control (glyceraldehyde-3-phosphate dehydrogenase).

Condition medium preparation. Subconfluent C4-2 cells were serum starved overnight and further treated with BKM1740 or DMSO in fresh serum-free T-medium for 48 h before condition media were collected (24).

ELISA. Vascular endothelial cell growth factor (VEGF) concentration was analyzed using a Quantikine ELISA kit (R&D Systems).

Apoptosis analysis. Cells treated with DMSO or BKM1740 were trypsinized and washed with PBS and resuspended in Annexin-binding buffer (BD PharMingen). Cells were then stained with both Annexin V-phycoerythrin and 7-amino-actinomycin for 15 min at room temperature. The stained samples for apoptosis assay were measured using a fluorescence-activated cell sorting (FACS) caliber bench-top flow cytometer (Becton Dickinson). The data were analyzed using FlowJo software (Tree Star, Inc.). The experiments were repeated at least three independently.

Assessment of in vivo effects of BKM1740 on human prostate tumor xenografts in mouse bone. All animal procedures were done in compliance with Emory University Institutional Animal Care and Use Committee and NIH guidelines. A total of 1.0×10^6 C4-2 cells were inoculated in mouse bilateral tibia using a previously established procedure (25, 26). Blood specimens (70 μ L) were obtained from the retro-orbital sinus vein every 2 wk for serum prostate-specific antigen (PSA) determination. Serum PSA was determined by microparticle ELISA using an Abbott IMx instrument (Abbott Laboratories). A total of 20 athymic male nude mice (BALB/c *nu/nu*; National Cancer Institute, Bethesda, MD) were divided into two groups: a vehicle control group ($n = 11$) and a BKM1740 treatment group ($n = 9$). The treatments were initiated at 4 wk after tumor cell inoculation with confirmed tumors in bone by X-ray and positive serum PSA (25, 27). BKM1740 was dissolved in 100% DMSO as a stock solution of 10 mg/mL. Mice were given BKM1740 every 2 d at 5 mg/kg via the i.p. route for an 8-wk period. Control mice received vehicle injections for the same duration. Mice were weighed every week and tumor growth in bilateral tibia was followed by serum PSA and X-ray every 2 wk. Mice were sacrificed 8 wk after the initiation of treatment. The bilateral tibia were removed, fixed in 10% neutralized formalin for 48 h, and decalcified in EDTA (pH 7.2) for 15 d. Tibia specimens were dehydrated and paraffin embedded for immunohistochemical analyses. To assess the systemic toxicity of BKM1740, athymic nude mice ($n = 4$) were administered at a high dose of 20 mg/kg via the i.p. route, twice per week, for 4 wk.

Immunohistochemical analysis. Survivin expression was analyzed in five human normal/benign prostatic glands, five each of well-differentiated and poorly differentiated primary PCa, and four bone metastatic PCa tissue specimens. Cell proliferation (Ki67), cell death (M30), and survivin in bone tumor specimens obtained from control and BKM1740-treated mice (two in each group) were conducted. Antibodies used were goat polyclonal antibody against Ki67 (1:500; Santa Cruz Biotechnology), mouse monoclonal antibody against M30 CytoDeath (1:500; DiaPharma Group, Inc.), and rabbit polyclonal antibody against survivin (1:200; Novus Biologicals). Tissues were deparaffinized, rehydrated, and subjected to 5-min pressure-cooking antigen retrieval, 10-min double endogenous enzyme block, and 30-min primary antibody incubation, and subjected to 30-min DakoCytomation EnVision+ horseradish peroxidase reagent (for M30 and survivin) or 15 min each of biotinylated link and streptavidin-peroxidase label reagents (for Ki67) incubation. Signals were detected by adding substrate hydrogen peroxide using diaminobenzidine as chromogen and counterstained by hematoxylin. All reagents were obtained from Dako Corp. Matching sera and IgG were used as negative controls. Relative expression of Ki67, M30, and survivin was shown as the number of positively stained cells in 200 cells \pm SE at three randomly selected areas at $\times 100$ magnification.

Data analysis. All data represent three or more experiments. Treatment effects were evaluated using a two-sided Student's *t* test. Errors are SE values of averaged results, and values of $P < 0.05$ were taken as a significant difference between means.

Results

Elevation of survivin is correlated to bone metastasis status in human PCa tumors. To investigate the clinicopathologic significance of survivin expression in human PCa progression, we analyzed the immunohistochemical protein expression of

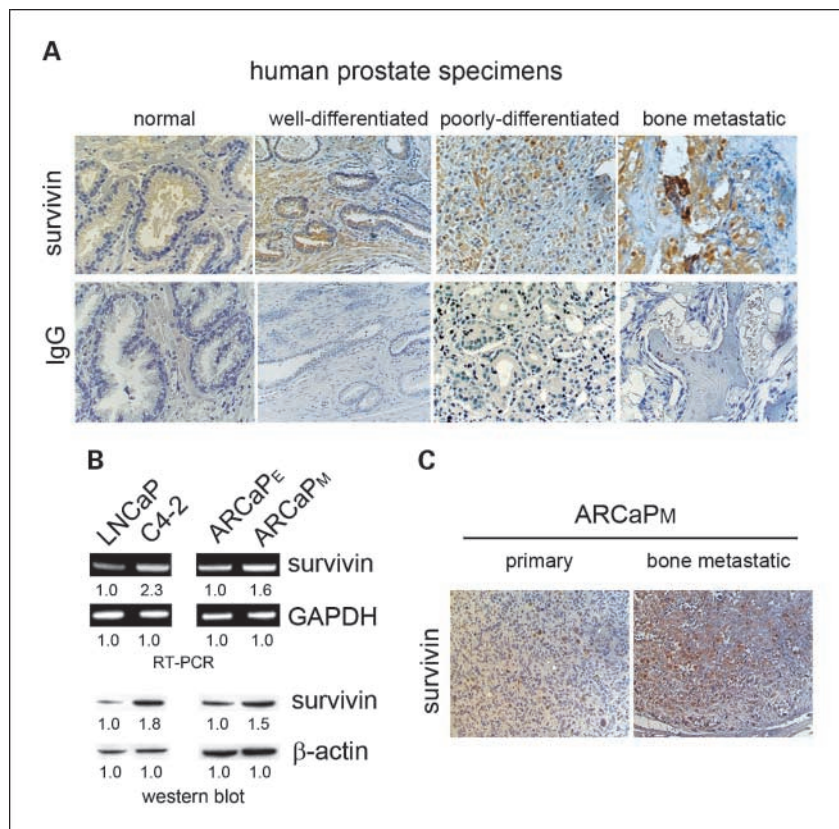


Fig. 1. Survivin expression is associated with bone metastasis in human PCa specimens and the ARCaP experimental model. **A**, survivin expression increased during PCa progression from normal to primary cancer to bone metastasis. **B**, RT-PCR and Western blot analyses of survivin expression in human PCa cell models. Survivin increased in bone metastatic C4-2 and ARCaPM cells compared with their parental LNCaP and ARCaPE cells. Relative expression was expressed as fold change compared with controls. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **C**, survivin expression in bone metastatic ARCaPM tumor was higher than in primary tumor.

survivin in primary and bone metastatic PCa tissue. We defined well-differentiated PCa as Gleason score ≤ 6 and poorly differentiated PCa as Gleason score ≥ 8 . Survivin expression was undetectable to marginal in all normal/benign glands ($n = 5$) and increased from well-differentiated cancer ($n = 5$) to poorly differentiated cancers ($n = 5$). Importantly, survivin was highly expressed in all bone metastatic PCa tumor specimens ($n = 4$; Fig. 1A). These data suggest that survivin expression is positively associated with PCa progression, particularly bone metastasis.

We have established several lines of human PCa cells that represent a continuum of PCa progression closely mimicking the clinical pathophysiology of bone metastasis (see ref. 28 for review). Two lineage-related sets of PCa cells were used in this study: the LNCaP-C4-2 model (19, 29) and the ARCaP_E-ARCaP_M model (20, 24, 30). RT-PCR and Western blotting analyses indicated that survivin expression was elevated in highly bone metastatic C4-2 and ARCaP_M PCa cell lines compared with the less invasive parental cell lines LNCaP and ARCaP_E (Fig. 1B). ARCaP_M cells were inoculated into athymic mice s.c., which resulted in metastases to bone tissues within a short latency period (20, 31). Survivin expression was examined by immunohistochemical staining of the ARCaP_M tumor specimens from either the primary site (s.c. injection) or metastatic bone. Consistently, survivin protein level was significantly increased in bone metastatic tumor compared with the primary tumor (Fig. 1C). These data obtained from *in vivo* PCa models validate a positive correlation between survivin expression and the bone metastatic propensity observed in clinical specimens.

BKM1740 induces apoptosis in metastatic PCa cells. BKM1740 is an acyl-tyrosine bisphosphonate amide derivative, which was developed from the key chemical structure of BKM-570, F5c-OC2Y [*N*-(2,3,4,5,6-pentafluorocinnamoyl)-*O*-(2,6-dichlorobenzyl)-tyrosine; refs. 18, 22]. BKM1740 is expected to have potent antitumor activity exhibited by the F5c-OC2Y moiety and can be selectively taken up and adsorbed to mineral surfaces in bone because of the introduction of the aminobisphosphonate moiety (32).

We first evaluated the cytotoxic effects of BKM1740 on bone metastatic PCa cells. C4-2 and ARCaP_M cells were exposed to the indicated concentrations of BKM1740 for various durations and cell proliferation was determined by MTS assay. BKM1740 was found to inhibit the *in vitro* growth of C4-2 (Fig. 2B) and ARCaP_M cells (Fig. 2C) in a dose- and time-dependent manner, with 50% inhibition (IC₅₀) observed at 2 and 9 $\mu\text{mol/L}$, respectively. Interestingly, compared with C4-2 cells, ARCaP_M only responded to BKM1740 treatment significantly within a narrow dose range (between 8 and 10 $\mu\text{mol/L}$), suggesting that this cell line is more resistant to the cytotoxicity of BKM1740 (Fig. 2C).

To further elucidate the mechanism for the effects of BKM1740 on PCa cell viability, we determined Annexin V expression, an indicator of apoptosis, in C4-2 cells treated with BKM1740 at the indicated concentrations for 24 h (Fig. 3A). Fluorescence-activated cell sorting analysis indicated that BKM1740 treatment significantly induced apoptosis in C4-2 cells in a dose-dependent manner. Greater than 40% cell death can be achieved in 24 h with 5 $\mu\text{mol/L}$ BKM1740 (Fig. 3B). Expression of caspases was determined by Western blot analysis of C4-2 cells treated with 5 $\mu\text{mol/L}$ BKM1740. Activation of

caspase-3, caspase-8, and caspase-9, as exhibited by increased cleaved protein bands at 17, 40, and 35 kDa, respectively, was observed after incubation with BKM1740 for 12 h. Cleavage of poly(ADP-ribose) polymerase, an indicator of apoptosis shown as a band at 89 kDa, also increased significantly (Fig. 3C). These data suggest that BKM1740 induces apoptosis in metastatic PCa cells through a caspase-dependent pathway.

BKM1740 specifically inhibits expression of survivin in metastatic PCa cells. Multiple factors are involved in the regulation of cell death by apoptosis (33). To elucidate the specific signaling pathway(s) mediating the cytotoxicity of BKM1740 in PCa cells, we analyzed the expression of several antiapoptotic proteins in C4-2 cells treated with BKM1740 (Fig. 4A and B). RT-PCR assay indicated that BKM1740 significantly inhibited survivin expression at the mRNA level. BKM1740 treatment did not significantly affect the expression of antiapoptotic protein Mcl-1 (34). Expression of VEGF, inhibited by treatment with BKM-570 in a previous study (22), was not affected by BKM1740 at either the mRNA (Fig. 4A) or protein level (Fig. 4B). Western blot analysis confirmed the inhibition of survivin protein expression following BKM1740 treatment in C4-2 and ARCaP_M cells (Fig. 4A and C). Basal expression of MMP-9, an important MMP implicated in PCa metastasis (22, 35), was not detected in C4-2 cells by Western blot analysis (data not shown) and not affected by BKM1740 treatment in ARCaP_M cells (Fig. 2C).

C4-2 cells were transiently transfected with a survivin-luciferase reporter (pSurvivin-luc1430) composed of a 1,430-bp region of human survivin promoter (23). The cells were further treated with BKM1740 at the indicated concentrations for 24 h before the luciferase activity assay was done. The data indicated that BKM1740 inhibited the survivin reporter activity in a dose-dependent manner (Fig. 4D), suggesting that survivin transcription was suppressed by BKM1740 treatment in C4-2 cells, which was consistent to the RT-PCR results (Fig. 4A). Taken together, these data showed that BKM1740 specifically inhibits survivin expression in bone metastatic PCa cells, which may mediate the activation of caspase-dependent apoptotic death caused by this compound.

BKM1740 treatment inhibits *in vivo* C4-2 tumor growth in mouse skeleton. To evaluate the *in vivo* effect of BKM1740 against the growth of bone metastatic PCa tumors, we treated athymic nude mice bearing intratibial C4-2 xenografts with BKM1740 at a dose of 5 mg/kg by the i.p. route, once every 2 days. The treatment started on day 28 (4 weeks) after tumor inoculation and continued for 8 weeks. Tumor growth and responsiveness to BKM1740 treatment were determined by serum PSA and skeletal X-ray. As shown in Fig. 5A, there was a significant reduction in serum PSA levels in the BKM1740-treated groups compared with vehicle control at 8 weeks ($P < 0.05$). Representative radiographs are shown in Fig. 5B. Compared with the vehicle control, C4-2 tumor-bearing bone treated with BKM1740 displayed improved architecture with reduced osteolytic destruction and osteoblastic lesions (Fig. 5B, left). These X-ray results were consistent with the inhibitory effects of BKM1740 treatment on serum PSA levels in C4-2 tumor-bearing mice. Mice treated with BKM1740 gained weight comparably with the controls (data not shown). To assess the potential *in vivo* toxicity of BKM1740 treatment, athymic nude mice without C4-2 tumor inoculation were treated with a high dose of BKM1740 (20 mg/kg) for 4 weeks. No systemic toxicity

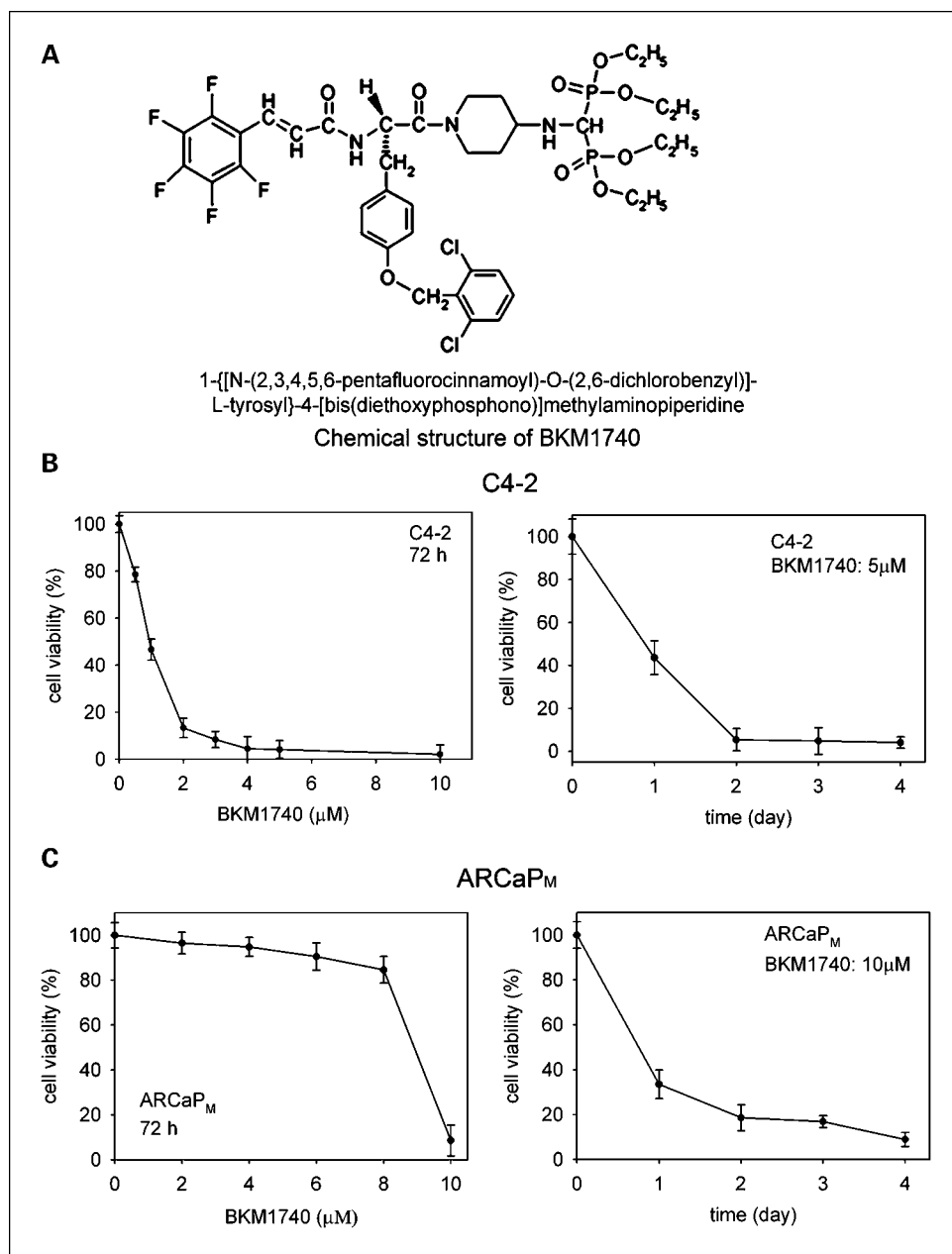


Fig. 2. BKM1740, an acyl-tyrosine bisphosphonate amide derivative, inhibits *in vitro* proliferation of C4-2 and ARCaP_M cells. **A**, chemical structure of BKM1740. **B** and **C**, BKM1740 effects on proliferation of C4-2 (**B**) and ARCaP_M cells (**C**). PCa cells were cultured in the presence of BKM1740 at indicated concentrations for various durations. The effects of BKM1740 treatment on cell numbers were evaluated using MTS assay.

was observed, and the mice gained body weight during the treatment (Supplementary Data). X-ray radiography showed intact bone architecture like that in normal mouse (Fig. 5B, right). These results suggested negligible *in vivo* acute toxicity of BKM1740 treatment.

Immunohistochemical analysis of human PCa xenografts subjected to BKM1740 treatment. The effects of BKM1740 treatment on C4-2 tumor growth in tibia were confirmed by immunohistochemical analyses of the harvested tumor specimens at the termination of the experiments. Immunohistochemical staining of mouse tibia indicated that compared with vehicle control, BKM1740 treatment resulted in (a) markedly decreased cell proliferation (Ki67) and massive apoptosis (M30) in tumor tissues and (b) significant inhibition of survivin expression (Fig. 6A). These differences are statistically significant (Fig. 6B). The data confirmed that the *in vivo* effects

of BKM1740 on C4-2 tumor growth were mediated by suppression of survivin expression and induction of apoptosis in PCa tumors.

Discussion

Current regimens treating metastatic PCa by conventional hormone therapy, chemotherapy, or radiation therapy have not resulted in improved patient survival (36). New approaches targeting bone with bisphosphonates to slow down skeletal events, and bone-directed chemotherapy and radiation therapy using strontium-89 or samarium-153, have been approved by the Food and Drug Administration for the clinical treatment of bone metastasis in PCa and breast cancer (37). In addition, "cotargeting" both the tumor and its stromal microenvironment using gene therapy approaches, and drug therapy

targeting osteoblasts, osteoclasts, marrow stromal cells, bone-derived endothelium, cell adhesion to extracellular matrices, or selected growth factor pathways (see refs. 2, 38 for reviews), has shown promise in a large number of bone metastasis models. In this study, we present evidence indicating that BKM1740, a novel bradykinin-related compound conjugated with aminobisphosphonate, induced massive apoptosis and retarded tumor growth in a human PCa bone metastasis model. These data suggest that BKM1740 is an attractive compound for evaluation as a PCa skeletal metastasis drug.

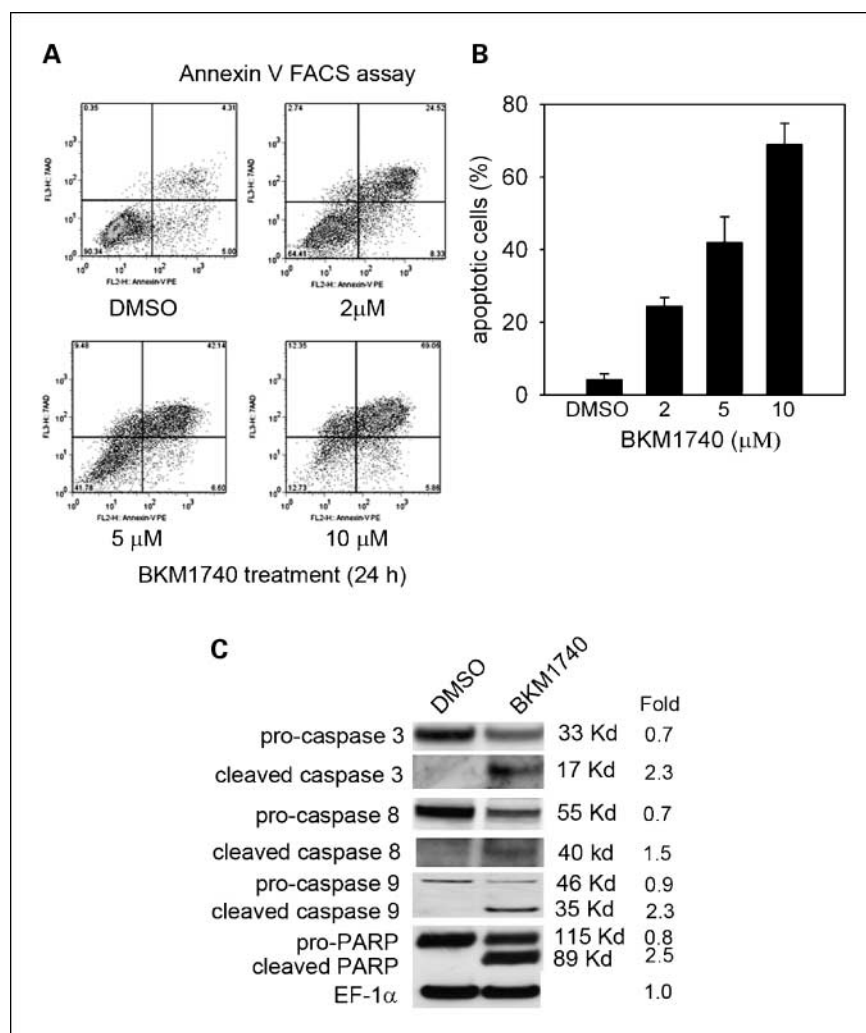
Aberrant signal transduction in both tumors and the bone microenvironment is critical in defining the invasiveness of PCa cells. "Targeted therapy" to interrupt specific signaling pathways implicated in PCa progression is a promising approach supported by recent experimental and clinical studies (39). Multiple signaling molecules have been identified as possible "targets" for rational drug design. Among them, survivin is considered uniquely promising for two reasons: (a) survivin intersects multiple signaling networks implicated in the inhibition of apoptosis and therefore blockade of the survivin signal will interrupt tumor progression regardless of the genetic background of the tumor and (b) survivin is overexpressed by virtually all solid tumors but undetectable or at very low levels in most terminally differentiated normal tissues; therefore,

survivin-based therapy may specifically target tumors with a favorable toxicity profile (3).

Although survivin expression has been correlated to advanced stages of PCa with higher Gleason scores and lymph node metastasis (4, 5), its role in PCa bone metastasis remains elusive. To validate survivin as a rational target for PCa bone metastasis, we first investigated the clinical significance of survivin in human PCa progression. The data showed that survivin expression is positively associated with higher Gleason scores in primary prostatic tumors, indicating that survivin is important in tumorigenesis. Intriguingly, survivin expression is further increased in bone metastatic PCa specimens, which we confirmed in PCa bone metastatic models of LNCaP-C4-2 and ARCaP. Despite the limited numbers of tumor specimens (which were extremely difficult to obtain), these results for the first time suggest a crucial role for survivin in the progression of advanced PCa toward bone metastasis. We hypothesize that overexpression of survivin may confer survival advantages to metastatic PCa cells that allow them to successfully disseminate and colonize. Inhibition of survivin expression may reverse this and induce regression of tumor growth in bone.

Bradykinin-related compounds are being explored as promising anticancer drugs. Several bradykinin antagonists and their mimetic have been found to effectively inhibit tumor growth in

Fig. 3. BKM1740 induces apoptosis in metastatic PCa cells. **A**, C4-2 cells were treated with BKM1740 at the indicated concentrations for 24 h, and Annexin V fluorescence-activated cell sorting (FACS) analysis was done. **B**, percentage of apoptotic cells induced by BKM1740 treatment. **C**, Western blot analysis of activation of caspase pathways. C4-2 cells were exposed to 5 $\mu\text{mol/L}$ BKM1740 for 12 h. Activated caspase-3, caspase-8, and caspase-9 and cleavage of poly(ADP-ribose) polymerase (PARP) were detected by the increased banding of proteins at 17, 40, 35, and 89 kDa, respectively. Relative expression was expressed as fold change compared with the EF1 α controls.



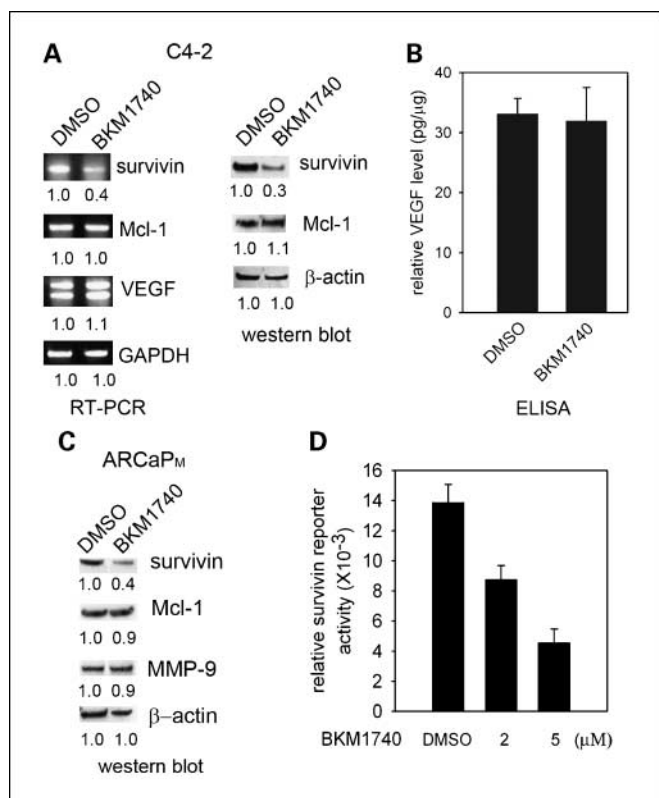


Fig. 4. BKM1740 inhibits survivin expression in metastatic PCa cells. **A**, BKM1740 specifically suppresses survivin expression at both RNA and protein levels in C4-2 cells. Left, C4-2 cells were exposed to 5 μ M/L BKM1740 for 12 h. Total RNA was collected and analyzed by RT-PCR for survivin, Mcl-1, and VEGF, with glyceraldehyde-3-phosphate dehydrogenase as loading control. Right, C4-2 cells were treated with 5 μ M/L BKM1740 for 24 h, and total lysates were analyzed for the expression of survivin and Mcl-1. Relative expression was expressed as fold change compared with the β -actin controls. **B**, ELISA of VEGF levels in conditioned medium of C4-2 cells treated with BKM1740 or vehicle for 48 h. Relative VEGF levels were normalized by dividing VEGF concentrations (pg/mL) by total protein concentrations (mg/mL) in condition medium. **C**, BKM1740 specifically suppresses survivin expression in ARCaP_M cells. ARCaP_M cells were treated with 10 μ M/L BKM1740 for 24 h, and total lysates were analyzed for the expression of survivin, Mcl-1, and MMP-9. Relative expression was expressed as fold change compared with the β -actin controls. **D**, C4-2 cells were cotransfected with pSurvivin-luc1430 and pRL-TK (internal control) for 48 h before exposure to BKM1740 at the indicated concentrations for a further 24-h incubation. Total lysates were analyzed for luciferase activity induced by the survivin promoter and normalized to the *Renilla* luciferase activity.

animal models of PCa and lung cancer (13–16, 18, 40). One of the peptide-based antagonists, CU201, is entering a phase I clinical trial for lung cancer. Mechanistic study showed that CU201 induced cancer cell apoptosis as a “biased” agonist by inhibiting $G\alpha_q$ activation and downstream events and by stimulating $G\alpha_{12,13}$ and downstream cascades (17). Intriguingly, these compounds may act as pluripotent molecules that could simultaneously inhibit cancer cell proliferation by inducing apoptosis, and interrupt angiogenesis by reducing VEGF expression (18). To develop novel anticancer reagents that specifically target PCa bone metastasis, we designed BKM1740 as an analogue that incorporates the key “anticancer” structure (F5c-OC2Y) of BKM-570 and an aminobisphosphonate group to improve specific delivery of the compound into bone, thereby increasing its bioavailability in tumor tissues residing in bone. The mechanism-based evidence presented here shows that (a) BKM1740 specifically inhibited survivin

expression at both the mRNA and protein levels and induced tumor regression in a mouse model of PCa bone metastasis, suggesting that BKM1740 and its derivatives could be novel small-molecule chemicals that effectively treat PCa bone metastasis, and (b) the design scheme for BKM1740 as a “pluripotent” compound could serve as a valuable principle in rational drug development.

Bisphosphonates are nonhydrolyzable pyrophosphate analogues, which have been shown to have inhibitory effects on metastasis-induced osteoclastic bone resorption (32, 41). Introduction of an aminobisphosphonate moiety in BKM1740 was expected to increase its bioavailability in bone metastatic tumor lesions and retain the inhibitory effects on bone resorption initiated by metastatic PCa. However, whether this compound retains the inhibitory activity of the bisphosphonate moiety on the osteolysis process is not clear. Future studies will examine the *in vitro* effects of BKM1740 on osteoclast activity and formation and assess the *in vivo* effects on osteolytic process and bone turnover using the model established in the current study. The results will validate the design strategy for BKM1740 and provide valuable information for developing alternative candidates.

Interestingly, recent studies suggest that bisphosphonates may have direct anticancer activity (42, 43). For example, zoledronic acid was found to be capable of inhibiting *in vitro* proliferation of LNCaP and PC-3 PCa cells (44). Because BKM1740 is conjugated with bisphosphonate, we do not

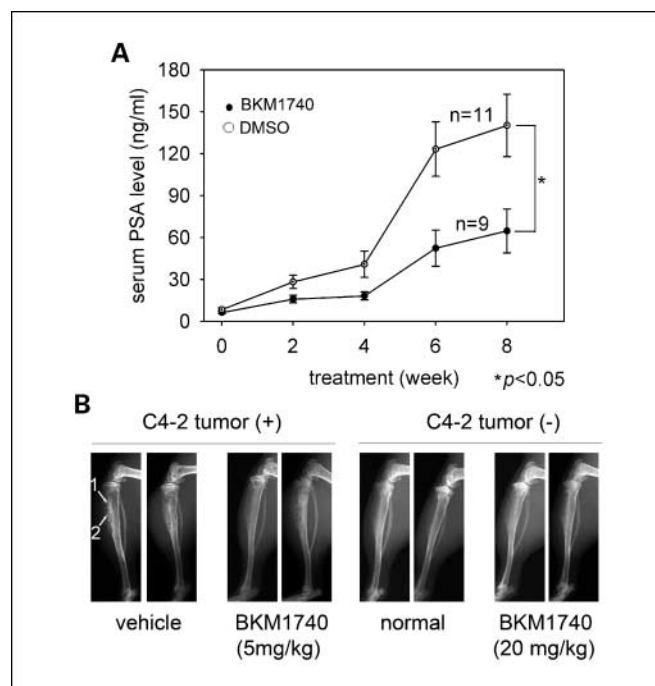
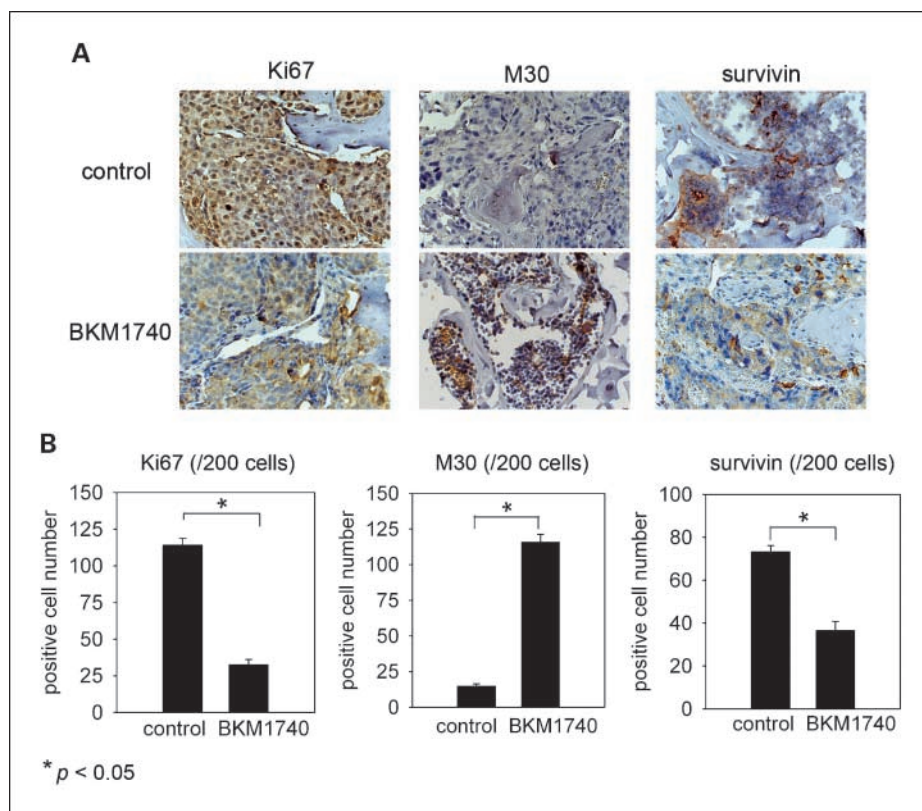


Fig. 5. BKM1740 induces regression of PCa skeletal tumor in C4-2 mouse xenografts. **A**, i.p. injection of BKM1740 reduced serum PSA in mice bearing C4-2 skeletal tumors compared with control group after 8 wk of treatments ($P < 0.05$). ○, vehicle control group; ●, BKM1740-treated group. **B**, representative chromatograms of the bones in each group as detected by X-ray, showing that BKM1740 treatment improves bone X-ray appearances in comparison with control group. Left, tumor-bearing athymic nude mice treated with either vehicle or BKM1740 at a dose of 5 mg/kg, every 2 d, for 8 wk. The osteolytic lesion and osteoblastic lesion in the tumor-bearing bone were indicated by arrows 1 and 2, respectively. Right, normal athymic nude mice or only treated with BKM1740 at a dose of 20 mg/kg, twice per week, for 4 wk.

Fig. 6. BKM1740 treatment exhibits growth-inhibitory and proapoptotic activity against C4-2 tumor xenografts in mice.

A. BKM1740 treatment inhibited cell proliferation (Ki67), induced apoptosis (M30), and suppressed survivin expression *in vivo* by immunohistochemical analysis. **B.** comparative quantification of BKM1740 treatment as opposed to controls on the expression of markers of cell proliferation, apoptosis, and survivin expression ($P < 0.05$).



exclude the possibility that its inhibitory effects on PCa cell survival were partially due to the bisphosphonate moiety. However, the zoledronic acid concentration used in the cited study was much higher (68 $\mu\text{mol/L}$ zoledronic acid in apoptosis analysis; ref. 44) than the BKM1740 concentration used in this report. Further, zoledronic acid only exhibited significant efficacy inhibiting PCa tumor growth in mouse bone, not in s.c. models (44), indicating that zoledronic acid activity on PCa skeletal growth is mainly attributable to indirect effects related to decreased osteolysis. These observations support our notion that the proapoptotic activity of BKM1740 may be primarily mediated by the effects of its F5c-OC2Y moiety on survivin expression.

Multiple growth factors and proteases are involved in PCa growth, survival, and invasion (2, 45, 46). A previous study by Stewart et al. (15) found that CU201 and BKM-570 significantly inhibited angiogenesis by reducing VEGF expression and decreased tissue permeability mediated by MMPs in a PC-3 model. Because BKM1740 is derived from BKM-570, we examined whether it has similar effects on the expression of VEGF and MMPs in C4-2 cells. The data indicated that BKM1740 treatment did not affect VEGF mRNA expression or protein secretion. Unlike in PC-3 cells, basal expression of MMP-9 was undetectable in C4-2 cells and not affected significantly by BKM1740 treatment in ARCaP_M cells. Taken together, these results suggested that BKM1740-induced C4-2 tumor regression in bone may be primarily mediated by specific inhibition of survivin and induction of apoptosis.

Survivin overexpression in human cancers has been associated with resistance to conventional chemotherapy and irradiation therapy (6–8). Furthermore, treatment with certain mitotic inhibitor-based drugs such as Taxol may result in

arresting the mitotic process and subsequent accumulation of survivin in the G₂-M phase of cell cycle, which eventually counteracts Taxol-induced apoptosis (47). Inhibition of survivin, particularly by small-molecule suppressants, could sensitize the resistant cells to apoptosis induction, thereby augmenting therapeutic responses (10). Several strategies are being pursued to down-regulate survivin expression and increase the efficacy of chemotherapy in PCa and breast cancer (7, 11). With the shown efficacy of BKM1740 in suppressing survivin and inducing apoptosis in invasive PCa cells, it can be expected that a combination treatment with BKM1740 and chemotherapeutic agents such Taxotere may have therapeutic advantages over a single regimen for the treatment of PCa bone metastasis.

In conclusion, our study shows that BKM1740 is a novel small acyl-tyrosine bisphosphonate amide analogue that specifically suppresses survivin expression and induces massive apoptosis in bone metastatic PCa cells. *In vivo* experiments validated its efficacy in inducing regression of pre-established bone metastatic PCa tumors without acute toxicity. Extensive studies of BKM1740 function and further development of its analogues could provide a novel therapeutic strategy for treating bone metastasis in PCa and other human cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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