Activation of the Wnt Pathway through AR79, a GSK3β Inhibitor, Promotes Prostate Cancer Growth in Soft Tissue and Bone

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Abstract

Due to its bone anabolic activity, methods to increase Wnt activity, such as inhibitors of dickkopf-1 and sclerostin, are being clinically explored. Glycogen synthase kinase (GSK3β) inhibits Wnt signaling by inducing β-catenin degradation, and a GSK3β inhibitor, AR79, is being evaluated as an osteoanabolic agent. However, Wnt activation has the potential to promote tumor growth; therefore, the goal of this study was to determine if AR79 has an impact on the progression of prostate cancer. Prostate cancer tumors were established in subcutaneous and bone sites of mice followed by AR79 administration, and tumor growth, β-catenin activation, proliferation, and apoptosis were assessed. Additionally, prostate cancer and osteoblast cell lines were treated with AR79, and β-catenin status, proliferation (with β-catenin knockdown in some cases), and proportion of ALDH+CD133+ stem-like cells were determined. AR79 promoted prostate cancer tumor growth, decreased phospho-β-catenin, increased total and nuclear β-catenin, and increased tumor-induced bone remodeling. Additionally, AR79 treatment decreased caspase-3 and increased Ki67 expression in tumors and increased bone formation in normal mouse tibiae. Similarly, AR79 inhibited β-catenin phosphorylation, increased nuclear β-catenin accumulation in prostate cancer and osteoblast cell lines, and increased proliferation of prostate cancer cells in vitro through β-catenin. Furthermore, AR79 increased the ALDH+CD133+ cancer stem cell-like proportion of the prostate cancer cell lines. In conclusion, AR79, while being bone anabolic, promotes prostate cancer cell growth through Wnt pathway activation.

Implications: These data suggest that clinical application of pharmaceuticals that promote Wnt pathway activation should be used with caution as they may enhance tumor growth. Mol Cancer Res; 11(12); 1597–610. ©2013 AACR.

Introduction

Bone loss is a common pathology that accompanies multiple diseases. Therefore, extensive research efforts have been aimed toward minimizing bone loss and have resulted in therapies that effectively decrease bone loss through inhibition of bone resorption. In addition to minimizing bone resorption, methods to promote bone formation (i.e., bone anabolic activity) have received great attention but clinical therapeutics in this area lag behind antiresorptives (1). For example, in the United States, teriparatide (parathyroid hormone, PTH 1-34) is the only U.S. Food and Drug Administration (FDA)–approved bone anabolic agent. However, its use is limited to 2 years due to preclinical findings of osteosarcoma in the rat model. Thus, continued efforts to identify bone anabolic agents are necessary to enhance efficacy and minimize their potential.

The Wnt pathway mediates bone development (2) and thus its manipulation serves as a potential bone anabolic therapy. Wnts mediate signaling through binding cell surface transmembrane low-density lipoprotein receptor-related (LRP5/6) and frizzled proteins, which results in β-catenin nuclear translocation and target gene activation (3). In the absence of Wnts, β-catenin is degraded in the cytoplasm by interacting with a protein complex consisting of axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β). Axin and APC are scaffold proteins that facilitate binding of β-catenin to GSK3β. GSK3β then phosphorylates β-catenin, which targets it for ubiquitination and degradation. Due to the key role of GSK3β in degrading

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β-catenin, inhibiting its activity should result in increased β-catenin activity. Thus, GSK3β has been considered as a therapeutic target for induction of β-catenin activity, which could ultimately result in bone anabolic activity. Along these lines, other compounds that promote Wnt activity through blocking inhibitors of Wnt signaling, such as sclerostin and dickkopf-1 (DKK1; ref. 4), are being evaluated for their bone anabolic effects, including the use of an anticalcitolbody antibody in clinical trials (5).

Although induction of bone anabolic activity through increased Wnt pathway signaling has potential therapeutic benefit, a major concern that should be considered is the impact of chronic Wnt activation on cancer progression. Increased β-catenin expression has been associated with multiple cancers (6, 7). Salmon calcitonin, which mediates the impact of chronic Wnt activation on cancer progression.

Increased β-catenin expression has been associated with multiple cancers (6, 7). Salmon calcitonin, which mediates degradation of GSK3β (8) and has been used therapeutically for bone anabolic activity, has been associated with increased cancer incidence, leading advisory panels of both the FDA to European regulatory committees to recommend discontinuing its use for osteoporosis. The majority of Wnt cancer-related action has been associated with early tumorigenesis. However, Wnt signaling may also play a role in established tumors, including maintenance of cancer stem cells (CSC; ref. 9). We have previously shown that modulation of the Wnt pathway with Wnt inhibitors, such as DKK1, promotes prostate cancer growth in soft tissue and bone (10). Because of the potential for modulation of the Wnt pathway to promote cancer, it is critical to evaluate any Wnt pathway therapies for the impact on cancer biology.

AR79 (AstraZeneca) is a potent inhibitor of GSK3β that was developed as a potential bone anabolic agent. AR79 upregulates β-catenin and bone mineralization in cell assays and produces an increase in bone mass in vivo (11). As AR79 modulates the Wnt pathway, we sought to determine whether it could impact the progression of prostate cancer in soft tissue and bone.

Materials and Methods

Cell culture

Human prostate cancer cell lines DU145 and PC3 were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 (Invitrogen Co.) supplemented with 10% FBS and 1% penicillin–streptomycin (Life Technologies). The C4-2B cell line, which is an LNCaP subline (kindly provided by Dr. Leland Chung, Cedars Sinai, Hollywood, CA), was maintained in T medium (80% Dulbecco’s Modified Eagle Medium (Life Technologies), 20% F12 (Invitrogen), 100 U/L penicillin G, 100 Ag/mL streptomycin, 5 µg/mL insulin, 13.6 pg/mL triiodothyronine, 5 µg/mL transferrin, 0.25 µg/mL biotin, and 25 µg/mL adenine) supplemented with 10% FBS. The human colorectal adenocarcinoma cell line HCT116 was purchased from ATCC and maintained in McCoy’s 5a Medium (Gibco Technology) supplemented with 10% heat-inactivated FBS (HyClone), 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen Life Technologies), 2 mmol/L L-glutamine (Invitrogen). The MC3T3-E1 (clone MC-4) cell line (kindly provided by Dr. Renny Franceschi, University of Michigan, Ann Arbor, MI), a preosteoblast cell line derived from murine calvariae that, when treated with ascorbate, expresses osteoblast-specific markers and produces a mineralized matrix, was routinely maintained in Minimal Essential Medium Alpha (α-MEM) containing 10% FBS and 1% penicillin–streptomycin (Life Technologies). The ST2 cell line, a mouse bone marrow stromal cell line, was obtained from RIKEN Cell Bank and maintained in α-MEM (Invitrogen) supplemented with 10% FBS, 1% penicillin–streptomycin (Life Technologies), and 2 mmol/L L-glutamine (Invitrogen). All cultures were maintained at 37°C, 5% CO2, and 100% humidity. Luiferase containing variants of the prostate cancer cell lines were made as previously described (12). Briefly, C4-2B and DU145 were transduced with retrovirus encoding the luciferase gene and selected using G418. Stable expression of luciferase was confirmed using bioluminescent imaging (BLI). Cell identities were confirmed using short tandem repeat mapping (Supplementary Table S1).

siRNA transfection

C4-2B and DU145 were plated at a density of 5 × 10^5 on 100 mm plates and then transfected with 100 mmol/L of two different sequences of β-catenin siRNAs (Cell Signaling Technology; signalSilence β-catenin siRNAIII, 6225, 6238) or scrambled control siRNA (Cell Signaling Technology; signalSilence control siRNA, 6568) using Lipofectamine RNAiMAX Reagent (Invitrogen, 13778). Transfection conditions were adjusted according to the manufacturer’s guidelines. After transfection for 72 hours, the cells were treated with AR79 (3 µg/mL) and rhWnt3a (60 ng/mL; R&D Systems) for 4 hours. Nuclear and cytoplasmic protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, 78835) following the manufacturer’s instructions.

Cell viability assay

DU145 and C4-2B cells and cells transfected with β-catenin siRNA were cultured in 96-well plates for overnight and then cells were treated with AR79 (3 µg/mL) and rhWnt3a (60 ng/mL; R&D Systems) and PBS for 24, 48, and 72 hours. Cell proliferation reagent WST-1 (Roche, 1644807) was added and incubated at 37°C and 5% CO2 for 4 hours. Absorbance was then measured at 440 nm with a plate reader (Multi-Mode Microplate Reader, SpectraMax M5, Molecular Devices; MDS Analytical Technologies).

TRACP 5b and osteocalcin assays

Whole blood was obtained and centrifuged to obtain serum that was frozen at −80°C until assayed. Mouse TRACP 5b (tartrate-resistant acid phosphatase 5b) and osteocalcin in mouse serum were measured using the Mouse-TRAP Assay (Immunodiagnostics Systems Inc.) and the Mouse Osteocalcin Assay (Biomedica Technologies Inc.), respectively, as recommended by the manufacturer.
Caspase-3/7 assay
C4-2B and DU145 cells were cultured into black 96-well plates at a concentration of 1 × 10⁴ (2 × 10⁵ cell/mL, 50 μL) per well. Cells were treated with 50 μL AR79 (0.03, 0.3, and 3 μg/mL) or vehicle (DMSO) or etoposide (100 nmol/L; Sigma) as a positive control after 12 hours and were allowed to grow for 24 hours, then 100 μL of Apo-ONE Caspase-3/7 Reagent (Promega) was added to each well and the cells were incubated for extended periods (>4 hours). Absorbance was determined at 499 and 521 nm using a plate reader (Multi-Mode Microplate Reader, SpectraMax M5, Molecular Devices; MDS Analytical Technologies).

Bioluminescence imaging in vitro
C4-2Bluc and DU145luc cells were plated into black, clear bottom 96-well plates (Costar) at a concentration of 3 × 10⁴ (for 24 and 48 hours test) or 2 × 10⁵ (for 72 hours test) per well in RPMI medium with 3% FBS. In some instances, after 12 hours, ST2 cells were added and plated with C4-2Bluc or DU145luc at a 1:6 or 1:3 ratio and cocultured for 12 hours. C4-2Bluc or DU145luc without ST2 was used as vehicle control group. The cells were treated with 0.03, 0.3, and 3 μg/mL AR79 and vehicle (DMSO), and were allowed to grow for 24, 48, and 72 hours, then 5 μL luciferin (40 mg/mL; Regis Technologies Inc.) was added to each well 5 to 10 minutes before imaging. BLI signal was quantified (IVIS System; Caliper Life Sciences). To determine the correlation between cell number and BLI signal, quantification was revealed using an HRP-conjugated anti-rabbit IgG (1:3,000; Cell Signaling Technology), or anti-mouse IgG (1:3,000; Amersham Pharmacia Biotech). Antibody complexes were detected using SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and exposure to X-Omat film (Kodak). The densities of the Western blot analysis bands were quantified using ImageJ software (version 1.40; NIH, Bethesda, MD).

Protein extraction and Western blot analysis
HT116, C4-2B, ST2, and MC3T3-E1 cell lines (1 × 10⁶ or 5 × 10⁵ cells/dish) were cultured in 100 mm dishes (1 × 10⁶ cells/dish for whole-cell protein, or 5 × 10⁵ for nuclear protein extraction). After 12 hours, the culture medium was changed to FBS-free medium for 12 hours and then the cells were treated with AR79 (0.03, 0.3, and 3 μg/mL) for 0, 15, 30, 60, and 120 minutes. Also, rhWnt3a (60 ng/mL) was treated with AR79 (0.03, 0.3, and 3 μg/mL) was allowed to grow for 24 hours, then 100 μL of Apo-ONE Caspase-3/7 Reagent (Promega) was added to each well and the cells were incubated for extended periods (>4 hours). Absorbance was determined at 499 and 521 nm using a plate reader (Multi-Mode Microplate Reader, SpectraMax M5, Molecular Devices; MDS Analytical Technologies).

HIPK2 assay
For immunoprecipitation of homeodomain-interacting protein kinase 2 (HIPK2), total protein (500 μg) was incubated with a polyclonal rabbit anti-human HIPK2 antibody (1 μL; Abcam) for a minimum of 1 hour at 4°C. Antibody-bound protein complexes were recovered via binding to mixed 50 μL protein A and protein G-Sepharose beads (Millipore) and subsequently incubated overnight at 4°C with gentle agitation. Afterward, the sepharose-bound immunoprecipitates were centrifuged at 8,000 × g rpm for 2 minutes followed by several washing steps with immunoprecipitation buffer. Immune complexes were eluted from beads into 30 μL of PBS and subsequent heating to 95°C for 10 minutes. After centrifugation, the supernatant containing the immunoprecipitated proteins was separated by SDS-PAGE (8%) and blotted onto polyvinylidene difluoride membrane (Bio-Rad). After blocking for 1 hour in 5% bovine serum albumin in TBS-T (TBS containing 0.05% Tween 20), blots were incubated with monoclonal mouse anti-human phosphotyrosine antibody (1:1,000; Cell Signaling Technology) overnight at 4°C followed by detection of binding by anti-mouse IgG (1:3,000; Amersham Pharmacia Biotech). Antibody complexes were detected using SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and exposure to X-Omat film (Kodak). The densities of the Western blot analysis bands were quantified using ImageJ software (version 1.40; NIH, Bethesda, MD).

In vivo prostate cancer models
Seven-week-old male NOD.CB17-Prkdcscid/NcrCrl (nonobese diabetic/severe combined immunodeficient, NOD/SCID) mice (Charles River Laboratories) were used and housed under pathogen-free conditions. All experimental protocols were approved by the University of Michigan Animal Care and Use Committee (Ann Arbor, MI). For subcutaneous injection, single-cell suspensions (1 × 10⁶ cells) of C4-2Bluc cells in T medium were injected in the flank at 100 μL per site using a 27-G3/8-inch needle under anesthesia with 2.5% isoflurane/air. Single-cell suspensions (3 × 10⁷ cells) of DU145luc cells with Matrigel (BD Biosciences) were injected in the flank at 100 μL per site using a 27-G3/8-inch needle. Subcutaneous tumor growth was monitored by palpation. Subcutaneous tumors were measured using BLI weekly. The tumor weights of the DU145luc injection group were measured at the end of the study. Animals in the C4-2Bluc injection group were measured using ImageJ software (version 1.40; NIH, Bethesda, MD).

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sacrificed at the end of 6 weeks and animals in the DU145luc injection group were sacrificed at the end of 9 weeks when tumors were harvested. Half of each tumor was kept for histologic analysis and the other half flash-frozen for molecular analysis. For intratibial injection, mice were anesthetized with 2.5% isoflurane/air, and both legs were cleaned with betadine and 70% ethanol. The knee was flexed, and a 27-G/3/8-inch needle was inserted into the proximal end of right tibia followed by injection of 20 μL single-cell suspensions of C4-2Bluc cells (5 × 10^5 cells). Tumor development in bone was evaluated weekly using BLI (methodology described in BLI section below) and radiography. All animals were sacrificed at the end of 9 weeks. At the time of euthanasia, serum was collected and necropsy was performed to evaluate for gross tumor lesions. Both tumor-injected and contralateral tibiae were collected, placed in formalin, radiographed and subject to dual-energy X-ray absorptiometry (DEXA), followed by processing for histologic analysis.

Immunohistochemistry
Both tibiae and subcutaneous tumors were fixed in 10% neutral buffered formalin for 24 hours. The tibiae were then decalcified for 48 hours in 10% EDTA and then both tibiae and subcutaneous tumors were processed for paraffin embedding. Of note, 5 μm sections were used for Hematoxylin and eosin staining and immunohistochemistry. Nonstained sections were deparaffinized and rehydrated then stained for Ki67 (1:200; Santa Cruz Biotechnology), caspase-3 (1:200; Cell Signaling Technology), and β-catenin (1:500; BD Biosciences).

Bioluminescence imaging in vivo
Mice were injected intraperitoneally with 100 μL luciferin (40 mg/mL in PBS), anesthetized with 1.5% isoflurane, and imaged 15 minutes after luciferin injection on the IVIS BLI system (Caliper Life Sciences) as previously described (14). Mice were imaged weekly after tumor injection to monitor tumor development. Bioluminescence generated by the luciferin/luciferase reaction served as a locator for cancer growth and was used for quantification using the Living Image software on a red (high intensity/cell number) to blue (low intensity/cell number) visual scale. A digital grayscale animal image was acquired followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photon counts emerging from active luciferase within the animal. Signal intensity was quantified as the sum of all detected photons within the region of interest during a 1-minute luminescent integration time.

Dual-energy X-ray absorptiometry measurement
Bone mineral content (BMC) and bone mineral density (BMD) of the excised tibiae were measured using DEXA on an Eclipse peripheral DEXA as previously described (13). Briefly, excised bones were subject to DEXA using pDEXA Sabre software version 3.9.4 in research mode (Norland Medical Systems). Bones were scanned at 2 mm/s with a resolution of 0.1 × 0.1 mm. Three 0.5 cm regions of interest were randomly selected for each fragment to determine BMC and BMD. Short-term precision (percentage of coefficient of variation) was approximately 3% for this technique.

Radiographic analysis
Magnified flat radiographs of hind limbs were obtained using a Faxitron machine (Faxitron X-ray Corp.).

Flow cytometry analysis of ALDH and CD133 expression
C4-2B and DU145 cells were dissociated with Accutase (Innovative Cell Technologies, Inc.) for aldehyde dehydrogenase (ALDH) staining, cells were subjected to the ALDEFLUOR Assay Kit (STEMCELL Technologies, Inc.) according to the manufacturer’s protocol. For CD133 staining, the cells were incubated with CD133/1 (AC133)-PE antibody (Miltenyi Biotec). One microgram per milliliter of 7-AAD (Sigma-Aldrich) was added to the samples before flow analysis to facilitate dead cell discrimination. Samples were analyzed on MoFlo XDP flow cytometer (Beckman Coulter, Inc.).

AR79 treatment in vivo
All animal studies were approved by the University of Michigan Animal Use and Care Committee. AR79 (AstraZeneca) was used at 15 and 50 mg/kg orally once daily based on in vivo studies in mice that demonstrated its inhibited GSK3 activity and increased bone mass in femurs (11). AR79 was formulated fresh daily in 0.5% hydroxypropylmethyl cellulose (HPMC; Sigma-H7509) with 0.1% Tween 80 (Sigma-P8074) in water. Vehicle consisted of the carrier (0.5% HPMC/0.1% Tween 80 in water).

Statistical analyses
All in vivo experiments were performed at least three times. Numerical data are expressed as mean ± SD. Statistical analysis was performed by analysis of one-way ANOVA and/or Student t-test for independent analysis. The value *P* < 0.05 was considered statistically significant.

Results
To determine whether Wnt activation promotes prostate cancer cell growth in soft tissue in vivo, we injected C4-2B cells into the subcutaneous tissue of mice and treated the mice with either vehicle or AR79 using low (15 mg/kg) and high (50 mg/kg) doses. Both doses of AR79 promoted prostate cancer growth over a 42-day period (Fig. 1A and B). We also evaluated Du145 prostate cancer cells using AR79 (50 mg/kg). Similar to the C4-2B cells, AR79 induced Du145 cell growth (Fig. 1C–F). In agreement with the BLI findings, individual tumor weights were greater in the Du145 tumors from mice that received AR79 (Fig. 1E and F). Mouse body weights were not impacted by treatment (Supplementary Fig. 3I).

In the basal state, GSK3 promotes phosphorylation and degradation of β-catenin. When Wnts are present, β-catenin dissociates from the GSK3 complex and is not phosphorylated, thus avoiding degradation resulting in increased β-catenin levels. AR79 mediates its activity through inhibiting
GSK3, which results in decreased β-catenin phosphorylation and increased nuclear β-catenin. Consequently, we next assessed whether AR79 impacted β-catenin expression in tumor tissues by measuring total and phospho-β-catenin. AR79 increased β-catenin in both C4-2B (Fig. 2A and B) and Du145 cells (Fig. 2D and E) and decreased total phospho-β-catenin expression in both C4-2B (Fig. 2A and C) and Du145 (Fig. 2D and F) cells. We evaluated additional prostate cancer cell lines (PC-3, ARCaP, and LNCaP) and in all instances, AR79 reduced phospho-β-catenin and increased total β-catenin (Supplementary Fig. S2).

In addition to impacting Wnt signaling, in vitro chemical pharmacology screening studies with AR79 have shown that this compound also inhibits HIPK2 with equipotency compared with GSK3β (11). To determine whether AR79 impacted HIPK2 in prostate cancer, we exposed multiple prostate cancer cell lines to AR79 and measured changes in phospho-HIPK2 expression. AR79 had no impact on phospho-HIPK2 expression, whereas it decreased GSK3 and phospho-β-catenin and increased total β-catenin (Supplementary Fig. S2).

To determine whether the observed effects of Wnt activation on tumor in soft tissue also extended to bone, the most common site of prostate cancer metastases, we injected C4-2B cells into the tibiae of mice and treated the mice with either vehicle or AR79. Similar to tumors in soft tissue sites, AR79 at both 15 and 50 mg/kg induced C4-2B tumor growth in bone (Fig. 3A and B). The increased tumor growth was accompanied by increased tumor-induced radiographic changes in the tibiae (Fig. 3C). AR79 in nontumor-bearing tibiae had no impact on the radiographic appearance of the tibiae (Supplementary Fig. S3A). To better define the impact of AR79 on tumor-induced bone changes, we subjected the tibiae to DEXA scan to measure bone mineral changes. The administration of AR79 had no impact on BMC but was associated with decreased BMC in the tibiae containing tumors (Fig. 3D and E), indicating an overall loss of bone. In contrast, AR79 (50 mg/kg) induced an increase in both

Figure 1. AR79 promotes prostate cancer cell growth in soft tissue in vivo. NOD/SCID mice were injected subcutaneously (SQ) with C4-2Bluc (1 × 10⁶, in 100 μL T medium) and DU145luc (3 × 10⁵ with Matrigel, in 100 μL RPMI). After establishment of tumor growth, treatment with either AR79 or vehicle was initiated and continued 6 weeks for C4-2Bluc and 9 weeks for DU145luc. Mice were imaged weekly using BLI after tumor injection to monitor tumor development. There were 15 NOD/SCID mice in the C4-2Bluc injection group (50 mg/kg of AR79, n = 5; 15 mg/kg of AR79, n = 5; vehicle, n = 5) and 20 NOD/SCID mice in the DU145luc injection group (50 mg/kg of AR79, n = 10; vehicle, n = 10). A and C, representative images of BLI. Note the increased signals in the AR79-treated SQ implanted compared with vehicle-treated mice. B and D, quantification of tumor volume as measured using BLI. *, P < 0.05 versus vehicle. E, pictures of the SQ DU145luc tumors. F, tumor weight of the DU145luc injection group.
BMD and BMC in tibiae without tumor (Supplementary Fig. S3B and S3C), indicating an increase in bone mineral within the bone itself. The AR79-induced tumor-mediated bone loss was associated with an overall increase of serum TRACP 5b, a marker of osteoclast activity (Fig. 3F), and had no impact on serum osteocalcin, a marker of bone turnover (Fig. 3G).

To explore cellular mechanisms that could account for AR79-mediated promotion of tumor growth, we assessed the tumor tissues for the expression of Ki67, a marker of cellular proliferation; caspase-3, a marker of apoptosis; and nuclear β-catenin. In both the C4-2B and Du145 subcutaneous tumors and the C4-2B intratibial tumors, the administration of AR79 increased Ki67 and decreased caspase-3, indicating that it overall promoted tumor growth through both increased proliferation and decreased apoptosis (Fig. 4A and B). In addition, AR79 induced nuclear β-catenin expression in all of these tumors, indicating that the Wnt pathway was activated (Fig. 4A and B).

The in vivo results suggested that AR79 stimulated proliferation of prostate cancer cells. To determine whether there was a direct effect of AR79 on prostate cancer or whether bone stroma was required for the proliferative effect, we exposed prostate cancer cells alone or in coculture with ST2 stromal cells. AR79 at 0.03 and 0.3 mg/mL had no impact; whereas, at 3.0 mg/mL induced C4-2B cell growth nearly 100% in vitro at both 48 and 72 hours (Fig. 5); but had no effect at any dose at 24 hours (not shown). The addition of ST2 cells at ratios of 3:1 or 6:1 (ST2:C4-2B) in the culture had no impact on cell number or AR79 effects (Fig. 5). To ensure that the effect of AR79 was not specific to C4-2B cells, the study was repeated with Du145 cells. Similar to the results observed with C4-2B cells, AR79 promoted Du145 cell growth (albeit only at 0.3 μg/mL) after 48 and 72 hours (Supplementary Fig. S4), but had no impact at 24 hours (not shown). These results confirm that AR79 directly induces prostate cancer growth.

We next determined whether the impact of AR79 on the Wnt pathway observed in vivo extended to C4-2B prostate cancer cells and the ST2 and MC3T3-E1 bone stromal cell lines in vitro. AR79 (3 mg/mL, but not lower doses) decreased phospho-β-catenin and increased total β-catenin in all cell lines evaluated, including the colon cancer HCT116 cell line that was used as a positive control (Fig. 6A). This effect was not observed until 60 minutes after treatment and continued through at least 120 minutes after treatment (Fig. 6B). A corresponding increase in nuclear β-catenin was observed in all cell lines evaluated, which was similar to the increase observed when the cells were treated with Wnt3a as a positive control (Fig. 6C and D; see NE for nuclear extracts).

As kinase inhibitors can have off-target effects, we further evaluated whether the proliferation-inducing activity of AR79 was dependent on upregulation of β-catenin. We used two different siRNA constructs to knockdown β-catenin expression in Du145 and C4-2B cells. Both constructs mediated more than 80% decreased in β-catenin protein expression in both cell lines compared with scrambled control (Supplementary Fig. S5). To ensure that knockdown was functional during administration of AR79, we assessed
the expression of nuclear β-catenin in the siRNA transfected cells. Administration of Wnt3a or AR79 increased nuclear β-catenin in the scrambled siRNA control cells lines compared with vehicle-treated cells; whereas neither Wnt3a nor AR79 increased nuclear β-catenin in the β-catenin siRNA cells compared with vehicle (Supplementary Fig. S3B). These results demonstrate that knockdown of β-catenin abrogates the ability of AR79 to increase nuclear β-catenin. To next assess whether blocking the ability of AR79 to induce β-catenin expression impacts its ability to promote prostate cancer proliferation, we evaluated the effect of AR79 on the cells with knockdown of β-catenin. At 24 hours, neither Wnt3a nor AR79 affected cell growth, as previously observed; however, at 48 and 72 hours, both Wnt3a and AR79 induced cell growth in the parental and scrambled siRNA cell lines and this effect was completely inhibited by knockdown of β-catenin (Fig. 7).

Wnts have been shown to promote maintenance of CSC-like cells (15–17; also called tumor initiating cells), indicating that modulation of Wnt activity may impact CSC. The definition of prostate cancer CSC is constantly being refined; however, the presence of positive staining for ALDH (18, 19) and CD133 (20, 21) has been associated with CSC-like phenotypes. Therefore, to determine whether AR79 modulated stemness of the prostate cancer cell population, we assessed for the impact of AR79 on the proportion of ALDH+/CD133+ cells in the C4-2B and Du145 populations. AR79 increased the proportion of ALDH+/CD133+ cells by approximately 100% and 200% in C4-2B (Fig. 8A and C) and Du145 (Fig. 8B and D), respectively.
Discussion

Induction of bone anabolic activity is a major therapeutic goal to diminish bone mineral loss. Toward that end, systemic administration of PTH (22, 23) and local administration of bone morphogenetic proteins with bone implants (24) have been used for their bone anabolic properties. However, because these therapies have limitations, including label warnings of cancer, there is a need to identify additional bone anabolic agents. In this light, manipulation of the Wnt pathway is being explored as a potential therapy due to the ability of Wnts to induce bone formation.

Wnt signaling plays a central role in osteoblast development and bone formation (25–27). Wnts promote the lineage commitment of mesenchymal precursor cells and the differentiation of progenitor cell lines into osteoblasts. They also stimulate direct effects on the formation and turnover of the mature skeleton. Mutations in components of the Wnt pathway are associated with human skeletal diseases and variation of BMD (28–31), demonstrating the functional relevance of Wnt pathway for human bone biology.

Canonical Wnt signaling is mediated through increasing nuclear β-catenin levels (3). GSK3β is a key regulator
of Wnt signaling through its ability to phosphorylate and target β-catenin for ubiquitination and degradation. Thus, inhibition of GSK3β is considered to be a viable and promising strategy to increase Wnt pathway activity through promotion of nuclear β-catenin expression. In agreement with the role of Wnts on bone production and inhibition of GSK3β on Wnt signaling, an orally available GSK3β inhibitor was shown to increase bone mass in rats (32). AR79 was developed to block GSK3β activity and hence induce bone anabolic activity as demonstrated by the stimulation of osteoblastogenesis with AR79 in vitro and a bone anabolic phenotype in rodents in vivo (11). However, before clinical evaluation of its bone anabolic potential, it was deemed important to determine its impact on prostate cancer, which is known to be influenced by Wnt signaling (33, 34).

Wnts may play a role in promotion of prostate cancer progression through several mechanisms. For example, Wnts, through β-catenin activation, have been shown to influence expression of the androgen receptor, a key regulator of prostate cancer (35). Furthermore, GSK3β has been shown to regulate the progression of prostate cancer to the advanced castration resistant form (36). In addition, it has been suggested that the activation of GSK3β may inhibit the proliferative effects of Wnt and β-catenin in prostate cancer (36). We have observed that as prostate cancer progresses to the bone metastatic phenotype, expression of the endogenous Wnt inhibitor DKK1 decreases, allowing for enhanced Wnt activity (37). This in turn can account for the osteoblastic phenotype of prostate cancer as demonstrated by the observation that the prostate cancer mediates bone production through activation of the Wnt receptor LRP5 (38). Taken together, these findings indicate that any therapy that modulates Wnt signaling could have an impact on prostate cancer progression.

Our results clearly demonstrate that AR79 promoted prostate cancer growth in soft tissue and bone in a murine model of prostate cancer. Furthermore, the increase of prostate cancer growth was associated with decreased phospho-β-catenin expression and increased total β-catenin expression. In addition, in vitro exposure of prostate cancer cells to AR79 decreased phospho-β-catenin and increased

Figure 5. AR79 increases numbers of C4-2B<sup>lac</sup> cells in the presence or absence of ST2 coculture. C4-2B<sup>lac</sup> cells were grown alone or cocultured with ST2 at 6:1 and 3:1. Then cells were treated with different concentrations (0.03, 0.3, and 3 μg/mL) of AR79 or vehicle (DMSO). Luciferase activity was measured after 24 (not shown), 48, and 72 hours by BLI. Data are presented as the mean ± SD from triplicate determinations.
nuclear β-catenin, providing further evidence that AR79 effectively activates the Wnt pathway. Taken together, these results suggest that AR79 promotes prostate cancer growth through activation of the canonical Wnt pathway due to its ability to block GSK3β activity. Our findings seem to conflict with an earlier report in which the GSK3 inhibitor CHIR99021 decreased growth of the 22Rv1 human prostate cancer cell line in vitro (39). However, differences in cell lines and compounds could account for differences. In addition, that report did not evaluate the in vivo impact of CHIR99021, which is critical for a thorough assessment due to the importance of microenvironment on cancer growth.

To determine cellular mechanisms through which AR79 promoted prostate cancer growth, we evaluated Ki67 expression, a marker of proliferation, and caspase-3/7, markers of apoptosis. The fact that AR79 administration was associated with both an increase of Ki67 and a decrease of caspase-3/7 expression in the tumor tissue indicated that it promoted overall tumor growth through both induction of cellular proliferation and inhibition of apoptosis. This finding is consistent with activation of the Wnt pathway, as Wnts are known to induce proliferation and inhibit apoptosis in a variety of cell systems (40). It should be pointed out that we measured these parameters only at the time of sacrifice; thus, this may not have been the optimal time to see the maximal changes. To further identify whether AR79 modified prostate cancer cell proliferation and apoptosis directly, we evaluated its impact on prostate cancer cells in vitro. However, we only identified an increase in cell proliferation and no impact on apoptosis. This finding suggests that AR79 modulates apoptosis in vivo through an indirect mechanism in the tumors. However, it may be that the basal...
apoptosis rate in a two-dimensional culture \textit{in vitro}, which is well oxygenated, is much lower than that in a three-dimensional tumor \textit{in vivo}, which will have areas of hypoxia and presumably more endogenous apoptosis. Thus, it is possible we could not identify an antiapoptotic effect \textit{in vitro} due to differences in the growth environment of the prostate cancer cells.

Because \textit{in vitro} pharmacology studies with AR79 indicated that this compound also inhibits HIPK2 with equipotency compared with GSK3\textbeta (11), we wanted to determine whether off-target effects of AR79 on HIPK2 could account for the impact on growth. HIPK2 is known to act as a tumor suppressor through activation of p53, which both (i) mediates induction of apoptosis (41) and (ii) inhibits the wnt/\beta-catenin pathway through miRNA-34 (42). Therefore, HIPK2 may have contributed to some of the responses observed in prostate cancer studies with AR79. However, our results indicated that AR79 had no impact on HIPK2 activation in prostate cancer cells. These data indicate that the response observed was not due to AR79 activity on HIPK2. Furthermore, the studies demonstrating that AR79 lost its ability to induce prostate cancer cell growth \textit{in vitro} upon knockdown of \beta-catenin provides strong evidence that AR79 mediates its proliferative effect through activation of canonical Wnt signaling. The mechanism through which Wnt pathway activation mediates prostate cancer cell growth is not clear at this time; however, it has been previously demonstrated that there is cross-talk between the Wnt pathway and the androgen receptor axis in prostate cancer (43, 44), which could contribute to the growth response.

In addition to a proproliferative effect, AR79 increased the proportion of CSC-like cells in two of the prostate cancer cell lines. This is consistent with the ability of Wnts to promote normal and CSC self-renewal and pluripotency (15–17, 45). This finding underscores that therapies that promote Wnt signaling could result in enhancing CSC-like activity. As CSCs are considered gain resistance to immunosurveillance (46), gain resistance to apoptosis and chemotherapy (47, 48), and have diminished induction of senescence (49), they may establish a nidus of cancer recurrence. Thus, therapies that result in the overall promotion of Wnt pathway activity
should be carefully considered before their use. A caveat to this concern is that it is not absolutely clear that the ALDH⁺/CD133⁺ cell population that was increased are definitively CSCs. Although there are reports that indicate that cells with these markers contribute to the CSC-like cell population (18–21), there are also indications that these markers may indicate the ability for these cells to have a greater proliferative potential as opposed to clear stem cell properties (50, 51). Even if these markers represent more of a proliferative capability as opposed to a clear CSC-like phenotype, the ability of AR79 to increase this component of the population would still be a major concern as they could contribute to increased tumor growth.

A key reason for the development of AR79 was to promote bone anabolic activity through activation of the canonical Wnt pathway. Along these lines, AR79 increased both BMD and BMC in normal bone, indicating an overall increase in bone production. Furthermore, AR79 activated the Wnt signaling pathway in osteoblast-like cells based on its ability to decrease phospho-β-catenin and increase total and nuclear β-catenin. Taken together, these results indicate that AR79 is an effective bone anabolic agent. However, in apparent contrast with its ability to induce bone production, the administration of AR79 decreased BMC in the bone containing tumor. The likely reason for this is that as AR79 induced prostate cancer cell growth, tumor-associated bone destruction occurred. This was evident from radiographs and paralleled by the measureable decrease in BMC.

A limitation of these experiments was the use of subcutaneous injection to model soft tissue growth. Ideally, orthotopic injection may have provided a model that more closely recapitulates human prostate cancer than subcutaneous injection. A challenge to orthotopic injection in the murine model is the presence of four biologically different and anatomically separated lobes of the prostate. Another limitation to this model is that the cell lines used do not completely recapitulate the osteoblastic bone.

Figure 8. AR79 increases the proportion of ALDH⁺/CD133⁺ cells in C4-2Bluc and DU145luc cell lines. C4-2Bluc and DU145luc (5 x 10⁶) were seeded into 10 mm plates and treated with indicated concentrations of AR79 or vehicle (DMSO) for 48 hours. Cells were dissociated with Accutase and flow cytometric analysis was performed for ALDH and CD133. A, flow cytometric analysis showing ALDH and CD133 expression in C4-2Bluc. B, flow cytometric analysis showing ALDH and CD133 expression in DU145luc. C and D, proportion of ALDH⁺/CD133⁺ in C4-2Bluc and DU145luc, respectively. Data are shown as the mean ± SD from two independent experiments. *, P < 0.05 versus vehicle.
response that is typical of clinical prostate cancer. Specifically, C4-2B cells induce mixed osteoblastic/osteolytic lesions and Du145 cells produce osteolytic lesions. It is unknown at this time whether the type of bone response induced by prostate cancer could influence the activity of AR79.

In summary, this research identified that inhibition of GSK3β using AR79 had a bone anabolic effect, but also stimulated prostate cancer growth in vivo a murine model. These findings suggest that therapeutic agents that modulate the Wnt pathway should be used with caution, particularly in the presence of prostate cancer. It is likely these effects would extend to other cancers as Wnts play a role in multiple cancers.

Disclosure of Potential Conflicts of Interest

E.T. Keller has received commercial research grant from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

References


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