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Pomegranate Extract Inhibits the Bone Metastatic Growth of Human Prostate Cancer Cells and Enhances the In Vivo Efficacy of Docetaxel Chemotherapy

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BACKGROUND. Docetaxel treatment is the only first-line chemotherapy with a survival benefit in metastatic castration-resistant prostate cancer (PCa). Nonetheless, most patients become docetaxel resistant and inevitably progress with no cure. In this study, we investigated the potential of pomegranate extract (PE) in targeting metastatic castration-resistant PCa and improving docetaxel chemotherapy.

METHODS. The in vitro and in vivo effect of POMx, a PE formula currently approved for clinical trials, in metastatic castration-resistant PCa cells was evaluated in experimental models.

RESULTS. We demonstrated that POMx exhibited potent in vitro cytotoxicity in metastatic castration-resistant PCa cells. Mechanistic studies identified survivin as a novel molecular target that may mediate the anti-cancer activity of POMx, presumably through the inhibition of signal transducer and activator of transcription 3. The in vivo administration of POMx treatment effectively inhibited survivin, induced apoptosis, retarded C4-2 tumor growth in skeleton and significantly enhanced the efficacy of docetaxel in athymic nude mice.

CONCLUSION. These results provide the first preclinical evidence that POMx may be effective in treating metastatic castration-resistant PCa and enhancing the efficacy of docetaxel chemotherapy. *Prostate 74:497–508, 2014.*

KEY WORDS: **prostate cancer; bone metastasis; pomegranate extract; docetaxel; animal model**

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INTRODUCTION

Bone metastasis contributes directly to prostate cancer (PCa) mortality and morbidity. More than 85% of PCa patients show evidence of skeletal metastasis at autopsy, with a median survival between 30 and 35 months [1,2]. The quality of life of PCa patients can be significantly compromised by skeletal complications. Currently, docetaxel-based treatment is the only first-line chemotherapy with a survival benefit in metastatic castration-resistant PCa (mCRPC) [3,4]. Nonetheless, despite some success in transiently controlling the clinical symptoms, most patients become docetaxel-resistant and inevitably progress with no cure. Although several second-line drugs (cabazitaxel, abiraterone, sipuleucel-T) are available for the management of mCRPC, these therapies can only extend median survival by approximately 3–4 months, and at high costs. Clearly, it is urgent to develop safe, efficacious and cost-effective therapies that can overcome docetaxel resistance and treat mCRPC [5].

Clinical evidence has demonstrated great promise of pomegranate (*Punica granatum*) in the therapeutic intervention of PCa [6–9]. In a recent multi-center, double-blind phase II trial [9], PCa patients with rising prostate-specific antigen (PSA) and without metastases were treated until progression for 18 months with POMx, a pomegranate extract (PE) product (POM Wonderful, LLC). POMx treatment markedly increased the PSA doubling time (PSADT) by over 6 months in both treatment arms (1 and 3 g daily), with no clinically significant toxicities.

The unique expression pattern and function of survivin in human cancers have positioned it as an ideal target for rational drug development [10]. As a genuine "onco-fetal" gene, survivin intersects multiple signaling networks and plays a pivotal role in the regulation of cell death and mitotic process. Targeting survivin can interrupt the progression of tumors regardless of their genetic backgrounds and without compromising safety profile [11]. In PCa, survivin overexpression has frequently been associated with high Gleason scores, poor clinical outcome, and resistance to hormone therapy, chemotherapy and radiation therapy [12–15]. Recent studies from our group and others have indicated that survivin is a potential prognostic biomarker and promising therapeutic target in metastatic PCa [16–18]. Nonetheless, currently available small molecule inhibitors of survivin (such as YM155) have only demonstrated modest response in patients with localized and castration-resistant PCa [19,20]. Identification of a natural and potent suppressor of survivin could greatly advance the clinical development of survivin-based therapy for the treatment of lethal mCRPC.

The biological activity of PE in bone metastatic PCa has not been reported previously. By using an intraosseous model of mCRPC, we demonstrated that POMx can effectively induce tumor regression in the mouse skeleton and significantly potentiate the in vivo efficacy of docetaxel. We further identified a novel mechanism of action by which POMx inhibits survivin, induces apoptosis and antagonizes docetaxel resistance in bone metastatic PCa cells. These results provided the first preclinical evidence suggesting that PE could be effective in treating PCa bone metastasis and enhancing docetaxel chemotherapy.

MATERIALS AND METHODS

Cell Culture and Reagents

Human PCa cell lines PC3, C4-2 and $ARCaP_M$ were routinely maintained in T-medium (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS) [21]. PrEC, a normal human prostate epithelial cell line, was purchased from Lonza Walkersville, Inc. (Walkersville, MD) and maintained in Prostate Epithelial Cell Medium BulletKit™. POMx capsules were purchased from POM Wonderful, LLC (Los Angeles, CA), and dissolved in 50% dimethyl sulfoxide (DMSO) (Sigma–Aldrich, St. Louis, MO). Docetaxel (Taxotere®) was obtained from LC Laboratories (Woburn, MA).

Cell Proliferation Assay

Cell proliferation was measured using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation (MTS) Assay kit (Promega, Madison, WI) according to the manufacturer's instruction. For cell viability assay, 4×10^3 cells per well were seeded on 96-well plates overnight, and treated with POMx, docetaxel or vehicle control at the indicated concentrations for 72 hr. A microplate reader (Bio-Rad Laboratories, Hercules, CA) was used to determine cell viability, which was expressed as relative survival with controls recorded as 100%. Cell numbers were also counted with trypan blue exclusion assay (Trypan Blue solution, Sigma–Aldrich) in the specified experiments.

Western Blot Analysis

Total cell lysates were prepared using radioimmunoprecipitation (RIPA) buffer (Santa Cruz Biotechnology). Immunoblotting analysis followed a standard procedure. Antibodies against poly (ADP-ribose) polymerase (PARP), signal transducer and activator of transcription 3 (Stat3), phosphorylated Stat3 (p-Stat3) (Ser727), p-Stat3(Tyr705), total c-Src, p-Src Family (Tyr416) were purchased from Cell Signaling (Danvers,

MA); survivin antibody was purchased from Novus Biologicals (Littleton, CO); hypoxia-inducible factor-1α (HIF-1α) antibody was purchased from Update (Billerica, MA); antibodies against myeloid cell leukemia-1 (Mcl-1), extracellular-signal-regulated kinases 1/2 (ERK1/2) and phosphorylated ERK1/2 (p-ERK1/2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); β-actin antibody was purchased from Sigma–Aldrich; and TATA-binding subunit of TFIID (TBP) antibody was obtained from Abcam (Cambridge, MA). ImageJ program was used to quantitate protein densitometry in certain Western blots.

Quantitative ReverseTranscription PCR (qRT-PCR)

Total RNA was prepared with Qiagen RNeasy Kit (Valencia, CA). The first-strand cDNA was synthesized using SuperScript® III First-Strand Synthesis System (Invitrogen). Quantitative PCR was performed by the Stratagene Mx3005P system (Agilent technologies) using a Brilliant® SYBR® Green QPCR Master Mix (Stratagene, Santa Clara, CA) according to the manufacturer's instructions. The human survivin-specific primer pairs were: 5′-CCACCGCATCTCTACATTCA-3′ (forward) and 5′-GCACTTTCTTCGCAGTTTC-3′ (reverse). The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously [22].

Plasmids and Transfection

Expression vector for human survivin (kindly provided by Dr. Lily Yang) and the control vector pcDNA3 were transfected into PCa cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Reporter Assay

PCa cells were seeded at a density of 1×10^5 cells per well in 24-well plates 24 hr before transfection. Human survivin reporters (pSurvivin-Luc1430 and pSurvivin-Luc230; kindly provided by Dr. Allen Gao) were transfected with pRL-TK (internal control; Promega) as described previously. Luciferase activities were measured at the indicated times using a Dual-Luciferase reporter assay system (Promega). Relative luciferase units were expressed as firefly luciferase intensity normalized to *Renilla* luciferase activity.

Apoptosis Analysis

Cells treated with 50% DMSO or PE were trypsinized and washed with PBS and resuspended in Annexin-binding buffer (BD Pharmingen, San Diego, CA). 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, Franklin Lakes, NJ). The data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Animal Study

A total of 20 athymic male nude mice (BALB/c *nu/nu*; 6-week old; National Cancer Institute, Bethesda, MD) were used. For each mouse, 1.0×10^6 C4-2 cells were inoculated into bilateral tibia using our established procedure [16]. Blood specimens were obtained from the retro-orbital sinus vein every 2 weeks for serum PSA determination using an ELISA kit from United Biotech, Inc. (Mountain View, CA). Four weeks later, tumor formation was confirmed by rising PSA levels, and tumor-bearing mice were randomly divided into four groups: vehicle control group ($n = 4$, one mouse died during the week 4 following tumor inoculation), docetaxel group ($n = 5$), POMx group ($n = 5$), docetaxel, and POMx combination group ($n = 5$). Each group of mice received the following injection via the i.p. route for a 12-week period: vehicle control group: 50% DMSO, three times per week; docetaxel group: 5 mg/kg body weight of docetaxel, once per week; POMx group: 60 mg/kg of PE, three times per week; combination group: 5 mg/kg of docetaxel, once per week, and 60 mg/kg of POMx, three times per week. Mice were weighed every week and tumor growth in bilateral tibia was followed by serum PSA and X-ray with a Faxitron MX20 digital radiography system (Faxitron Bioptics, LLC; Tucson, AZ) every 2 weeks. At the endpoint, the bilateral tibia were removed, fixed in 10% neutralized formalin for 48 hr, and decalcified in EDTA (pH 7.2) for 15 days. Tibia specimens were dehydrated and paraffin embedded. All animal procedures were performed in compliance with Emory University Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health guidelines.

Immunohistochemistry (IHC) Assay

Expression of survivin, p-Stat3(Ser727) and p-Stat3 (Tyr705) in C4-2 skeletal tumor tissues were analyzed by IHC staining using rabbit polyclonal antibodies against survivin (Novus Biologicals; 1:300 dilution), p-Stat3(Ser727), and p-Stat3(Tyr705) (Cell Signaling Technology; 1:100 dilution). Briefly, tissues were deparaffinized, rehydrated, and subjected to 5-min pressure-cooking antigen retrieval, 10-min double

endogenous enzyme block, and overnight primary antibody incubation, and subjected to prediluted biotinylated pan-specific universal secondary antibody for 10 min. Signals were detected by adding 3,3′-diaminobenzidine (DAB) substrate hydrogen peroxide and counterstained by hematoxylin QS. All reagents were obtained from Vector Laboratories (Burlingame, CA). Positive expression was defined as >15% positive staining in cell population.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

TUNEL assay was performed according to the manufacturer's instructions (TUNEL Apoptosis Detection Kit, GenScript, Piscataway, NJ). The slides were routinely dewaxed, hydrated, then enzymatically digested with 20 μg/ml protease K for 30 min at room temperature. Slides then were washed in PBS and placed in 3% H_2O_2 in methanol for 10 min at room temperature. After washing in PBS, 50 μl TUNEL reaction mixture was added to the tissues and incubated for 60 min at 37°C. Slides were washed in PBS and 50 μl Streptavidin-HRP solution was added to the samples, incubated for 30 min at 37°C. After washing in PBS, DAB working solution was applied to the tissues for 3 min, then slides were routinely counterstained with hematoxylin and dehydrated for coverslipping with Permount.

Statistical Analysis

Treatment effects at specific time-points were evaluated using a two-sided Student's *t*-test at each measurement time-point. To assess the longitudinal effect of treatment, a mixed model was employed to test the overall difference across all groups as well as between each pair of groups during the whole study period. The significance levels were set at 0.05 for all tests. The SAS statistical package V9.2 (SAS Institute, Inc., Cary, NC) was used for data management and analysis.

RESULTS

POMx Exhibits In Vitro Cytotoxicity in Metastatic Castration-Resistant PCa Cells

We examined the in vitro effects of POMx in several established metastatic castration-resistant PCa cell lines that recapitulate the aggressive phenotypes of advanced PCa [23,24]. The half maximal inhibitory concentration (IC_{50}) of POMx was determined as 42, 78, and 161 μg/ml in C4-2, PC3, and $ARCaP_M$ cells, respectively (Fig. 1A, left panel). In comparison, PrEC, a normal human prostatic epithelial cell line, was much less sensitive to POMx treatment (Fig. 1A, right panel). FACS assay further showed that POMx effectively induced apoptosis in a dose-dependent manner in PCa cells (Fig. 1B). The inhibition of PCa cell viability was associated with increased cleavage of PARP and caspase 3 (Fig. 1C), two general indicators of an activated apoptotic process. These data indicate that POMx can effectively inhibit proliferation and induce apoptosis in metastatic castration-resistant PCa cells.

POMx Is an Inhibitor of Survivin in Metastatic Castration-Resistant PCa Cells

To understand the mechanism by which POMx activates apoptosis in PCa cells, we examined the effect of POMx on the expression of survivin and Mcl-1, two anti-apoptotic proteins positively associated with clinical PCa bone metastasis [16,18,25]. As shown in Figure 2A, POMx treatment resulted in a significant reduction of survivin protein in PCa cells, but only modestly affected Mcl-1expression. Quantitative PCR analysis further showed a rapid inhibition of survivin transcripts upon POMx treatment (Fig. 2B). These data indicate that survivin is a novel molecular target of POMx, whose inhibition may mediate the cytotoxicity of POMx in metastatic castration-resistant PCa cells. Supporting this hypothesis, ectopic expression of survivin effectively attenuated the inhibitory effect of POMx on the viability of C4-2 cells (Fig. 2C).

POMx Inactivates Stat3-Dependent Transcription of Survivin

Figure 2A and B suggested that the inhibition of survivin by POMx may primarily occur at the transcriptional level. Given the importance of Stat3 signaling in PCa bone metastasis and the regulation of survivin expression [26,27], we examined the effect of POMx on the activation status of Stat3 in PCa cells. As shown in Figure 3A, POMx treatment significantly reduced the nuclear levels of total Stat3 and p-Stat3 (Ser727) in all the tested PCa cell lines. Although the phosphorylation of Stat3 at Tyr705 was markedly inhibited in C4-2 and PC3 cells, it was only marginally affected by POMx in $ARCaP_M$ cells. In comparison, nuclear expression of HIF-1α, a transcription factor implicated in survivin regulation [28], was not significantly altered by POMx treatment. Interestingly, quantification of protein densitometry showed that in the total lysate samples, POMx reduced total Stat3 proteins in C4-2 cells, whereas slightly (in $ARCaP_M$) or significantly (in PC3 cells) increased total Stat3 at the time points of 4 or 6 hr (Table I). The inductive effect of POMx on Stat3 in PC3 total cell lysates is somewhat

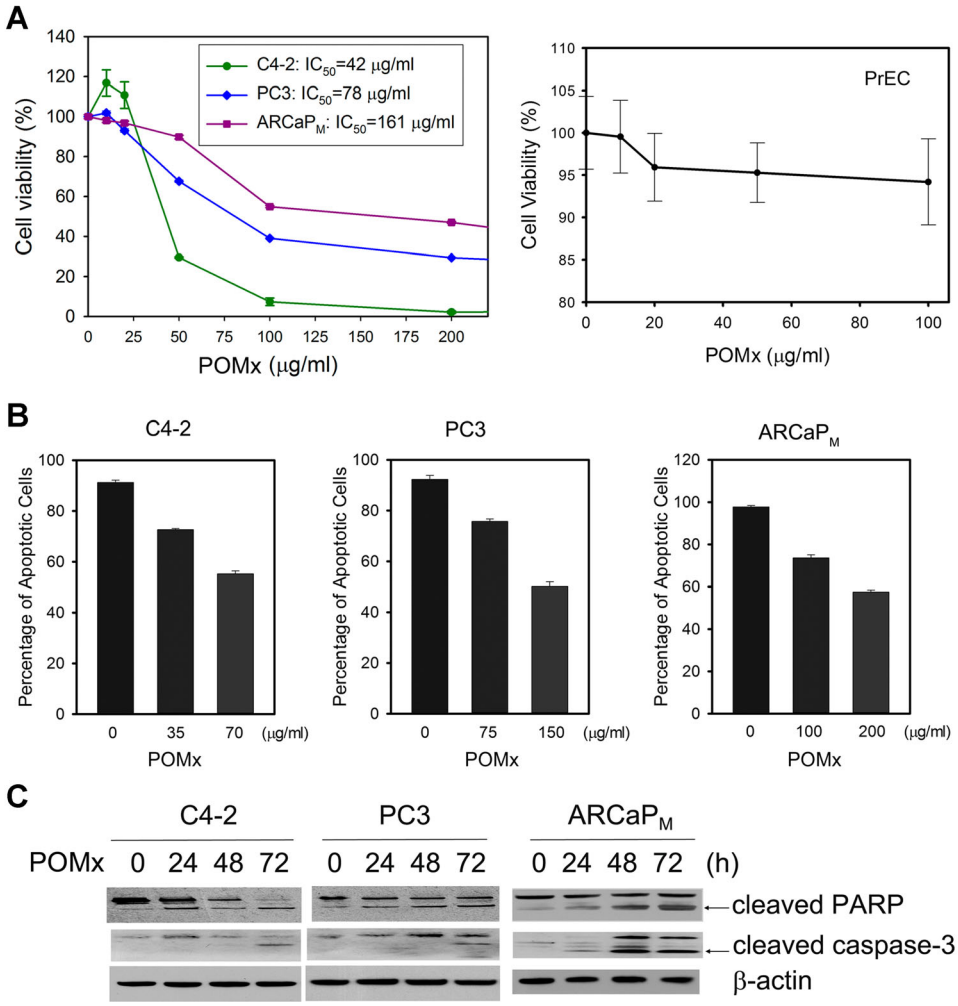

Fig. 1. POMx induces apoptosis in metastatic castration-resistant PCa cells. **A**: POMx treatment (72 hr) effectively inhibits the viability of metastatic castration-resistant PCa cells with IC_{50} ranging between 42 and 161 μg/ml. In comparison, PrEC is less sensitive to treatment with POMx. **B**: POMx treatment (48 hr) dose-dependently induces apoptosis in metastatic castration-resistant PCa cells. **C**: POMx treatment (72 hr) activates apoptotic signaling in metastatic castration-resistant PCa cells. Note different doses were used (C4-2: 35 μg/ml; PC3: 100 μg/ml; $ARCaP_M$: 150 μg/ml).

confusing, which may be partially due to the intrinsic semi-quantitative properties of Western blotting and densitometry-based analysis [29]. Nonetheless, these data indicated that POMx can inhibit the activity (phosphorylation) of Stat3 in these PCa cells.

Several *cis*-elements have been identified in the human survivin promoter, including two Stat3-binding sites (located between −1,174 to −1,166 and −1,095 to −1,087) and a hypoxia-responsive element (HRE) (located between −85 and −81) [28,30]. To further address the role of Stat3 in mediating the POMx inhibition of survivin transcription, we transiently transfected C4-2 cells with two human survivin luciferase reporters, that is, pSurvivin-Luc1430 containing both the Stat3 binding sites and HRE, and pSurvivin-Luc230, a mutant with the Stat3 sites deleted (Fig. 3B, left panel). The basal reporter activity of pSurvivin-Luc230 was significantly lower than that of pSurvivin-Luc1430, suggesting the importance of the Stat3-containing region. Consistently, POMx effectively inhibited the luciferase activity of pSurvivin-Luc1430, but not that of pSurvivin-Luc230 (Fig. 3B, right panel). These results indicate that Stat3 may mediate the inhibition of survivin transcription upon POMx treatment.

We further examined the effect of POMx on the activity of ERK1/2 and Src kinase family, which have been shown to mediate the phosphorylation of Stat3 at Ser727 and Tyr705, respectively [31–33]. POMx treatment rapidly inhibited the phosphorylation of ERK1/2 in all the tested PCa cell lines. In comparison, POMx significantly reduced phosphorylated Src kinases in

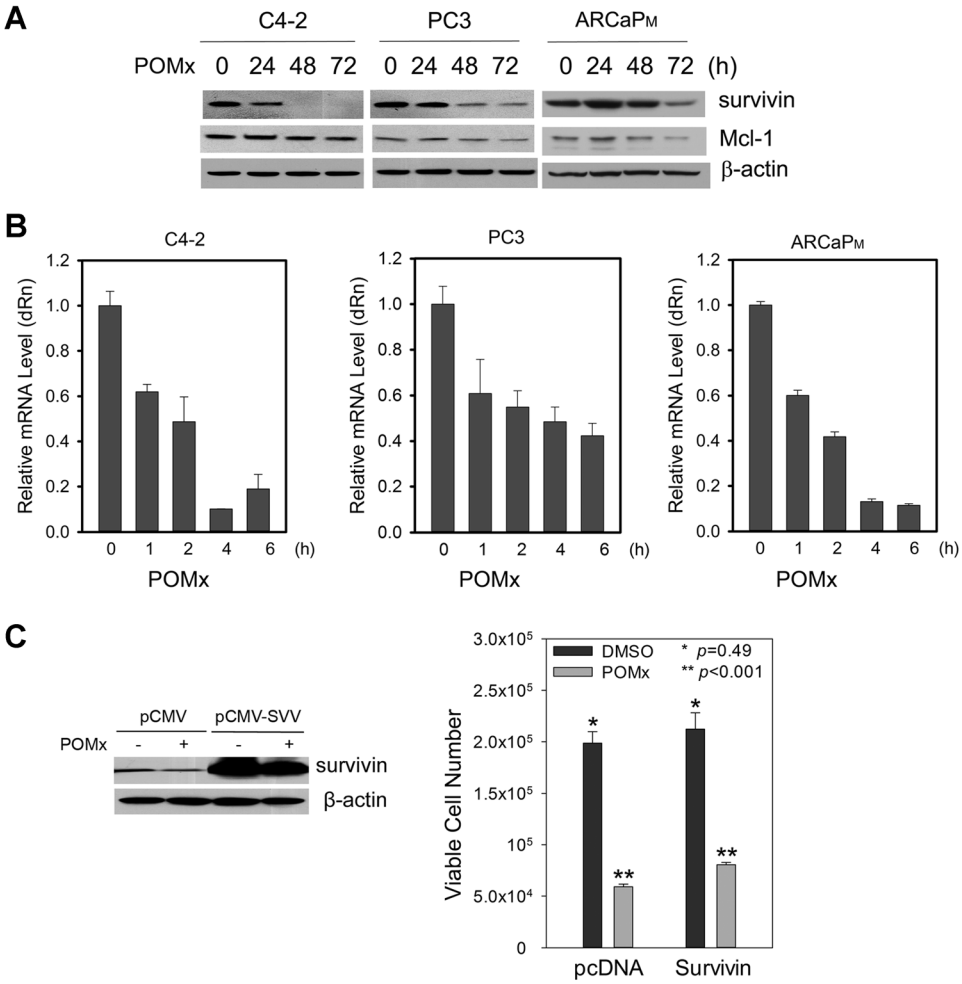

Fig. 2. POMx inhibits survivin expression in metastatic castration-resistant PCa cells. **A**: POMx effectively inhibits survivin protein in PCa cells. Total lysates from C4-2 and PC3 are the same as those in Figure IC. **B**: POMx rapidly inhibits survivin mRNA levels in a time-dependent manner. Note different doses were used (C4-2: 35 μg/ml; PC3: 100 μg/ml; ARCaP$_M$: 150 μg/ml). **C**: Ectopic expression of human survivin antagonizes the inhibitory effect of POMx on PCa cell viability. **Left**: Western blot analysis of survivin expression in C4-2 cells transfected with pcDNA-Survivin or control vector (2 days) and further treated with POMx (35 μg/ml, 2 days); **Right**: Trypan blue exclusion assay of viable C4-2 cells (n = 3).

C4-2 and PC3 cells, but to a lesser degree in ARCaP$_M$ cells (Fig. 3C).

POMx Antagonizes the Survivin-Inducing Effect of Docetaxel

Interestingly, treatment with low concentrations of docetaxel in PCa cells resulted in a temporary but striking increase in survivin protein, starting at as early as 4 hr, reaching a peak at 16 hr and returning to the baseline after 48 hr (Fig. 4A). On the other hand, POMx effectively attenuated the inductive effect of docetaxel on survivin protein and increased the cleavage of PARP and caspase-3 (Fig. 4B). These results indicate that POMx can antagonize docetaxel-induced survivin, activate apoptotic signaling and sensitize PCa cells to docetaxel treatment. Indeed, POMx significantly potentiated the in vitro cytotoxicity of docetaxel in all the tested cell lines (Fig. 4C), suggesting that the combination of POMx and docetaxel is more effective than docetaxel alone in inducing apoptosis in AI and metastatic PCa cells.

POMx Retards the Skeletal Growth of C4-2 Tumors and Enhances the In Vivo Efficacy of Docetaxel

We evaluated the in vivo efficacy of POMx in treating bone metastatic PCa and enhancing docetaxel response in an intraosseous model of C4-2, which expresses high levels of PSA that can be a surrogate marker for total tumor burden in mouse skeletons [16].

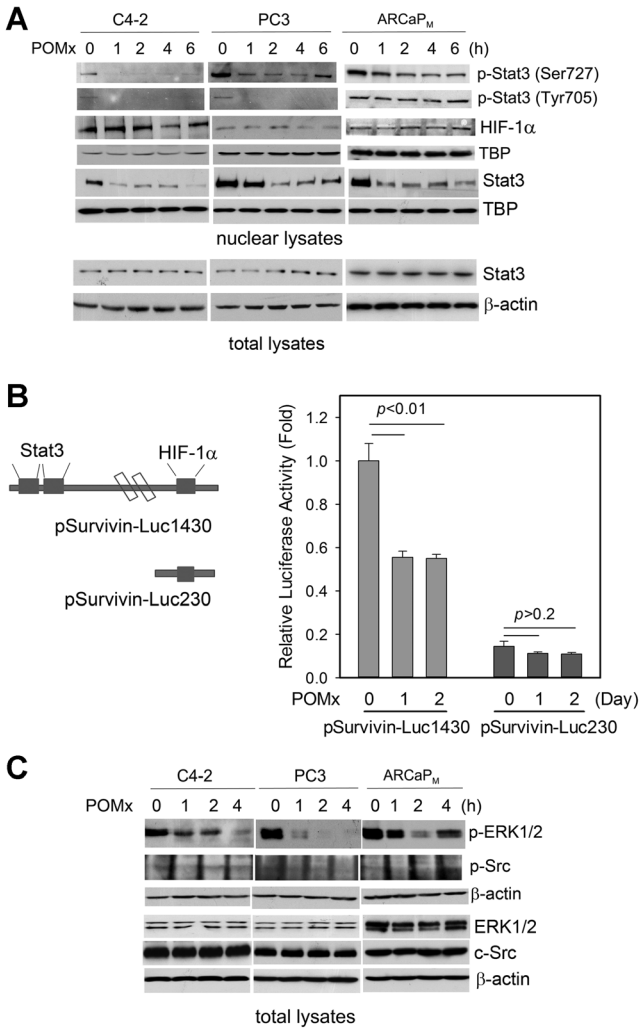

Fig. 3. POMx inhibits Stat3-dependent expression of survivin in metastatic castration-resistant PCa cells. **A**: POMx rapidly inhibits the phosphorylation of Stat3 at Ser727 and/or Tyr705 in a cell type-dependent manner. Note different doses were used (C4-2: 35 μg/ml; PC3: 100 μg/ml; ARCaP$_M$: 100 μg/ml). **B**: (**Left**) Human survivin promoter contains the *cis*-elements for binding Stat3 and HIF-1α. Note the two putative Stat3-binding motifs are absent in pSurvivin-Luc230. **Right**: POMx effectively inhibits the reporter activity of pSurvivin-Luc1430, whereas not significantly affecting that of pSurvivin-Luc230. C4-2 cells were transfected with reporter constructs and after 2 days treated with 35 μg/ml of POMx for the indicated times. pRL-TK was used as an internal control. **C**: POMx rapidly inhibits the phosphorylation of ERK1/2 and Src kinases in a cell type-dependent manner.

Four weeks after tumor inoculation, athymic nude mice bearing intratibial C4-2 xenografts were treated with vehicle control, docetaxel, POMx or the combination of POMx and docetaxel for 12 weeks. The average PSA level in each group at the endpoint was 60.53 ± 7.5 ng/ml (control), 54.06 ± 4.67 ng/ml (docetaxel), 40.4 ± 6.39 ng/ml (POMx), and 11.57 ± 4.12 ng/ml (POMx and docetaxel combination), respectively (Fig. 5A). Statistical analysis using a mixed model showed that there was a significant difference in PSA values among different treatment groups ($P = 0.0003$). Pairwise comparison in PSA levels over time between any two groups found that, compared with the control, low-dose docetaxel did not significantly inhibit C4-2 tumor growth in mouse bone ($P = 0.9168$). However, POMx alone ($P = 0.0438$) or the combination treatment ($P = 0.0003$) exhibited significant inhibitory effect on PSA levels during the whole experiment period. Moreover, the PSA values of the POMx group ($P = 0.0419$) and combination group ($P = 0.0002$) were significantly different from that of the docetaxel group. There was no significant difference in PSA levels between the POMx group and the combination group ($P = 0.084$). Consistently, X-ray radiography showed that, compared with the control group, C4-2 tumor-bearing bone treated with either POMx or the combination regimen displayed improved architecture with reduced osteolytic destruction and osteoblastic lesions (Fig. 5B), indicating an inhibitory effect of POMx or the combination treatment on PCa growth in mouse bones. Compared to the control group, no significant acute toxicity was observed in any of the treatment groups ($P = 0.1116$), as demonstrated by body weight (Fig. 5C).

POMx Inhibits Tissue Expression of Survivin and Induces Apoptosis in C4-2 Skeletal Tumor

The in vivo effects of the treatments on tissue expression of survivin and phosphorylated Stat3 were analyzed in C4-2 tumor specimens harvested at the specified endpoints (Fig. 6). Compared with the control group, docetaxel treatment resulted in a similar level of survivin expression but significantly reduced the phosphorylation of Stat3 at both Ser727 and Tyr705 sites. The immunoreactivity of survivin, p-Stat3 (Ser727) and p-Stat3(Tyr705) was dramatically decreased in C4-2 tumors receiving POMx or the combination regimen. Consistently, TUNEL staining was significantly higher in tumor tissues treated with docetaxel or POMx, and further increased in those from the combination group. These data indicate that POMx effectively inhibited Stat3-survivin signaling and induced apoptosis at the tissue level, which may contribute to the suppression of tumor growth in mouse bone and the enhanced efficacy of docetaxel chemotherapy.

DISCUSSION

Pomegranate has recently gained great interest for its clinical potential in the treatment of PCa [7,8]. In a randomized phase II trial in patients with recurrent

TABLE I. ImageJ Quantification of Protein Densitometry (Fig. 3A)

Time (hr)	0	1	2	4	6
Effect of POMx on Stat3 in total cell lysates (upper panel)					
C4-2					
pStat3(Ser)	1	−0.69645	−0.14058	0.302299	0.398898
pStat3(Tyr)	1	−6.30973	−0.56757	−0.50495	0.375149
Total Stat3	1	0.161211	0.325473	0.342945	0.086391
PC3					
pStat3(Ser)	1	0.360522	0.178382	0.100631	0.40471
pStat3(Tyr)	1	0.729301	−0.16755	0.220799	0.167384
Total Stat3	1	0.872059	0.207135	0.348418	0.498834
$ARCaP_M$					
pStat3(Ser)	1	0.695595	0.506489	0.386634	0.409768
pStat3(Tyr)	1	0.804647	0.975764	0.869942	1.202545
Total Stat3	1	0.320663	0.331071	0.43351	0.267999
Effect of POMx on Stat3 in total cell lysates (bottom panel)					
C4-2					
Total Stat3	1	1.082016	0.87286	0.747043	0.766163
PC3					
Total Stat3	1	0.631452	1.03862	1.460677	1.750568
$ARCaP_M$					
Total Stat3	1	0.990157	1.137296	1.074012	1.298142

and non-metastatic PCa, Paller et al. observed a significant inhibition of PCa progression following POMx treatment. The median PSADT lengthened in the low-dose group from 11.9 to 18.8 months and from 12.2 to 17.5 months in the high-dose group, with no significant difference between dose groups [9]. Although a large-scale clinical trial still lacks, these encouraging results demonstrate the promise of POMx as an efficacious and safe remedy in the management of localized PCa. In this report, we further evaluated the in vivo effects of POMx on the growth of mCRPC in an intraosseous model. To our knowledge, this is the first preclinical evidence demonstrating that POMx can effectively inhibit tumor growth and significantly enhance the in vivo efficacy of docetaxel in experimental models of bone metastatic PCa.

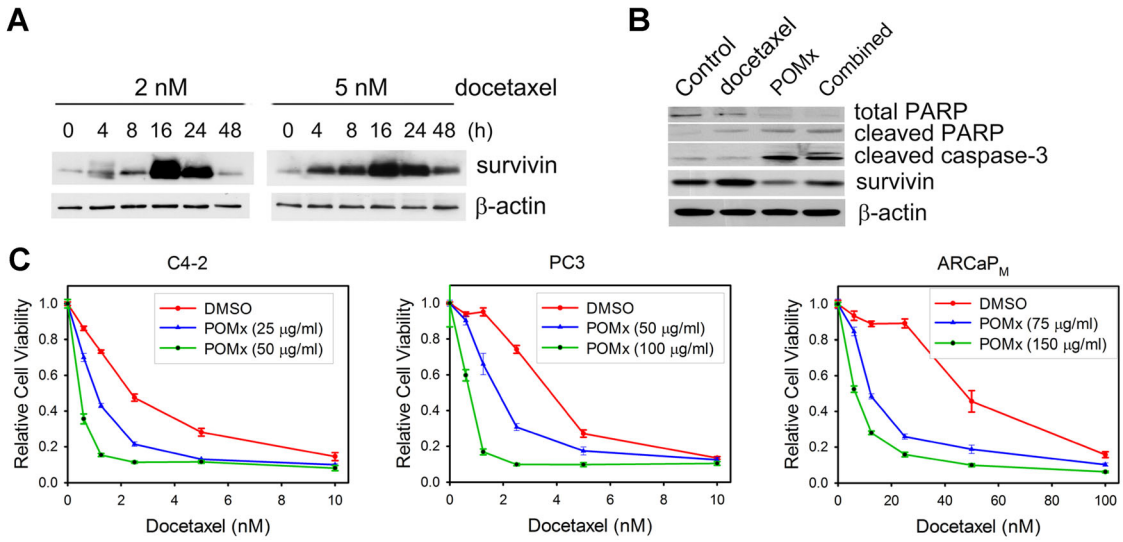

Fig. 4. POMx antagonizes docetaxel induction of survivin and enhances the in vitro cytotoxicity of docetaxel in metastatic castration-resistant PCa cells. **A**: Docetaxel at low doses temporarily increases survivin protein in C4-2 cells. **B**: POMx (35 μg/ml) antagonizes survivin induction by docetaxel (5 nM) and activates apoptosis in C4-2 cells. **C**: POMx treatment (72 hr) sensitizes PCa cells to docetaxel treatment.

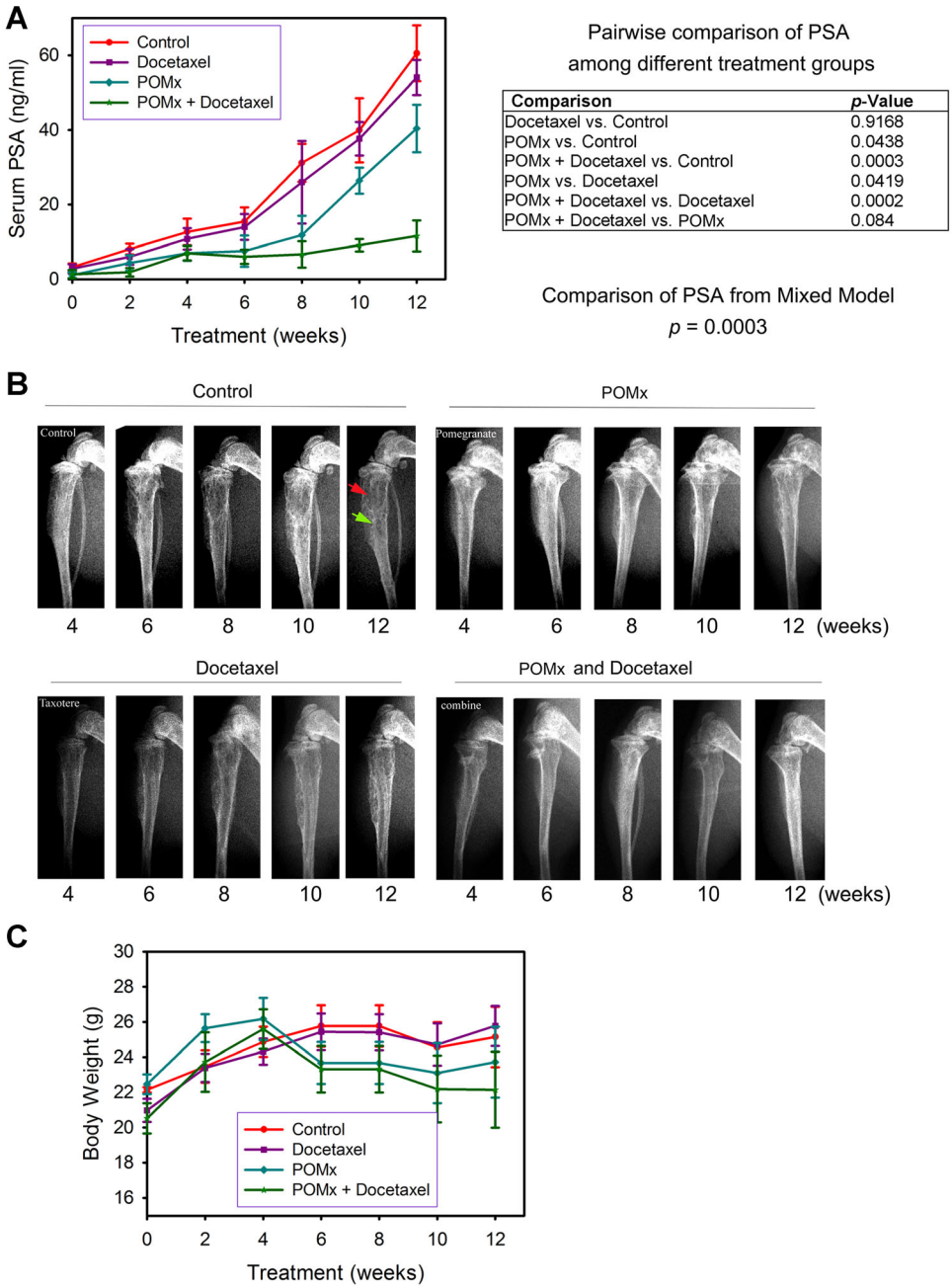

Fig. 5. POMx inhibits C4-2 tumor growth in mouse skeletons and enhances the in vivo efficacy of docetaxel. **A**: POMx alone or combined with docetaxel significantly reduces serum PSA in C4-2 intratibial tumors. **B**: POMx treatment improves the bone architecture in C4-2 tumor-bearing mice, as shown by X-ray radiography. Red arrow: osteolytic lesion; green arrow: osteoblastic lesion. **C**: The effect of treatments on the body weight of mice.

As a rich source of polyphenolic ingredients and anti-cancer compounds (including γ-tocopherol, catechins, anthocyanidins, flavols, and flavones) [34], PE could inhibit multiple oncogenic processes (including proliferation, invasion, and angiogenesis) while activating apoptotic signals. Experimental evidence indicated that PE can affect various signaling pathways and molecular targets in a cell context-dependent manner. The biological activity of PE in PCa models has been associated with the inhibition of NF-κB, cyclins, c-myc, Bcl-XL, Bcl-2, protein kinase C, and androgen-synthesizing genes, and the induction of p21, p27, Bax, Bak, and activation of caspase-3 [35–39]. Nonetheless, the effect and mechanism of action of PE in bone metastatic PCa cells remain largely elusive. Given the close correlation between survivin

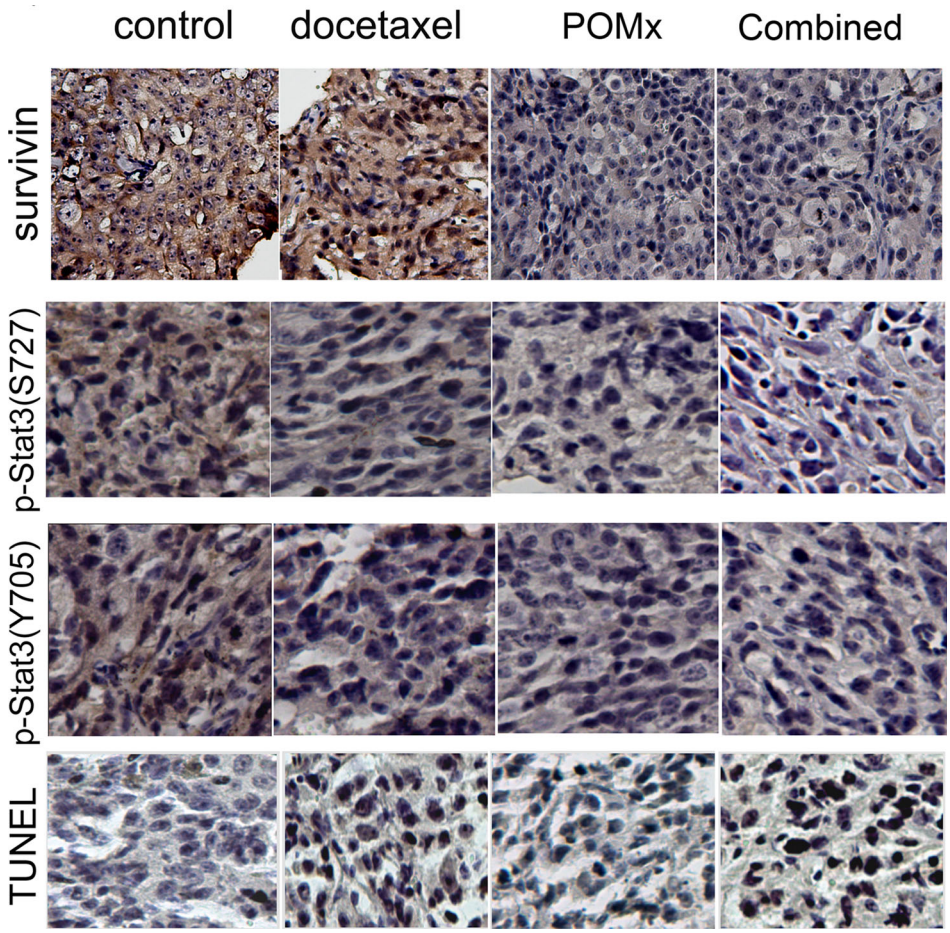

Fig. 6. POMx inhibits the in vivo expression of survivin and phosphorylated Stat3 and induces apoptosis in C4-2 skeletal tumor. IHC was performed in C4-2 tumor tissues at the endpoint of animal experiment for the expression of survivin, p-Stat3(Ser727), and p-Stat3(Tyr705). TUNEL assay was performed to examine the effect of treatments on apoptotic cells.

overexpression and the aggressive phenotypes (therapeutic resistance, bone metastasis) of PCa, we examined the effect of POMx on survivin signaling and identified survivin as a novel target of POMx in PCa cells. The in vitro and in vivo evidence presented here clearly support the inhibition of survivin as a crucial mechanism of action for the pro-apoptotic and chemosensitizing abilities of POMx in bone metastatic PCa.

Constitutive activation of Stat3 signaling has been correlated to pathologic stage and Gleason score in PCa [40], and Stat3 inhibition induced apoptosis and suppressed the in vivo growth of PCa cells [41]. Two Stat3 *cis*-elements have been identified to be critical to the activation of the survivin promoter [30]. By using two human survivin reporters that differ in the presence of Stat3 sites, we demonstrated that these binding motifs are required for POMx inhibition of survivin transcription. Interestingly, although the optimal regulation of Stat3 transcription activity could be achieved by the phosphorylation at two amino acid residues, that is, Tyr705 and Ser727 [31], it appears that at least in certain PCa cells (such as $ARCaP_M$), POMx selectively inhibits the phosphorylation of Stat3 at Ser727, and consistently, the activity of its major upstream regulator ERK1/2. These observations suggest that the Ser727 residue may play a more essential role in mediating the POMx inhibition of survivin in PCa cells. Taken together, although other transcription factors (such as NF-κB) may also be involved, our data indicate an indispensible role of Stat3 in mediating the inhibitory effect of POMx on survivin expression.

Multiple mechanisms could contribute to the acquisition of docetaxel resistance in PCa cells [42]. Interestingly, short-term exposure to docetaxel resulted in a significant increase in survivin expression in PCa cells, which is in line with previous reports that paclitaxel could acutely induce survivin in a manner independent of cell cycle in human PCa and breast cancer cells [43–45]. Although the exact mechanism of survivin induction remains unknown, it may occur rapidly at the post-translational level and partially be attributed to the potent inhibitory effect of docetaxel on microtubule

disassembly and mitotic division. The resulting aberrant accumulation of survivin protein can provide an "emergent" protective mechanism and counteract docetaxel-induced apoptosis in cancer cells. Conversely, POMx effectively inhibits survivin expression (presumably at the transcriptional level) and eventually antagonizes docetaxel-induced survivin, thereby sensitizing PCa cells to chemotherapy. This inhibitory effect of POMx on the endogenous pool of survivin in PCa cells could be a key mechanism of action for the enhanced in vitro cytotoxicity and in vivo efficacy of docetaxel in the presence of POMx.

As one of the few known nodal proteins, that is, pivotal in the progression of human cancers, survivin has been actively pursued as an ideal target for cancer treatment [10,11]. Current strategies include vaccination approaches to generate an antigen-specific immune response against survivin-bearing tumor cells, the development of antisense oligonucleotides, ribozymes, or siRNA molecules targeting survivin, and small molecule inhibitors of survivin function. At least one small molecule inhibitor (YM155) has entered clinical trials in patients with localized, castration-resistant PCa. However, only modest activity has been observed. For example, a 25% PSA response and 25% partial response rate were reported in a recent phase I/II combination trial with standard-dose docetaxel [19,20,46]. Identification of POMx as a potent inhibitor of survivin could provide a novel and alternative strategy to effectively inhibit the expression and function of survivin in advanced PCa. Given the demonstrated safety profile of POMx in PCa patients [6–9], this PE formula could be readily tested in a clinical setting for its efficacy in enhancing docetaxel chemotherapy and treating mCRPC.

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