Bisphosphonates Inhibit Stellate Cell Activity and Enhance Antitumor Effects of Nanoparticle Albumin–Bound Paclitaxel in Pancreatic Ductal Adenocarcinoma

Vianey Gonzalez-Villasana1, Cristian Rodriguez-Aguayo1, Thiruvengadam Arumugam2, Zobeida Cruz-Monserrate3, Enrique Fuentes-Mattei3, Defeng Deng2, Rosa F. Hwang4, Huamin Wang5, Cristina Ivan6, Raul Joshua Garza7, Evan Cohen7, Hui Gao7, Guillermo N. Armaiz-Pena2,6, Paloma del C. Monroig-Bosque1,8, Bincy Philip2, Mohammed H. Rashed1,9, Burcu Aslan1, Mumin Alper Erdogan1, Yolanda Gutierrez-Puente10, Gabriel Lopez-Berestein1,11

Abstract
Pancreatic stellate cells (PSC) have been recognized as the principal cells responsible for the production of fibrosis in pancreatic ductal adenocarcinoma (PDAC). Recently, PSCs have been noted to share characteristics with cells of monocyte-macrophage lineage (MML cells). Thus, we tested whether PSCs could be targeted with the nitrogen-containing bisphosphonates (NBP; pamidronate or zoledronic acid), which are potent MML cell inhibitors. In addition, we tested NBP's treatment combination with nanoparticle albumin–bound paclitaxel (nab-paclitaxel) to enhance antitumor activity. In vitro, we observed that PSCs possess α-naphthyl butyrate esterase (ANBE) enzyme activity, a specific marker of MML cells. Moreover, NBP's inhibited PSCs proliferation, activation, release of macrophage chemoattractant protein-1 (MCP-1), and type I collagen expression. NBP's also induced PSCs apoptosis and cell-cycle arrest in the G1 phase. In vivo, NBP's inactivated PSCs; reduced fibrosis; inhibited tumor volume, tumor weight, peritoneal dissemination, angiogenesis, and cell proliferation; and increased apoptosis in an orthotopic murine model of PDAC. These in vivo antitumor effects were enhanced when NBP's were combined with nab-paclitaxel but not gemcitabine. Our study suggests that targeting PSCs and tumor cells with NBP's in combination with nab-paclitaxel may be a novel therapeutic approach to PDAC. Mol Cancer Ther; 13(11); 2583–94. ©2014 AACR.

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death and one of the most lethal human cancers (1). The prognosis of patients with PDAC is extremely poor, with a median survival time of 6 months and an overall 5-year survival rate of less than 5% (1–3). Recently, the contribution of stromal elements to the initiation and progression of this disease has come to be appreciated (3–5). Targeting tumor microenvironment represents a potential strategy for improving the delivery and efficacy of chemotherapeutic agents for PDAC and, hence, the prognosis. The activated stroma of PDAC comprises extracellular matrix and several cell types, including stellate cells, endothelial cells, nerve cells, and immune cells (4). The principal cells responsible for the production of fibrosis in PDAC are the pancreatic stellate cells (PSC; refs. 6, 7). In normal pancreas, PSCs are quiescent, but when pancreatic injury or inflammation occurs, these cells undergo morphologic and functional changes to become activated, expressing α-smooth muscle actin (α-SMA) and secreting growth factors, a wide variety of proinflammatory cytokines, such as macrophage chemoattractant protein-1 (MCP-1) and interleukin-8 (IL8), and excessive amounts of extracellular matrix proteins such as type I collagen (5, 6, 8). If the injury or inflammation is sustained or repeated, PSCs maintain permanent activation, allowing the development of pancreatic fibrosis and tumor progression (7).
The origination of PSCs is unclear. However, it has been demonstrated that PSCs display phagocytic activity (9) and express TLR2, TLR4, TLR3, and TLR5 (10), which suggests that these cells may play a macrophage-like role in the pancreas that is comparable with the role of Kupffer cells in liver. Highly phagocytic cells, such as macrophages and osteoclasts, which share the same lineage, are the major target of bisphosphonates (11, 12). Thompson and colleagues (13) showed that bisphosphonates are internalized by J774 murine macrophages by fluid-phase endocytosis and vesicular acidification, suggesting that highly phagocytic cells, such as macrophages, can internalize bisphosphonates, which makes them ideal targets for these drugs. There are two classes of bisphosphonates, those that contain nitrogen and those that do not (14). Nitrogen-containing bisphosphonates (NBP), such as pamidronate, alendronate, and ibandronate, have been shown to inhibit cell proliferation, reduce cell viability, and cause the apoptotic cell death of macrophages (11). In this study, we explored the use of NBP s to reduce PSCs-mediated fibrosis as a way to reduce the burden of PDAC and its use in combination with nanoparticle albumin-bound paclitaxel (nab-paclitaxel), which is known to enhance drug delivery in tumors.

Materials and Methods

Reagents
Pamidronate (Pam) was purchased from Sigma-Aldrich, zoledronic acid (ZA) from Novartis Pharmaceuticals Corporation, gemcitabine (Gem) from APP Pharmaceuticals, LLC, and nab-paclitaxel from Abraxis BioScience, Inc.

Cell culture
Human PSCs isolated by Dr. Rosa Hwang [The University of Texas MD Anderson Cancer Center (MDACC), Houston, TX; ref. 15] were maintained in DMEM/F12 (Invitrogen Corporation) supplemented with 10% FBS. Capan-2 human PDAC cells [American Type Culture Collection (ATCC)] were transfected with luciferase and maintained in McCoy 5A medium (Invitrogen Corporation) with 10% FBS in 5% CO2/95% air at 37°C. Peripheral blood monocytes (PBM) isolated from buffy coats of healthy adult donors (MDACC) and THP-1 human monocytic leukemia cells (ATCC) were both maintained in RPMI-1640 medium (Invitrogen) with 10% FBS. The ATCC uses the Promega PowerPlex system to authenticate cell lines. All cell lines were expanded, cryopreserved, used within 6 months after resuscitation, and screened for Mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lanza Rockland, Inc.) as described by the manufacturer.

Isolation of PBMs by adherence
PBMs were isolated from buffy coats of three human donors by Ficoll-Hypaque density-gradient centrifugation. The mononuclear fraction was washed in PBS, counted, adjusted at 2.5 × 10^6 cells/mL in serum-free RPMI-1640 medium, and incubated for 3 hours. We next removed the medium and washed the adherent cell layer twice with serum-free RPMI-1640 medium. Cells were then cultured with 10% FBS RPMI-1640 medium for 6 days.

Isolation of PBMs by CD14 microbeads
PBMs were isolated from buffy coats of human donors by Ficoll-Hypaque density-gradient centrifugation, and then were counted and centrifuged at 300 × g for 10 minutes. The pellet was resuspended in running buffer (MACS Miltenyi Biotec), and then 20 μL of CD14 microbeads (MACS Miltenyi Biotec) per 1 × 10^7 total cells was added and the cells were incubated for 15 minutes at 4°C. Then, the cells were washed with running buffer and centrifuged at 300 × g for 10 minutes at 4°C, the supernatant was removed, and the cells were resuspended in running buffer. Magnetic separation was conducted using the MACS separator (MACS Miltenyi Biotec). The collected CD14-positive cells were counted, adjusted at 12 × 10^6 cells in RPMI-1640 medium with 10% FBS, and incubated at 37°C.

ANBE activity
PSCs, THP-1, and PBMs were harvested, counted, and adjusted at 5 × 10^4 cells in their respective medium. Samples were centrifuged in a Shandon CytoSpin 3 centrifuge at 600 rpm for 2 minutes and then fixed in a citrate-acetone-formaldehyde solution. Staining was performed with an α-naphthyl acetate esterase kit (Sigma-Aldrich) according to the manufacturer’s protocol (pH of the incubating medium adjustment to 6.3). Cells were viewed with an inverted microscope (Nikon Corporation Instruments Company) at ×10 magnification.

Western blotting
Cells were lysed with ice-cold lysis buffer. Lysates were centrifuged, supernatants were collected, and protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories). Samples were electrophoresed using 10% and 4% to 15% gradient polyacrylamide gels (Bio-Rad) and then transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked, rinsed, and incubated with primary antibodies against α-SMA (Dako), unprenylated Rap1A (Santa Cruz Biotechnology, Inc.), transglutaminase 2 (TG2; Abcam), cleaved caspase-3 (eBioscience), cleaved PARP-1 (Cell Signaling), fibronectin (Santa Cruz Biotechnology, Inc.), laminin (Thermo Fisher Scientific, Inc.), caveolin-1 (BD Bioscience), farnesyltransferase beta (FNTB; Abcam), and type I collagen (SouthernBiotech). After overnight incubation at 4°C, membranes were washed and incubated with their corresponding secondary antibody conjugated with horseradish peroxidase (HRP). Proteins bands were detected with enhanced chemiluminescence detection kit (GE Healthcare). Immunoblots were scanned by an Alpha Imager densitometer (Alpha Inotech Corp.) for quantification of protein expression. β-Actin (Sigma-Aldrich) and vinculin (Santa Cruz Biotechnology, Inc.) were used as a loading control.
Cell viability assay
Cells were seeded on 96-well plates at 1,250 cells per well and incubated overnight. Cells were then treated for 72 hours with pamidronate. The MTS cell proliferation assay (Promega Corporation) was performed according to the manufacturer’s instructions. The results are expressed in terms of percentage of growth inhibition with respect to untreated (control) cells.

Apoptosis
Cell apoptosis was measured with an FITC apoptosis detection kit (BD Biosciences), according to the manufacturer’s instructions. Apoptotic cells were analyzed with a FACScalibur flow cytometer (BD Biosciences). The CellQuest Pro software (BD Biosciences) was used to determine the number of apoptotic cells. Apoptosis was also assessed using freshly cut frozen tissue and a DeadEnd fluorometric TUNEL system kit (Promega) according to the manufacturer’s instructions. The number of terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL)-positive cells was counted in five random fields per slide (one slide per mouse, 10 slides/group) at ×200 magnification.

Cell-cycle analysis
Cells were fixed with 70% cold ethanol for 24 hours, then were collected and resuspended in PBS containing 50 μg/mL propidium iodide and 100 U/mL of RNase A. Cells were incubated at 37°C for 30 minutes in the dark and then analyzed by a FACS Calibur flow cytometer (BD Biosciences). The CellQuest Pro software (BD Biosciences) was used to determine the number of cells in each phase of the cell cycle.

Measurement of MCP-1 levels
We measured the MCP-1 levels in the supernatant of PSCs treated with pamidronate by multiplex bead immunoassay using a Luminex kit (Millipore Corp.).

Orthotopic tumor implantation and drug treatment
At week 0, 8-week-old female athymic nude mice purchased from Taconic were anesthetized with Nembutal [20 mg/kg body weight (BW)/i.p.], and Capan-2 cells transfected with luciferase were injected (1×10⁶ cells/100 μL) directly into tail of the pancreas. One week after implantation (week 1), bioluminescence was measured using the Living Image software (Caliper Life Sciences). Mice were sacrificed the fifth week after treatment began. The complete pancreas was weighed to obtain tumor weight. Tumor tissue was fixed in formalin and embedded in paraffin for immunohistochemical analysis and staining with hematoxylin and eosin (H&E). The animal experiments were conducted in accordance with the American Association for Laboratory Science (AALAS) regulations and the approval of the MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Immunohistochemical analysis
Unstained sections of mouse tissues were deparaffinized and rehydrated. Antigen retrieval was performed with DAKO antigen retrieval solution (Dako). Endogenous peroxidase was blocked by hydrogen peroxide (3%). For protein blocking, cold-water fish skin gelatin 40% (Electron Microscopy Sciences) in PBS was applied for 1 hour. Tissues were then incubated with HRP streptavidin solution (Vector Labs) for 30 minutes. For Ki67, the goat anti-rabbit HRP secondary antibody (Vector Labs) was incubated for 20 minutes. Slides were then incubated with HRP streptavidin solution (Vector Labs) for 30 minutes. For Ki67, the goat anti-rabbit HRP secondary antibody (Vector Labs) was incubated for 20 minutes. For Ki67, the goat anti-rabbit HRP secondary antibody (Vector Labs) was incubated for 20 minutes. Slides were then incubated with HRP streptavidin solution (Vector Labs) for 30 minutes. For Ki67, the goat anti-rabbit HRP secondary antibody (Vector Labs) was incubated for 20 minutes. Slides were then incubated with HRP streptavidin solution (Vector Labs) for 30 minutes. For Ki67, the goat anti-rabbit HRP secondary antibody (Vector Labs) was incubated for 20 minutes.

Statistical analysis
Statistical analyses were conducted using the Student t test. P < 0.05 was considered statistically significant for
all assays. The nonparametric test Kruskal-Wallis was applied to assess the relationship between tumor volume and treatment. A box-and-whisker plot [Box plot represents first (lower bound) and third (upper bound) quartiles, whiskers represent 1.5 times the interquartile range] was used to visualize the data.

To examine the interaction index between gemcitabine and zoledronic acid, an isobologram analysis was used as described by Vivas-Mejia and colleagues (19) using the following equation:

\[
\text{Interaction index} = \left( \frac{\text{Gem}}{\text{Gem}_a} \right) + \left( \frac{\text{ZA}}{\text{ZA}_a} \right)
\]

where Gem and ZA are concentrations of gemcitabine and zoledronic acid, respectively, that produce some specified effect when used alone (a) or in combination (c). The interaction is considered synergistic when the interaction index value is lower than 1.0 and antagonistic when the value is higher than 1.0.

**Results**

**α-Naphthyl butyrate esterase activity in PSCs**

Recently, PSCs have been noted to share characteristics with cells of monocyte-macrophage lineage (MML cells; refs. 7, 9). We first assessed whether PSCs display α-naphthyl butyrate esterase (ANBE) activity, which is a specific enzyme present in MML cells. PSCs, undifferentiated PBMs, differentiated PBMs, and THP-1 cells all expressed ANBE (Fig. 1A), suggesting that PSCs share a key marker with MML cells.

**PSCs express α-SMA and TG2 when treated with 12-O-tetradecanoyl-phorbol-13-acetate**

12-O-Tetradecanoyl-phorbol-13-acetate (TPA) is a tumor promoter and protein kinase C (PKC) activator that induces the differentiation of leukemic cells such as THP-1 to monocytic lineage (20–22). To determine whether PSCs can be differentiated such as monocytes, PSCs, and THP-1, cells were treated with TPA at 50 and 100 ng/mL, respectively, for 72 hours, which was followed by immunoblotting to determine TG2 and α-SMA expression levels in the presence and absence of TPA treatment. TG2 is a highly selective marker of monocyte differentiation (23, 24), and α-SMA is the hallmark marker of PSCs activation. We found that TPA induced the expression of TG2 and α-SMA in PSCs, PBMs, and THP-1 cells (Fig. 1B). These results indicate that PSCs follow a similar pattern of differentiation markers as MML cells do.

**Pamidronate inhibits PSCs proliferation and leads to apoptosis and cell-cycle arrest**

Our observations that PSCs behave in a similar fashion as MML cells suggested that they might be susceptible to MNL inhibitors such as NBPs (11, 12). For this reason, we targeted the PSCs with pamidronate. Seventy-two hours of treatment with pamidronate (0.1, 1, 10, and 100 μmol/L) led to a dose-dependent reduction in cell proliferation (Fig. 2A). We examined the effect of pamidronate in proliferation at 24, 48, and 72 hours and because we observed the best response at 72 hours, we decided to use these time point for the rest of the experiments. It is conceivable that the concentrations used maybe achieved in bone marrow but not in peripheral organs.

We next determined whether the inhibition of PSCs proliferation by pamidronate was through apoptosis. The maximal induction of apoptosis was observed at 100 μmol/L pamidronate (89.7% of apoptotic cells; Fig. 2B). Our results were confirmed by Western blotting, which showed that pamidronate induced the expression of cleaved caspase-3 and cleaved PARP-1 in a dose-dependent manner; maximum induction of these apoptotic markers was at 100 μmol/L pamidronate (Fig. 2C).

We also assessed the effect of pamidronate on the distribution of PSCs in the cell cycle. Pamidronate resulted in G1 arrest at a concentration of 100 μmol/L (Fig. 2D). Western blotting confirmed that compared with
untreated cells, cdk2, a key protein required for transition from the G1-phase to the S-phase, was downregulated by 40%, whereas p27, which blocks cdk2, was upregulated by almost 300% in PSCs treated with 100 μmol/L pamidronate (Fig. 2E). Taken together, our observations suggest that inhibition of PSCs proliferation by pamidronate was due to both apoptosis and cell-cycle arrest in the G1-phase.

Pamidronate and zoledronic acid treatment inactivates PSCs and inhibits the mevalonate pathway

One key marker of PSCs activation is α-SMA expression. To determine whether pamidronate and zoledronic acid inactivate PSCs, we treated these cells with different concentrations of pamidronate (0.1, 1, 10, and 100 μmol/L) or zoledronic acid (0.1, 1, 2.5, 5, and 7.5 μmol/L) for 72 hours. The level of α-SMA was substantially decreased at all doses of pamidronate (Fig. 3A) and zoledronic acid (Fig. 3B), as indicated by Western blot analysis. The greatest reductions were observed at 10 and 100 μmol/L pamidronate (by 53.3% and 59.7%, respectively) compared with untreated cells.

Detection of increased levels of unprenylated Rap1A is a common surrogate measurement of NBP uptake by cells in vitro and inhibition of the mevalonate pathway (25–27). The level of unprenylated Rap1A in PSCs increased as pamidronate concentration increased (by 137.8% at 0.1 μmol/L to 297.5% at 10 μmol/L compared with untreated cells; Fig. 3A). However, the level of unprenylated Rap1A decreased precipitously at 100 μmol/L pamidronate likely because at this concentration most of the PSCs underwent apoptosis, as we observed by Annexin V and Western blot analysis (Fig. 2B and C). The treatment with zoledronic acid increased the levels of unprenylated Rap1A in a dose-dependent manner in PSCs (Fig. 3B).

Activated PSCs produce extracellular matrix components, such as type I collagen, and secrete the...
proinflammatory cytokine MCP-1, which is one of the most potent macrophage recruitment factors (28–30). We performed Western blotting of PSCs treated with 0.1, 1, 10, and 100 μmol/L pamidronate or 0.1, 1, 2.5, 5, and 7.5 μmol/L zoledronic acid for 72 hours and Luminex assay with the supernatant of these cells to determine whether pamidronate- and zoledronic acid–induced inactivation of PSCs could reduce the expression level of type I collagen and MCP-1, respectively. The level of MCP-1 decreased in a dose-dependent manner, with significant reductions at 10 and 100 μmol/L pamidronate (Fig. 3C) and 2.5, 5, and 7.5 μmol/L zoledronic acid (Fig. 3D) compared with no treatment. The type I collagen level was decreased by 60% in PSCs at the highest concentration of pamidronate (Fig. 3E) but not in a dose response fashion. Similar to pamidronate, zoledronic acid inhibited the levels of type I collagen (Fig. 3F). These results are important because MCP-1 and type I collagen are
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important contributors in the development of pancreatic fibrosis.

We performed qRT-PCR, as is described in Supplementary Materials and Methods, to determine the mRNA levels of α-SMA, Rap1A, and MCP-1 (see Supplementary Table S1 for primer sequences) in tissues isolated from the four groups of mice included in the in vivo study. As expected, the levels of α-SMA only decreased in the group of mice treated with zoledronic acid and zoledronic acid plus nab-paclitaxel (Supplementary Fig. S2A). This decrease was statistically significant ($P = 0.0378$) when zoledronic acid was combined with nab-paclitaxel. Regarding the levels of Rap1A, we did not observe any significant differences in the mRNA levels in any of the four groups (Supplementary Fig. S2B). This is consistent with our predictions, because treatment with zoledronic acid inhibits prenylation at a posttranslational level. Finally, MCP1 mRNA levels were not detected by qRT-PCR in any of the four groups (data not shown).

**Inhibition of PSCs function improved in vivo therapeutic effectiveness of nab-paclitaxel but not gemcitabine**

On the basis of our observations of reduced PSCs function after treatment with the NBP’s pamidronate and zoledronic acid, we next evaluated the effectiveness of combining this treatment with cytotoxic chemotherapeutic agents in vivo. For the in vivo experiments, we selected the Capan-2 orthotopic animal model of PDAC, which develops tumors with extensive fibrosis accompanied by PSCs (31, 32). Twenty-four days after tumor implantation, nude mice bearing Capan-2 luciferase cells ($n = 10$ mice/group) were treated with saline solution, the NBP zoledronic acid, gemcitabine (a first-line treatment for patients with PDAC), or the combination of zoledronic acid plus gemcitabine as described in Materials and Methods. We selected zoledronic acid rather than pamidronate because it is safer and more potent but influences the same mechanisms (33).

The results showed that single-agent gemcitabine or zoledronic acid decreased tumor volume but that when these agents were combined, the tumor volume was similar to that of the saline solution controls (Supplementary Fig. S3A). Similar results were observed for α-SMA expression, which was significantly reduced by zoledronic acid alone compared with saline solution ($P < 0.05$) but not in mice treated with the combination (Supplementary Fig. S3B). These findings were supported by the MTS proliferation assay, which showed a dose-dependent inhibition of PSCs proliferation when the cells were treated with zoledronic acid alone (Supplementary Fig. S4A), gemcitabine alone (Supplementary Fig. S4B), or nab-paclitaxel alone (Supplementary Fig. S4C). Capan-2 cells were also treated with zoledronic acid alone (Supplementary Fig. S4D), gemcitabine alone (Supplementary Fig. S4E), and nab-paclitaxel alone (Supplementary Fig. S4F). Moreover, the isobologram analysis revealed an antagonistic effect when zoledronic acid was combined with gemcitabine in PSCs (Supplementary Fig. S5A), whereas the combination of zoledronic acid with nab-paclitaxel was synergistic (Supplementary Fig. S5B). Similar results were observed in Capan-2 cells, the combination of zoledronic acid with gemcitabine was antagonistic (Supplementary Fig. S5C), whereas synergistic effect was found when zoledronic acid was combined with nab-paclitaxel (Supplementary Fig. S5D).

On the basis on these data, we decided to use nab-paclitaxel as a chemotherapeutic agent instead of gemcitabine for further in vivo experiments. Nab-paclitaxel is an albumin-bound paclitaxel-nanoparticle formulation that has been suggested to promote endothelial transcytosis through its binding to a gp60 receptor (34). Paclitaxel is a highly hydrophobic molecule, which is a limitation for its clinical applications. Nab-paclitaxel is bound to albumin that is a natural carrier of hydrophobic molecules. Moreover, albumin stabilizes the drug particle at an average size of 130 nm, which prevents the potential risk of capillary obstruction (34). Preclinical studies in breast cancer demonstrated that nab-paclitaxel has higher penetration into tumor cells and antitumor activity, compared with an equal dose of paclitaxel (35).

One week after mice were injected orthotopically with Capan-2 luciferase tumor cells, we assessed the first IVIS images to verify tumor implantation. Luciferase bioluminescence data derived from week 7 IVIS images revealed that zoledronic acid decreased tumor volume slightly more than nab-paclitaxel did and that the combination of these agents resulted in the greatest decrease of tumor volume as compared with saline solution ($P < 0.0005$; Fig. 4A).

After 5 weeks of treatment, mice were sacrificed and tumor weight and peritoneal tumor dissemination was assessed. Compared with treatment with saline solution only, tumor weight was lower with zoledronic acid alone ($P < 0.001$) or nab-paclitaxel alone ($P < 0.05$) but even more so with both drugs ($P < 0.0001$; Fig. 4B). Likewise, zoledronic acid alone ($P < 0.001$), nab-paclitaxel alone ($P < 0.001$), and their combination ($P < 0.0001$) decreased peritoneal dissemination (Fig. 4C). Minimal variation was observed in tumor weight single distribution (Fig. 4D). In this orthotopic murine model, no skeletal metastases develop.

**Zoledronic acid inactivated PSCs and reduced fibrosis in vivo**

Immunohistochemical analysis of α-SMA was performed to assess the state of activation of PSCs in the tumor tissue. Compared with mice that received saline solution, α-SMA expression decreased significantly in mice treated with zoledronic acid alone ($P < 0.05$) or combined with nab-paclitaxel ($P < 0.05$), but not in the mice treated with nab-paclitaxel alone (Fig. 5A), suggesting that PSCs inactivation occurs through the effect of zoledronic acid treatment. Using conventional H&E staining, we also determined that mice treated with the...
A combination of zoledronic acid and nab-paclitaxel exhibited significant reduction of fibrotic tissue compared with the saline controls \((P < 0.05; \text{Fig. 5B})\). We also found a reduction in the levels of fibronectin in PSCs treated with zoledronic acid combined with nab-paclitaxel (Supplementary Fig. S6A) and reduction of laminin levels in PSCs treated with the combination of the two drugs (Figures 4C and D).

Figure 4. *In vivo* therapeutic efficacy of zoledronic acid and nab-paclitaxel. Mice treated with zoledronic acid (1 mg/kg BW/i.p.), nab-paclitaxel (10 mg/kg BW/i.v.), or both for 5 weeks exhibited lower tumor volume (A), tumor weight (B), and peritoneal dissemination (C) compared with treatment with saline solution. Because the data were non-normally distributed, the median was used to graph tumor volume. The nonparametric test Kruskal–Wallis was applied to assess the relationship between tumor volume and treatment. A box-and-whisker plot (Box plot represents first (lower bound) and third (upper bound) quartiles; whiskers represent 1.5 times the interquartile range) was used to visualize the data. For tumor weight and peritoneal dissemination, values are presented as means ± SD. **, \(P < 0.001\); ***, \(P < 0.0001\) versus saline solution. D, tumor weight single distribution \((n = 10\) mice/group).

Figure 5. *In vivo* inactivation of PSCs and reduction of fibrosis by zoledronic acid. Mice were treated with zoledronic acid (1 mg/kg BW/i.p.), nab-paclitaxel (10 mg/kg BW/i.v.), or both for 5 weeks. Representative sections of α-SMA immunohistochemical staining (A) and H&E staining for fibrosis (B) from each treatment group are shown. Percentage of α-SMA expression or fibrosis was determined by our pathologist. Total magnification = 200. *, \(P < 0.05\); error bars, SD \((n = 10\) mice/group).
mice treated with the combination of both drugs (Supplementary Fig. S6B).

**Effect of zoledronic acid and nab-paclitaxel on cell proliferation, apoptosis, and angiogenesis**

The effects of zoledronic acid combined with nab-paclitaxel on cell proliferation, apoptosis, and angiogenesis were also analyzed. Cell proliferation was determined by staining for the nuclear marker Ki67. Compared with the group that received saline solution alone, zoledronic acid \((P < 0.0001)\) and nab-paclitaxel \((P < 0.0001)\) each induced a significant decrease in the Ki67 index as single agents; the combination of these agents further decreased proliferation \((P < 0.0001);\) Fig. 6A). Apoptosis was determined using TUNEL analysis, which revealed the same pattern but in the opposite direction, with each treatment increasing the level of apoptosis \((P < 0.0001\) for each treatment group; Fig. 6B). Angiogenesis was analyzed by determining the number of CD31-positive cells. Compared with the saline solution group, zoledronic acid and nab-paclitaxel each resulted in a significant decrease in the number of CD31-positive cells, which was further reduced by combining zoledronic acid and nab-paclitaxel \((P < 0.0001\) for each treatment group; Fig. 6C). These results suggest that NBPs have a dual effect targeting PSCs and tumor cells.

Our study demonstrated that the combination of zoledronic acid and nab-paclitaxel provided superior results to either treatment alone. This combination enhanced the inactivation of PSCs, which resulted in enhanced reduction of fibrosis. The combination also enhanced the inhibition of tumor volume, tumor weight, peritoneal dissemination, angiogenesis, and cell proliferation while increasing apoptosis in the Capan-2 orthotopic murine model of PDAC. Supplementary Fig. S7A shows that nab-paclitaxel alone and zoledronic acid alone decrease caveolin-1 and this effect was enhanced when zoledronic acid is combined with nab-paclitaxel. We also found that the silencing of caveolin-1 (Supplementary Fig. S7B) and FNTB (farnesyltransferase beta; Supplementary Fig. S7C) increases the unprenylated Rap1A levels. These results indicate that there is a mechanistic association between zoledronic acid and nab-paclitaxel in which the inhibition of the mevalonate pathway and caveolin-1 is crucial.

**Discussion**

PSCs are the main cells mediating fibrosis in PDAC (5). Phagocytic activity (9) and the expression of TLRs by PSCs suggest that these cells might also act as resident phagocytic cells during pancreatic injury (7, 9). In the current study, we found that PSCs also possess ANBE activity (a selective monocyte marker; ref. 36) and are differentiated by TPA in a similar way as monocytes are.

Furthermore, NBPs (such as pamidronate) have been observed to induce PSCs death by apoptosis at a concentration \((100 \mu\text{mol/L})\) previously shown to have this effect on macrophages (11), suggesting that PSCs can be treated in the same manner that macrophages are.
NBPs also have antitumor activity. For example, zoledronic acid can induce antiproliferative and apoptotic effects in PDAC cell lines (e.g., BxPC-3, CFPAC-1, and PANC-1; ref. 2). In our study, we observed that zoledronic acid reduced the volume, weight, and cell proliferation of tumors, suggesting that in our in vivo model zoledronic acid targeted both PSCs and tumor cells.

It has been reported that bisphosphonates such as incadronate and alendronate inhibit peritoneal dissemination in pancreatic and ovarian cancer, respectively, and the authors suggest that this antitumor effect may result from the suppression of migration and invasion by Rho inhibition (37, 38). In this study, we observed that zoledronic acid alone and nab-paclitaxel alone decreased angiogenesis (CD31; marker for angiogenesis) and peritoneal dissemination, these effects were enhanced in mice treated with zoledronic acid combined with nab-paclitaxel. This effect in peritoneal dissemination may be derived from the inhibition of angiogenesis by zoledronic acid and nab-paclitaxel, because angiogenesis is also a crucial step to allow peritoneal dissemination.

We demonstrated in vitro and in vivo that the NBPs pamidronate and zoledronic acid can inactivate PSCs while inducing cell-cycle arrest and apoptosis. In addition, the levels of MCP-1 and type I collagen were reduced when PSCs were inactivated by pamidronate in vitro. However, in our in vivo model, a significant reduction of fibrosis in the tumor tissue was observed only with the combination of zoledronic acid and nab-paclitaxel. An explanation for this finding could be that Capan-2 cells are less sensitive to zoledronic acid than PSCs are, as we observed in vitro. The data suggest that whereas zoledronic acid has a dual effect on tumor cells and PSCs, Capan-2 cells are less affected by this treatment and can produce significant fibrosis, although activated PSCs are considered the major source of type I collagen in fibrotic pancreas (15, 39), the tumor cells themselves also produce collagen and other matrix proteins. Capan-2 cells produce collagen types I, III, and IV as well as fibronectin, laminin, vitronectin, and undulin in vitro and in vivo (40, 41). Moreover, MCP-1, which is one of the major chemoattractants for monocytes leading to inflammation in tumors, also acts as a fibrosis-promoting chemokine (by stimulating collagen gene expression via endogenous upregulation of transforming growth factor β in rat lung fibroblasts; refs. 42, 43). The expression of MCP-1 has been reported for several tumor types, including human melanoma, ovarian cancer, esophageal cancer, and PDAC (44–47). In our study, we also observed that Capan-2 cells are more sensitive to nab-paclitaxel than zoledronic acid in vitro. However, the combination of zoledronic acid and nab-paclitaxel resulted in synergistic effect in vitro and provided maximum efficacy in vivo, suggesting that this is a clinically applicable regimen that could be easily translated to into phase I studies.

The reduction of fibrosis by zoledronic acid may result from targeting not only PSCs but also tumor-associated macrophages (TAMs), because TAMs regulates fibrosis in part by promoting inflammation, angiogenesis, and the recruitment of macrophages and fibroblasts to the tumor site (48). And both PSCs and TAMs are highly phagocytic cells that can internalize zoledronic acid, which makes them ideal targets for these drugs (13).

On the other hand, it was previously reported that the combination of the NBP zoledronic acid with gemcitabine significantly reduced the growth, angiogenesis, and metastasis of SUIT-2 PDAC cells orthotopically implanted into nude mice (49). In our study, however, this combination was antagonistic in vitro and in vivo. On the basis of our in vitro observation that zoledronic acid induced cell-cycle arrest in the G1-phase and the fact that gemcitabine is effective only in the S-phase (50), zoledronic acid could be preventing cells from reaching the S-phase, where gemcitabine can act.

The differences observed between the combination of zoledronic acid and nab-paclitaxel and the combination of zoledronic acid and gemcitabine are likely explained by the differences in mechanisms of action of these two chemotherapeutic agents. Nab-paclitaxel is a mitotic inhibitor, (34), whereas gemcitabine is a modified nucleotide that blocks DNA synthesis (50). The results of this study suggest targeting PSCs and tumor cells with zoledronic acid in combination with a chemotherapeutic agent; nab-paclitaxel could be a potential option in treating PDAC. The use of gemcitabine combined with nab-paclitaxel does not preclude the use of zoledronic acid plus nab-paclitaxel. They can be either used in an alternating or sequential regimen or in second- or third-line treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Gonzalez-Villasana, C. Rodriguez-Aguayo, R.F. Hwang, R.J. Garza, B. Aslan, A.K. Sood, G. Lopez-Berestein
Study supervision: B. Ozpolat, A.K. Sood, C. Logsdon
Other (doing some experiments involved in the article, e.g., Western blotting, preparing the samples for Lumines assay, and interpretation of the results of these experiments): M.H. Rashed
Other (in vivo experiments and in vivo data): M.A. Erdogan
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References

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