Rac1/Pak1/p38/MMP-2 Axis Regulates Angiogenesis in Ovarian Cancer


Abstract

**Purpose:** Zoledronic acid is being increasingly recognized for its antitumor properties, but the underlying functions are not well understood. In this study, we hypothesized that zoledronic acid inhibits ovarian cancer angiogenesis preventing Rac1 activation.

**Experimental Design:** The biologic effects of zoledronic acid were examined using a series of *in vitro* (cell invasion, cytokine production, Rac1 activation, reverse-phase protein array) and *in vivo* (orthotopic mouse models) experiments.

**Results:** There was significant inhibition of ovarian cancer (HeyA8-MDR and OVCAR-5) cell invasion as well as reduced production of proangiogenic cytokines in response to zoledronic acid treatment. Furthermore, zoledronic acid inactivated Rac1 and decreased the levels of Pak1/p38/matrix metalloproteinase-2 in ovarian cancer cells. In *vivo*, zoledronic acid reduced tumor growth, angiogenesis, and cell proliferation and inactivated Rac1 in both HeyA8-MDR and OVCAR-5 models. These *in vivo* antitumor effects were enhanced in both models when zoledronic acid was combined with nab-paclitaxel.

**Conclusions:** Zoledronic acid has robust antitumor and antiangiogenic activity and merits further clinical development as ovarian cancer treatment.

**Introduction**

Ovarian cancer is the most common cause of death from gynecologic malignancy. At present, the mainstay in advanced ovarian cancer is debulking surgery, chemotherapy (particularly with taxanes and platinum compounds) and radiotherapy. However, most patients with ovarian cancer eventually have a relapse after a median disease-free survival of 18 months (1, 2), thus highlighting the need for new avenues of therapy that target key processes in tumor progression. Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a complex and critical process for tumor growth and development of metastasis (3, 4). Current antiangiogenic therapies are showing promise in ovarian cancer but display limited efficacy and encounter tumor resistance (5, 6). These limitations underscore the need for additional molecular targets and effective therapies for ovarian cancer.

Bisphosphonates have been widely used in the treatment of patients with benign and malignant bone diseases to inhibit osteoclast-mediated bone resorption. Recently, nitrogen-containing bisphosphonates (NBP), such as zoledronic acid, have been used in the treatment of metastatic bone disease in women with breast cancer and have been reported to possess potent antiangiogenic properties (7, 8). NBPs inhibit farnesyl pyrophosphate synthase (FPPS) and the downstream enzyme geranylgeranyl disphosphate synthase, which are required for the prenylation of small GTPases such as Rac1 (9–11). Rac1, a member of the Rho GTPase family, plays a central role in angiogenesis through the regulation of endothelial cell migration, tube formation, adhesion, invasion, and proliferation (12, 13).

In this study, we hypothesized that zoledronic acid inhibits ovarian cancer angiogenesis by preventing the activation of Rac1. To test this hypothesis, we treated human ovarian cancer HeyA8-MDR and OVCAR-5 cells with zoledronic acid to assess its effects on cell invasion, matrix metalloproteinase (MMP) expression, and angiogenic factors (Milliplex assay). We performed reverse-phase protein array (RPPA) analysis to determine the pathway through which Rac1 inhibits angiogenesis that was validated by Western blotting. We also determined Rac1 activation by pull-down assay: HeyA8-MDR and OVCAR-5 orthotopic murine models of ovarian cancer were assessed for expression of CD31, Ki67,
Ovarian cancer is the most common cause of death from gynecologic malignancy. Although current antiangiogenic therapies are showing promise in ovarian cancer, they display limited efficacy and resistance. This highlights the need for additional molecular targets and effective therapies. In this study, we found that zoledronic acid reduced tumor growth, angiogenesis, cell proliferation, and inactivated Rac1, in ovarian cancer models, showing potent antitumor and antiangiogenic effects that were enhanced with the combination of zoledronic acid and nab-paclitaxel. We also found that these effects are mediated by the Rac1/Pak1/p38/MMP-2 pathway in HeyA8-MDR ovarian cancer cells. These findings open the door for the use of the combination of nab-paclitaxel with zoledronic acid as a novel therapeutic approach in ovarian cancer and other solid tumors.

Materials and Methods

Reagents
Zoledronic acid was purchased from Novartis Pharmaceuticals, Corporation and nab-paclitaxel from Abraxis BioScience, Inc.

Cell culture
The human ovarian cancer cell line HeyA8-MDR was maintained and propagated in RPMI-1640 medium (Invitrogen Corporation) supplemented with 10% FBS and 400 ng/mL of paclitaxel (Bedford Laboratories). A2780-CP20 cell line was developed and maintained as previously described (14). SKOV3-TR cell line was maintained in RPMI-1640 medium (Invitrogen Corporation) supplemented with 10% FBS and 150 ng/mL taxol. The OVCAR-5 cell line was maintained in DMEM (Invitrogen) with 10% FBS. The cells used are taxane-sensitive and -resistant. OVCAR-5 cells are cisplatin- and taxane-sensitive. The resistant ones are either HeyA8-MDR, A2780-CP20 (cisplatin-resistant cells), or SKOV3-TR (taxane-resistant cells). The immortalized human endothelial RF24 (EC-RF24) cells were expanded, cryopreserved, and used within 6 months after resuscitation. The authentication of all cell lines was done by short tandem repeat (STR) method. Briefer, HeyA8-MDR and OVCAR-5 cells were collected, cryopreserved, and used within 6 months after resuscitation. The authentication of all cell lines was done by short tandem repeat (STR) method.

Invasion assay
The invasiveness of HeyA8-MDR and OVCAR-5 cells treated with zoledronic acid was determined as previously described (15). Briefly, HeyA8-MDR and OVCAR-5 cells were collected and washed with serum-free media. Cells (4 × 10⁵) were resuspended in 1 mL of serum-free RPMI-1640 and DMEM respectively and added onto 6-well plate transwell inserts (8-μm pore size; Fisher Scientific) coated with a Matrigel basement membrane (0.7 mg/mL; BD Biosciences). Lower chambers were filled with 2 mL of medium supplemented with 10% FBS. HeyA8-MDR and OVCAR-5 cells in the Transwell inserts were then treated with different concentrations of zoledronic acid in serum-free media. Seventy-two hours later, noninvading cells on the upper surface of the filter were removed with cotton swabs. Cells that invaded through the Matrigel onto the lower side of the filter were fixed, stained with the Hema-3 Stain System (Fisher Scientific), and photographed. For each filter, the number of invaded cells was counted in 9 fields and expressed as the mean number of cells from triplicate measurements.

Tube formation assay
EC-RF24 cells were treated with different concentrations of zoledronic acid and nab-paclitaxel for 72 hours. Forty-eight-well plates were coated with 200 μL of BD Matrigel (BD Biosciences) and incubated at 37°C for 30 minutes. EC-RF24 cells were trypsinized, adjusted at 2 × 10⁵ cells, and plated onto coated plates and incubated for an additional 6 hours at 37°C. Experiments were performed in triplicate. Using an Olympus IX81 inverted microscope, 5 images per well were taken at 100× magnification. The number of nodes (defined as when at least 3 cells formed a single point) per image was quantified as previously described (16).

Measurement of angiogenic factors and MMP
Angiopoietin-2, BMP-9, EGF, endoglin, endothelin-1, FGF-1, FGF-2, follistatin, G-CSF, HB-EGF, hepatocyte growth factor (HGF), IL8, leptin, PLGF, VEGF-A, VEGF-C, VEGF-D, MMP-1, MMP-10, MMP-2, MMP-7, and MMP-9 levels were detected in the supernatant of HeyA8-MDR and OVCAR-5 cells treated with zoledronic acid respectively by multiplex bead immunoassay using a Luminex kit (Millipore Corp.).

Active Rac1 pull-down
HeyA8-MDR and OVCAR-5 cells were treated with different concentrations of zoledronic acid for 72 hours. After cell incubation, the Active Rac1 Pull-down and Detection Kit was used according to the manufacturer’s instructions (Thermo Scientific).

siRNA transfection
EC-RF24 cells were plated at a density of 1.5 × 10⁶ cells per well in a 6-well plate. The next day, cells were transfected with 5 μg control or Rac1 siRNA (Sigma-Aldrich) using HiPerFect transfection reagent (Qiagen) for 48 hours.

RPPA
The RPPA is a high-throughput antibody-based technique that measures levels of protein expression, as well as protein modifications such as phosphorylation. The RPPA was done by the MDACC/RPPA Core Facility. Briefly, HeyA8-MDR was treated with zoledronic acid for 72 hours. After protein extraction and denaturation by 1% SDS, serial dilutions of each sample were performed. Samples were arrayed on nitrocellulose-coated slides. The slides were probed with 161 validated antibodies detected by 3,3’-diaminobenzidine (DAB) colorimetric reaction. The slides were scanned, analyzed, and quantified using a customized software MicroVigene (VigeneTech, Inc.) to generate spot intensity. The fitted curve for each dilution was generated using the.
super curve fitting, a logistic model developed by the Department of Bioinformatics and Computational Biology at MDACC. The fitted curve was plotted with the signal intensities on the y-axis and log₂ concentration of proteins on the x-axis. The protein concentrations were normalized using median polish that was corrected across samples using linear expression values and the median expression levels of all antibody experiments to calculate a loading correction factor for each sample.

Western blotting
Lyases were centrifuged, supernatants were collected, and protein concentration was determined using a DC Protein Assay (Bio-Rad Laboratories). Samples were electrophoresed using 4% to 15% gradient PAGE (Bio-Rad) and then transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked, rinsed, and incubated with primary antibodies against unpreylated Rap1A (Santa Cruz Biotechnology, Inc.); p-P38, total P38, p-Pak1, total Pak1 (Cell Signaling Technology, Inc.); cleaved PARP-1 (Cell Signaling); and cleaved caspase-3 (eBioscience). After overnight incubation at 4°C, membranes were washed and incubated with their corresponding secondary antibody conjugated with horseradish peroxidase (HRP). Protein bands were detected with an enhanced chemiluminescence detection kit (GE Healthcare). β-Actin (Sigma Aldrich) and vinculin (Santa Cruz) were used as loading controls.

Orthotopic tumor implantation and drug treatment
Female athymic nude mice (Ncr-nu, 8–12 weeks old.) were purchased from Taconic (Hudson). HeyA8-MDR [1 x 10⁶ cells/0.2 mL Hanks’ Balanced Salt Solution (HBSS; Gibco)] and OVCAR-5 (1 x 10⁶ cells/0.2 mL HBSS) cells were injected into the peritoneal cavity. One week after implantation, mice bearing HeyA8-MDR and OVCAR-5 tumors were randomly divided into 4 treatment groups (n = 10 mice per group): (i) saline solution (200 μL intraperitoneally), (ii) zoledronic acid alone [1 mg/kg body weight (BW)/once per week/ intraperitoneally], (iii) nab-paclitaxel alone [10 mg/kg BW/once per week/intraperitoneally], or (iv) a combination of zoledronic acid [1 mg/kg BW/once per week/intraperitoneally] and nab-paclitaxel [10 mg/kg BW/once per week/intraperitoneally]. Mice were sacrificed the fourth week after treatment onset. Tumor weight and number of tumor nodules were determined. Tumor tissue was fixed in formalin for paraffin embedding or frozen in optimal cutting temperature (OCT) media to prepare frozen slides. The animal experiments were conducted in accordance with the American Association for Laboratory Animal Science (AALAS) regulations and with the approval of the MDACC Institutional Animal Care and Use Committee. All drugs were purchased from Taconic (Hudson). HeyA8-MDR [1 x 10⁶ cells/0.2 mL Hanks’ Balanced Salt Solution (HBSS; Gibco)] and OVCAR-5 (1 x 10⁶ cells/0.2 mL HBSS) cells were injected into the peritoneal cavity. One week after implantation, mice bearing HeyA8-MDR and OVCAR-5 tumors were randomly divided into 4 treatment groups (n = 10 mice per group): (i) saline solution (200 μL intraperitoneally), (ii) zoledronic acid alone [1 mg/kg body weight (BW)/once per week/ intraperitoneally], (iii) nab-paclitaxel alone [10 mg/kg BW/once per week/intraperitoneally], or (iv) a combination of zoledronic acid [1 mg/kg BW/once per week/intraperitoneally] and nab-paclitaxel [10 mg/kg BW/once per week/intraperitoneally]. Mice were sacrificed the fourth week after treatment onset. Tumor weight and number of tumor nodules were determined. Tumor tissue was fixed in formalin for paraffin embedding or frozen in optimal cutting temperature (OCT) media to prepare frozen slides. The animal experiments were conducted in accordance with the American Association for Laboratory Animal Science (AALAS) regulations and with the approval of the MDACC Institutional Animal Care and Use Committee. All drugs were used in a clinically relevant doses, based on the equivalent surface area dosage conversion factors described by Freireich and colleagues (17) and in the Drug Guidances by the FDA (18).

Immunohistochemical analysis
The immunohistochemical analysis was performed as previously described (19). Briefly, unstained sections of mouse tissues were deparaffinized and rehydrated. Antigen retrieval was performed with DAKO antigen retrieval solution (DAKO, North America Inc.). Endogenous peroxidase was blocked by hydrogen peroxide (3%). For protein blocking, IgG blocking from a Vector M.O.M. Kit (Vector Laboratories, Inc.) was applied for 1 hour (for active Rac1-GTP) or 5% normal horse serum and 1% normal goat serum in PBS were used (for Ki67 and CD31). Primary antibodies against active Rac1-GTP (Neweast Biosciences), Ki67 (Thermo/Lab Vision), and anti-CD31 (Pharmaingen) were incubated overnight at 4°C. For active Rac1-GTP, a M.O.M. kit anti-mouse biotinylated secondary antibody (Vector) was incubated for 30 minutes. Slides were then incubated with Vectastain elite ABC solution (Vector) for 30 minutes. For Ki67, goat anti-rabbit HRP secondary antibody and for CD31, goat anti-rat HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc.) diluted in blocking solution were added, and incubated for 1 hour at room temperature.

Slides were developed with DAB substrate (Vector Labs) and counterstained with Gill’s no. 3 hematoxylin solution. For active Rac1, the slides were imaged by an ACIS III image analysis system (DAKO) and the percentage of active Rac1 intensity was quantified in 5 random fields per slide (one slide per mouse, 5 slides per group). The Rac1 staining was reviewed by Dr. Huamin Wang (pathologist at The University of Texas MDACC). To quantify Ki67 and CD31 expression, the number of positive (DAB-stained) cells was counted in 5 random fields per slide (one slide per mouse, 10 slides per group) at 200× magnification, and the percentage of cells that were Ki67- and CD31-positive was calculated for each group. A single microvessel was defined as a discrete cluster or single cell stained positive for CD31, and the presence of a lumen was required for scoring as a microvessel (20). Cell apoptosis was determined by immunohistochemical analysis as described previously (19).

Statistical analysis
Statistical analyses were performed in R and the statistical significance was set to 0.05. The Shapiro–Wilk test was applied to verify if the data follows a normal distribution. Accordingly, Student’s t test and ANOVA test together with post hoc Tukey test, or the nonparametric test Kruskal–Wallis test followed by a Nemeyni post hoc test, was applied to assess the relationship between groups.

Results
Effect of zoledronic acid and nab-paclitaxel on angiogenesis, cell proliferation, and apoptosis
First, we examined the effects of zoledronic acid using orthotopic mouse models of ovarian cancer. In the OVCAR-5 model, 4 weeks after treatment, onset mice were sacrificed and tumor weight and number of tumor nodules were quantified. We found a significant decrease in tumor weight in mice treated with zoledronic acid (P < 0.05) alone or nab-paclitaxel (P < 0.05) alone and enhanced activity when the drugs were combined (P < 0.001; Fig. 1A) compared with saline solution group. A decrease in the number of nodules was found in the groups treated with zoledronic acid alone (P < 0.001), nab-paclitaxel alone (P < 0.001), and a combination of both (P < 0.001; Fig. 1B). The tumor weight single distribution showed a low variance (Fig. 1C). The number of CD31-positive cells resulted in a significant reduction after treatment with zoledronic acid or nab-paclitaxel and the combination of zoledronic acid with nab-paclitaxel (P < 0.0001 for each treatment group; Fig. 1D), compared with saline solution group. Cell proliferation was also decreased significantly in mice treated with zoledronic acid (P < 0.0001) or nab-paclitaxel (P < 0.0001) alone, and the combination of these agents further decreased proliferation (P < 0.0001; Fig. 1E), compared with the
group that received saline solution only. We found a significant decrease in Ki67 expression when nab-paclitaxel was combined with zoledronic acid (\( P < 0.05 \) and \( P < 0.001 \)) compared with nab-paclitaxel alone and zoledronic acid alone, respectively. Moreover, we observed that zoledronic acid, nab-paclitaxel, and the combination of both agents induced apoptosis in OVCAR-5 \textit{in vivo} model (Supplementary Fig. S1A).

Given that many patients will develop chemotherapy-resistant disease, we also tested the effects of zoledronic acid in the HeyA8-MDR model. After 4-week treatment, onset mice were sacrificed, and tumor weight and number of tumor nodules were quantified. We found that compared with saline solution group, tumor weight was lower in the mice treated with zoledronic acid alone (\( P < 0.05 \)) or nab-paclitaxel alone (\( P < 0.001 \)), and these effects were further enhanced after treatment with both drugs (\( P < 0.0001 \); Fig. 2A). The same decrease was found in the number of nodules, zoledronic acid alone (\( P < 0.001 \)), nab-paclitaxel alone (\( P < 0.05 \)), and their combination (\( P < 0.001 \); Fig. 2B). The tumor weight single distribution is shown in Fig. 2C. Angiogenesis was analyzed by determining the number of CD31-positive cells. Compared with results in the saline solution–treated group, zoledronic acid alone, nab-paclitaxel alone, or a combination of the 2 resulted in a significant decrease in the number of CD31-positive cells, (\( P < 0.0001 \) for each treatment group; Fig. 2D). Cell proliferation was determined by staining for the nuclear marker Ki67. Zoledronic acid (\( P < 0.0001 \)) and nab-paclitaxel (\( P < 0.0001 \)) each induced a significant decrease in the Ki67 index as single agent; the combination of these agents further decreased proliferation (\( P < 0.0001 \); Fig. 2E). Also, we found a significant comparison of nab-paclitaxel alone or zoledronic acid alone with the combination of both agents in tumor weight and CD31-positive cells. There was a significant reduction of tumor weight in the combination treatment (\( P < 0.05 \)) as compared with either nab-paclitaxel or zoledronic acid alone. Blood vessel density was significantly decreased in the combination group (\( P < 0.0001 \) and \( P < 0.001 \)) as compared with either nab-paclitaxel or zoledronic acid alone, respectively. In addition, we observed that zoledronic acid, nab-paclitaxel, and the combination of both agents induced apoptosis in HeyA8-MDR \textit{in vivo} model (Supplementary Fig. S1B) and \textit{in vitro} (Supplementary Fig. S1C and S1D).

**Zoledronic acid prevents activation of Rac1 \textit{in vivo}**

To address whether zoledronic acid prevents Rac1 activation \textit{in vivo}, we assessed immunohistochemistry using an active Rac1-GTP antibody. In OVCAR-5 cells (Fig. 3A), we observed a significant (\( P < 0.0001 \)) decrease in the expression of active Rac1 in the groups treated with zoledronic acid alone compared with results.
in the mice treated with saline solution. In HeyA8-MDR cells (Fig. 3B), we observed the same results. Moreover, we found that when we combined zoledronic acid and nab-paclitaxel, the prevention of Rac1 activation was enhanced. These results suggest that zoledronic acid inhibits in vivo angiogenesis and thus cell proliferation by preventing activation of Rac1. In addition, in

Figure 2.
In vivo therapeutic efficacy of zoledronic acid (ZA) and nab-paclitaxel. Mice bearing HeyA8-MDR ovarian tumor and treated with ZA (1 mg/kg BW/intraperitoneally), nab-paclitaxel (10 mg/kg BW/i.v.), or both for 5 weeks exhibited lower tumor weight (A), number of nodules (B), tumor weight single distribution (C), CD31-positive cells (D), and Ki67 index (E) compared with treatment with saline solution. Means ± SD. *, P < 0.05; **, P < 0.001; ***, P < 0.0001. NS, no significant versus saline solution, n = 10 mice per group.

Figure 3.
Rac1 inactivation by zoledronic acid (ZA) in vivo. Representative images of immunohistochemical staining for active Rac1, showing inactivation of Rac1 by ZA in OVCAR-5 (A) and HeyA8-MDR (B) in vivo models. Means ± SD. ***, P < 0.0001. NS, not significant versus saline solution, n = 10 mice per group.
both in vivo models, a significant \( P < 0.0001 \) decreased in the expression of active Rac1 was observed when we compared the mice treated with the combination of zoledronic acid and nab-paclitaxel with nab-paclitaxel alone but not in the mice treated with zoledronic acid alone, suggesting that the prevention of Rac1 activation occurs through the effect of zoledronic acid treatment.

Zoledronic acid inhibits tube formation and invasion in vitro

To examine the effect of zoledronic acid on angiogenesis in vitro, we performed tube formation and invasion assays as well as Luminex assay to determine the levels of MMP and angiogenic factors. To assess tube formation, EC-RF24 cells were treated with 5, 10, 50, and 100 nmol/L zoledronic acid for 72 hours. After 6 hours of incubation in Matrigel, we observed a significant dose-dependent decrease in the number of nodes in the endothelial cells treated with zoledronic acid compared with the number of nodes in the untreated cells (Fig. 4A), indicating that zoledronic acid inhibits in vitro angiogenesis. Because we also observed a reduction on angiogenesis in vitro by nab-paclitaxel, we examined the effect of nab-paclitaxel on angiogenesis in vitro by tube formation assay. EC-RF24 cells were treated with 5, 10, 50, and 100 nmol/L nab-paclitaxel for 72 hours. After 6 hours of incubation in Matrigel, we observed a significant dose-dependent decrease in the number of nodes in the cells treated with nab-paclitaxel compared with the number of nodes in the untreated cells (Supplementary Fig. S2), indicating that nab-paclitaxel inhibits in vitro angiogenesis.

To determine the effect of zoledronic acid on ovarian cancer invasion, we treated HeyA8-MDR cells with 10 and 50 \( \mu \text{mol/L} \) zoledronic acid and OVCAR-5 cells with 60 and 100 \( \mu \text{mol/L} \) zoledronic acid. After 72 hours, cells were harvested and counted for the invasion assay. In HeyA8-MDR cells, we observed that at 10 and 50 \( \mu \text{mol/L} \) of zoledronic acid the percentages of invading cells were 40.8\% \( (P < 0.0001) \) and 10.2\% \( (P < 0.0001) \), respectively, compared with untreated cells (Fig. 4B). In the presence of 60 and 100 \( \mu \text{mol/L} \) zoledronic acid, the percentages of invading OVCAR-5 cells were 36.5\% \( (P < 0.0001) \) and 17.9\% \( (P < 0.0001) \), respectively, compared with the untreated cells (Fig. 4C). These results indicate that zoledronic acid decreases the invasiveness of both ovarian cancer cell lines in a dose-dependent manner.

Zoledronic acid inhibits ovarian cancer angiogenesis through the prevention of Rac-1 activation in vitro

RPPA analysis was used to study proteins that were affected in HeyA8-MDR after treatment with 10 and 50 \( \mu \text{mol/L} \) zoledronic acid. For assessing the significance of protein expression change, \( t \) test was performed. Significantly changed proteins were analyzed with Ingenuity Pathway Analysis (IPA) to find pathways and molecular functions. The RPPA showed a significant decrease in phospho-p38 \( (P < 0.05) \) in treated cells compared with nontreated cells (Fig. 5A). There was also a significant decrease in the levels of proteins related with angiogenesis such as paxillin, PI3K, and EGFR \( (P < 0.05) \) and proteins related to apoptosis such as cleaved caspase-7 and -9 as it is shown in (Supplementary Fig. S3). However, IPA showed that p38 is related to the Rac1 pathway (Fig. 5B); p38 MAPK plays a role in invasion, migration (21, 22), and angiogenesis (23, 24). On the basis of these data, we focused and started to validate by Western blotting the proteins involved in the Rac1/p38 pathway shown in blue color in Fig. 5B. First, we performed an active Rac1 pull-down in HeyA8-MDR cells. We found the absence of active Rac1 in the samples treated with GDP (negative control) and the
presence of active Rac1 in the samples to which we added GTP (positive control). In the samples without any control, we found a decrease in active Rac1 in HeyA8-MDR (Fig. 6A). Then, we detected the levels of p21-activated kinase 1 (Pak1), a potential downstream mediator of Rac1 (12, 25). We observed a decrease in the levels of phospho-Pak1 in the HeyA8-MDR cell line after treatment with zoledronic acid (Fig. 6B). Pak1 has been reported to activate the p38 MAPK pathway (25, 26). We next determined the levels of phospho-p38 (Fig. 6C), and we found a decrease in the levels of phospho-p38. p38 is reported to mediate MMP-2
activity (27, 28). We finally determined by Luminex assay MMP-2 secretion in HeyA8-MDR treated with zoledronic acid. We found a reduction in the secretion of MMP-2 in HeyA8-MDR (Fig. 6D). These data suggest that zoledronic acid inhibits the production of angiogenic factors in HeyA8-MDR by preventing the activation of the Rac1/Pak1/p38/MMP-2 pathway.

The most differentially expressed proteins in HeyA8-MDR cells after treatment with zoledronic acid have their biologic functions in cellular development, growth, and proliferation; cell death and survival; cell cycle; cellular movement, assembly and organization; cellular function, maintenance, and cell morphology (Supplementary Fig. S4).

Zoledronic acid inhibits angiogenic factors in vitro

Angiogenic factors were analyzed by Luminex assay, zoledronic acid significantly decreased the levels of VEGF-C in HeyA8-MDR cells (Supplementary Fig. S5A) and in OVCAR-5 cells significantly decreased the levels of VEGF-C, HGF, IL8, G-CSF, follistatin, and PLGF (Supplementary Fig. S5B). These data suggest that zoledronic acid decreases the secretion of important angiogenic factors in HeyA8-MDR and OVCAR-5 cells.

Detection of increased levels of unprenylated Rap1A is a surrogate measurement of NBP uptake by cells in vitro and of inhibition of prenylation (29–31). To confirm that the inhibition of angiogenesis occurs through the effect of zoledronic acid, we measured the levels of unprenylated Rap1A by Western blotting. The level of unprenylated Rap1A in HeyA8-MDR increased as zoledronic acid concentration increased (by 497.9% at 10 μmol/L and by 608% at 50 μmol/L compared with untreated cells); similar increases were obtained in OVCAR-5 cells (by 436.1% at 60 μmol/L zoledronic acid and by 666.6% at 100 μmol/L compared with untreated cells; Supplementary Fig. S5C).

To demonstrate the relevance of zoledronic acid–mediated prenylation inhibition, we studied 2 additional chemotherapy-resistant cell lines A2780 CP20 and SKOV3-TR, cisplatin- and taxol-resistant cells, respectively. We first demonstrated the inhibition of prenylation by zoledronic acid in a dose-dependent manner in both cell lines (Supplementary Fig. S5D). Decreased invasion was observed at similar concentrations in both cell lines (Supplementary Fig. S6A and S6B). These data demonstrated that inhibition of prenylation is a key factor in reduction of invasiveness in chemoresistant cells.

To examine the effect of Rac1 on angiogenesis in vitro, we transfected EC-RF24 cells with control and Rac1 siRNA for 48 hours to perform tube formation. After 6-hour incubation of cells in Matrigel, we observed a significant decrease in the number of nodes in the endothelial cells treated with Rac1 siRNA compared with the control cells (Supplementary Fig. S6C), indicating that Rac1 is required for endothelial cell tube formation. Western blotting was performed to verify Rac1 silencing (Supplementary Fig. S6D).

Discussion

The key findings from this study are that zoledronic acid has potent antitumor and antiangiogenic effects in ovarian cancer models and that these effects are mediated by the Rac1/Pak1/p38/MMP-2 pathway in HeyA8-MDR ovarian cancer cells. Pak1 has been reported as an upstream kinase that activates p38 (25, 32), those reports are consistent with our RPPA and Western blotting findings that zoledronic acid decreased levels of Pak1 and p38.

MMPs are a family of proteolytic enzymes that degrade components of the extracellular matrix (ECM; refs. 33, 34). MMPs have been implicated in cancer cell growth, differentiation, apoptosis, migration and invasion, and angiogenesis (33). In the
angiogenesis process, MMPs play an important role not only by degrading basement membrane and other ECM components and facilitating endothelial cell migration but also by releasing ECM-bound proangiogenic factors (b-FGF, VEGF, and TGFβ; ref. 35). Endogenous and synthetic MMP inhibitors have been shown to be potent inhibitors of angiogenesis both in vivo and in vitro (33, 36–38). It has been reported that MMP-2 is required for the switch to the angiogenic phenotype during the development of chondrosarcoma (36). Also, it has been shown that MMP-2 is produced by endothelial cells during tube formation, and the exogenous addition of MMP-2 enhances this process (39). Moreover, it was found that in an Mmp2-deficient mice, the tumor angiogenesis and growth were reduced compared with wild-type mice (40). All of these published data support our data and the important role of MMP-2 on angiogenesis.

Zoledronic acid has antitumor and antiangiogenic effects, but its underlying mechanisms are not fully understood. However, inhibition of the prenylation of GTPases is thought to be the main molecular mechanism involved directly in the antitumor and antiangiogenic effects of NBPs. Therefore, we carried out the blocking of prenylation by zoledronic acid. Our results showed that zoledronic acid increased the levels of unprenylated Rap1A protein and prevented the activation of Rac1 in HeyA8-MDR and OVCAR-5 cell lines; our findings were consistent with findings reported earlier on the zoledronic acid–mediated inhibition of prenylation of small GTPases such as Rac1, process that is required for the activation of GTPases (9, 41, 42).

Rac1 activation requires prenylation and eventual membrane localization (41, 42). Prenylation is a posttranslational modification of the C-terminal CAAX motif (where C is cysteine, A is any aliphatic amino acid, and X is any amino acid) by the addition of farnesyl or geranylgeranyl isoprenoid lipid tail to the cysteine residue. This modification increases protein hydrophobicity facilitating membrane association (41, 42). In our in vitro and in vivo results, we observed that zoledronic acid decreased the expression levels of active Rac1 protein. We did not observe changes in the cellular location of Rac1 protein between control group and the groups treated with zoledronic acid because we used an antibody that specifically recognized the active form of Rac1 (GTP-Rac1), thus the changes occur just in the expression level of Rac1, suggesting that although there are 2 distinct and additional sequence elements upstream of the CAAX motif necessary to the membrane association and activation of the GTPases (42), the inhibition of prenylation prevents the activation of GTPases like Rac1.

In vivo, we observed that zoledronic acid decreased the activation of Rac1 and decreased angiogenesis and cell proliferation. The inhibition of cell proliferation could be the consequence of: (i) angiogenesis inhibition because angiogenesis is absolutely required for tumor growth; (ii) inhibition of Rac1 which is also involved in cell proliferation (43), or (iii) zoledronic acid treatment, we previously showed that zoledronic acid not only has antiangiogenic activity but also antiproliferative and proapoptotic effects (19). In this study, we observed that zoledronic acid induces apoptosis in OVCAR-5 in vivo model (Supplementary Fig. S1A) and in HeyA8-MDR both in vitro and in vivo (Supplementary Fig. S1B–S1D).

When we silenced Rac1 expression, we observed less effect on endothelial tube formation that observed with zoledronic acid treatment. An explanation for this finding could be that zoledronic acid works through several mechanisms including prevention of Rac1 activation, which maybe predominant; however, zoledronic acid also inhibits other proteins involved in angiogenesis like paxillin, PI3K, and EGFR as shown in the heatmap from the RPPA in Supplementary Fig. S3.

Rac1 is a GTPase involved in tumorogenesis, angiogenesis, invasion, and metastasis (12, 13) and is emerging as a potential target for angiogenesis. It has been shown that NSC23766, an inhibitor of Rac1, inhibits migration and reverse cellular phenotypes of trastuzumab resistance in lung (44) and breast cancers (45). However, studies about the efficacy and toxicity of NSC23766 are not available.

Nevertheless, zoledronic acid, the most potent of the NBPs, was already approved by the FDA to treat patients with multiple myeloma or patients with bone metastases from breast cancer or other solid tumors (46, 47). NBPs are associated with side effects such as nephrotoxicity and osteonecrosis; however, we have previously shown that neither toxicity or overt deleterious clinical effects were present after prolonged administration of zoledronic acid in mice (19).

Nab-paclitaxel, an albumin-bound paclitaxel nanoparticle, has a better therapeutic window than paclitaxel in metastatic breast cancer, non–small lung cancer, and pancreatic cancer, where this agent has been approved by the FDA (48, 49). In a phase II evaluation, weekly administration of nab-paclitaxel in highly resistant patients with ovarian cancer showed better efficacy than weekly administration of paclitaxel (49). Other agents such as gossypol have been combined with zoledronic acid, enhancing the induction of apoptosis and the down-regulation of mRNA of angiogenic factors such as FGF, platelet-derived growth factor PDGF), and VEGF (50) in ovarian cancer.

Our data support the important role of zoledronic acid–mediated inhibition on Rac1 activity and of the antiangiogenesis pathway. These findings open the door for the therapeutic use of a nab-paclitaxel plus zoledronic acid combination in ovarian cancer and other solid tumors.

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

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