The Activity of Zoledronic Acid on Neuroblastoma Bone Metastasis Involves Inhibition of Osteoclasts and Tumor Cell Survival and Proliferation

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Abstract

Metastasis to the bone is seen in 56% of patients with neuroblastoma and contributes to morbidity and mortality. Using a murine model of bone invasion, we have reported previously that neuroblastoma cells invade the bone by activating osteoclasts. Here, we investigated the antitumoral and antiosteolytic activities of zoledronic acid, a bisphosphonate inhibitor of osteoclasts, in combination with cytotoxic chemotherapy in our model. We first show that zoledronic acid given at the same time (early prevention) or 2 weeks after tumor cell injection (late prevention) significantly prevented the formation of severe osteolytic lesions. It also prevented formation of these lesions when given 4 weeks after tumor cell injection (intervention) when combined with chemotherapy including cyclophosphamide and topotecan. The combination of zoledronic acid + cyclophosphamide/topotecan also significantly improved survival ($P < 0.001$). In mice treated with zoledronic acid, we observed a marked inhibition of osteoclasts inside the bone associated with a decrease in tumor cell proliferation and increase in tumor cell apoptosis. *In vitro*, zoledronic acid inhibited neuroblastoma cell proliferation and induced apoptosis, and these effects were significantly enhanced by the addition of 4-hydroxyperoxycyclophosphamide (4-HC). The proapoptotic effect of zoledronic acid and zoledronic acid in combination with 4-HC on tumor cells was associated with an increase in caspase-3 activity and a decrease in phosphorylated Bcl-2, Bcl-2, and Bcl-X_L expression. Zoledronic acid inhibited the association of Ras with the plasma membrane and activation of c-Raf, Akt, and extracellular signal-regulated kinase 1/2. The data indicate that zoledronic acid, in addition to inhibiting osteoclasts, is active against tumor cells and suggest that zoledronic acid in combination with cytotoxic chemotherapy may be effective in children with neuroblastoma that has metastasized to the bone. [Cancer Res 2007;67(19):9346–55]

Introduction

Neuroblastoma is a tumor derived from the neural crest and the most common extracranial solid neoplasm in children (1). Although the event-free survival of patients with nonmetastatic and noninvasive tumors (stage I or II) is in the range of 90% to 95%, patients with high-risk disease have only a 3-year event-free survival of 42% when treated with intensive myeloablative chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-*cis*-retinoic acid (2). Metastasis at diagnosis is common in patients with neuroblastoma and sites most frequently involved include bone marrow, bone, lymph nodes, liver, and intracranial and orbital sites. Neuroblastoma rarely metastasizes to the lungs or the brain (3).

The mechanisms involved in the formation of bone metastasis in neuroblastoma have now begun to be elucidated. It is documented that, as observed in breast cancer and multiple myeloma, neuroblastoma bone metastases are predominantly osteolytic (4, 5). The formation of osteolytic lesions by metastatic neuroblastoma cells involve the recruitment and activation of osteoclasts, which can occur through two mechanisms (6). A first mechanism consists of the direct activation of osteoclasts by neuroblastoma cells. For example, when cultured in the presence of murine bone marrow cells, neuroblastoma cells express the receptor activator of nuclear factor-κB ligand (RANKL), a potent stimulator of RANK-expressing osteoclasts. This effect is abrogated in the presence of the RANKL inhibitor, osteoprotegerin (soluble RANK or OPG; refs. 7, 8). This mechanism of osteoclast activation is used by breast cancer cells as they metastasize to the bone (9, 10). A second mechanism reported recently by our laboratory involves the contribution of bone marrow–mesenchymal stem cells (BM-MSC), which in the presence of neuroblastoma cells express interleukin-6 (IL-6), a potent stimulator of osteoclasts (11). This mechanism was also reported in myeloma cells, which on contact with bone marrow stromal cells stimulate IL-6 expression (12), but in neuroblastoma, stimulation of IL-6 expression does not require cell-cell contact. At the convergence of these two mechanisms are the activation of osteoclasts and the degradation of mineralized bone (13).

Bisphosphonates are a family of synthetic compounds, whose structure is derived from pyrophosphoric acid, which binds with high affinity to the bone matrix (14). These compounds are potent inhibitors of osteoclast activity that are currently used in the clinic for a variety of benign and malignant conditions associated with increased bone resorption, such as postmenopausal osteoporosis (15), malignant hypercalcemia (16), and bone metastasis (17). Bisphosphonates have been proposed as potential therapeutic agents in bone metastasis in patients with breast, prostate, and myeloma tumors, and clinical trials have shown efficacy and minimal systemic toxicity (18). Among the most potent bisphosphonate compounds are nitrogen-containing bisphosphonates

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(N-BP), which exert their effect on cells by inhibiting farnesyl diphosphate synthase, a key enzyme in the prenylation of proteins (19). Zoledronic acid is a N-BP that has been approved recently by the Food and Drug Administration for the treatment of bone metastases in patients with breast and prostate cancers (20).

Here, we have tested the activity of zoledronic acid given in combination with cytotoxic chemotherapy including cyclophosphamide and topotecan, in a preclinical xenotransplanted model of neuroblastoma bone invasion. The data show that zoledronic acid is not only a potent inhibitor of osteoclasts but also has a direct inhibitory activity on tumor cell proliferation and survival and that when used in combination with chemotherapy, it significantly prolongs survival.

Materials and Methods

Cell cultures. The human neuroblastoma cell lines CHLA-255 and SMS-SAN obtained from C.P.R. were grown in Iscove's modified Dulbecco's medium (Bio-Whittaker) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 μg/mL streptomycin sulfate (Irvine Scientific). Cells were passaged approximately every 5 days by trypsinization. CHLA-255 Luc20 cells were obtained after transfection of CHLA-255 cells with a lentiviral vector containing a *firefly* luciferase reporter gene obtained from Dr. D. Kohn (Children's Hospital Los Angeles, Los Angeles, CA). This clone was derived from stable transfected cells selected first *in vitro* and then *in vivo* after intraosseous injection into the femur of an immunodeficient mouse (21).

Bone invasion model. BALB/c *nu/nu* mice purchased from Harlan Sprague-Dawley were anesthetized with 2.5% isoflurane in 97.5% O_2 and tumor cells (2×10^5 cells suspended in 2 μL of serum-free culture medium) were injected locally into the bone marrow cavity of the right femur as described previously (6). Mice were then monitored weekly by doing radiographs of both femurs using a Faxitron X-ray instrument (MX-20, Faxitron X-ray Corp.). These high-resolution images were then blindly evaluated by a radiologist (M.D.N.) and graded according to a preestablished grading system in which a grade 1 lesion represents a normal bone, a grade 2 lesion indicates an asymmetric, nonprogressive radiolucent lesion of the distal femur typically related to the trauma, a grade 3 lesion represents an asymmetrical and progressive osteolytic lesion extending beyond the distal femur, and a grade 4 lesion indicates the presence of a pathologic fracture or a cortical breach in the bone cortex (6). Mice showing signs of distress or developing a local extraosseous tumor were sacrificed by CO_2/O_2 euthanasia. When indicated, mice were injected i.p. 1 h before sacrifice with 2 mg bromodeoxyuridine (BrdUrd). This protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Children's Hospital Los Angeles.

Bioluminescence imaging. Mice were anesthetized and injected i.p. with 0.3 mL of D-luciferin (5 mg/mL) in sterile PBS. Bioluminescence imaging was initiated 15 min after injection using a charge-coupled device camera (IVIS, Xenogen). Images were obtained with a 15-cm field of view, a binning (resolution) factor of 10, 4/f stop, and open filter (21).

***In vivo* treatments.** Zoledronic acid was obtained from Novartis Pharma AG and maintained as a stock solution at a concentration of 40 μg/mL in sterile 0.9% NaCl. Mice were injected s.c. with 4 μg (100 μL) zoledronic acid 5 days/wk. Treatment with zoledronic acid was started either at the time of tumor cell injection (early prevention), 2 weeks after tumor cell injection when mice showed no evidence of progressive osteolytic lesions (late prevention), or 4 weeks after tumor cell injection when mice had developed grade 3 or 4 osteolytic lesions (intervention). Cyclophosphamide was diluted fresh in sterile PBS at a concentration of 31.25 mg/mL and given i.p. daily at 156 mg/kg (3.1 mg/mouse) for 5 days. Topotecan was diluted in sterile PBS at a concentration of 0.15 mg/mL and given i.p. daily at 0.75 mg/kg (0.015 mg/mouse) for 5 days.

Histology and histochemistry. The entire leg of the sacrificed mouse was harvested en bloc and fixed overnight in 10% formaldehyde containing 2% sucrose and decalcified in 0.37% unbuffered formaldehyde containing 5.5% EDTA (pH 6.0) for 1 to 2 weeks. When decalcified, the samples were embedded in paraffin and 5-μm sections were obtained and stained by H&E. Detection of osteoclasts was done by staining for tartrate-resistant acid phosphatase (TRAP) using leukocyte acid phosphatase (Sigma) at 37°C for 1 h and counterstaining with H&E as published previously (6). To quantify the amount of osteoclasts present in the femur, we delineated the surface of the bone and the surface covered by TRAP-positive cells on digital images from histologic sections stained for TRAP. The percentage of the bone surface covered with TRAP-positive cells was calculated using NIH image software (version 1.62). Staining for nuclear BrdUrd was done by immunohistochemistry on histologic sections that were treated with 0.01% trypsin for 3 min at 37°C. Slides were incubated with a murine anti-BrdUrd monoclonal antibody (dilution, 1:2,000; BrdUrd Labeling and Detection kit, Roche Diagnostic Corp.) at 4°C overnight. After washing with PBS, the slides were incubated with a biotinylated goat anti-mouse IgG secondary antibody for 1 h at room temperature. Streptavidin peroxidase-conjugated (ABC Elite, Vector Laboratories) was then applied and the reaction was developed in 3,3′-diaminobenzidine (Sigma). The percentage of BrdUrd-positive cells was determined by counting the number of cells with nuclear stain in six fields (×40 magnification) of three separate histologic sections. Counting was done blindly by two independent investigators.

Proliferation assay *in vitro*. To examine the effect of zoledronic acid on cell proliferation *in vitro*, cells were cultured in eight-chamber culture slides for 48 h (Nalge Nunc International) to reach 70% confluence. The cells were then incubated with 10 μmol/L BrdUrd for 30 min at 37°C, before being fixed in methanol, and stained for BrdUrd using a BrdUrd Labeling and Detection kit according to the instructions of the manufacturer. Counting of BrdUrd-positive cells was done blindly by two independent observers.

Detection of apoptosis. Apoptosis in histologic sections was examined by immunohistochemistry using the *In situ* Cell Death Detection kit (Roche Diagnostic). Paraffin-embedded tissue sections were treated with proteinase K (10 μg/mL) in 10 mmol/L Tris-HCl (pH 7.5) at 37°C for 20 min followed by blocking in 10% goat serum and 0.2% Triton X-100 for 1 h. The slides were then incubated with terminal deoxynucleotidyl transferase and a nucleotide mixture at 37°C for 1 h before being incubated with a horseradish peroxidase (HRP)–conjugated anti-alkaline phosphatase antibody at room temperature for 30 min. After counterstain with hematoxylin, the number of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive cells in six fields (×40 magnification) of three individual histologic sections was blindly counted by two independent investigators. For apoptosis, *in vitro* cells treated with zoledronic acid for 24 h in serum-containing medium were harvested, fixed with 1% paraformaldehyde, washed with PBS, and kept overnight in 70% ethanol at −20°C. The cells were then stained using the TUNEL APO-Direct kit (BD Biosciences). Analysis was done by flow cytometry using a FACScan flow cytometer (Beckman Coulter Epics Elite ESP) and Expo 32 software for quantitative analysis (Applied Cytometry Systems). Caspase-3 and caspase-8 activities were determined in cell lysates using caspase-3/CPP32 and caspase-8/CPP32 colorimetric protease assay kits (Biosource International) according to the instructions of the manufacturer. Readings were done at 405 nm in a Synergy HT microplate reader (Bio-Tek Instruments).

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] containing a cocktail of protease inhibitors (Roche Diagnostic). When indicated, cell membranes were separated from the cytosol using the FOCUS Membrane Protein Extraction kit (Geno Technology, Inc.). After incubation for 15 min and centrifugation, the protein concentration in the supernatant was measured by a BCA Protein Quantification kit (Pierce). Twenty micrograms of each sample were then loaded on 0.1% SDS and 4% to 12% gradient polyacrylamide gels (Invitrogen). After electrophoresis, gels were blotted on a nitrocellulose membrane (Bio-Rad), and the membranes were probed with one of the following antibodies obtained from Cell Signaling Technology, Inc.: a rabbit polyclonal anti–phosphorylated Bcl-2 (phospho-Bcl-2) antibody (dilution, 1:1,000); a rabbit polyclonal anti-Bcl-2 antibody (dilution, 1:1,000); a rabbit polyclonal anti-Bcl-X_L antibody (dilution, 1:1,000); a rabbit polyclonal

anti-Bax antibody (dilution, 1:1,000); a rabbit polyclonal anti-Bad antibody (dilution, 1:1,000); a rabbit polyclonal anti-Ras antibody (dilution, 1:1,000); a mouse monoclonal anti–phosphorylated c-Raf (phospho-c-Raf) antibody (dilution, 1:1,000); a mouse monoclonal anti–phosphorylated Akt (phospho-Akt) antibody (dilution, 1:1,000); a mouse monoclonal anti-Akt antibody (dilution, 1:1,000); a mouse monoclonal anti–phosphorylated extracellular signal-regulated kinase 1/2 (phospho-ERK1/2) antibody (dilution, 1:1,000); a rabbit polyclonal anti-ERK1/2 antibody (dilution, 1:1,000); a rabbit polyclonal anti–caveolin-1 antibody (dilution, 1:1,000); and a rabbit monoclonal anti-actin antibody (dilution, 1:2,500) as control for protein loading. After overnight incubation with the primary antibody at 4°C, the membranes were washed thrice with TBS-Tween and blotted with a HRP-conjugated antimouse or antirabbit IgG antibody (dilution, 1:2,000) at room temperature for 1 h. The immunoreactive bands were detected using an enhanced chemiluminescence plus detection system (Amersham Biosciences).

Statistical analysis. In each of the *in vivo* experiments, 5 or 10 mice were used for each experimental condition; 10 animals were evaluated if the outcome was time to developing a grade 4 lesion or survival, except on occasion in a control group where only 5 animals were used. The log-rank test was used to compare the animals according to the treatment groups. Pair-wise comparisons of the treatment groups were also done using the log-rank test, if the overall test was significant at the 0.05 level; the Pike estimates of relative risks were calculated with the use of observed and expected number of events as calculated in the log-rank test. The life table method was used to estimate the probability of remaining free of grade 4 lesions or survival, using a weekly interval. ANOVA was used to investigate the effect of cyclophosphamide/topotecan and zoledronic acid on cell proliferation and apoptosis *in vivo*. The interaction effects were also tested. The least significant difference method was used for pair-wise comparisons, once the overall F test was significant at the 0.05 level. The 95% confidence interval was calculated for each treatment group. Triplicate samples were used in each treatment group for the effect of cyclophosphamide/topotecan and zoledronic acid on cell proliferation and apoptosis. The analysis of dose effects of zoledronic acid on cell proliferation and apoptosis in CHLA-255 neuroblastoma cells *in vitro* was done using a logistic growth (nonlinear) model. Three replicates were used at each dose. All reported P values are two sided.

Results

Zoledronic acid inhibits the formation of osteolytic lesions in mice locally injected with neuroblastoma cells. To test the inhibitory effect of zoledronic acid on the formation of osteolytic lesions by neuroblastoma cells *in vivo*, we injected *nu/nu* mice (10 mice in each group) with 2×10^5 CHLA-255 or SMS-SAN human neuroblastoma cells into the bone marrow cavity of the femur and treated them with either zoledronic acid or PBS (control vehicle). Mice were treated with zoledronic acid (4 μg; 5 days/wk) either immediately after injection (early prevention) or 2 weeks after injection of the tumor cells (late prevention). High-resolution

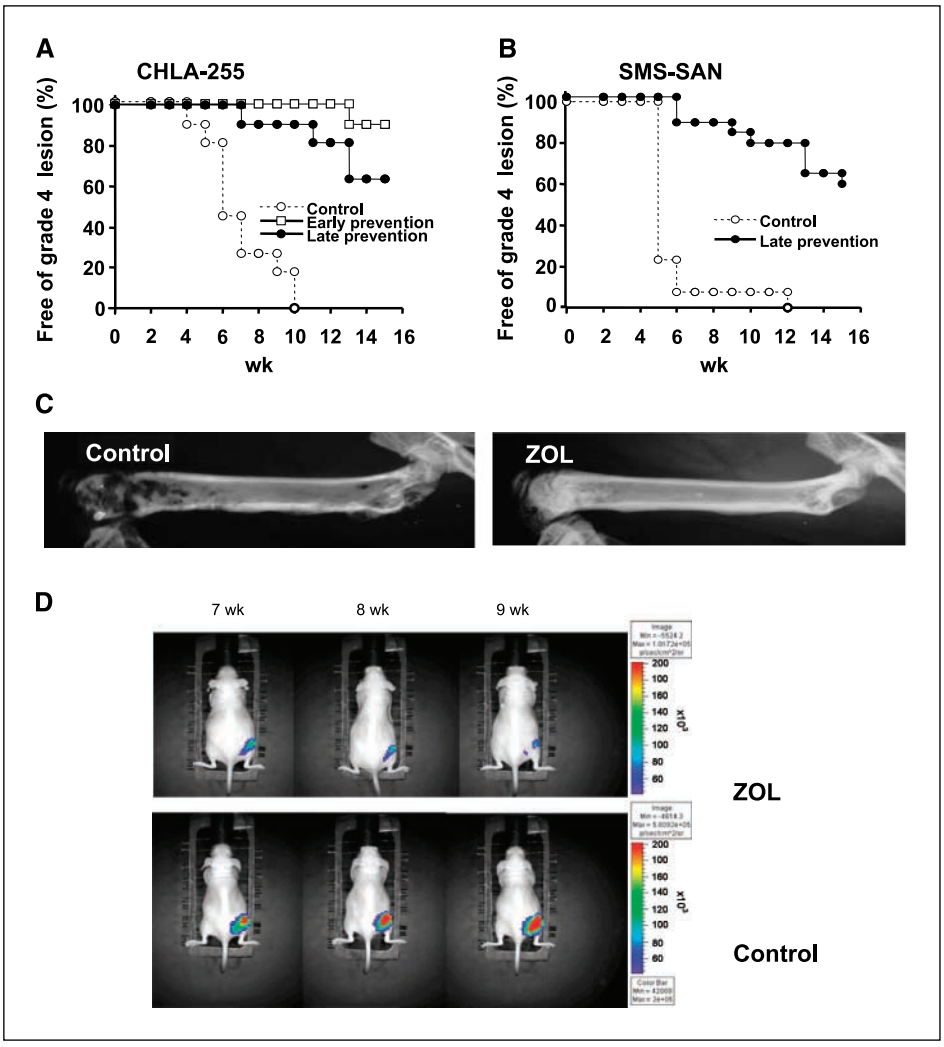

Figure 1. Effect of zoledronic acid (*ZOL*) on the prevention of osteolytic lesions in mice injected into the femur with neuroblastoma cells. *A* and *B,* life table method was used to estimate the percentage of mice free of grade 4 lesions in each experimental group ($n = 10$). *C,* representative radiographs of the femur obtained 4 wk after tumor cell implantation in control and treated mice. *D,* D-luciferin–induced bioluminescence obtained in two mice 7, 8, and 9 wk after intrafemoral injection of CHLA-255 Luc20 cells and treated with zoledronic acid or 0.9% NaCl (control). The images are representative of seven mice in the zoledronic acid group and six mice in the control group.

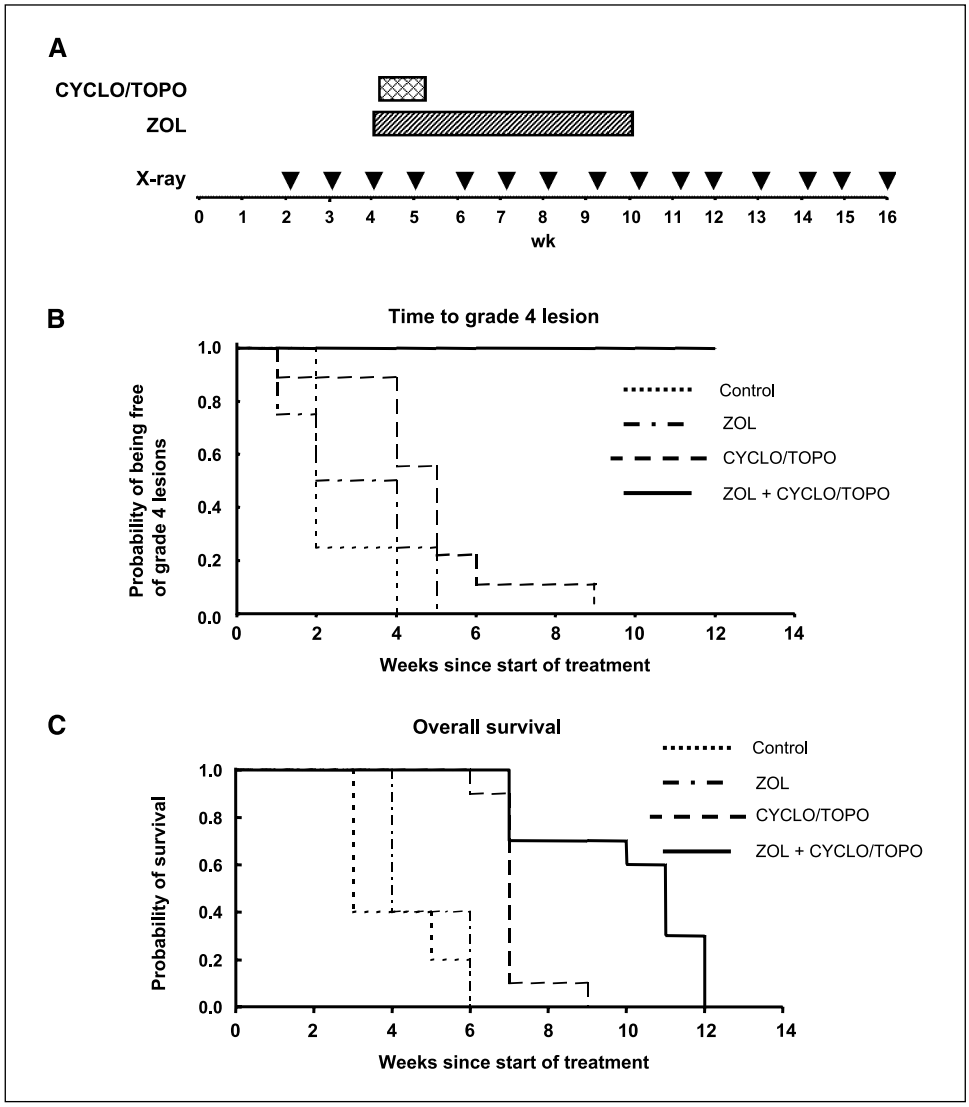

Figure 2. Effect of zoledronic acid, cyclophosphamide (*CYCLO*)/topotecan (*TOPO*), and their combination on the formation of osteolytic lesions and on the survival in mice injected with CHLA-255 cells. *A,* protocol for the administration of the therapeutic agents. *B,* life table estimates of the percentage of mice free of grade 4 lesions in the three intervention treatment groups and the control group. *C,* life table estimates of the time to death or sacrifice in the four experimental groups. The statistical analysis of outcomes is shown in Supplementary Table S1.

radiographs of the femurs were obtained every week and the time to develop severe osteolytic lesions characterized by a cortical breach or pathologic fracture (grade 4 lesion) was the end point used to evaluate the effect of the treatment (6). Mice were sacrificed by CO_2/O_2 euthanasia when they had developed a grade 4 lesion. In the control group, 100% of the mice developed grade 4 lesions by 10 (CHLA-255 cells; Fig. 1*A*) or 12 (SMS-SAN cells; Fig. 1*B*) weeks after injection of tumor cells. In contrast, 60% of mice injected with CHLA-255 or SMS-SAN cells in the late prevention groups and 90% of the mice injected with CHLA-255 in the early prevention group remained free of grade 4 lesions up to 15 weeks after implantation of tumor cells. The radiological evaluation of the femur of the mice treated with zoledronic acid consistently revealed a marked increase in bone density in the cortex and the diaphysis of the bone (Fig. 1*C*). The effect of zoledronic acid on tumor cells in this model was also evaluated using CHLA-255 Luc20 cells that express the *firefly* luciferase and D-luciferin–induced bioluminescence. This experiment indicated a marked decrease in D-luciferin–induced bioluminescence in mice treated with zoledronic acid (early prevention) when compared with the control group (Fig. 1*D*) between 7 and 9 weeks after tumor cell implantation. The data suggest that zoledronic acid has not only a potent inhibitory activity on bone resorption but also an antitumor effect as indicated by its inhibitory activity on D-luciferin–induced bioluminescence.

Combination of zoledronic acid and cyclophosphamide/topotecan improves survival in mice with established osteolytic lesions. We then determined if zoledronic acid would have a therapeutic effect when used in an intervention protocol in mice who had developed osteolytic lesions, a situation that more closely mimics its potential clinical use in neuroblastoma patients with established bone metastases. We did an experiment in which the treatment of mice injected into the femur with CHLA-255 cells was delayed until 4 weeks after injection of tumor cells, a time when all animals had developed at least a grade 3 lesion (radiological evidence of osteolysis without breach in the cortex). In this experiment, we compared treatment with zoledronic acid alone (n = 5) with a 5-day treatment with cyclophosphamide and topotecan (n = 10) and with a combination of both zoledronic acid and cyclophosphamide/topotecan (n = 10; Fig. 2*A*). In the control group (n = 10), mice were treated with a sterile s.c. injection of 0.9% NaCl and i.p. injection of PBS. To determine the effect of

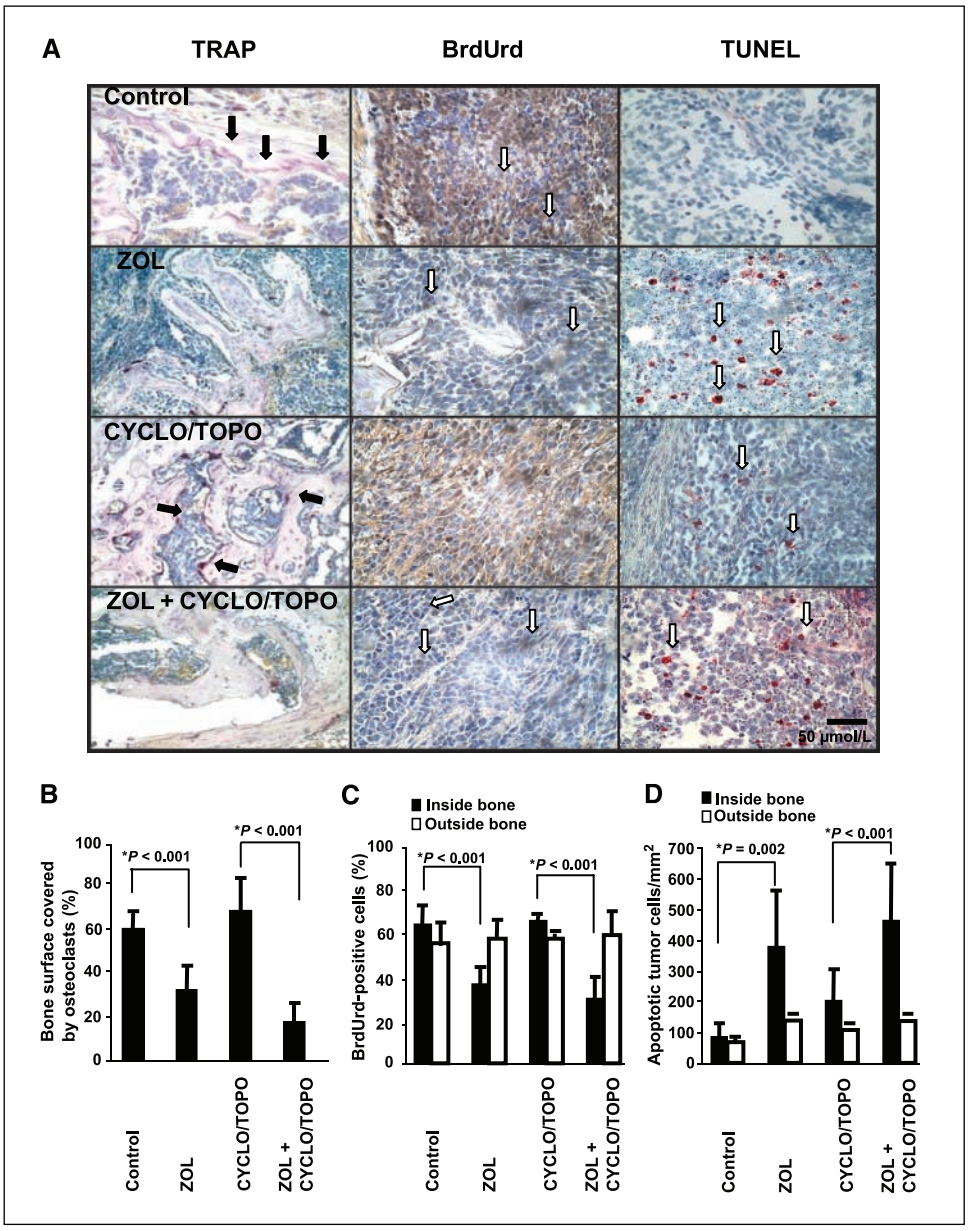

Figure 3. Effect of zoledronic acid on osteoclast formation and on tumor cell proliferation and apoptosis in mice. *A,* representative histologic sections stained for TRAP, BrdUrd, and TUNEL in tumors obtained in mice 10 wk after injection of CHLA-255 cells in the femur. Treatment was started 4 wk after tumor cell injection as shown in Fig. 2*A*. *Black arrows,* osteoclasts; *white arrows,* BrdUrd- and TUNEL-positive neuroblastoma cells. *B,* quantitative analysis of the percentage of bone surface covered by osteoclasts. *Columns,* mean percentage of the surface covered with TRAP-positive cells from a total of four sections examined; *bars,* SD. *C,* quantitative analysis of the percentage of BrdUrd-positive tumor cells inside (*black columns*) and outside (*white columns*) the bone. *Columns,* mean percentage of BrdUrd-positive cells in 12 histologic sections; *bars,* SD. *D,* quantitative analysis of apoptotic tumor cells inside (*black columns*) and outside the bone (*white columns*). *Columns,* mean number of TUNEL-positive cells per millimeter square of 12 histologic sections; *bars,* SD.

these treatments on survival, in addition to an effect on the progression of radiological lesions, the animals were kept alive, closely monitored, and euthanized when they had developed an extraosseous tumor that impaired movement or when they became moribund. When examining the effect on bone degradation (Fig. 2*B*), we observed that the combination treatment group (zoledronic acid + cyclophosphamide/topotecan) had a significant lower risk of developing grade 4 lesions than either the control or the two single-treatment groups (all $P < 0.001$). The risk of developing grade 4 lesions was not significantly different between the two single-treatment groups ($P = 0.14$), although there was a trend that cyclophosphamide/topotecan was better than zoledronic acid alone as shown by the relative hazard ratios (Supplementary Table S1). When examining the effect on overall survival, the effect of treatment with zoledronic acid alone was not significantly different from control ($P = 0.32$; Fig. 2*C*), but both cyclophosphamide/topotecan and zoledronic acid + cyclophosphamide/topotecan had a significant effect on survival with the combination having the greatest effect on overall survival ($P < 0.0011$) decreasing the relative hazard ratio from 0.26 to 0.15 (Supplementary Table S1) and extending the 50% survival time from 7 to 11 weeks since the beginning of the treatment (Fig. 2*C*). In this group of mice, treated with zoledronic acid and cyclophosphamide/topotecan, all animals had developed a tumor outside the bone invading the adjacent muscle without radiological evidence of a grade 4 lesion at the time of death or sacrifice. The data indicate that treatment with zoledronic acid alone is of little therapeutic benefit when initiated in mice with established osteolytic lesions but has a significant effect when used in combination with cytotoxic agents, such as cyclophosphamide and topotecan, in preventing the progression of osteolytic lesions and prolonging overall survival.

Zoledronic acid inhibits proliferation and increases apoptosis in tumor cells inside the bone. To assess the effect of these

treatments on osteolysis, tumor cell proliferation, and apoptosis, we did a third experiment in which mice (n = 5 in each group) treated with the intervention protocol were sacrificed 10 weeks after intraosseous tumor cell injection (therefore 6 weeks after treatment was initiated) or earlier if they had to be euthanized. The femurs were then examined by immunohistochemistry for the presence of osteoclasts, tumor cell proliferation (BrdUrd incorporation), and apoptosis (TUNEL; Fig. 3*A*). At the time of sacrifice in the majority of the mice, the tumor had directly extended outside the bone into the adjacent muscles. This tumor was resected en bloc with the femur and similarly examined for tumor cell proliferation and apoptosis. Consistent with the anticipated inhibitory activity of zoledronic acid on osteoclasts, we observed a significant decrease in the number of osteoclasts present inside the femoral bone of mice treated with zoledronic acid or with zoledronic acid + cyclophosphamide/topotecan when compared with the control group or the cyclophosphamide/topotecan group (P < 0.001; Fig. 3*B*). There was also a significant decrease in the number of BrdUrd-positive tumor cells inside the femur of mice treated with zoledronic acid or a combination of zoledronic acid + cyclophosphamide/topotecan when compared with the control group or the cyclophosphamide/topotecan group (P < 0.001; Fig. 3*C*). Interestingly, zoledronic acid had no effect on BrdUrd incorporation by tumor cells outside the bone (P = 0.18). We also observed a significant increase in the number of apoptotic tumor cells in the femur of mice treated with zoledronic acid (alone or in combination) when compared with mice treated with PBS control (P = 0.002 and P < 0.001, respectively; Fig. 3*D*). As was the case for proliferation, there was no difference in the number of TUNEL-positive tumor cells among the four groups in the sections of the tumors that had extended outside the femur (Fig. 3*D, white columns*). The inhibition of tumor cell proliferation and increase in tumor cell apoptosis observed inside the bone of mice treated with zoledronic acid was consistent with the inhibition of D-luciferin–induced bioluminescence observed in our previous experiment (Fig. 1*D*) and suggested that zoledronic acid could have a direct effect on tumor cell proliferation and survival. The absence of effect of cyclophosphamide/topotecan alone on tumor cell proliferation and apoptosis was due to the fact that the analysis was done 6 weeks after administration of chemotherapy when mice had recovered from cytotoxicity. This analysis therefore could not evaluate whether the administration of cyclophosphamide/topotecan would add to the toxic effect of zoledronic acid.

Zoledronic acid inhibits proliferation and stimulates apoptosis in CHLA-255 neuroblastoma cells. To test the possibility that zoledronic acid could have a direct antiproliferative and proapoptotic effect on tumor cells, we examined the effect of zoledronic acid on the proliferation and apoptosis of CHLA-255 neuroblastoma cells *in vitro*, by exposing cultured cells to zoledronic acid and measuring the effect on BrdUrd incorporation (proliferation) or TUNEL positivity (apoptosis). We observed a dose-dependent inhibition of BrdUrd incorporation by CHLA-255 cells treated with zoledronic acid at concentrations between 12.5 and 50 μmol/L (P < 0.001; Fig. 4*A*). Treatment of the cells with zoledronic acid also significantly increased the number of TUNEL-positive cells within the same range of concentrations (P < 0.001; Fig. 4*B*). The addition of 4-hydroxyperoxycyclophosphamide (4-HC; 1 μg/mL), an active derivative of cyclophosphamide, to zoledronic acid (12.5 μmol/L) significantly further decreased BrdUrd incorporation (Fig. 4*C*) and increased the percentage of apoptotic tumor cells (Fig. 4*D*). In these two assays, the combination had a stronger effect than any agent used alone, which is consistent with our *in vivo* experiments (Fig. 2). These data thus show that, at micromolar concentrations, zoledronic acid inhibits tumor cell proliferation and induces apoptosis in tumor cells and that this antitumor activity is enhanced in the presence of a cytotoxic agent like cyclophosphamide.

Zoledronic acid increases caspase-3 activity and inhibits Bcl-2. The effect of zoledronic acid on apoptosis was further examined by measuring its effect on caspase-3 and caspase-8

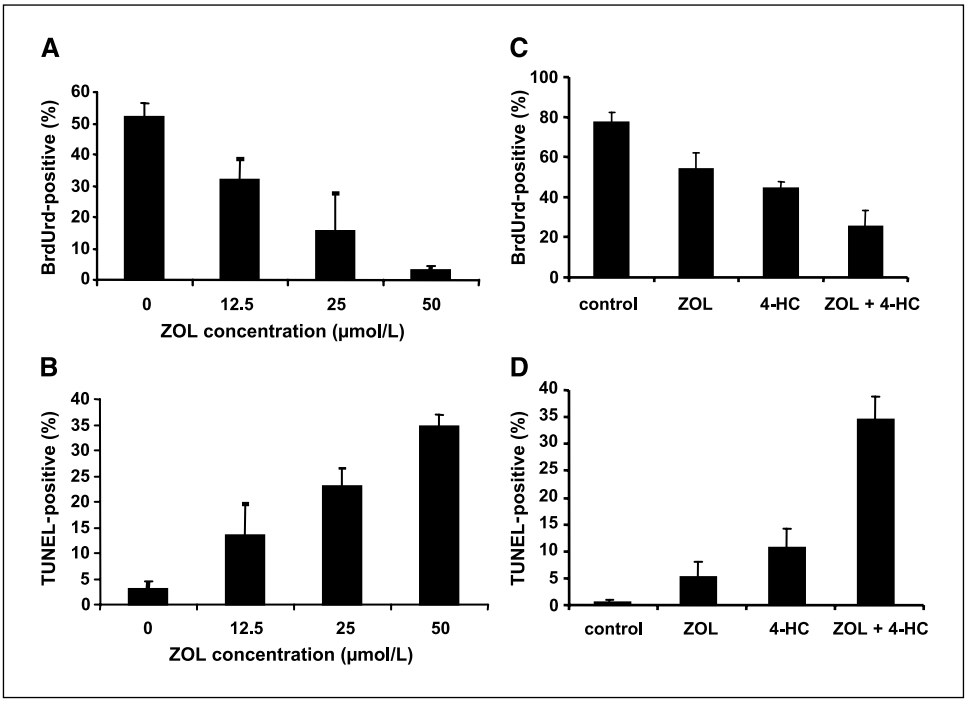

Figure 4. Zoledronic acid alone and in combination with 4-HC inhibits proliferation and induces apoptosis in CHLA-255 neuroblastoma cells *in vitro. A,* cells were exposed to zoledronic acid for 24 h and BrdUrd for 30 min before being examined. *Columns,* mean percentage of BrdUrd-positive tumor cells from a total of three separate tissue culture dishes; *bars,* SD. *B,* cells were treated with zoledronic acid for 24 h before being harvested and examined by fluorescence-activated cell sorting (FACS) for TUNEL positivity. *C* and *D,* similar experiments as in (*A* and *B*) were done by exposing CHLA-255 cells to zoledronic acid (12.5 μmol/L), 4-HC (1 μg/mL), or a combination of both. Representative of two independent experiments showing similar results. *Columns,* mean percentage of tumor BrdUrd-positive (*C*) and TUNEL-positive (*D*) cells; *bars,* SD.

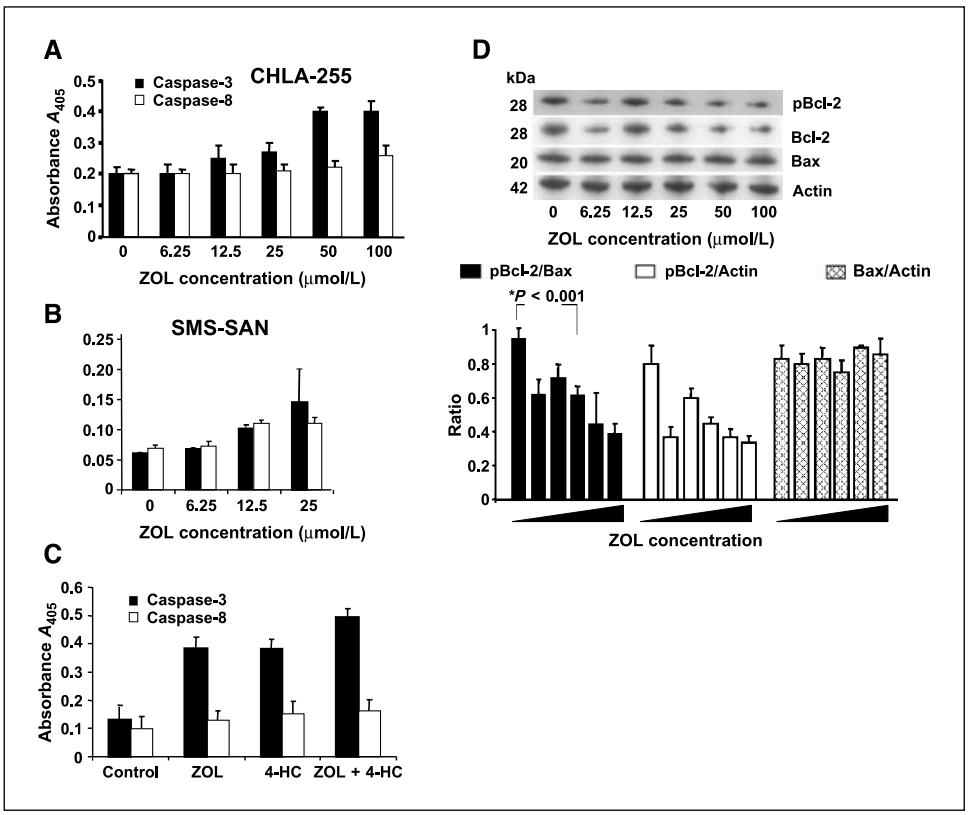

Figure 5. Zoledronic acid induces apoptosis by activating the intrinsic apoptotic pathway. *A* and *B,* activity of caspase-3 and caspase-8 in lysates of CHLA-255 (*A*) and SMS-SAN (*B*) cells treated for 24 h with zoledronic acid determined by colorimetric protease assay. Representative of three experiments showing similar results. *Columns,* mean absorbance values of three separate samples; *bars,* SD. *C,* activity of caspase-3 and caspase-8 in lysates of CHLA-255 cells treated for 24 h with zoledronic acid (12.5 μmol/L), 4-HC (1 μg/mL), or a combination of both. Representative of two experiments showing similar data. *Columns,* mean absorbance values of triplicate samples; *bars,* SD. *D, top,* levels of phospho-Bcl-2 (*pBcl-2*), Bcl-2, and Bax in CHLA-255 cell lysates were examined by Western blot analysis. Cells were incubated with zoledronic acid for 24 h before being harvested and analyzed. *Bottom,* quantitative measurements of the data. *Columns,* mean ratios of triplicate measurements; *bars,* SD.

activity. Consistent with an absence of involvement of the extrinsic apoptotic pathway, we observed no effect of zoledronic acid treatment of CHLA-255 cells on caspase-8 activity but a stimulatory effect on caspase-3 activity (Fig. 5*A*). In SMS-SAN cells, we similarly observed an increase in caspase-3 activity but also an increase in caspase-8 activity at zoledronic acid concentrations up to 25 μmol/L. At higher concentrations, zoledronic acid was toxic and resulted in >80% cell death (Fig. 5*B*). The increase in caspase-3 activity observed in CHLA-255 cells was associated with a decrease in expression of Bcl-2 and phospho-Bcl-2 in the absence of changes in Bax expression, resulting in a decrease in the phospho-Bcl-2/Bax ratio in favor of the proapoptotic Bax protein (Fig. 5*D*). Consistent with a synergistic effect of zoledronic acid and 4-HC on apoptosis, we observed a further increase in caspase-3 activity and decrease in the antiapoptotic proteins Bcl-2 and Bcl-X_L when cells were exposed to zoledronic acid (12.5 μmol/L) and 4-HC (1 mg/mL) in the absence of changes in the expression of the proapoptotic proteins Bax and Bad (Supplementary Fig. S5*E*). These data are consistent with zoledronic acid inducing apoptosis in neuroblastoma cells via the intrinsic apoptotic pathway and having its effect further enhanced with 4-HC.

Zoledronic acid inhibits Ras-mediated Akt and ERK1/2 activation. N-BPs have a known inhibitory activity on protein prenylation, farnesylation and geranylgeranylation, post-translational modifications that promote their localization to the plasma membrane, and subsequent activation (14, 22). We therefore tested whether zoledronic acid could inhibit the translocation of Ras to the plasma membrane, an event associated with its activation. We first compared the presence of Ras in the plasma membrane and cytosolic fractions of CHLA-255 cells 15 and 30 min after treatment with zoledronic acid (25 μmol/L). On treatment with zoledronic acid, we found a significant decrease in membrane-associated Ras when compared with the amount of caveolin-1, a plasma membrane-associated protein, in the absence of changes in cytosolic Ras (Fig. 6*A–C*). Consistent with this effect being associated with inhibition of Ras activation, we observed a simultaneous decrease in phosphorylation of c-Raf, a downstream target of Ras. To determine the signaling pathways downstream of Ras and c-Raf affected by zoledronic acid, we examined the effect of zoledronic acid treatment of CHLA-255 cells on Akt and ERK1/2 phosphorylation. The data (Supplementary Fig. S6*D* and *E*) indicated a significant inhibition of ERK1/2 phosphorylation at 15 min (P = 0.001) and 30 min (P = 0.049) after treatment with zoledronic acid. Inhibition of Akt phosphorylation was also significant but only 15 min after exposure to zoledronic acid. The data suggest that zoledronic acid may inhibit proliferation and stimulate apoptosis in tumor cells via inhibition of Ras-mediated Akt and ERK1/2 activation.

Discussion

Current upfront treatment of high-risk metastatic neuroblastoma focuses on targeting malignant cells with a combination of surgery, radiation therapy, high-dose myeloablative chemotherapy, and bone marrow transplantation (2). Among these therapies, a combination of high-dose myeloablative chemotherapy followed by bone marrow transplantation and high-dose 13-*cis*-retinoic acid improved 3-year disease-free survival from 21% to 42%. Much less attention however has been placed on testing agents specifically targeted toward bone metastasis, a major factor of mortality and morbidity in patients with metastatic disease. Our recent understanding of the mechanisms involved in bone invasion

in neuroblastoma pointed to the important role played by osteoclasts and suggested that they may be suitable target for therapeutic intervention (4). Bisphosphonates, and in particular N-BPs, are among the leading agents with well-demonstrated antiosteoclastic activity and have shown promising results in several diseases associated with increased bone remodeling like osteoporosis, osteoarthritis, and metastatic cancer (17). Their unique ability to bind to the bone matrix explain their short half-life in the plasma and normal tissues, their low systemic toxicity, and their high therapeutic index (23). Among those, zoledronic acid in particular has been studied because of its excellent bioavailability, great efficacy, low toxicity, and convenient administration schedule in patients (4 mg i.v. every 4 weeks; refs. 24, 25). However, the clinical activity of zoledronic acid in pediatric cancer has not been studied.

The observation that zoledronic acid inhibits the formation of osteolytic lesions in mice locally injected with neuroblastoma cells is consistent with other reports in tumor models of breast, prostate, and myeloma, where osteoclasts play an active and causal role in the formation of osteolytic lesions. Here, we provide evidence that zoledronic acid has also a potent antitumoral activity by directly inhibiting tumor cell proliferation and promoting apoptosis. *In vivo*, this effect was observed only inside the bone and *in vitro*, it required zoledronic acid concentrations ranging from 12.5 to 50 μmol/L. In patients treated with a standard dose of 4 mg zoledronic acid, the maximum zoledronic acid concentration achieved in the plasma is only 1 μmol/L (309 ± 71 ng/mL; ref. 26). When given to rat at 0.16 mg/kg i.v., plasma concentrations of 4.47 μmol/L were achieved after 5 min (27). Zoledronic acid plasma disposition is multiphasic with half-lives of 0.2 and 1.4 h with 38% of the dose being excreted in the urine within 24 h, consistent with the extensive uptake by and slow release from bone (28). These plasma concentrations are therefore too low to have any significant inhibitory effect on tumor cell proliferation and apoptosis outside the bone, and this is consistent with the absence of effect that zoledronic acid had on tumor cell proliferation and apoptosis in sections of tumors that had extended outside the femur of mice treated with zoledronic acid. However, zoledronic acid accumulates in the bone where it can reach concentrations in the 10 to 50 μmol/L ranges. For example, when tested for its binding affinity to human bone *in vitro* in competition binding assays, zoledronic acid was found to have a K_i of 81 μmol/L, a concentration above the ones we used in our *in vitro* experiments (29), and to bind to calcified surfaces at concentrations ranging from 1 to 50 μmol/L (30). The concentration of zoledronic acid achieved in the bone marrow space of mice treated with zoledronic acid in our study could not be determined; however, based on published data and our observation of a significant inhibition of proliferation and increase in apoptosis in tumor cells inside the bone, one could reasonably assume that they reached the micromole per liter range where zoledronic acid was shown to have activity *in vitro*. Although we show a direct effect of zoledronic acid on neuroblastoma cells *in vitro*, we cannot rule out the possibility that, *in vivo*, zoledronic acid could have an additional indirect effect on tumor cells in the bone, through its inhibitory activity against osteoclasts. By inhibiting osteoclast proliferation and activity, and therefore osteolysis, zoledronic acid inhibits the release of growth factors bound to the bone matrix (10, 17). Among these is insulin-like growth factor-I, which has a growth stimulatory activity on neuroblastoma cells (31, 32). And vice versa, it is also conceivable that the antitumoral effect of zoledronic acid on neuroblastoma cells in the bone inhibits the stimulatory effect of neuroblastoma cells on the production of the osteoclast-activating factor IL-6 by bone marrow–derived stromal cells (11). These possibilities are currently under investigation in our laboratory.

The cytotoxicity of NB-Ps like alendronate and zoledronic acid in neuroblastoma cells *in vitro* has been reported recently (33). Similar to our observations, the data indicated a 50% inhibition of cell proliferation *in vitro* on 72 h of exposure to alendronate and zoledronic acid at concentrations ranging from 3.97 to 34.1 μmol/L,

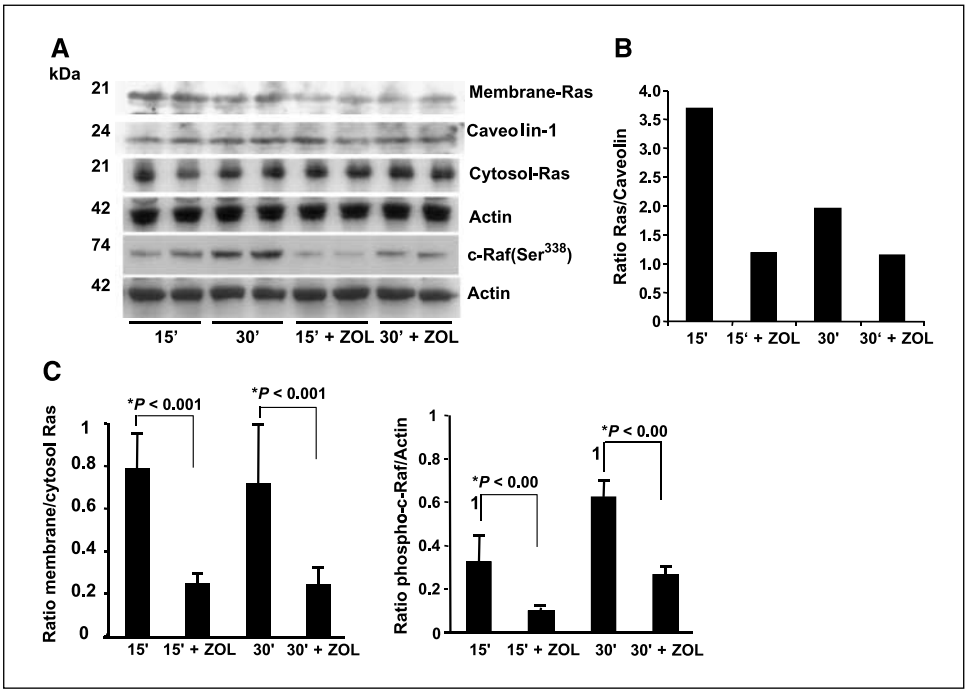

Figure 6. Zoledronic acid inhibits activation of Ras, c-Raf, ERK1/2, and Akt. CHLA-255 cells were incubated overnight in serum-free medium before being exposed to 10% FBS in the presence or absence of zoledronic acid (25 μmol/L). The cells were then washed and lysed 15 and 30 min after treatment. *A,* presence of Ras in membrane and cytosol extracts and of phospho-c-Raf in lysates of duplicate samples taken 15 and 30 min after zoledronic acid treatment and in controls. *B,* ratio of membrane-associated Ras/caveolin-1 in plasma membrane extracts obtained from indicated conditions were calculated by scanning densitometry analysis of the Western blot data shown in (*A*). *C,* quantitative analysis of the data shown in (*A*) was done by scanning. *Columns,* mean ratios from two separate samples in three experiments; *bars,* SD.

depending on the cell line. These authors also reported that NB-Ps induce differentiation and apoptosis; however, neither the mechanism of action of these agents nor their efficacy in preclinical models was examined. In a recent article, Dickson et al. (34) also reported that zoledronic acid inhibits proliferation and increases apoptosis in human neuroblastoma cells exposed *in vitro* to concentrations ranging from 10 to 100 μmol/L and inhibits tumor growth in mice injected into the femur and treated weekly with zoledronic acid starting 10 days after tumor cell inoculation. An increased inhibition of breast cancer cell proliferation *in vitro* by ibandronate and zoledronic acid at a concentration of 30 μmol/L in combination with cytotoxic anticancer agents, such as cyclophosphamide, methotrexate, 5-fluorouracil, epirubicin, and paclitaxel, has also been reported recently (35). At low concentrations (1 μmol/L), zoledronic acid inhibits actin skeleton organization and cell migration in a RhoA-dependent manner and inhibits cyclooxygenase-2 expression (36). Induction of apoptosis in prostate cancer cells by zoledronic acid in a caspase-dependent mechanism involving the intrinsic apoptotic pathway has also been reported previously (37). Here, we provide evidence that similar to prostate cancer cells, the mechanism may involve activation of caspase-3 and alteration in the Bcl-2/Bax ratio in favor of the proapoptotic Bax protein. It is interesting to note that in CHLA-255 cells, zoledronic acid affects caspase-3 and not caspase-8 activity considering that loss of caspase-8 expression has been shown recently to potentiate neuroblastoma metastasis (38), suggesting therefore that loss of caspase-8 activity would not prevent the apoptotic activity of zoledronic acid on metastatic neuroblastoma cells inside the bone. We also show that consistent with the known inhibitory activity of N-BPs on protein prenylation, zoledronic acid inhibits the membrane localization of Ras and consequently Ras-dependent signaling. We also show that inhibition of Ras activation by zoledronic acid is associated with inhibition of c-Raf activation and ERK1/2 and Akt activation, suggesting, although not proving, an involvement of these two signal pathways in proliferation and apoptosis respectively (39).

In these experiments, we have used an intensive treatment with zoledronic acid given 5 days/wk, without observing any acute toxicity in mice. Subsequently, we have also tested a weekly administration schedule and observed a similar effect on inhibition of osteolysis and on mice survival (data not shown). Similarly, Daubine et al. (40) have shown that whether zoledronic acid is given daily or weekly, it is effective in inhibiting skeletal tumor growth and is not toxic in a mouse model of breast cancer bone metastasis.

Our data also point to the beneficial effect of using a combination of chemotherapy and zoledronic acid in patients with established bone metastases. In our experiments, we selected a combination of cyclophosphamide and topotecan because it is among the combinations of cytotoxic agents that have shown efficacy in upfront treatment of phase II clinical trials in children with metastatic neuroblastoma, is well tolerated, and has an acceptable hematopoietic toxicity (41, 42). It is presently a combination of choice in children with recurrent metastatic disease.[6] Although zoledronic acid given in a prevention regimen alone inhibited the formation of osteolytic lesions, it failed to do so in an intervention protocol in mice with established osteolytic lesions. In contrast, when given in combination with cyclophosphamide/topotecan, it inhibited the progression of these lesions to grade 4 in 100% of the mice treated, an effect not observed with chemotherapy alone. Combination of zoledronic acid and chemotherapy also had a significant effect on survival without disabling morbidity. Similarly, the administration of zoledronic acid alone to mice injected into the tibia with PC-3M12 human prostate cancer cells produced significant bone preservation but did not inhibit tumor progression (43). In contrast, when combined with imatinib (Gleevec) and paclitaxel, it decreased tumor incidence and lymph node metastasis and produced apoptosis in tumor cells as well as tumor and bone-associated endothelial cells. An enhancement in tumor regression in a rat model of osteosarcoma was also observed when zoledronic acid was given in combination with ifosfamide (44). The potential benefit of combining zoledronic acid with a cytotoxic agent, such as cyclophosphamide, is further shown by our *in vitro* data showing a significant enhancement of the antiproliferative effect and the apoptotic effect of zoledronic acid when combined with 4-HC, an active metabolite of cyclophosphamide.

In summary, our data show that zoledronic acid has a dual antiosteoclastic and antitumoral activity and provide data suggesting that the antitumoral activity of zoledronic acid involves inhibition of Ras, c-Raf, and ERK1/2 and Akt activation. The data show that when used in combination with cytotoxic chemotherapy to mice with established osteolytic lesions, zoledronic acid in addition to preventing bone degradation significantly extends survival. The data emphasize the unique effect of zoledronic acid in the bone microenvironment and support its use in combination with cytotoxic chemotherapy in children with neuroblastoma that has metastasized to the bone.

[6] R. Seeger, (Children's Hospital Los Angeles, Los Angeles, CA), personal communication.

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