Bisphosphonates Inhibit the Growth of Mesothelioma Cells

In vitro and In vivo

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Abstract

Purpose: Bisphosphonates (such as risedronate and zoledronate) are widely used inhibitors of bone resorption. Despite their in vitro antiproliferative effects in various cancer cells, bisphosphonates have not exhibited significant antitumor efficacy in animal models of visceral cancer, which may be due to their poor bioavailability. The diagnostic use of radioactive bisphosphonates has revealed the accumulation of bisphosphonates in mesothelioma, which prompted us to test the antitumor efficacy of bisphosphonates in this disease.

Experimental Design and Results: Treatment with either risedronate or zoledronate (2 × 10^{-4} to 2 × 10^{-6} mol/L) inhibited the growth of AB12 and AC29 mouse mesothelioma cells and induced the accumulation of unprenylated Rap1A in these cells. Both these in vitro effects were reversed by geranylgeraniol, an end product of the mevalonate pathway that these bisphosphonates inhibit. Both bisphosphonates also induced the phosphorylation of the p38 mitogen-activated protein kinase in AB12 and AC29 cells. The inhibition of p38 augmented bisphosphonate-induced growth inhibition in these cells. Bisphosphonate-induced p38 phosphorylation was not reversible by geranylgeraniol. Risedronate (15 mg/kg) and zoledronate (0.5 mg/kg) inhibited the growth of s.c. tumors and increased the median survival of mice with i.p. mesothelioma tumors in vivo.

Discussion: In conclusion, risedronate and zoledronate inhibit the mevalonate pathway and induce p38 activation in mesothelioma cells in vitro. The effects on the mevalonate pathway dominate because the net result is growth inhibition. Both bisphosphonates also inhibit mesothelioma tumor growth in vivo and prolong the survival of mesothelioma-bearing mice. These results support further study of bisphosphonates in the management of mesothelioma.

Mesothelioma, an asbestos-related neoplasm of the pleural and peritoneal space, occurs in ~10,000 patients worldwide (1). Due to the long latency period for tumor development and the widespread use of asbestos for many years, the incidence is expected to rise until the year 2020 (2). The biological behavior is distinct from other solid tumors in that mesothelioma tends to grow in a sheet-like fashion, covering the surface of the pleura or peritoneum. It shows little tendency to invade, especially early in the course of the disease (3). Mesothelioma typically recurs even after the most aggressive attempts at surgical resection and is poorly responsive to radiotherapy and chemotherapy. Multimodality approaches have had a relatively small effect on the majority of the patients and have been associated with toxicity. The survival of patients with mesothelioma ranges between 4 and 12 months (4, 5). Clearly, new treatment modalities are needed.

Bisphosphonates are synthetic analogues of the naturally occurring pyrophosphate. Depending on their molecular structure, these drugs can be divided into pyrophosphate-resembling (p-bisphosphonates, such as clodronate) and nitrogen-containing bisphosphonates (n-bisphosphonates, such as risedronate and zoledronate; ref. 6). At the cellular level, the different bisphosphonates have different mechanisms of action; n-bisphosphonates inhibit the mevalonate pathway, whereas the effects of p-bisphosphonates are mediated via intracellular ATP-like analogues. The main effect of all bisphosphonates is their ability to inhibit osteoclast-mediated bone resorption. These drugs are therefore widely clinically used in the treatment of metabolic bone diseases that are due to increased bone resorption, such as osteoporosis (7). Bisphosphonates also inhibit the osteolytic complications of bone metastases of solid tumors and multiple myeloma (8). Data from animal models suggest that in addition to osteoclast inhibition at the site of bone metastasis, these drugs may also inhibit cancer cell proliferation in bone (9, 10). In particular, the newer n-bisphosphonates have also been suggested to actually inhibit the cancer
spread to bones in animal models (11). Although these drugs significantly inhibit the growth of various cancer cells in vitro, they have not, however, proven effective as single agents in preventing tumor growth at visceral sites in various animal models of cancer (9–16). This may be due to their poor bioavailability to the tumors; bisphosphonates are poorly absorbed from the gastrointestinal tract and when given i.v., they are quickly cleared from the circulation and adsorbed to bone matrix hydroxyapatite, where they are retained for prolonged periods (17). It is indeed thought that this bone-seeking propensity of the otherwise very hydrophilic drugs makes them available for the cancer cells residing in the bone microenvironment.

The uptake of the bone scan agent 99Tcm diphosphonate, which is structurally similar to the actual bisphosphonate drugs, in malignant pleural effusions and, rarely, in nonmalignant pleural effusions is well established. Although the exact mechanism of uptake in these conditions remains unclear, passive transudation has been implicated (18). There are also several reports of uptake of 99Tcm diphosphonate by cancers localized at the soft tissue sites (19–21). Because mesothelioma has been associated with such an accumulation of 99Tcm diphosphonate, we hypothesized that bisphosphonates may also exhibit direct antitumor activity against this tumor type in vitro (22, 23). We show here that the new nitrogen-containing bisphosphonates, risedronate and zoledronate, effectively inhibit the proliferation of mesothelioma cells in vitro and the growth of mesothelioma tumors in vivo. Furthermore, we show that administration of these drugs after tumor formation can significantly extend the survival of tumor-bearing mice in experimental models of mesothelioma.

Materials and Methods

Bisphosphonates. Risedronate (a gift from Leiras OY, Turku, Finland) was dissolved in PBS and the stock solution was set to pH 7.4 with NaOH. Zoledronate (Novartis, Geneva, Switzerland) was obtained from the pharmacy and diluted into the cell culture medium. For animal studies, both bisphosphonates were diluted into sterile 0.9% saline.

Cell culture. The mouse mesothelioma cell lines AB12 and AC29 were provided by Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA). These cell lines were originally generated by Dr. Bruce Robinson at the Queen Elizabeth II Medical Center (Nedlands, Perth, Western Australia) by i.p. implantation of asbestos fibers in BALB/c and CBA/L mice, respectively, and have been well characterized (24). AB12 and AC29 cells were cultured and maintained in complete medium consisting of high-glucose DMEM (Mediatech, Washington, DC) supplemented with 10% heat-inactivated FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine (Sigma, St. Louis, MO). All cell cultures were plated in incubators in a 37°C atmosphere of 5% CO2/95% air.

In vitro growth assay. Mesothelioma cells were plated in 96-well plates in normal culture medium, and treated for the indicated periods of time with various concentrations of zoledronate, risedronate, or PBS with or without the p38 inhibitor SB202190 or the inactive control compound SB207420 (both at the final concentration of 10−5 mol/L; Calbiochem, San Diego, CA), 25 μmol/L geranylgeraniol (cold, all trans, American Radiolabeled Chemicals, St. Louis, MO), or the same volume of ethanol as a vehicle control. DNA synthesis was measured as an indication of cell proliferation, using nonisotopic bromodeoxyuridine (BrdU) incorporation immunoassays (Exalpha Biologicals, Watertown, MA), according to the manufacturer’s instructions. Briefly, 104 cells were plated onto 96-well plates in 100 μL of normal culture medium. The cells were then treated with the indicated agents for various times. BrdU was added to the wells for the final 24 hours and incorporated BrdU was detected with sequential additions of monoclonal mouse anti-BrdU antibody and horseradish peroxidase–conjugated anti-mouse antibody. After addition of the substrate for horseradish peroxidase, the intensity of the colored reaction product, which is proportional to the amount of BrdU incorporated into the cells, was read with a spectrophotometer at 450 nmol/L.

Western blotting. AB12 and AC29 cells were plated on six-well plates in normal culture medium until near confluency. The cells were then rinsed with sterile PBS and cultured for a further 24 hours in serum-free culture medium, in the presence or absence of 2 × 10−4 to 10−3 mol/L risedronate, zoledronate, or PBS control, with or without 25 μmol/L of geranylgeraniol, or the same volume of ethanol as a vehicle control. Culture medium was discarded and the cells were harvested in lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium PPI, 1 mmol/L β-glycerolphosphate, 1 mmol/L Na3VO4, 1 μg/mL leupeptin; Cell Signaling, Beverly, MA] and clarified by centrifugation. After boiling the supernatants in reducing SDS sample buffer, equal amounts of protein (~50 μg) were loaded per lane and the samples were electrophoresed on 10% polyacrylamide SDS gel and transferred to a nitrocellulose membrane. Unprenylated Rap1A was detected with the antibody SC-1482 and total Rap1 (both prenylated and unprenylated forms of both Rap1A and Rap1B) was detected with the antibody SC-65 (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s recommendations (25, 26). The phosphorylation status of p38 was studied with anti-phospho-p38 and anti-total p38 antibodies (Cell Signaling, as recommended by the manufacturer (27)). The protein bands were visualized by chemiluminescence using SuperSignal West Pico enhanced chemiluminescence kit (Pierce, Rockford, IL).

In vivo mesothelioma models. Female BALB/c mice, 4 to 8 weeks of age, were obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD) and were housed in the Pathogen-Free Rodent Shared Facility (Comprehensive Cancer Center, University of Alabama at Birmingham). All animal procedures were done in accordance with recommendations for the proper care and use of laboratory animals and were approved by the local IACUC. S.c. and i.p. mouse mesothelioma models were evaluated. In the s.c. model, 3 × 105 AB12 cells were first injected s.c. into cohorts of BALB/c mice. Treatments with i.p. bisphosphonates or vehicle were started when tumors became palpable on day 10 and continued every 6 days for a total of four treatments. Tumor size was measured bidimensionally with calipers every 2 to 3 days, and tumor volume was calculated by the formula (length × width2) / 2. Mice were euthanized before tumors reached the size of 2,000 mm3. In the i.p. model, AC29 or AB12 cells (5 × 105 / 0.5 mL) were injected i.p. into cohorts of 10 to 12 BALB/c mice using a 26-gauge needle. Treatment was initiated 6 days after tumor inoculation and the mice were followed for survival. In the i.p. model, risedronate (15 mg/kg), zoledronate (0.5 mg/kg), or PBS were given i.p. thrice a week for 2 weeks.

Statistical analysis. Kaplan-Meier survival curves were analyzed with the Mantel-Cox Log-rank test. Fisher exact test was used to examine differences in the proportion of tumors responding and proportion of mice surviving. Student’s t test (two-tailed) was used to examine differences in growth assays and for the time to death/sacrifice. Results are expressed as mean ± SD. P < 0.05 was considered to be statistically significant.

Results

Risedronate and zoledronate effects on unprenylated Rap1A accumulation and growth inhibition can be partially reversed by geranylgeraniol in mesothelioma cells. Nitrogen-containing bisphosphonates have previously been shown to inhibit the growth of various epithelial cancer cells in vitro via inhibiting the mevalonate pathway (28, 29). This inhibition results in the depletion of intracellular prenyl-groups, such as
geranylgeraniol, which are needed for the posttranslational modification and activation of small GTP-binding proteins, such as Ras, Rho, Rac, and Rap (7). For example, treatment with n-bisphosphonates has been shown to result in the accumulation of unprenylated Rap1A in CaCo-2 and leukemia cells (25, 30). To investigate whether risedronate and zoledronate similarly inhibit the mevalonate pathway in mesothelioma cells, AB12 and AC29 cells were treated for 24 hours with PBS or with $2 \times 10^{-4}$ to $2 \times 10^{-6}$ mol/L risedronate or zoledronate, with 25 μmol/L geranylgeraniol, or the same volume of ethanol as a vehicle control. The cells were then lysed and prepared for Western blot analysis. Accumulation of unprenylated Rap1A was used as a surrogate marker for the inhibition of the mevalonate pathway (26). Zoledronate and risedronate induced a dose-dependent accumulation of unprenylated Rap1A in both cell lines. Risedronate-induced accumulation of unprenylated Rap1A was almost completely reversed by 25 μmol/L geranylgeraniol in both cells. Zoledronate-induced accumulation of unprenylated Rap1A was partially reversed in both cells. Stripping and reblotting the membranes with the anti-total Rap1 antibody clearly indicated that the findings were not due to uneven loading of the gels (Fig. 1). Higher concentrations of geranylgeraniol were also tested and found effective, but because they compromised cell viability, they were not routinely used. Geranylgeraniol also reversed the bisphosphonate-induced inhibition of DNA synthesis in both cells, but the extent of this reversal was dependent on the cell line and the bisphosphonates used (Fig. 2).

Inhibition of p38 augments n-bisphosphonate induced growth inhibition. We have previously shown that in addition to the inhibitory effects on the mevalonate pathway, in particular, the n-bisphosphonates also activate the p38 mitogen-activated protein kinase in breast cancer cells. This activation signals for resistance against bisphosphonate-induced growth inhibition because blocking of the p38 mitogen-activated protein kinase pathway augments the growth-inhibitory effects of bisphosphonates (27). To study whether a similar mechanism operates in mesothelioma cells, we first investigated the effects of risedronate or zoledronate on p38 phosphorylation in these cells. Using phospho-p38-specific and total p38 antibodies in Western blotting, we show here that both risedronate and zoledronate induce a dose-dependent increase of p38 phosphorylation in AB12 and AC29 cells. Unlike the accumulation of unprenylated Rap1A, this effect was, however, not reversible by excess geranylgeraniol (25 μmol/L). Increasing the geranylgeraniol dose did not affect the bisphosphonate-induced, increased phosphorylation status of p38 either (data not shown for higher geranylgeraniol concentrations; Fig. 3). AB12 and AC29 cells were then cultured with risedronate or zoledronate, with or without the specific p38 inhibitor SB202190 (10 μmol/L), or with the same concentration of an inactive control compound SB202474. Inhibition of p38 augmented both risedronate- and zoledronate-induced growth inhibition in both cell lines, even though there were cellspecific differences between the bisphosphonate concentrations at which these effects were seen. In general, AC29 cells were more sensitive to the effects of p38 inhibition (Fig. 4).

Risedronate and zoledronate inhibit mesothelioma growth in vivo. The antitumor activity of n-bisphosphonates was tested in vivo in a s.c. tumor model using AB12 cells, which are syngeneic in BALB/C mice, because AB12 tumors are more

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### Fig. 1. n-Bisphosphonates induce the accumulation of unprenylated Rap1A in mesothelioma cells.

Accumulation of unprenylated Rap1A was detected in AB12 (A) and in AC29 (B) cells after treatment for 24 hours with PBS or with the indicated concentrations of risedronate or zoledronate, with 25 μmol/L geranylgeraniol (GG), or the same volume of ethanol as a vehicle control. The cells were then lysed and prepared for Western blot analysis. The levels of unprenylated Rap1A (top) were used as a surrogate marker to detect the inhibition of the mevalonate pathway, using the antibody SC-1482. The blots, which represent replicate experiments, were stripped and total Rap1 was detected with the antibody SC-65, to show that the effects were not due to a loading error.

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Cancer Therapy: Preclinical
aggressive than the AC29 cells and have been resistant to most cancer chemotherapeutics in vivo (31). Groups of 10 BALB/c mice were inoculated s.c. with AB12 cells. Ten days later, when the tumors were palpable and between 100 and 175 mm³ in size, the mice were treated with risedronate or zoledronate, using higher doses and more infrequent dosing schedules than previously applied in mouse tumor models. Inoculations with PBS served as a vehicle control (9, 32). Tumor volume was measured over time. Mice were sacrificed when tumors reached 2,000 mm³. Both risedronate (\(P < 0.02\)) and zoledronate (\(P < 0.003\)) inhibited s.c. tumor growth (Fig. 5A). However, neither of these bisphosphonates-treated tumors completely regressed, and after initial suppression, most tumors grew at a rate comparable to control tumors (data not shown). The effects of risedronate and zoledronate on survival were examined in vivo in an i.p. tumor model. In particular, AB12 cells form diffuse tumors throughout the peritoneal cavity following i.p. injection, a pattern similar to the presentation of human peritoneal mesothelioma (31). Six days after i.p. inoculation of AB12 or AC29 cells, groups of 10 to 12 mice were treated by i.p. injection of either risedronate, zoledronate, or PBS. Administration of zoledronate led to a significant increase in median survival (43 days for zoledronate versus 26 days for PBS; \(P < 0.001\)). Median survival in the risedronate group was 30 days, which was not statistically significantly different from the PBS group. All PBS-treated mice died by day 35. In contrast, there were three long-term (>60 days) and two long-term (>85 days) survivors in the risedronate and zoledronate-treatment groups, respectively (Fig. 5B). A similar survival experiment was also done with mice bearing AC29 cells. Although the differences were not statistically significant (\(P = 0.08\)) after a total of six inoculations with the drug, the median survival of mice in the zoledronate-treatment group was 39 days, whereas in the control group, the median survival was 26.5 days (Fig. 5C).

Discussion

The in vivo anticaner effects of bisphosphonates have been previously detected in models of bone metastases. In this setting, the growth-inhibitory effects of these drugs have been attributed to their ability to inhibit osteoclasts and thereby, to the inhibition of growth factor liberation from the bone matrix during bone resorption (9, 10, 16). We show here for the first time that both risedronate and zoledronate inhibit mesothelioma tumor growth in vitro and in vivo. Treatment of mesothelioma-bearing mice with these agents also promoted their survival. Thus, bisphosphonates might also have bone-independent, direct anticancer effects against certain tumors.

Our in vivo results with mesothelioma tumors established at soft tissue sites seem to be superior to those achieved with bisphosphonates in other tumor models. For example, bisphosphonates have not been shown to inhibit tumor growth in a mouse breast cancer model using s.c. injection of human MDA-MB-231 breast cancer cells (9, 10). Furthermore, in a...
mouse model of breast cancer metastasis, zoledronate increased the overall survival of the tumor-bearing mice by, at most, 4 days, whereas the median survival in our studies was increased by 17 days with zoledronate (32). Although given less frequently, the bisphosphonate doses used in our study were much higher than those used in the earlier studies. Therefore, despite the less frequent dosing, which mimics the use of i.v. bisphosphonates in oncology, the cumulative doses are high and possibly not directly translatable into clinical use (8).

Our findings are surprising in light of the poor bioavailability of bisphosphonates to visceral tumors. For example, serum...
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Concentrations of 1 to 3 μmol/L are maintained only for a few hours after a 4 mg dose of zoledronate (33). Bisphosphonates are rapidly adsorbed from the circulation to bone tissue hydroxypapitate, where they are retained for prolonged periods (17). The fact that mesothelioma tumors have been reported to accumulate Tc-99m hydroxymethylene diphosphonate in patients with mesothelioma suggests that these cells may possess a yet to be identified mechanism to accumulate bisphosphonates, which may also explain their in vivo sensitivity to these drugs. Such an extracellular accumulation of the bone scanning agent has also been described for several other epithelial cancer cells, including small cell lung cancer (19, 22, 34). Interestingly, it was shown recently that treatment with zoledronate also significantly inhibited the growth of small cell lung cancer cells in the flanks of nude mice (35). The mechanism by which cancer cells accumulate Tc-99m hydroxymethylene diphosphonate is not understood, although tumor necrosis and calcification have been suggested to have a role in this process (20, 21). Recent studies conducted in macrophages suggest the presence of an active, yet uncharacterized uptake mechanism, for which n-bisphosphonates and p-bisphosphonates compete (26). Further studies are required to characterize whether a similar mechanism is also active in mesothelioma and other cancer cells.

n-Bisphosphonates have also recently been shown to inhibit angiogenesis in several models (36–39). n-Bisphosphonate treatment also resulted in decreased amounts of circulating mediators of angiogenesis in patients with breast cancer (40, 41). Therefore, another possible mechanism through which the n-bisphosphonates may have inhibited mesothelioma growth in our study is through inhibited angiogenesis.

n-Bisphosphonate-induced cellular effects, such as inhibition of osteoclast activity, have been suggested to be due to inhibition of the mevalonate pathway (42–45). This inhibition results in the lack of geranylgeraniol and thereby, in defective protein prenylation in cells. Inversely, excess geranylgeraniol has been shown to reverse all the inhibitory effects of n-bisphosphonates in osteoclasts (42, 44). Geranylgeraniol was also shown to partially reverse the antiproliferative and mineralization-promoting effects of zoledronate, but not of pamidronate, in human fetal osteoblasts (46). Geranylgeraniol also reversed the inhibitory effects of n-bisphosphonates on migration, in human ovarian and breast cancer cells (47, 48). n-Bisphosphonate-induced decrease in breast cancer viability was not, however, reversed by geranylgeraniol (46). Our results showed that geranylgeraniol also reversed, completely or partially, the n-bisphosphonate-induced accumulation of unlabeled Rap1A in the studied mesothelioma cells. The effects of risedronate on the accumulation of unlabeled Rap1A were more completely reversed by geranylgeraniol than those of zoledronate, which may represent potency differences between these two drugs (49). Furthermore, the inhibitory effects on proliferation were more completely reversed by geranylgeraniol than the inhibitory effects on accumulation of unlabeled Rap1A (for example with zoledronate in AC29 cells). This suggests that the function of the small GTPases controlling various aspects of cell behavior are differentially regulated by n-bisphosphonates. Similar findings have also been reported by other investigators recently (48, 50).

We further discovered that similar to the effects seen in breast cancer cells, these drugs induce the phosphorylation of the p38 mitogen-activated protein kinase in mesothelioma cells as well (27). Furthermore, similar to breast cancer cells, the n-bisphosphonate-induced p38 activation results in resistance against the growth-inhibitory effects of these drugs, because inhibition of p38 also augments bisphosphonate-induced growth inhibition in mesothelioma cells. Whether n-bisphosphonate-induced p38 phosphorylation promotes survival or increases cell cycling, remains to be studied. The effects on the mevalonate pathway dominate over the activation of the p38-mediated pathway, because the net result of the bisphosphonate-treatment is decreased growth.

In conclusion, our demonstration that n-bisphosphonates inhibit the growth of mesothelioma cells in vitro and in vivo, and prolong the survival of mesothelioma-bearing mice in vivo support further study of bisphosphonates in the management of mesothelioma.

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*In vitro* and *In vivo*

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