The bisphosphonate pamidronate is a potent inhibitor of Ewing's sarcoma cell growth *in vitro*

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The MTT assay was used to measure the effects of pamidronate, clodronate and mevastatin on the cell viability of Ewing's sarcoma cell lines 6647, CADO-ES-1, ES-2, ES-3, RD-ES, SK-ES-1, STA-ET-2.1 and VH-64. Treatment of these cells with pamidronate inhibited cell viability in a time- and dose-dependent manner. After a 72-h incubation period with 50 μM pamidronate, cell numbers were reduced by up to 80%, whereas the monophosphonate analog 3-aminopropyl phosphonate had no effect at concentrations up to 2 mM. Clodronate reduced cell viability by maximally 40% at 1 mM. These data provide the first evidence for a direct growth-inhibitory effect of pamidronate on Ewing's sarcoma cells. Hence, pamidronate definitely merits a more thorough exploration into its potential use in the therapy of patients with Ewing's sarcoma. *Anti-Cancer Drugs* 14:767–771 © 2003 Lippincott Williams & Wilkins.

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Introduction

Bisphosphonates (BPs) are an important class of drugs for the therapy of bone diseases [1]. In clinical practice, they are the most effective inhibitors of osteoclast-mediated bone resorption, and thus are widely used for the treatment of osteoporosis and Paget's disease. Moreover, BPs are now the standard treatment of cancer-induced osteolysis. Notably, they are highly useful for reducing bone pain and the rate of skeletal morbidity—with consequent improvements in the quality of life—in tumors metastatic to bone [2,3].

BPs are analogs of pyrophosphate with a P–C–P backbone whose antiresorptive potency is determined by the two side chains attached to the central carbon atom [4]. They can be divided into two distinct classes defined by the nature of the R2 side chain. BPs lacking a nitrogen within R2 (e.g. clodronate) can be metabolically incorporated into non-hydrolyzable analogs of ATP, thereby inhibiting ATP-dependent enzymes [5]. BPs having a nitrogen in the R2 side chain are substantially more potent at suppressing bone resorption. These nitrogen-containing BPs (N-BPs), such as pamidronate and alendronate, are not metabolized, instead they inhibit farnesyldiphosphate synthase, an enzyme in the mevalonate pathway [6,7]. Inhibition of farnesyldiphosphate synthase prevents the synthesis of farnesyldiphosphate and its derivative geranylgeranyldiphosphate, which are required for the prenylation of small GTPases. Prenylation is necessary for the proper function of these proteins that control activities crucial for osteoclast function [8]. N-BPs including pamidronate were shown to induce apoptosis in osteoclasts [9]; preventing of protein prenylation appears to be one route by which N-BPs can lead to apoptosis [6]. This is consistent with the observation that statins (e.g. mevastatin), which are approved for blocking the mevalonate pathway, cause apoptosis of osteoclasts [6].

Thus, the beneficial effects of BP treatment on skeletal metastases associated with excessive osteoclastic activities are well appreciated. Yet, potential direct activities of BPs on primary bone tumors still await elucidation. We addressed this issue in a systematic *in vitro* approach. In a recent study, we demonstrated that the N-BP pamidronate was capable of effectively inhibiting cell growth of cultivated osteosarcoma cells while sparing normal human fibroblasts [10]. These studies were extended to another bone neoplasm, Ewing's sarcoma, the second most common malignant bone cancer of children and young adults [11]. Here, we report that the viability of Ewing's sarcoma cells (6647, CADO-ES-1, ES-2, ES-3, RD-ES, SK-ES-1, STA-ET-2.1 and VH-64) could be potently reduced by treatment with pamidronate.

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Methods

Reagents

The BPs pamidronate (3-amino-1-hydroxy-propylidene bisphosphonate) and clodronate (dichloromythelene bisphosphonate) were gifts from Novartis Pharma (Wehr, Germany) and Roche Diagnostics (Mannheim, Germany), respectively. 3-Aminopropyl phosphonate and mevastatin were obtained from Sigma-Aldrich Chemie (Taufkirchen, Germany). Stock solutions of BPs and 3-aminopropyl phosphonate were prepared in phosphate-buffered saline (PBS), adjusted to pH 7.4 and sterilized by filtration. Mevastatin was converted from the lactone form as described [6]. Other chemicals and reagents were of analytical grade.

Cell lines and culture maintenance

The following Ewing's sarcoma cell lines were studied: ES-2 and ES-3 were a gift from A. T. Look (Memphis, TN), 6647 from T. J. Triche (Los Angeles, CA), and STA-ET-2.1 from H. Kovar (Vienna, Austria). RD-ES and SK-ES-1 were obtained from ATCC (Rockville, MA), CADO-ES-1 from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). VH-64 has been previously established by one of us (F. v. V.). All cell lines were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B and 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. The cells were grown in collagen-coated (5 µg/cm^2) tissue culture flasks and passaged twice a week.

Cell viability assay

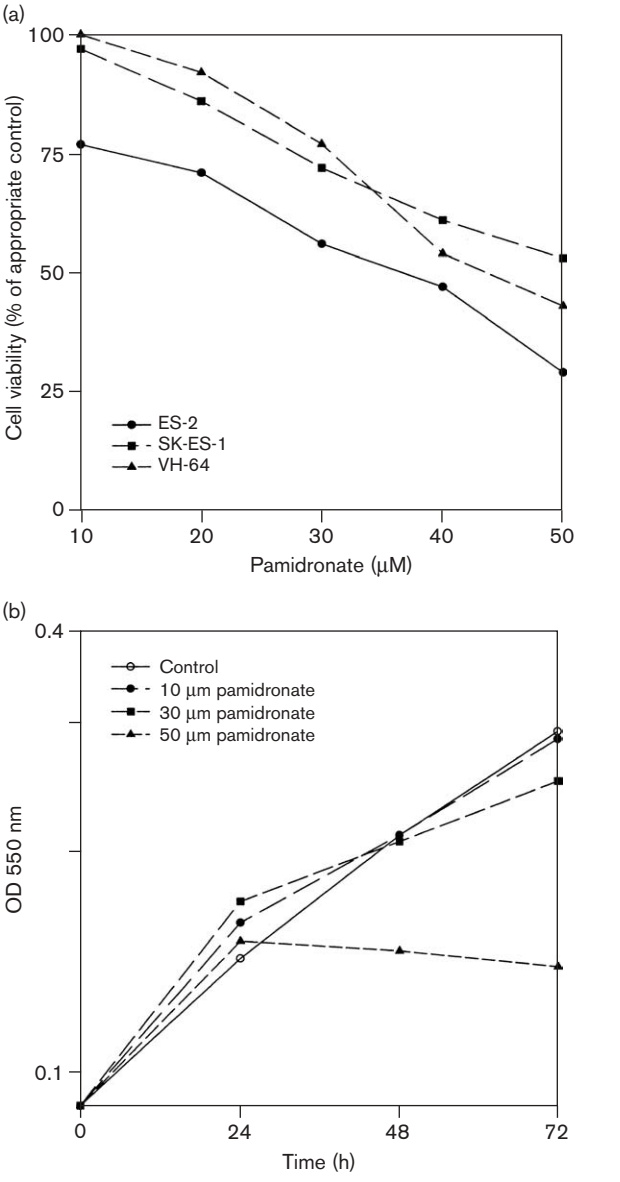
The assays were performed in collagen-coated 96-well flat-bottom microtiter plates. Cells were seeded depending on the growth-rate at densities of 5000–10000 cells/well in 100 µl of complete medium. At 24 h after inoculation, the medium was replaced by medium containing the indicated compounds or vehicle; each group was tested in four replicate wells. The cells were then incubated for 24–72 h, after which MTT reagent in PBS was added to a final concentration of 0.5 mg/ml. After an incubation at 37°C for an additional 4 h, the insoluble product was dissolved by addition of 100 µl of 50% dimethylformamide in 10% SDS. The absorbance of the wells was measured at 550 nm using a Dynatech MR7000 microplate reader.

Results

Sensitivity of Ewing's sarcoma cell lines to pamidronate

Eight Ewing's sarcoma cell lines (6647, CADO-ES-1, ES-2, ES-3, RD-ES, SK-ES-1, STA-ET-2.1 and VH-64) were tested for their sensitivity to pamidronate, a nitrogen-containing BP. In initial experiments, the effects of pamidronate on the viabilities of ES-2, SK-ES-1 and VH-64 cells were monitored by MTT assay over 24–72 h (Fig. 1). All three cell lines responded in a dose- and time-dependent manner: cell viabilities of cultures treated with 50 µM pamidronate for 72 h were reduced to 29, 53 and 43% for ES-2, SK-ES-1 and VH-64 cells, respectively. Since the cytotoxic effects of treatments for 24–48 h were not very pronounced, subsequent experiments were generally carried out over a 72-h period. As shown in Table 1, cell viabilities of all Ewing's sarcoma cell lines examined were significantly decreased in the presence of 50 µM pamidronate. Cell viability after a 72-h

Fig. 1



Effect of pamidronate on cell viability of Ewing's sarcoma cells as assessed by MTT assay: (a) ES-2, SK-ES-1 and VH-64 cells were incubated with the indicated concentrations of pamidronate for 72 h; the means of three experiments in quadruplicate are shown. (b) ES-2 cells were incubated for 24–72 h in the absence or presence of pamidronate; one representative experiment is shown.

incubation ranged from 20% for STA-ET-2.1 to 68% for 6647 cells compared to untreated control cultures. Of note, in a previous report, we have shown that the viability of normal human fibroblasts is only slightly impaired by pamidronate: cultivation of fibroblasts in the presence of 50 μM pamidronate for 72 h reduced cell viability by 21% only [10].

The effect of pamidronate was compared with that of its monophosphate analog 3-aminopropyl phosphonate. A 72-h treatment of Ewing's sarcoma cells with this compound at concentrations up to 2 mM had no effect on cell viability (not shown).

Sensitivity of Ewing's sarcoma cell lines to clodronate

Next, we extended our studies to clodronate, a representative of BPs lacking a nitrogen within the R2 side chain, for its effects on Ewing's sarcoma cells. As demonstrated in Figure 2, treatment with clodronate led only to a minor reduction in cell viability. After a 72-h incubation with 1 mM clodronate, cell viabilities compared to control cultures were 61, 87 and 78% for ES-2, SK-ES-1 and VH-64 cells, respectively. Clodronate at concentrations up to 100 μM disclosed no significant decline in cell viability. In five additional Ewing's sarcoma cell lines cell numbers ranged between 60% for ES-3 and STA-ET-2.1 and 92% for RD-ES cells following exposure to 1 mM clodronate for 72 h (Table 1).

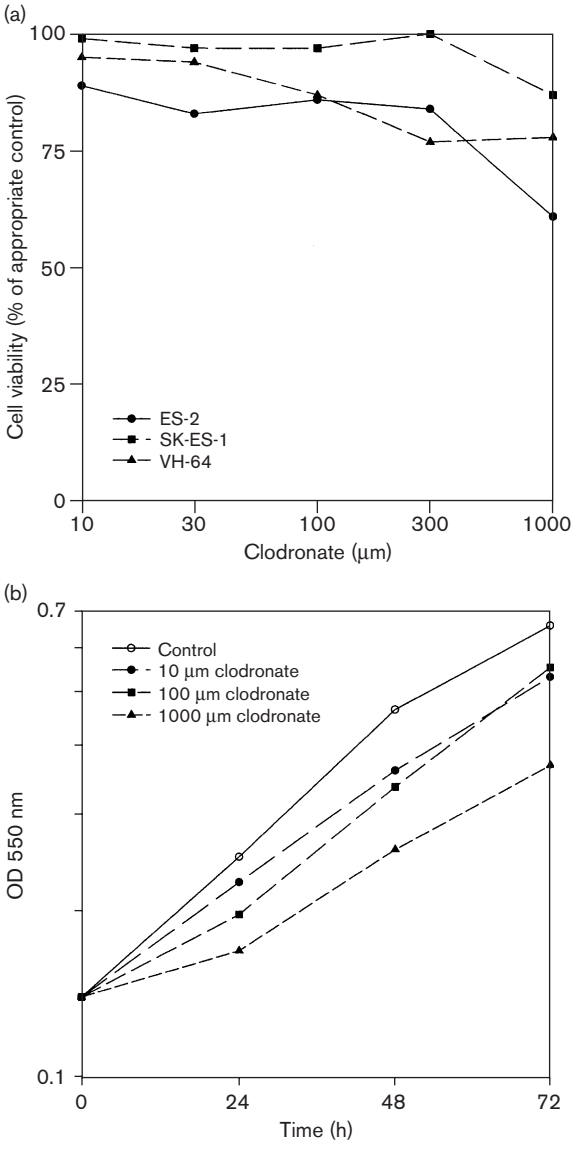
Sensitivity of Ewing's sarcoma cell lines to mevastatin

Bisphosphonates have previously been demonstrated to inhibit the mevalonate pathway, which is responsible for the biosynthesis of cholesterol and isoprenoid lipids. Concentrations of 1–100 μM mevastatin, which blocks the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonate [12], caused a dose-dependent reduction of cell number in the Ewing's sarcoma cells. As shown in Figure 3, there was a significant loss of viability in ES-2, SK-ES-1 and VH-64 cells upon exposure to 100 μM mevastatin for 72 h. As shown in the experiments with pamidronate, all other Ewing's sarcoma cell lines tested were also highly sensitive to mevastatin: cultivation of cells in the presence of 100 μM mevastatin for 72 h reduced cell numbers to levels between 6 and 31% compared to those of untreated cultures (Table 1).

Discussion

A number of studies report an effect of BPs on osteoclasts. So, BPs are implied to impede the function of osteoclasts by different mechanisms including inhibition of recruitment, proliferation and differentiation of pre-osteoclasts as well as suppression of the bone-resorbing action of mature osteoclasts; in addition, BPs are shown to trigger apoptosis in osteoclasts [4]. It has thus been concluded that their proven efficacy in the

Fig. 2



Effect of clodronate on cell viability of Ewing's sarcoma cells as assessed by MTT assay: (a) ES-2, SK-ES-1 and VH-64 cells were incubated with the indicated concentrations of clodronate for 72 h; the means of two experiments in quadruplicate are shown. (b) ES-2 cells were incubated for 24–72 h in the absence or presence of clodronate; one representative experiment is shown.

Table 1 Effect of pamidronate, clodronate and mevastatin on cell viability of Ewing's sarcoma cells

Cell line	Cell viability (% of control)		
	50 μM pamidronate	1 mM clodronate	100 μM mevastatin
6647	68	69	31
CADO-ES-1	38	91	19
ES-3	49	60	19
RD-ES	53	92	31
STA-ET-2.1	20	60	6

Cells were incubated for 72 h; data were compiled from two (clodronate, mevastatin) or three (pamidronate) experiments in quadruplicate.

treatment of bone malignancies may mainly rest on the inhibition of osteoclastic activities, with only indirect action on tumor cells.

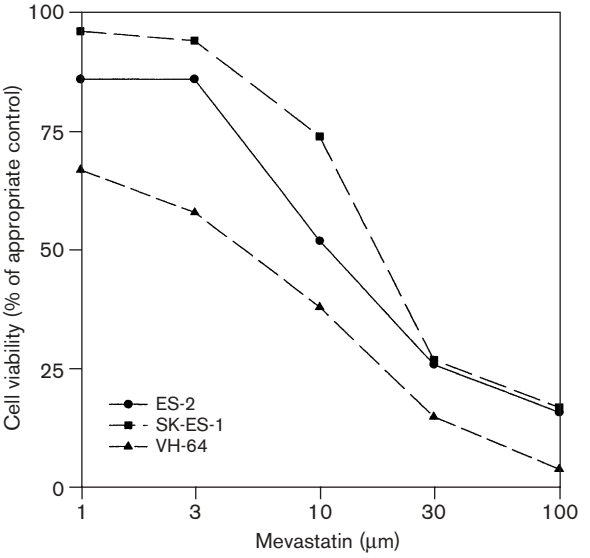
However, there is a growing body of evidence pointing to a direct effect of BPs including pamidronate on neoplastic cells. Recent studies on different cancer cell types such as myeloma [13], breast cancer [14], prostate cancer [15] and melanoma [16] demonstrate such direct effects of BPs. They indicate that BPs are capable of inducing cytostasis and/or apoptosis of tumor cells, significantly inhibiting their growth *in vitro*. Additionally, *in vitro* data suggest that BPs can prevent invasion of breast and prostate cancer cells [17] as well as ovarian cancer cells [18].

Little information exists on BP action in primary bone cancers. Therefore, we performed a systematic study to investigate the putative effect of two BPs, clodronate and the N-BP pamidronate, on cell growth of two bone malignancies, osteosarcoma and Ewing's sarcoma, *in vitro*. In a previous report, we provided clear evidence that BPs act directly on osteosarcoma cells by potently reducing their viability [10]. Here, we demonstrate that treatment with pamidronate also inhibits cell growth of Ewing's sarcoma cells, a highly malignant tumor of bone and soft tissue that occurs in children and young adults [11].

We examined the effects of pamidronate and clodronate on the survival of eight Ewing's sarcoma cell lines. Pamidronate caused a time- and dose-dependent decrease of cell number of all eight cell lines at relatively low concentrations, in the range of 20 to 50 μM (Fig. 1 and Table 1). In contrast, clodronate merely displayed weak effects at high doses only (in the range of 0.3–1 mM; Fig. 2 and Table 1). This is in concordance with our study on osteosarcoma cells [10] and other previous reports. For example, in studies with MDA-MB-231 breast cancer cells, IC_{50} values for pamidronate and clodronate were 40 and 700 μM, respectively [14]. This differential potency of these two BPs might be attributable to their different mechanisms of action. Clodronate is metabolized to a non-hydrolyzable cytotoxic analog of ATP [5], whereas pamidronate inhibits the mevalonate pathway, resulting in the disruption of intracellular signaling [6]. The observed anti-proliferative effects of mevastatin (Fig. 3 and Table 1), an established inhibitor of the mevalonate pathway [12], are consistent with a similar mechanism of pamidronate on Ewing's sarcoma cell growth.

However, one caveat to our study might be that the concentrations required to attain growth inhibitory effects may not be achieved *in vivo*. Serum levels of pamidronate are reported to reach 10 μM [19–21]. However, the strong affinity of BPs for bone mineral may lead to much higher local concentrations in bone [1]. The doses applied in our work may therefore reflect the *in vivo* situation. Notably, the recommended dosages of pamidronate (90 mg every 3–4 weeks) [22] and clodronate (1600 mg/day) [23] in the adjuvant setting in breast cancer patients correspond to the different potencies of these drugs in our study.

Fig. 3



Effect of mevastatin on cell viability of Ewing's sarcoma cells as assessed by MTT assay: ES-2, SK-ES-1 and VH-64 cells were incubated with the indicated concentrations of mevastatin for 72 h; the means of two experiments in quadruplicate are shown.

In conclusion, our study provides the first evidence that BPs directly affect the growth of Ewing's sarcoma cells. The N-BP pamidronate reduced cell viability in all eight cell lines examined. This finding is consistent with our previous work showing that pamidronate inhibits the proliferation of osteosarcoma cells [10]. Taken together, these studies support the notion that BPs warrant a more in-depth investigation for their potential use in adjuvant treatment strategies of childhood bone cancer, all the more since adverse side-effects of BP therapy in children appear to be very low [24].

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