Anti-Cancer Drugs 2001, **12,** pp. 459–465

Report

The bisphosphonate pamidronate is a potent inhibitor of human osteosarcoma cell growth *in vitro*

Jürgen Sonnemann,[1] Vera Eckervogt,[1] Borna Truckenbrod,[1] Joachim Boos,[2] Winfried Winkelmann[1] and Frans van Valen[1]

[1]Labor für Experimentelle Orthopädie/Zellbiologie, Klinik und Poliklinik für Allgemeine Orthopädie, and [2]Abteilung für Pädiatrische Hämatologie/Onkologie, Klinik und Poliklinik für Kinderheilkunde, Universitätsklinikum Münster, Domagkstraße 3, 48149 Münster, Germany.

Bisphosphonates (BPs), such as pamidronate and clodronate, are an important class of drugs for the treatment of bone diseases. It is widely recognized that they inhibit bone resorption by suppressing the action of osteoclasts through antagonizing the mevalonate pathway, thereby reducing osteolytic bone metastases derived from different cancers, i.e. breast carcinoma and multiple myeloma. In contrast, the effects of BPs on primary bone tumors is an issue still to be resolved. Therefore, a systematic approach was set up to test the hypothesis that BPs could act directly on osteosarcoma cells. The effects of pamidronate and clodronate on seven osteosarcoma cell lines (HOS, MG-63, OST, SaOS-2, SJSA-1, U_2OS and ZK-58) were studied. Pamidronate inhibited cell growth in a time- and dose-dependent manner, and decreased proliferation for up to 73% at 50 μM after 72 h, whereas its monophosphonate analog 3-aminopropyl phosphonate did not reduce cell viability at concentrations up to 2 mM. Clodronate showed less inhibitory effects (maximally 38% reduction at 1 mM after 72 h). Importantly, cell growth of fibroblasts was only very weakly affected by treatment with pamidronate. These results suggest that pamidronate may be a useful agent for the treatment of patients with osteosarcoma. [© 2001 Lippincott Williams & Wilkins.]

Key words: Bisphosphonates, bone tumor, clodronate, mevastatin, osteosarcoma, pamidronate.

Introduction

Bisphosphonates (BPs) are powerful inhibitors of bone resorption. As such, they are routinely used for the treatment of bone diseases including osteoporosis, Paget's disease and metastatic cancer in bone. They reduce the occurrence of pathological fractures, bone pain and hypercalcemic episodes without causing major side effects or complications, and are thus considered to be ideal drugs, especially for use in a palliative setting.

BPs are analogs of endogenous pyrophosphate in which a carbon atom replaces the central oxygen.[1] The two additional side chains attached to the germinal carbon give rise to a huge number of potential structures. Due to their high affinity for bone mineral, BPs concentrate to bone surfaces where they act on osteoclasts. They decrease the function of these cells by various mechanisms including inhibition of recruitment, proliferation and differentiation of preosteoclasts as well as suppression of the bone-resorbing activities of mature osteoclasts. Moreover, they have been reported to induce apoptosis in osteoclasts. The way these effects are brought about by different BPs is mainly dependent on the nature of their R2 side chains.[2]

It is now widely accepted that BPs exert their activities on osteoclasts by being taken up into the cytosol.[3] With respect to the mode of action, BPs can be divided into two classes.[2] BPs belonging to the first class (e.g. clodronate) can be metabolized by intracellular enzymes into non-hydrolyzable analogs of ATP likely leading to the inhibition of several ATP-dependent intracellular reactions.[4] BPs belonging to the second, more potent, category are characterized by an amino group in the R2 side chain. These BPs (e.g. pamidronate and alendronate) are not metabolized, but interfere with the mevalonate pathway which is responsible for the biosynthesis of cholesterol and isoprenoid lipids, thereby inhibiting the prenylation of proteins such as small GTPases.[5] Prenylation is required for the correct function of these important signaling proteins that regulate a variety of cell

Correspondence to J Sonnemann, Labor für Experimentelle Orthopädie/Zellbiologie, Klinik und Poliklinik für Allgemeine Orthopädie, Universitätsklinikum Münster, Domagkstraße 3, 48149 Münster, Germany.
Tel: (+49) 251 8356756; Fax: (+49) 251 8356758;
E-mail: sonneman@uni-muenster.de

activities relevant for osteoclast function. By blocking this pathway, amino group-containing BPs also trigger apoptosis of osteoclasts.[6] In concordance with this finding statins such as mevastatin and lovastatin, which are potent inhibitors of the mevalonate pathway, cause osteoclast apoptosis.[5,7]

Whereas BPs are widely used clinically for the treatment of diseases in which osteoclastic bone resorption is in excess, their effects on primary bone tumors is an issue still to be resolved. Therefore, we set up a systematic approach to test the hypothesis that BPs could act directly on osteosarcoma cells. We chose pamidronate and clodronate for investigation because they are the most frequently used BPs in oncology.[8] In this report, we demonstrate that pamidronate potently reduces the viability of osteosarcoma cells (HOS, MG-63, OST, SaOS-2, SJSA-1, U_2OS and ZK-58) *in vitro*, while sparing normal human fibroblastic cells.

Material and 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. Bonefos® was kindly provided by Medac (Hamburg, Germany). 3-Aminopropyl phosphonate and mevastatin were obtained from Sigma-Aldrich Chemie (Taufkirchen, Germany). The pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVADfmk) was from Calbiochem-Novabiochem (Bad Soden, Germany). Stock solutions of BPs and 3-aminopropyl phosphonate were prepared in phosphate-buffered saline, adjusted to pH 7.4 and sterilized by filtration. Mevastatin was converted from the lactone form as described.[5] Other chemicals and reagents were of analytical grade.

Cell lines and culture maintenance

The following human osteosarcoma cell lines were studied: HOS, MG-63, OST, SaOS-2, SJSA-1 and U_2OS were obtained from ATCC (Rockville, MA), and ZK-58 was provided by M Bürger (Münster, Germany). Primary human fibroblasts were a gift from S Winters (Münster, Germany). 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 passaged twice a week.

Cell viability assay

The assays were performed in 96-well flat-bottom microtiter plates. Cells were seeded depending on the growth rate at densities of 5000–10 000 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 phosphate-buffered saline was added to a final concentration of 0.5 mg/ml. After 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.

Fluorocytometric analysis of phosphatidylserine expression

Cell-surface exposure of phosphatidylserine was identified by staining with annexin V conjugated to fluorescein (Roche Diagnostics, Mannheim, Germany) and quantitated fluorocytometrically according to the manufacturer's instructions. The staining was analyzed on a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest software.

DNA fragmentation analysis

For analysis of genomic DNA, attached and non-attached cells were pooled, then lyzed in 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1.5 mM $MgCl_2$ and 0.25% (v/v) NP-40 at 4°C for 30 min. Nuclei were pelleted at 3000 *g* for 5 min, and subsequently lyzed in 10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 0.5% (w/v) SDS and 100 μg/ml proteinase K at 37°C for 3 h. Samples were extracted with phenol–chloroform and precipitated by ethanol. DNA was dissolved in TE buffer and RNA digested with 50 μg/ml RNase A at 37°C for 30 min prior to electrophoresis. Aliquots of 4 μg of each sample were electrophoresed, together with molecular weight markers of 123 bp multiples (Gibco, Karlsruhe, Germany), on a 1.5% agarose gel containing 1 μg/ml ethidium bromide.

Results

Sensitivity of osteosarcoma cell lines to pamidronate

In initial experiments, possible cytotoxic effects induced by pamidronate, a nitrogen-containing BP

belonging to the second class, on MG-63 osteosarcoma cells were examined by MTT assay. As shown in Figure 1, treatment with pamidronate caused a significant time- and dose-dependent cytotoxicity in MG-63 osteosarcoma cells: cell viability was reduced to 28% after incubation for 72 h. Since the effects of 24- and 48-h incubations were not very pronounced, cells were subsequently incubated in general for 72 h. Further experiments monitored the cytotoxicity of pamidronate on six additional osteosarcoma cell lines (HOS, OST, SaOS-2, SJSA-1, U_2OS and ZK-58): the cell viability after a 72-h treatment with 50 μM pamidronate ranged from 27% for OST to 64% for ZK-58 cells (Table 1). Importantly, human fibroblast cultures displayed a very weak sensitivity towards pamidronate: incubation with 50 μM pamidronate for 72 h caused a reduction in cell viability of only 21% (Figure 1A).

It has been reported that the nitrogen-containing BP alendronate stimulates formation of osteoblast precursors in human bone marrow cultures at concentrations below 100 nM.[9] Therefore, the osteosarcoma cell lines were treated with pamidronate at concentrations from 10^{-12} to 10^{-6} M for 24–72 h. No effect on cell growth could be detected in any of the cell lines (not shown).

The effect of pamidronate was compared with that of its monophosphate analog 3-aminopropyl phosphonate. Upon administration of this compound at concentrations up to 2 mM to osteosarcoma cell cultures no reduction of cell viability could be observed after 72 h (not shown).

Assessment of apoptosis in pamidronate-treated cells

Pamidronate causes apoptosis in murine osteoclasts,[10] in murine macrophages,[11] in human myeloma cells[12] and in human breast cancer cells.[13,14] In light of these findings, an apoptotic process might also account for the observed cytotoxic effects of pamidronate on osteosarcoma cells. By the criteria we applied, pamidronate did not induce apoptosis in osteosarcoma cell lines. Firstly, apoptosis was assessed by a fluorocytometric-based assay using annexin V as an indicator for phosphatidylserine exposed on the surface of cells undergoing apoptosis. Pamidronate did not induce annexin V binding to the cell surface, whereas treatment with TRAIL led to annexin V staining (not shown).[15] Secondly, apoptosis was measured by DNA fragmentation analysis. No DNA cleavage could be observed in pamidronate-treated MG-63 cells (Figure 2A, lane 3). In contrast, cells showed endonucleosomal DNA degradation when exposed to mevastatin (Figure 2A, lane 4). Thirdly, we investigated whether caspase-like proteases, im-

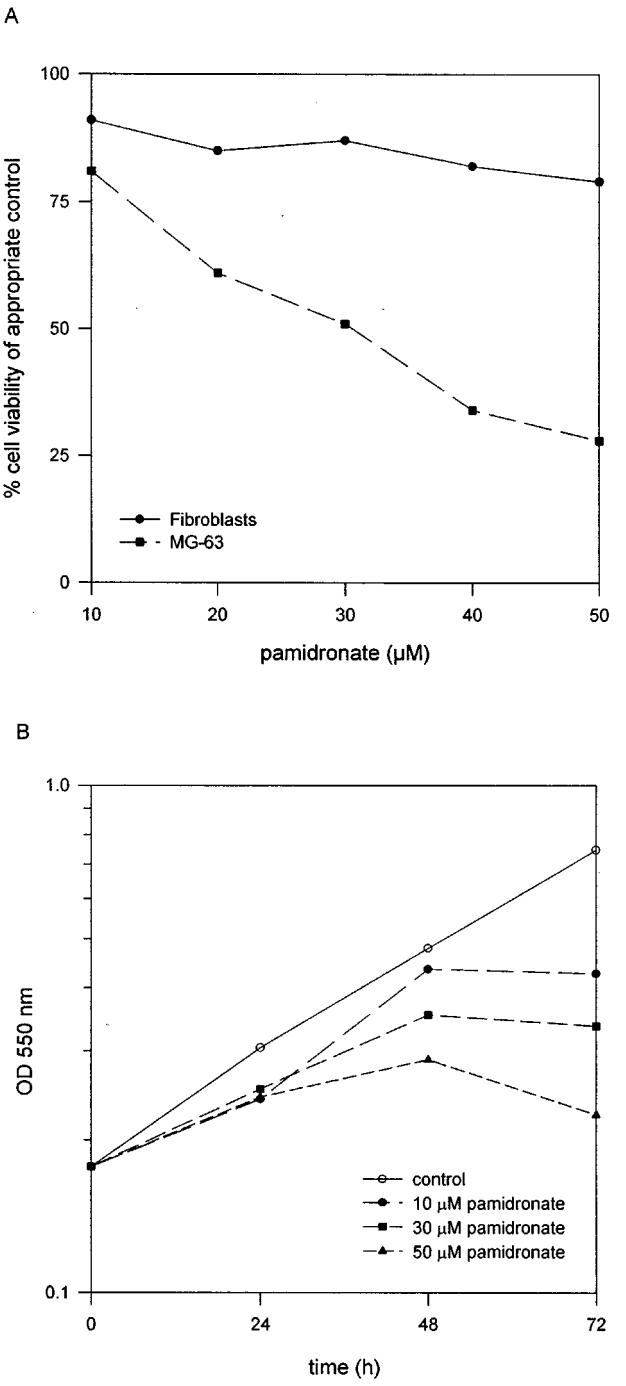

Figure 1. Effect of pamidronate on cell viability of osteosarcoma cells and fibroblasts as assessed by MTT assay. (A) Cells were incubated with the indicated concentrations of pamidronate for 72 h; the means of three experiments in quadruplicate are shown. (B) MG-63 osteosarcoma cells were incubated for 24–72 h in the absence or presence of pamidronate; one representative experiment is shown.

Table 1. Effect of pamidronate, clodronate and mevastatin on cell viability of osteosarcoma cells

Cell line	Cell viability (% of control)		
	50 μM pamidronate	1 mM clodronate	100 μM mevastatin
HOS	30	77	2
OST	27	85	16
SaOS-2	44	76	4
SJSA-1	47	81	11
U_2OS	37	62	2
ZK-58	64	69	15

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

portant effectors in apoptotic cell death pathways,[16] might be involved in pamidronate-induced cytotoxicity. As demonstrated in Figure 2(B), the pan-caspase antagonist zVADfmk was incapable of protecting osteosarcoma cells against the cytotoxic effects of pamidronate.

Sensitivity of osteosarcoma cell lines to clodronate

In further studies, we tested clodronate, a representative of the first class of BPs, for its effects on osteosarcoma cells. As displayed in Figure 3, the viability of cells in the presence of clodronate was not very intensely affected. After treatment with 1 mM clodronate for 72 h, cell viability compared to those of control cultures was 73% for MG-63 cells. No significant decline in cell number could be detected at 100 μM clodronate. Fibroblasts also showed a weak sensitivity towards clodronate (Figure 3A). In six additional osteosarcoma cell lines cell viabilities after exposure to 1 mM clodronate for 72 h were between 62% for U_2OS and 85% for OST cells (Table 1).

Sensitivity of osteosarcoma cell lines to mevastatin

Mevastatin, which blocks the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonate,[17] caused a time- and dose-dependent reduction of cell viability of MG-63 cells (Figure 4). After a 72-h treatment with 100 μM mevastatin, the viability of MG-63 cells was almost abolished, whereas cell viability of fibroblasts was still 65% (Figure 4A). As in the experiments with pamidronate, all osteosarcoma cell lines (HOS, OST, SaOS-2, SJSA-1, U_2OS and ZK-58)

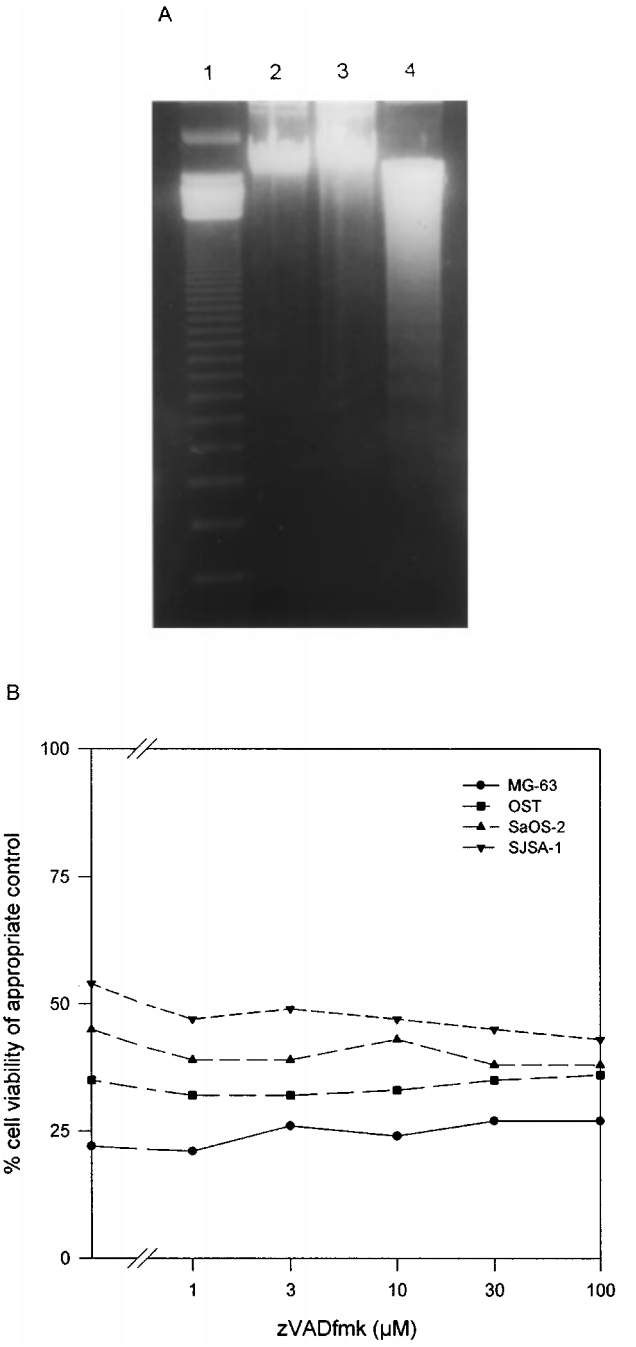

Figure 2. Pamidronate does not induce apoptosis in osteosarcoma cells. (A) Electrophoretic analysis of intranucleosomal DNA fragmentation in MG-63 osteosarcoma cells: lane 1, 123 bp DNA ladder; lane 2, untreated culture; lane 3, 50 μM pamidronate for 72 h; lane 4, 10 μM mevastatin for 72 h. (B) Cells were incubated with 50 μM pamidronate and the indicated concentrations of zVADfmk for 72 h.

were also highly sensitive to mevastatin: cultivation of cells in the presence of 100 μM mevastatin for 72 h

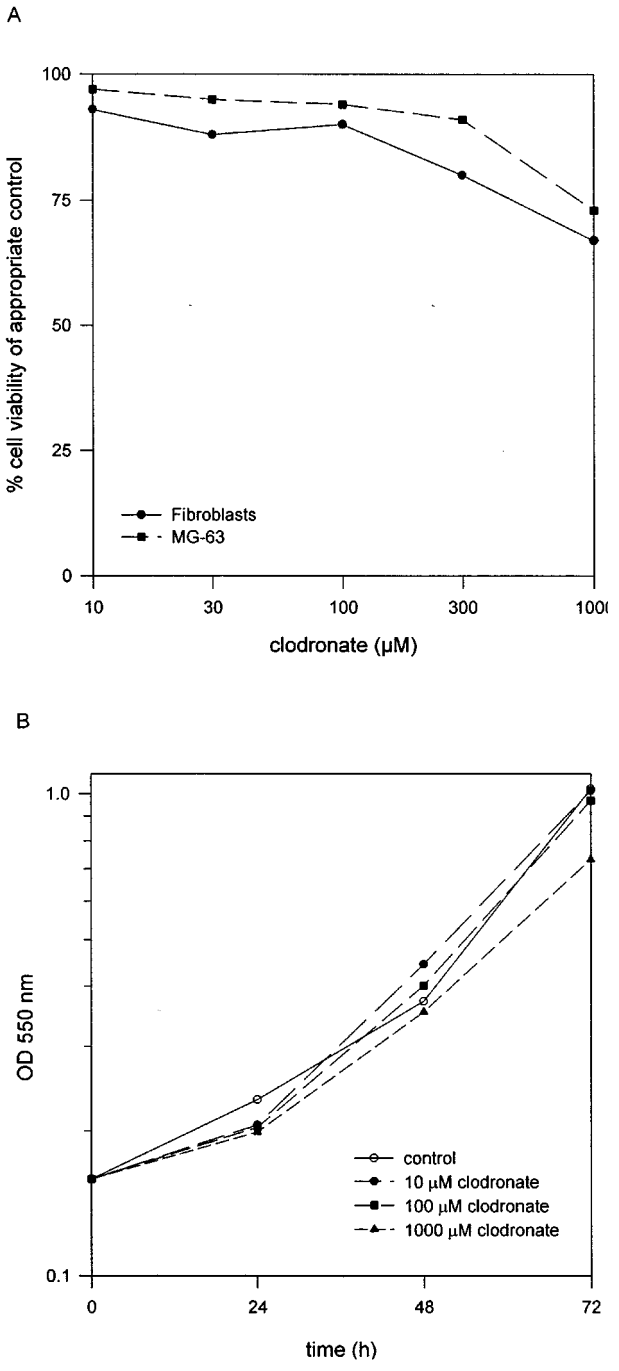

Figure 3. Effect of clodronate on cell viability of osteosarcoma cells and fibroblasts as assessed by MTT assay. (A) Cells were incubated with the indicated concentrations of clodronate for 72 h; the means of two experiments in quadruplicate are shown. (B) MG-63 osteosarcoma cells were incubated for 24–72 h in the absence or presence of clodronate; one representative experiment is shown.

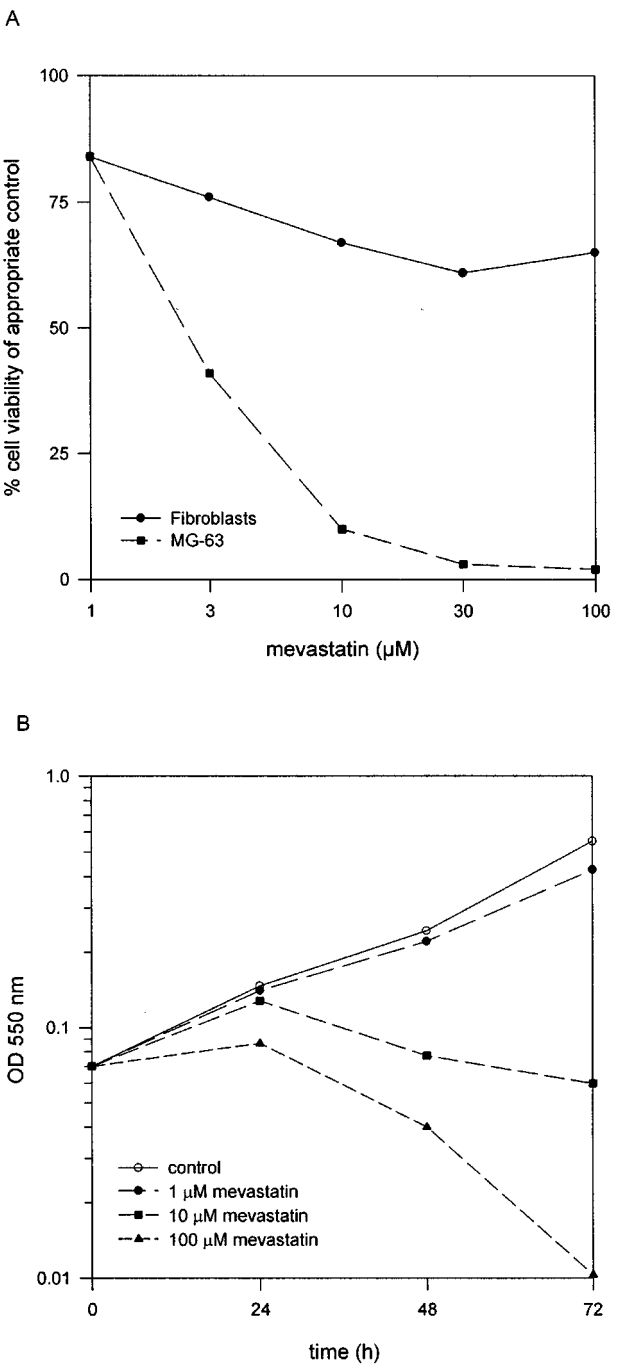

Figure 4. Effect of mevastatin on cell viability of osteosarcoma cells and fibroblasts as assessed by MTT assay. (A) Cells were incubated with the indicated concentrations of mevastatin for 72 h; the means of two experiments in quadruplicate are shown. (B) MG-63 osteosarcoma cells were incubated for 24–72 h in the absence or presence of mevastatin; one representative experiment is shown.

reduced cell viabilities of these osteosarcoma cell lines to levels below 20% compared to those of untreated cultures (Table 1). In concordance with a previous report,[5] but in contrast to pamidronate, mevastatin caused oligonucleosomal DNA fragmentation (Figure 2A, lane 4).

Discussion

The most common clinical indication for BPs is osteoporosis, but their use in osteolytic bone disease has increased remarkably during the past decade. In particular, pamidronate is now widely used in the treatment of patients with osteolytic metastases: clinical studies indicate that pamidronate reduces significantly the mean skeletal morbidity rate in patients with myeloma or breast cancer.[8] Since BPs inhibit osteoclast activity, their efficacy in diseases with increased osteoclastic bone resorption is not surprising. However, in addition to these effects on osteoclasts, BPs may also affect other cells, such as cells of the osteoblastic lineage. Indeed, recent studies suggest that BPs can influence osteoblast function,[9,18–20] but the observed effects differ, depending on the BP and the model system used. Here, we report the antiproliferative actions of two BPs, pamidronate and clodronate, on cultured osteosarcoma cells.

Seven osteosarcoma cell lines were examined and the proliferation of all seven cell lines was decreased by pamidronate in a dose-dependent manner (Figure 1 and Table 1). Half-maximal inhibition was observed at 20–50 μM pamidronate, depending on the cell line. The actual doses of BPs that bone tumor cells or other cells in the organism are exposed to under clinical conditions are unknown. However, after i.v. administration, concentrations of pamidronate have been reported to reach 10 μM in sera.[21–23] Whereas these peak serum levels are transient, their strong affinity for bone mineral leads to rapid clearance of BPs from the circulation and to accumulation at high levels in bone.[1] Hence, the concentrations of pamidronate used in our study reasonably correspond to the *in vivo* situation.

In clinical practice, pamidronate and clodronate exhibit different potencies. For example, for the treatment of tumor-induced hypercalcemia, recommended dosages of pamidronate and clodronate are 90 and 1500 mg, respectively.[8] In our *in vitro* study, clodronate was less potent at 1000 μM than pamidronate at 50 μM (Table 1). These differences likely reflect their different molecular mechanisms of action. Clodronate is metabolized into non-hydrolyzable analogs of ATP that may suppress ATP-dependent intracellular reactions,[4] whereas pamidronate antagonizes the mevalonate pathway, thereby blocking a multitude of activities critical for cell function.[5] The latter mechanism might also apply for the observed effects of pamidronate on osteosarcoma cell growth. This is supported by the finding that the proliferation of these bone tumor cells is efficiently repressed by mevastatin (Figure 4 and Table 1), an inhibitor of the mevalonate pathway.[17]

The monophosphonate analog of pamidronate was totally inactive at reducing cell viability at concentrations up to 2 mM, further contradicting a mere unspecific toxic action of pamidronate. However, the inhibitory effect of pamidronate was independent of apoptosis induction (Figure 2). This is in contrast to findings showing murine osteoclasts[10] and myeloma cells[12] to undergo apoptosis upon treatment with high doses of different BPs including pamidronate. It must be taken into account, however, that in our experiments BP treatment was carried out in the presence of serum, which is a survival factor for osteosarcoma cells. Thus, serum might counteract the potential pro-apoptotic effect of pamidronate.

The sensitivity of osteosarcoma cells to pamidronate was compared with that of normal human fibroblasts. Interestingly, the viability of fibroblasts was much less affected by pamidronate treatment (Figure 1A). This is consistent with a previous report showing that alendronate, which potently reduced macrophage viability,[11] did affect viability of osteoblasts only at concentrations equal to or higher than 100 μM.[18]

Several recent studies suggest direct effects of BPs on human osteoblastic cells, but the reported effects differ.[9,18–20] Thus, Plotkin *et al.*[19] describe an anti-apoptotic effect of BPs on osteoblasts and Giuliani *et al.*[9] report a stimulatory effect on osteoblastic cell precursors. Nevertheless, concerns that BPs might also stimulate proliferation of osteosarcoma cells seem to be unsubstantiated. Evidently, in our experiments, no stimulatory effect on cell growth of pamidronate at concentrations ranging from 10^{-12} to 5×10^{-5} M could be observed.

Conclusion

Our present data support observations that BPs can directly act on the growth of tumor cells such as shown for myeloma cells[12] and breast cancer cells.[13,14] Our report demonstrates for the first time that the viability of cultured osteosarcoma cells is significantly reduced by pamidronate. The clinical relevance of these *in vitro* findings remains to be verified. However, since it has been convincingly shown that BPs can reduce metastatic bone pain in a clinically significant manner,[8] they may also exert analgesic effects in osteosarcoma patients. In addition, the observed direct inhibitory action of pamidronate on osteosarcoma cell growth might contribute beneficially to the treatment of osteosarcomas. Since the rate of complications and adverse effects caused by BPs is

extremely low,[24] an adjuvant BP therapy of osteosarcoma appears not to be contra-indicated and definitely merits further evaluation.

References

1. Fleisch H. Bisphosphonates: mechanisms of action. *Endocr Rev* 1998; **19**: 80–100.
2. Russel RGG, Rogers MJ. Bisphosphonates: from the laboratory to the clinic and back again. *Bone* 1999; **25**: 97–106.
3. Felix R, Guenther HL, Fleisch H. The subcellular distribution of [^{14}C]dichloromethylenebisphosphonate and [^{14}C]1-hydroxyethylidene-1,1-bisphosphonate in cultured calvaria cells. *Calcif Tissue Int* 1984; **36**: 108–13.
4. Frith JC, Mönkkönen J, Blackburn GM, Russell RGG, Rogers MJ. Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5′-(β,μ-dichloromethylene) triphosphate, by mammalian cells *in vitro*. *J Bone Miner Res* 1997; **12**: 1358–67.
5. Luckman SP, Hughes DE, Coxon FP, Russell RGG, Rogers MJ. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. *J Bone Miner Res* 1998; **13**: 581–9.
6. Reszka AA, Halasy-Nagy JM, Masarachia PJ, Rodan GA. Bisphosphonates act directly on the osteoclast to induce caspase cleavage of Mst1 kinase during apoptosis. *J Biol Chem* 1999; **274**: 34967–73.
7. Fisher JE, Rogers MJ, Halasy JM, *et al*. Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation *in vitro*. *Proc Natl Acad Sci USA* 1999; **96**: 133–8.
8. Body JJ, Bartl R, Burckhardt P, *et al*. Current use of bisphosphonates in oncology. *J Clin Oncol* 1998; **16**: 3890–9.
9. Giuliani N, Pedrazzoni M, Negri G, Passeri G, Impicciatore M, Girasole G. Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures *in vitro* and promote early osteoblastogenesis in young and aged mice *in vivo*. *Bone* 1998; **22**: 455–61.
10. Hughes DE, Wright KR, Uy HL, *et al*. Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. *J Bone Miner Res* 1995; **10**: 1478–87.
11. Luckman SP, Coxon FP, Ebetino FH, Russell RGG, Rogers MJ. Heterocycle-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: evidence from structure–activity relationships in J774 macrophages. *J Bone Miner Res* 1998; **13**: 1668–78.
12. Shipman CM, Rogers MJ, Apperley JF, Russell RGR, Croucher PI. Bisphosphonates induce apoptosis in human myeloma cell lines: a novel anti-tumour activity. *Br J Haematol* 1997; **98**: 665–72.
13. Fromigue O, Lagneaux L, Body JJ. Bisphosphonates induce breast cancer cell death *in vitro*. *J Bone Miner Res* 2000; **11**: 2211–21.
14. Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. Bisphosphonates induce apoptosis in human breast cancer cell lines. *Br J Cancer* 2000; **82**: 1459–68.
15. van Valen F, Fulda S, Truckenbrod B, *et al*. Apoptotic responsivness of the Ewing's sarcoma family of tumours to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). *Int J Cancer* 2000; **88**: 252–9.
16. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998; **281**: 1312–6.
17. Endo A, Kurada M, Tanzawa K. Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B, fungal metabolites having hypocholesterolemic activity. *FEBS Lett* 1976; **72**: 323–6.
18. Garcia-Moreno C, Serrano S, Nacher M, *et al*. Effect of alendronate on cultured normal human osteoblasts. *Bone* 1998; **22**: 233–9.
19. Plotkin LI, Weinstein RS, Parfitt AM, Robertson PK, Manalogas SC, Bellido T. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. *J Clin Invest* 1999; **104**: 1363–74.
20. Reinholz GG, Getz B, Pederson L, *et al*. Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. *Cancer Res* 2000; **60**: 6001–7.
21. Leyvraz S, Hess U, Flesch G, *et al*. Pharmacokinetics of pamidronate in patients with bone metastases. *J Natl Cancer Inst* 1992; **84**: 788–92.
22. Oiso Y, Tomita A, Hasegawa H, *et al*. Pamidronate treatment with tumor-associated hypercalcemia: pharmacological effects and pharmacokinetics. *Endocr J* 1994; **41**: 655–61.
23. Berenson JR, Rosen L, Vescio R, *et al*. Pharmacokinetics of pamidronate disodium in patients with cancer with normal or impaired renal function. *J Clin Pharmacol* 1997; **37**: 285–90.
24. Diel IJ. Antitumour effects of bisphosphonates. *Drugs* 2000; **59**: 391–9.

(Received 27 February 2001; accepted 13 March 2001)