Bisphosphonate-induced ATP analog formation and its effect on inhibition of cancer cell growth

Hannu Mönkkönen, Johanna Kuokkanen, Ingunn Holen, Alyson Evans, Diane V. Lefley, Marjo Jauhiainen, Seppo Auriola and Jukka Mönkkönen

Bisphosphonates (BPs) are effective inhibitors of tumor-induced bone resorption. Recent studies have demonstrated that BPs inhibit growth, attachment and invasion of cancer cells in culture and promote apoptosis. The mechanisms responsible for the observed anti-tumor effects of BPs are beginning to be elucidated. Recently, we reported that nitrogen-containing bisphosphonates (N-BPs) induce formation of a novel ATP analog (ApppI) as a consequence of the inhibition of farnesyl diphosphate synthase in the mevalonate pathway. Similar to AppCp-type metabolites of non-N-BPs, ApppI is able to induce apoptosis. This study investigated BP-induced ATP analog formation and its effect on cancer cell growth. To evaluate zoledronic acid (a N-BP)-induced ApppI accumulation, inhibition of protein prenylation and clodronate (a non-N-BP) metabolism to AppCCl$_2$p, MCF-7 and MDA-MB-436 breast cancer cells, MCF-10A nonmalignant breast cells, PC-3 prostate cancer cells, MG-63 osteosarcoma cells, RPMI-8226, and NCI-H929 myeloma cells were treated with 25 μmol/l zoledronic acid or 500 μmol/l clodronate for 24 h. The inhibition of cell growth by zoledronic acid and clodronate was studied in MCF-7, MDA-MB-436, and RPMI-8226 cells by exposing the cells with 1–100 μmol/l zoledronic acid or 10–2000 μmol/l clodronate for 72 h. Marked differences in zoledronic acid-induced ApppI formation and clodronate metabolism between the cancer cell lines were observed. The production of cytotoxic ATP analogs in tumor cells after BP treatment is likely to depend on the activity of enzymes, such as farnesyl diphosphate synthase or aminoacyl-tRNA synthetases, responsible for ATP analog formation. Additionally, the potency of clodronate to inhibit cancer cell growth corresponds to ATP analog formation. *Anti-Cancer Drugs* 19:391–399 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2008, **19**:391–399

Keywords: ATP analog, clodronate, metabolism, mevalonate pathway, zoledronic acid

Department of Pharmaceutics, University of Kuopio, Kuopio, Finland

Correspondence to Dr Hannu Mönkkönen, PhD, Department of Pharmaceutics, University of Kuopio, PO Box 1627, 70211 Kuopio, Finland
Tel: +358 50 361 1726; e-mail: hannu.monkkonen@uku.fi

Received 21 September 2007 **Revised form accepted** 31 December 2007

Introduction

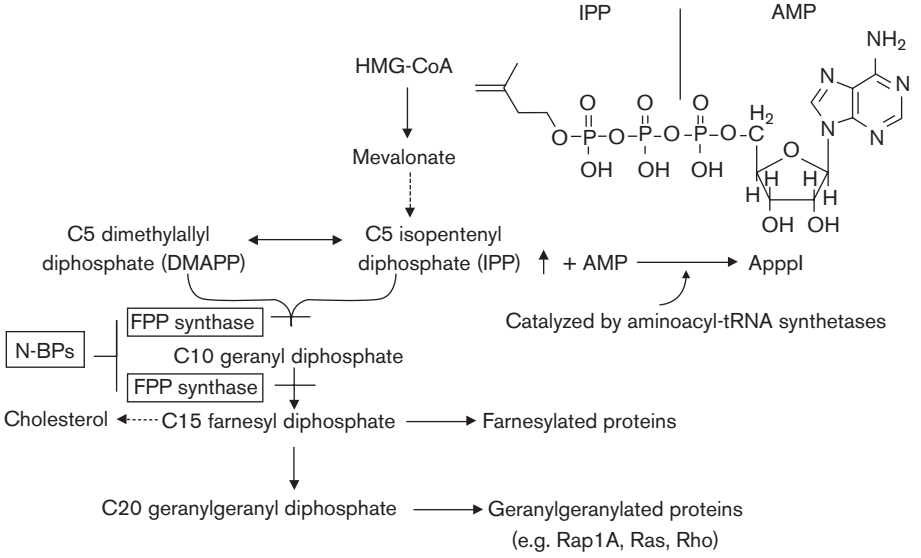
Bisphosphonates (BPs) are pyrophosphate analogs that effectively inhibit osteoclastic bone resorption and are widely used in the treatment of metabolic bone diseases, such as Paget's disease [1], hypercalcemia [2], and postmenopausal osteoporosis [3]. Additionally, BPs are effective inhibitors of tumor-induced bone resorption and significantly reduce the incidence of skeletal complications in patients with bone metastases, especially from breast cancer [4].

According to the chemical structure, BPs can be divided into two classes with distinct molecular mechanisms of action. Low-potency BPs lacking a nitrogen group (non-N-BPs), such as clodronate, are metabolized to cytotoxic ATP analogs [5,6] resulting in the induction of apoptosis by inhibiting the mitochondrial ADP/ATP translocase [7]. The formation of this metabolite is catalyzed by aminoacyl-tRNA-synthetases [8]. By contrast, the more potent nitrogen-containing BPs (N-BPs) are not metabolized [5,9] but act primarily by inhibiting farnesyl diphosphate (FPP) synthase, a key enzyme in the mevalonate pathway [9,10]. The activity of FPP synthase is required for the posttranslational prenylation of small GTPases, such as Ras and Rap families. As prenylation is required for the localization of these GTPases to subcellular membranes, N-BPs disrupt the function of small GTPases that are essential for osteoclast activity and survival. In contrast, recent studies suggest that the antiresorptive effect of N-BPs on osteoclasts may actually be due to the accumulation of unprenylated small GTPases in their active state (i.e. causing inappropriate activation of downstream signalling pathways) rather than loss of the prenylated proteins [11,12].

Our recent research has revealed a new mechanism of action for N-BPs, establishing that the potent N-BPs, such as zoledronic acid, induce formation of a new type of ATP analog, ApppI *in vitro* [13] and *in vivo* [14]. ApppI production results from the inhibition of FPP synthase in the mevalonate pathway and subsequent accumulation of the intracellular isopentenyl diphosphate (IPP) (Fig. 1). ApppI formation from IPP is probably catalyzed by the same enzyme family (i.e. aminoacyl-tRNA synthetases) as the ATP analogs of non-N-BPs, but ApppI does not contain a BP structure. Similar to AppCp-type metabolites of non-N-BPs [7], ApppI is able to induce direct apoptosis through blockade of the mitochondrial ADP/ATP translocase [13].

0959-4973

Fig. 1



Biosynthesis of ApppI results from inhibition of FPP synthase by N-BPs in the mevalonate pathway and consequent accumulation of IPP. FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate.

The molecular mechanisms whereby N-BPs inhibit bone resorption are well established, whereas the mechanisms underlying antitumor activity of these compounds remain to be clarified. A large amount of preclinical evidence suggests that BPs may have direct effects on tumors, inhibiting tumor cell invasion and adhesion to bone [15,16], as well as inducing tumor cell apoptosis [17–19].

The purpose of this study was to evaluate the role of apoptotic ATP analogs induced by BPs in the antitumor properties of these drugs. Therefore, we explored IPP/ApppI accumulation induced by zoledronic acid, protein prenylation and clodronate metabolism to $AppCCl_2p$ in various cancer cell lines. This was also expected to clarify the formation mechanism of ApppI from IPP, and the role of aminoacyl-tRNA synthetase in the reaction. The cellular uptake of zoledronic acid and clodronate in selected cancer cell lines was investigated to determine whether there is a correlation between drug uptake and subsequent ATP analog accumulation. In addition, the inhibition of cell growth by zoledronic acid and clodronate in selected cancer cell lines was studied to clarify the role of ATP analogs in cell growth. This is the first study to show that BPs induce the accumulation of ATP analogs, ApppI and $AppCC_2p$ in tumor cells.

Materials and methods

Chemicals

Zoledronic acid [2-(imidazol-1-yl)-hydroxy-ethylidene-1, 1-bisphosphonic acid, disodium salt, 4.75 hydrate] and [^{14}C]zoledronic acid were provided by Novartis Pharma AG (Basel, Switzerland). Clodronate (dichloromethylene-1,1-bisphosphonate) and [^{14}C]clodronate by Schering Oy (Bayer Schering Pharma AG, Berlin, Germany). Stock solutions of BPs were prepared in phosphate-buffered saline (PBS; pH 7.4; Gibco, Paisley, UK) and solutions were filter-sterilized before use. Clodronate metabolite ($AppCCl_2p$) was synthetized by Professor A. Azhayev (University of Kuopio, Finland). ApppI was synthesized as described previously [13]. IPP and AppCp were purchased from Sigma (St Louis, Missouri, USA). Sodium fluoride was from Riedel-de-Haën (Seelze, Germany) and sodium orthovanadate from Sigma.

Cell lines

The human estrogen-dependent breast cancer cell line MCF-7, the human osteosarcoma cell line MG-63, and the human myeloma cell lines NCI-H929 and RPMI-8226 were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The human estrogen-independent breast cancer cell line MDA-MB-436, the human androgen-independent prostate cancer cell line PC-3 and the human nonmalignant breast cell line MCF-10A were obtained from the American Type Culture Collection (Manassas, Virginia, USA). All other cells, except MCF-10A, were cultured at 37°C in RPMI-1640 media with L-glutamine (BioWhittaker, Cambrex Bioscience, Verviers, Belgium), supplemented with 10% of fetal calf serum (Invitrogen, Paisley, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). MCF-10A cells were cultured in DMEM:F12 (1 : 1) (Invitrogen), supplemented with 5% of horse serum (Invitrogen), 100 U/ml penicillin and 100 μg/ml strepto-

mycin (Invitrogen), 100 ng/ml cholera toxin (Calbiochem, Darmstadt, Germany), 20 ng/ml EGF (Sigma), 0.1 μmol/l hydrocortisol (Sigma) and 10 μg/ml insulin (Sigma). Cells were harvested using trypsin (Invitrogen). Plastics were supplied by Costar (High Wycombe, UK).

Isopentenyl diphosphate/ApppI production and clodronate metabolism

For studies on the zoledronic acid-induced IPP/ApppI production and clodronate metabolism to $AppCCl_2p$, the MCF-7, MDA-MB-436, PC-3, MG-63, MCF-10A, RPMI-8226, and NCI-H929 cells were seeded into six-well plates at a density of 1×10^6 cells/well and left to adhere for 2 h, then treated with 25 μmol/l zoledronic acid or 500 μmol/l clodronate for 24 h. After treatment, the cultured cells were scraped off the wells and washed in ice-cold PBS. Extracts from cells were prepared by using ice-cold acetonitrile as described previously [20].

Analysis of isopentenyl diphosphate/ApppI and $AppCCl_2p$

The samples were redissolved in 150 μl of water containing sodium fluoride (0.25 mmol/l) and sodium orthovanadate (0.25 mmol/l) as phosphatase inhibitors for preventing the degradation of ATP analogs. AppCp was used as internal standard. The molar amounts of IPP, ApppI and $AppCCl_2p$ in cell extracts were determined by high-performance liquid chromatography (HPLC) negative ion electrospray ionization mass spectrometry [20]. IPP, ApppI and $AppCCl_2p$ are very hydrophilic compounds and therefore the use of dimethylhexylamine (DMHA) as an ion-pair agent was necessary to retain these compounds into a reversed-phase column. HPLC separation was performed using a Phenomenex Gemini C_{18} column (2.0×50.0 mm, 5 μm) and an eluent system consisting 20 mmol/l DMHA buffer (pH 7) (eluent A) and 80% methanol with 2 mmol/l DMHA (pH 7) (eluent B). The flow rate was 200 μl/min and injection volume 45 μl. After HPLC separation, negative ion mass spectra for IPP, ApppI and $AppCCl_2p$ were acquired using an LTQ quadrupole ion trap mass spectrometer equipped with an electrospray ionization source (Thermo Electron Corporation, San Jose, California, USA). Selected reaction monitoring was used for analysis of the compounds in the sample and quantitation was based on characteristic fragment ions. Following transitions were monitored: m/z 245→159 for IPP, m/z 574→408 for ApppI, m/z 572→225 and m/z 574→227 for $AppCCl_2p$ (^{35}Cl and ^{37}Cl), and m/z 504→406 for internal standard AppCp. The standard curve was created by spiking extracts from untreated cells with synthesized IPP, ApppI, or $AppCCl_2p$. The concentrations of the samples were determined using the peak areas of the selected reaction monitoring chromatograms and the standard curve.

Cellular uptake

For studies on the cellular uptake of zoledronic acid and clodronate, MCF-7, RPMI-8226, and MDA-MB-436 cells were seeded into six-well tissue culture plates at a density of 1×10^6 cells/well and allowed to adhere for 2 h, then treated with 25 μmol/l [^{14}C] zoledronic acid or 500 μmol/l [^{14}C] clodronate for 24 h. After treatment, the medium was collected, and the wells were rinsed five times with PBS solution. All wash solutions were collected. Finally, cells were carefully scraped off from the wells (two wells were pooled together) and extracted with acetonitrile (300 μl) and water (200 μl). The soluble and precipitated protein fractions were separated by centrifugation (13 000*g*, 1 min). Cell precipitates were digested with 1 ml of 1 mol/l NaOH at 60°C for 2 h. The soluble acetonitrile/water extracts were evaporated in a vacuum centrifuge. For the radioactivity measurements, the evaporated samples were redissolved in 120 μl of Milli-Q water. The cellular uptake percentage of BP was counted by comparing the radioactivity of cell extracts (acetonitrile/water and digested protein fractions) to the total radioactivity (cell extracts, medium and washes). Radioactivity was measured by a scintillation counter (Wallac Microbeta, TriLux) after mixing with OptiPhase HiSafe3 scintillation cocktail (Wallac, Turku, Finland).

Western blot analysis

To determine the effect of zoledronic acid on protein prenylation in different cancer cells, accumulation of unprenylated Rap1A were analyzed by Western blot. One million MCF-7, MDA-MB-436, PC-3, MG-63, MCF-10A, RPMI-8226, and NCI-H929 cells were treated with 25 μmol/l zoledronic acid on the six-well plate for 24 h and then the cells were lysed in 500 μl of cell precipitation buffer [Mammalian Cell Lysis Kit, Sigma (MCL-1)]. Ten milligrams of protein/lane were electrophoresed on 10% SDS–polyacrylamide gels followed by transfer onto nitrocellulose membrane (Millipore Corporation, IPVH00010, Boston, Massachusetts, USA). Nonspecific antibody binding was blocked by incubating the membrane in 5% nonfat milk in PBS with 0.1% Tween before incubation with goat polyclonal anti-Rap1A antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA; SC-1482 clone C-17) used at a dilution of 1/200 overnight at 4°C. The secondary antibody was HRP-conjugated antigoat from Autogen Bioclear used at a concentration of 1/50 000 (ABN021HRP; Calne, UK). Membranes were developed using Supersignal (Pierce, 34075, Tattenhall, UK). After visualizing the chemiluminescent signal, the membrane was then stripped in PBS pH 2, neutralized in PBS pH 7.5 and then incubated in mouse monoclonal antiactin antibody (from Autogen Bioclear, ABJ1275), followed by a 1/30 000 dilution of antimouse HRP conjugate from Amersham (Little Chalfont, UK). The blots were again visualized with a Supersignal. Densitometric analysis of blots was carried out using Quantity One software by BioRad (Hemel Hempstead, UK).

Inhibition of cell growth

For studies on the inhibition of cell growth by zoledronic acid and clodronate, MCF-7, RPMI-8226, and MDA-MB-436 cells were seeded into 96-well tissue culture plates at a density of 5000, 7500, and 2500 cells/well, respectively. The cells were incubated overnight and exposed to 1–100 μmol/l zoledronic acid or 10–2000 μmol/l clodronate. The cell growth was assayed 72 h later using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay as described previously [21,22]. The cell culture medium was replaced with 100 μl of serum-free culture medium and 0.5 mg/ml 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (Sigma) followed by incubation for a further 1 h at 37°C. The formazan precipitate was dissolved by the addition of 100 μl of SDS–*N*,*N*-dimethyl formamide buffer (20% w/v sodium dodecyl sulfate in a solution of 50% *N*,*N*-dimethyl formamide, pH 4.7) and overnight incubation at 37°C. Absorbances (Abs) were measured at 560 nm using Automated Microplate Reader (Dynex Technologies Inc., Chantilly, Virginia, USA). The percentage of viable cells was calculated using the following formula:

$$\frac{(\mathrm{Abs}_{\mathrm{sample}} - \mathrm{Abs}_{\mathrm{blank}}) \times 100\%}{\mathrm{Abs}_{\mathrm{control}} - \mathrm{Abs}_{\mathrm{blank}}}$$

where $\mathrm{Abs}_{\mathrm{sample}}$, absorbance of the cells treated with drug; $\mathrm{Abs}_{\mathrm{blank}}$, absorbance of solutions used; $\mathrm{Abs}_{\mathrm{control}}$, absorbance of the cells treated with 1% of PBS (zoledronic acid) or 2% of PBS (clodronate).

Statistical analysis

For cellular uptake and inhibition of cell growth studies one-way analysis of variance with Tukey's multiple comparison tests were used to assess significant differences in drug uptake or IC_{50} values between different cell lines.

Results

Isopentenyl diphosphate/ApppI production varies between different cancer cell types

Before this study, there were no reports of ApppI production by tumor cells, and as zoledronic acid is used to treat tumor-induced bone disease in multiple myeloma, breast and prostate cancer we investigated the IPP/ApppI formation in a range of different cancer cell lines. Both IPP and ApppI production varied between cell lines following drug treatment. IPP levels in cancer cell lines were 511 (MCF-7), 374 (RPMI-8226), 174 (MG-63), 87 (MDA-MB-436), 71 (PC-3), 7 (NCI-H929), and 2 pmol (MCF-10A) (Fig. 2a). The capability of zoledronic acid to induce ApppI production in these cells was 177 (MCF-7), 30 (MG-63), 11 (PC-3), 3 (RPMI-8226), and ~0.03 pmol (MDA-MB-436). MCF-10A and NCI-H929 cells did not produce detectable levels of ApppI after zoledronic acid treatment (Fig. 2c).

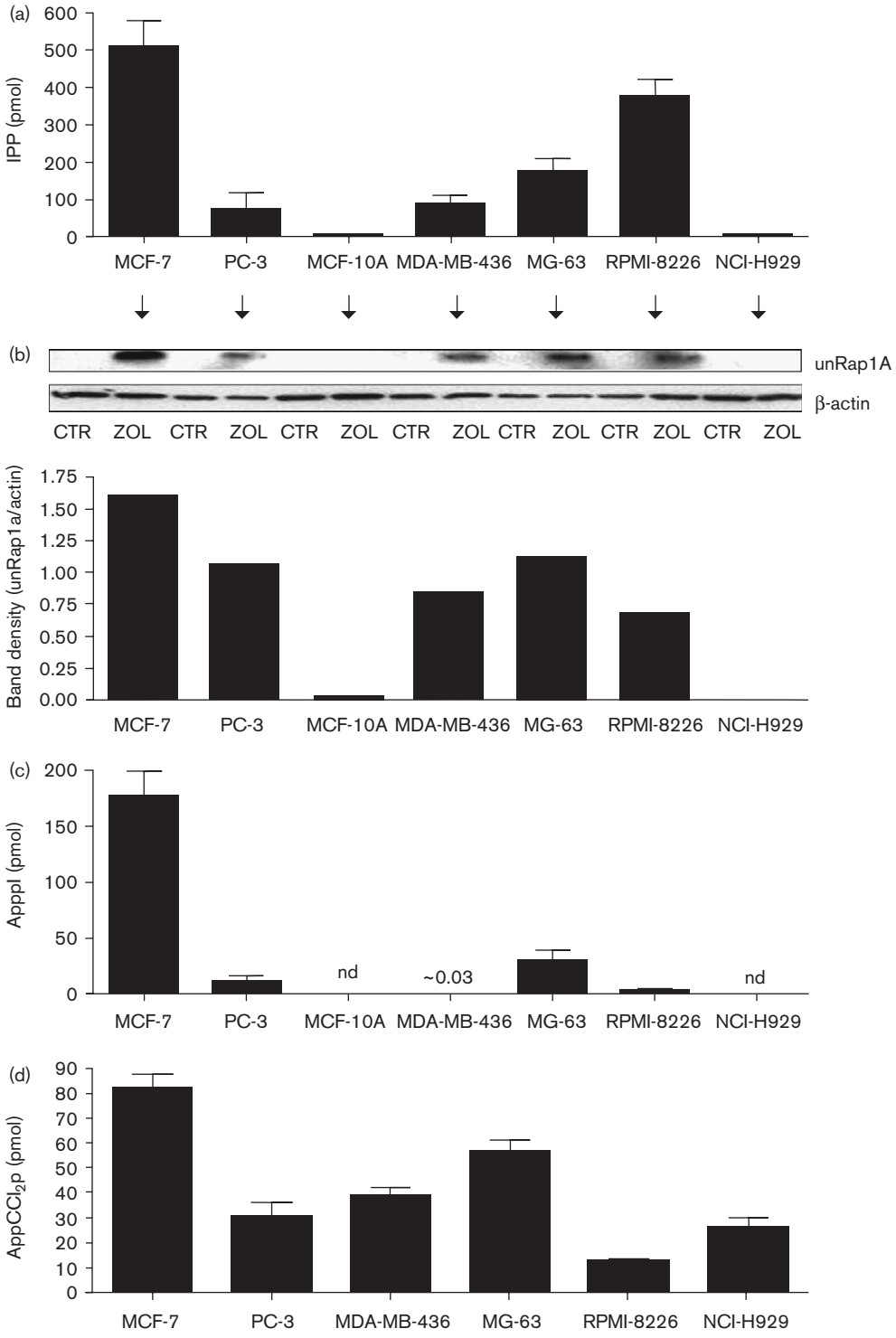
Isopentenyl diphosphate production correlates with the capacity of zoledronic acid to inhibit Rap1A prenylation, but does not correlate with cellular uptake of the drug

The differences between IPP and ApppI production detected in the various cell lines in the initial experiments may be due to the differential uptake of zoledronic acid by the cells. This possibility was addressed by studying the cellular uptake of zoledronic acid in MCF-7, MDA-MB-436, and RPMI-8226 cells. The levels of cellular uptake of zoledronic acid (Fig. 3a) did not correlate with IPP accumulation in these cells (Fig. 2a). This finding raised the question of whether there is any connection between IPP accumulation induced by zoledronic acid and the activity of FPP synthase. We therefore determined the inhibition of Rap1A prenylation after zoledronic acid treatment in the different cell lines. By inhibiting the FPP synthase in the mevalonate pathway, N-BPs, such as zoledronic acid, prevent the biosynthesis of isoprenoid lipids (FPP and GGPP), which are essential for the posttranslational prenylation of small GTPase signalling proteins, such as Rap1A (Fig. 1). In this study, the measurement of accumulation of unprenylated Rap1A was used for estimating the activity of FPP synthase in different cancer cell lines. This experiment indicated that IPP production (Fig. 2a) correlated with the capacity of zoledronic acid to inhibit Rap1A prenylation (Fig. 2b).

Clodronate metabolism correlates with the efficiency of isopentenyl diphosphate to ApppI conversion

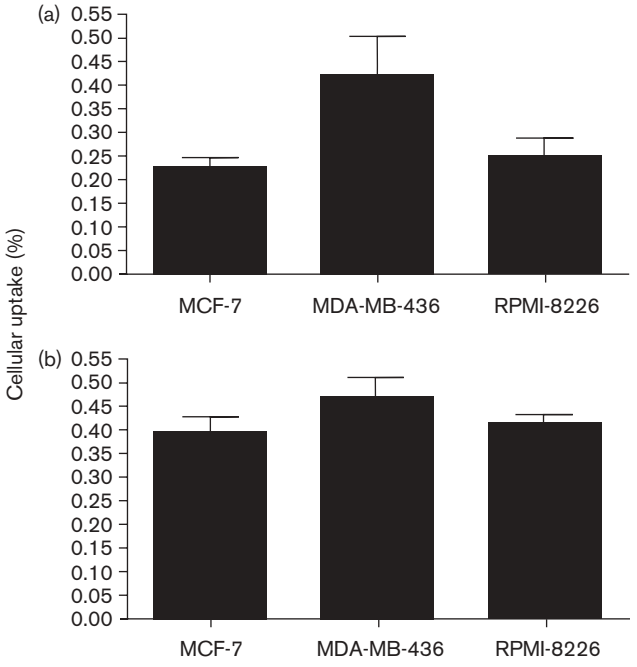
The formation of AppCp-type metabolites of non-N-BPs (such as clodronate) is catalyzed by aminoacyl-tRNA synthetases [8]. We have previously shown that clodronate decreases the ApppI production, and reciprocally, risedronate (a N-BP) decreases the metabolism of clodronate to its $AppCCl_2p$ metabolite after the cotreatment in J774 macrophages [13]. Therefore, we hypothesized that aminoacyl-tRNA synthetases may also be involved in ApppI production induced by N-BPs. To compare the N-BP-induced ApppI formation and the metabolism of non-N-BP, clodronate metabolism was studied in different cancer cell lines following exposure to 500 μmol/l clodronate for 24 h. The metabolism of clodronate to $AppCCl_2p$ in different cells was 82 (MCF-7), 57 (MG-63), 39 (MDA-MB-436), 31 (PC-3), 26 (NCI-H929), and 13 pmol (RPMI-8226) (Fig. 2d). No significant differences in the clodronate uptake between cell lines ($P > 0.05$) were observed (Fig. 3b). Therefore, as was the case for zoledronic acid-induced IPP accumulation, the differences in clodronate metabolism were not due to different levels of uptake of BP between the cell lines. The efficiency of ApppI production from IPP in different cells followed the order of potency; MCF-7 > MG-63 > PC-3 > RPMI-8226. This order correlated with the potency of clodronate metabolism to $AppCCl_2p$ metabolite ($R^2 = 0.9461$) (Fig. 4). These results support the hypothesis that the N-BP-induced

Fig. 2



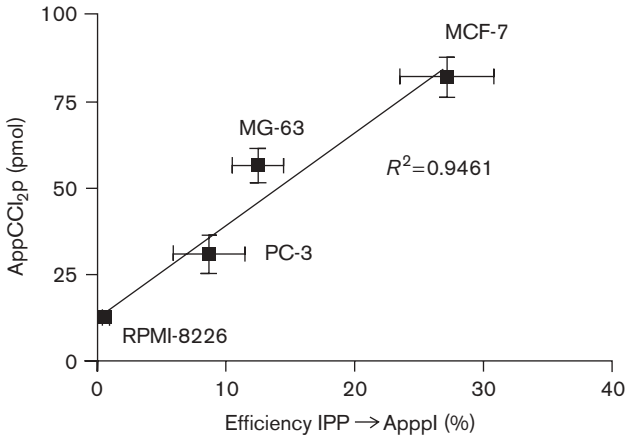
Zoledronic acid-induced IPP/ApppI formation, inhibition of Rap1A prenylation and clodronate metabolism to $AppCCl_2p$ in different cancer cell lines. IPP and ATP analogs were analyzed by HPLC-ESI-MS and Rap1A prenylation by Western blotting for unprenylated Rap1A. IPP accumulation (a), unprenylated Rap1A and β-actin (b), ApppI production (c), clodronate metabolism to $AppCCl_2p$ (d) in cancer cells after treatment with 25 μmol/l zoledronic acid or 500 μmol/l clodronate for 24 h (mean ± SEM, $n = 6–9$). HPLC-ESI-MS, high-performance liquid chromatography negative ion electrospray ionization mass spectrometry; IPP, isopentenyl diphosphate; nd, not detected; ZOL, zoledronic acid; CTR, control.

Fig. 3



The differences in cellular uptake of zoledronic acid or clodronate between cell lines are not significant. The cells were treated with ^{14}C-labelled drugs, 25 μmol/l zoledronic acid (a) and 500 μmol/l clodronate (b), for 24 h (mean ± SEM, $n=6$). $P>0.05$ using Tukey's multiple comparision test.

Fig. 4



Clodronate metabolism correlates with the efficacy of ApppI formation from IPP (isopentenyl diphosphate).

ApppI production is catalyzed by aminoacyl-tRNA synthetases, similar to the metabolism of non-N-BPs to the AppCp metabolite. As non-N-BPs (pCp) and IPP resemble pyrophosphate in the structure, the reverse reaction can also take place but with pCp or IPP replacing pyrophosphate, to form an AppCp metabolite or ApppI, respectively (Fig. 5).

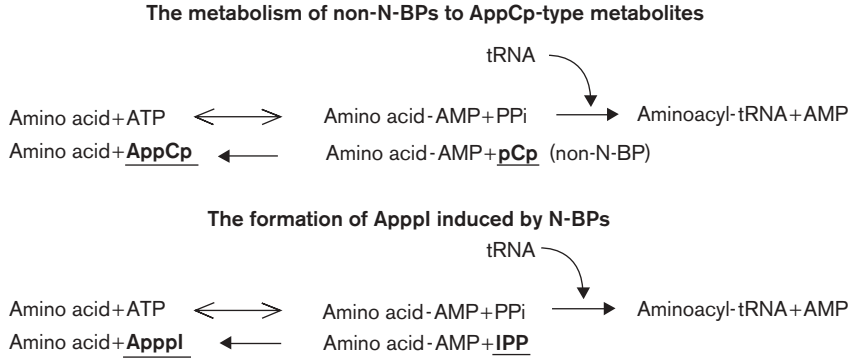
Potency of clodronate to inhibit cancer cell growth corresponds to ATP analog formation

It has been suggested that AppCp-type metabolites are mainly responsible for the effect of non-N-BPs [12]. The relationship between N-BP-induced ApppI accumulation and cellular effect, however, is unknown. One main strategy in cancer therapy is to inhibit cancer cell growth. To compare the BP-induced ATP analog formation and its effect on cell growth inhibition, cell growth was studied in MCF-7, MDA-MB-436, and RPMI-8226 cells following exposure to 1–100 μmol/l zoledronic acid or 10–2000 μmol/l clodronate for 72 h. These cell lines were chosen for this experiment because of their diverse potency to produce ATP analogs (Figs 2c and d). MCF-7 and MDA-MB-436 breast cancer cells were more sensitive to the effect of zoledronic acid on cell growth inhibition than RPMI-8226 myeloma cells (Fig. 6a). IC_{50} values (50% inhibition of cell growth) of zoledronic acid for MCF-7, MDA-MB-436, and RPMI-8226 cells were 7.0, 6.0, and 18.1 μmol/l, respectively. No significant differences in IC_{50} values between MCF-7 and MDA-MB-436 cells ($P>0.05$) were observed (Fig. 6c). Instead, IC_{50} value of RPMI-8226 cells was significantly higher compared with breast cancer cells ($P<0.001$) (Fig. 6c). The inhibition of cell growth by clodronate was quite similar between these cell lines (Fig. 6b). IC_{50} values of clodronate for MCF-7, MDA-MB-436, and RPMI-8226 cells were 893, 1035, and 1179 μmol/l, respectively. A significant difference in IC_{50} values between MCF-7 and RPMI-8226 cells ($P<0.01$) was observed (Fig. 6d). These observations show that clodronate metabolism to $AppCCl_2p$ (Fig. 2d) corresponds better than zoledronic acid-induced ApppI formation (Fig. 2c) to the potency of BP to inhibit cell growth (Fig. 6).

Discussion

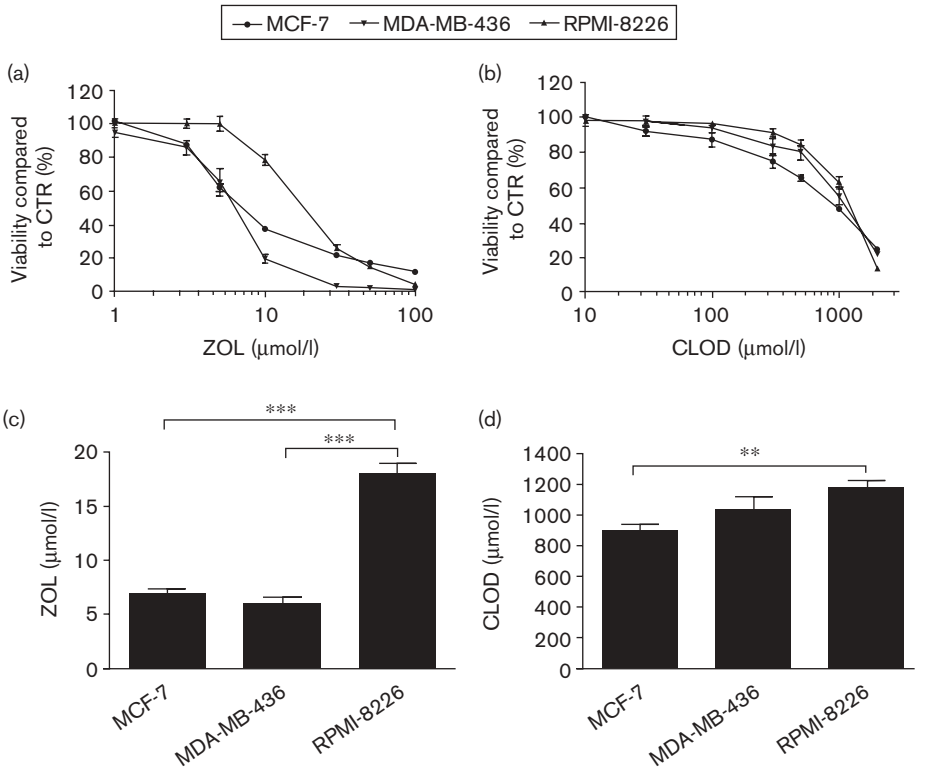
BPs have a high affinity for the bone mineral and are selectively internalized by osteoclasts, where they disrupt the processes involved in bone resorption [23]. N-BPs inhibit the mevalonate pathway [24], whereas non-N-BPs are converted to intracellular (AppCp-type) metabolites by aminoacyl-tRNA synthetase enzymes [8], with both mechanisms ultimately leading to osteoclast apoptosis. In addition to their inhibitory effect on osteoclast function, there is considerable preclinical evidence to suggest that BPs also have antitumor activity *in vitro*, through direct effects on tumor cells [25]. The mechanisms responsible for the observed antitumor effects of BPs are beginning to be elucidated. One of the primary mechanisms responsible for the direct antitumor activity of BPs is the inhibition of cancer cell growth. Mechanisms by which BPs inhibit cancer cell growth might be through the production of cytotoxic ATP analogs. N-BPs possess a capacity to induce intracellular ATP analog (ApppI)

Fig. 5



The metabolism of non-N-BP to AppCp-metabolite and the formation of ApppI induced by N-BPs is catalyzed by aminoacyl-tRNA synthetases. AMP, ampicillin; IPP, isopentenyl diphosphate.

Fig. 6



Inhibition of cell growth by zoledronic acid and clodronate. The cells were treated with 1–100 μmol/l zoledronic acid or 10–2000 μmol/l clodronate for 72 h. Cell growth was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay. The effect of zoledronic acid (a) and clodronate (b) on cell growth inhibition. IC_{50} values of zoledronic acid (c) and clodronate (d) (mean ± SEM, $n=12$). $^{**}P<0.01$; $^{***}P<0.001$ using Tukey's multiple comparision test. ZOL, zoledronic acid; CLOD, clodronate; CTR, control.

production, which results from covalent binding of ampicillin and IPP. Similar to the $AppCCl_2p$ metabolite of clodronate, ApppI is able to induce direct apoptosis in cells [13]. Therefore, zoledronic acid and other potent N-BPs evoke apoptosis by at least two distinct mechanisms: indirectly through the inhibition of protein isoprenylation [10] and also directly through the inhibition of mitochondrial ADP/ATP translocase by ApppI [13].

We evaluated the ATP analog formation in various cancer cell lines (such as breast and prostate cancer and myeloma cell lines) after treatment with zoledronic acid or clodronate. Interestingly, we observed that zoledronic acid-induced IPP/ApppI accumulation and clodronate metabolism to $AppCCl_2p$ were remarkably cell line-dependent. To clarify the reasons for the variation of BP-induced IPP and ATP analog accumulation between different cancer cell lines, drug uptake studies were carried out using ^{14}C-labelled drugs, zoledronic acid, and clodronate. For this purpose, we selected three cancer cell lines that differed significantly from each other in their BP-induced IPP and ATP analog expression. No significant differences, however, in BP uptake between selected cell lines were observed ($P > 0.05$). Therefore, these data demonstrated that IPP accumulation and apoptotic ATP analog formation are independent from the cellular uptake of BP. Regarding BP uptake and ATP analog formation in tumor cells *in vivo*, it is more likely that BPs as bone-targeting drugs can reach tumor cells on bone surface rather than in soft tissues. Osteoclasts can become exposed to very high concentrations of BPs *in vivo*, as has been shown for alendronate (N-BP), which reaches about 1 mmol/l concentration in the resorption space beneath an osteoclast [26]. It is therefore possible that the tumor cells on the bone surface may be exposed to relatively high concentrations of BPs and be capable of the production of cytotoxic ATP analogs.

N-BPs have been shown to inhibit the key enzyme, FPP synthase, in the mevalonate pathway causing intracellular accumulation of unprenylated forms of small GTP-binding proteins [10]. As the cellular uptake of zoledronic acid did not correspond to IPP accumulation, it raised the question whether there is any connection between IPP accumulation induced by zoledronic acid and the activity of FPP synthase? For this purpose, unprenylated form of GTPase Rap1A, which is a surrogate marker of the FPP synthase inhibition, was used to demonstrate the effect of 25 µmol/l zoledronic acid to prevent protein prenylation in cells. The results of Western blot analysis evidenced that the unprenylated form of Rap1A could be only detected in cell lines that produced relatively high levels of IPP. This demonstrated that IPP production correlated with the capacity of zoledronic acid to inhibit Rap1A prenylation (i.e. FPP synthase). Taken together, the data from studies of prenylation and drug uptake strongly suggest that the efficiency of zoledronic acid in inhibiting protein prenylation is dependent on the activity of FPP synthase in tumor cells. Additionally, the measurement of IPP levels by mass spectrometry provides a very sensitive new technique to study the FPP synthase inhibition by N-BPs.

Earlier, we have shown that ApppI production correlated with the increase in the IPP concentration in J774 macrophages [13]. ApppI production from IPP, however, did not correspond to IPP accumulation between different cancer cells. Therefore, the efficiency of ApppI production from IPP seems to vary with cell type. We hypothesized that the novel ATP analog, ApppI, is produced by the same metabolic pathway as the metabolite of non-N-BP. The hypothesis was tested by comparing the N-BP-induced ApppI formation from IPP to formation of ATP analog of a non-N-BP in the same cancer cell lines. We found that the efficiency of ApppI production from IPP correlated with clodronate metabolism in the investigated cell lines. The MDA-MB-436 cell line, however, does not fit the hypothesis that the aminoacyl-tRNA synthetases catalyze ApppI formation because zoledronic acid-induced IPP accumulation and clodronate metabolism to $AppCCl_2p$ were relatively high in this cell line. Thus, this result partly supports the hypothesis that the aminoacyl-tRNA synthetase family, which catalyzes the formation of AppCp-type metabolites from non-N-BPs [8], also seems to be involved in the ApppI production induced by N-BPs.

BP-induced ATP-analog formation and its relationship with the inhibition of cell growth were tested in selected cancer cell lines. The effect of clodronate on cell growth inhibition corresponds to the potency of clodronate metabolism to $AppCCl_2p$. This finding supports the hypothesis that AppCp-type metabolites are responsible for the effects of non-N-BPs [12]. Zoledronic acid-induced ApppI formation does not correspond to the effect of the drug on cell growth inhibition. In addition to cytotoxic ApppI production [13,14], the inhibition of FPP synthase by N-BPs, such as zoledronic acid, causes a lack of essential prenylated proteins [12]. As N-BPs having a more complex mechanism of action compared with non-N-BPs, a direct connection between ApppI formation and cellular effects may not be found. It is possible that the N-BP-induced IPP accumulation prior to ApppI formation plays a role in cell function. Particularly, as Gober *et al.* [27] have reported that the treatment of tumor cells with N-BPs *in vitro* also causes the accumulation of IPP, which can activate γδ T cells capable of tumor cell killing. Therefore, N-BPs could be used as an immune therapy, whereas N-BP-induced IPP accumulation in tumor cells is a powerful danger signal that activates T-cell-mediated immune response against tumors [27,28]. The present data proved the considerable variation of IPP accumulation between different cancer cells after zoledronic acid treatment. This suggests that N-BPs may have differences in capability to induce cancer cell killing.

In conclusion, the results of this study suggest that BPs may have diverse antitumor effects. This was seen as a significant variation between IPP/ApppI formation and clodronate metabolism to $AppCCl_2p$. We conclude that the differences may be related to different activities of the enzymes responsible for IPP accumulation and ATP analog formation, such as FPP synthase or

aminoacyl-tRNA synthetases. Therefore, the production of cytotoxic ATP analogs in different tumor cells after BP treatment is likely to depend on the activity of the appropriate pathways involved in BP metabolism. Taken together, the ability of the cancer cells to metabolize the cytotoxic compounds varies, implying a potentially new mechanism contributing to the specificity of BPs against different tumor cell types.

Acknowledgements

The authors thank Dr Jonathan Green (Novartis Pharma AG, Basel, Switzerland) for his valuable comments on the manuscript. This work was financially supported by the Academy of Finland, Finnish Cultural Foundation, Saastamoinen Foundation and Oskar Huttunen Foundation. Novartis Pharma AG donated the zoledronic acid and Schering AG the clodronate.

References

1 Roux C, Dougados M. Treatment of patients with Paget's disease of bone. *Drugs* 1999; **58**:823–830.
2 Fleisch H. Bisphosphonates. Pharmacology and use in the treatment of tumour-induced hypercalcaemic and metastatic bone disease. *Drugs* 1991; **42**:919–944.
3 Delmas PD. Treatment of postmenopausal osteoporosis. *Lancet* 2002; **359**:2018–2026.
4 Coleman RE. The role of bisphosphonates in breast cancer. *Breast* 2004; **13** (**Suppl 1**):S19–S28.
5 Auriola S, Frith J, Rogers MJ, Koivuniemi A, Mönkkönen J. Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography-electrospray mass spectrometry. *J Chromatogr B Biomed Sci Appl* 1997; **704**:187–195.
6 Frith JC, Mönkkönen J, Blackburn GM, Russell RG, Rogers MJ. Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5′-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells *in vitro*. *J Bone Miner Res* 1997; **12**:1358–1367.
7 Lehenkari PP, Kellinsalmi M, Näpänkangas JP, Ylitalo KV, Mönkkönen J, Rogers MJ, *et al*. Further insight into mechanism of action of clodronate: inhibition of mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing metabolite. *Mol Pharmacol* 2002; **61**:1255–1262.
8 Rogers MJ, Brown RJ, Hodkin V, Blackburn GM, Russell RG, Watts DJ. Bisphosphonates are incorporated into adenine nucleotides by human aminoacyl-tRNA synthetase enzymes. *Biochem Biophys Res Commun* 1996; **224**:863–869.
9 Benford HL, Frith JC, Auriola S, Mönkkönen J, Rogers MJ. Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates: biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. *Mol Pharmacol* 1999; **56**:131–140.
10 Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G, 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**:581–589.
11 Dunford JE, Rogers MJ, Ebetino FH, Phipps RJ, Coxon FP. Inhibition of protein prenylation by bisphosphonates causes sustained activation of Rac, Cdc42, and Rho GTPases. *J Bone Miner Res* 2006; **21**:684–694.
12 Russell RGG, Xia Z, Dunford JE, Opermann U, Kwaasi A, Hulley PA, *et al*. Bisphosphonates. An update on mechanisms of action and how these relate to clinical efficacy. *Ann N Y Acad Sci* 2007; **1117**:209–257.
13 Mönkkönen H, Auriola S, Lehenkari P, Kellinsalmi M, Hassinen IE, Vepsäläinen J, *et al*. A new endogenous ATP analog (ApppI) inhibits the mitochondrial adenine nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-containing bisphosphonates. *Br J Pharmacol* 2006; **147**:437–445.
14 Mönkkönen H, Ottewell PD, Kuokkanen J, Mönkkönen J, Auriola S, Holen I. Zoledronic acid-induced IPP/ApppI production *in vivo*. *Life Sci* 2007; **81**:1066–1070.
15 Boissier S, Ferreras M, Peyruchaud O, Magnetto S, Ebetino FH, Colombel M, *et al*. Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. *Cancer Res* 2000; **60**:2949–2954.
16 Van der Pluijm G, Vloedgraven H, van Beek E, van der Wee-Pals L, Lowik C, Papapoulos S. Bisphosphonates inhibit the adhesion of breast cancer cells to bone matrices *in vitro*. *J Clin Invest* 1996; **98**:698–705.
17 Jagdev SP, Coleman RE, Shipman CM, Rostami-H A, Croucher PI. The bisphosphonate, zoledronic acid, induces apoptosis of breast cancer cells: evidence for synergy with paclitaxel. *Br J Cancer* 2001; **84**:1126–1134.
18 Senaratne SG, Colston KW. Direct effects of bisphosphonates on breast cancer cells. *Breast Cancer Res* 2002; **4**:18–23.
19 Shipman CM, Rogers MJ, Apperley JF, Russell RG, Croucher PI. Bisphosphonates induce apoptosis in human myeloma cell lines: a novel anti-tumour activity. *Br J Haematol* 1997; **98**:665–672.
20 Mönkkönen H, Moilanen P, Mönkkönen J, Frith JC, Rogers MJ, Auriola S. Analysis of an adenine nucleotide-containing metabolite of clodronate using ion pair high-performance liquid chromatography electrospray ionization mass spectrometry. *J Chromatogr B* 2000; **738**:395–403.
21 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; **65**:55–63.
22 Mönkkönen J, Taskinen M, Auriola SOK, Urtti A. Growth inhibition of macrophage-like and other cell types by liposome-encapsulated, calcium-bound, and free bisphosphonates *in vitro*. *J Drug Target* 1994; **2**:299–308.
23 Rogers MJ, Xiong X, Brown RJ, Watts DJ, Russell RG, Bayless AV, *et al*. Structure–activity relationships of new heterocycle-containing bisphosphonates as inhibitors of bone resorption and as inhibitors of growth of Dictyostelium discoideum amoebae. *Mol Pharmacol* 1995; **47**: 398–402.
24 Amin D, Cornell SA, Gustafson SK, Needle SJ, Ullrich JW, Bilder GE, *et al*. Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase and cholesterol biosynthesis. *J Lipid Res* 1992; **33**:1657–1663.
25 Neville-Webbe HL, Holen I, Coleman RE. The anti-tumour activity of bisphosphonates. *Cancer Treat Rev* 2002; **28**:305–319.
26 Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, *et al*. Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. *J Clin Invest* 1991; **88**:2095–2105.
27 Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, De Libero G. Human T cell receptor gamma delta cells recognize endogenous mevalonate metabolites in tumor cells. *J Exp Med* 2003; **197**:163–168.
28 Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T *et al*. γδ T cells for immune therapy of patients with lymphoid malignancies. *Blood* 2003; **102**:200–206.