0026-895X/99/010131-10$3.00/0

MOLECULAR PHARMACOLOGY, **56:**131–140 (1999).

Farnesol and Geranylgeraniol Prevent Activation of Caspases by Aminobisphosphonates: Biochemical Evidence for Two Distinct Pharmacological Classes of Bisphosphonate Drugs

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Received November 30, 1998; accepted April 12, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

Recently, advances have been made in understanding the molecular mechanisms by which bisphosphonate drugs inhibit bone resorption. Studies with the macrophage-like cell line J774 have suggested that alendronate, an amino-containing bisphosphonate, causes apoptosis by preventing post-translational modification of GTP-binding proteins with isoprenoid lipids. However, clodronate, a nonaminobisphosphonate, does not inhibit protein isoprenylation but can be metabolized intracellularly to a cytotoxic, β-γ-methylene (AppCp-type) analog of ATP. These observations raise the possibility that bisphosphonates can be divided into two groups with distinct molecular mechanisms of action depending on the nature of the R_2 side chain. We addressed this question by directly comparing the ability of three aminobisphosphonates (alendronate, ibandronate, and pamidronate) and three nonaminobisphosphonates (clodronate, etidronate, and tiludronate) to inhibit protein isoprenylation and activate caspase-3-like proteases or to be metabolized to AppCp-type nucleotides by J774 cells. All three aminobisphosphonates inhibited protein isoprenylation and activated caspase-3-like proteases. Apoptosis and caspase activation after 24-h treatment with the aminobisphosphonates could be prevented by addition of farnesol or geranylgeraniol, confirming that these bisphosphonates inhibit the metabolic mevalonate pathway. No AppCp-type metabolites of the aminobisphosphonates could be detected by mass spectrometry. The three nonaminobisphosphonates did not inhibit protein isoprenylation or cause activation of caspase-3-like proteases, but were incorporated into AppCp-type nucleotides. Taken together, these observations clearly demonstrate that bisphosphonate drugs can be divided into two pharmacological classes: the aminobisphosphonates, which act by inhibiting protein isoprenylation, and the less potent nonaminobisphosphonates, which act through the intracellular accumulation of AppCp-type metabolites.

Excessive bone resorption is the major pathological feature of a number of common bone diseases, including Paget's disease, tumor-associated osteolysis, and postmenopausal osteoporosis (Mundy, 1995). Postmenopausal osteoporosis affects approximately 30% of women over the age of 50 and therefore is of particular social and economic importance. Bisphosphonates are among the most important drugs used in the clinical management of these diseases, because they are powerful inhibitors of bone resorption (Fleisch, 1991).

H.L.B. is a recipient of the Ann Stansfield Fellowship from the National Association for the Relief of Paget's Disease (NARPD). J.C.F. is supported by a studentship from the Medical Research Council. This work was also funded by the Technical Development Centre, Finland.

Bisphosphonates are a class of pyrophosphate analogs containing a phosphate-carbon-phosphate (P-C-P) backbone. This structure confers the ability to chelate calcium ions and, consequently, the ability to target to bone mineral in vivo. The geminal carbon of the P-C-P group has two side chains, R_1 and R_2. The R_1 side chain is usually a hydroxyl group, because this enhances the affinity of the compounds for bone mineral but has little influence on the antiresorptive potency (van Beek et al., 1994; Rogers et al., 1995). The major determinant of antiresorptive potency is the structure and conformation of the R_2 side chain (van Beek et al., 1994; Rogers et al., 1995). First generation of bisphosphonate drugs have a short R_2 side chain, such as —CH_3 (as in etidronate) or —Cl

ABBREVIATIONS: Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; Ac-YVAD-AMC, *N*-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin; FPLC, fast performance liquid chromatography; MS, mass spectrometry; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; CLO, dichloromethylene-1,1-bisphosphonate; ETI, 1-hydroxyethylidene-1,1-bisphosphonate; TIL, chloro-4-phenylthiomethylene-1,1-bisphosphonate; ALN, 4-amino-1-hydroxybutylidene-1,1-bisphosphonate; PAM, 3-amino-1-hydroxypropylidene-1,1-bisphosphonate; IBA, 1-hydroxy-3(methylpentylamino)-propylidene-1,1-bisphosphonate; FOH, farnesol; GGOH, geranylgeraniol; ACN, acetonitrile; ESI, electrospray ionization.

(as in clodronate). These bisphosphonates, together with tiludronate (which has a chlorophenylthiomethylene R_2 side chain), are 10- to 1000-fold less potent than the second-generation bisphosphonates, which have an R_2 side chain containing a primary amino group (as in pamidronate and alendronate). The third-generation bisphosphonate ibandronate is an analog of pamidronate that contains a tertiary amino group and is 10,000-fold more potent than the first-generation compounds (Geddes et al., 1994) (Table 1).

Bisphosphonates seem to inhibit bone resorption by directly affecting bone-resorbing osteoclasts (Flanagan and Chambers, 1991), preventing processes required for resorption (Sato et al., 1991; Murakami et al., 1995), or by promoting osteoclast apoptosis (Hughes et al., 1995). Bisphosphonates may also inhibit bone resorption by preventing the formation of osteoclasts from hematopoietic precursors (Boonekamp et al., 1986) or through effects on osteoblasts (Sahni et al., 1993). Until recently, however, the molecular basis for these effects was unclear. We have been using the J774 macrophage-like cell line to study the molecular properties of bisphosphonates, because these cells also undergo apoptosis after treatment with some bisphosphonates (Rogers et al., 1996b). Apoptosis in J774 cells induced by alendronate involves the activation of caspase-3-like enzymes (Coxon et al., 1998). Caspases are a family of cysteine proteases that have recently been shown to play an important role in the initiation and execution of apoptosis (Cohen, 1997). Caspases can be divided into two major groups depending on their homology to interleukin-1β-converting enzyme (caspase-1) or CPP32 (caspase-3), which differ in their ability to cleave the fluorogenic peptide substrates *N*-acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin (Ac-YVAD-AMC) and *N*-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC), respectively (Cohen, 1997). This permits quantification of active caspase-1-like or caspase-3-like proteases in cell lysates.

Several studies have suggested that aminobisphosphonates have a molecular mechanism of action different from or in addition to that of the nonaminobisphosphonates (Reitsma et al., 1982; Boonekamp et al., 1986). We found previously (Rogers et al., 1992, 1994a; Frith et al., 1997) that clodronate could be metabolized intracellularly by eukaryotic cells in vitro to a nonhydrolyzable analog of ATP ($AppCCl_2p$), which could be detected in cell extracts by using fast-performance liquid chromatography (FPLC). More recently, we developed a highly sensitive technique to identify bisphosphonate metabolites in cell extracts, combining HPLC and tandem mass spectrometry (MS) (Auriola et al., 1997). Using the latter procedure, we confirmed that clodronate could be metabolized to $AppCCl_2p$ (Auriola et al., 1997). This clearly raises the possibility that some bisphosphonates could act because of the accumulation of AppCp-type metabolites. By contrast with clodronate, we recently proposed that the aminobisphosphonate alendronate inhibits enzymes of the mevalonate pathway, resulting in the loss of isoprenoid intermediates [farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP)] required for post-translational modification (isoprenylation) of small GTP-binding proteins (Coxon et al., 1998; Luckman et al., 1998). It is likely that apoptosis induced by alendronate is the consequence of loss of isoprenylated proteins (Coxon et al., 1998). Consistent with this notion, we found that apoptosis induced by alendronate could be partially suppressed by the addition of farnesyl pyrophosphate and geranylgeranyl pyrophosphate (Luckman et al., 1998).

Taken together, these observations suggest that bisphosphonates may be tentatively classified into two groups with distinct molecular mechanisms of action; those that can be metabolically incorporated into analogs of ATP (the nonaminobisphosphonates) and those that inhibit protein isoprenylation (the aminobisphosphonates). To examine this hypothesis, we directly compared the effects of three nonaminobisphosphonates [dichloromethylene-1,1-bisphosphonate (CLO), 1-hydroxyethylidene-1,1-bisphosphonate (ETI), and chloro-4-phenylthiomethylene-1,1-bisphosphonate (TIL)] and three aminobisphosphonates [4-amino-1-hydroxybutylidene-1,1-bisphosphonate (ALN), 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (PAM), and 1-hydroxy-3(methylpentylamino)propylidene-1,1-bisphosphonate (IBA)] on J774 macrophages. Specifically, we compared 1) the ability of the bisphosphonates to activate caspase-3- or caspase-1-like proteases and to induce apoptosis; 2) the ability of cell-permeable analogs of FPP and GGPP, farnesol (FOH), and geranylgeraniol (GGOH), respectively (Crick et al., 1997), to suppress caspase activation and apoptosis; 3) the ability of bisphosphonates to inhibit protein isoprenylation; and 4) the ability of J774 cells to metabolize these compounds to AppCp-type analogs of ATP.

TABLE 1
Structure and relative potencies of bisphosphonates

Bisphosphonate	Side Chain		LED values[a]
	R_1	R_2	
			mg P/kg/day
ETI	$-OH$	$-CH_3$	1.0
CLO	$-Cl$	$-Cl$	0.3
TIL	$-H$	$-S-C_6H_5Cl$	0.3
PAM	$-OH$	$-CH_2-CH_2-NH_2$	0.03
ALN	$-OH$	$-CH_2-CH_2-CH_2-NH_2$	0.001
IBA	$-OH$	$-CH_2-CH_2-N(CH_3)(C_5H_{11})$	0.0001

[a] LED values are from Geddes et al., 1994.

Materials and Methods

Reagents. ALN, IBA, PAM, CLO, ETI, and TIL were kindly provided by Procter and Gamble Pharmaceuticals (Cincinnati, OH). Stock solutions (10 mM or 50 mM) were prepared in PBS, pH 7.4, then filter-sterilized using a 0.2-μm filter. Caspase substrates (Ac-DEVD-AMC and Ac-YVAD-AMC) were purchased from Alexis (Zurich, Switzerland) and caspase-3 inhibitor (Z-DEVD-fmk) was from Calbiochem (Nottingham, UK). Stock solutions (10 mM) of the caspase substrates and caspase inhibitor were prepared in dry dimethyl sulfoxide. [^{14}C]Mevalonolactone was purchased from Amersham (Aylesbury, UK). Mevastatin was purchased from Sigma Chemical Co. (Poole, UK) and prepared as described previously (Luckman et al., 1998). All other reagents were purchased from Sigma Chemical Co. (Poole, UK).

Cell Culture. J774 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured at 37°C in Dulbecco's modified Eagle's medium (GIBCO, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM L-glutamine in a 5% CO_2 atmosphere.

Determination of Caspase Activity. Caspase-3- and caspase-1-like enzyme activity was measured by proteolytic cleavage of the

fluorogenic substrates Ac-DEVD-AMC and Ac-YVAD-AMC, respectively. J774 cells were seeded in 6-well plates at a density of 7×10^5 cell/well and left to adhere for 24 h. Cells were treated with 100 μM ALN, IBA, or PAM or 750 μM CLO, ETI, or TIL for 16 h. Adherent and nonadherent cells were then harvested and pooled, washed in PBS, and lysed in 70 μl of lysis buffer as described previously (Coxon et al., 1998). For the caspase assay, 50 μl of cell lysates were made up to 1.5 ml with lysis buffer containing 5 mM cysteine and the respective substrate (40 μM) and incubated at 37°C for 1 h. The release of amino-4-methylcoumarin was determined on a Perkin-Elmer fluorometer (Perkin-Elmer, Norwalk, CT) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The results were corrected for protein content of the samples (bicinchoninic acid assay; Pierce, Rockford, IL) and expressed as percentage of control.

The effect of FOH and GGOH on caspase-3-like activity was investigated by treating J774 cells with 100 μM bisphosphonate ± 50 μM FOH or 50 μM GGOH for 24 h. Caspase-3-like activity was determined as above and results were expressed as percentage of control.

Quantification of Apoptosis. J774 cells were seeded into 24-well tissue culture plates (1×10^5 cells/well) and left overnight to adhere. The cells were then treated for up to 48 h with 100 μM ALN, 100 μM IBA, or 100 μM PAM alone or in combination with 50 μM GGOH or 50 μM FOH (quadruplicate wells for each treatment). After 24 and 48 h, adherent and nonadherent cells in each well were pooled and centrifuged (3200*g*, 5 min) and the percentage of apoptotic cells was assessed based on nuclear morphology after staining nuclei with 4,6-diamidino-2-phenylindole, as described previously (Rogers et al., 1996b).

To determine the effect of a caspase-3 inhibitor on apoptosis, J774 cells were seeded into 48-well plates (5×10^4 cells/well) and treated the following day with 100 μM ALN or 100 μM ALN + 200 μM Z-DEVD-fmk for 30 h. Adherent and nonadherent cells were harvested and pooled. The percentage of apoptotic cells was assessed based on nuclear morphology after staining with 4,6-diamidino-2-phenylindole (Rogers et al., 1996b). Caspase-3-like activity was also determined in lysates from these cells by measuring the cleavage of Ac-DEVD-AMC, as described above.

Incorporation of [^{14}C]Mevalonate into Isoprenylated Proteins. The ability of amino- and nonaminobisphosphonates to affect protein isoprenylation in J774 cells was investigated by measuring metabolic incorporation of [^{14}C]mevalonolactone into proteins that had been post-translationally modified with farnesyl and geranylgeranyl groups, using the method of Luckman et al. (1998). In short, cells were depleted of mevalonate by incubation with 5 μM mevastatin for 4 h. Cells were then treated for 24 h with 7.5 μCi/ml [^{14}C]mevalonolactone (specific activity, 57 mCi/mmol); 5 μM mevastatin; and either 100 μM ALN, IBA, or PAM or 750 μM CLO, ETI, or TIL. Cells were lysed in radioimmunoprecipitation assay buffer and 50 μg of protein (as determined by Pierce bicinchoninic acid assay) of each sample were electrophoresed on 12% polyacrylamide-SDS gels. These were then stained with Coomassie blue to confirm equal loading of wells and dried. Radiolabeled bands were visualized using a Bio-Rad Personal FX Imager (Bio-Rad, Hercules, CA).

Identification of Bisphosphonate Metabolites by HPLC-Tandem MS. Approximately 2.5×10^6 J774 cells were seeded into 162-cm^2 tissue culture flasks (one flask per treatment). The cells were cultured until approximately 70% confluent and then treated for 24 h with either 250 μM CLO, 750 μM ETI, 100 μM TIL, 100 μM ALN, 100 μM IBA, 100 μM PAM, or an equivalent volume of PBS. These concentrations were selected because they either cause a significant reduction in J774 cell viability as assessed by the MTT assay (for CLO, ETI, TIL, data not shown) or induce a significant increase in apoptosis in cultures of J774 cells (for ALN, IBA, PAM; Fig. 4). After treatment, the cells were scraped from the flask, centrifuged (220*g*, 5 min) and washed in cold PBS. The cell pellets were kept on ice and were resuspended in 300 μl of acetonitrile (ACN). Within 2 min, 200 μl of ice-cold distilled water was added, the extracts were centrifuged (14,000*g*, 2 min, 4°C), and the supernatants were transferred to fresh Eppendorf tubes. The ACN was evaporated under a stream of nitrogen (approximately 5 min) and the aqueous samples that remained were dried down in a SpeedVac concentrator (Savant, Holbrook, NY) and then stored at −20°C.

On-line HPLC-electrospray ionization (ESI)-MS measurements were carried out as described previously (Auriola et al., 1997) with the following modifications to improve sample capacity. The reversed-phase column was a Genesis C18 (50 × 2 mm; Jones Chromatography, Lakewood, CO), which was eluted with a mobile phase at a rate of 200 μl/min. Eluent A was 20 mM dimethylhexamide formate with the pH adjusted to 7.0 with formic acid. Eluent B was 50% methanol containing 20 mM dimethylhexamide formate, pH 7.0. The gradient was 20 to 100% of B in 4 min and then it remained at 100% B for 8 min. The identification of samples by collision-induced dissociation and tandem MS was carried out as described previously (Auriola et al., 1997).

Statistical Analysis. Changes in the percentage of apoptotic cells in treated cultures were analyzed by the Mann-Whitney *U* test or by ANOVA.

Results

Aminobisphosphonates Cause Activation of Caspase-3-Like Proteases in J774 Cells. After treatment of cultures of J774 macrophages with 100 μM ALN, IBA, or PAM (the aminobisphosphonates) for 16 h, a 12- to 15-fold increase in caspase-3-like activity (determined by the cleavage of the fluorogenic substrate Ac-DEVD-AMC) could be detected in cell lysates (Fig. 1A). By contrast, treatment of J774 macrophages with 750 μM CLO, ETI, or TIL (the nonaminobisphosphonates) for 16 h did not cause activation of caspase-3-like proteases (Fig. 1A).

Neither Aminobisphosphonates nor Nonamino-Bisphosphonates Cause Activation of Caspase-1-Like Proteases in J774 Cells. After treatment of cultures of J774 macrophages for 16 h with 100 μM ALN, IBA, or PAM (the aminobisphosphonates) or 750 μM CLO, ETI, or TIL (the nonaminobisphosphonates), there was no detectable increase in caspase-1-like activity in cell lysates (determined by the cleavage of the fluorogenic substrate Ac-YVAD-AMC) (Fig. 1B).

Inhibition of Caspase-3-Like Proteases Suppresses Alendronate-Induced Apoptosis. Treatment for 30 h with 100 μM ALN caused a significant increase in the proportion of cells with morphologically apoptotic nuclei (14.6%) compared with cells from control cultures (1.0%), in accord with our previous observations (Rogers et al., 1996b). When cultures were coincubated with 100 μM ALN and 200 μM caspase-3 inhibitor (Z-DEVD-fmk), the proportion of apoptotic cells was significantly reduced to 9.2% (Fig. 2). After coincubation of J774 cells with 100 μM ALN and 200 μM Z-DEVD-fmk for 24 h, however, there was no increase in caspase-3-like activity in subsequent cell lysates (data not shown). These observations suggest that Z-DEVD-fmk is both cell permeable and an effective inhibitor of caspase-3-like proteases but did not completely prevent apoptosis.

FOH and GGOH Prevent Activation of Caspase-3-Like Proteases and Induction of Apoptosis by Aminobisphosphonates. As described above, treatment of J774 macrophages for 24 h with the aminobisphosphonates ALN, IBA, or PAM caused a substantial increase in caspase-3-like activity compared with control. Coincubation of J774 cells with 100 μM ALN, IBA, or PAM and 50 μM FOH for 24 h,

significantly reduced the increase in caspase-3-like activity (Fig. 3). Similarly, addition of 50 μM GGOH to aminobisphosphonate-treated cultures effectively reduced caspase-3-like activity, to the levels observed in untreated cultures in the case of ALN + GGOH, or IBA + GGOH (Fig. 3). After 48 h of treatment, GGOH was still effective at preventing activation of caspase-3-like proteases. However, FOH was completely ineffective (data not shown).

Treatment with all of the aminobisphosphonates caused an increase in the level of apoptosis from 1% in control cultures to 24, 9, and 29% in cultures treated for 24 h with ALN, IBA, and PAM, respectively (Fig. 4A). When J774 macrophages were treated with the aminobisphosphonates in the presence of 50 μM FOH for 24 h, the levels of apoptosis were significantly reduced to 5, 1, and 15%, respectively (Fig. 4A). Similarly, addition of 50 μM GGOH reduced the levels of apoptosis after 24 h from 18, 5, and 24% after treatment with ALN, IBA, or PAM, respectively, to 1, 1, and 18% (Fig. 4B). After 48 h of treatment, GGOH was still effective at preventing apoptosis. However, FOH was completely ineffective after 48 h (data not shown). These results are consistent with the ability of both FOH and GGOH to prevent activation of caspase-3-like protease(s) after 24 h of treatment, and the ability of GGOH (but not FOH) to prevent caspase activation after 48 h of treatment.

Aminobisphosphonates Inhibit Protein Isoprenylation, whereas Nonaminobisphosphonates Do Not. After metabolically labeling J774 cells with [^{14}C]mevalonolactone for 24 h, radiolabeled isoprenylated proteins in cell lysates could be separated by electrophoresis into major bands of 21 to 26 kDa (mostly geranylgeranylated small GTP-binding proteins, but also farnesylated Ras proteins) and 60 to 70 kDa (farnesylated lamin B and prelamin A), in accord with previous studies (Luckman et al., 1998). When J774 cells were treated with ALN, IBA, or PAM (all at 100 μM) for the 24-h labeling period, the incorporation of [^{14}C]mevalonolactone into the protein bands (especially the small GTP-binding proteins) was markedly inhibited (a representative gel is shown in Fig. 5). ALN and IBA seemed to be consistently more effective than PAM. In addition, ALN, IBA, and PAM inhibited the incorporation of [^{14}C]mevalonolactone into the major band at the dye front, which we previously suggested consists of low-molecular-mass isoprenoid intermediates of the mevalonate pathway, such as GGPP (Luckman et al., 1998). By contrast, when J774 cells were incubated with 750 μM CLO or ETI for the 24-h labeling period, there was no marked effect on the incorporation of radiolabel into the isoprenylated protein bands or into the low-molecular-mass compounds at the dye front. TIL (750 μM) slightly inhibited protein isoprenylation (although this concentration, unlike CLO or ETI, substantially reduced cell viability) but did not reduce the incorporation of label into isoprenoid compounds at the dye front.

Nonaminobisphosphonates Are Metabolized to Nonhydrolyzable Analogs of ATP, but Aminobisphosphonates Are Not. A concentration of aminobisphosphonates was chosen (100 μM) that induces significant increases in the level of apoptosis in cultures of J774 cells (Fig. 4). When extracts of J774 cells previously treated with the aminobisphosphonates (ALN, IBA, or PAM) were analyzed by HPLC-ESI-MS, there were no peaks containing ions with the predicted molecular weights for AppCp-type metabolites (*m/z* 577, 647, and 563, respectively) that were not present in control cell extracts (Fig. 6). This demonstrated that these bisphosphonates were not metabolized to AppCp-type metabolites.

By contrast, as suggested previously (Auriola et al., 1997) the nonaminobisphosphonates are metabolized to AppCp-type nucleotides. Concentrations of nonaminobisphosphonates were chosen that significantly reduce J774 cell viability. Analysis of extracts from J774 cells previously treated with 250 μM CLO for 24 h showed the appearance of a new peak on the total ion HPLC chromatogram eluting at 6.07 min, which was not present in control extracts (Fig. 7A). When the compounds within the peak were analyzed by MS, the full-scan MS profile showed a molecular ion of *m/z* 572

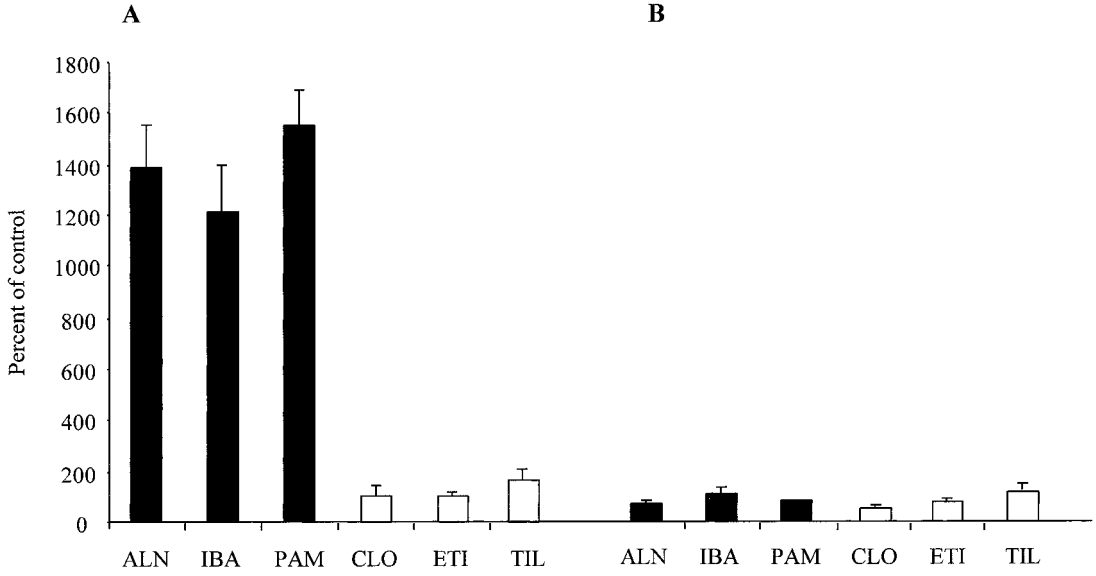

Fig. 1. A, the effect of three aminobisphosphonates (100 μM ALN, IBA, or PAM) and three nonaminobisphosphonates (750 μM CLO, ETI, or TIL) on caspase-3-like activity (cleavage of Ac-DEVD-AMC) in J774 cells. B, the effect of the bisphosphonates on caspase-1-like activity (cleavage of Ac-YVAD-AMC) in J774 cells. Values are the mean ± S.E.M. (n = 3) expressed as a percentage of caspase activity in control cells.

and 574 ($AppCCl_2p$) and the fragment ions produced by collision-induced dissociation were of *m/z* 225 and 227 (clodronate). Extracts from 750 μM ETI-treated and 100 μM TIL-treated cultures both contained new peaks on the extracted total ion HPLC chromatogram compared with control. The new peak in extracts from cells previously treated with etidronate eluted at 5.83 min and contained a molecular ion of *m/z* 534 ($AppC(OH)(CH_3)p$). After collision-induced dissociation of this ion, a fragment ion was observed of *m/z* 186 (etidronate) (Fig. 7B). The new peak in extracts from TIL-treated J774 cells eluted at 9.78 min and contained molecular ions of *m/z* 646 and 648 ($AppCC_6H_5Clp$) which, upon collision-induced dissociation, gave rise to fragment ions of *m/z* 299 and 301 (tiludronate) (Fig. 7C). The *m/z* doublet from clodronate and tiludronate peaks arises from the occurrence of the ^{35}Cl and ^{37}Cl isotopes of chlorine. These results confirm that these three nonaminobisphosphonates are metabolized to AppCp-type analogs of ATP by J774 cells.

In the present study, the cell extraction procedure after treatment with bisphosphonates was optimized from a previous method (Auriola et al., 1997). This improved the overall performance of the HPLC-ESI-MS system for detection of bisphosphonate metabolites. In previous studies, cells were extracted with perchloric acid. The perchlorate salts present within the final extracts interfered with the HPLC separation, causing high levels of background noise, and the peaks eluted were wide and fronting. The extraction method used here involved extracting the cells with ACN, which resulted in a cleaner HPLC separation with less background noise. The ACN extraction procedure was also much quicker, taking just 15 min in total, compared with 2 h for the perchloric acid extraction method.

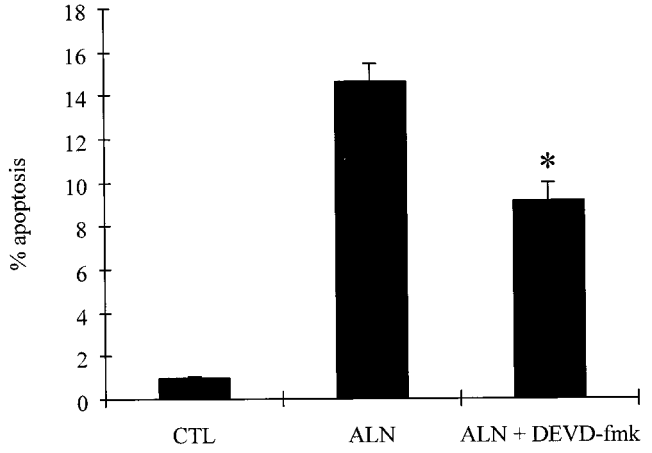

Fig. 2. Percentage of apoptotic J774 cells after culture for 30 h without bisphosphonates (CTL), with 100 μM alendronate, or with 100 μM alendronate and 200 μM Z-DEVD-fmk. The proportion of apoptotic cells was assessed by changes in nuclear morphology. Values are expressed as the mean ± S.E.M. (n = 4). * denotes values significantly different from treatment with alendronate alone ($p < .05$, ANOVA).

Discussion

Ever since the first use of bisphosphonates to inhibit bone resorption (Fleisch et al., 1969; Smith et al., 1971), efforts have been made to identify the molecular mechanisms involved. It is now clear that bisphosphonates inhibit bone resorption by affecting osteoclasts, the specialized multinucleated bone resorbing cells. It is also possible that effects on cells of the osteoblast lineage or on osteoclast precursors may contribute to the overall inhibition of bone resorption in vivo (Ebetino et al., 1998; Fleisch, 1998).

More than 10 years ago, Boonekamp et al. (1986) proposed that bisphosphonates containing a nitrogen group in the R_2 side chain (such as PAM) had a mode of action in addition to that of the bisphosphonates that do not contain a nitrogen group (such as CLO). More specifically, they proposed that CLO affected only mature osteoclasts, whereas PAM at low concentrations could affect osteoclast precursors. Higher concentrations of PAM could affect both mature osteoclasts and osteoclast precursors. Similar results were later obtained with another nitrogen-containing bisphosphonate (Boonekamp et al., 1987; Lowik et al., 1988). Reitsma et al. (1982), also presented evidence that CLO and PAM differed in their cytotoxic effects on macrophages. However, the molecular basis for these differences remained unknown.

We recently demonstrated that CLO can be metabolically incorporated into a nonhydrolyzable analog of ATP ($AppCCl_2p$)

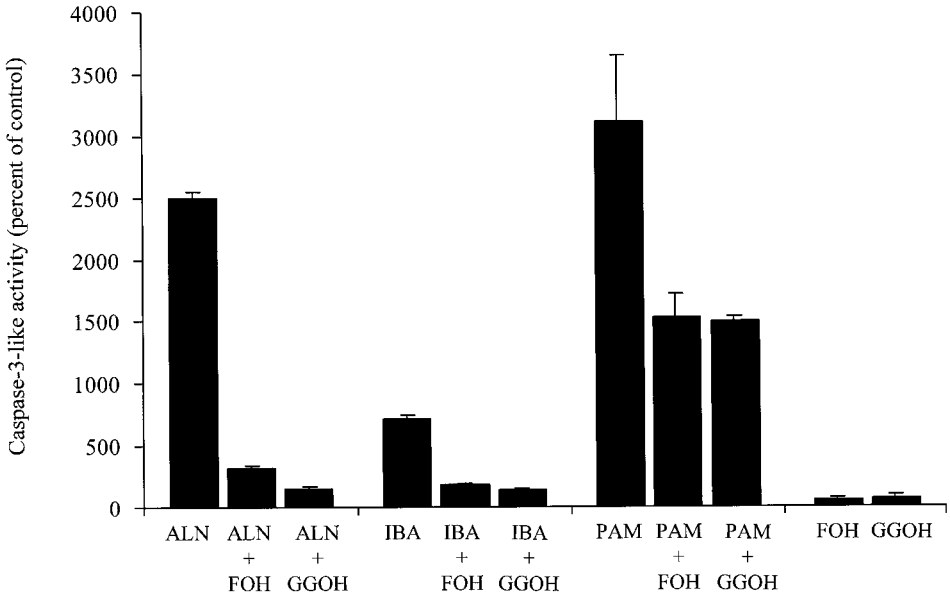

Fig. 3. The effect of 50 μM FOH or 50 μM GGOH on caspase-3-like activity induced in J774 cells after 24-h treatment with 100 μM ALN, IBA, and PAM. Values are the mean ± S.E.M. (n = 3) expressed as a percentage of caspase activity in control cells.

in the cytoplasm of J774 macrophages in vitro (Auriola et al., 1997; Frith et al., 1997). The intracellular accumulation of the metabolite is the likely cause of the growth inhibitory effect and cytotoxicity of these bisphosphonates toward macrophages (Frith et al., 1997). Metabolites of aminobisphosphonates could not be detected by UV absorbance using the technique of FPLC to separate nucleotides in cell extracts

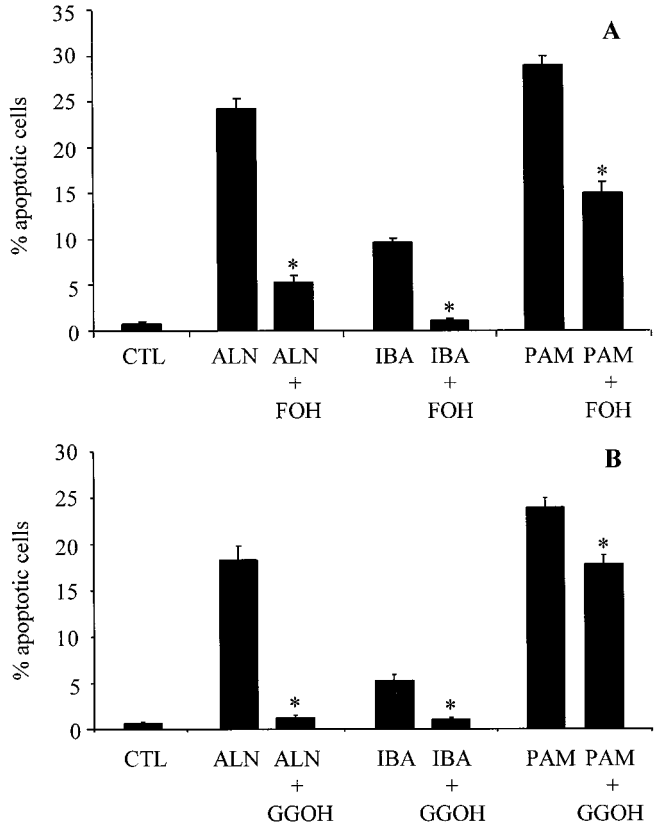

Fig. 4. The effect of (A) 50 μM FOH or (B) 50 μM GGOH on J774 apoptosis induced by 100 μM ALN, IBA, and PAM. Values are the mean ± S.E.M. (n = 4). * denotes values significantly different from treatment with bisphosphonate alone ($p < .05$, Mann-Whitney U test).

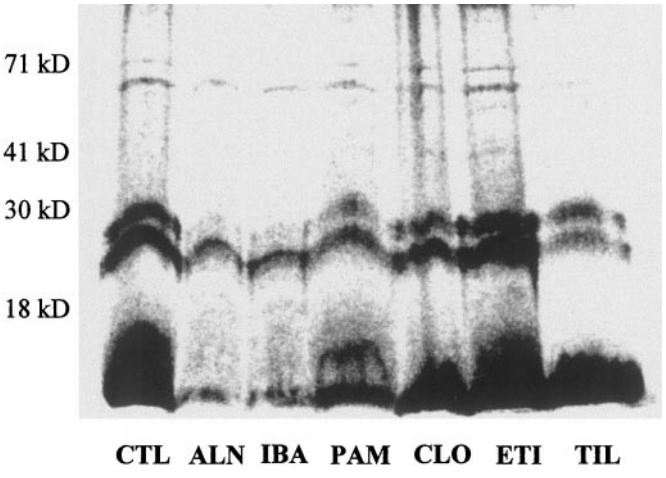

Fig. 5. The effect of aminobisphosphonates and nonaminobisphosphonates on incorporation of [^{14}C]mevalonolactone into isoprenylated proteins. Cell lysates were from control cells (CTL) or cells treated for 24 h with 100 μM ALN, IBA, and PAM or with 750 μM CLO, ETI, and TIL. Wells were equally loaded with 50 μg of protein. The positions of molecular mass markers are also indicated.

(Frith et al., 1997). However, we recently found that aminobisphosphonates could inhibit enzymes of the mevalonate pathway in J774 macrophages, thus leading to loss of isoprenoid intermediates (FPP and GGPP) required for the post-translational modification of GTP-binding proteins with isoprenoid groups (Luckman et al., 1998). Loss of isoprenylated proteins is the likely cause of apoptosis induced by these bisphosphonates, involving the activation of caspase-3-like proteases (Coxon et al., 1998). In this study, we directly compared the ability of three nonaminobisphosphonates and three aminobisphosphonates to cause activation of caspases in J774 macrophages. Clearly, all of the aminobisphosphonates caused an increase in the activity of caspase-3-like proteases, whereas the nonaminobisphosphonates did not (even at concentrations known to reduce cell viability or inhibit cell proliferation). This is consistent with our previous studies, which found that aminobisphosphonates were much more effective at causing apoptosis of J774 cells than nonaminobisphosphonates (Rogers et al., 1996b). Neither aminobisphosphonates nor nonaminobisphosphonates caused an increase in the activity of caspase-1-like proteases.

Activation of caspase-3-like enzymes is thought to be the irreversible step in the pathway leading to apoptotic cell death and to be required for some of the morphological and biochemical changes associated with the process (Cohen, 1997). Accordingly, we found that an inhibitor of caspase-3 significantly reduced the proportion of apoptotic cells coincubated with ALN. The caspase-3 inhibitor did not completely prevent apoptosis. However, treatment with the inhibitor did completely prevent the increase in activity of caspase-3-like proteases. Thus, caspase-3 or caspase-3-like proteases seem to be involved in, but not essential for, apoptosis induced by aminobisphosphonates. Studies with caspase inhibitors and other apoptosis-inducing agents have led to similar conclusions (Inayat-Hussain et al., 1997).

FOH and GGOH, cell permeable analogs of the mevalonate-derived compounds FPP and GGPP, respectively, effectively prevented the activation of caspase-3-like proteases by all three of the aminobisphosphonates. Similarly, FOH and GGOH effectively prevented apoptosis induced by these bisphosphonates and were more effective than FPP or GGPP (Luckman et al., 1998). Interestingly, in a recent preliminary report (Reszka et al., 1998), GGOH was also shown to prevent the ALN-induced and PAM-induced activation of Mst 1, a kinase that is cleaved and activated in osteoclasts by caspase-3-like proteases. These observations are consistent with our recent hypothesis that aminobisphosphonates cause apoptosis as a consequence of the loss of farnesylated and/or geranylgeranylated proteins (Luckman et al., 1998). It is likely that the addition of exogenous FOH or GGOH rescues the cells from apoptosis by replenishing the cytosolic pool of isoprenoid substrates required for protein isoprenylation. FOH and GGOH can be converted to FPP and GGPP, respectively, and consequently used for protein farnesylation and geranylgeranylation (Crick et al., 1997). Although both FOH and GGOH were effective at preventing caspase activation and apoptosis after 24 h of treatment with aminobisphosphonates, only GGOH was effective after 48 h of treatment. This suggests that geranylgeranylated proteins (such as Rho and Rac) rather than farnesylated proteins (such as Ras) may be particularly important for preventing apoptosis. It is possible that the protective effect of FOH after 24 h may result from

the conversion of some FOH to GGPP via FPP, although it is currently thought that FOH cannot be converted to GGPP (Crick et al., 1997). Alternatively, it is possible that proteins normally geranylgeranylated were farnesylated (using FOH), resulting in temporary rescue of cells from apoptosis.

Because the nonaminobisphosphonates CLO, ETI, and TIL did not cause activation of caspase-3-like proteases in J774 cells, we could not examine any suppressive effect of FOH or GGOH. However, we previously demonstrated that CLO can cause necrotic cell death and a reduction in total cell viability, which can be detected using the MTT assay (Frith et al., 1997). In the present study, we also found that treatment for 48 h with 100 μM TIL, CLO, and ETI reduced total cell viability to 3, 44, and 81% of control, respectively. Furthermore, cotreatment with 50 μM FOH or GGOH had no significant effect on the reduction in cell viability (data not shown), supporting the view that these bisphosphonates do not act by preventing protein isoprenylation.

To confirm that aminobisphosphonates, but not nonaminobisphosphonates, inhibit protein isoprenylation, J774 macrophages were metabolically labeled with [^{14}C]mevalonate. After a 24-h labeling period in the presence of 100 μM each of the aminobisphosphonates, the incorporation of [^{14}C]mevalonolactone into both farnesylated and geranylgeranylated small GTP-binding proteins was substantially inhibited. ALN and IBA seemed to be slightly more effective than PAM, consistent with the difference in antiresorptive potency of these compounds. By contrast, the nonaminobisphospho-

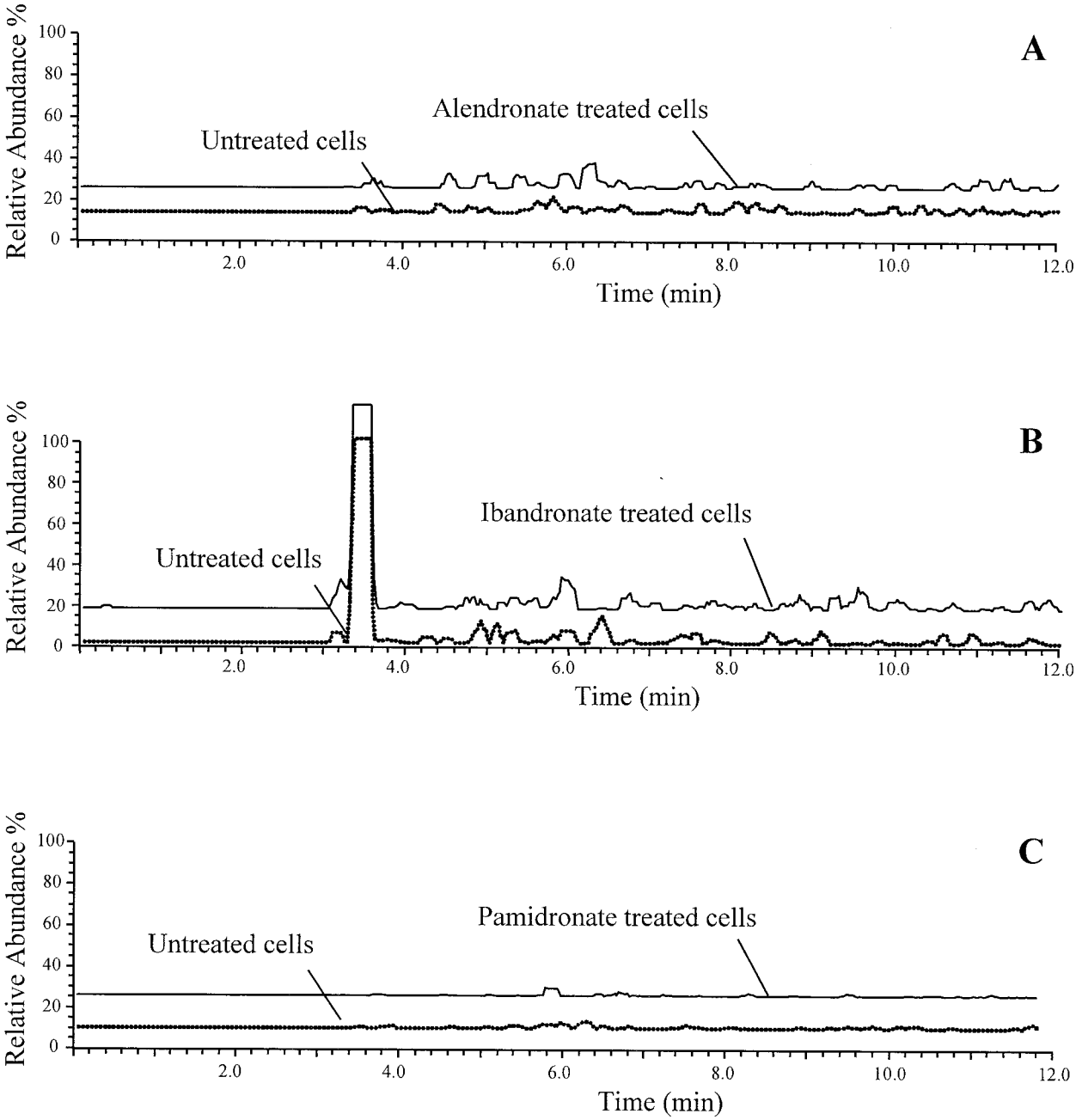

Fig. 6. Extracted HPLC ion chromatograms of ACN extracts from J774 cells previously treated with 100 μM ALN (scan region of *m/z* 577) (A), 100 μM IBA (scan region of *m/z* 647) (B), and 100 μM PAM (scan region of *m/z* 563) (C) compared with control extracts (bottom lines).

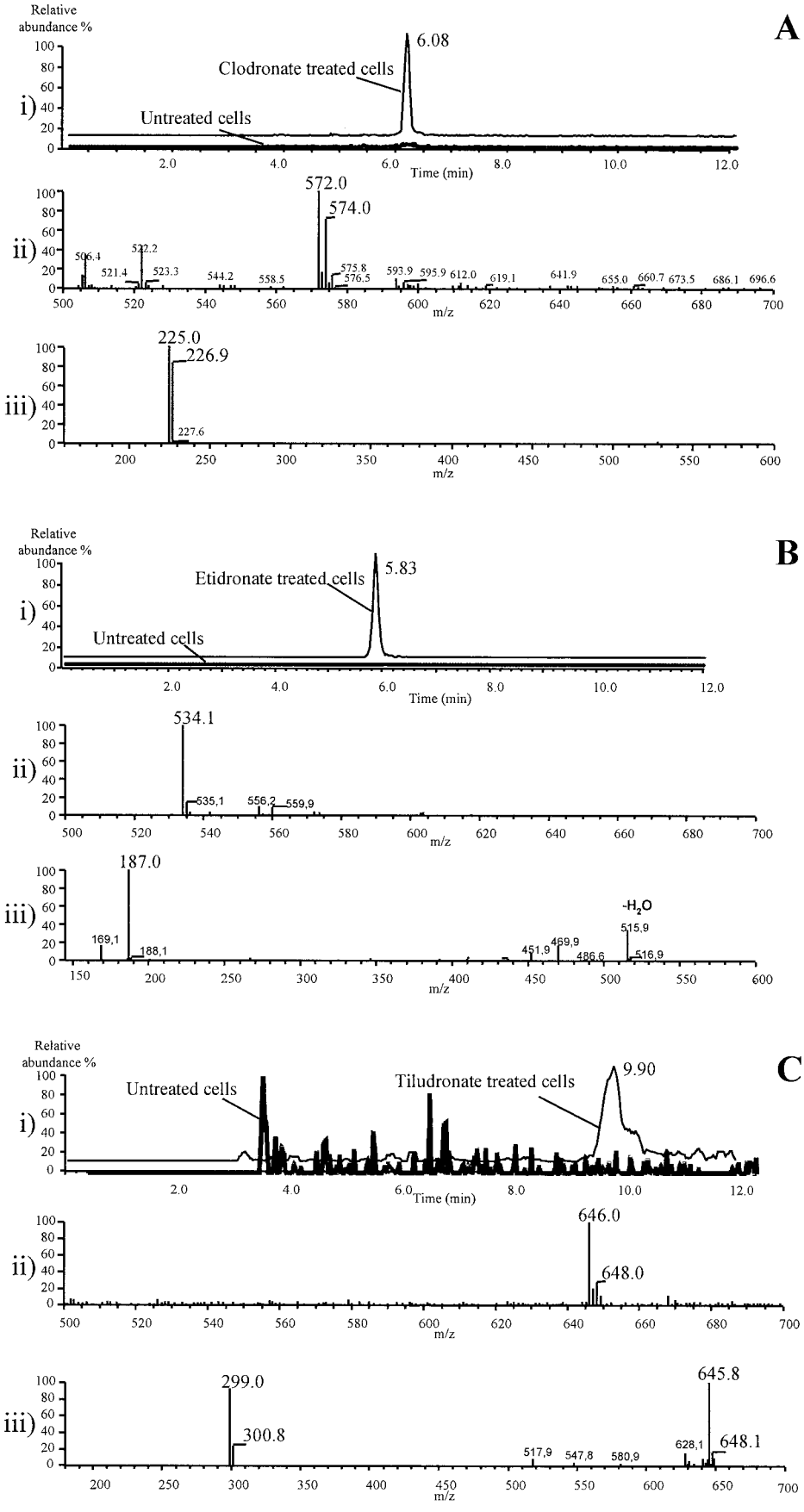

Fig. 7. Analysis of ACN extracts of J774 cells previously treated with 250 μM CLO (A) showing i) extracted HPLC ion chromatogram of *m/z* 571–576, ii) full-scan MS of the peak at 6.08 min, and iii) MS/MS of *m/z* 571–576; 750 μM etidronate (B) showing i) extracted HPLC ion chromatogram of *m/z* 534, ii) full-scan MS of the peak at 5.83 min, and iii) MS/MS of *m/z* 534; and 100 μM tiludronate (C) showing i) extracted HPLC ion chromatogram of *m/z* 646, ii) full-scan MS of the peak at 9.90 min, and iii) MS/MS of *m/z* 643–649.

nates CLO and ETI did not inhibit the incorporation of [^{14}C]mevalonate into small GTP-binding proteins, even at the much higher concentration of 750 μM. This concentration of TIL slightly inhibited protein isoprenylation (probably the result of reduced cell viability) but did not inhibit the formation of low-molecular-mass isoprenoid compounds. Together with the evidence that FOH and GGOH prevent aminobisphosphonate-induced apoptosis and caspase activation, these data clearly demonstrate that aminobisphosphonates, but not nonaminobisphosphonates, inhibit enzymes of the mevalonate pathway required for protein isoprenylation.

We previously suggested that nonaminobisphosphonates and aminobisphosphonates also differ in their ability to be metabolized to AppCp-type nucleotides (Rogers et al., 1996a). In previous studies, we were unable to detect metabolites of the aminobisphosphonates ALN and PAM on the basis of UV absorbance after FPLC separation (Rogers et al., 1994a, 1996a; Frith et al., 1997). However, this did not rule out the possibility that metabolites of these bisphosphonates could be formed but at concentrations below the limit of detection by FPLC. More recently, we developed a more sensitive technique for detecting bisphosphonate metabolites using HPLC-MS (Auriola et al., 1997). In the present study, we optimized the procedure for preparation of cell extracts for HPLC-MS analysis and directly compared the ability of nonaminobisphosphonates and aminobisphosphonates to be metabolized. Using this technique, we found that all three of the nonaminobisphosphonates examined could be metabolically incorporated into AppCp-type nucleotides. However, no AppCp-type metabolites of any of the three aminobisphosphonates could be detected using the sensitive HPLC-MS technique. It is unlikely that the lack of detection of metabolites of aminobisphosphonates was attributable to the lower concentration of aminobisphosphonates used to treat the J774 cultures (100 μM aminobisphosphonates versus 100 μM TIL, 250 μM CLO, and 750 μM ETI). We previously showed that metabolites of aminobisphosphonates cannot be detected even when cell-free lysates are incubated with 500 μM aminobisphosphonates (conditions in which 500 μM nonaminobisphosphonates are metabolized; Rogers et al., 1996a). Thus, we conclude that aminobisphosphonates are not metabolized by J774 macrophages.

These observations provide conclusive evidence for two pharmacological classes of bisphosphonates. Those that lack an amino group in the R_2 side chain can be metabolically incorporated into nonhydrolyzable nucleotide analogs, which may compete with ATP in enzymatic reactions. It is likely that the growth-inhibitory and cytotoxic effects of these bisphosphonates on macrophages and other cells, including osteoclasts (Flanagan and Chambers, 1991; Mönkkönen et al., 1994; Rogers et al., 1994b; Frith et al., 1997), are the result of the cytoplasmic accumulation of the metabolites. By contrast, the amino-containing bisphosphonates are not metabolized but can inhibit enzymes of the mevalonate pathway, indirectly preventing the isoprenylation (and hence the function) of small GTP-binding proteins. These proteins are involved in signaling pathways regulating apoptosis and processes vital for osteoclastic bone resorption, such as organization of the actin cytoskeleton (Zhang et al., 1995), membrane ruffling (Ridley et al., 1992), and vesicular trafficking (Olkkonen and Stenmark, 1997). Therefore, it is likely that this is the mechanism by which aminobisphosphonates cause morphological changes to macrophages and osteoclasts, inhibit osteoclast function (Sato et al., 1991; Murakami et al., 1995), and cause apoptosis (Hughes et al., 1995; Rogers et al., 1996b). This is supported by our finding that caspase activation and macrophage apoptosis induced by all three aminobisphosphonates tested could be prevented by addition of FOH and, in particular, GGOH, which can be used as substrates for protein isoprenylation. Although the exact enzymes of the mevalonate pathway that are inhibited by these bisphosphonates remain to be identified, it is likely that the presence of a nitrogen function in the R_2 side chain is crucial for the ability of these bisphosphonates to interact with enzymes of the mevalonate pathway.

Nonaminobisphosphonates and aminobisphosphonates are known to differ in their ability to cause an acute phase response in patients receiving the drugs for the first time (Adami et al., 1987; Sauty et al., 1996). In addition, studies in vitro and in animal models have suggested that nonaminobisphosphonates seem to have anti-inflammatory properties at concentrations that are not cytotoxic (Pennanen et al., 1995; Makkonen et al., 1996; Nakamura et al., 1996), whereas aminobisphosphonates may be proinflammatory (Pennanen et al., 1995; Nakamura et al., 1996). Identification of the molecular mechanism of action of these two classes of bisphosphonates may help in understanding the basis for these differences in clinical practice.

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