Inhibition of Growth of *Dictyostelium discoideum* Amoebae by Bisphosphonate Drugs Is Dependent on Cellular Uptake

Michael J. Rogers,[1,5] Xiaojuan Xiong,[2] Xiaohui Ji,[2] Jukka Mönkkönen,[3] R. Graham G. Russell,[1] Michael P. Williamson,[2] Frank H. Ebetino,[4] and Donald J. Watts[2]

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Purpose. The aim of the study was to determine whether bisphosphonates are internalised by *Dictyostelium* amoebae and whether cellular uptake is required for their growth-inhibitory effects. Bisphosphonates inhibit growth of amoebae of the slime mould *Dictyostelium discoideum*, by mechanisms that appear to be similar to those that cause inhibition of osteoclastic bone resorption.

Methods. Cell-free extracts prepared from amoebae that had been incubated with bisphosphonates were analysed by ^{31}P-n.m.r. spectroscopy or ion-exchange f.p.l.c., to identify the presence of bisphosphonates or bisphosphonate metabolites respectively. The growth-inhibitory effect of bisphosphonates towards *Dictyostelium* amoebae was also examined under conditions in which pinocytosis was inhibited.

Results. All of the bisphosphonates studied were internalised by *Dictyostelium* amoebae, probably by fluid-phase pinocytosis, and could be detected in cell-free extracts. Amoebae that were prevented from internalising bisphosphonates by pinocytosis were markedly resistant to the growth-inhibitory effects of these compounds. In addition, bisphosphonates encapsulated within liposomes were more potent growth inhibitors of *Dictyostelium* owing to enhanced intracellular delivery of bisphosphonates.

Conclusions. All bisphosphonates inhibit *Dictyostelium* growth by intracellular mechanisms following internalisation of bisphosphonates by fluid-phase pinocytosis. It is therefore likely that bisphosphonates also affect osteoclasts by interacting with intracellular, rather than extracellular, processes.

KEY WORDS: bisphosphonates; *Dictyostelium*; endocytosis; growth inhibition; ^{31}P-n.m.r.; osteoclasts.

INTRODUCTION

Bisphosphonates are synthetic analogues of pyrophosphate that contain P-C-P rather than P-O-P bonds (1). By virtue of their ability to inhibit osteoclast-mediated bone resorption, bisphosphonates have become established as an important class of drugs for the treatment of diseases that cause high bone turnover or excessive bone resorption such as Paget's disease, hypercalcaemia due to malignancy and osteolysis resulting from bone metastases (2,3) and, more recently, in the treatment of osteoporosis (4).

Although the exact molecular targets for bisphosphonate drugs have not been identified, bisphosphonates probably inhibit bone resorption *in vivo* primarily by a direct effect on osteoclast cells (5,6), through mechanisms that can lead to osteoclast apoptosis (7). *In vitro*, bisphosphonates have also been shown to cause apoptosis or to have anti-proliferative effects on other cell types, including macrophages (8,9) and connective tissue cells (10). However, since bisphosphonates are non-physiological compounds having a large negative charge, it is unclear how bisphosphonates pass through the cell plasma membrane and therefore whether bisphosphonates act extracellularly or intracellularly.

Bisphosphonates are also inhibitors of growth of amoebae of the slime mould *Dictyostelium discoideum* (11,12). Furthermore, for a wide range of bisphosphonates with different side chains, there is a remarkable correlation between the order of potency of the bisphosphonates as inhibitors of growth of *D. discoideum* and the order of potency of the bisphosphonates as anti-resorptive agents *in vivo* (12,13). This suggests that bisphosphonates have molecular targets which are common to both osteoclasts and *Dictyostelium*. Several bisphosphonates of low anti-resorptive potency can be internalised and metabolised by *Dictyostelium* into potentially toxic, methylene-containing analogues of ATP, by a back-reaction catalysed by several cytosolic aminoacyl-tRNA synthetase enzymes (11,14,15). This is evidence that at least some bisphosphonates are internalised and enter the cytosol in *Dictyostelium*. However, more potent bisphosphonates are not metabolised and therefore appear to act by a different mechanism (14). We have sought to determine whether the non-metabolised bisphosphonates are also internalised by *Dictyostelium* and whether cellular uptake is required for the growth-inhibitory effect of bisphosphonates on *Dictyostelium* amoebae. If bisphosphonates act intracellularly in *Dictyostelium*, it is likely that these agents also affect osteoclasts through an intracellular mechanism.

Dictyostelium amoebae are capable of both fluid-phase pinocytosis and phagocytosis (16). In axenic growth medium (without bacteria) the amoebae internalise nutrients by pinocytosis. In the presence of suspensions of bacteria the amoebae feed exclusively by phagocytosis, and pinocytosis is inhibited

[1] Institute for Bone and Joint Medicine, Department of Human Metabolism and Clinical Biochemistry, University of Sheffield Medical School.

[2] Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, PO Box 594, Firth Court, Western Bank, Sheffield S10 2UH, UK.

[3] Department of Pharmaceutics, University of Kuopio, Finland.

[4] Procter & Gamble Pharmaceuticals, Cincinnati, Ohio.

[5] To whom correspondence should be addressed. (e-mail: m.j.rogers@sheffield.ac.uk)

ABBREVIATIONS: 3-PHEBP, 2-(3-pyridinyl)-1-hydroxyethylidene-1,1-bisphosphonic acid; ADP, adenosine 5′-diphosphate; AHBuBP, 4-amino -1-hydroxybutylidene-1,1-bisphosphonic acid; AHPrBP, 3-amino -1-hydroxypropylidene-1,1-bisphosphonic acid; Ap_4A, 5′, 5‴-diadenosyl-P^1,P^4-tetraphosphate; $AppCH_2p$, adenosine 5′-(β,γ-methylene)triphosphate; $AppCH_2ppA$, diadenosine 5′5‴-P^1,P^4-(P^2,P^3-methylene)tetraphosphate; ATP, adenosine 5′-triphosphate; Cl_2MBP, dichloromethylene-1,1-bisphosphonic acid; CPTMBP, chloro-4 phenylthiomethylene-1,1-bisphosphonic acid; EBP, ethylidene-1,1-bisphosphonic acid; f.p.l.c., fast protein liquid chromatography; FITC, fluorescein isothiocyanate; FMN, flavin mononucleotide; GTP, guanosine 5′-triphosphate; HEBP, 1-hydroxyethylidene-1,1-bisphosphonic acid; HMBP, 1-hydroxymethylene-1,1-bisphosphonic acid; MBP, methylene-1,1-bisphosphonic acid; $Me_2AHPrBP$, dimethyl-3-amino-1-hydroxypropylidene-1,1-bisphosphonic acid; n.m.r., nuclear magnetic resonance; NAD, nicotinamide adenine dinucleotide; PCA, perchloric acid; Pi, inorganic phosphate; ppm, parts per million.

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(16). Fluid-phase pinocytosis is thus a likely route of cellular uptake of bisphosphonates from axenic medium. We therefore examined the effect of inhibitors of fluid-phase pinocytosis, such as orthovanadate (17) and caffeine (18), on the uptake and potency of bisphosphonates. In addition, we compared the effect of bisphosphonates on amoebae grown in the absence or presence of a bacterial food source.

Liposomes provide an efficient method of intracellular drug delivery to phagocytic cells and can markedly increase the growth-inhibitory potency of bisphosphonates towards macrophages (9). Hence, we also examined whether liposome-encapsulated bisphosphonates are more potent inhibitors of *Dictyostelium* growth than free bisphosphonates.

MATERIALS AND METHODS

Growth of *Dictyostelium discoideum*

Amoebae of the Ax-2 strain were grown either axenically in HL5-glucose medium, as previously described (12), or with a shaken suspension of *Klebsiella aerogenes* in 50mM phosphate buffer pH6.5 (19). Cell proliferation was assessed daily using a Coulter counter or a haemocytometer.

Chemicals

MBP was from Aldrich Chemical Co. Ltd. Cl_2MBP (clodronate), HMBP, EBP, HEBP (etidronate), Me_2AHPrBP (olpadronate), 3-PHEBP (risedronate) and CPTMBP (tiludronate) were from Procter and Gamble Pharmaceuticals, Cincinnati, USA. AHPrBP (pamidronate) and AHBuBP (alendronate) were from Gentili S.p.A., Pisa, Italy. Other chemicals were obtained from Sigma Chemical Co. Ltd, Poole, UK.

Determination of Bisphosphonates in Cell Extracts of *Dictyostelium* by Using ^{31}P-n.m.r.

^{31}P-n.m.r. analysis was used to determine whether bisphosphonates were present in cell extracts prepared from *Dictyostelium* amoebae that had been incubated with bisphosphonate. PCA extracts were prepared from approximately 10^{10} axenically-grown amoebae that had been incubated with 5mM bisphosphonate in 20mM 2-[N-morpholino]ethanesulfonic acid buffer, pH 6.5, for 2h at 22°C, and analysed on a Bruker AMX-500 spectrometer as previously described (14). As a control, extracts were also prepared from amoebae immediately after addition of bisphosphonate. Extracts were also prepared from amoebae that had been incubated for 2h with bisphosphonate in the presence of 10mM caffeine or 5mM sodium orthovanadate, both of which have been shown to inhibit pinocytosis in *Dictyostelium* (17,18) or that had been incubated with bisphosphonate for 2h at 4°C rather than at 22°C.

Comparison of the Potencies of Bisphosphonates, and the Metabolism of MBP into Adenine Nucleotides, by Axenically- and Bacterially-Grown Amoebae

In order to determine whether amoebae internalise and metabolise MBP, PCA extracts were prepared from amoebae that had been grown for 96h in the presence of 500μM MBP either in axenic culture (without bacteria) or in the presence of *K. aerogenes*. 70ml cultures were inoculated with amoebae to an initial density of 1×10^4 cells/ml and amoebae were harvested after 96h. PCA extracts were analysed by anion-exchange chromatography as previously described (14,15).

The potency of HEBP, AHBuBP and 3-PHEBP as growth inhibitors of axenic or bacterially-grown *Dictyostelium* amoebae was determined as previously described (12). Cultures to be grown axenically or with suspensions of *K. aerogenes* were inoculated with amoebae to a density of 10^4 cells/ml and contained various concentrations of bisphosphonate. The IC_{50} for each bisphosphonate was determined as the concentration of bisphosphonate which halted growth after half the number of cell divisions that would have occurred in the absence of bisphosphonate. Since bacterially-grown amoebae grew twice as rapidly as axenically-grown amoebae (a phenomenon that could affect the values of IC_{50}), we also identified the lowest concentrations of bisphosphonates that totally inhibited growth in bacterial or axenic cultures.

Liposome-Encapsulated Bisphosphonates

Cultures of *Dictyostelium* amoebae were also grown in the presence of Cl_2MBP or AHPrBP contained within unilamellar, negatively-charged, distearoylphosphatidylglycerol-containing liposomes. Liposomes containing Cl_2MBP or AHPrBP were prepared by reverse phase evaporation as described by Mönkkönen and Heath (20). Stock suspensions of these liposomes contained 10.7 mM Cl_2MBP or 16.1 mM AHPrBP i.e. disruption of the liposomes gave rise to these concentrations of bisphosphonates in solution.

RESULTS

^{31}P-n.m.r. Analysis of the Uptake of Bisphosphonates by *Dictyostelium* Amoebae

The appearance of ^{31}P-n.m.r. spectra of cell extracts prepared from *Dictyostelium* amoebae have previously been described (11,14,15,21). The major signals in the n.m.r. spectrum all occur upfield of the signals due to phosphomonoesters (e.g. glucose-6-phosphate, fructose-1,6-bisphosphate) at +4 to +5 ppm, and generally consist of inorganic phosphate (P_i) at around +3 ppm, a cluster of peaks due to inositol hexakisphosphate at +1 ppm to +3 ppm, phosphodiesters (probably glycerophosphorylcholine) at 0 ppm, the γ, α, and β phosphates of nucleoside triphosphates at −5 ppm, −10 ppm and −20 ppm respectively, and the β and α peaks of nucleoside diphosphates at −5 ppm and −10 ppm respectively.

^{31}P-n.m.r. spectra obtained from PCA extracts of amoebae that had been incubated for 2h with 5 mM bisphosphonate (MBP, Cl_2MBP, CPTMBP, EBP, HMBP, HEBP, AHPrBP, Me_2AHPrBP or 3-PHEBP) were found to contain an extra peak (Fig. 1B), resonating far downfield of any other signals, that was not observed in the spectra of extracts prepared from amoebae washed immediately after addition of bisphosphonate (Fig. 1A). The new downfield signal was identified as that of bisphosphonate by comparison with the chemical shift of samples of authentic bisphosphonate. The chemical shifts of several bisphosphonates in cell extracts of *D. discoideum* are given in Table 1. All of the compounds examined could be identified in cell extracts prepared from *Dictyostelium* amoebae following a 2h incubation in bisphosphonate. Since MBP,

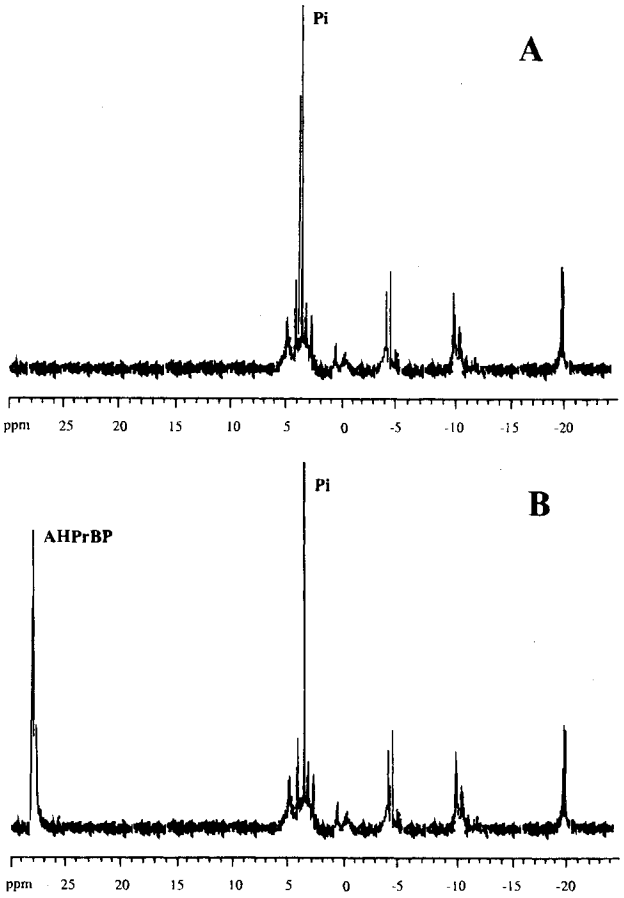

Fig. 1. ^{31}P NMR spectra of cell extracts prepared from Dictyostelium amoebae that had been (A) washed immediately after addition of 5 mM AHPrBP, (B) washed following a 2 h incubation in 5 mM AHPrBP.

Cl_2MBP, EBP, HMBP and CPTMBP are metabolised into AppCp-type nucleotides by *Dictyostelium* (14), the spectra of PCA extracts from amoebae incubated with these bisphosphonates also contained additional smaller signals (upfield of the signal due to bisphosphonate) which represent the α phosphate and β and γ phosphonate groups of the AppCp-type metabolites of the bisphosphonates e.g. signals for the γ and β phosphonates of the $AppCH_2p$ metabolite of MBP at +12.5 ppm and +14 ppm respectively (Fig. 2A).

Table 1. ^{31}P-n.m.r. Chemical Shifts of Bisphosphonates in PCA Extracts Prepared from *Dictyostelium* Amoebae that Had Been Incubated for 2 Hours with 5 mM Bisphosphonate

Bisphosphonate	Chemical shift (ppm)
MBP	+17
Cl_2MBP	+11
HMBP	+16
EBP	+22
HEBP	+20
AHPrBP	+28
Me_2AHPrBP	+18
3-PHEBP	+18.5
CPTMBP	+14

It was found that the bisphosphonate signal was virtually absent from the n.m.r. spectra of extracts from amoebae that had been incubated with bisphosphonate in the presence of 5 mM sodium orthovanadate (Fig. 2B) or 10 mM caffeine. Similarly, the bisphosphonate signal was markedly diminished when cells had been incubated with bisphosphonate at 4°C (Fig. 2C). *Dictyostelium* growth is inhibited by 5 mM orthovanadate and 10 mM caffeine (17,18), since the amoebae cannot internalise nutrients from the extracellular medium. However, the decrease in the amount of MBP in cell extracts from vanadate- or caffeine-treated cells was not the result of decreased cell growth, since the amoebae were only incubated with MBP for 2h.

Bacterially-Grown Amoebae Are Resistant to the Effects of Bisphosphonates and Fail to Metabolise MBP

The IC_{50} for inhibition of growth of amoebae was determined from dose-response curves for three bisphosphonates in

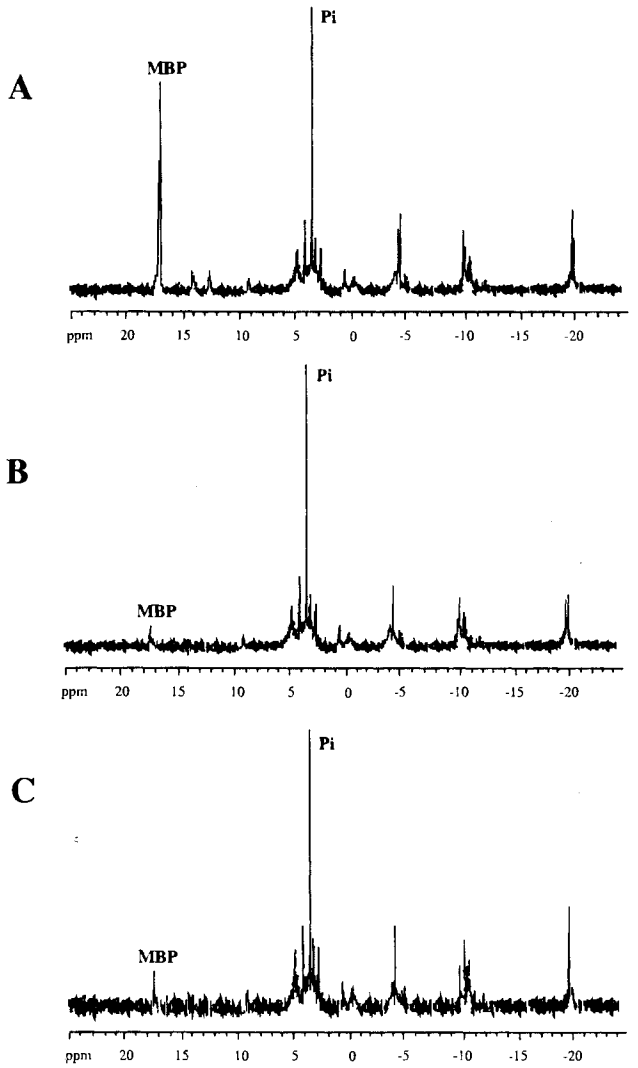

Fig. 2. ^{31}P NMR spectra of cell extracts prepared from Dictyostelium amoebae that had been (A) incubated for 2 h with 5 mM MBP, (B) incubated for 2 h with 5 mM MBP and 5 mM sodium orthovanadate, (C) incubated for 2 h with 5 mM MBP at 4°C. Extracts were prepared from similar numbers of cells.

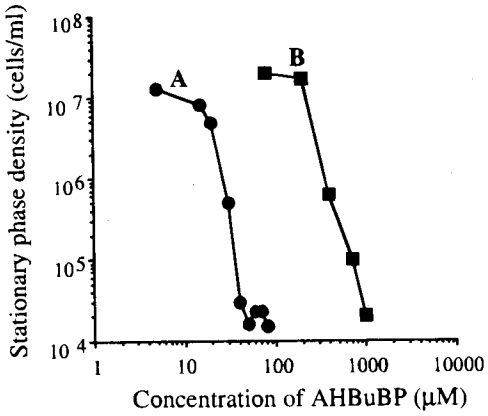

Fig. 3. Dose-response curves for inhibition of growth of *Dictyostelium* amoebae by AHBuBP, either in axenic medium (A) or in the presence of bacterial food source (B).

the presence or absence of bacteria (e.g. for AHBuBP, Fig.3). In the presence of bacteria, the values for IC_{50} were increased from 380 μM to 8000 μM for HEBP, from 30 μM to 510 μM for AHBuBP, and from 12 μM to 67 μM for 3-PHEBP. Thus, by comparison with amoebae grown axenically, amoebae grown with bacteria were approximately 20-fold resistant to HEBP and AHBuBP, and over 5-fold resistant to the more potent bisphosphonate, 3-PHEBP. A similar conclusion was reached by comparison of the minimum concentrations of bisphosphonates that totally inhibited growth in bacterial or axenic cultures (Table 2). To determine whether the resistance to bisphosphonates could be a result of removal of bisphosphonate from the medium by the bacteria (rather than inhibition of pinocytosis), we used ^{31}P-n.m.r. to compare the amount of bisphosphonate present in the culture medium in the presence or absence of *K. aerogenes*. After 3 days in the presence of live bacteria, the total concentration of bisphosphonate in the medium decreased by 16–30% (not shown). This decrease did not occur in the presence of dead (autoclaved) bacteria. Hence, the bacteria probably internalised some of the bisphosphonates. However, the decrease in the amount of bisphosphonate present in the medium was insufficient to account for the marked resistance (up to 20 fold) of *Dictyostelium* to the growth-inhibitory effects of bisphosphonates in the presence of bacteria.

Axenically-grown amoebae metabolised MBP into $AppCH_2p$ and $AppCH_2ppA$ nucleotides, which were detected in cell extracts by using f.p.l.c. Cell extracts prepared from amoebae that had been grown with MBP in the presence of a bacterial substrate contained barely detectable amounts of such metabolites (Fig. 4).

Table 2. Comparison of the Lowest Concentration of Bisphosphonates that Totally Inhibited Growth of *Dictyostelium* Amoebae When Grown Either in Axenic Culture (i.e., in the Absence of Bacteria) or with a Suspension of *K. aerogenes*

Bisphosphonate	Grown in axenic medium	Grown with bacteria
HEBP	700 μM	14000 μM
AHBuBP	50 μM	900 μM
3-PHEBP	30 μM	100 μM

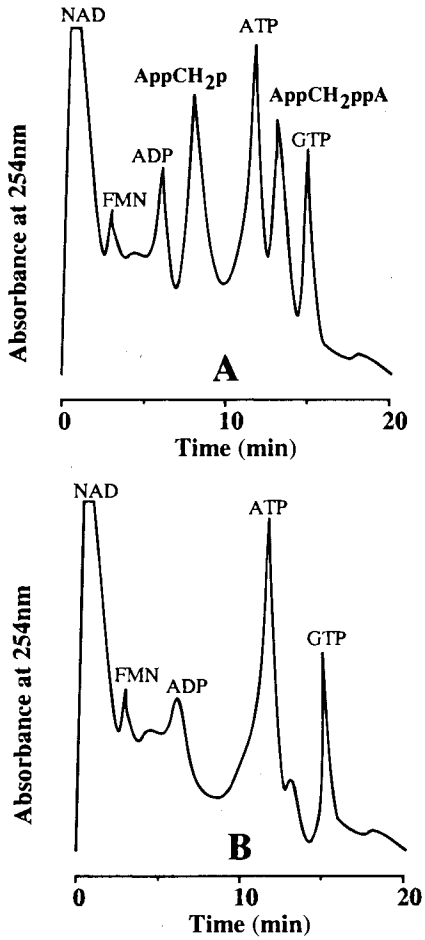

Fig. 4. FPLC elution profiles of deproteinised cell extracts prepared from *Dictyostelium* amoebae that had been (A) grown for 96 h in axenic medium with 500 μM MBP, (B) grown for 96 h with 500 μM MBP in the presence of bacterial food source. The extracts were prepared from similar numbers of amoebae.

Liposome-Encapsulated Bisphosphonates Are More Potent Growth Inhibitors than Free Bisphosphonates

Both Cl_2MBP and AHPrBP were considerably more potent towards *Dictyostelium* when contained within liposomes. While up to 800 μM free Cl_2MBP was required inhibit *Dictyostelium* growth (Fig. 5A), the same effect could be achieved with about 50 μM (final concentration) liposome-encapsulated Cl_2MBP (Fig. 5B). Similarly, 200 μM free AHPrBP completely inhibited growth (Fig. 5C), while only 10 μM (final concentration) liposome-encapsulated AHPrBP had the same effect (Fig. 5D). Empty liposomes had no effect on cell growth or viability (not shown).

DISCUSSION

We have previously shown that bisphosphonates, a class of anti-resorptive drugs, are inhibitors of growth of *Dictyostelium* amoebae (12). While some bisphosphonates may inhibit growth by metabolic incorporation into ATP analogues or inhibition of aminoacyl-tRNA synthetase enzymes (11,14,15), the more potent bisphosphonates apparently act by affecting specific cellular targets (13). In order to determine whether these targets

for bisphosphonates in *Dictyostelium* may be intracellular we have examined whether *Dictyostelium* amoebae are capable of internalising bisphosphonates, and whether such internalisation is necessary for the inhibitory effect of bisphosphonates on cell growth.

The uptake of bisphosphonates was examined by using ^{31}P-n.m.r. analysis to determine whether bisphosphonates were present in cell extracts from amoebae that had been incubated in axenic medium containing bisphosphonates. By contrast with extracts prepared from cells that had been incubated for 2 h with bisphosphonates, extracts from cells that had been washed immediately after addition of the bisphosphonate did not contain any of the drug. Furthermore, cell extracts from amoebae that had been incubated with bisphosphonate at 4°C contained negligible drug. The presence of bisphosphonate in cell extracts could not therefore be accounted for simply as a consequence of binding of bisphosphonate to the cell surface, since this would not be inhibited at 4°C, but must have been due to internalisation of the compounds. Uptake of bisphosphonates could also be inhibited in the presence of 5 mM orthovanadate or 10 mM caffeine. Orthovanadate has been shown to inhibit fluid-phase pinocytosis of FITC-dextran by *Dictyostelium* amoebae (17), while Gonzalez et al. (18) reported that caffeine could inhibit FITC-dextran uptake in *Dictyostelium*, possibly as a result of the perturbation of intracellular calcium levels. The concentrations of orthovanadate or caffeine required to prevent uptake of bisphosphonate are in close agreement with those concentrations which were found to inhibit fluid-phase pinocytosis in *Dictyostelium* (17,18). *Dictyostelium* amoebae growing in axenic medium take up nutrients exclusively by fluid-phase pinocytosis (16). We have recently reported that a fluorescently-labelled derivative of AHBuBP also appears to be internalised into endocytic vacuoles by *Dictyostelium*, macrophages and osteoclasts *in vitro* (22). This confirms that the initial uptake of AHBuBP from the extracellular medium occurs by pinocytosis.

By contrast with axenically-grown amoebae, amoebae grown with bacteria in the presence of MBP synthesised only negligible amounts of the $AppCH_2p$ and $AppCH_2ppA$ metabolites of MBP. This indicates that the bacterially-grown amoebae failed to internalise free MBP. In addition, it was found that the amoebae grown with bacteria (i.e. conditions in which bisphosphonates could not be internalised by pinocytosis) showed resistance to the growth-inhibitory effects of bisphosphonates. The extent of the resistance apparently became progressively less as the potency of the bisphosphonates increased, suggesting that a small degree of uptake (possibly owing to adsorption of bisphosphonates to the bacterial surface) may have occurred during phagocytosis of bacteria. For the highly potent bisphosphonates such as 3-PHEBP, such a small degree of uptake may be sufficient to affect cell growth. All these results are consistent with the conclusion that free bisphosphonates are efficiently taken up by *Dictyostelium* amoebae only by pinocytosis and such uptake is essential for the bisphosphonates to inhibit amoebal growth. This latter point was further confirmed by studies making use of liposome-encapsulated Cl_2MBP and AHPrBP. The encapsulated bisphosphonates were almost 20-fold more potent at inhibiting *Dictyostelium* growth than the free bisphosphonates and this could have been only because the encapsulated bisphosphonates were taken up by the amoebae and then released intracellularly where they exerted their growth-inhibitory effects. A similar increase in the potency of liposome-encapsulated bisphosphonates compared with free bisphosphonates has been found with other highly phagocytic cells (9,20).

Since cellular uptake is essential for bisphosphonates to inhibit *Dictyostelium* growth, the site of action of the bisphosphonates must be either in the pinocytic vacuoles, the cytosol or other compartments of the cytoplasm. For bisphosphonates (such as MBP) that are metabolised, a cytosolic site of action is indicated by the evidence that these bisphosphonate may act by being metabolised into methylene-containing analogues of ATP by the action of some of the cytosolic aminoacyl-tRNA synthetase enzymes (14). Furthermore, a mutant strain of *Dictyostelium* which is resistant to MBP appears to be defective in the uptake of this compound by endocytosis (23,24).

Since the molecular mechanisms by which bisphosphonates inhibit osteoclast-mediated bone resorption have not yet been identified, it is not clear whether bisphosphonates exert their effects on osteoclasts intra- or extra-cellularly (5). Studies with two radiolabelled bisphosphonates have confirmed that fibroblasts and calvarial cells are capable of internalising bisphosphonates, which were found to accumulate largely in the cytosol (10,25), while autoradiography of bone sections from rats treated with tritiated AHBuBP has also shown that the bisphosphonate was selectively internalised by osteoclasts and appeared to be localised to the cystosol, nuclei, mitochondriae and especially vacuoles (26). Carano *et al.* (27) and Emonds-Alt *et al.* (28) have also suggested that bisphosphonates act intracellularly, as metabolic inhibitors of osteoclasts and chondrocytes. In addition to all of these observations, it is clear that the potency of bisphosphonates toward different types of mammalian cells appears to be dependent on the ability of the cells to internalise bisphosphonates. The particular sensitivity of macrophages and osteoclasts to bisphosphonates may be ascribed to the ability of these cells to internalise bisphosphonates by pinocytosis or by phagocytosis. Thus, free bisphosphonates in solution appear to affect only cells that are capable of pinocytosis, such as macrophages (29), osteoclasts and fibroblasts (27), while bisphosphonates bound to bone particles or bone slices (27,30), bisphosphonates complexed with calcium or iron, or bisphosphonates within liposomes (9,20) are only

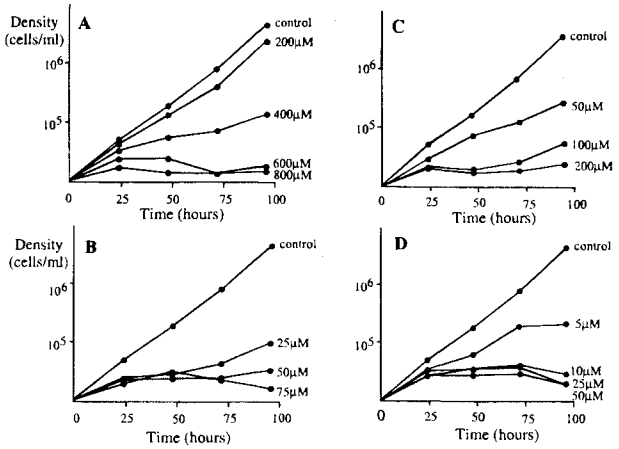

Fig. 5. Growth inhibition of *Dictyostelium* amoebae by (A) free Cl_2MBP, (B) liposome-encapsulated Cl_2MBP, (C) free AHPrBP, (D) liposome-encapsulated AHPrBP.

potent toward cells such as macrophages and osteoclasts that can internalise bisphosphonates in these forms either by phagocytosis or (in the case of osteoclasts) by release of bisphosphonates during resorption of bisphosphonate-coated bone. These observations all suggest that bisphosphonates act intracellularly.

Amoebae of the slime mould *Dictyostelium* appear to have targets for bisphosphonates that are very similar to those in osteoclasts. We have now shown that these targets appear to be intracellular and that *Dictyostelium* amoebae are susceptible to the effects of bisphosphonates following internalisation of bisphosphonates by fluid-phase pinocytosis. This is further evidence that bisphosphonates also have an intracellular mechanism of action in osteoclasts.

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