Structural aspects of the effectiveness of bisphosphonates as competitive inhibitors of the plant vacuolar proton-pumping pyrophosphatase

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The bisphosphonates (general structure PO_3-R-PO_3) competitively inhibit soluble and membrane-bound inorganic pyrophosphatases (PPases) with differing degrees of specificity. Aminomethylenebisphosphonate (AMBP; $HC(PO_3)_2NH_2$) is a potent, specific inhibitor of the PPase of higher plant vacuoles (V-PPase). To explore the possibility of constructing photoactivatable probes from bisphosphonates to label the active site of V-PPase we analysed the effects of different analogues on the hydrolytic and proton pumping activity of the enzyme. Bisphosphonates with a range of structures inhibited competitively and the effects on PP_i hydrolysis correlated with the effects on proton pumping. Low-molecular-mass bisphosphonates containing hydrophilic groups (α-NH_2 or OH) were the most effective, suggesting that the catalytic site is in a restricted polar pocket. Bisphosphonates containing a benzene ring were less active but the introduction of a nitrogen atom into the ring increased activity. Compounds of the general formula $NH_2(CH_2)_nC(PO_3)_2OH$ were more inhibitory than compounds of the $H(CH_2)_nC(PO_3)_2NH_2$, $NH_2(CH_2)_nC(PO_3)_2NH_2$ or $OH(CH_2)_nC(PO_3)_2NH_2$ series, with activity decreasing as n increased. A nitrogen atom in the carbon chain increased activity but activity was decreased by the presence of an oxygen atom. An analogue with a ring attached via a four-carbon chain, which included an amide linkage and a hydroxy group on the α-carbon atom, inhibited competitively ($K_i = 62.0\ \mu M$), suggesting that it may be possible to design bisphosphonate inhibitors which contain a photoactivatable azido group for photoaffinity labelling of V-PPase active site.

Key words: aminomethylenebisphosphonate, photoactivatable probe, catalytic site.

INTRODUCTION

The bisphosphonates are a family of compounds with the general structure PO_3-R-PO_3. They affect a variety of significant cellular processes and their action on bone resorption has rendered them important as therapeutic drugs [1,2]. It is believed that they may exert their action *in vivo* by substituting for pyrophosphate (PP_i) [3], and that their effectiveness as pharmacological agents can be attributed to their enhanced stability compared with PP_i, due to the presence of the P-C-P structure, which is resistant to enzymic hydrolysis and chemical breakdown [4]. Several bisphosphonate analogues have been shown to competitively inhibit the soluble mammalian, yeast and bacterial inorganic pyrophosphatases (PPases) [5] and the membrane-bound PPases from the higher plant vacuole (V-PPase) and *Rhodospirillum rubrum* [6], but they were found to differ greatly in their effectiveness and specificity.

The bisphosphonates have been used as specific inhibitors to help address the physiological role of the V-PPase [6]. This unique enzyme is structurally distinct from other known PPases with the exception of the PP_i synthase from *R. rubrum*, to which it is immunologically and functionally related [7]. Isolation and cloning of the major catalytic subunit of the enzyme from a number of species [8–12] revealed that it consisted of a single 88 kD molecule capable of all recognised functions of V-PPase when heterologously expressed in yeast [13]. Kinetic studies suggest that it contains at least four regulatory binding sites [14], although none have been unequivocally identified, and that its substrate is the complex Mg_2PP_i [14–16]. V-PPase is present in the vacuolar membrane of all higher plants where it is believed to be responsible for maintaining the vacuolar proton gradient [17], however, because it coexists within the tonoplast with a second, ATP-dependent proton pump [17,18] it has been suggested that V-PPase may possess a different function. Bisphosphonates may help to identify this role. Previously, two PP_i analogues, methylenebisphosphonate (MBP) and imidobisphosphate, were used to characterize the activity of the enzyme [19] but more recently the effects of aminomethylenebisphosphonate (AMBP) have been studied [6]. This compound is a more potent inhibitor of V-PPase and, provided it is used under strictly controlled conditions, is highly specific. It has virtually no effect on the vacuolar ATPase and its enhanced action on V-PPase is believed to be due the interaction of the amino group with a carboxy group near the active site [7].

To understand the relationship between structure and inhibitory capacity of the bisphosphonates towards V-PPase from mung bean (*Vigna radiata* L.), we have embarked on a systematic analysis of the effects of a wide range of different bisphosphonates on the activity of the enzyme. By determining which properties influence the inhibitory efficiency of the compounds we hoped to obtain information about the active site of the enzyme and to identify compounds that are both effective as competitive inhibitors and which contain functional groups which might be used to develop photoactivatable probes for structural studies of V-PPase .

MATERIALS AND METHODS

All chemicals were from Sigma or B.D.H. (Poole, Dorset, U.K.), except for the bisphosphonate analogues which were obtained from the pharmaceutical industry. Seeds of mung bean

Abbreviations used: AMBP, aminomethylenebisphosphonate; MBP, methylenebisphosphonate; PPase, pyrophosphatase; PP_i, pyrophosphate; V-PPase, vacuolar pyrophosphatase.

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(*V. radiata* L.) were germinated and grown on water-saturated vermiculite, in the dark, at 25 °C for 4 days; the temperature was then decreased to 4 °C [20] and the seedlings were harvested after a further 24 h. Tonoplast vesicles were isolated as described previously [21]. The hydrolytic activity of V-PPase was measured using a method reported previously [14], except that 100 mM imidazole, pH 8.0, was used as the pH buffer [22], and enzyme activity is reported as the amount of PP_i hydrolysed (P_i released divided by 2). Where low PP_i concentrations were required, the assay time was reduced to 12 min to ensure that PP_i was not depleted. Vacuolar ATPase assays were performed as described previously [23], and proton transport in isolated tonoplast vesicles was measured using quinacrine fluorescence to monitor the development of an acidic pH within the vesicles [14]. The bisphosphonates were added to the assay media at the concentrations indicated in the Figure legends. Protein was assayed by the method of Appleroth and Angsten [24] with BSA as a standard.

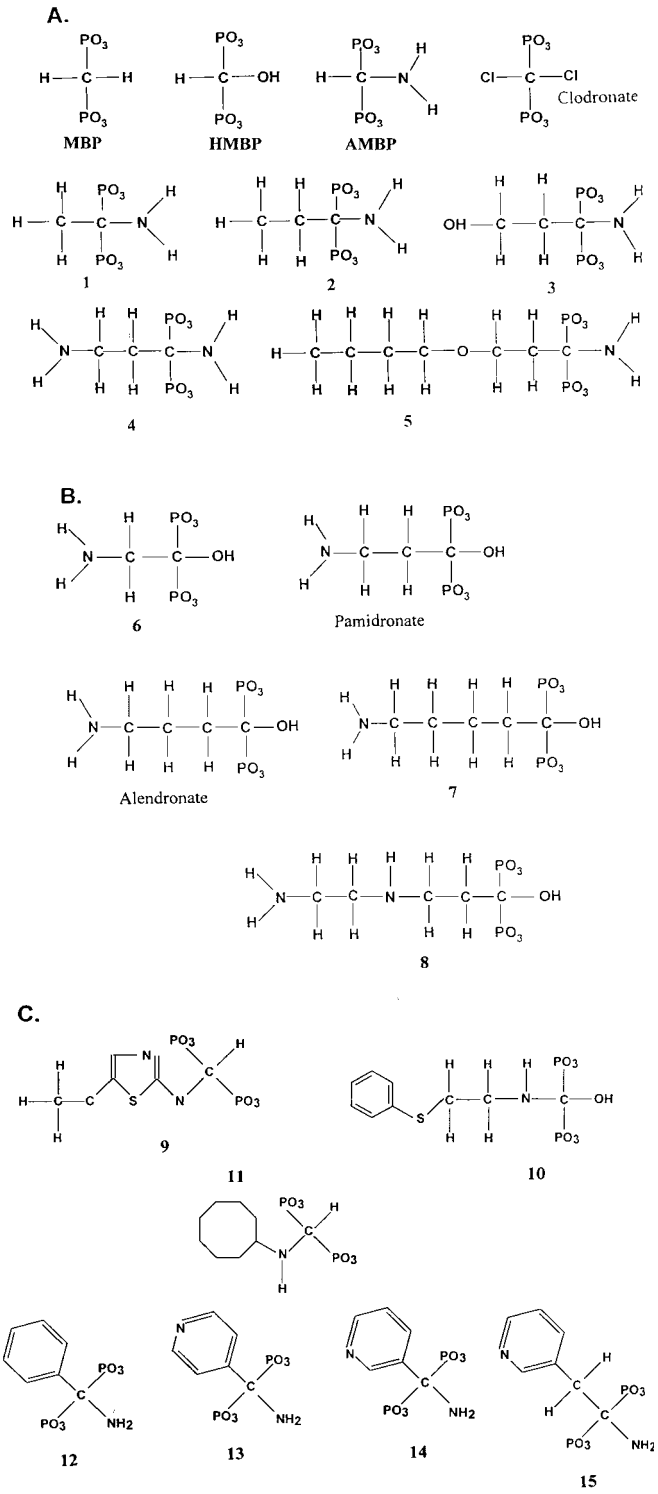

Figure 1 Structures of bisphosphonate compounds tested for inhibition of V-PPase activity

(**A**) Compounds with simple aliphatic side chains; (**B**) compounds of the $NH_2(CH_2)_nC(PO_3)_2OH$ series; (**C**) compounds with side chains containing ring structures.

RESULTS

The concentration-dependent inhibition of PP_i hydrolysis by a selection of bisphosphonate analogues with a variety of different side chains was studied (see Figure 1 for structures). Three low molecular mass compounds, AMBP [$HC(PO_3)_2NH_2$], MBP [$HC(PO_3)_2H$], and compound 1 [$CH_3C(PO_3)_2NH_2$]; four further compounds, compound 6 [$NH_2CH_2C(PO_3)_2OH$], compound 3 [$OHCH_2CH_2C(PO_3)_2NH_2$] and pamidronate [$NH_2CH_2CH_2C(PO_3)_2OH$], which contain hydroxy and amino groups and different length carbon chains; and three compounds containing ring structures, compound 9, compound 10 and compound 11, were tested. AMBP, compound 6 and pamidronate were the most inhibitory and compound 1, MBP and compound 3 were slightly less active (Figure 2). Much greater concentrations of the aromatic compounds and compound 9 were required for

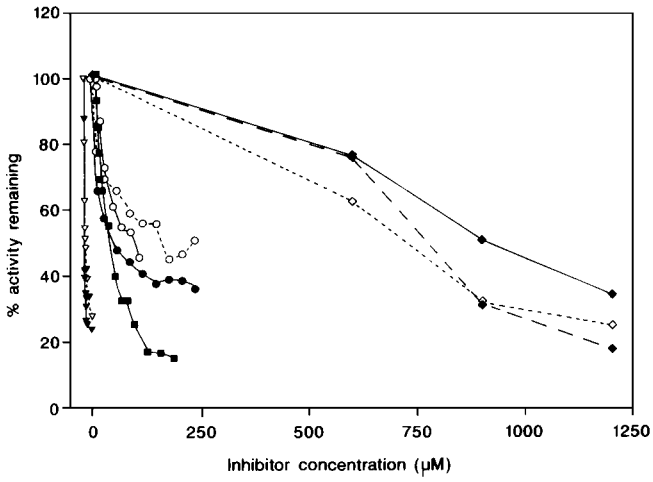

Figure 2 Concentration-dependence of inhibition of V-PPase by various bisphosphonate compounds

Hydrolysis of PP_i was measured in a reaction medium containing 0.3 mM PP_i-Bis-Tris propane, 1.3 mM $MgSO_4$, 50 mM KCl, 250 mM glycerol, 5 μM gramicidin D, 100 mM imidazole/HCl, pH 8.05, 10 $\mu g \cdot ml^{-1}$ L-α-lysophosphatidylcholine, 300 $\mu g \cdot ml^{-1}$ Triton X-100. The bisphosphonates were added to the assay media at the concentrations shown on the Figure. The specific activity (means $\pm$ S.E.) of V-PPase in control experiments was 22.1 $\pm$ 3.21 μmol PP_i hydrolysed $\cdot$ mg of protein$^{-1} \cdot h^{-1}$). The compounds tested were: AMBP (▼), MBP (○, solid line), compound 6 (▽), pamidronate (■), compound 1 (●), compound 3 (○, broken line), compound 10 (◇), compound 11 (◆, broken line) and compound 9 (◆, solid line).

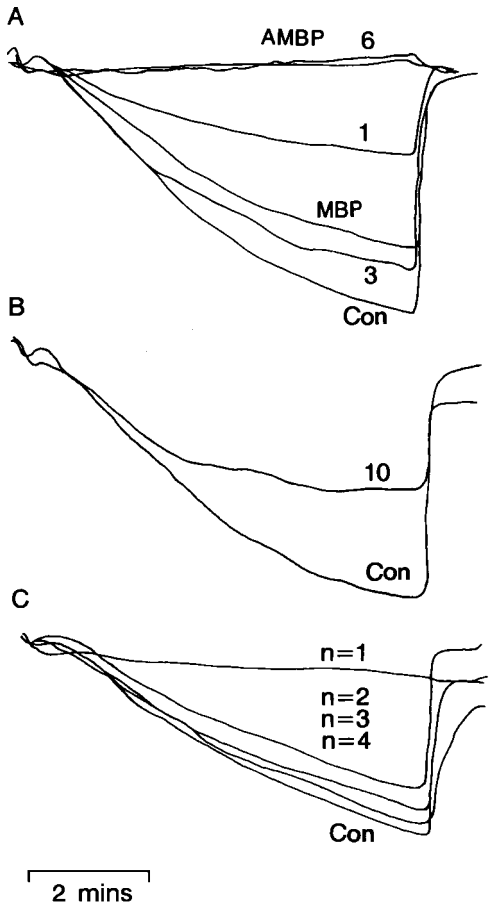

Figure 3 Inhibition of PP_i-dependent proton transport by bisphosphonate compounds in isolated tonoplast vesicles

Activity was measured by quenching of quinacrine fluorescence, as described previously [14], in the presence of 0.3 mM PP_i-Bis-Tris propane (assay volume 1 ml). The bisphosphonate compounds indicated in the Figure were added to the incubation media at concentrations of 30 μM (**A** and **C**) or 250 μM (**B**). (**C**) Bisphosphonate compounds of the series $NH_2(CH_2)_nC(PO_3)_2OH$, where n = carbon chain length. Con, control traces performed in the absence of inhibitor. Vesicles were added (50–100 μg protein) and the reaction was started by the addition of 20 μl of 0.6 M $MgSO_4$ and 10 μl of 0.5% gramicidin D was added to collapse the gradient.

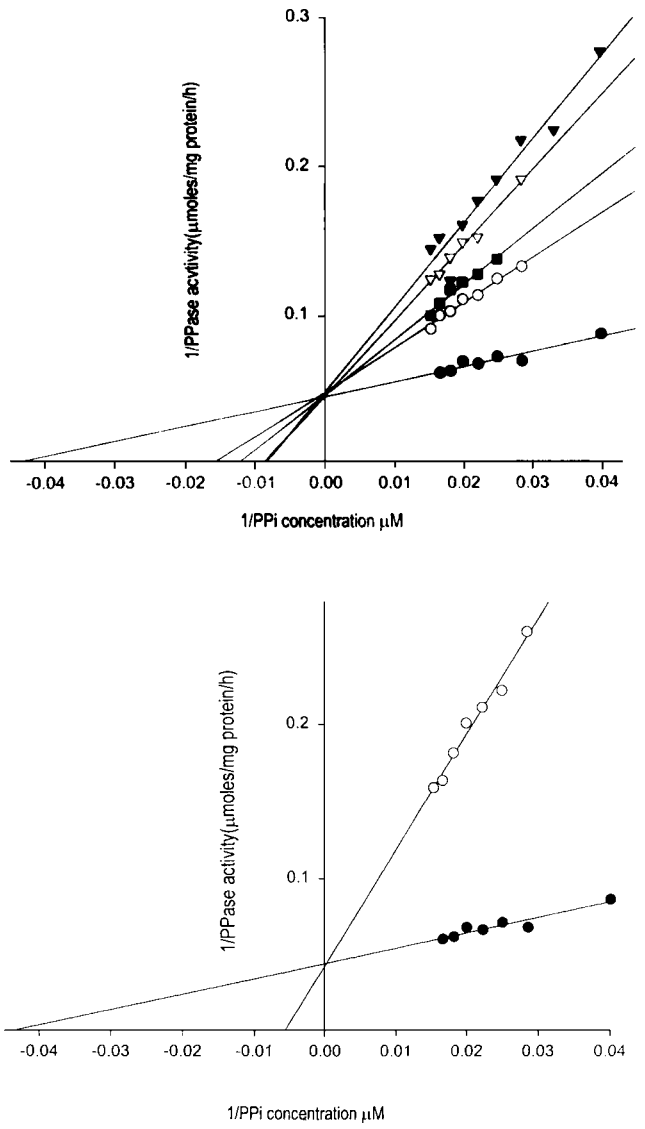

Figure 4 Double-reciprocal plots of PP_i concentration and mung bean V-PPase activity in the presence of various bisphosphonates

The assay was performed in the absence of the inhibitor (●) or in the presence of: (upper panel) 3 μM AMBP (▼), 3 μM compound 6 (▽), 10 μM pamidronate (■) or 90 μM compound 3 (○); (lower panel) 250 μM compound 10 (○).

maximum inhibition, and extension of the carbon chain reduced inhibition (compare compound 1 with AMBP).

To confirm that the compounds reduced hydrolytic activity by specifically inhibiting V-PPase, their effect on the hydrolytic activity of vacuolar ATPase [6] and PP_i-dependent proton pumping activity was measured. None of the compounds tested (AMBP, compound 1 and compound 3 at 120 μM or compound 10 at 900 μM) had any effect on ATP hydrolysis (results not shown). AMBP and compound 6 provided full inhibition of PP_i-dependent quenching of quinacrine fluorescence, a measure of proton pumping activity, at concentrations of 30 μM (Figure 3A). The rank order in which compound 1, MBP and compound 3, at 30 μM, affected proton pumping was similar to their effect on PP_i hydrolysis (compare Figure 3A with Figure 2). Approx. 50% inhibition of proton pumping activity was brought about by 250 μM compound 10 (Figure 3B), although this hydrophobic compound inhibited hydrolytic activity by only 20% at this concentration. The enhanced effect on proton pumping may be due to disruption of the membrane by the presence of large concentrations of the hydrophobic compound in the incubation medium. These results demonstrate that the nature of the side chain strongly affects the effectiveness of these compounds as inhibitors, with both size and hydrophobicity playing a significant negative role.

For photoactivatable probes to be effective, the compounds must interact exclusively with the catalytic site and thus it was important to confirm that each compound inhibited the enzyme competitively. All of the compounds tested displayed competitive kinetics (Figure 4, upper panel), including compound 10, which has a side chain containing a ring structure (Figure 4, lower panel).

The presence of an aromatic ring is advantageous for the introduction of both a photoactivatable group and a radioactive label

Table 1 Effect of carbon side-chain length (*n*) on the $K_{i,app}$ of bisphosphonates which contain amino or hydroxy groups

Each of the compounds tested inhibited V-PPase activity in a competitive manner and the $K_{i,app}$ values were derived from eqn. (1). For structures see Figures 1(A) and (B). Values are the means ± S.E. of three independent measurements. n.a., compound not available.

	$K_{i,app}$ (μM)			
n	$NH_2(CH_2)_nC^*OH$	$H(CH_2)_nC^*NH_2$	$NH_2(CH_2)_nC^*NH_2$	$OH(CH_2)_nC^*NH_2$
0	Unstable	1.32 ± 0.05	n.a.	n.a.
1	1.92 ± 0.06	18.7 ± 1.22	n.a.	n.a.
2	10.2 ± 0.51	313 ± 21.1	173 ± 19.2	63.2 ± 1.22
3	33.7 ± 2.39	n.a.	n.a.	n.a.
4	37.2 ± 1.84	n.a.	n.a.	n.a.

* Link carbon atom attached to phosphate groups.

into the molecule but it was shown to have a negative effect on inhibitory activity (Figure 2). The apparent inhibition constant ($K_{i,app}$) of a series of aromatic compounds with an α-amino group attached to the link carbon atom (Figure 1C; compounds 12, 13, 14 and 15), which has been shown to enhance inhibitory activity [5], were compared to see if the presence of the amino group overcame the negative effect of the aromatic ring. The $K_{i,app}$ for each compound could be derived from the relationship in eqn. (1):

$$v = v_o/(1 + [I]/2K_{i,app}) \quad (1)$$

where [I] is the concentration of the inhibitor.

It was found that a bisphosphonate compound in which the benzene ring was attached directly to the link carbon atom (Figure 1C, compound 12) did not inhibit V-PPase, but that the introduction of a nitrogen atom into the ring (Figure 1C, compound 14) generated an active compound ($K_{i,app}$ = 252.0 ± 17.4, mean ± S. E.). The positioning of the nitrogen atom in the *para* position (Figure 1C, compound 13) was slightly more effective than in the *ortho* position ($K_{i,app}$ = 227.0 ± 17.0), and separation of the benzene ring from the link carbon atom by a methyl group (Figure 1C, compound 15) also provided a very slight increase in activity ($K_{i,app}$ = 185.0 ± 12.9). The $K_{i,app}$ values were derived from eqn. (1).

The $K_{i,app}$ values of a series of compounds of the general formula $NH_2(CH_2)_nC(PO_3)_2OH$ (see Figure 1B) were studied to compare the effect of increasing the aliphatic carbon chain length on V-PPase activity. As shown in Table 1, increasing the length of the carbon chain (from $n = 0$ to $n = 4$) within this family of compounds progressively increased the $K_{i,app}$ values of the compounds. The effect on proton pumping was in the same order and the compound $n = 0$ provided full inhibition of quinacrine quenching at 30 μM, whereas the compound with a carbon chain length of $n = 4$ caused only a slight reduction in quenching at this concentration (Figure 3C). The activities of some analogues with α-amino groups [$H(CH_2)_nC(PO_3)_2NH_2$] (see Figure 1A; AMBP, compound 1 and compound 2) were also compared with these compounds and those of the $NH_2(CH_2)_nC(PO_3)_2OH$ series were consistently more active. Furthermore, compound 3 (Figure 1A), in which the position of the hydroxy and amino groups are reversed, was 6-fold less active than the equivalent compound of the $NH_2(CH_2)_nC(PO_3)_2OH$ series, and the presence of an amino group at both ends of the carbon chain, where $n = 2$ (Figure 1A, compound 4), produced a compound with even weaker inhibitory activity.

The introduction of an amino group linking the second and third carbon atoms of the aliphatic chain of the $n = 4$ member of the $NH_2(CH_2)_nC(PO_3)_2OH$ series [$NH_2(CH_2)_2NH(CH_2)_2C(PO_3)_2OH$; Figure 1B, compound 8] reduced the $K_{i,app}$ from 37.2 ± 1.84 μM for $NH_2(CH_2)_nC(PO_3)_2OH$ to 6.58 ± 0.37 μM (means ± S.E.). However, the compound $CH_3(CH_2)_3O(CH_2)_2C(PO_3)_2NH_2$ (Figure 1A, compound 5) was a very weak inhibitor ($K_{i,app}$ = 425 ± 31.2 μM). A further compound, which also contained an amino linkage, with a benzene ring attached to the carbon chain via a sulphur atom (Figure 1C; compound 10), had a $K_{i,app}$ of 62.0 ± 2.85 μM, which was significantly lower than any of the other ring-containing compounds tested.

DISCUSSION

In this study we have tried to ascertain some of the factors which influence the ability of bisphosphonate compounds to competitively inhibit the plant V-PPase. Our results confirm that the presence of an hydroxy or an amino group attached to the α-carbon atom plays a major role in conferring activity, possibly because of interaction of these groups with carboxy or other charged residues near the catalytic site [5]. Small molecules were more active and the presence of aromatic and other cyclic structures decreased activity, implying that size or hydrophobicity affect the ability of the compound to gain access to the active site. Taken together these observations suggest that the catalytic site may be located in a small hydrophilic pocket within the protein.

AMBP has been shown to be a significantly more active inhibitor of V-PPase than either $HC(PO_3)_2OH$ (HMBP, Figure 1A) (K_i = 5.7 μM) [6] or MBP (K_i = 68 μM) [6], whereas clodronate ($Cl_2C(PO_3)_2$) was almost inactive [6]. However, in agreement with the findings of Chanson and Pilet [19], the $K_{i,app}$ obtained for MBP (14.0 ± 0.811 μM) was considerably lower. The most active inhibitors of the mung bean V-PPase were the compounds of the $NH_2(CH_2)_nC(PO_3)_2OH$ series, possibly due to the presence of the α-hydroxy group, which increases metal ion chelating capacity. V-PPase has been shown to use Mg_2PP_i as its substrate [15] and Mg^{2+}-chelated forms of the bisphosphonate analogues are believed to interact with mammalian soluble PPases [5]. Consequently the affinity of the compounds for metal ions would be expected to influence their ability to bind to the enzyme. However, the enhanced activity of $NH_2(CH_2)_nC(PO_3)_2OH$ compared with $OH(CH_2)_nC(PO_3)_2NH_2$ may, in view of the greater effectiveness of AMBP compared with hydroxymethylenebisphosphonate [6], be due to the presence of the amino group at the end of the aliphatic chain, which may be optimally placed for hydrogen bond formation. These compounds were also more effective than extended forms of AMBP [for example, $H(CH_2)_nC(PO_3)_2NH_2$ where $n > 1$] and $NH_2(CH_2)_nC(PO_3)_2NH_2$, but the compound of this series where $n = 0$ [$NH_2C(PO_3)_2OH$], potentially even more effective than AMBP, is highly unstable.

The bond angle between the two phosphate groups is not affected by the nature of the substituents and is hence unlikely to contribute to differences in potency [25]. We found the bond angle to be the same for two compounds of widely differing potency (AMBP and compound 9), when their structures were compared using the computer program Alchemy III (Tripos Associates, Inc., Milton Keynes, U.K.) (results not shown).

A wide range of bisphosphonates have been tested for their effects as inhibitors of osteoclast-mediated bone resorption and of growth of *Dictyostelium discoideum* [26] with the same target protein for bisphosphonate action. However, although the

$NH_2(CH_2)_nC(PO_3)_2OH$ series of compounds have been found to be effective both as an inhibitor of V-PPase and of bone resorption, an increase in the aliphatic chain length of compound 3 members enhanced inhibition of bone resorption activity and still larger compounds retained considerable potency [26]. This contrasts with their effect on V-PPase activity (see Table 1). Similarly, compounds containing aromatic ring structures are particularly effective as inhibitors of bone resorption [27], with compounds containing shorter aliphatic chains linking the aromatic ring to the link carbon atom being more potent than those with extended chains [28]. Hydrophobic compounds may partition into the membrane and be more readily internalized by osteoclasts [29].

Although the presence of hydrophobic groups on the bisphosphonate compounds decreases inhibition of V-PPase , where they are located at a distance from the α-carbon atom (compare compound 12 with compound 15) or, where groups which favour hydrogen-bond formation are introduced into the molecule, interaction with the active site still appears to take place. We found that the introduction of a nitrogen atom into the ring, which decreases the ring size and the hydrophobicity of the molecule and may also favour hydrogen bond formation (see above), enhanced the effect of the compounds against V-PPase activity (see the Results section). A similar effect has been shown in bone resorption assays with bisphosphonates containing side chains with secondary or tertiary amine groups [27,28,30]. Particularly effective is the presence of an amino group within the carbon chain, illustrated by comparison of compound 8 ($K_i = 6.58 \pm 0.37\ \mu M$) with compound 7 ($K_i = 37.2 \pm 1.84\ \mu M$) and compound 5 ($K_i = 425 \pm 31.4\ \mu M$), where the presence of the nucleophilic oxygen atom within the carbon chain may interfere with hydrogen bond formation with carboxy groups. Accordingly, compound 10 was more active than other aromatic-ring-containing compounds even though it contained a sulphur linkage, which may produce an effect similar to the presence of an oxygen atom within the carbon chain. It may therefore be possible to design a bisphosphonate inhibitor which contains a photoactivatable azido group and which has sufficiently high affinity for V-PPase to enable it to be used for photoaffinity labelling studies. Our results suggest that the compound would contain an α-hydroxy or amino group and a benzene ring connected to the α-carbon atom via a carbon chain containing a nitrogen atom.

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