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Structure-Activity Relationships of New Heterocycle-Containing Bisphosphonates as Inhibitors of Bone Resorption and as Inhibitors of Growth of *Dictyostelium discoideum* Amoebae

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SUMMARY

The mechanisms by which bisphosphonate drugs inhibit osteoclast-mediated bone resorption are unclear. Effects of bisphosphonates on cellular enzymes, metabolic pathways, and osteoclast morphology have previously been described and could culminate in a generalized cytotoxic effect or a decreased capacity of osteoclasts to resorb bone. Recent studies of the structure-activity relationship for the bisphosphonate side chain indicate, however, that at least the newer generations of nitrogen-containing bisphosphonates probably act by binding to a specific target at a site that is complementary in structure to the bisphosphonate side chain. We have previously proposed that such a target for bisphosphonates is also present in amoebae of the cellular slime mold *Dictyostelium discoideum*, because growth of this microorganism is inhibited by a wide range of bisphosphonates in a manner that closely reflects the antiresorptive potencies of the bisphosphonates *in vivo*. We have added support for this view by examining the potency towards *Dictyostelium* of bisphosphonates in which slight changes in the structure of the side chain or conformational restrictions to the side chain have marked effects on antiresorptive potency. The changes in the side chain that affected the *in vivo* antiresorptive potency of the bisphosphonates consistently affected in a similar manner the potency of the bisphosphonates as inhibitors of the growth of *Dictyostelium* amoebae. These observations confirm that bisphosphonate drugs have a molecular target that is common to both *Dictyostelium* amoebae and osteoclasts.

BPs are synthetic analogues of pyrophosphate in which two phosphate groups are separated by a stable methylene group, i.e., PO_3H_2-CR^1R^2-PO_3H_2 (where R^1 and R^2 denote side chains of variable structure attached to the geminal carbon atom). Like pyrophosphate, BPs have high affinity for bone mineral and can act as inhibitors of both the growth and the dissolution of calcium phosphate crystals (1, 2). In contrast to pyrophosphate, however, some BPs are also powerful inhibitors of osteoclast-mediated bone resorption (1–3) and have become clinically important agents used to inhibit bone resorption in a variety of widespread disorders of mineral metabolism (4), including Paget's disease (5), hypercalcemia of malignancy (6), and, more recently, postmenopausal and glucocorticosteroid-induced osteoporosis (7, 8).

The mechanism of action of BPs remains unclear (9) but may include a cytotoxic effect on mature osteoclasts that resorb BP-coated bone (10, 11). Inhibition of enzymes or metabolic pathways (12–14) or changes in osteoclast ultrastructure (15, 16) could result in a loss of the ability of osteoclasts to resorb bone. Formation of mature osteoclasts from hemopoietic precursors may also be inhibited by BPs (17–20). We have previously shown that BPs also have growth-inhibitory and cytotoxic effects on amoebae of the cellular slime mold *Dictyostelium discoideum* and that the order of potency of a wide range of BPs as growth inhibitors of *Dictyostelium* closely matches the order of potency of these compounds as antiresorptive agents *in vivo* (21). This suggests that BPs may have

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ABBREVIATIONS: BP, bisphosphonate; LED, lowest effective dose; MES, 2-(*N*-morpholino)ethanesulfonic acid; NE10501, (6,7-dihydro-5*H*-2-pyrindin-7-yl)hydroxymethylenebisphosphonic acid; NE11728, 3-(2-pyridinyl)propylidene-1,1-bisphosphonic acid; NE11807, *N*-(2-pyridyl) aminomethane-1,1-bisphosphonic acid; NE11808, 2-pyridylaminoethane-1,1-bisphosphonic acid; NE11809, *N*-(3-picolyl)aminoethane-1,1-bisphosphonic acid; NE58025, *cis*-octahydro-1-pyrindine-6,6-bisphosphonic acid; NE58051, 3-(3-pyridinyl)propylidene-1,1-bisphosphonic acid; NE58086, 6,7-dihydro-5*H*-1-pyrindine-6,6-bisphosphonic acid; NE58095, 2-(3-pyridyl)ethylidene-1,1-bisphosphonic acid (Risedronate); NE80702, (6,7-dihydro-5*H*-1-pyrindin-7-yl)methylene-1,1-bisphosphonic acid; NE97220, *N*-(3-picolyl)aminomethane-1,1-bisphosphonic acid; NE97221, 2-(2-pyridyl)ethylidene-1,1-bisphosphonic acid.

a mechanism of action that is similar in osteoclasts and *Dictyostelium* amoebae.

It has recently become clear that BPs with a heterocycle-containing side chain are generally very potent antiresorptive agents (22, 23). In addition, particular three-dimensional conformations of the heterocyclic group appear to be critical for high antiresorptive activity (24, 25), and small changes in the structure of the heterocyclic group or in the length of the 'spacer' chain between the heterocyclic group and the geminal carbon atom also markedly alter the antiresorptive potency (22). To examine more closely the hypothesis that BPs have cellular effects on osteoclasts and *Dictyostelium* amoebae by the same mechanism, we have examined the antiresorptive potency of 12 BPs that have a nitrogen-containing heterocyclic side chain but that differ either in the length of the chain between the geminal carbon atom and the heterocyclic group, in the position of a methyl group in the heterocyclic group, or by having heterocyclic groups with restricted or unrestricted conformations (Table 1). Because these changes in the structure of the side chain affected the antiresorptive potency of the heterocycle-containing BPs, it was expected that, if the mechanism of action of BPs was the same in both osteoclasts and *Dictyostelium*, then such changes in the structure of the BP side chain would also affect the potency of these BPs as growth inhibitors of *Dictyostelium* amoebae.

Materials and Methods

BPs. All BPs were synthesized by Procter and Gamble Pharmaceuticals (Cincinnati, OH). Pairs of BPs differed either in the length of the spacer between the heterocyclic group and the geminal carbon atom (three pairs of BPs) or in the presence or absence of a methyl group in the heterocyclic ring (two pairs of BPs) or had a heterocyclic group in a restricted or unrestricted conformation (three pairs of BPs). The structures of the BP side chains, R^1 and R^2, are shown in Table 1.

Determination of the antiresorptive potency of BPs. Antiresorptive potency was assessed histomorphometrically by determination of the LED to inhibit bone resorption in the Schenk growing rat model (26). Values are expressed as milligram of phosphorus/kilogram of body weight (mg P/kg).

Determination of the potency of BPs as growth inhibitors of *D. discoideum*. Amoebae of *D. discoideum*, strain Ax-2, were grown axenically in HL5-glucose medium in sterile, six-well, tissue culture plates (Falcon, NJ), as described previously (21). Sterile solutions of BPs dissolved in 20 mM MES buffer, final pH 6.4, were added to cultures inoculated at 10^4 cells/ml, and growth of the cultures was monitored using a model ZM Coulter counter. Dose-response curves were plotted, using logarithmic/logarithmic scales, as the final cell density at the stationary phase of growth (for a given concentration of BP) versus BP concentration. Three experiments were carried out with each concentration of BP; hence, each point on the dose-response curve is the mean of three experiments. The IC_{50} values were determined from the dose-response curves and were defined as the concentrations of BP that allowed only half the number of cell divisions that would have occurred in the absence of BP (21).

Results

Inhibition of *Dictyostelium* growth by BPs. All of the BPs examined were inhibitors of growth of *Dictyostelium* amoebae. This is shown, for example, for NE11808 and NE11809 in Fig. 1. The IC_{50} values were obtained from dose-response curves (representative curves are shown for NE58025 and NE58086 in Fig. 2) and are listed in Table 1.

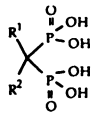
Effects of side chain length. Three pairs of BPs were examined in which the lengths of the chains linking the heterocyclic group to the geminal carbon differed by two or three carbon or nitrogen atoms. The 3-pyridylhydroxyethane-BP (NE58095), with a two-carbon chain, was one of the most potent inhibitors of *Dictyostelium* growth (IC_{50}, 13 μM), whereas the corresponding BP with a three-carbon chain (3-pyridylhydroxypropane-BP, NE58051) was much less potent (IC_{50}, 310 μM). NE58095 was also a very potent inhibitor of bone resorption (LED, 0.0003 mg P/kg), whereas NE58051 showed little activity (LED, 1.0 mg P/kg).

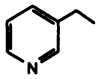
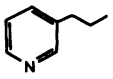
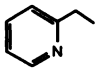
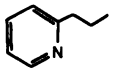
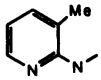
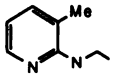
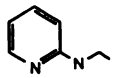
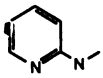
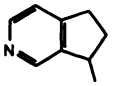
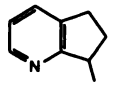
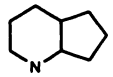
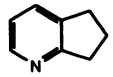
Similarly, the 2-pyridylethane-BP (NE97221) was a potent inhibitor of *Dictyostelium* growth (IC_{50}, 20 μM), whereas the

TABLE 1

Side chain structures, and LED values to inhibit bone resorption in the Schenk rat model, and IC_{50} values to inhibit *Dictyostelium* growth for the 12 heterocycle-containing BPs

IC_{50} values were determined from dose-response curves and were defined as the concentrations of BP that allowed only half the number of cell divisions that would have occurred in the absence of BP (21). The structure of a geminal BP is shown.



BP	R^1	R^2[a]	LED	IC_{50}
			mg of phosphorus/kg	*μM*
NE58095	OH		0.0003	13
NE58051	OH		1.0	310
NE97221	H		0.01	20
NE11728	H		>1.0	150
NE97220	H		0.001	9
NE11809	H		1.0	40
NE11808	H		0.01	15
NE11807	H		0.01	12
NE10501	OH		0.01	36
NE80702	H		0.1	38
NE58025			0.01	13
NE58086			>1.0	1200

[a] Me, methyl.

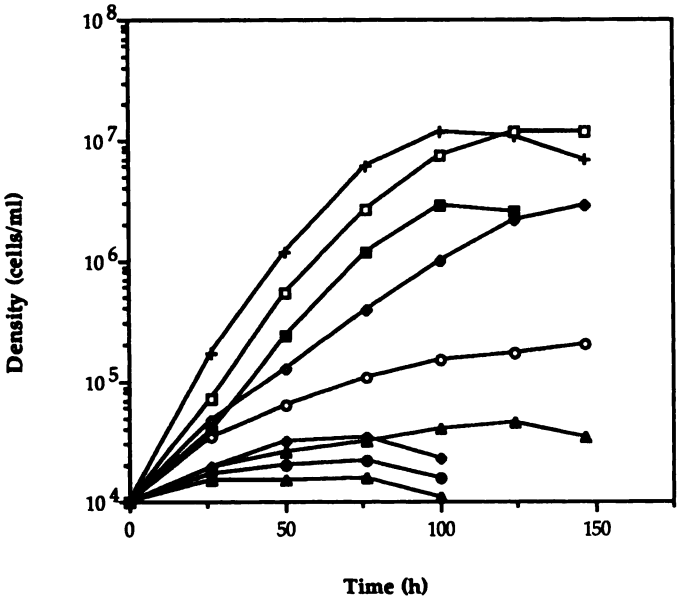


Fig. 1. Inhibition of growth of *Dictyostelium* amoebae in the presence of NE11808 (*filled symbols*) or NE11809 (*open symbols*). +, Control; *squares*, 5 μM; *diamonds*, 25 μM; *circles*, 50 μM; *triangles*, 100 μM.

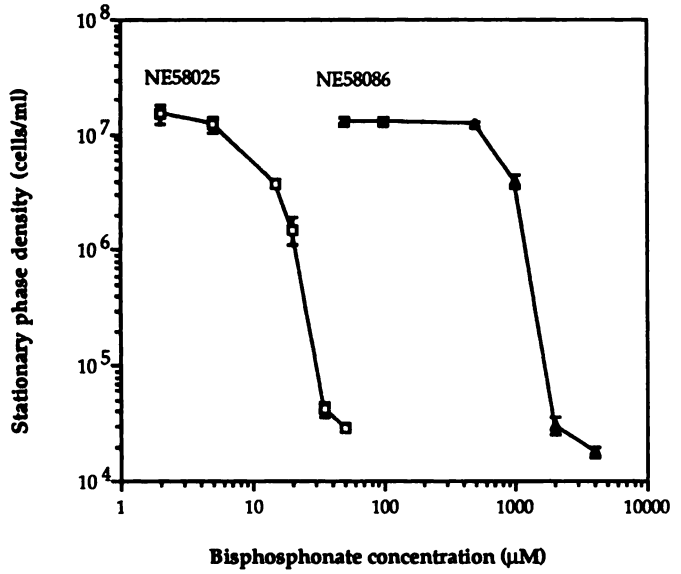


Fig. 2. Dose-response relationship for the effects of NE58025 and NE58086 effects on *D. discoideum*. Each *point* represents the mean ± standard error for three experiments. For points that do not appear to have error bars, the standard error is smaller than the symbol.

2-pyridylpropane-BP (NE11728) was far less potent (IC_{50}, 150 μM). As inhibitors of bone resorption *in vivo*, NE97221 was very potent (LED, 0.01 mg P/kg), whereas NE11728 was inactive (LED, >1.0 mg P/kg) (22).

The 3-picolylaminomethane-BP (NE97220) was the most potent inhibitor of *Dictyostelium* growth of the BPs studied (IC_{50}, 9 μM), whereas the 3-picolylaminoethane-BP (NE11809) was 4-fold less potent (IC_{50}, 40 μM). NE97220 was also a very potent antiresorptive BP (LED, 0.001 mg P/kg), whereas NE11809 was of low potency (LED, 1.0 mg P/kg).

Effects of ring methylation. NE11808, a BP with a 2-pyridylaminoethane side chain, was a potent inhibitor of *Dictyostelium* growth (IC_{50}, 15 μM) and of bone resorption (LED, 0.01 mg P/kg). In contrast, the corresponding BP methylated at the 3-position of the heterocyclic ring (to form a 3-picolylaminoethane side chain) (NE11809) was less potent both as a growth inhibitor of *Dictyostelium* (IC_{50}, 40 μM) and as an antiresorptive BP (LED, 1.0 mg P/kg).

Of the other pair of BPs that differed in the methylation of the heterocyclic ring, the methylated BP NE97220 (a 3-picolylaminomethane-BP) was the more potent inhibitor of *Dictyostelium* growth (IC_{50}, 9 μM) and of bone resorption (LED, 0.001 mg P/kg), although the corresponding nonmethylated BP (NE11807, with a 2-pyridylaminomethane side chain) was also a potent growth inhibitor (IC_{50}, 12 μM) and antiresorptive compound (LED, 0.01 mg P/kg).

Effects of conformational restrictions to the heterocyclic group. NE10501 is an analogue of the potent BP NE58095 (22). In the two compounds, the nitrogen atom in the heterocyclic group is at a similar distance from the geminal carbon atom. However, the bicyclic group of NE10501 is maintained in a fixed, rigid, three-dimensional conformation, whereas NE58095 adopts a less constrained, somewhat more flexible, chair conformation (25). NE10501 was a potent growth inhibitor (IC_{50}, 36 μM) and antiresorptive compound (LED, 0.01 mg P/kg), although less so than NE58095 (IC_{50}, 13 μM; LED, 0.0003 mg P/kg).

Similarly, NE80702, which has a heterocyclic group of fixed conformation, is an analogue of NE97221 that has a 2-pyridylethylidene side chain. The distance between the nitrogen atom in the heterocyclic group and the geminal carbon atom is similar in the two compounds, but NE97221 has a more flexible conformation. Again, the BP with the less flexible conformation (NE80702) was a less potent inhibitor of *Dictyostelium* growth (IC_{50}, 38 μM) and bone resorption (LED, 0.1 mg P/kg) than the BP with the more flexible conformation (NE97221) (IC_{50}, 20 μM; LED, 0.01 mg P/kg).

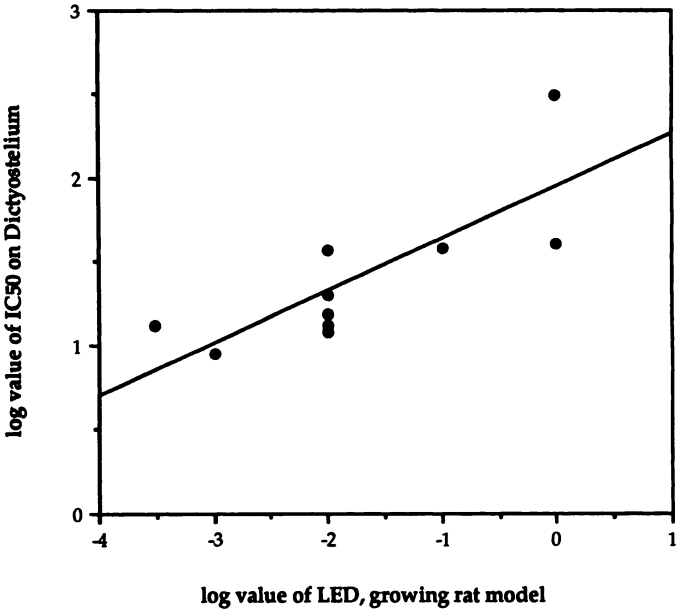


Fig. 3. Comparison of the effects of the BPs in Table 1 (except NE11728 and NE58086, for which no precise value of LED was determined) on *Dictyostelium* and in the growing rat (Schenk) model. Linear regression analysis was performed using log IC_{50} values for inhibition of growth of *Dictyostelium* plotted versus corresponding log LED values for inhibition of bone resorption in the growing rat model. The r^2 value was 0.63.

The BPs NE58086 and NE58025 both have a bicyclic group containing a nitrogen atom at an equivalent position in the bicyclic ring. However, the unsaturated ring of NE58086 adopts a three-dimensional conformation that is completely different from that of the pyrindine ring of NE58025 (23–25). The latter BP was a very potent inhibitor both of *Dictyostelium* growth (IC_{50}, 13 μM) (Fig. 2) and of bone resorption (LED, 0.01 mg P/kg), whereas NE58086 was of extremely low potency as a growth inhibitor (IC_{50}, 1200 μM) and as an inhibitor of bone resorption (LED, >1.0 mg P/kg).

Discussion

Although the exact mechanisms by which BPs inhibit osteoclast-mediated bone resorption have yet to be identified, it is clear that it is the structures of the BP side chains that impart bioactivity to these compounds (21, 22, 27). Some progress has been made in recent years toward clarifying the structure-activity relationships of the BP side chains. The presence of a basic primary amine group in an aliphatic side chain was originally found to markedly enhance the antiresorptive potency, relative to BPs that lack the amine group or have short side chains (26, 27). This observation has been followed by the development of even more potent BPs with side chains that contain a secondary amine group (28), a tertiary amine group (29, 30), or a nitrogen atom within a heterocyclic group (22, 31, 32). The BPs with a heterocyclic group are among the most potent antiresorptive BPs. Further studies with these compounds have revealed that minor changes in the structure of the heterocyclic group, such as substitutions within the ring or alteration of the length of the side chain by one $-CH_2$ group, markedly affect the antiresorptive potency (22). Furthermore, isomers of the same BP, or BPs that have similar chemical structures but very different three-dimensional conformations, also have very different potencies (25). These observations suggest the possibility that, rather than acting through nonspecific effects such as chelation of calcium or iron (21, 33–35) or alteration of membrane permeability (16, 21, 36), at least the more potent BPs may act by binding to a specific cellular target that has a binding site that is capable of recognizing certain stereochemical features of the BP side chain.

We have previously shown that BPs are inhibitors of the growth of amoebae of the cellular slime mold *D. discoideum* (21). At sufficiently high concentrations BPs are also cytotoxic to the amoebae. Antiproliferative and cytotoxic effects of BPs on macrophages, osteoclasts, fibroblasts, and other connective tissue cells have also been described (10, 14, 37–40), although often there is a poor correlation between effects on cell viability and antiresorptive potency. However, in *Dictyostelium* there is a remarkable correlation between the order of potency of a range of BPs as growth inhibitors and as inhibitors of bone resorption (21). Hence, BPs with side chains of simple structure are the least potent growth inhibitors; BPs with an aminoalkyl side chain are much more potent, whereas substitution into the amine group further increases potency. The heterocycle-containing BPs are among the most potent growth inhibitors of *Dictyostelium*. These observations suggest that BPs may have a cellular target that could be common to *Dictyostelium* and to osteoclasts. To substantiate this idea, we compared the potency, as growth inhibitors of *Dictyostelium*, of pairs of BPs that have similar structures but very different antiresorptive potencies. The effects of BPs with defined three-dimensional conformations were also investigated.

An increase of only one $-CH_2$ in the length of the side chain between the geminal carbon atom and the heterocyclic (2-pyridyl, 3-pyridyl, or 3-picolyl) group consistently decreased the potency of all three pairs of BPs, both as inhibitors of *Dictyostelium* growth and as inhibitors of bone resorption (Table 1). The detrimental effect of increasing chain length on antiresorptive potency was previously reported for one pair of these compounds (NE97221 and NE11728) by Sietsema *et al.* (22). The length of the side chain therefore appears important in determining the ability of the BPs to bind to a target in both *Dictyostelium* amoebae and BP-responsive cells in bone (presumably osteoclasts).

Substitution of a methyl group into the 2-pyridyl ring of BPs decreased the antiresorptive potency when the ring was attached to the geminal carbon via an aminoethane chain but increased the antiresorptive potency when the ring was attached via an aminomethane chain (22). The same effect was observed for potency of the BPs as inhibitors of *Dictyostelium* growth, indicating that the presence of a methyl group in the 3-position of the pyridine ring affects the binding of the BPs to a target in both *Dictyostelium* amoebae and osteoclasts.

A definitive way of determining the specificity of ligand binding to potential targets is to compare the binding of ligands that have similar chemical structures but different three-dimensional conformations. This has been achieved for BPs by the synthesis of the cyclic BP NE58086 and its hydrogenated analogue NE58025. The latter is a potent antiresorptive BP, whereas the dihydropyrindine analogue is inactive (24). Conformational analysis of NE58086 and NE58025 has revealed that the inactive dihydropyrindine adopts a fixed, relatively flat conformation that is very different from the fixed chair conformation of the potent antiresorptive octahydropyrindine-BP (24, 25). In *Dictyostelium* the potent antiresorptive octahydropyrindine-BP was also a very potent inhibitor of amoebal growth, whereas the dihydropyrindine, which is not antiresorptive, was 400-fold less potent towards *Dictyostelium* growth.

The antiresorptive potency of conformationally restricted analogues of two other potent BPs (NE58095 and NE97221) has previously been examined (25), with the expectation that the conformationally restricted analogues would also be potent antiresorptive BPs. NE10501 is an analogue of NE58095 in which the heterocyclic ring is in a similar but fixed conformation, relative to the more flexible conformation of NE58095. Similarly, NE80702 is an analogue of NE97221 in which the heterocyclic group is maintained in a fixed conformation. Both NE10501 and NE80702 are potent inhibitors of bone resorption, but slightly less so than NE58095 and NE97221, respectively (25). It was therefore concluded by Ebetino *et al.* (25) that some flexibility in the conformation of the heterocyclic group may be important for binding of the BPs to the cellular target. We have also observed this effect in *Dictyostelium*, because NE10501 is also a potent inhibitor of *Dictyostelium* growth but is almost 3-fold less potent than NE58095, whereas NE80702 is also a potent inhibitor of *Dictyostelium* growth but is approximately 2-fold less potent than NE97221. Thus, the conformation and flexibility of the heterocyclic group that impart high antiresorptive potency likewise impart high potency as inhibitors of *Dictyostelium* growth.

It is therefore clear that the structure and three-dimensional

conformation of the BP side chain are important factors in determining the potency of BPs as inhibitors of *Dictyostelium* growth, as well as their potency as antiresorptive agents, and that there appears to be similar stereospecific recognition between BPs and their targets in *Dictyostelium* and osteoclasts. Slight changes in the structure of the BP side chain that markedly alter the antiresorptive potency similarly alter potency of the BPs as inhibitors of *Dictyostelium* growth. Although the magnitudes of the changes in potency were greater in the rat model of bone resorption than in *Dictyostelium*, we have previously noted this effect and suggested that it is due to factors, such as clearance of BPs from the circulation and adsorbance and subsequent release of BPs from bone surfaces, that influence the *in vivo* antiresorptive efficacy of BPs but are irrelevant in *Dictyostelium* (21). Despite these considerations, it can hardly be fortuitous that every change in the structure of the BP side chain that affects the *in vivo* antiresorptive potency of BPs also affects in the same way the potency of BPs towards *Dictyostelium* (Fig. 3). This strongly supports the idea that the molecular target for BPs in *Dictyostelium* may be similar to that in osteoclasts. *Dictyostelium* therefore appears to be a very convenient model with which to screen BPs for high antiresorptive potency and may be a useful model with which to identify potential targets for the potent antiresorptive BPs. Work toward this goal is currently in progress.

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