Molecular Mechanisms of Action of Bisphosphonates: Current Status

Anke J. Roelofs, Keith Thompson, Sharon Gordon, and Michael J. Rogers

Abstract

Purpose: Bisphosphonates are currently the most important class of antiresorptive agents used in the treatment of metabolic bone diseases, including tumor-associated osteolysis and hypercalcemia. These compounds have high affinity for calcium ions and therefore target bone mineral, where they are internalized by bone-resorbing osteoclasts and inhibit osteoclast function.

Experimental Design: This article reviews the pharmacology of bisphosphonates and the relationship between chemical structure and antiresorptive potency. We also describe new insights into their intracellular molecular mechanisms of action, methods for assessing the effects of bisphosphonates on protein prenylation, and their potential as direct antitumor agents.

Results: Nitrogen-containing bisphosphonates act intracellularly by inhibiting farnesyl diphosphate synthase, an enzyme of the mevalonate pathway, thereby preventing prenylation of small GTPase signaling proteins required for normal cellular function. Inhibition of farnesyl diphosphate synthase also seems to account for their antitumor effects observed *in vitro* and for the activation of γ,δ T cells, a feature of the acute-phase response to bisphosphonate treatment in humans. Bisphosphonates that lack a nitrogen in the chemical structure do not inhibit protein prenylation and have a different mode of action that seems to involve primarily the formation of cytotoxic metabolites in osteoclasts.

Conclusions: Bisphosphonates are highly effective inhibitors of bone resorption that selectively affect osteoclasts *in vivo* but could also have direct effects on other cell types, such as tumor cells. After >30 years of clinical use, their molecular mechanisms of action on osteoclasts are finally becoming clear but their exact antitumor properties remain to be clarified.

Bisphosphonates remain the most widely used and effective antiresorptive agents for the treatment of diseases in which there is an increase in the number or activity of osteoclasts, including tumor-associated osteolysis and hypercalcemia (1). This brief review summarizes our current understanding of the molecular mechanisms of action of bisphosphonates on osteoclasts and their potential to affect other cell types, such as tumor cells, via the same molecular mechanisms.

General Properties of Bisphosphonates

Bisphosphonates are synthetic, nonhydrolyzable analogues of PP_i (Fig. 1). The P-C-P structure of bisphosphonates imparts the ability to bind divalent metal ions, such as Ca^{2+} (2). For this reason, bisphosphonates are rapidly cleared from the circulation (3, 4) and bind to bone mineral surfaces *in vivo* at sites of active bone remodeling, particularly areas undergoing osteoclastic resorption (5). The targeting of bisphosphonates to bone, localized release during osteoclastic bone resorption, and efficient uptake into osteoclasts by endocytosis explains why bisphosphonates seem to have a highly selective effect on osteoclasts (2). However, this does not exclude the possibility that small amounts of these drugs are internalized by neighboring cells (such as osteoblasts, bone marrow cells, or tumor cells), particularly with repeated administration over extended periods.

Metabolites of Simple Bisphosphonates Induce Osteoclast Apoptosis

Following earlier studies on slime mould amoebae (6), mammalian cells were found to convert some bisphosphonates (only the first-generation bisphosphonates, which closely resemble PP_i, such as clodronate and etidronate) intracellularly into methylene-containing (AppCp type) analogues of ATP (Fig. 2; ref. 7). These AppCp-type metabolites accumulate to high concentrations in the cytosol of osteoclasts and other cell types that can effectively internalize bisphosphonates (8). The accumulation of the $AppCCl_2p$ metabolite of clodronate in osteoclasts *in vitro* inhibits bone resorption by inducing osteoclast apoptosis (Fig. 2; ref. 9), most likely by inhibiting

Authors' Affiliation: Bone Research Group, Institute of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom

Received 4/5/06; revised 7/21/06; accepted 8/2/06.

Grant support: Arthritis Research Campaign, Scottish Hospital Endowments Research Trust, Leukaemia Research Fund, Medical Research Council, National Association for the Relief of Paget's Disease, Novartis, Procter and Gamble, and Merck.

Presented at the First Cambridge Conference on Advances in Treating Metastatic Bone Cancer, a symposium held in Cambridge, Massachusetts, October 28-29, 2005.

Requests for reprints: Michael J. Rogers, Bone Research Group, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom. Phone: 44-1244-554842; Fax: 44-1224-559533; E-mail: m.j.rogers@abdn.ac.uk.

doi:10.1158/1078-0432.CCR-06-0843

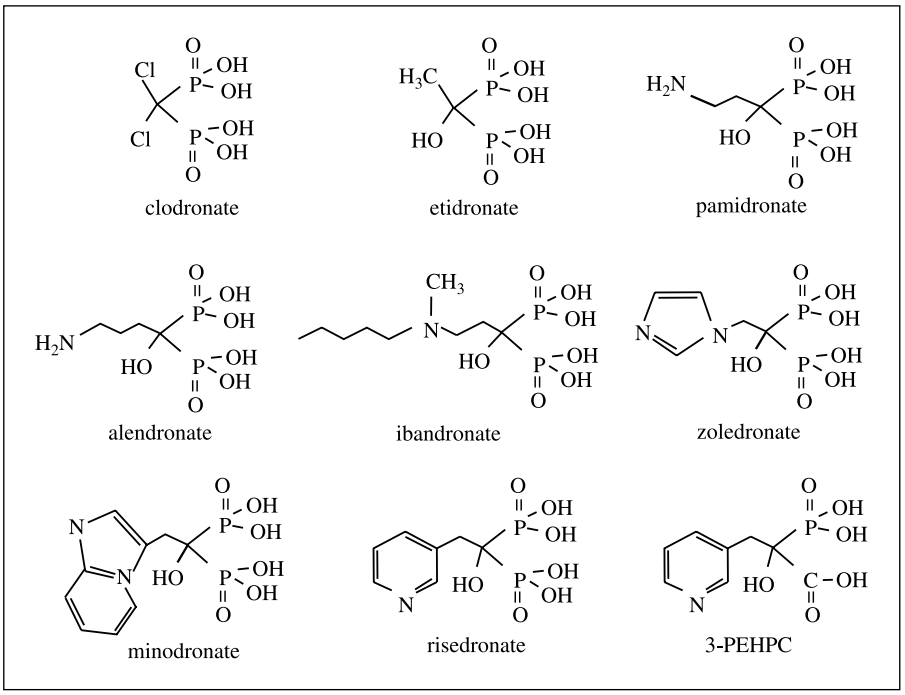

Fig. 1. The structure of simple bisphosphonates (clodronate and etidronate), N-BPs, and the phosphonocarboxylate analogue 3-PEHPC (also known as NE10790).

ATP-dependent enzymes, such as the adenine nucleotide translocase, a component of the mitochondrial permeability transition pore (10). Induction of osteoclast apoptosis seems to be the primary mechanism by which the simple bisphosphonates inhibit bone resorption because the ability of clodronate and etidronate to inhibit resorption *in vitro* can be overcome when osteoclast apoptosis is prevented using a caspase inhibitor (11).

Nitrogen-Containing Bisphosphonates Act by Inhibiting Farnesyl Diphosphate Synthase

Nitrogen-containing bisphosphonates (N-BPs), which are several orders of magnitude more potent at inhibiting bone resorption *in vivo* than the simple bisphosphonates (2, 12), are not metabolized to toxic analogues of ATP (13). Instead, they act by inhibiting farnesyl diphosphate (FPP) synthase, a key enzyme of the mevalonate pathway (Fig. 3). This enzyme is inhibited by nanomolar concentrations of N-BPs (Table 1; refs. 14–16). Zoledronic acid and the structurally similar minodronate are extremely potent inhibitors of FPP synthase (16) and inhibit the enzyme even at picomolar concentrations. Importantly, studies with recombinant human FPP synthase revealed that minor modifications to the structure and conformation of the R^2 side chain that are known to affect antiresorptive potency (16) also affect the ability to inhibit FPP synthase (2). These studies strongly suggest that FPP synthase is the major pharmacologic target of N-BPs in osteoclasts *in vivo*

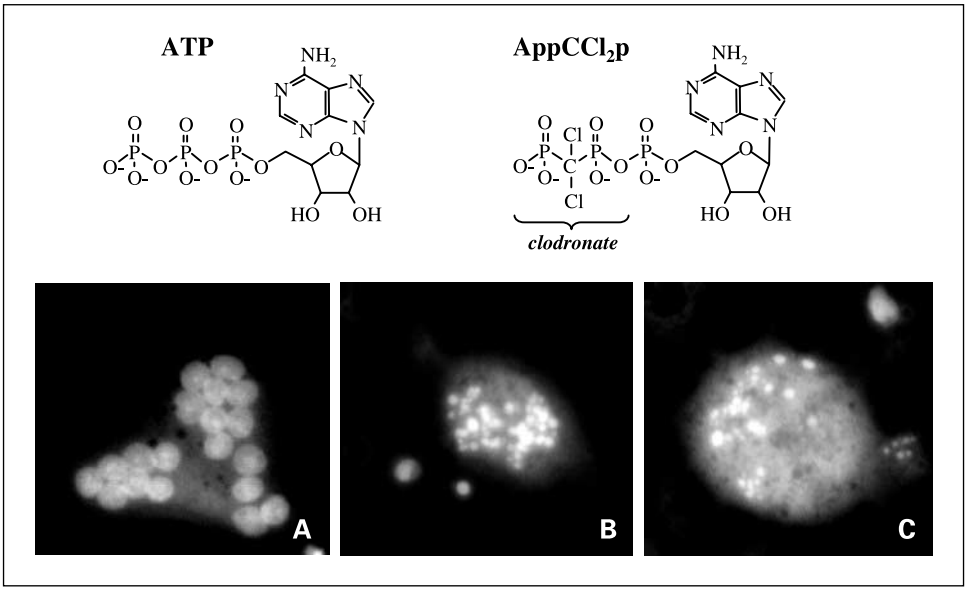

Fig. 2. The structure of ATP and the AppCp-type metabolite of clodronate ($AppCCl_2p$). Bottom, rabbit osteoclasts were treated with empty liposomes (*A*), clodronate-containing liposomes (*B*), or $AppCCl_2p$-containing liposomes (*C*) and then stained with 4′,6-diamidino-2-phenylindole to visualize nuclear morphology (a single osteoclast is shown at the same magnification). Both clodronate and $AppCCl_2p$ cause nuclear condensation and fragmentation characteristic of apoptotic cell death. Reproduced from Frith et al. (Arth Rheum 2001;44:2201-2210) with permission of the American College of Rheumatology.

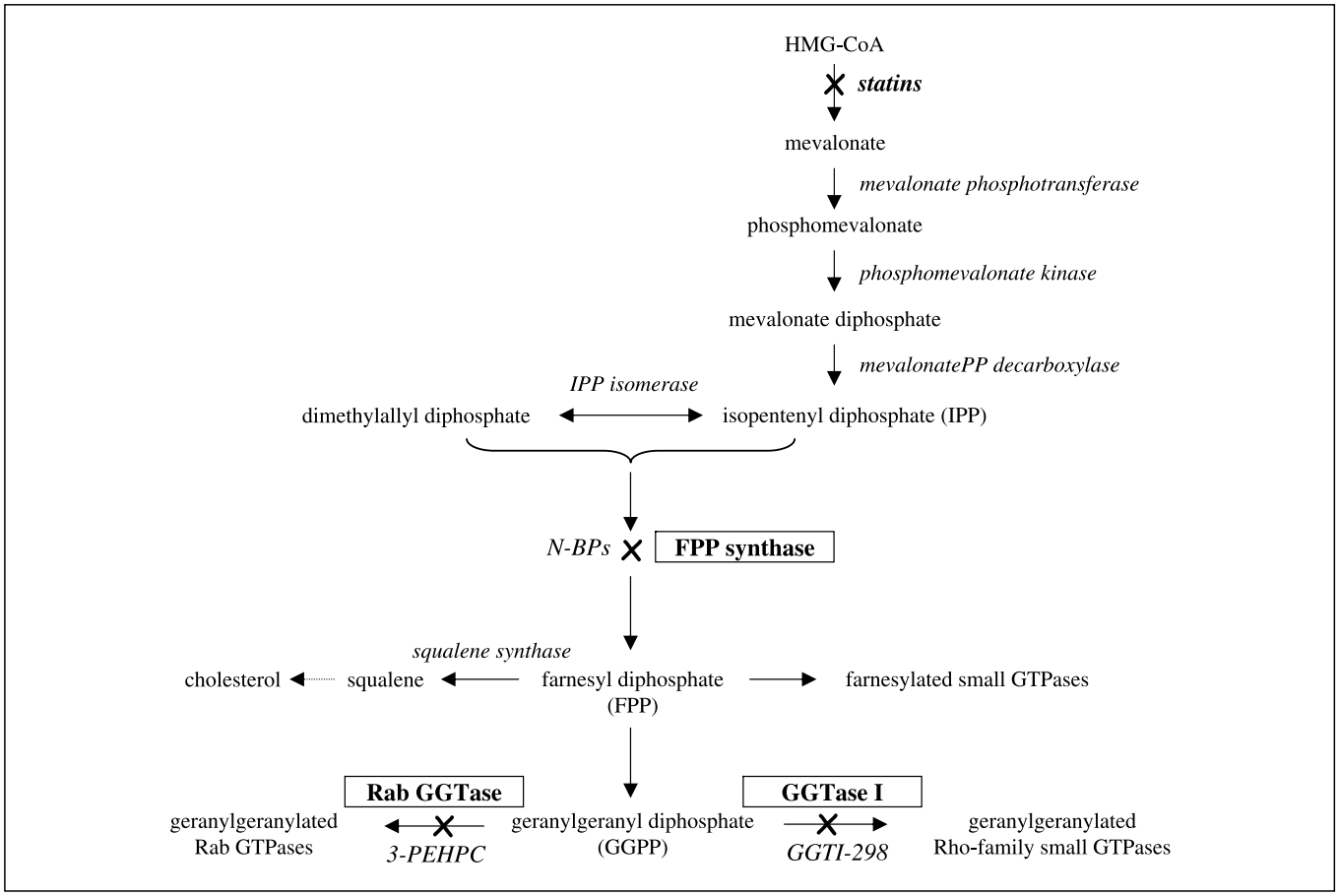

Fig. 3. Schematic diagram of the mevalonate pathway. N-BPs inhibit FPP synthase, thereby preventing the synthesis of FPP and geranylgeranyl diphosphate (*GGPP*) required for protein prenylation. Statins, GGTI-298, and 3-PEHPC also prevent protein prenylation in osteoclasts *in vitro* [by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase (*HMG-CoA reductase*), geranylgeranyltransferase I (*GGTase I*), or Rab geranylgeranyltransferase (*Rab GGTase*), respectively] and mimic the effects of N-BPs on osteoclasts, which are dependent on geranylgeranylated proteins.

and help to explain the relationship between bisphosphonate structure and antiresorptive potency.

The exact mechanism by which N-BPs inhibit FPP synthase is only just becoming clear. The recent generation of X-ray crystal structures of the human FPP synthase enzyme, cocrystallized with risedronate or zoledronic acid (17, 18), revealed that N-BPs bind in the geranyl diphosphate (GPP) binding site of the enzyme, with stabilizing interactions occurring between the nitrogen moiety of the N-BP and a conserved threonine and lysine residue in the enzyme. This is consistent with the earlier suggestion by Oldfield et al. (19) that N-BPs mimic the structure of the natural isoprenoid pyrophosphate substrates of the enzyme, GPP and dimethylallyl diphosphate, and compete for binding at the GPP/dimethylallyl diphosphate substrate binding pocket. N-BPs also seem to inhibit bacterial FPP synthase in a similar manner (20). Enzyme kinetic analysis with human FPP synthase indicates that the interaction with N-BPs is highly complex

Table 1. Potency of N-BPs for inhibiting FPP synthase

Bisphosphonate	IC_{50} (nmol/L), recombinant human enzyme*	IC_{50} (nmol/L), purified recombinant human enzyme†	K_i (nmol/L)‡
Pamidronate	200	500	ND
Alendronate	50	340	ND
Ibandronate	20	ND	ND
Risedronate	10	3.9	0.34
Zoledronate	3	ND	0.07
Minodronate	3	ND	ND

Abbreviation: ND, not determined.
*Values of IC_{50} are from Dunford et al. (16) using partially purified recombinant enzyme.
†Values of IC_{50} are from Bergstrom et al. (15) using purified enzyme.
‡Values of the overall dissociation constant, K_i, are from Kavanagh et al. (18) using purified enzyme.

and characteristic of "slow tight binding" inhibition (18). Initially, N-BPs seem to compete directly with dimethylallyl diphosphate or GPP for binding to the dimethylallyl diphosphate/GPP binding pocket. This is followed by more complex interactions that promote binding of isopentenyl diphosphate (IPP) in the second isoprenoid binding site of the enzyme, causing conformational changes that stabilize the final ternary complex, helping to explain the extraordinary inhibitory potency of some N-BPs toward this enzyme. These studies are therefore beginning to provide key insights, at the atomic level, into the reasons why minor changes to the structure of the N-BP side chain or the phosphonate groups markedly influence antiresorptive potency (2).

Inhibition of FPP Synthase Prevents the Prenylation of Small GTPases

By inhibiting FPP synthase, N-BPs prevent the synthesis of FPP and its downstream metabolite geranylgeranyl diphosphate (Fig. 3). These isoprenoid lipids are the building blocks for the production of a variety of metabolites, such as dolichol and ubiquinone (21), but are also required for post-translational modification (prenylation) of proteins, including small GTPases (22, 23). The loss of synthesis of FPP and geranylgeranyl diphosphate therefore prevents the prenylation of small GTPases, the majority of which are geranylgeranylated (24–26). Inhibition of protein prenylation by N-BPs can be shown by measuring the incorporation of [^{14}C]mevalonate into farnesylated and geranylgeranylated proteins (13, 27). Risedronate almost completely inhibits protein prenylation in J774 cells at a concentration of 10 μmol/L, which is similar to the concentration that affects osteoclast viability *in vitro* (28, 29) and has been predicted to be achieved within the osteoclast resorption lacuna *in vivo* (30). More recently, we and others confirmed that N-BPs (e.g., ≥10 μmol/L zoledronic acid; Fig. 4) inhibit the incorporation of [^{14}C]mevalonate into prenylated small GTPase proteins in purified osteoclasts *in vitro* (15, 31). Alternatively, the inhibitory effect of N-BPs on the mevalonate pathway can be shown by detecting accumulation of the unprenylated form of the small GTPase Rap1A, which acts as a surrogate marker for inhibition of FPP synthase and which accumulates in cells exposed to N-BPs (Fig. 5A; ref. 32). We have detected the unprenylated form of Rap1A in osteoclasts purified from alendronate-treated rabbits using immunomagnetic beads (9, 33), thereby showing that N-BPs inhibit protein prenylation *in vivo*.

Inhibition of Protein Prenylation by N-BPs in Other Cell Types

Because FPP synthase is a highly conserved, ubiquitous enzyme, N-BPs have the potential to affect any cell type *in vitro*. We have shown the ability of N-BPs to inhibit the prenylation of Rap1A in cultures of all types of primary cells and cell lines studied thus far (Fig. 5), including osteoclasts, osteoblasts, macrophages, epithelial and endothelial cells, and breast, myeloma, and prostate tumor cells. Macrophages and osteoclasts seem to be the most sensitive to low concentrations of N-BPs (1-10 μmol/L) *in vitro*. In macrophages, treatment with 100 μmol/L N-BP causes the detectable accumulation of unprenylated Rap1A within a few hours (lower concentrations have a delayed effect; the more potent the N-BP, the more rapid the effect; Fig. 5B). In other cell types, such as myeloma cells, the unprenylated form of Rap1A can also be detected within hours of treatment *in vitro*, but higher concentrations are sometimes required (Fig. 5C). The sensitivity of different cell types to N-BPs most likely depends largely on their ability to internalize sufficient amounts of N-BP to inhibit FPP synthase. Recent studies with a fluorescently labelled bisphosphonate have shown that macrophages and osteoclasts internalize bisphosphonates into membrane-bound vesicles by fluid-phase endocytosis (34). Subsequent acidification of endocytic vesicles is required for bisphosphonates to enter the cytosol, by reducing the negative charge on the phosphonate groups of bisphosphonates and thereby allowing either diffusion or transport of bisphosphonates across the vesicular membrane (34). This mechanism of uptake results in large amounts of N-BP in intracellular vesicles but probably only very small amounts of bisphosphonate in the cytosol or other organelles are available for inhibition of FPP synthase, although the relatively poor uptake of bisphosphonates into the cell cytosol is overcome by their extremely potent inhibition of FPP synthase (16, 18).

Consequences of Inhibiting Protein Prenylation

Prenylated small GTPases, such as those of the Ras, Rho, and Rab families, are important signaling proteins that regulate a variety of cell processes important for osteoclast function (35). Inhibition of the mevalonate pathway and loss of prenylated proteins, particularly geranylgeranylated small GTPases, seem to be the major mechanism of action of N-BPs because bypassing inhibition of FPP synthase and replenishing cells

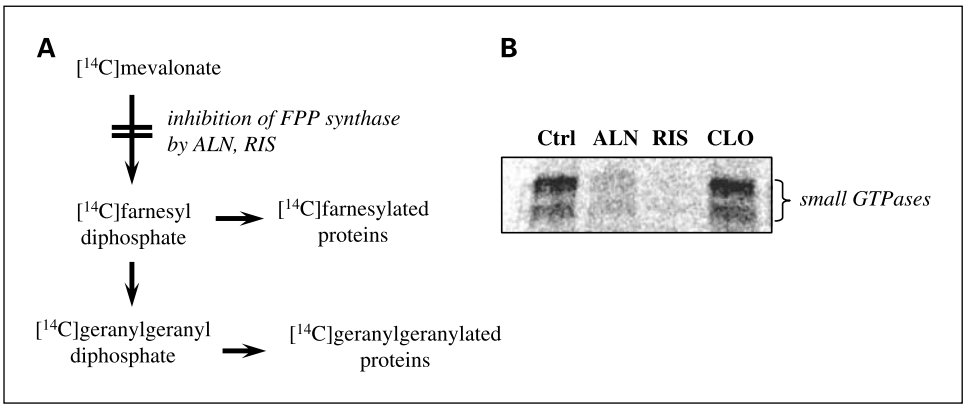

Fig. 4. N-BPs inhibit protein prenylation in osteoclasts *in vitro*. *A,* purified rabbit osteoclasts were incubated with [^{14}C]mevalonate, which becomes incorporated into [^{14}C]-labeled, prenylated proteins. *B,* prenylated small GTPase proteins can then be detected by autoradiography following electrophoretic separation. Both alendronate (*ALN*) and risedronate (*RIS*) prevent the incorporation of [^{14}C]mevalonate into prenylated proteins, whereas clodronate (*CLO*) has no effect. Reproduced from Coxon et al. (J Bone Miner Res 2000;15:1467-1476) with permission of the American Society for Bone and Mineral Research.

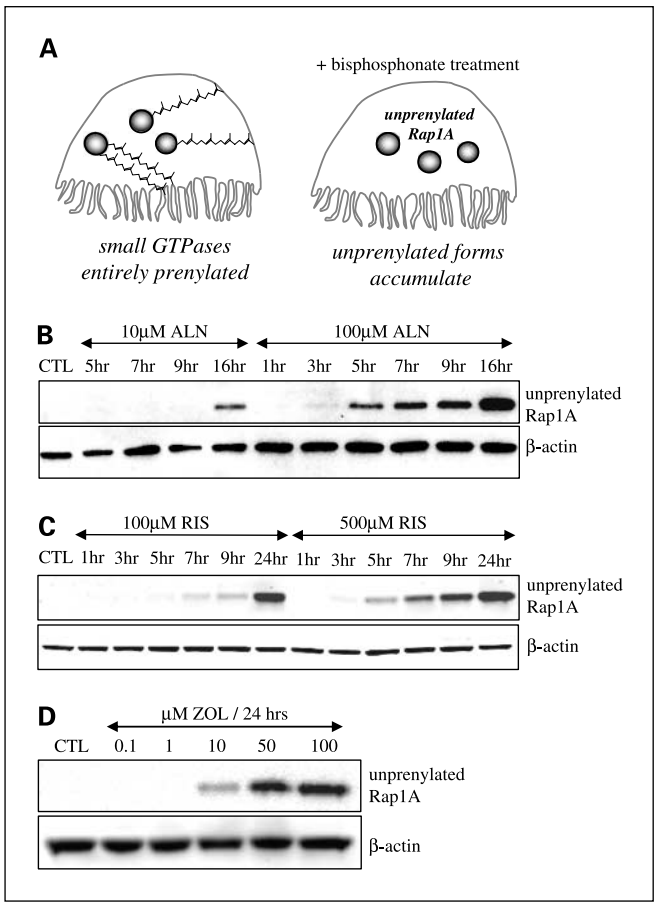

Fig. 5. *A,* by inhibiting FPP synthase, N-BPs cause the accumulation of the unprenylated forms of small GTPases, such as Rap1A. Unprenylated Rap1A can be detected in response to N-BP treatment in J774 macrophages (*B*), JJN-3 human myeloma cells (*C*), and human umbilical vascular endothelial cells (*D*) by Western blotting using a goat polyclonal antibody that specifically recognizes unprenylated Rap1A. β-Actin was analyzed as a control for total protein. Unprenylated Rap1A is undetectable in untreated cells (*CTL*) but is clearly detectable after 16 hours of treatment with 10 μmol/L alendronate or after 5 hours of treatment with 100 μmol/L alendronate in J774 macrophages or in JJN-3 myeloma cells after 7 hours of treatment with 100 μmol/L risedronate or 5 hours of treatment with 500 μmol/L risedronate. Unprenylated Rap1A is also clearly detectable in human umbilical vein endothelial cells after 24 hours of treatment with 10 μmol/L zoledronate (*ZOL*).

with an isoprenoid lipid substrate that restores geranylgeranylation can overcome the effects of N-BPs on osteoclast formation, apoptosis, and bone resorption (36–38). In addition, other inhibitors of protein geranylgeranylation, such as statins or GGTI-298 (Fig. 3), mimic the effects of N-BPs (27, 31). However, recent studies by van Beek et al. (39) suggest that pamidronate may have an additional, as yet unidentified, molecular target in osteoclasts because (unlike with other N-BPs) the antiresorptive effect of pamidronate could not be overcome effectively by replenishing cells with a substrate for protein prenylation.

A recent report suggests another intriguing mechanism by which N-BPs could disrupt osteoclast function via effects on the mevalonate pathway. Inhibition of FPP synthase causes the accumulation of the upstream substrate IPP, which seems to become conjugated to AMP to form a novel ATP analogue (ApppI; ref. 40). This metabolite, as with the AppCp-type metabolites of simple bisphosphonates (10), can inhibit mitochondrial adenine nucleotide translocase and induce osteoclast apoptosis. However, the pharmacologic significance of this is unclear because restoring prenylation with a substrate for protein geranylgeranylation overcomes the antiresorptive effects of bisphosphonates *in vitro* (37) but would be unlikely to affect levels of ApppI. Furthermore, unlike the simple bisphosphonates that act by inducing osteoclast apoptosis, the antiresorptive effect of N-BPs is not dependent on apoptosis at least *in vitro* (11). Hence, inhibition of protein prenylation remains the most likely explanation for the antiresorptive effects of N-BPs.

Following the discovery that N-BPs inhibit FPP synthase and prevent protein prenylation, it has been assumed that the antiresorptive effects of N-BPs result from the loss of signaling pathways downstream of prenylated (particularly geranylgeranylated) small GTPases. However, we have shown recently that the unprenylated forms of Rho, Rac, and Cdc42 that accumulate following treatment with N-BPs are in the active, GTP-bound form most likely due to their inability to interact with regulatory proteins, such as Rho GTPase-activating protein (41). Unprenylated small GTPases may therefore affect normal cellular function by inappropriate and sustained activation, rather than inhibition, of downstream signaling pathways, such as p38 (41, 42). A dominant effect of the accumulation of unprenylated proteins, as opposed to the loss of prenylated proteins, would explain why ongoing protein synthesis is required for bisphosphonates to exert their cytotoxic effects (43) because, in all cells, the accumulation of unprenylated proteins is dependent on *de novo* protein synthesis. Further studies are clearly required to elucidate in more detail the effects of unprenylated proteins on cell function.

Effects of N-BPs on Tumor Cells

Numerous studies have described the ability of N-BPs to reduce the survival, proliferation, adhesion, migration, and invasion of tumor cells *in vitro* (44, 45). Most, if not all, of these antitumor effects of N-BPs *in vitro* are due to inhibition of FPP synthase because the effects of N-BPs can be largely overcome by replenishing cells with isoprenoid substrates (farnesol or geranylgeraniol) required for protein prenylation (46–49). Furthermore, the structure-activity relationships of N-BPs for affecting tumor cell adhesion and invasion match the structure-activity relationships for inhibiting FPP synthase (50, 51). Similarly, some N-BPs have been shown to affect the viability, migration, and activity of endothelial cells *in vitro*. Some of these effects could be overcome by replenishing cells with geranylgeranylpyrophosphate and therefore appear to be due to loss of protein prenylation (52, 53). We have recently confirmed that the concentrations of zoledronic acid that affect endothelial cells *in vitro* ($\geq$10 μmol/L) do indeed inhibit protein prenylation (Fig. 5D). It therefore becomes of increasing importance to determine the *in vivo* relevance of these *in vitro* observations.

A variety of intriguing studies in mouse models have shown that treatment with N-BPs can inhibit skeletal metastasis or reduce tumor burden in bone or even at extraskeletal sites *in vivo* (45, 53–60). In addition, bisphosphonates have been shown to inhibit angiogenesis in experimental models and in animal models of tumorigenesis (45, 55, 61) and to lower circulating levels of proangiogenic vascular endothelial growth

factor and platelet-derived growth factor in cancer patients (62, 63). However, it has not yet been unequivocally proven that these effects are due to inhibition of protein prenylation following direct internalization of N-BPs by tumor, endothelial, or other cells, such as macrophages, *in vivo*. Effects of N-BPs on skeletal metastasis, tumor burden in bone, or even angiogenesis could result from inhibition of bone resorption alone (58, 64). Therefore, although N-BPs clearly have the potential for direct antitumor effects, whether this is achievable with clinically relevant doses remains a highly controversial issue and depends on whether tumor cells are able to internalize sufficient amounts of the drugs *in vivo*.

Adding weight to the possibility that N-BPs may have direct effects on tumor cells *in vivo* is the demonstration that an i.v. N-BP infusion results in a sufficiently high peripheral blood concentration [e.g., 1 μmol/L with zoledronic acid (4)] to allow entry into monocytes and/or other highly endocytic cells. This is the cause of the flu-like acute-phase reaction (65) that typically occurs in approximately one third of patients receiving i.v. N-BP treatment for the first time. Inhibition of FPP synthase in these circulating peripheral blood mononuclear cells causes intracellular accumulation of IPP, which is presented to $V\gamma9V\delta2^+$ T cells by an as yet unidentified mechanism. This results in the activation and proliferation of these γ,δ T cells (66–68), triggering cytokine release and thus causing flu-like symptoms (69, 70). Similarly, treatment of tumor cells with N-BPs *in vitro* also causes the accumulation of IPP, which can activate γ,δ T cells capable of tumor cell killing (67). These observations raise the possibility that N-BPs could be used as an immune therapy, having indirect antitumor effects *in vivo* via the activation of γ,δ T cells (71). However, this mechanism does not seem to explain the antitumor activity of N-BPs in mouse models of tumorigenesis because rodents lack an analogous subset of γ,δ T cells that can be activated by IPP (72).

Direct uptake of N-BPs by epithelial cells in the gastrointestinal tract followed by inhibition of FPP synthase and loss of prenylated proteins may also explain the ability of some orally administered N-BPs to cause esophagitis and ulceration (2). Taken together, the studies described above suggest that, under certain circumstances, cells other than osteoclasts are indeed capable of internalizing bisphosphonates *in vivo*. Much therefore remains to be learned about the concentrations of bisphosphonate that occur *in vivo* in the bone microenvironment and in other tissues, which cell types are capable of internalizing sufficient N-BP to affect protein prenylation, and what effects this might cause, for example, on tumor cells. Importantly, recent studies have shown that bisphosphonates can act in synergy with a variety of currently used anticancer agents at least *in vitro* (45, 73, 74). This raises the possibility that combination treatment with other cytotoxic drugs could lower the concentration of bisphosphonates needed to directly affect tumor cells and thus increase the potential for N-BPs to have direct antitumor effects *in vivo*.

Development of New Bisphosphonate Analogues

It has become clear recently that changes to the structure of N-BPs might give rise to compounds capable of inhibiting other enzymes of the mevalonate pathway that use isoprenoid lipids. For example, we recently found that replacement of one of the phosphonate groups of risedronate with a carboxylate group, giving rise to a phosphonocarboxylate analogue (3-PEHPC; Fig. 1), confers the novel ability to specifically inhibit Rab geranylgeranyltransferase (Fig. 3), thereby selectively preventing the prenylation and membrane localization of Rab GTPases without affecting Rho or Ras family GTPases (75, 76). 3-PEHPC is a weak inhibitor of bone resorption, probably by disrupting Rab-dependent vesicular trafficking in osteoclasts (75, 76), induces apoptosis in human myeloma cells (77), and inhibits invasion of breast and prostate cancer cells (51).

Interestingly, the structure-activity relationships of several phosphonocarboxylate analogues for inhibiting Rab geranylgeranyltransferase do not match the structure-activity relationships of the parent bisphosphonates for inhibiting FPP synthase (76), indicating that phosphonocarboxylates represent a new class of antiresorptive and/or antitumor agents with a defined and specific molecular target. Unlike N-BPs, 3-PEHPC does not cause activation of γ,δ T cells *in vitro* (the basis of the acute-phase response to N-BPs; ref. 66) and may therefore have a different adverse effect profile. Furthermore, 3-PEHPC treatment of myeloma cells does not induce the S-phase arrest characteristic of N-BPs (77). Although more potent phosphonocarboxylates than 3-PEHPC would be required for further development, the lower bone affinity of such agents compared with the parent N-BPs (78) might be an attractive property in situations where long-term retention in bone is undesirable, for example, in the treatment of pediatric bone disease. In addition, N-BPs with a lower affinity for bone mineral may display higher equilibrium concentrations in the bone marrow microenvironment compared with high-affinity compounds (79), raising the possibility that low bone affinity compounds could act more effectively on tumor cells residing in the bone marrow.

Summary

Bisphosphonates can be grouped into two general classes according to their chemical structure and molecular mechanism of action. The simple bisphosphonates can be metabolically incorporated into nonhydrolyzable analogues of ATP that accumulate intracellularly in osteoclasts, resulting in induction of osteoclast apoptosis. By contrast, the more potent N-BPs inhibit FPP synthase, an enzyme in the mevalonate pathway. Inhibition of this enzyme in osteoclasts prevents the biosynthesis of isoprenoid lipids that are essential for the prenylation of small GTPase signaling proteins. Inhibition of FPP synthase also seems to account for the adverse effects of N-BPs *in vivo* and for the antitumor effects of N-BPs *in vitro*. Although N-BPs have been shown to have antitumor activity in various animal models, it remains to be confirmed whether this is directly due to the inhibition of protein prenylation in tumor cells, endothelial cells, or other nonosteoclast cell types *in vivo*.

Open Discussion

Dr. Boyce: Are there any data that show that accumulation of some unprenylated GTPases could be stimulating tumor cells? There is some evidence that outside the skeleton tumor cell growth may be enhanced, at least in animal models.

Dr. Rogers: What we found in macrophages at least is that activation of Rac and downstream activation of p38 has an antiapoptotic effect. If we treat macrophages with a bisphosphonate and a p38 inhibitor, we get more apoptosis. So, depending on the cell type, if you activate some of them, you might get a prosurvival effect and an antiapoptotic effect, but perhaps activation of others, like Rho, might be proapoptotic, depending again on the cell type. So, yes, under some circumstances it might have antiapoptotic effects on some cell types.

Dr. Boyce: It could vary from one cell type or one tumor type to another.

Dr. Rogers: Absolutely. This effect of p38 activation we found in macrophages prevents apoptosis, but it has the opposite effect in myeloma cells. Certainly, it could be cell type dependent.

Dr. Roodman: Do you think that this activation of p38 MAP kinase explains why bisphosphonates don't totally inhibit osteoclasts?

Dr. Rogers: That's a distinct possibility, although it's difficult to dissect out what all of these GTPases are doing when they accumulate in the unprenylated form.

Dr. Roodman: Have you shown that p38 is activated in osteoclasts?

Dr. Rogers: We're doing those studies now, but we have looked in a variety of tumor cell types. We see Rho, Rac, and Cdc42 activation in all cell types studied so far. However, what might be different between cell types are the particular signaling pathways then get activated downstream, like p38 or JNK.

Dr. Suva: There was a lot of interest a couple of years ago in osteosarcoma and bisphosphonates. Do you know if anything has come of that? It seems to me like that's a reasonable cancer target for a bisphosphonate.

Dr. Rogers: I'm not sure that's been followed or that anyone has reproduced that.

Dr. Coleman: One of the reasons we may not have seen antitumor activity in the clinical situation is because obviously bisphosphonates target so exquisitely to bone. What's been done about adjusting the affinity to bone, perhaps using the FPP synthase activity alone?

Dr. Rogers: The problem is that the phosphonate groups are essential for binding to FPP synthase because the phosphonate groups bind to those magnesium ions. So as soon as you start modifying the phosphonate groups, most of those compounds no longer inhibit FPP synthase. However, there's one compound that does inhibit FPP synthase even though it has modified phosphonate groups. These sorts of compounds could be interesting to test in animal models because they could have less propensity to bind to mineral and might detach more readily in a bone microenvironment. Perhaps they could then reach higher local concentrations around tumor cells.

Dr. Weilbaecher: Regarding clodronate, we can't prove it has a direct antitumor effect, but there certainly is compelling evidence in some of the adjuvant trials with this weak bisphosphonate that there might be effects outside the bone on cancer cells. How does clodronate work if it doesn't get this pathway on osteoclasts?

Dr. Rogers: Clodronate, like etidronate and tiludronate, does not inhibit FPP synthase or inhibits it very weakly. It has no detectable effects on protein prenylation at sensible concentrations *in vitro*. Clodronate gets metabolized intracellularly to a nonhydrolyzable ATP analogue. That ATP analogue accumulates in the cytosol of cells and probably inhibits all sorts of ATP-dependent enzymes and seems to trigger apoptosis. If we, for example, use liposomes to introduce the synthetic metabolite into cells, it triggers osteoclast apoptosis. Interestingly, clodronate has lower bone affinity than most of the nitrogen bisphosphonates. On the other hand, it has little effect on tumor cells. It kills endocytic cells like macrophages and osteoclasts, but it doesn't have the same effects on tumor cell adhesion, invasion, and migration that the nitrogen bisphosphonates do presumably because it doesn't inhibit prenylation.

Dr. Suva: From the crystal structure of FPP synthase, is there any evidence for other binding pockets on the surface?

Dr. Rogers: In the molecular modeling studies, it looked as if one molecule of bisphosphonate is bound to each of those substrate-binding pockets in the enzyme. However, in the crystal structures, it looks as if the bisphosphonates only binds to the GPP pocket and not to the IPP pocket.

Dr. Suva: Theoretically, you could target a molecule that might target the other pocket.

Dr. Rogers: Yes. We've been trying to do some enzyme kinetic studies, but this is a really complicated enzyme to study.

Dr. Suva: Has anybody been able to take advantage of the conformational change and develop allosteric inhibitors?

Dr. Rogers: Maybe that's for the future.

Dr. Body: What are the effects of these drugs on osteoblasts and on the dialog between osteoblasts and osteoclasts?

Dr. Rogers: We found effects on apoptosis in tumor cells, but knowing now that these compounds inhibit a ubiquitous metabolic enzyme, it's not surprising that you get apoptosis and growth inhibition of any cell type *in vitro* with high enough concentrations, including osteoblasts. At the moment, we are limited by the sensitivity of the method for detecting changes in protein prenylation because we can only see an effect on prenylation above about 1 μmol/L or 5 μmol/L of zoledronate. However, almost certainly below those concentrations, there are still very subtle effects on prenylation that we can't detect. It's a limitation of the Western blot approach. So are these effects relevant *in vivo*? Do osteoblasts take up enough bisphosphonates *in vivo* to have an effect on prenylation? We don't have those answers yet.

Dr. Bruland: Did you achieve these molar concentrations by the standard approved doses? Is it realistic to expect antitumor activity?

Dr. Rogers: Studies with zoledronate show that about 1 to 3 μmol/L is the maximum circulating concentration with a standard dose of zoledronate in patients. Concentrations above that, for example, 10, 50, or 100 μmol/L, are almost certainly not relevant, at least not in the peripheral circulation. We still don't know what's happening in the bone microenvironment. Beneath the resorbing osteoclasts, there may well be hundreds of micromolar perhaps even millimolar concentrations of bisphosphonates in the resorption lacuna, but we don't know what sort of concentrations are reached around a resorbing osteoclast. Certainly, 1 μmol/L of zoledronate or risedronate is sufficient to affect tumor cell adhesion and migration and invasion *in vitro*.

Dr. Vessella: Why don't we know what the concentrations of the bisphosphonates are in the bone? If we take a bone

biopsy specimen after giving a course of bisphosphonates, isn't there a way of determining how much bisphosphonate is in the bone?

Dr. Rogers: The problem is, what does that mean? You can estimate how much is bound to bone, but when it's bound to bone it's pharmacologically inactive. It's only when it's released and it can be taken up by cells that it becomes active. The bisphosphonates are constantly desorbing from the bone surface and reattaching and then you have a resorbing osteoclast that's releasing lots of bisphosphonate. Does some of that diffuse into the bone marrow? Does it then reattach? Does it enter the circulation? Is it recycled?

Dr. Weilbaecher: Once you've given your dose of bisphosphonate and have presumably inhibited osteoclasts, would you then have less release of this bisphosphonate, because you don't have the osteoclasts to resorb the bone anymore?

Dr. Rogers: Yes, in other words they probably inhibited their own release eventually. However, it gets more complicated, because endocytosis, which is the way that these bisphosphonates get into cells, is dependent on small GTPases, and when you block prenylation of those, you get less endocytosis. Therefore, bisphosphonates also inhibit their own uptake in cells, such as macrophages.

Dr. Lipton: You commented that 10-minute exposure is enough for maximum inhibition. Have you done that with multiple cell lines? We did some crude experiments years ago with the breast cancer cell line where it looked like you needed a 24-hour period of prolongation to get maximum cell inhibition.

Dr. Rogers: We haven't done an extensive comparison. Certainly, with endocytic cells like macrophages, and probably osteoclasts, you only need a short exposure because they're so endocytic and internalize bisphosphonate very quickly. The less endocytic the cell type (such as tumor cells), the longer you need to expose them to get sufficient bisphosphonate into the cell to have an effect.

Acknowledgments

We thank Drs. Julie Crockett, Fraser Coxon, and Jim Dunford for their important contributions and many helpful discussions.

References

1. Coleman RE. Metastatic bone disease: clinical features, pathophysiology, and treatment strategies. Cancer Treat Rev 2001;27:165–76.
2. Rogers MJ. New insights into the molecular mechanisms of action of bisphosphonates. Curr Pharm Des 2003;9:2643–58.
3. Lin JH. Bisphosphonates: a review of their pharmacokinetic properties. Bone 1996;18:75–85.
4. Chen T, Berenson J, Vescio R, et al. Pharmacokinetics and pharmacodynamics of zoledronic acid in cancer patients with bone metastases. J Clin Pharmacol 2002;42:1228–36.
5. Masarachia P, Weinreb M, Balena R, Rodan GA. Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones. Bone 1996;19:281–90.
6. Rogers MJ. From molds and macrophages to mevalonate: a decade of progress in understanding the molecular mode of action of bisphosphonates. Calcif Tissue Int 2004;75:451–61.
7. Frith JC, Monkkonen J, Blackburn GM, Russell RG, Rogers MJ. Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5′-(β,γ-dichloromethylene) triphosphate, by mammalian cells *in vitro*. J Bone Miner Res 1997; 12:1358–67.
8. Monkkonen H, Rogers MJ, Makkonen N, Niva S, Auriola S, Monkkonen J. The cellular uptake and metabolism of clodronate in RAW 264 macrophages. Pharm Res 2001;18:1550–5.
9. Frith JC, Monkkonen J, Auriola S, Monkkonen H, Rogers MJ. The molecular mechanism of action of the anti-resorptive and anti-inflammatory drug clodronate: evidence for the formation *in vivo* of a metabolite that inhibits bone resorption and causes osteoclast and macrophage apoptosis. Arthritis Rheum 2001; 44:2201–10.
10. Lehenkari PP, Kellinsalmi M, Napankangas JP, et al. Further insight into mechanism of action of clodronate: inhibition of mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing metabolite. Mol Pharmacol 2002;61:1255–62.
11. Halasy-Nagy JM, Rodan GA, Reszka AA. Inhibition of bone resorption by alendronate and risedronate does not require osteoclast apoptosis. Bone 2001;29: 553–9.
12. Green JR, Rogers MJ. Pharmacologic profile of zoledronic acid: a highly potent inhibitor of bone resorption. Drug Dev Res 2002;55:210–24.
13. Benford HL, Frith JC, Auriola S, Monkkonen J, Rogers MJ. Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates: biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. Mol Pharmacol 1999;56:131–40.
14. van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. Biochem Biophys Res Commun 1999; 264:108–11.
15. Bergstrom JD, Bostedor RG, Masarachia PJ, Reszka AA, Rodan G. Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. Arch Biochem Biophys 2000;373:231–41.
16. Dunford JE, Thompson K, Coxon FP, et al. Structure-activity relationships for inhibition of farnesyl diphosphate synthase *in vitro* and inhibition of bone resorption *in vivo* by nitrogen-containing bisphosphonates. J Pharmacol Exp Ther 2001;296:235–42.
17. Rondeau JM, Bitsch F, Geiser M, et al. Structural basis for the exceptional *in vivo* efficacy of bisphosphonate drugs. J Med Chem 2006;1:267–73.
18. Kavanagh K, Guo K, Dunford JE, et al. The molecular mechanism of nitrogen-containing bisphosphonates as anti-osteoporosis drugs: crystal structure and inhibition of farnesyl pyrophosphate synthase. Proc Natl Acad Sci U S A 2006;103:7829–34.
19. Martin MB, Arnold W, Heath HT, Urbina JA, Oldfield E. Nitrogen-containing bisphosphonates as carbocation transition state analogs for isoprenoid biosynthesis. Biochem Biophys Res Commun 1999; 263:754–8.
20. Hosfield DJ, Zhang Y, Dougan DR, et al. Structural basis for bisphosphonate-mediated inhibition of isoprenoid biosynthesis. J Biol Chem 2003;279:8526–9.
21. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990;343:425–30.
22. McTaggart SJ. Isoprenylated proteins. Cell Mol Life Sci 2006;63:255–67.
23. Lane KT, Beese LS. Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. J Lipid Res 2006;47:681–99.
24. Zhang FL, Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem 1996;65:241–69.
25. Wright LP, Philips MR. Thematic review series: lipid posttranslational modifications. CAAX modification and membrane targeting of Ras. J Lipid Res 2006;47: 883–91.
26. Leung KF, Baron R, Seabra MC. Thematic review series: lipid posttranslational modifications. geranylgeranylation of Rab GTPases. J Lipid Res 2006;47: 467–75.
27. Luckman SP, Hughes DE, Coxon FP, Russell RGG, Rogers MJ. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J Bone Miner Res 1998;13:581–9.
28. Sato M, Grasser W. Effects of bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. J Bone Miner Res 1990;5:31–40.
29. Breuil V, Cosman F, Stein L, et al. Human osteoclast formation and activity *in vitro*: effects of alendronate. J Bone Miner Res 1998;13:1721–9.
30. Sato M, Grasser W, Endo N, et al. Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. J Clin Invest 1991;88: 2095–105.
31. Coxon FP, Helfrich MH, van 't Hof RJ, et al. Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. J Bone Miner Res 2000;15: 1467–76.
32. Reszka AA, Halasy-Nagy J, Rodan GA. Nitrogen-bisphosphonates block retinoblastoma phosphorylation and cell growth by inhibiting the cholesterol biosynthetic pathway in a keratinocyte model for esophageal irritation. Mol Pharmacol 2001;59:193–202.
33. Staal A, Frith JC, French MH, et al. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. J Bone Miner Res 2003;18:88–96.
34. Thompson K, Rogers MJ, Coxon FP, Crockett JC. Cytosolic entry of bisphosphonate drugs requires acidification of vesicles after fluid-phase endocytosis. Mol Pharmacol 2006;69:1624–32.
35. Coxon FP, Rogers MJ. The role of prenylated small GTP-binding proteins in the regulation of osteoclast function. Calcif Tissue Int 2003;72:80–4.
36. Reszka AA, Halasy-Nagy JM, Masarachia PJ,

Rodan GA. Bisphosphonates act directly on the osteoclast to induce caspase cleavage of mst1 kinase during apoptosis. A link between inhibition of the mevalonate pathway and regulation of an apoptosis-promoting kinase. J Biol Chem 1999;274:34967–73.
37. Fisher JE, Rogers MJ, Halasy JM, et al. Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation *in vitro*. Proc Natl Acad Sci U S A 1999;96:133–8.
38. van Beek E, Lowik C, Van der Pluijm G, Papapoulos S. The role of geranylgeranylation in bone resorption and its suppression by bisphosphonates in fetal bone explants *in vitro*: a clue to the mechanism of action of nitrogen-containing bisphosphonates. J Bone Miner Res 1999;14:722–9.
39. van Beek ER, Cohen LH, Leroy IM, Ebetino FH, Lowik CW, Papapoulos SE. Differentiating the mechanisms of antiresorptive action of nitrogen containing bisphosphonates. Bone 2003;33:805–11.
40. Monkkonen H, Auriola S, Lehenkari P, et al. A new endogenous ATP analog (Apppl) inhibits the mitochondrial adenine nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-containing bisphosphonates. Br J Pharmacol 2006;147:437–45.
41. Dunford JE, Rogers MJ, Ebetino FH, Phipps RJ, Coxon FP. Inhibition of protein prenylation by bisphosphonates causes sustained activation of Rac, Cdc42, and Rho GTPases. J Bone Miner Res 2006;21:684–94.
42. Coxon FP, Thompson K, Rogers MJ. Recent advances in understanding the mechanism of action of bisphosphonates. Curr Opin Pharmacol 2006;6:307–12.
43. Coxon FP, Benford HL, Russell RGG, Rogers MJ. Protein synthesis is required for caspase activation and induction of apoptosis by bisphosphonate drugs. Mol Pharmacol 1998;54:631–8.
44. Clezardin P, Ebetino FH, Fournier PG. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. Cancer Res 2005;65:4971–4.
45. Green JR. Bisphosphonates: preclinical review. Oncologist 2004;9 Suppl 4:3–13.
46. Shipman CM, Croucher PI, Russell RGG, Helfrich MH, Rogers MJ. The bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells *in vitro* by inhibiting the mevalonate pathway. Cancer Res 1998;58:5294–7.
47. Virtanen SS, Vaananen HK, Harkonen PL, Lakkakorpi PT. Alendronate inhibits invasion of PC-3 prostate cancer cells by affecting the mevalonate pathway. Cancer Res 2002;62:2708–14.
48. Coxon JP, Oades GM, Kirby RS, Colston KW. Zoledronic acid induces apoptosis and inhibits adhesion to mineralized matrix in prostate cancer cells via inhibition of protein prenylation. BJU Int 2004;94:164–70.
49. Sawada K, Morishige K, Tahara M, et al. Alendronate inhibits lysophosphatidic acid-induced migration of human ovarian cancer cells by attenuating the activation of rho. Cancer Res 2002;62:6015–20.
50. Boissier S, Magnetto S, Frappart L, et al. Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. Cancer Res 1997;57:3890–4.
51. Boissier S, Ferreras M, Peyruchaud O, et al. Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Res 2000;60:2949–54.
52. Bezzi M, Hasmim M, Bieler G, Dormond O, Ruegg C. Zoledronate sensitizes endothelial cells to tumor necrosis factor-induced programmed cell death: evidence for the suppression of sustained activation of focal adhesion kinase and protein kinase B/Akt. J Biol Chem 2003;278:43603–14.
53. Yamagishi S, Abe R, Inagaki Y, et al. Minodronate, a newly developed nitrogen-containing bisphosphonate, suppresses melanoma growth and improves survival in nude mice by blocking vascular endothelial growth factor signaling. Am J Pathol 2004;165:1865–74.
54. Hiraga T, Williams PJ, Ueda A, Tamura D, Yoneda T. Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model. Clin Cancer Res 2004;10:4559–67.
55. Giraudo E, Inoue M, Hanahan D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. J Clin Invest 2004;114:623–33.
56. Matsumoto S, Kimura S, Segawa H, et al. Efficacy of the third-generation bisphosphonate, zoledronic acid alone and combined with anti-cancer agents against small cell lung cancer cell lines. Lung Cancer 2005;47:31–9.
57. Hashimoto K, Morishige K, Sawada K, et al. Alendronate inhibits intraperitoneal dissemination in *in vivo* ovarian cancer model. Cancer Res 2005;65:540–5.
58. Gao L, Deng H, Zhao H, et al. HTLV-1 tax transgenic mice develop spontaneous osteolytic bone metastases prevented by osteoclast inhibition. Blood 2005;106:4294–302.
59. Wakchoure S, Merrell MA, Aldrich W, et al. Bisphosphonates inhibit the growth of mesothelioma cells *in vitro* and *in vivo*. Clin Cancer Res 2006;12:2862–8.
60. Ory B, Heymann MF, Kamijo A, Gouin F, Heymann D, Redini F. Zoledronic acid suppresses lung metastases and prolongs overall survival of osteosarcoma-bearing mice. Cancer 2005;104:2522–9.
61. Croucher PI, De Hendrik R, Perry MJ, et al. Zoledronic acid treatment of 5T2MM-bearing mice inhibits the development of myeloma bone disease: evidence for decreased osteolysis, tumor burden, and angiogenesis, and increased survival. J Bone Miner Res 2003;18:482–92.
62. Santini D, Vincenzi B, Dicuonzo G, et al. Zoledronic acid induces significant and long-lasting modifications of circulating angiogenic factors in cancer patients. Clin Cancer Res 2003;9:2893–7.
63. Santini D, Vincenzi B, Hannon RA, et al. Changes in bone resorption and vascular endothelial growth factor after a single zoledronic acid infusion in cancer patients with bone metastases from solid tumours. Oncol Rep 2006;15:1351–7.
64. van der Pluijm G, Que I, Sijmons B, et al. Interference with the microenvironmental support impairs the *de novo* formation of bone metastases *in vivo*. Cancer Res 2005;65:7682–90.
65. Adami S, Bhalla AK, Dorizzi R, et al. The acute-phase response after bisphosphonate administration. Calcif Tissue Int 1987;41:326–31.
66. Thompson K, Rogers MJ. Statins prevent bisphosphonate-induced γ,δ-T-cell proliferation and activation *in vitro*. J Bone Miner Res 2004;19:278–88.
67. Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, De Libero G. Human T cell receptor γδ cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 2003;197:163–8.
68. Hewitt RE, Lissina A, Green AE, Slay ES, Price DA, Sewell AK. The bisphosphonate acute phase response: rapid and copious production of proinflammatory cytokines by peripheral blood gd T cells in response to aminobisphosphonates is inhibited by statins. Clin Exp Immunol 2005;139:101–11.
69. Kunzmann V, Bauer E, Wilhelm M. γ/δ T-cell stimulation by pamidronate. N Engl J Med 1999;340:737–8.
70. Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M. Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood 2000;96:384–92.
71. Kunzmann V, Wilhelm M. Anti-lymphoma effect of γδ T cells. Leuk Lymphoma 2005;46:671–80.
72. Hayday AC. γδ cells: a right time and a right place for a conserved third way of protection. Annu Rev Immunol 2000;18:975–1026.
73. Woodward JK, Coleman RE, Holen I. Preclinical evidence for the effect of bisphosphonates and cytotoxic drugs on tumor cell invasion. Anticancer Drugs 2005;16:11–9.
74. Budman DR, Calabro A. Zoledronic acid (Zometa) enhances the cytotoxic effect of gemcitabine and fluvastatin: *in vitro* isobologram studies with conventional and nonconventional cytotoxic agents. Oncology 2006;70:147–53.
75. Coxon FP, Helfrich MH, Larijani B, et al. Identification of a novel phosphonocarboxylate inhibitor of Rab geranylgeranyl transferase that specifically prevents Rab prenylation in osteoclasts and macrophages. J Biol Chem 2001;276:48213–22.
76. Coxon FP, Ebetino FH, Mules EH, Seabra MC, McKenna CE, Rogers MJ. Phosphonocarboxylate inhibitors of Rab geranylgeranyl transferase disrupt the prenylation and membrane localization of Rab proteins in osteoclasts *in vitro* and *in vivo*. Bone 2005;37:349–58.
77. Roelofs AJ, Hulley PA, Meijer A, Ebetino FH, Russell RG, Shipman CM. Selective inhibition of Rab prenylation by a phosphonocarboxylate analogue of risedronate induces apoptosis, but not S-phase arrest, in human myeloma cells. Int J Cancer 2006;119:1254–61.
78. van Beek ER, Lowik CW, Ebetino FH, Papapoulos SE. Binding and antiresorptive properties of heterocycle-containing bisphosphonate analogs: structure-activity relationships. Bone 1998;23:437–42.
79. Nancollas GH, Tang R, Phipps RJ, et al. Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. Bone 2006;38:617–27.