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Review

Biochemical and molecular mechanisms of action of bisphosphonates

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ABSTRACT

This review describes the key discoveries over the last 15 years that have led to a clearer understanding of the molecular mechanisms by which bisphosphonate drugs inhibit bone resorption. Once released from bone mineral surfaces during bone resorption, these agents accumulate intracellularly in osteoclasts. Simple bisphosphonates such as clodronate are incorporated into non-hydrolysable analogues of adenosine triphosphate, which induce osteoclast apoptosis. The considerably more potent nitrogen-containing bisphosphonates are not metabolised but potently inhibit farnesyl pyrophosphate (FPP) synthase, a key enzyme of the mevalonate pathway. This prevents the synthesis of isoprenoid lipids necessary for the post-translational prenylation of small GTPases, thereby disrupting the subcellular localisation and normal function of these essential signalling proteins. Inhibition of FPP synthase also results in the accumulation of the upstream metabolite isopentenyl diphosphate, which is incorporated into the toxic nucleotide metabolite ApppI. Together, these properties explain the ability of bisphosphonate drugs to inhibit bone resorption by disrupting osteoclast function and survival. These discoveries are also giving insights into some of the adverse effects of bisphosphonates, such as the acute phase reaction that is triggered by inhibition of FPP synthase in peripheral blood monocytes.

This article is part of a Special Issue entitled Bisphosphonates.

Contents

Introduction

Despite being in clinical use for four decades, it is only in the last 10 years that the exact molecular mechanisms of action of bisphosphonates (BPs) have become clear. These agents, by virtue of their backbone P-C-P structure and ability to chelate calcium ions, target rapidly to bone mineral. This review describes the biochemical and molecular actions of these drugs on osteoclasts that have ingested BPs from the bone surface, but similar effects will occur in any cell type capable of internalising BPs (for example, most cell types cultured *in vitro*).

Skeletal targeting and intracellular uptake

The preferential uptake of BPs in the skeleton, by adsorption to mineral surfaces in bone, brings them into close extracellular contact with osteoclasts and probably some osteocytes [1], but prevents prolonged exposure to most other cell types. During bone resorption by osteoclasts, the acidic pH in the resorption lacuna causes the dissociation of BP from the bone mineral surface, followed by intracellular uptake of the BP (most likely as a complex with calcium

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ions) into osteoclasts by fluid-phase endocytosis [2]. Since cells other than osteoclasts are unable to acidify the bone surface to release bone-bound BPs, osteoclasts appear to be the only cell type capable of internalising substantial amounts of these drugs *in vivo*, although it is becoming increasingly clear that other endocytic cells such as monocytes and macrophages may internalise BPs that are present transiently in the circulation [3,4].

Fluorescently-labelled BPs have proved extremely useful tools to visualize the skeletal distribution and cellular uptake of BPs *in vivo*, as well as the localization of BPs in osteoclasts in intracellular endocytic vesicles (Fig. 1), which may enter the transcytotic pathway used by osteoclasts to release the products of bone matrix degradation [2]. Acidification of these intracellular vesicles appears to be necessary to allow the movement of BPs from the vesicles into the cytosol [5] and presumably then into other organelles such as peroxisomes, where these drugs exert their biochemical effect.

Intracellular metabolism of simple BPs

A breakthrough in understanding, at the molecular level, how the simple BPs (clodronate and etidronate) affect osteoclasts came about in 1989 through observations utilising the simplest BP, methylene bisphosphonate (medronate), to measure intracellular pH in *Dictyostelium* slime mould amoebae. Using ^{31}P-NMR Klein et al. noticed that medronate could be metabolically incorporated into methylene-containing analogues of adenosine triphosphate (ATP) and diadenosine tetraphosphate (Ap_4A) [6]. The metabolite analogue of ATP ie $AppCH_2p$, contains the BP (P-C-P) moiety of medronate in place of the β,γ pyrophosphate (P-O-P) moiety of ATP. As a result, $AppCH_2p$ resembles ATP but is resistant to hydrolytic breakdown and release of phosphate. Although this initial study only showed that medronate could be metabolised, we [7,8] and others [9] soon found that clodronate and other BPs of simple chemical structure that closely resemble PPi, such as etidronate, could also be metabolised by *Dictyostelium* amoebae to methylene-containing (AppCp-type) analogues of ATP (Fig. 2). Furthermore, the accumulation of AppCp-type metabolites of BPs was associated with cytotoxicity and inhibition of proliferation of *Dictyostelium* amoebae [6,8–10].

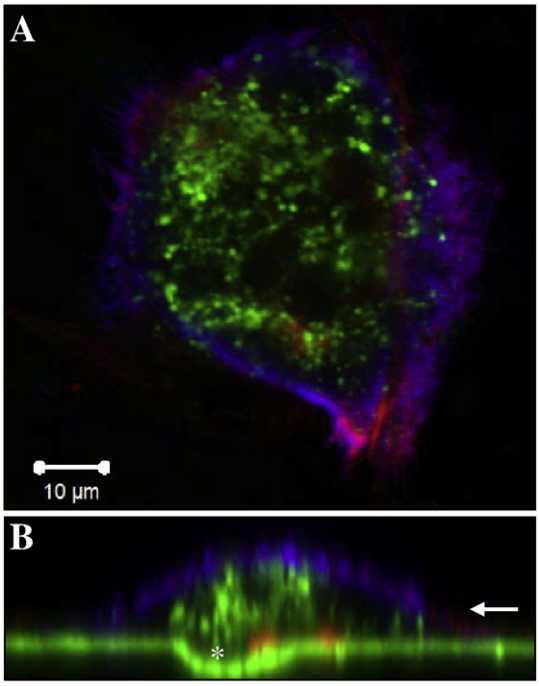

Fig. 1. Uptake of a fluorescent BP (FL-ALN) from the surface of dentine *in vitro*. Rabbit osteoclasts were cultured for 24 h on dentine slices that had been pre-coated with FL-ALN (green). Cells were fixed in 4% formaldehyde, and counterstained for F-actin (red) and the vitronectin receptor (blue) before examining by laser scanning confocal microscopy. (A) 1 micron *xy* section of an osteoclast (at the position denoted by the arrow in (B)), showing abundant intracellular vesicles containing FL-ALN. (B) *zx* section of the same osteoclast. Note the intense labelling of the dentine surface, in particular the resorption pit (asterisk). The actin ring can be seen as two distinct (red) regions just above the dentine surface in this sectional view.

Combining the relatively insensitive techniques of ^{31}P-NMR with analysis of cell extracts using FPLC and uv absorbance, we extended our study from slime moulds to extracts of human cell and to murine J774 macrophages [11,12], and showed that mammalian cells could also metabolise simple BPs to AppCp-type compounds [12,13]. The identity of these metabolites of clodronate, etidronate and tiludronate (Fig. 2B) was later confirmed using highly sensitive HPLC-electrospray ionisation tandem mass spectrometry [13,14], which allowed the detection of the AppCp-type metabolites of BPs in small quantities of cell lysate.

The incorporation of simple BPs into nucleotide analogues appears to be brought about by members of the family of Type II class of aminoacyl-tRNA synthetases (Fig. 2A) [8,11], although a recent report has suggested that other intracellular enzymes such as T4 RNA ligase may also be capable of metabolising simple BPs [15]. In the case of aminoacyl-tRNA synthetases, it appears that simple BPs (with short side chains ie clodronate and etidronate but also, surprisingly, tiludronate) resemble PPi sufficiently well to be accommodated into the enzyme active site in place of PPi. This allows a back-reaction involving the condensation of a BP with AMP (derived from an aminoacyladenylate) to form an AppCp-type nucleotide. However, the sterically bulkier N-BPs, such as alendronate, pamidronate and ibandronate, cannot be metabolised by these enzymes and we were unable to detect AppCp-type metabolites of N-BPs in lysates from cultures of BP-treated cells [13].

Twelve years after the first report that medronate could be metabolised by *Dictyostelium* amoebae, we confirmed (using immunomagnetic beads to isolate osteoclasts *ex vivo* from rabbits) that osteoclasts *in vivo* do indeed metabolise clodronate to $AppCCl_2p$ [16]. The AppCp-type metabolites of BPs such as clodronate probably accumulate to high concentrations (up to 1 mM; [17]) in the cytosol of osteoclasts, macrophages, *Dictyostelium* amoebae or other cell types that can effectively internalise BPs by endocytosis. Owing to the non-hydrolysable nature of the ATP analogues, their intracellular accumulation is likely to inhibit numerous intracellular metabolic enzymes, thus having detrimental effects on cell function and inducing apoptosis [18–23]. In accord, using liposome-encapsulated preparations, we found that treatment of cultured osteoclasts with the clodronate metabolite $AppCCl_2p$ inhibits bone resorption and causes osteoclast apoptosis to the same extent as treatment with clodronate [16] (Fig. 2C).

One of the molecular pathways by which AppCp-type metabolites of BPs can cause osteoclast apoptosis (Fig. 2C) appears to involve the adenine nucleotide translocase (ANT), a component of the mitochondrial permeability transition pore. The metabolite of clodronate, $AppCCl_2p$, inhibits the ANT [24] and prevents translocation of ATP across inner mitochondrial membranes. This causes initial hyperpolarization of the inner mitochondrial membrane in osteoclasts [24], followed by breakdown of the mitochondrial membrane potential [25], thus causing the mitochondrial permeability transition [26], activation of caspase-3 [25], and subsequent caspase-mediated cleavage of Mst-1, an apoptosis-promoting kinase [27]. Studies by Reszka and colleagues suggest that induction of osteoclast apoptosis is the primary mechanism by which the simple BPs inhibit bone resorption, since the ability of clodronate and etidronate to inhibit resorption *in vitro* can be overcome when osteoclast apoptosis is prevented using a caspase inhibitor [28]. Recently, we and others have shown that RANKL and TNFα can also rescue osteoclasts from the apoptosis-inducing effects of BPs, at least in part by inducing expression of the anti-apoptotic Bcl2-family members Mcl-1 and Bcl-xL. This may explain why BPs have not been as effective as

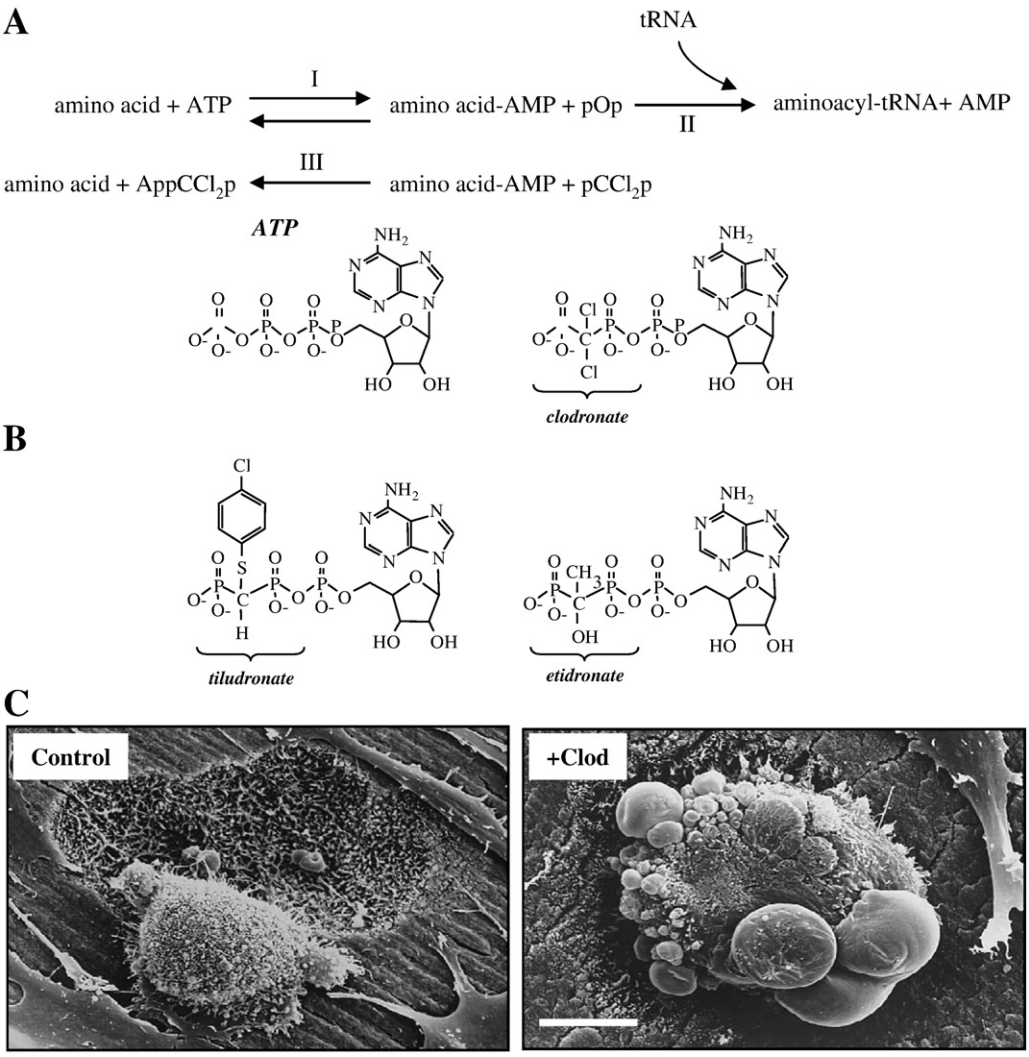

Fig. 2. (A) The formation of AppCp-type metabolites of bisphosphonates is catalysed by aminoacyl-tRNA synthetases. An amino acid condenses with ATP (Appp) to form an aminoacyl-adenylate (amino acid-AMP), releasing pyrophosphate (pOp) in a reversible reaction (I). The aminoacyl-adenylate then condenses with a molecule of tRNA to form aminoacyl-tRNA (reaction II). Since simple bisphosphonates (eg clodronate, pCCl2p) resemble pyrophosphate in structure, the reverse reaction of (I) can occur with pCCl2p in place of pOp, to form an analogue of ATP (AppCCl2p) containing the bisphosphonate. (B) The structure of ATP and the AppCp-type metabolite of clodronate, etidronate and tiludronate. (C) Clodronate treatment induces apoptosis in cultured rabbit osteoclasts. Compared to untreated osteoclasts (left), clodronate causes the appearance of rounded cells with membrane blebbing (right), characteristic of apoptosis. Treatment with the clodronate metabolite AppCCl2p, encapsulated in liposomes, causes the same morphological changes. Reproduced in part from Sutherland et al. [29].

expected in some inflammatory models of bone loss in which there are high levels of RANKL and TNFα [29,30].

Together, these studies provide convincing evidence that clodronate, etidronate and tiludronate act as prodrugs, being converted to AppCp-type metabolites following intracellular uptake by osteoclasts *in vivo*. The accumulation of these metabolites has a cytotoxic effect on osteoclasts, thus inhibiting bone resorption by causing osteoclast apoptosis [22,24,25,28,29]. The targeting of BPs to bone and their selective uptake by osteoclasts accounts for the ability of these BPs to selectively cause apoptosis of osteoclasts, but not other bone cells *in vivo*.

Nitrogen-containing BPs inhibit the mevalonate pathway

The nitrogen-containing BPs (N-BPs) pamidronate, alendronate, ibandronate, risedronate and zoledronate are up to several orders of magnitude more potent than the simple BPs at inhibiting bone resorption in preclinical models, but are not metabolised to AppCp-type nucleotides like simple BPs [13]. The first clue that led to clarification of the mechanism of action of N-BPs appeared in 1992, when Amin et al. reported that, in a study to identify novel inhibitors of cholesterol biosynthesis, the N-BPs ibandronate and incadronate inhibited squalene synthase and possibly other enzymes of the mevalonate (cholesterol synthesis) pathway in J774 macrophages [31]. Pamidronate and alendronate inhibited cholesterol synthesis in these cells but did not inhibit squalene synthase, whilst clodronate and etidronate did not affect cholesterol synthesis [31,32]. The fact that alendronate and pamidronate inhibited sterol biosynthesis but were not potent inhibitors of squalene synthase suggested that these N-BPs inhibit an enzyme further upstream in the mevalonate pathway (now known to be farnesyl pyrophosphate synthase; see later). Further studies were lacking until, several years later, we found that the order of potency of N-BPs for inducing apoptosis in J774 cells matched the order of potency for inhibiting bone resorption [33]. Even small changes to the R^2 side chain that were known to affect anti-resorptive potency also affected the ability to cause apoptosis of J774 cells. Hence, this macrophage cell line appeared to be an appropriate and convenient model in which to study the mechanism of N-BP-induced

apoptosis. Around that time, the publication of several reports that statins (which inhibit HMG-CoA reductase, the proximal enzyme in the mevalonate pathway) (Fig. 3) could also induce apoptosis led us to the realisation that inhibition of this metabolic pathway could explain the ability of N-BPs to induce apoptosis in osteoclasts and hence inhibit bone resorption. This was supported by the close similarity between induction of J774 macrophage apoptosis by statins and BPs; in both cases apoptosis is dependent on protein synthesis and occurs after a lag period of 15–24 h [34].

Inhibition of the mevalonate pathway prevents the prenylation of essential small GTPases in osteoclasts

The primary function of the mevalonate pathway is the production of cholesterol as well as the synthesis of isoprenoid lipids such as farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). These isoprenoid lipids are the building blocks for the production of a variety of metabolites, such as dolichol and ubiquinone [35], but are also required for post-translational modification (prenylation) of proteins (Fig. 3). The process of prenylation involves the transfer of the 15-carbon chain or 20-carbon chain isoprenoid groups from FPP or GGPP, respectively, onto a cysteine residue in characteristic carboxy-terminal motifs of specific target proteins [36,37]. The resulting farnesylated and geranylgeranylated proteins [38] comprise up to 2% of mammalian proteins (the "prenylome"), predominantly small GTPase signalling proteins but also γ subunits of heterotrimeric G proteins, phosphodiesterase subunits, and nuclear lamins. Prenylation is required for the correct function of these proteins, since it serves to anchor the proteins in cell membranes, and is involved in their interactions with other proteins such as (in the case of small GTPases) regulatory GAPs and GDIs [37,39]. The correct interaction of small GTPases with the latter proteins is essential for the normal regulation of small GTPase function. Since prenylated small GTPases act as molecular switches, their activity must be tightly controlled. Recent studies suggest that unprenylated small GTPases, which accumulate after exposure of cells to N-BPs, accumulate in their active (GTP-bound) state, causing inappropriate activation of downstream signalling kinases such as p38 [40].

The process of prenylation can be followed in cultured cells by measuring the incorporation of radiolabelled mevalonate, FPP or GGPP into both farnesylated and geranylgeranylated proteins (Fig. 4A).

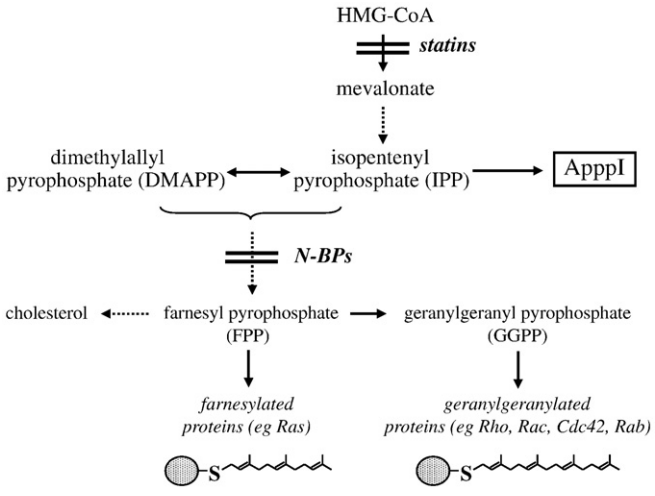

Fig. 3. Schematic diagram of the mevalonate pathway. Nitrogen-containing bisphosphonates (N-BPs) are potent inhibitors of FPP synthase, thereby preventing the synthesis of FPP and GGPP required for the prenylation of proteins that are crucial for osteoclast function and survival. Inhibition of FPP synthase also causes the accumulation of IPP, which is incorporated into the cytotoxic metabolite ApppI. Statins inhibit HMG-CoA reductase and therefore also prevent protein prenylation and mimic the effect of N-BPs on osteoclasts *in vitro*.

Inhibition of proximal enzymes in the mevalonate pathway (for example inhibition of HMG-CoA reductase by statins, or inhibition of FPP synthase by N-BPs) prevents the synthesis of FPP and GGPP and thus indirectly prevents the prenylation of small GTPases, the majority of which are geranylgeranylated [37]. Continuing to use J774 macrophages as a model of N-BP action, we showed, in 1998, that N-BPs prevent the incorporation of [^{14}C]mevalonate into both farnesylated and geranylgeranylated proteins in intact J774 cells, whereas the simple BPs (ie clodronate and etidronate) had no effect [13,41]. Risedronate almost completely inhibited protein prenylation in J774 cells at a concentration of 10^{-5} M, which is similar to the concentration that affects osteoclast viability *in vitro* [42–44] and could be achieved within the osteoclast resorption lacuna [45]. We and others confirmed that N-BPs (for example, 100 μM alendronate or risedronate, or ≥10 μM zoledronate, Figs. 4B,C) inhibit the incorporation of [^{14}C]mevalonate into prenylated small GTPase proteins in purified osteoclasts *in vitro* [46,47] and prevent protein prenylation in osteoclasts *in vivo* [16,47,48]. The latter studies measured the level of the unprenylated form of the small GTPase Rap1A which (like other small GTPases) accumulates in cells that are starved of FPP or GGPP, the substrates for prenylation (Fig. 4D). Fisher et al. have presented further evidence that N-BPs affect the mevalonate pathway in osteoclasts *in vivo*. Rats treated with alendronate, ibandronate or risedronate showed suppressed levels of HMG-CoA reductase in osteoclasts, presumably due to feedback regulation of the mevalonate pathway [49]. However, the reduction in HMG-CoA reductase expression is rather surprising, since SRE-mediated transcriptional upregulation of this enzyme is known to occur when cholesterol synthesis is inhibited [35,50].

Prenylated small GTPases such as those of the Ras, Rho, Rac, Cdc42 and Rab families are important signalling proteins that regulate a variety of cell processes important for osteoclast function, including cytoskeletal arrangement, membrane ruffling, trafficking of intracellular vesicles, and apoptosis [51,52]. Inhibition of the mevalonate pathway [41], leading to loss of prenylated proteins (and *loss* of downstream signalling) and/or accumulation of unprenylated proteins (and therefore *inappropriate activation* of downstream signalling pathways) (Fig. 5), could therefore account for most, if not all, of the various effects of N-BPs on osteoclasts that have been described. For example, loss of prenylation of Rho, Rac or Cdc42 could lead to loss of the osteoclast ruffled border, which is absent in osteoclasts treated with BPs *in vitro* or *in vivo* [42]. Since Rho, Rac and Cdc42 are required for cytoskeletal organisation in osteoclasts [52], loss of prenylation of these small GTPases could also cause the loss of actin rings, a characteristic effect of BP treatment. Rab GTPases are crucial regulators of vesicular trafficking and several are known to be required for osteoclast function [51,53,54]. Loss of prenylation of these proteins would therefore likely affect formation of the ruffled border, trafficking of lysosomal enzymes and transcytosis of degraded bone matrix [55]. Loss of prenylation of small GTPases such as Rac, and disruption of downstream signalling pathways promoting cell survival, are also the likely routes by which osteoclasts undergo apoptosis when exposed to sufficiently high concentrations of N-BP [56]. However, unlike for the simple BPs, induction of osteoclast apoptosis does not account for the inhibition of bone resorption caused by N-BPs, since preventing osteoclast apoptosis *in vitro* using a caspase inhibitor did not prevent N-BPs from inhibiting bone resorption, unlike with simple BPs [28]. Furthermore, inhibition of bone resorption by N-BPs is not always associated with signs of cell toxicity or a decrease in osteoclast number except at high concentrations or doses [42,43]. Indeed, N-BP therapy can lead to the formation of "giant" osteoclasts, identified histologically as hypernucleated cells that are usually detached from the bone surface or associated with superficial resorption lacunae [57,58]. These presumably occur as a result of accumulation of functionally inactive osteoclasts, that continue to undergo cell fusion.

The importance of prenylated proteins for osteoclast function has been confirmed using specific inhibitors that prevent either protein farnesylation (FTI-277, an inhibitor of farnesyl transferase) or protein

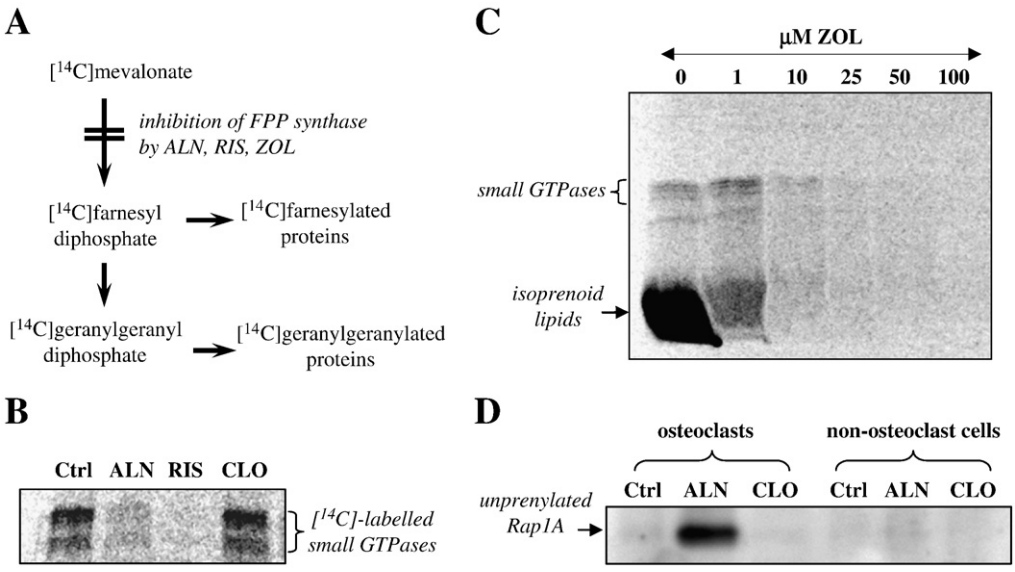

Fig. 4. (A) Inhibition of protein prenylation in osteoclasts by nitrogen-containing bisphosphonates can be demonstrated *in vitro* by culturing cells with [^{14}C]mevalonate, which becomes incorporated into ^{14}C-labelled, prenylated proteins. Radiolabelled, prenylated proteins can then be detected by autoradiography following electrophoretic separation. (B) Both alendronate (ALN) and risedronate (RIS) prevent the incorporation of [^{14}C]mevalonate into prenylated proteins in purified rabbit osteoclasts, whereas clodronate (CLO) has no effect. (C) Purified osteoclasts were metabolically-labelled with [^{14}C]mevalonate in the presence of 1–100 μM zoledronate (ZOL). 1 μM zoledronate inhibits the synthesis of radiolabelled isoprenoid lipids at the dye-front (arrowhead), but ≥10 μM zoledronate also inhibits prenylation of small GTPases. Reproduced from Coxon et al., J Bone Miner Res 2000; 15: 1467–1476 with permission of the American Society for Bone and Mineral Research. (D) Neonatal rabbits were injected with 10 mg/kg alendronate or clodronate. 24 h later, osteoclasts were purified using immunomagnetic beads and cell lysates were analysed by western blotting for the presence of unprenylated Rap1A. Alendronate (but not clodronate) treatment *in vivo* causes the accumulation of unprenylated protein in osteoclasts. The lack of effect in non-osteoclast cells demonstrates that alendronate specifically affects osteoclasts. Reproduced from Frith et al., Arth Rheum 2001; 44: 2201–2210 with permission of the American College of Rheumatology.

geranylgeranylation (GGTI-298, an inhibitor of geranylgeranyl transferase I). Whereas loss of farnesylated proteins in osteoclasts has little effect, loss of geranylgeranylated proteins causes disruption of actin rings, inhibits bone resorption and stimulates osteoclast apoptosis [46], clearly indicating the fundamental importance of geranylgeranylated small GTPases rather than farnesylated proteins in osteoclasts. This is supported by the finding that replenishing cells with an isoprenoid lipid substrate that restores geranylgeranylation can overcome the effects of N-BPs on osteoclast formation, apoptosis and bone resorption [27,59,60]. Interestingly, loss of geranylgeranylation of Rac is probably the cause of the regular inflammatory episodes associated with mevalonate kinase deficiency. This rare hereditary disorder (encompassing mevalonic aciduria, a severe and often fatal multisystemic disease, and the more benign hyper-IgD/HIDS and periodic fever syndromes) is caused by a variety of autosomal recessive mutations in the gene encoding mevalonate kinase, a proximal enzyme in the mevalonate pathway [61,62]. Decreased activity of the mutant enzyme, as well as feedback upregulation of HMG-CoA reductase, can lead to massive accumulation of mevalonate. However, it is likely that the shortage of isoprenoid lipids (and abnormal protein prenylation, particularly lack of geranylgeranylation), rather than the excess of mevalonate, is the pathogenic factor [63,64]. Recent studies suggest that the recurrent episodes of fever, arthralgia and skin rash in individuals with mevalonate kinase deficiency may be caused by lack of Rac

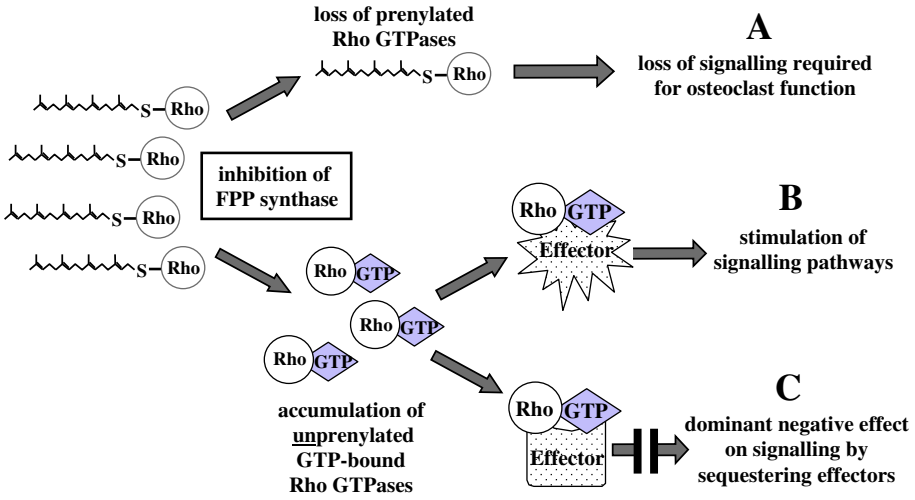

Fig. 5. Potential mechanisms by which N-BPs could affect signalling, following inhibition of prenylation of Rho family GTPases. (A) By inhibiting FPP synthase, N-BPs deplete the levels of prenylated proteins, perhaps leading to loss of downstream signalling pathways required for osteoclast function. Alternatively, accumulation of GTP-bound unprenylated GTPases might either (B) cause inappropriate activation of downstream signalling pathways or (C) exert a dominant negative effect on signalling by sequestering effectors in nonproductive cytoplasmic complexes. These potential effects could also apply to prenylated GTPases other than those of the Rho family, although this remains to be studied.

prenylation, leading to abnormal activation of Rac and increased caspase-1 activity, thus generating increased levels of mature IL-1β following minor infections [65].

FPP synthase is the major molecular target in the mevalonate pathway

Following the recognition in 1998 that N-BPs most likely act by inhibiting the mevalonate pathway and altering protein prenylation, a flurry of papers [47,66–68] identified FPP synthase as the major enzyme of the pathway that was inhibited in J774 macrophages and osteoclasts (Fig. 3), although some N-BPs are also much weaker inhibitors of other enzymes in the mevalonate pathway, including IPP isomerase, squalene synthase and GGPP synthase [31,69,70]. More detailed studies with recombinant human FPP synthase demonstrated that it is inhibited by nanomolar concentrations of N-BPs [68,71] and that there is a highly significant correlation between the order of potency for inhibiting human FPP synthase *in vitro* and anti-resorptive potency *in vivo*. Importantly, minor modifications to the structure and conformation of the R^2 side chain that were known to affect anti-resorptive potency were shown to affect the ability to inhibit FPP synthase [68]. These observations have helped explain the relationship between BP structure and anti-resorptive potency, strongly suggesting that FPP synthase is indeed the major pharmacologic target of N-BPs in osteoclasts *in vivo*. The exact mechanism by which N-BPs inhibit FPP synthase is reviewed by Dunford et al. in this edition of *Bone*.

Studies with *Dictyostelium* slime mould amoebae have provided further evidence that FPP synthase is the major target of N-BPs. As with J774 macrophages, the order of potency of N-BPs for inhibiting the proliferation of *Dictyostelium* amoebae closely matches the order of potency for inhibiting bone resorption [10,72,73], indicating that N-BPs affect the same, ubiquitous molecular target in *Dictyostelium* as in mammalian cells. Spontaneous mutant strains of *Dictyostelium* [74], as well as tumour cells cultured in low concentrations of N-BPs [75,76], are resistant to the growth-inhibitory or cytotoxic effect of the drugs and have been found to have increased FPP synthase activity. Whether patients may also acquire resistance to N-BPs or have inter-individual differences in the response to N-BP therapy, due to mutations or SNPs in FPP synthase, remains a largely unexplored question. A study of 283 Caucasian women suggested that a SNP (rs2297480) in FPP synthase may be a genetic marker for bone mineral density (BMD), since statistically significant differences were found between the C/C or C/A genotypes at the spine, trochanter, distal radius, and proximal ulna after adjustment for age and BMI, although there were no significant differences in bone turnover markers [77]. However, an explanation at the molecular/cellular level for such an effect of FPP synthase on BMD remains to be provided.

The high degree of evolutionary conservation of this enzyme explains why N-BPs also inhibit FPP synthase from *Dictyostelium* [74] and other eukaryotes such as *Leishmania* and *Trypanosome* parasites [78–81]. The exact mechanism of inhibition of FPP synthase has become clear through characterisation of the X-ray crystal structure of FPP synthase and detailed measurements of enzyme kinetics, indicating that the N-BPs mimic the structure of the enzyme's natural isoprenoid pyrophosphate substrates GPP/DMAPP and compete for binding at the GPP/DMAPP substrate binding site of the enzyme.

Although N-BPs can have dramatic anti-tumour effects *in vitro* and in some *in vivo* models of cancer [82], mediated by inhibition of FPP synthase in tumour cells, macrophages or other tumour-associated cells [4], the effectiveness of N-BPs as anti-tumour agents *in vivo* is likely restricted by their rapid targeting to bone surfaces and hence low bioavailability to cells other than osteoclasts and monocytes. Removal of the phosphonate groups of N-BPs would prevent binding to bone mineral and could increase drug accessibility to other cells, however it is clear that even subtle modifications to the phosphonate groups (such as methylation) that reduce binding to bone mineral also decrease the ability to inhibit protein prenylation [83] since the phosphonate groups are important for interaction with magnesium ions in the GPP/DMAPP pocket of FPP synthase [84,85]. Interestingly, replacement of a phosphonate group with a carboxylate group also substantially reduces mineral affinity and the ability to inhibit FPP synthase, but creates compounds that selectively inhibit Rab GGTase and therefore specifically prevent the prenylation of Rab GTPases [55,86–88]. As a result, these phosphonocarboxylate drugs disrupt the osteoclast ruffled border (since maintenance of this membrane domain is dependent on Rab-regulated vesicular trafficking), but have no effect on the integrity of the actin ring, since they do not affect prenylation of Rho family GTPases [55,86]. There is some evidence that phosphonocarboxylates possess anti-tumour activity *in vivo* [89], but this may be limited by the fact that these compounds still target to bone due to the presence of one phosphonate group. More recently, an exciting study by Jahnke and colleagues [90] has identified novel, non-BP inhibitors of FPP synthase that bind to a previously unknown allosteric site on the enzyme. These inhibitors lack the phosphonate moieties of N-BPs and hence do not bind to bone mineral, offering the prospect of new classes of FPP synthase inhibitors that may be of clinical use as anti-tumour or anti-parasitic agents or in even more diverse therapeutic applications. For example, inhibition of FPP synthase via N-BP treatment, in combination with a statin to further inhibit the mevalonate pathway, was recently found to reduce the aging symptoms and to increase longevity in a mouse model of Hutchinson–Gilford progeria syndrome (HGPS), by preventing the farnesylation or geranylgeranylation and hence abnormal membrane localisation and function of mutant lamin A in the nucleus of HGPS cells [91].

Inhibition of FPP synthase causes the accumulation of IPP and the formation of ApppI

As well as preventing the prenylation of small GTPase proteins, inhibition of FPP synthase by N-BPs causes accumulation of IPP, the metabolite immediately upstream of FPP synthase in the mevalonate pathway (Fig. 3). This accumulation of IPP appears to be the cause of the acute phase reaction to N-BPs, a common adverse effect involving fever and "flu-like" symptoms that are transient and usually occur soon after first intravenous (IV) administration of the drug. It appears that, after IV infusion, uptake of N-BP by monocytes in peripheral blood [3] causes inhibition of FPP synthase and rapid accumulation of IPP [92,93], which is known to be a ligand for the most common subset of γ,δ-T cells in humans, Vγ9Vδ2 T cells. Although the precise mechanism by which IPP is released or "presented" to these γ,δ-T cells remains unknown, activation of the γ,δ-T cells causes the release of TNFα and thereby initiates the pro-inflammatory acute phase response [94]. The activation of γ,δ-T cells by N-BPs can be completely overcome *in vitro* by co-treating cells with statins, which prevent the accumulation of IPP [92].

The accumulation of IPP in cells that have internalised N-BP, such as cultured tumour cells, and osteoclasts and macrophages *in vivo* [95–97], leads to the production of a new metabolite, ApppI [98] (Fig. 3). Similarly, the concurrent accumulation of DMAPP appears to lead to the formation of ApppD, at an even higher rate than ApppI [99]. The exact mechanism by which ApppI and ApppD are generated remains uncertain. Some evidence suggests that aminoacyl-tRNA synthetases, the same enzymes that metabolise simple BPs, may also be able to conjugate IPP with AMP to form ApppI [100], although this has not been confirmed using purified enzymes. However, like the AppCp-type metabolite of simple N-BPs, ApppI inhibits the mitochondrial ANT and can cause osteoclast apoptosis [98]. Hence, inhibition of FPP synthase by N-BPs can cause osteoclast apoptosis (and thereby inhibit bone resorption) by two routes, inhibition of protein prenylation and accumulation of ApppI [100], although the other morphological changes associated with exposure of osteoclasts to N-BPs (such as loss of the

ruffled border, disruption of the actin cytoskeleton and altered vesicular trafficking) are best explained by the effect on protein prenylation.

Conclusions

After 40 years of clinical use, the biochemical and molecular actions of BPs on osteoclasts are now clear and can be divided into two distinct mechanisms. Whereas BPs of simple chemical structure are incorporated into toxic, non-hydrolysable ATP analogues, the more potent, nitrogen-containing BPs inhibit the enzyme FPP synthase, thereby disrupting the production of isoprenoid lipids in the mevalonate pathway, preventing the prenylation of small GTPase proteins necessary for osteoclast function and causing accumulation of a toxic, isoprenoid-containing metabolite. Although additional molecular targets for BPs may exist, these two main mechanisms of action account for the anti-resorptive effects of these agents on osteoclasts, as well as for some of the adverse effects of BPs such as the acute phase response. Further studies are required to fully understand the (perhaps subtle) effects *in vivo* of BPs on other cell types such as osteocytes, monocytes and tumour cells, but these are described in other reviews in this special edition of *Bone*.

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