Mechanism of Action of Bisphosphonates

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In recent years, substantial progress has been made in understanding the mechanism for bisphosphonate suppression of bone turnover. Bisphosphonates can now be distinguished based on their molecular and cellular mechanisms of action. Simple bisphosphonates such as clodronate and etidronate inhibit bone resorption through induction of osteoclast apoptosis. Clodronate, and perhaps etidronate, triggers apoptosis by generating a toxic analog of adenosine triphosphate, which then targets the mitochondria, the energy center within the cell. For nitrogen-containing bisphosphonates, the direct intracellular target is the enzyme farnesyl diphosphate synthase in the cholesterol biosynthetic pathway. Its inhibition suppresses a process called protein geranylgeranylation, which is essential for the basic cellular processes required for osteoclastic bone resorption. Although nitrogen-containing bisphosphonates can induce osteoclast apoptosis, this is not necessary for their inhibition of bone resorption.

Introduction

Bisphosphonates, in particular alendronate and risedronate, are the only nonhormonal agents shown so far to reduce the risk of spinal and nonvertebral osteoporotic fractures. They are widely used for the treatment and prevention of osteoporosis in postmenopausal women, in men, and in glucocorticoid-treated patients. Bisphosphonates reduce the number of bone remodeling sites where excessive osteoclastic destruction of bone takes place. Bisphosphonate-induced suppression of bone turnover leads to improvement of bone strength, reflected in a reduction of fracture risk, which can be attributed to improvements in bone mass, mineralization, and architecture [1•,2].

Bisphosphonates are analogs of pyrophosphate (P-O-P) where the geminal oxygen has been substituted by carbon (P-C-P) (Fig. 1). A main feature of the P-C-P backbone is that by adhering to the hydroxyapatite component of bone, it localizes these compounds to the target tissue. The geminal carbon, in addition to bridging the two phosphorus atoms, can form two additional covalent bonds (designated R^1 and R^2) to hydrogen or substituents such as hydroxyls (OH), methyl groups (CH_3), or other more complex moieties (Fig. 1). The substituents bound to the geminal carbon of the bisphosphonate can influence the affinity for bone and the potency and efficacy in suppressing bone resorption. The attachment of a hydroxyl at position R^1 increases a bisphosphonate's affinity to bone [3]. Meanwhile, at position R^2 , the presence of small nitrogen-containing hydrocarbon chains, or ring structures, can greatly enhance antiresorptive potency, provided that the nitrogen is at a distance of approximately four positions from the geminal carbon. Individual R^2 modifications have no effect on binding to bone ex vivo [3]. Thus, the various carbon and nitrogen side chains can generate a large family of compounds (Fig. 1) with different physicochemical and pharmacologic properties.

Bisphosphonate Uptake by the Osteoclast

To suppress osteoclastic bone resorption, bisphosphonates must be internalized by the osteoclast in order to interact with their intracellular target. The pharmacokinetic parameters of bisphosphonates are well characterized, and bioavailability is limited to less than 1% to 2%. It may seem surprising that such small quantities can have such strong protective effects on bone (*eg,* 0.7% of once weekly dosing of 35 to 70 mg alendronate, currently available for clinical use). For the potent bisphosphonates, this relates, in part, to the specific targeting of bisphosphonates to bone. Not only does this involve rapid binding to the hydroxyapatite component of bone, but also preferential localization to sites of osteoclastic bone resorption, as documented for alendronate [4–6]. In comparison, the less potent bisphosphonate, etidronate, showed a more diffuse distribution over the bone surface at its higher pharmacologic relevant dose. By inference, all of the potent nitrogen-containing bisphosphonates (N-BPs) should exhibit selective binding properties similar to those of alendronate, although this has not been verified.

The exact mechanism of bisphosphonate uptake from the bone surface into the osteoclast cytoplasm is less clearly understood. Earlier work suggests that the acid secreted onto the bone surface as part of the active resorption process could release the bisphosphonate from the

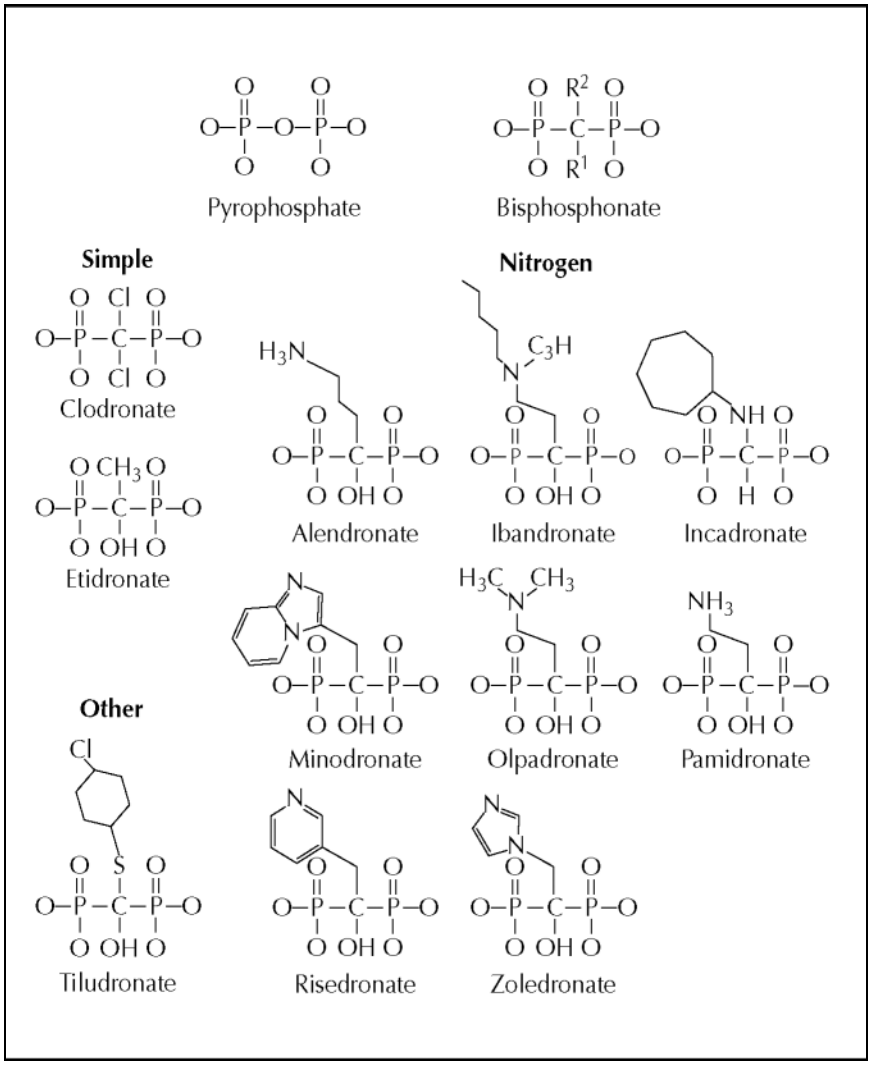

Figure 1. Structure of the bisphosphonates. *Top*, Comparison of the structure of a generic bisphosphonate and that of pyrophosphate. *Left,* Structures of simple bisphosphonates and tiludronate. These likely act by forming toxic adenosine triphosphate analogs. *Right*, Structure of the nitrogen-containing bisphosphonates. These have been proven to act through inhibition of farnesyl diphosphate synthase.

bound state and enable uptake through the ruffled border adjacent to the resorption lacuna [4]. In support of this, others have shown that inactive oc/oc osteoclasts, which have no ruffled border and cannot resorb bone, do not respond to tiludronate treatment in vitro, unless the bisphosphonate is microinjected directly into the cell's cytoplasm [7]. More recently, however, incadronate was shown to affect osteoclast bone attachment and lifespan in vivo in the oc/oc mouse [8], suggesting that active bone resorption was not required. However, for these studies, incadronate was injected at a comparatively high dose (1 mg/kg), and thus the response may not represent the pharmacologic action of potent bisphosphonates. For comparison, the human oral dose of 70 mg alendronate once a week, corrected for absorption, represents the injected equivalent of less than 10 µg/kg.

Earlier work shows that after administration of pharmacologic relevant doses of radioactive alendronate in vivo, the bisphosphonate can be detected inside the osteoclast within 4 hours [4,6]. Assuming that acid secretion onto the bone surface is a prerequisite for the osteoclast to internalize the relatively low doses of alendronate and other highly potent bisphosphonates, there is likely to be a mechanism for uptake through the plasma membrane. Osteoclasts gather the content of the resorption lacunae through the ruffled border and translocate it through the cell by a process of transcytosis [9]. Although internalized vesicles are physically located within the cytoplasm, they remain separated from cytoplasmic contents by the vesicular membrane (originally derived from the ruffled border membrane). One process for crossing these membranes could involve passive diffusion, although recent evidence suggests that the process may involve active or facilitated transport [10,11]. First, bisphosphonates' effects on osteoclasts attached to nonmineralized bone are suppressed when the active process of pinocytosis is inhibited [10]. Pinocytosis is somewhat analogous to transcytosis because it involves the uptake of fluid by invagination and pinching off of the plasma membrane. More recently, the effects of alendronate on its target intracellular pathway within the osteoclast were found to be suppressed by combining alendronate with increasing amounts of clodronate [11], which acts through its own independent mechanism of action. This showed that the mechanism for entry into the

cytoplasm was subject to competition, suggesting the involvement of a structure-specific process. Radiolabel studies suggest that ibandronate uptake could be partially suppressed by combining it with unlabeled clodronate. A reasonable interpretation of the small but significant reductions in ibandronate uptake is that pinocytosis remained unaffected, whereas transmembrane transport was inhibited by the added clodronate.

Taken together, bone resorption seems to be required for uptake of potent bisphosphonates, likely through the ruffled border. Once internalized in transcytotic vesicles, bisphosphonates may cross the vesicular membrane into the cytoplasm through an active process. However, because the N-BPs, acting through their respective intracellular targets, ultimately suppress resorption and the transcytotic process, this may also limit excessive exposure to the bisphosphonate. This could then reduce the likelihood that the osteoclast undergoes apoptosis. For N-BPs, such as alendronate and risedronate, this seems to be the case [12••]. Thus, for N-BPs there seem to be two built-in negative feedback loops for limiting their action. These loops are the availability of resorption surfaces to localize on, and active resorption required for osteoclast bisphosphonate uptake. However, for clodronate and etidronate, their different mechanism of action precludes the sparing of the osteoclast.

Clodronate and Etidronate

Over the years, bisphosphonates have been shown to affect several biochemical pathways, especially those involving phosphate. Intracellular metabolism involving the phosphate moiety was first documented for methylenebisphosphonate (medronate) in dictyostelium discoideum [13]. Methylene-containing analogs of adenosine triphosphate (ATP) and diadenosine tetraphosphate were detected where the bisphosphonate had substituted for pyrophosphate. This suggested that other bisphosphonates might also be metabolized to similar compounds. Therefore, a most probable mechanism of action for clodronate involves the incorporation of the P-C-P backbone into the β,γ positions of ATP, ultimately generating a toxic metabolite, $AppCCl_2p$ [14]. Etidronate and tiludronate were similarly metabolized, although to a far lesser degree. For clodronate, conversion to $AppCCl_2p$ seems to be the primary mechanism. This process involves its substitution for two of three phosphates, suggested to take place in the reverse reaction of ATP-dependent transfer RNA (tRNA) formation [15••]. Recent in vivo studies have demonstrated the presence of $AppCCl_2p$ in enriched osteoclast preparations isolated from the bone marrow of rabbits treated with clodronate [16]. Injection of rats with liposome-encapsulated clodronate to target macrophages also resulted in the accumulation of $AppCCl_2p$ in these cells. Consistent with different mechanisms for different bisphosphonates, no metabolism of alendronate was detected in osteoclasts or macrophages after similar administration.

Several forms of evidence suggest that the conversion of clodronate to $AppCCl_2p$ is responsible for its induction of osteoclast apoptosis and inhibition of bone resorption. Clodronate and etidronate have been shown to induce apoptosis in purified osteoclast cultures through direct action on the osteoclast itself [12••,16–20]. A strong link between clodronate and etidronate induction of osteoclast apoptosis and their antiresorptive effects has also been established [12••]. Therefore, simply blocking the apoptotic process can counteract the effects of these simple bisphosphonates, but not alendronate or risedronate, on osteoclastic bone resorption. Other evidence strongly suggests that the formation of the $AppCCl_2p$ clodronate/ATP analog is sufficient to induce osteoclast apoptosis [15••,16,20]. This is based on the finding that direct delivery of $AppCCl_2p$ to osteoclasts and macrophages results in the induction of apoptosis. Apoptosis is likely to be induced by $AppCCl_2p$ through inhibition of the osteoclast mitochondrial adenine nucleotide translocase [20]. This results in the collapse of the mitochondrial membrane potential, leading to the release of cytochrome–C, caspase activation, and other steps of the apoptotic program.

Several forms of evidence suggest that clodronate acts through formation of a toxic ATP analog, $AppCCl_2p$, which disrupts the mitochondrial membrane potential and induces osteoclast apoptosis. The subsequent loss of viable osteoclasts leads to a net reduction in bone turnover and an increase in bone mass. Etidronate may act through a similar mechanism, but further evidence is needed.

Farnesyl Diphosphate Synthase As the Molecular Target of the Nitrogen-containing Bisphosphonates

Approximately 15 years ago, it was shown that certain bisphosphonate derivatives (isoprenoid [phosphinylmethyl] phosphonates) could inhibit the cholesterol biosynthetic enzyme, squalene synthase [21]. Since then, numerous studies have described the search for more potent bisphosphonate inhibitors of this enzyme or capitalized on squalene synthase inhibitory activity to measure N-BP levels in clinical serum samples. Despite that all bone-active N-BPs suppress the mevalonate/cholesterol biosynthetic pathway (Fig. 2), squalene synthase is not the relevant target for inhibition of bone resorption because repletion of cholesterol does not block N-BP (or statin) effects on osteoclasts [22••]. Instead, a number of studies suggest that an enzyme upstream in this pathway controlling both cholesterol synthesis and isoprenylation is the critical target of the N-BPs in the osteoclast [12••,19,22••,23,24•]. This led to the identification of FPP synthase as the relevant molecular target [25–27]. This molecular action has recently been confirmed in vivo for several N-BPs, including alendronate, risedronate, and ibandronate, but not for the simple bisphosphonates, clodronate and etidronate [16,28••].

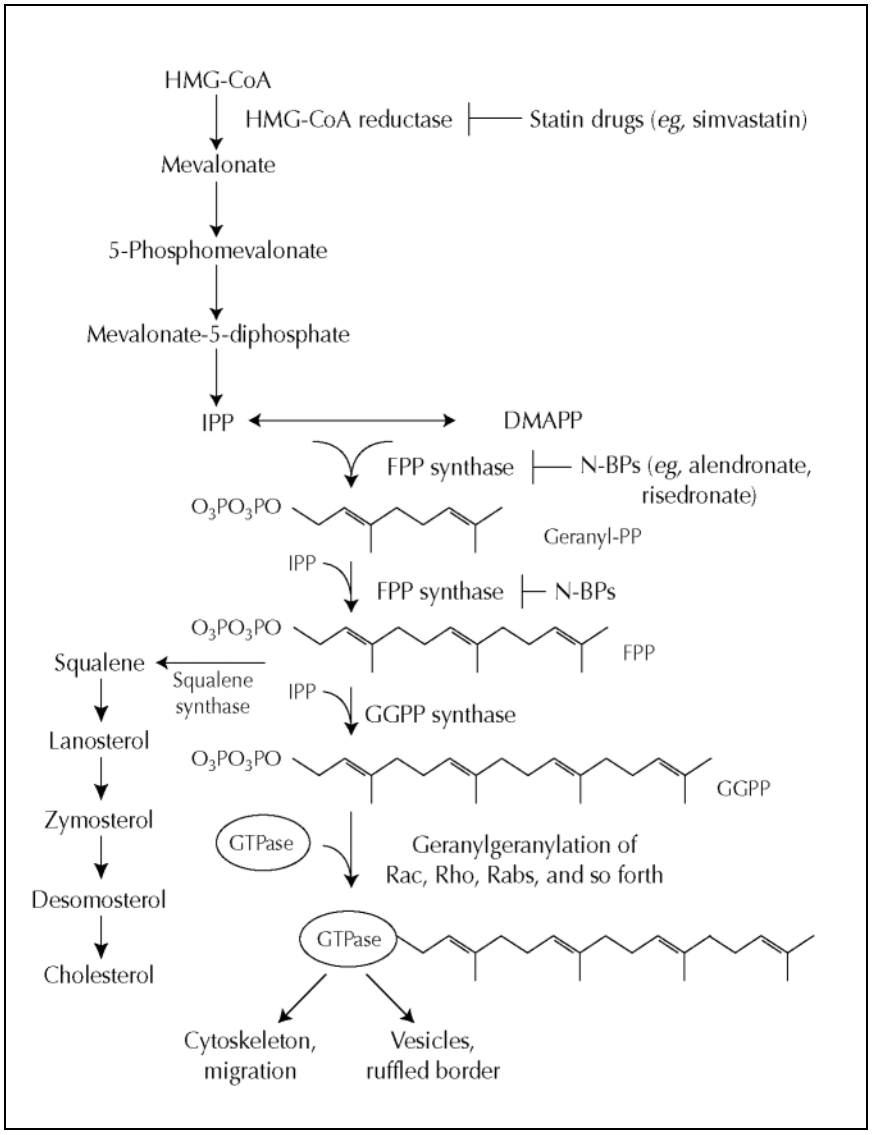

Figure 2. Schematic of the cholesterol biosynthetic pathway. Sites of inhibition for the nitrogen-containing bisphosphonates (N-BPs; farnesyl diphosphate [FPP] synthase) and statins (3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase) are indicated. The basic structures of FPP and geranylgeranyl diphosphate (GGPP) are also shown. DMAPP—dimethyallyl diphosphate; GTPase—guanosine triphosphate; IPP—isopentenyl diphosphate.

Farnesyl diphosphate (FPP) synthase is an enzyme of the cholesterol biosynthetic pathway (Fig. 2) responsible for producing the isoprenoid lipids FPP (15 carbon) and geranylgeranyl diphosphate (GGPP; 20 carbon). While the FPP isoprenoid can be condensed to form squalene and ultimately cholesterol, its conversion to GGPP is the critical step for N-BP suppression of osteoclastic bone resorption. Isoprenylation involves the transfer of a farnesyl (farnesylation) or geranylgeranyl (geranylgeranylation) lipid group onto a cysteine amino acid residue in characteristic carboxy-terminal motifs (*eg*, Cys-Ala-Ala-X). N-BPs suppression of FPP synthase causes a decline in the levels of FPP and consequently GGPP. The subsequent loss of protein geranylgeranylation leads to osteoclast inactivation. Most of the isoprenylated proteins identified to date are small geranylgeranylated regulatory proteins, named guanosine triphosphatases (GTPases), and these are important for the control of a variety of cell processes required for osteoclast function, including cytoskeletal regulation, formation of the ruffled border, and regulation of cell survival.

Bisphosphonate inhibition of FPP synthase and its relation to protein isoprenylation provide the best documentation for a cause-effect relationship between a molecular target and a functional consequence in the osteoclast. Because FPP synthase is required for the synthesis of metabolites that are further processed and modified by downstream enzymes, simply replenishing the missing metabolites can restore the isoprenylation system. Among the various downstream metabolites, only GGPP, which can be replenished by the addition of the lipid alcohol, geranylgeraniol, prevents inhibition of osteoclast formation and bone resorption by the N-BPs [12••,22••,24•]. The geranylgeraniol effect to prevent N-BP inhibition of bone resorption has been demonstrated in the presence of inhibitory concentrations of alendronate, ibandronate, or risedronate. Furthermore, N-BP effects can be mimicked by the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)

reductase inhibitors, lovastatin and mevastatin (upstream of FPP synthase), and this too can be blocked by addition of geranylgeraniol [22••,29]. Meanwhile, mevalonate, a metabolite that feeds into this pathway between sites of statin and N-BP action, can completely rescue inhibition of resorption by the statins but not the N-BPs. N-BPs competitively inhibit FPP synthase [30], suggesting that increasing amounts of upstream metabolites could have some effect on restoring osteoclast function. Consistent with this, some partial effects of mevalonate have been reported [22••,31]. Taken together, these observations strongly suggest that N-BP inhibition of bone resorption is a consequence of competitive FPP synthase inhibition with resulting loss of protein geranylgeranylation.

Substantial evidence has accumulated to link the loss of geranylgeranylation to induction of osteoclast apoptosis, disruption of the actin cytoskeleton, and altered membrane trafficking [12••,19,32,33]. It was reported that bisphosphonates induce osteoclast apoptosis, both in vitro and in vivo, in normal mice and in mice with increased bone resorption [17]. Induction of apoptosis in purified osteoclast cultures by alendronate and risedronate (not clodronate or etidronate) can be blocked by the addition of geranylgeraniol, but not farnesyl, suggesting that, like with bone resorption responses, only geranylgeranylation was critical [12••,19]. Geranylgeraniol was also effective in blocking statin-induced osteoclast apoptosis. Consistent with this, an inhibitor of geranylgeranylation (GGTI-298), but not farnesylation (FTI-277), can induce osteoclast apoptosis in vitro [32].

The signaling pathways involving geranylgeranylated small GTPases that are affected by bisphosphonates and that lead to osteoclast apoptosis remain to be determined. Perhaps most proximal to the GTPases is the mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase (S6K) signaling pathway [34]. Signaling through this path is suppressed when geranylgeranylation is blocked in the osteoclast. Furthermore, specific inhibition of mTOR by rapamycin causes induction of osteoclast apoptosis over a similar time course to that of the N-BPs. Downstream consequences of N-BP or rapamycin treatment include activation of caspases, pro-apoptotic kinase, and mammalian sterile-zo-like kinase (MST1). MST1 was identified as a pro-apoptotic signaling intermediate downstream of the bisphosphonates that is activated during apoptosis by N-BPs, lovastatin, and clodronate [19]. MST1 kinase acts as a substrate for caspases 3, 7, and 9 and as an activator of these caspases [35,36]. MST1 is thus responsive to and an inducer of the apoptotic process. Caspase cleavage of MST1 results in the formation of an endogenously, highly active kinase species. This is most likely mediated through caspase 3, the major effector caspase activated in osteoclasts undergoing apoptosis following treatment with a range of bisphosphonates in vitro [37].

Although induction of apoptosis will lead to a decrease in the number of osteoclasts and thus suppress resorption, this is usually seen only after longer treatment with bisphosphonates. Brief 48-hour treatments with alendronate, risedronate, or ibandronate, but not clodronate and etidronate, were found to increase osteoclast number in vivo [28••]. In vitro, N-BP suppression of resorption is seen prior to reductions in osteoclast number [12••,33], suggesting direct inhibition of osteoclast function. Suppression of the apoptotic process had no effect on N-BP disruption of the cytoskeleton or on their inhibition of bone resorption [12••]. It was reported that following bisphosphonate administration, osteoclasts show changes in morphology and appear inactive [38]. The changes are numerous and include alterations in the cytoskeleton, including actin and vinculin and disruption of the ruffled border [4,38,39,40]. In comparison with their effects on survival, N-BPs were shown to disrupt the actin cytoskeleton and vesicular trafficking in vitro prior to or separately from induction of apoptosis [12••,33].

Based on these observations, means of suppressing osteoclastic bone resorption, other than apoptosis, seem more likely for the N-BPs when administered at clinically relevant doses. Based on our understanding of the functions of geranylgeranylated proteins, we suggest that regulation of vesicular trafficking (*eg*, formation of the ruffled border) and cytoskeletal structures are the primary targets. The ruffled border is a convoluted membrane that faces the bone surface and acts as a hallmark of active osteoclasts. Disappearance of the ruffled border provides morphologic evidence for mechanism-based osteoclast inactivation and could explain the lack of acid extrusion caused by alendronate in isolated osteoclasts [41]. Ruffled border formation is a process that is highly dependent on cytoskeletal function and strongly regulated by geranylgeranylated GTPases, such as Rac and Rho. Moreover, the vesicles normally located above (that disappear after nitrogen bisphosphonate treatment) are needed for the formation of the ruffled border, and the trafficking of these vesicles is largely under the control of the Rab GTPases, which are also geranylgeranylated. Because the ruffled border and vesicles disappear after alendronate treatment, several GTPases could be implicated in the response.

Although our understanding of the roles of geranylgeranylated GTPases is limited, several have been shown to play a functional role in the osteoclast. The Rabs play a key role in controlling vesicular trafficking, and at least seven different Rab proteins have been identified in rat osteoclasts. These GTPases are physically associated with the ruffled border and vesicular structures [42]. Suppression of one of these (Rab7) by blocking expression within the osteoclast disrupts the targeting of vesicles to the ruffled border with ultrastructural changes remarkably similar to those seen after treatment with alendronate [4,43]. The functional consequence of this is a reduction in the number and size of bone resorption pits. With respect to cytoskeletal organization and migration, three Rho family proteins, Cdc42, Rac, and Rho, have been shown to play a regulatory role in the osteoclast. Inactive mutants of either Rac or RhoA suppress bone resorption, associated with dis-

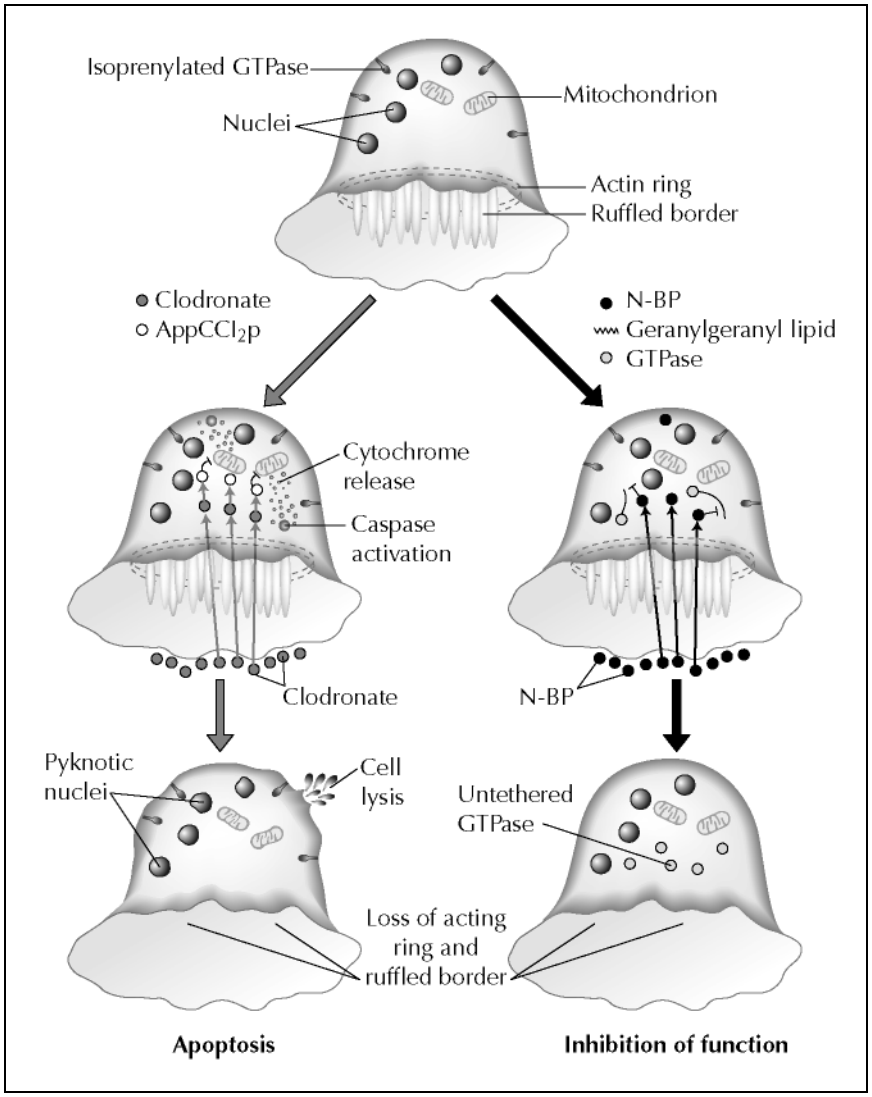

Figure 3. Schematic for nitrogen-containing bisphosphonate (N-BP) and clodronate molecular actions in suppressing osteoclastic bone resorption. GTPase—guanosine triphosphates; IPP—isopentenyl diphosphate; DMAPP—dimethylallyl diphosphate.

ruptions in osteoclast morphology, motility, and cytoskeletal organization [44–46].

The loss of function of either Rab or Rho family proteins caused by N-BPs should correlate with their normal rate of turnover within the cell because there is no evidence to suggest that the lipid component can be lost before the protein is degraded. As each geranylgeranylated protein is synthesized, it is processed to include one or two geranylgeranyl lipids. Therefore, effects of lost geranylgeranylation depend on the normal lifespan of each affected protein. For RhoA, the half-life of the protein has been estimated at 31 hours [47], suggesting that effects of N-BPs, if mediated through this protein, should be observed within a similar or longer time frame. However, time-course analyses suggest that, at least for alendronate, suppression of bone resorption is much quicker, with effects seen within the first 24 hours of treatment [12••,33]. For Cdc42, the half-life is shorter, approximately 15 hours, which could suggest a role for this protein in the overall response, and overexpression of Cdc42 has been shown to alter cytoskeletal structure in the osteoclast [46]. Less is known about the respective half-lives of other geranylgeranylated proteins with known function in the osteoclast. We speculate that the identification of the rate-limiting geranylgeranylated protein with the shortest half-life could lead to the identification of the protein(s) involved in the earliest responses to N-BP treatment.

Conclusions

With recent advances in basic research tools, we have gained substantial information regarding the molecular and cellular mechanisms of action of bisphosphonates. Simple bisphosphonates such as clodronate and etidronate act at the cellular level by inducing osteoclast apoptosis (Fig. 3). The consequent reduction in viable osteoclast number results in a net reduction in bone turnover. At the molecular level, strong evidence suggests that clodronate acts as a sort of "pro-drug" that is metabolized intracellularly to form $AppCCl_2p$, a toxic ATP analog that inhibits the mitochondrial adenine nucleotide translocase, thus inducing osteoclast apoptosis. The molecular mechanism for etidronate induction of apoptosis remains to be firmly established.

In stark contrast to the simple bisphosphonates, N-BPs do not require apoptosis in order to suppress osteoclastic bone resorption. Substantial data show inhibition of bone resorption in the absence of any apoptotic response (Fig. 3). Inhibition of osteoclast function seems to be the consequence of GTPase inactivation and is achieved through loss of protein geranylgeranylation. While we wait for further information regarding exactly which GTPases are responsible, vesicular and cytoskeletal analyses suggest that the responsible agents may be GTPases of the Rho and Rab families. The process of narrowing the possibilities to one or few GTPases is hampered by the fact that the N-BPs inhibit FPP synthase, with a resulting broad inhibition of geranylgeranylation, along with farnesylation and cholesterol synthesis. To that extent, the major advances over the past few years have related to the identification of this metabolic pathway, specifically FPP synthase, as the target of the N-BPs and the identification of geranylgeranylation as the key downstream process suppressed by these agents. Future identification of key GTPase targets that lie further downstream may provide additional possibilities for the development of novel osteoporosis therapies.

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- •• Of major importance

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