Nitrogen-Containing Bisphosphonate Mechanism of Action

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Abstract: The current paradigm for drug discovery requires the identification of a target involved in the disease process (e.g. enzyme or receptor) and the development of an appropriate ligand (activator, inhibitor or selective modulator). Selection of ligands for clinical development is based on the therapeutic window between efficacy vs. safety and ADME (absorption, distribution, metabolism and elimination) considerations. For bisphosphonates (BPs) the process has not followed that paradigm. BPs have very low absorption and are retained in bone, their target tissue. A few have been used on a limited basis for over 20 years in diseases of rapid bone destruction (e.g. post-menopausal osteoporosis, Paget's disease, bone metastases, etc.), without understanding their molecular mechanism of action. The nitrogen-containing BPs (N-BPs) are the latest and most potent addition to this family of compounds and have the widest use. They have high potency, are specifically targeted to the osteoclast on bone and are used at very low doses (5-10 mg clinically). Over the last four years, there was significant progress in elucidating the mechanism of action of BPs, both lacking and containing nitrogen. This review will focus on the mechanism of action of the N-BPs, specifically alendronate (ALN) and risedronate (RIS), the two agents most widely used. For these and all other N-BPs, the molecular target is the isoprenoid biosynthetic enzyme, farnesyl diphosphate synthase, in the cholesterol biosynthesis pathway. Although inhibition of this enzyme by N-BPs results in the suppression of sterol biosynthesis, it is actually disruption of a branch pathway, isoprenylation, that is responsible for N-BP pharmacological activity. Isoprenylation involves covalent linkage of the 15 or 20 carbon isoprene moiety farnesyl diphosphate or geranylgeranyl diphosphate, respectively, to the carboxy-terminus of regulatory proteins, including the small GTPases Ras, Rac, Rho and Cdc42. The latter three, as well as numerous others, are geranylgeranylated and play a rate-limiting role in the activity of the bone-resorbing osteoclast. This targeted osteoclast inhibition accounts for the potency of the N-BPs and for their ability to elicit the desired therapeutic response of suppressing bone turnover. The occasional gastrointestinal irritation caused by N-BPs appears to be mechanism-based and is also briefly reviewed.

INTRODUCTION

Until recently, the mechanism of action of bisphosphonates (BPs) on bone, especially at the molecular/biochemical level, was not well understood. Advances over the last several years have provided new insights that are covered in this review. For didactic simplicity, we shall summarize the mechanism at the molecular, cellular and tissue levels, starting with BP structure.

The bisphosphonates (BPs), in particular alendronate (ALN) and risedronate (RIS), are the only non-hormonal agents shown so far to reduce the risk of both spine and non-vertebral osteoporotic fractures. Depending on the BP, they are widely used for the treatment and prevention of osteoporosis in postmenopausal women, in men and in glucocorticoid-treated patients [1-13]. Osteoporosis is defined as a reduction in bone mass, measurable by dual-beam x-ray absorptiometry (DXA), and a change in bone microarchitecture, associated with increased bone fragility and fracture risk. The most common cause of osteoporosis is estrogen deficiency following menopause, a condition characterized by increased bone turnover and excessive bone resorption (destruction), relative to bone formation. The imbalance between the two processes causes bone loss of up to 4-5%/year during the first years post menopause. Extensive cross-sectional and prospective epidemiological studies as well as more recent therapeutic trials have shown a close correlation between the reduction in bone mineral density and the increase in fracture risk [14-17]. A similar correlation between increased bone turnover and increased fracture risk was also reported [1, 18, 19]. Incidence of fracture increases with age, an independent contributor to fracture risk. The earliest osteoporotic fractures are in the wrist. The most common fractures occur in the spine. Their incidence increases significantly in women in the seventh decade of life and in men about a decade later. The most serious fractures are those of the hip, they increase exponentially with age and reach an incidence of about 5%/year in the ninth decade of life. The lifetime risk of osteoporotic fractures in 50 year old Caucasian women in the US is 45% [20]. About 25-30% of all hip fractures occur in men. With the continued increase in life expectancy, it is projected that the incidence of osteoporotic fractures will reach epidemic proportions within the next couple of decades if effective means to combat them are not developed or implemented. BPs, the most effective treatments available to date, have been shown to reduce the risk of vertebral and hip fractures by up to 60%, depending on the specific BP and regimen [3, 4, 7, 8, 10, 11, 13, 21-23]. The mode of action of some of these compounds is the subject of this review.

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BP STRUCTURE

BPs are analogs of pyrophosphate (P-O-P) in which the geminal oxygen has been substituted by carbon (Fig. (**1**)). There is no enzyme capable of cleaving the P-C-P bond, which minimizes the possibility for metabolism, and none has been detected for ALN [24, 25]. A main feature of the P-C-P backbone is that by adhering to the hydroxyapatite component of bone it localizes these compounds in the target tissue. While the affinity is low (Kd in the low to sub-millimolar range), the skeleton has a large surface area and virtually an unsaturable capacity for these compounds.

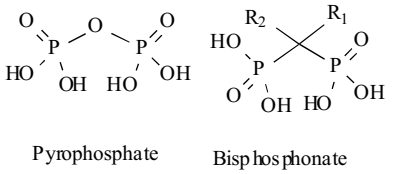

Fig. (1).

Substituents of the geminal carbon of the bisphosphonate can enhance both affinity for bone and efficacy in suppressing bone resorption. In particular, the presence of a hydroxyl at R1 has been shown to increase BP affinity to bone [26], while the presence of a nitrogen approximately 3-4 positions from the geminal carbon can greatly enhance antiresorptive potency (discussed below). The various carbon side chains thus generate a large family of compounds (Fig. (**2**)) with different pharmacological and toxicological properties.

PHARMACOKINETICS

The P-C-P backbone of the BP group endows the entire class with several common properties, especially regarding pharmacokinetics. The bulky and highly charged phosphonate moieties limit absorption in the gut to 2% or less. Following absorption, BPs are rapidly cleared from the circulation, as about 50% binds to the hydroxyapatite bone mineral and 50% being excreted in the urine in part by an active secretion process. The half-life in the circulation is around one hour. Based on studies of alendronate (ALN),

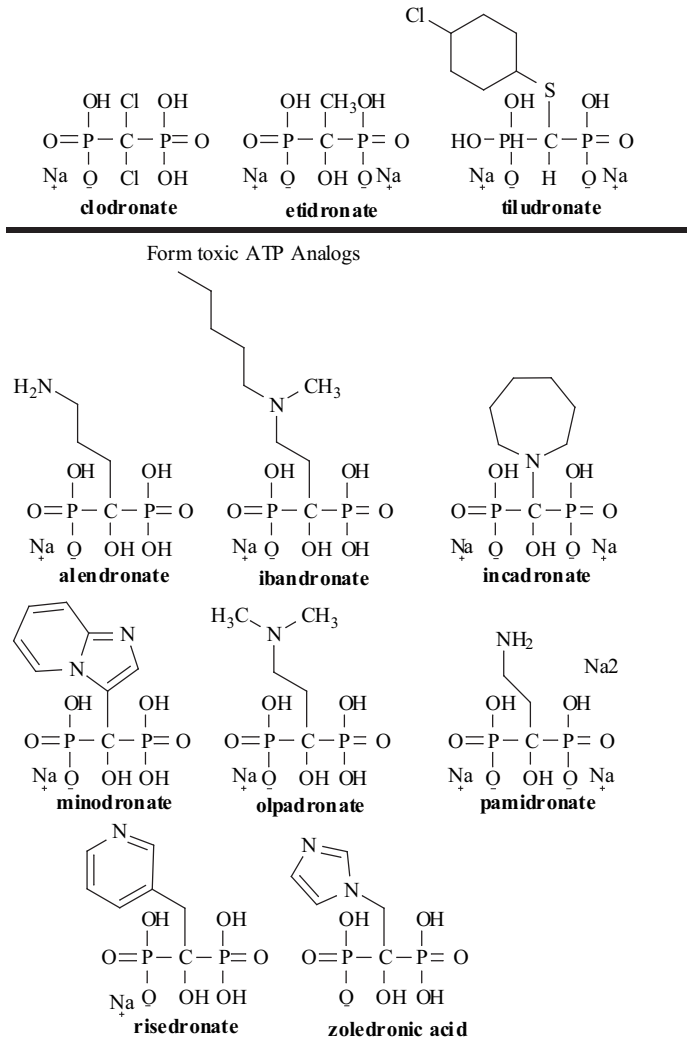

Fig. (2).

over 90% of the BP (not retained on the bone surface) is excreted within the first 24 hours during the first elimination phase. In the second elimination phase, ALN has a half life of about 10 days. The hydrophilic phosphonate moieties limit the penetration of these molecules through cellular lipid bilayer membranes to undetectable levels, thus distribution is essentially limited to bone.

On bone BPs bind, as mentioned, to the surface, absorbing to the exposed mineral calcium hydroxyapatite. It was shown that sites of active bone resorption are the preferential sites for ALN uptake in bone at pharmacologically relevant doses [27-29]. However, for the weaker bisphosphonates, such as etidronate, the required dosing is substantially higher. This results in essentially non-selective distribution on bone at resorption, formation and quiescent sites [29]. The BP that has localized on the resorption surface is eventually covered as a result of subsequent bone formation. It remains covered until it is released back into the circulation as part of the normal turnover of that bone. Rates of turnover from both cortical and cancellous bone determine not only the subsequent release of BPs but also the relative uptake and distribution of BPs when administered. The cancellous bone takes up a relatively larger proportion of the absorbed BP than the cortical bone, since cancellous bone is subject to substantially higher turnover. Accurate assessments of terminal half-life require a substantial follow-up, since the curve for elimination from bone remains non-linear for months. The average terminal elimination half-life of ALN from the skeleton, estimated by urinary excretion in an 18 month follow-up study, is about 10 years. Nonetheless, low dosing and low absorption translate into a very low body burden of the potent BPs.

BISPHOSPHONATE ACTION AT THE MOLECULAR LEVEL

Although used clinically for over two decades, the molecular targets of the BPs have only recently been identified. Over the years, BPs were shown to affect several biochemical pathways, especially those involving phosphate. For example, the BP tiludronate was reported to inhibit the vacuolar ATPase [30] and numerous BPs inhibit the activity of several protein tyrosine phosphatases [31-35]. These actions occur usually at the upper range of pharmacologically relevant concentrations and do not correlate with the pharmacological potency of these agents. Although these activities could be involved in the mechanism of action of some BPs, more compelling proof was obtained for the molecular targets responsible for BP inhibition of osteoclastic bone resorption described below.

Conversion of BPs to Toxic ATP Analogs

A most probable mechanism of action for clodronate involves the incorporation of the P-C-P backbone into the β,γ positions of adenosine triphosphate, ultimately generating a toxic ATP metabolite, $AppCCl_2p$. This was first documented for methylenebisphosphonate (medronate) metabolism by *Dictyostelium discoideum* [36]. Methylene-containing analogs of ATP and diadenosine tetraphosphate (Ap_4A) were identified, suggesting that other BPs might also be similarly metabolized. It was found that clodronate can be converted into $AppCCl_2p$, whereby it substitutes for pyrophosphate in the reverse reaction of ATP-dependent tRNA formation [37]. There is also solid evidence that microinjection of the metabolite, or its introduction via liposomes, into osteoclasts leads to induction of osteoclast apoptosis.

The more potent BPs, which contain nitrogen in their structure, failed to incorporate into ATP, suggesting a different mechanism. However the above mechanism has been implied for the action of etidronate and possibly tiludronate, although the respective metabolites for these BPs accumulate to a far lesser degree, perhaps due to instability of those metabolites.

BP Inhibitors of Cholesterol Synthesis

Over ten years ago it was shown that certain BP derivatives (isoprenoid [phosphinylmethyl] phosphonates) weakly inhibit the cholesterol biosynthetic enzyme, squalene synthase [38]. The search for more potent inhibitors that might block cholesterol production revealed that the N-BPs incadronate (YM175) and ibandronate potently inhibit squalene synthase [39]. Subsequent studies examined the structure-activity relationship for inhibition of squalene synthase [40-42]. *In vivo* testing showed that certain compounds suppressed serum cholesterol in rodents [40]. Other cholesterol lowering bisphosphonates were shown to cause degradation of hydroxymethylglutaryl coenzyme A [43, 44]. In the same context, utility of squalene synthase inhibition by bisphosphonate was also used for the development of an assay to measure zoledronate levels in animals and clinical serum samples [45].

FPP Synthase as the Molecular Target of the N-BPs

While N-BP potency for inhibition of bone resorption does not correlate with inhibition of squalene synthase, these studies raised the possibility that the cholesterol biosynthetic pathway could be a target for the effects of N-BPs on bone resorption. For instance, it was originally found that ALN and pamidronate failed to affect squalene synthase, yet they did inhibit sterol biosynthesis with IC_{50}s of 170 and 420 nM, respectively [39], suggesting potential inhibition of a target upstream of squalene synthase in the mevalonate pathway (Fig. (**3**)). In recent studies, the enzyme inhibited by all N-BPs examined to date was identified as farnesyl diphosphate (FPP) synthase [46-49]. Modeling of the interaction between bisphosphonate and FPP synthase suggests binding to the geranyl diphosphate site [50], where they act as transition-state analogs, or to the binding site of isopentenyl diphosphate [51]. Enzymological studies suggest that inhibition of FPP synthase is indeed complex [52]. Both competitive and non-competitive inhibition is seen, depending on the substrate used in the assay, isopentenyl diphosphate or geranyl diphosphate, respectively. Other studies have centered on the structure-activity relationship for bisphosphonate inhibition of FPP synthase. While RIS is a potent inhibitor of FPP synthase, modifications (e.g. addition of a methyl group) to the structure of the side chain give rise to analogs that are markedly less potent inhibitors of FPP synthase and less

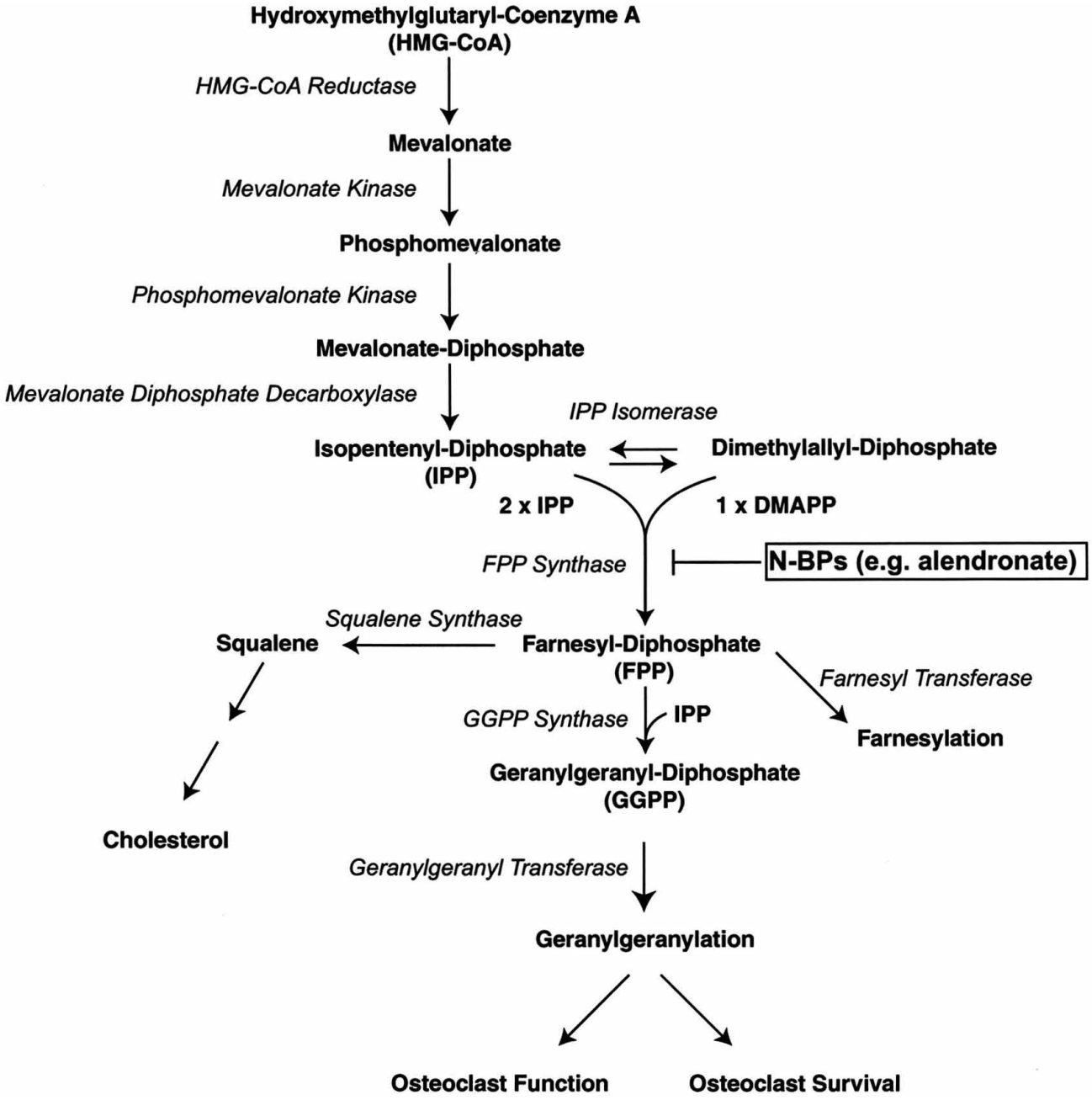

Fig. (3).

effective inhibitors of bone resorption *in vivo* [49]. The variable conferring potency against FPP synthase relates to the position of the nitrogen group relative to the phosphonate groups. A modification in one of the phosphonate groups of RIS, while drastically reducing FPP synthase inhibition, gave rise to new activity against type II geranylgeranyl transferase [53]. This derivative has substantially less antiresorptive activity than RIS *in vivo*, although this is in part due to reduced binding to bone [54]. This suggests that FPP synthase is the better target for suppression of osteoclastic bone resorption. Indeed, for a wide range of N-BPs, there is a significant correlation between potency for inhibition of FPP synthase and anti-resorptive potency *in vivo* [49].

The most potent anti-resorptive N-BPs, such as zoledronate, are extremely potent inhibitors of FPP synthase with IC_{50}s in the low nanomolar range [49]. However, this alone cannot accurately predict activity in the clinical setting, where the range of potency is much narrower than that seen *in vitro* or in animal models. For instance, zoledronate is about 70-fold more potent than pamidronate in the inhibition of FPP synthase [49], up to1000-fold more potent than pamidronate in the rat [55], but clinical testing suggests a potency ratio of about 20-fold [56].

Inhibition of FPP Synthase Blocks Protein Isoprenylation and Sterol Synthesis

FPP synthase is responsible for the production of isoprenoid lipids FPP (15 carbon) and geranylgeranyl diphosphate (GGPP) (20 carbon). While FPP can be used to synthesize cholesterol, it is actually another process called protein isoprenylation that is critical for N-BP effects on

suppressing osteoclastic bone resorption. Isoprenylation involves the transfer of a farnesyl or geranylgeranyl lipid group onto a cysteine amino acid residue in characteristic carboxy-terminal (e.g. CAAX) motifs [57, 58]. Most of the isoprenylated proteins identified to date are small GTPases that are geranylgeranylated [57]. These signaling proteins are important for the regulation of a variety of cell processes required for osteoclast function, including cytoskeletal regulation, formation of the ruffled border and regulation of apoptosis [59-62].

The ability of N-BPs to inhibit the cholesterol biosynthetic pathway and protein isoprenylation was first demonstrated in J774 macrophages [39, 63]. RIS almost completely inhibited protein isoprenylation at a concentration similar to the concentration that affects osteoclast viability *in vitro* [64-66] and could be achieved within the osteoclast resorption lacuna [27]. N-BPs (zoledronate, RIS, ibandronate, ALN, and pamidronate) also inhibit protein isoprenylation in osteoclasts *in vitro*. Alendronate inhibits incorporation of [^{14}C]mevalonate into either isoprenylated proteins or sterols in purified murine osteoclasts [46], while another study found several N-BPs, but not the non-N-BPs (clodronate, etidronate or tiludronate), prevented incorporation of mevalonate into isoprenylated proteins in purified rabbit osteoclasts [67].

Evidence for Molecular Mechanisms *in Vivo*

The molecular actions of the N-BPs, described above, have recently been confirmed *in vivo* using surrogate markers [68, 69]. In one study, the previously documented feedback regulation of HMG-CoA reductase expression by mevalonate pathway metabolites was examined [68]. The N-BPs alendronate, ibandronate and risedronate, but not clodronate or etidronate (which lack nitrogen), suppressed expression of HMG-CoA reductase in osteoclasts from the proximal tibia. While all three N-BPs induced changes in HMG-CoA reductase expression in the osteoclast, no changes were seen in other bone or marrow-associated cells, which is consistent with the observed targeting of alendronate to the osteoclast [27, 29]. This effect on HMG-CoA reductase is consistent with earlier observations showing that the bisphosphonate SR-12813 induces its degradation [43]. The effect observed in the osteoclast appeared to be mediated, in part, by the accumulation of metabolites upstream of the BP-inhibited enzyme FPP synthase, since co-administration of simvastatin along with ALN partially blocked the effect. The loss of HMG-CoA reductase expression along with inhibition of FPP synthase in the osteoclast could potentially have additive effects on the mevalonate/cholesterol biosynthetic pathway. In another study, osteoclasts were examined for the *in vivo* actions of both ALN and clodronate [69]. In osteoclasts purified after ALN treatment, geranylgeranylation of the small GTPase Rap1A was suppressed.

The mechanism of action for clodronate was also documented *in vivo* measuring the generation of the $AppCCl_2p$ metabolite [69]. Animals injected with clodronate, but not alendronate, accumulated $AppCCl_2p$ in osteoclasts but not other cells found in bone marrow. Similarly, liposome-encapsulated clodronate, which targets the drug to macrophages, caused accumulation of $AppCCl_2p$. In light of the convincing *in vitro* and *in vivo* data, there is strong support for dividing the bisphosphonates so far into two general classes based on their mechanism of action. These include inhibitors of FPP synthase that suppress protein isoprenylation (the N-BPs) and ATP mimics in the aminoacyl-tRNA synthetase pathway that form cytotoxic ATP analogs (the non-N-BPs).

MECHANISM OF ACTION AT THE CELLULAR LEVEL

The relationship between molecular action and anti-resorptive effects has been documented for BPs lacking and containing nitrogen. For the non-N-BPs, such as clodronate, the induction of apoptosis is likely due to the formation of toxic ATP analogs, as discussed above. The ability of $AppCCl_2p$ to cause osteoclast apoptosis is likely related to its inhibition of the adenine nucleotide translocase [70], a component of the permeability transition pore complex in the inner mitochondrial membrane.

Perhaps best documentation for a cause-effect relationship has been established for the N-BPs, where inhibition of FPP synthase and inhibition of bone resorption were linked. Evidence is based on the ability of downstream metabolites to suppress the inhibitory effects of N-BPs. Among the downstream metabolites, only geranylgeraniol, a lipid alcohol that can replenish geranylgeranyl diphosphate (GGPP), prevents inhibition of osteoclast formation and bone resorption by the N-BPs, as demonstrated in the presence of inhibitory concentrations of ALN or ibandronate [71, 72]. Mevalonate, an upstream metabolite, can partially rescue inhibition of resorption, although this effect disappears with increasing concentration of bisphosphonate [63, 71]. This is consistent with very recent data suggesting, in part, competitive inhibition of FPP synthase by N-BPs [52]. Furthermore, a selective inhibitor of protein geranylgeranylation (GGTI-298) also inhibits bone resorption *in vitro* [67]. Other metabolites feeding into farnesylation or sterol synthesis are without effect. Finally, inhibition of bone resorption, osteoclast formation and disruption of the osteoclast actin cytoskeleton by lovastatin and mevastatin can also be blocked by addition of geranylgeraniol to osteoclast cultures [71, 73]. Taken together, these observations strongly suggest that N-BP inhibition of bone resorption is a consequence of loss of geranylgeranylated proteins.

In addressing the cellular mechanisms related to suppression of bone resorption, substantial evidence has accumulated to link loss of geranylgeranylation to induction of osteoclast apoptosis, disruption of the actin cytoskeleton and altered membrane trafficking [67, 74-77]. It was reported that bisphosphonates induce osteoclast apoptosis, both *in vitro* and *in vivo*, both in normal mice and in mice with increased bone resorption [78]. The apoptotic action of both N-BPs and BPs lacking nitrogen results from intracellular action within the osteoclast, as opposed to other indirect actions via osteoblasts [74]. The likelihood that N-BPs cause apoptosis by interfering with isoprenylated proteins in osteoclasts was demonstrated by blocking the effect simply by replacing GGPP. Induction of osteoclast apoptosis by ALN and RIS, but not clodronate or etidronate, was blocked by addition of geranylgeraniol, but not farnesol, suggesting

that only geranylgeranylation was critical [74]. Consistent with this, an inhibitor of geranylgeranylation (GGTI-298), but not farnesylation (FTI-277), can induce osteoclast apoptosis *in vitro* [67]. In macrophages on the other hand, both FPP and GGPP can prevent apoptosis, perhaps suggesting easier conversion of FPP to GGPP in these cells [63, 79-81].

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 [82]. 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 and a pro-apoptotic kinase, MST1. Caspase 3 is the major effector caspase activated in osteoclasts undergoing apoptosis following treatment with a range of bisphosphonates *in vitro* [81]. MST1 kinase acts as both a substrate for caspases 3, 7 and 9 and as an activator of these caspases [83-85]. MST1 was identified as a pro-apoptotic signaling intermediate downstream of the bisphosphonates that is activated during apoptosis by N-BPs, lovastatin and clodronate [74]. Caspase cleavage of MST1 results in the formation of an unregulated, highly active kinase species, shown to cause nuclear condensation [86].

While 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 bisphosphonate. More often, suppression of resorption is seen prior to reductions in osteoclast number, suggesting direct inhibition of osteoclast function by the bisphosphonate. It was reported long ago that, following bisphosphonate administration, osteoclasts show changes in morphology and appear inactive [87]. The changes are numerous [64] and include alterations in the cytoskeleton, including actin and vinculin as well as disruption of the ruffled border [27, 87-89]. An observation that defines the N-BPs as a class is the increase in osteoclast number found *in vivo* within about 48 hr after treatment with alendronate, ibandronate and risedronate, but not clodronate or etidronate [68]. Previous studies reported that alendronate treatment increased osteoclast number along with bone surface [90, 91], however, recently an early increase was seen as soon as inhibition of bone resorption occurred [68]. Consistent with direct inhibition of the osteoclast, N-BPs, ALN and RIS, were shown to disrupt the actin cytoskeleton, a marker for disrupted function, prior to induction of apoptosis [75]. On the other hand, with etidronate, a non-N-BP, the two effects were simultaneous. In separate studies, electron microscopic examination revealed apoptotic osteoclasts associated with resorption inhibition by clodronate, while with alendronate, morphology was altered (retracted cells, loss of microvilli) without substantial evidence of apoptosis [76]. Importantly Z-VAD-FMK, an inhibitor of apoptosis, which maintains osteoclast numbers *in vitro*, cannot suppress ALN or RIS inhibition of bone resorption [75]. However, for clodronate and etidronate, interference with the induction of apoptosis was sufficient to significantly and substantially increase bone resorption. Therefore, while a post-apoptotic osteoclast would be incapable of bone resorption, it is important to note that apoptosis may serve as the primary mechanism of bone resorption inhibition only for clodronate and etidronate and the secondary mechanism for ALN, RIS and other N-BPs.

Based on these observations, other means of suppressing osteoclastic bone resorption seem more likely for the N-BPs when administered at clinically-relevant doses. As mentioned above, all BPs are rapidly taken up by the skeleton and localize preferentially on exposed mineral at bone resorption surfaces. Osteoclasts, the bone resorbing cells, attach to the exposed mineral and start the bone resorption process. The result of the intracellular action of nitrogen-containing BPs, shown for pamidronate and ALN [27, 92], is disappearance of the ruffled border, while osteoclast morphology shifts towards the generation of large and plump cells [68]. The ruffled border is convoluted membrane, which faces the bone surface and acts as a hallmark of active osteoclasts. Ruffled border formation is a process that is highly dependent on cytoskeletal function, strongly regulated by geranylgeranylated GTP binding proteins, such as Rac, Rho, etc. Moreover, the vesicles normally located above (that disappear after nitrogen-BP treatment) are needed for the formation of the ruffled border and the trafficking of these vesicles is largely under the control of the Rabs, which are also geranylgeranylated. Disappearance of the ruffled border therefore, provides morphological evidence for mechanism-based osteoclast inactivation and could explain the lack of acid extrusion caused by ALN in isolated osteoclasts [93].

It was recently shown that during resorption osteoclasts internalize the content of the resorption lacunae *via* the ruffled border and translocate it through the cell by a process of transcytosis [94]. Several years ago it was documented by microradiography that following radioactive ALN administration *in vivo*, the BP can be detected inside the osteoclast four hours later [27, 29], consistent with the recently shown transcytotic uptake. Other studies have pointed to a requirement for cellular BP uptake for its ultimate effect. It was shown *in vitro* that osteoclasts that have lost the ability to take up material from their surroundings, due to a mutation oc/oc do not respond to tiludronate, as measured by disruption of their actin ring [89]. This effect was produced, however, by microinjecting the BP into the cells. Ruffled border is not required, however, for incadronate (YM-175) to induce osteoclast apoptosis when injected at high dose (1 mg/kg) into oc/oc mice [95]. It is possible, therefore, for bisphosphonates to enter the osteoclast *via* a second route. Finally, slime mold growth inhibition by BPs is reduced when pinocytosis is inhibited [96]. Taken together, the ruffled border seems to be required for N-BP uptake. However, since the N-BPs subsequently suppress the formation and function of this cellular structure, inhibition of the transcytosis process not only results in suppression of osteoclastic bone resorption, but may also limit intracellular exposure to the N-BP. This then might reduce exposure and the likelihood that the osteoclast undergoes apoptosis.

MODE OF ACTION AT THE TISSUE LEVEL

Osteoporosis and other types of bone loss are associated with increased bone turnover and elevated levels of bone resorption. Osteoclastic bone resorption occurs in the first stage of the bone remodeling process, which can be effectively slowed by inhibiting osteoclast generation, osteoclast activity or both. BPs are to date the most effective inhibitors of bone resorption. BP improvement of mechanical strength, reflected in a reduction in fracture risk, is caused by an increase in bone mass and mineralization (see below) as well as by an improvement in architecture, attributable to a reduction in bone turnover. A higher number of bone remodeling sites, where excessive osteoclastic destruction of bone takes place, leads to formation of areas of stress concentration and thus increased fracture risk. By reducing turnover of bisphosphonates can reverse this condition. Effects on bone turnover are estimated by measuring either *C*-terminal or *N*-terminal collagen degradation products in the urine or in the blood. BP-induced suppression of these markers can be detected within days, and maximal effects are reached within a few weeks when levels stabilize and remain suppressed for the duration of treatment, followed up to ten-years for ALN so far [97]. Bone formation is also suppressed, albeit later than resorption, as part of the reduction in bone turnover, reaching a nadir at three to six months. This is probably a reflection of the so-called "coupling" between resorption and formation whereby, through mechanisms that have not been fully elucidated, changes in resorption engender changes in formation in the same direction. Another mechanism for increased bone strength is the increase in mineralization associated with lower bone turnover [98-100]. This has been described in alendronate treated baboons [98] and more recently in osteoporotic women [99, 100]. Lower turnover lengthens the life span of the BMU (basic multicellular unit), thus permitting it to mineralize more completely and increase mineral content. BMD (bone mineral density) or BMC (bone mineral content), measure the combined BP effects on bone mass and mineralization. The initial rise in bone mass measured by dual beam X-ray absorptiometry is caused by the continued rebuilding of BMUs that were initiated prior to bisphosphonate treatment. BPs also reduce the number of new BMUs, and at individual BMUs, they act by decreasing the depth of resorption and possibly increasing wall width during the formation phase [101]. A continuous increase in spinal BMD was observed during 10 years treatment of postmenopausal women with ALN [97]. Increases in BMD, mineralization and crystal formation are associated with improvements in bone strength. Increased bone strength following BP treatment has been documented in experimental animals by ex vivo biomechanical testing [102-106] and is reflected in the reduction in fracture risk observed in clinical trials.

It is important to note here that very high doses of ALN and RIS (six times above clinical dosing) administered for a period of one year can suppress bone turnover in dogs by up to 95% and cause accumulation of microdamage in both cortical and cancellous bone [107]. Interestingly, for both ALN and RIS, microdamage in dog bones was not associated with any extrinsic biomechanical property, although it was associated with an increase in compressive strength. At such high doses, the distribution of BP is more likely to be non-selective for the osteoclast surface [29], as would otherwise be seen with clinically relevant dosing. Indeed, the documented changes in distribution of BPs at low and high doses makes difficult the process of modeling long-term effects by brief administration to animals at high doses. Our best comparator for this type of modeling comes from the ten-year data for ALN (extension of Phase III clinical trial), which show no increase in non-vertebral fracture risk when years 8-10 are compared to years 1-3 [97]. This suggests either absence of microdamage accumulation or a lack of relevance of microdamage to fracture risk. Consistent with the latter, elderly women with and without femoral neck fractures were found to have the same degree of microdamage accumulation [108], suggesting that microdamage itself is not a predictor of fracture risk.

RELATIONSHIP BETWEEN MECHANISM OF ACTION AND TOXICOLOGY

Toxicological animal studies have been published on ALN, clodronate, etidronate, incadronate, pamidronate, and tiludronate. When bisphosphonates are administered subcutaneously, local toxicity can occur, with local necrosis. This is especially the case for the N-BPs. This is consistent with anecdotal and clinical evidence on irritation of the gastric lining in occasional patients [109, 110].

Following the appearance in humans of gastrointestinal adverse events, after oral administration of *N*-containing bisphosphonates [111], the effects of oral bisphosphonates were examined in special studies in animals. ALN, given orally to rats at suprapharmacological doses, has been reported to induce gastric and esophageal erosions and ulcerations and delay healing of indomethacin-induced gastric erosions. These effects were not attributable to changes in gastric acid secretion, or prostaglandin synthesis, but are thought to be due to a topical irritant effect. Similar effects were reported with etidronate, risedronate, and tiludronate when given at pharmacologically equivalent doses [112-114].

To address the issue of mechanism, recent studies have examined apoptosis and suppression of cell growth in *in vitro* models of the esophageal stratified epithelium and the large intestine [115-118]. In CACO-2 intestinal epithelium cells and in Ch1.Es esophageal fibroblasts, the N-BPs induced apoptosis, which could be blocked by the addition of geranylgeraniol [115, 117, 118]. This suggested that N-BP inhibition of protein geranylgeranylation was instrumental in the apoptotic response. In normal human epidermal keratinocytes [116] and, as a second phenotype, in CACO-2 cells, growth suppression was observed in response to N-BP treatment. In the keratinocyte, used as a model for stratified squamous epithelium lining the esophagus, this was associated with both suppression of cholesterol biosynthesis and protein geranylgeranylation (See Fig. (**2**)). Reduced cell growth was linked to a block in regulation of proteins (cyclin-dependent kinases) that control the cell cycle [116]. These recent in vitro studies suggest that N-BP-induced gastrointestinal irritation and / or delayed repair of irritation are mediated by inhibition of FPP synthase in the affected tissues. These findings have recently been validated *in vivo*, where irritation at the site of subcutaneous injection was blocked by co-administration of an agent that causes accumulation of isoprenoids (e.g. FPP and GGPP) [118].

In conclusion, recent data have identified the mevalonate pathway enzyme, farnesyl diphosphate synthase, as the primary molecular target of the potent N-BPS. Inhibition of this enzyme reduces the isoprenylation of regulatory proteins in osteoclasts, causing their inactivation. Osteoclasts are the only cells exposed to high enough N-BP concentrations to show this response, except for GI cells, which occasionally are also subject to analogous mechanism-based suppressive effects.

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