Bisphosphonate Mechanism of Action

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Abstract: Nitrogen-containing bisphosphonates (N-BPs) are potent inhibitors of bone resorption widely used in the treatment of osteoporosis and other bone degrading disorders. At the tissue level, N-BPs reduce bone turnover, increase bone mass and mineralization, measured clinically as a rise in bone mineral density, increase bone strength and reduce fracture risk. At the cellular level, N-BPs, localize preferentially at sites of bone resorption, where mineral is exposed, are taken up by ostoclasts and inhibit osteoclast activity. The bone formation that follows incroporates the N-BP in the matrix, where it becomes pharmacologically inactive until released at a future time during bone remodeling. At the molecular level, N-BPs inhibit an enzyme in the cholesterol synthesis pathway, farnesyl diphosphate synthase. As a result, there is a reduction in the lipid geranylgeranyl diphosphate, which prenylates GTPases required for cytoskeletal organization and vesicular traffic in the osteoclast, leading to osteoclast inactivation.

Keywords: Bisphosphonates, osteoporosis, alendronate, cholesterol pathway, prenylation, osteoclasts, bone remodeling

Bisphosphonates (BPs), in particular the nitrogen-containing BPs alendronate (ALN) and risedronate (RIS) are the only pharmacological agents shown so far to reduce the risk of both spine and non-vertebral osteoporotic fractures. BPs 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, resulting in 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 epidemiological studies as well as more recent prospective studies have shown a close correlation between the reduction in bone mineral density and the increase in fracture risk [14,15]. A similar, albeit less firm, correlation between increased bone turnover and increased fracture risk was also reported [1,16]. By the same token randomized, placebo-controlled therapeutic trials with BPs have shown that increased bone density and decreased bone turnover correlate with reduced fracture risk. Incidence of fracture increases with age, an independent contributor to fracture risk. 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. 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 [2-4,7,8,10,11,13,17,18].

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, which will be briefly reviewed here. For didactic simplicity, we shall summarize the mechanism at the tissue, cellular and molecular levels, starting with BP structure and pharmacokinetics.

BISPHOSPHONATE STRUCTURE AND PHARMACOKINETICS

BPs are analogs of pyrophosphate (P-O-P) in which the bridging oxygen has been substituted by carbon. 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 [19,20]. The carbon side chains generate a large family of compounds with different pharmacological

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and toxicological properties. Some of these compounds inhibit bone resorption and have been or are being developed for this therapeutic indication. The BP group endows this whole class with several common properties, especially regarding pharmacokinetics. The bulky and highly charged phosphonate moieties limit absorption in the gut to around 1% [20]. Following absorption BPs, which are very hydrophilic, are rapidly cleared from the circulation, about 50% binding 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 about one hour or less. Based on studies of ALN, over 90% of the BP not retained on the bone surface is excreted within the first 24 hours. The bulky charged phosphonate moieties limit the penetration of these molecules through cellular lipid bilayer membranes to undetectable levels, thus virtually precluding exposure of all organs to these drugs, except bone.

In bone, BPs bind, as mentioned, to the bone surface, absorbing to the exposed mineral calcium hydroxyapatite. The most accessible exposed mineral surfaces are at sites of bone resorption, and it was shown that these are the preferential sites for ALN uptake in bone when administered at pharmacologically relevant doses [21,22]. 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. Turnover of 30% per year was estimated to occur for the cancellous bone, which constitutes 20% of the skeleton, while 3% per year is the estimated turnover of cortical bone, which constitutes the remaining 80% of the skeleton [23]. These rates of turnover from these two compartments determine not only the release of BPs but also the relative uptake and distribution of BPs when administered, a relatively larger proportion being taken up by the cancellous bone than by cortical bone. The average terminal elimination half-life of ALN from the skeleton, estimated by urinary excretion in an 18 months follow up study, is about 10 years.

Thus, to summarize this section, absorption of BPs is very low, and their half-life in the circulation is short, with about half being excreted in the urine and the other half taken up by bone. On bone they concentrate preferentially on resorption surfaces and are later buried by the new bone formed on top of the original resorption site. There is no metabolism of the Pi-C-Pi bonds and there is minimal exposure to BPs of other tissues than bone. The body burden of the potent BPs is thus minute and the terminal elimination time is long, corresponding approximately to the overall skeletal turnover.

MODE OF ACTION AT THE TISSUE LEVEL

As mentioned above, postmenopausal 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 and were shown to reverse bone loss, with ALN causing an increase in bone mineral density in the spine of 11% (average) by seven years [24]. Both increased BMD and reduced bone turnover resulting from BP treatment have been extensively documented. BMD, estimated by DXA, was shown to increase most in the spine, which contains a relatively large component of cancellous bone (5% in the first year for ALN), with similar increases in the trochanter and somewhat less in the femoral neck, which has a higher content of cortical bone [8,12,18,24,25]. Effects on bone turnover were 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 seven years for ALN so far [24].

Bone formation is also suppressed, albeit later than resorption, as part of the reduction in bone turnover, reaching a nadir at approximately three 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.

As mentioned above, BP reduction of bone turnover is associated with increased bone mineral density, shown to be due in part to improved mineralization [26,27], believed to contribute to the increase in bone strength. Increased bone strength following BP treatment has been documented in experimental animals by ***ex vivo*** biomechanical testing [28-31] and is reflected in the reduction in fracture risk observed in clinical trials.

To summarize this section, at the tissue level BPs: (i) inhibit bone resorption (extensively documented for ALN) and, consequently, (ii) bone turnover and (iii) increase bone density (up to 11% over 7 years in the spine) and (iv) bone strength, assessed by a reduction in fractures and by ***ex vivo*** mechanical testing in animals.

MODE OF ACTION AT THE CELLULAR LEVEL

As mentioned above, 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. It was recently shown that during resorption osteoclasts internalize the content of the resorption lacunae and

translocate it through the cell by a process of transcytosis [32]. 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 [22,33], 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 [34]. 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 [35]. 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 [36].

The result of the intracellular action of nitrogen-containing BPs, shown for pamidronate and ALN [33,37], is disappearance of the ruffled border. This convoluted membrane, which faces the bone surface, is a hallmark of active osteoclasts. Disappearance of the ruffled border is therefore morphological evidence for osteoclast inactivation and could explain the lack of acid extrusion caused by ALN in isolated osteoclasts [38].

As reported, based on ***in vitro*** and ***in vivo*** findings, risedronate increases osteoclast apoptosis [39]. Various BPs were also shown to induce apoptosis in macrophages and tumor cells [40-42]. It was also shown that all clinically used BPs tested can stimulate caspase-dependent cleavage and activation of a signaling protein kinase Mst1 in isolated osteoclasts [43]. The ability to induce apoptosis in isolated osteoclasts suggests direct action. It was therefore generally assumed that induction of osteoclast apoptosis is a cellular mechanism through which BPs suppress bone resorption. However, recent observations on ALN and other nitrogen-containing BPs have indicated that the disruption of the osteoclast cytoskeleton and the associated osteoclast inactivation occurs separately and prior to induction of apoptosis [44]. This study has shown that prevention of apoptosis by pharmacological inhibitors of caspases, whose activity is necessary for BP-induced apoptosis, does not eliminate osteoclast inactivation caused by nitrogen BPs such as ALN and RIS. On the other hand, for BPs that do not contain nitrogen, such as etidronate and clodronate, induction of apoptosis seemed to be the primary mechanism for inhibiting osteoclastic bone resorption. With these BPs bone resorption was significantly increased in the presence of the caspase inhibitor. Thus the BPs can be divided into two categories based on their cellular level of action. For some, such as etidronate and clodronate, induction of apoptosis is a primary means of inhibiting resorption. For others, such as ALN and RIS, the cytoskeleton or ruffled border appears to be the primary site of inhibition.

BISPHOSPHONATE ACTION AT THE MOLECULAR LEVEL

Over the years, BPs were shown to affect several biochemical pathways, especially those involving phosphates. For example, BPs were reported to inhibit alkaline phosphatase, the vacuolar ATPase [45] and all BPs tested inhibited protein tyrosine phosphatases [46-50]. Although these actions occur at pharmacologically relevant concentrations, more compelling proof was obtained for different rate-limiting actions in BP inhibition of osteoclastic bone resorption. Significant insights into the identification of molecular targets for BP action were obtained in the last few years.

It was found that clodronate can be very effectively incorporated into ATP, substituting for pyrophosphate in the reverse reaction of the ATP-dependent tRNA formation, ultimately generating a toxic ATP metabolite [51]. This mechanism of action for clodronate was further confirmed by documenting the generation of this metabolite ***in vivo*** [52]. There is also solid evidence that microinjection of the metabolite, or its introduction via liposomes, into osteoclasts leads to induction of osteoclast apoptosis. A similar 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 the metabolite. Meanwhile, the more potent BPs, which contain nitrogen in their structure, failed to incorporate into ATP, suggesting a different mechanism.

About 10 years ago, it was shown that the nitrogen-containing compounds inhibit cholesterol synthesis in a cell-free system, and some inhibit the cholesterol pathway enzyme squalene synthase [53-57]. Others, such as ALN, are not actual inhibitors of squalene synthase but nonetheless do inhibit cholesterol synthesis, suggesting inhibition of an upstream enzyme in this metabolic pathway (Figure **1**). More recently, Luckman et al. showed that the apoptotic effects of nitrogen-containing BPs can be mimicked by statins [58]. This supports the hypothesis that enzyme(s) in the mevalonate to cholesterol pathway are molecular target(s) for the nitrogen-containing BPs. Using J774 macrophages as a surrogate model for the osteoclast, it was shown that statins, like nitrogen-containing BPs can induce apoptosis in these cells. This can be blocked, but not for etidronate or clodronate, when metabolites from the mevalonate pathway are included in the cultures. Furthermore, the nitrogen-containing compounds inhibited the incorporation of radioactive mevalonate into proteins with a molecular weight of around 20 kilodaltons, supporting the

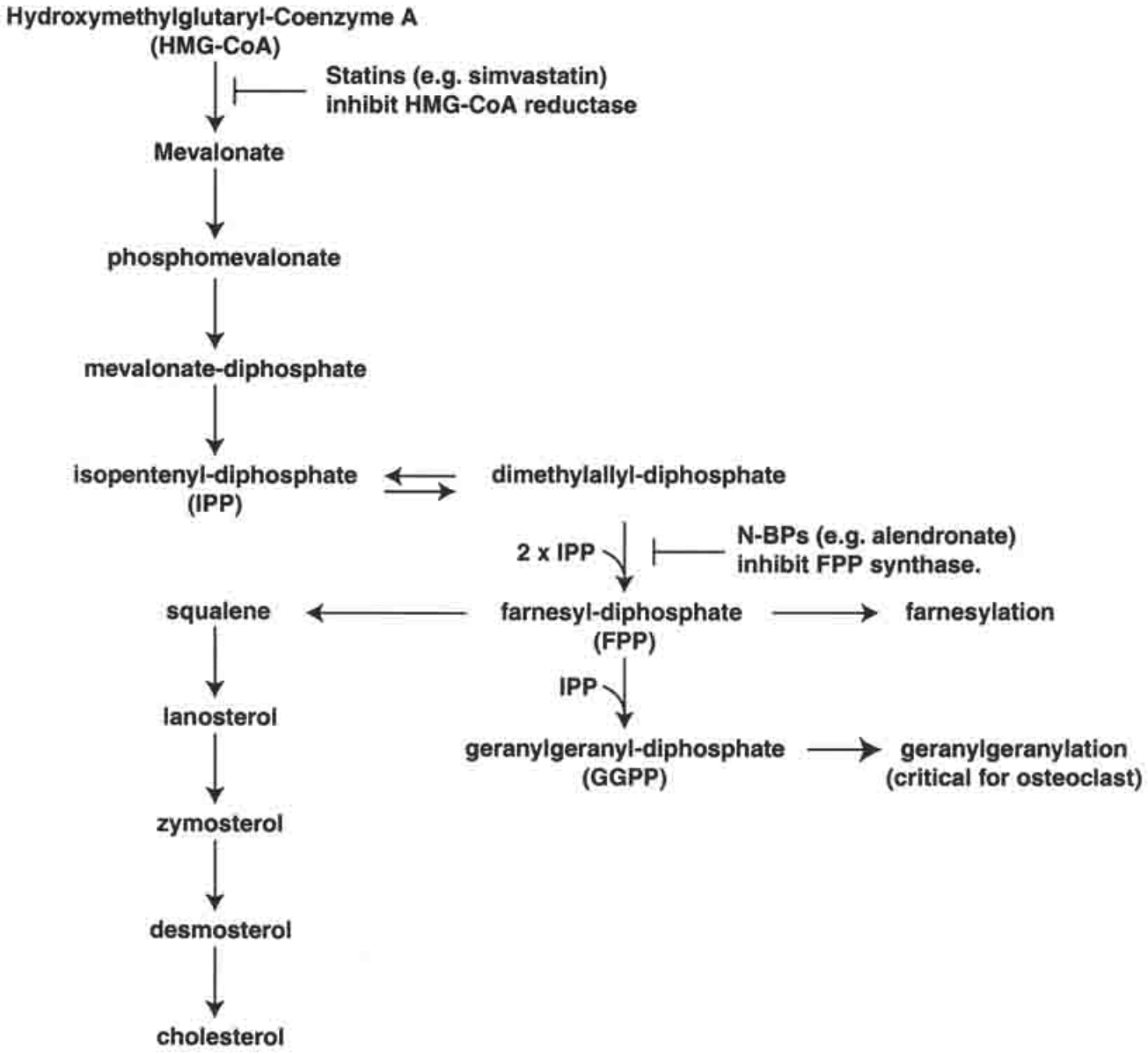

Figure 1. Schematic of the biosynthetic pathway targeted by the N-BPs.

hypothesis that statins and BPs induce apoptosis by blocking prenylation of GTP binding proteins, such as Rho, Rac and Cdc42, which fall into this size range. These observations were followed by the demonstration that statins and N-BPs inhibit osteoclast formation and osteoclastic bone resorption in bone explants and isolated osteoclasts and that geranylgeraniol, but not farnesol, can prevent this effect [59]. Exogenously applied geranylgeraniol is converted in the cell to geranylgeranyl diphosphate, a substrate for geranylgeranyl transferase-I and II, two of the three enzymes involved in protein isoprenylation. Meanwhile, farnesol is converted to farnesyl diphosphate, which is a substrate for the third enzyme, farnesyl transferase. The selective response of the osteoclast to geranylgeraniol contrasts somewhat to that of the macrophage, where both farnesyl diphosphate and geranylgeranyl diphosphate suppress induction of apoptosis [43,58]. These findings suggest that the target for inhibition by any N-BP is located in the mevalonate pathway and upstream of geranylgeranyl diphosphate generation.

More recently, biochemical studies of the mevalonate to cholesterol pathway enzymes identified farnesyl diphosphate synthase as the enzyme inhibited by N-BPs but not by clodronate or etidronate [60-64]. IC50s for the N-BPs fall in the nanomolar range, while for those lacking nitrogen, the IC50 is high micromolar or no inhibition is observed at all [60,63]. That this is indeed the target of the N-BPs is bolstered by the ability of not only

statins but also inhibitors of geranylgeranyl transferase, primarily GGTI-298, to suppress osteoclastic bone resorption and induce osteoclast apoptosis [65]. Furthermore, a separate line of evidence independently supported these conclusions. In analyses of osteoclastic biochemical changes induced by BPs, it was shown that all BP inhibitors of bone resorption tested stimulated the caspase-dependent cleavage and activation of the serine/threonine protein kinase Mst-1 [43]. Caspase activation of this enzyme is associated with the induction of apoptosis in these and other cells. This effect was mimicked by statins. For both the N-BPs and statins, but not clodronate or etidronate, Mst1 cleavage and activation can be prevented by addition of geranylgeraniol, indicating that this also results from inhibition of geranylgeranylation.

Consistent with observation that apoptosis may be a secondary effect for the N-BPs, as noted above, short-term ***in vivo*** treatment with N-BPs was found to suppress the mevalonate pathway and increase, rather than decrease osteoclast numbers [66]. These findings are highly interesting in relation to the cellular observation that the ruffled border (and the vesicles located above it) is disrupted by N-BP treatment [33,37]. 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 N-BP treatment) are needed for the formation of the ruffled border. The control of vesicular trafficking is largely under the control of the Rabs, which are also geranylgeranylated.

Taken together, these findings converge to point to suppression of isoprenylation, in particular geranylgeranylation, resulting from inhibition of farnesyl diphosphate synthase as a primary rate-limiting step in the molecular action of nitrogen-containing BPs, leading both to disruption of the ruffled border and eventually to apoptosis.

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