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PERSPECTIVES

An Update on the Pharmacology of Bisphosphonates and Analogues with Lower Bone Affinity

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Abstract

It has become clear that the pharmacological characteristics of nitrogen-containing bisphosphonates (N-BPs) are the result of both their ability to inhibit farnesyl diphosphate synthase in osteoclasts and their affinity for bone mineral. Both of these properties differ for every N-BP, helping to explain the differences in both potency and duration of action within this class of drugs. The mechanistic basis for the different characteristics of N-BPs is highlighted in this review. In addition, numerous N-BP analogues have been synthesized that exhibit different target enzyme specificity and much larger variations in bone affinity than can be achieved with N-BPs. These are also discussed, with particular emphasis on the potential applications and advantages that such compounds could offer over existing N-BPs. *IBMS BoneKEy*. 2008 October;5(10):357-369.

Introduction

Bisphosphonates (BPs) are the most widely used and effective treatments for diseases in which there is an increase in osteoclastic resorption, including post-menopausal osteoporosis and tumor-associated osteolysis. These drugs comprise two phosphonate groups linked by non-hydrolyzable P-C bonds to a central carbon atom, to which two covalently-bonded side chains (R_1 and R_2) that differ between the various BPs are also attached (Fig. 1). The more potent BPs are characterized by a nitrogen moiety in their R_2 side chain, either in an alkyl chain, *e.g*., alendronate (ALN) and ibandronate (IBA), or within a heterocyclic ring structure, *e.g*., risedronate (RIS) and zoledronate (ZOL), and are therefore often termed nitrogen-containing BPs (N-BPs). These N-BPs disrupt osteoclast function by inhibiting farnesyl diphosphate synthase (FPPS), an enzyme of the mevalonate pathway (Fig. 2). As a consequence, cells become depleted of the isoprenoid lipids FPP and geranylgeranyl diphosphate (GGPP), which are required for the post-translational prenylation of small GTPases (1-3). N-BPs therefore inhibit resorption by preventing the prenylation of small GTPases that are essential for osteoclast activity and survival, reviewed extensively elsewhere (4-7).

The pharmacological characteristics of N-BPs depend not only on the ability to inhibit FPPS, but also on affinity for bone mineral, and it has recently become apparent that both these properties differ between N-BPs. In addition, studies of novel BP analogues, in particular phosphonocarboxylates (PCs), have revealed that it is possible to synthesize compounds that have much lower bone affinity than N-BPs but that retain the ability to inhibit enzyme(s) of the mevalonate pathway and block protein prenylation.

Differences in FPP Synthase Inhibition Between N-BPs

The recent generation of X-ray crystal structures of human FPP synthase co-crystallized with N-BPs (8;9), together with detailed kinetic analysis of the recombinant

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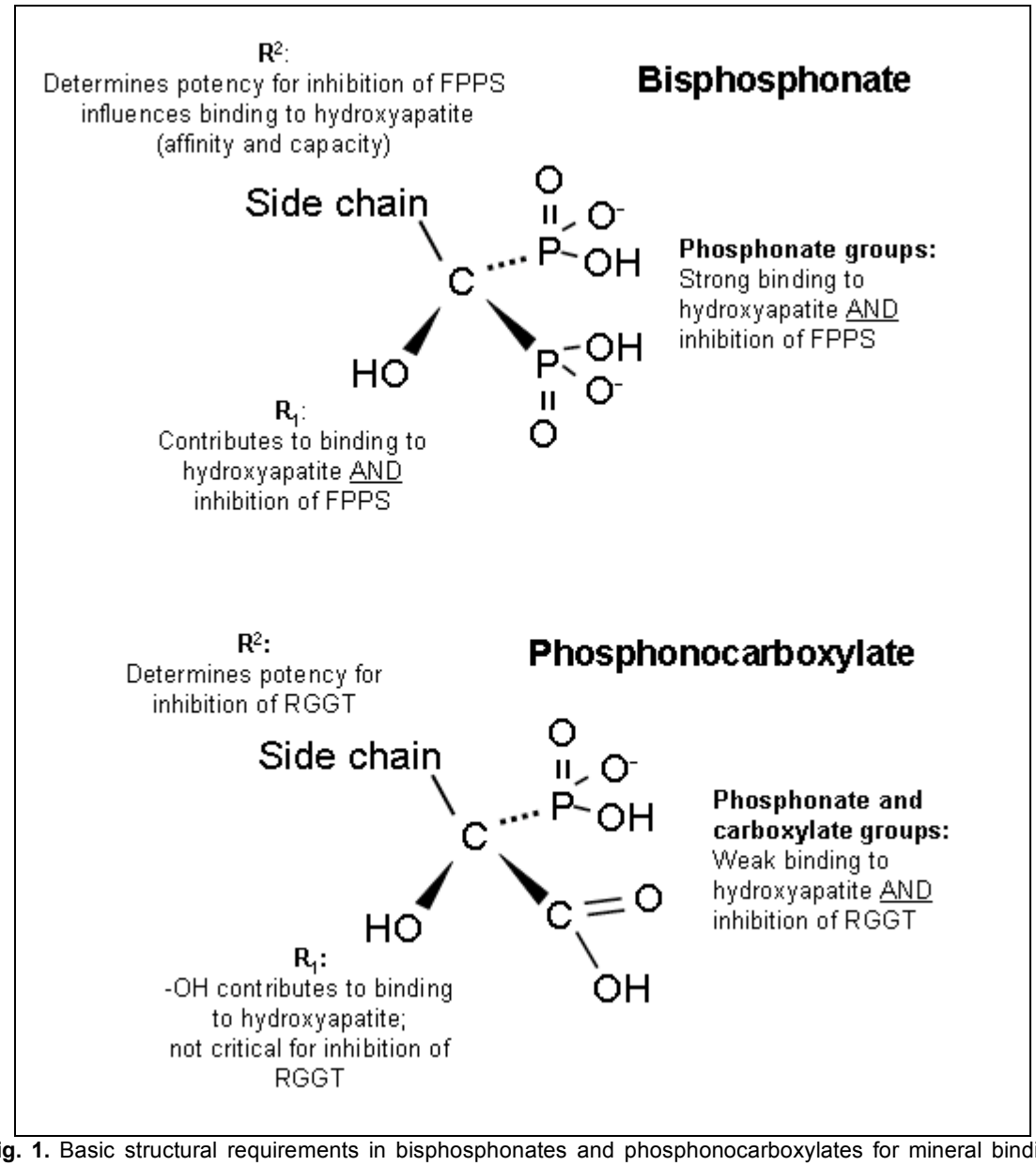

Fig. 1. Basic structural requirements in bisphosphonates and phosphonocarboxylates for mineral binding and target enzyme inhibition. FPPS: farnesyl diphosphate synthase; RGGT: Rab geranylgeranyl transferase.

human enzyme, indicates that the interaction is characteristic of 'slow-tight binding' inhibition (8). Initially, N-BPs appear to compete for binding with the isoprenoid lipid substrates DMAPP and GPP, their two phosphonates coordinating via magnesium ions to an aspartic acid-rich region of the FPPS enzyme. The nitrogen in the R_2 side-chain of the N-BP then forms interactions with conserved threonine and lysine residues in the enzyme, explaining why the spatial position of the nitrogen is critical for potent inhibition. This is associated with a conformational change in the enzyme that promotes the binding of the second substrate, IPP, which then causes further conformational changes leading to the final "locked" complex of N-BP-bound enzyme. This tight complex is likely to exist for several hours (10), and helps to explain the very high potency of some BPs, such as zoledronate, for inhibiting FPPS (11). The

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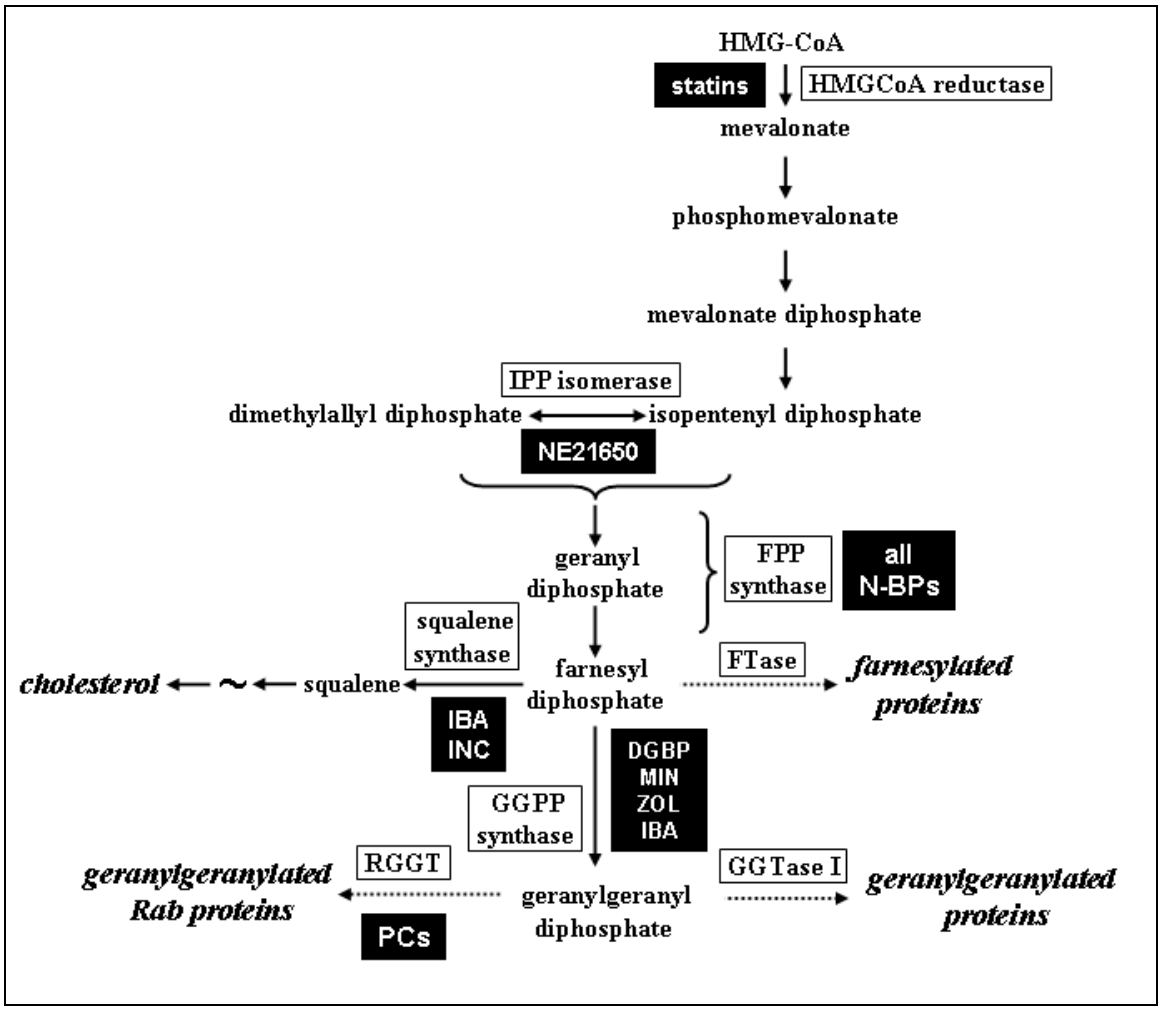

Fig. 2. The mevalonate pathway, showing sites of inhibition by N-BPs and analogues. Note that the enzymes between squalene and cholesterol are not shown. Solid arrows: mevalonate pathway steps; dashed arrows: protein prenylation steps. PC: phosphonocarboxylate; DGBP: digeranyl bisphosphonate; INC: incadronate; IBA: ibandronate; MIN: minodronate; ZOL: zoledronate; ALN: alendronate.

ability of N-BPs to cause this conformational change in FPPS correlates well with the Ki for inhibition of the enzyme, therefore highlighting the importance of this structural alteration for the inhibitory activity of these compounds (10). This is illustrated by the difference in potency between RIS and ZOL, since these BPs have very similar abilities to compete with geranyl diphosphate or dimethylallyl diphosphate for binding, but ZOL is a much stronger inducer of structural changes in the enzyme (10). While it remains to be confirmed whether such modifications are crucial for the effects of N-BPs in intact cells, observations that incubation of cells with N-BP for as little as two hours is sufficient to inhibit protein prenylation and to cause cytotoxic effects up to 48 hours later (12) support this model. Furthermore, N-BPs lacking a geminal –OH group are often better competitive inhibitors of FPPS as compared to their parent N-BPs, but have much reduced ability to induce conformational changes in the enzyme (10), and consistently have reduced potency in cell-based assays (13;14).

Inhibition of Other Enzymes of the Mevalonate Pathway by Nitrogen-Containing Bisphosphonates

N-BPs are also able to inhibit other enzymes of the mevalonate pathway that utilize isoprenoid diphosphates as substrate. Both ibandronate and incadronate inhibit squalene synthase (15), which is involved in cholesterol biosynthesis (but not in protein prenylation; Fig. 2). More recently, an analogue of RIS has been shown to inhibit IPP isomerase (16), while several N-BPs, including zoledronate and minodronate, can inhibit geranylgeranyl pyrophosphate synthase (GGPPS) (17;18). A recent study has shown that different N-BPs bind to either the FPP substrate site or to the GGPP product site, and in some cases to both sites, in this enzyme (19). Although inhibition of IPP isomerase and/or GGPPS also results in inhibition of protein prenylation, the much lower potency of N-BPs towards these enzymes compared to FPPS suggests that these effects are unlikely to contribute to the overall pharmacological effects of N-BPs *in vivo*.

Interestingly, a novel BP that has recently been synthesized, which has two geranyl groups in place of the R_1 and R_2 side chains, has been shown to inhibit GGPPS without affecting FPPS (20). This compound reduces GGPP levels and inhibits protein geranylgeranylation as potently as ZOL in RPMI-8226 myeloma cells. Since the anti-resorptive effects of N-BPs are mediated predominantly through inhibition of protein geranylgeranylation (21;22), such novel GGPPS inhibitors might therefore be expected to be equally effective as anti-resorptive agents, although the bone affinity or anti-resorptive potency of this compound has not been reported.

Bone Affinity and Recycling of Bisphosphonates

The P-C-P backbone structure enables BPs to bind avidly to Ca^{2+}, and as a result BPs have a high affinity for bone mineral, and are rapidly retained *in vivo* at sites of active bone remodeling. This bone targeting property helps to explain why BPs have a highly selective effect on osteoclasts *in vivo,* despite being able to affect most, if not all, cell types *in vitro* (6;23).

The traditional view was that mineral binding is simply conferred by the P-C-P group, together with the R_1 group if it comprises an –OH, enabling tridentate binding to Ca^{2+} (24). Indeed, the importance of the R_1 group has been highlighted by a recent study, in which subtle modifications to this group significantly affected mineral affinity (14). However, it is now apparent that the R_2 side chain of bisphosphonates also contributes to mineral affinity, as a result of the ability of the nitrogen moiety to interact with the crystal surface of bone mineral. Although one study did not find major affinity differences between N-BPs (25), studies using assay systems involving both binding to hydroxyapatite and dissolution of carbonated apatite (which more closely resembles bone mineral) have found substantial differences, with the rank order of affinity for the most common clinically used BPs being ZOL>ALN>IBA>RIS (26;27). These differences are thought to result from the different orientations of the nitrogen atoms in the R_2 side-chain, which influences N-H-O bond formation at the crystal surfaces.

The strong correlation between FPPS inhibition *in vitro* with anti-resorptive potency *in vivo* indicates that potency for inhibition of FPPS is the dominant driver of anti-resorptive efficacy *in vivo*, particularly between the N-BPs that are clinically used (11). However, differences in mineral affinity between these N-BPs are also important, since this correlation can be improved by also taking account of bone affinity (28).

Differences in the electrical charge on the nitrogen atom of N-BPs may also affect the local binding of additional BP molecules, which will influence the capacity of any given surface of bone mineral to adsorb different BPs (26). In addition, molecular size (particularly in bulky BPs) also contributes to the capacity differences between BPs (29). However, whether such differences contribute significantly to the effect of different N-BPs *in vivo* is unclear, since it is unlikely that pharmacological doses of these compounds result in saturation of bone surfaces.

All BPs will naturally desorb from mineral surfaces, and therefore exist in equilibrium between the adsorbed component and the soluble, desorbed component (30). An important concept is that following this desorption from the mineral surface, a greater proportion of a high-affinity BP will be recycled by binding back to the mineral surface (23;26;31). Indeed, it has been shown using *in vitro* studies that a fluorescent BP reattaches avidly to newly exposed mineral surfaces following osteoclastic resorption, whereas fluorescent compounds that have much lower bone affinity, such as calcein, do not display this recycling activity (23). In addition, BPs with high bone affinity appear to recycle to newly formed bone surfaces *in vivo* more than compounds with low bone affinity (32). Such recycling is likely to have an important impact (together with the amount of BP loaded on to bone immediately following administration) on the duration of action of a single dose of BP, and may help to explain why single doses of very high affinity N-BPs such as ZOL can suppress bone turnover for a year or more (7;33).

Uptake by Cells Other Than Osteoclasts

Although BPs predominantly affect osteoclasts *in vivo*, there is evidence that they can be taken up by some other cell types. For example, N-BPs induce an acute phase reaction via the activation of γ,δ-T cells, which occurs as a result of IPP accumulation in peripheral blood mononuclear cells following inhibition of FPPS (comprehensively reviewed by Thompson and Rogers (34)). Following an intravenous infusion, N-BP reaches sufficiently high concentrations in the circulation to allow highly endocytic monocytes to internalize enough BP to inhibit FPPS (35).

Since N-BPs can reduce the adhesion, migration, proliferation and survival of tumor cells *in vitro* as a result of inhibition of protein prenylation (6;36), they have the potential for direct effects on tumor cells *in vivo,* provided that these cells can take up sufficient amounts of N-BP. While it is well-established that N-BPs can reduce skeletal metastasis or tumor burden in animal models (6), it remains controversial whether these anti-tumor effects occur indirectly as a result of inhibition of bone resorption, or whether they involve direct effects on tumor cells (or other cell types in the bone marrow that may mediate the anti-tumor effects of BPs, such as endothelial cells) (37). However, the ability of N-BPs to reduce tumor burden at extraskeletal sites in some preclinical studies is suggestive of an anti-tumor effect independently of inhibition of bone resorption (6;38;39).

If anti-tumor effects do indeed occur independently of the anti-resorptive effect, compounds that retain the ability to inhibit FPPS but have lower bone affinity would be expected to act more effectively on tumor cells, or other cells in the bone marrow environment. However, such compounds are difficult to design, since the phosphonate groups of N-BPs are critical for FPPS inhibition as well as bone affinity (8;10) (Fig. 1). Therefore, although alterations to these groups effectively reduce bone affinity, they also result in dramatically reduced enzyme inhibition (5). Modification to the R_1 side-chain is also limited by the same problem, for example, in BP analogues in which the optimal hydroxyl group is lacking or replaced by a halogen group (14).

Low Affinity Phosphonocarboxylate Analogues of N-BPs

Phosphonocarboxylate (PC) analogues, in which one of the phosphonate groups of BPs is replaced with a carboxylate group, have a bone affinity about 50 times lower than that of their N-BP counterparts (40). Interestingly, although these analogues are unable to affect FPPS, they specifically inhibit a different enzyme of the mevalonate pathway that uses an isoprenoid lipid as a substrate, the protein:prenyl transferase Rab geranylgeranyl transferase (RGGT; Fig. 2). Since this enzyme exclusively prenylates the Rab GTPases, PCs inhibit the prenylation and membrane localization of this subfamily of GTPases only (41;42). Although the first PC to be described is a weak inhibitor of

RGGT and a very weak anti-resorptive agent (41), a PC that is much more potent has since been identified (43). Importantly, the ability of these compounds to inhibit RGGT can be separated from their affinity for bone, and modifications to the R_1 side-chain can actually increase potency for enzyme inhibition while further reducing affinity for bone (14). Part of the explanation for this could be that, unlike BP inhibitors of FPPS, PCs may not require coordination with a divalent metal ion in the active site of RGGT (in support of this, GGPP does not interact with a divalent metal ion in RGGT (44)).

The Potential of Low Affinity Compounds Such as PCs for the Treatment of Cancer

The combination of low bone affinity and inhibition of RGGT suggests that PCs may be effective against tumors that metastasize to bone, since Rab proteins have been implicated in the pathogenesis of certain cancers, including breast cancer (45). Moreover, a recent study revealed that drugs that were designed to inhibit farnesyl transferase, and have shown anti-cancer activity *in vivo*, most likely act through inhibition of RGGT (46). The potential of RGGT inhibitors is further highlighted by the fact that the anti-proliferative effect of a specific inhibitor of GGPPS in K562 leukemia cells is actually antagonized by an inhibitor of GGTase I, suggesting that loss of prenylation of Rabs is crucial to the effects of this compound (47). In this case, the antagonism could occur as a result of inhibition of GGTase I increasing the pool of GGPP available for prenylation of Rab proteins

Accordingly, in addition to inhibiting bone resorption by osteoclasts, PCs can reduce the viable number of J774 macrophages as a result of inhibition of Rab prenylation (41;42), and can inhibit invasion of breast cancer cells and induce apoptosis of myeloma cells *in vitro* (48;49). Intriguingly, it has been reported that a PC reduces tumor burden in an animal model of breast cancer metastasis at a concentration that does not affect bone resorption (50), suggesting a possible direct anti-tumor effect. However, further *in vivo* studies are required to establish whether such effects are indeed the result of direct inhibition of Rab prenylation in tumor cells.

Potential Advantages of Low Affinity Compounds Such as PCs

N-BP analogues that have low bone affinity may offer other potential advantages in addition to possible increased availability to tumor cells. One possibility relates to beneficial effects of BPs on the osteocyte network. It has been shown that low concentrations of BPs can prevent glucocorticoid-induced osteocyte apoptosis through a mechanism independent of the mevalonate pathway, involving opening of connexin 43 hemichannels (51;52). Since apoptotic osteocytes stimulate bone remodeling, the preservation of osteocyte viability by BPs could reduce remodeling in cortical bone and contribute to the anti-fracture efficacy of N-BPs (in conjunction with effects on osteoclasts). The anti-apoptotic effect of N-BPs is also seen *in vivo* (53;54), and the significance of its contribution to bone strength should be revealed by *in vivo* studies using BPs that are able to inhibit osteocyte apoptosis without affecting osteoclast activity (55). Affinity for bone is likely to influence the extent to which BPs can penetrate the vast network of osteocyte canaliculi, since compounds that have high affinity will be adsorbed to the bone at the first point of contact (7). Compounds with low bone affinity are likely to be able to access a much greater proportion of this network and therefore inhibit osteocyte apoptosis *in vivo* to a greater extent. However, it is important to stress that it is yet to be determined whether PCs, or other low-affinity analogues of BPs, are also able to inhibit osteocyte apoptosis.

The reduced skeletal retention of low affinity compounds may also be beneficial when there are concerns regarding long-term suppression of bone remodeling by BPs, for example in pediatric use (such as treatment of osteogenesis imperfecta) (31). In addition,

long-term suppression of bone turnover by N-BPs such as ALN can hinder the anabolic response to subsequent treatment with PTH, in patients with osteoporosis (56). Compounds with reduced bone affinity and shorter duration of action would be expected to interfere less, and in support of this, the anabolic response to PTH is greater in patients previously treated with RIS (which has lower affinity for bone than ALN) than in patients previously treated with ALN (57).

Potential Advantages of Inhibition of RGGT by PCs

The fact that PCs inhibit an enzyme downstream of FPPS in the mevalonate pathway may offer further advantages. Firstly, inhibition of cholesterol synthesis (as with an FPPS inhibitor) results in loss of negative feedback signals to HMG-CoA reductase and other mevalonate pathway enzymes (58), potentially reducing the effectiveness of such inhibitors at inhibiting protein prenylation. This is illustrated by the increased HMG-CoA reductase expression in cells treated with lovastatin, which can be prevented by apomine (which down-regulates HMG-CoA reductase), resulting in enhancement of the inhibition of protein prenylation caused by lovastatin alone (59). Drugs that act by inhibiting enzymes downstream of FPPS in the prenylation pathway, which do not affect cholesterol synthesis, could avoid such feedback effects, as is the case with a specific GGPPS inhibitor (47).

Secondly, some of the adverse effects of N-BPs are the result of inhibition of FPPS, such as the acute phase reaction that often occurs following intravenous administration of N-BPs. Interestingly, unlike N-BPs, PCs do not activate γδ T-cells *in vitro* (60), since they do not affect FPPS and therefore do not cause accumulation of intracellular IPP. As a result, these compounds are unlikely to cause an acute phase reaction *in vivo*. In addition, PC inhibitors of RGGT could lack the gastrointestinal irritation associated with some N-BPs, since there is evidence that this effect is the result of inhibition of prenylation of proteins modified by GGTase I in gastrointestinal epithelial cells (61).

Finally, compounds that inhibit an enzyme target in the mevalonate pathway other than FPPS may be useful in the situation of apparent acquired resistance to BPs, which can occur in patients with Paget's disease undergoing treatment with N-BPs (62). Although the mechanistic basis behind this resistance remains unclear, it could involve increased expression of FPPS, since prolonged culture of myeloma cell lines with ALN, or of osteosarcoma cell lines with ZOL, increases FPPS activity and induces resistance to the N-BPs (63;64). In this scenario, switching treatment to a compound that inhibits a different molecular target from FPPS, such as PCs, would be expected to overcome such resistance.

Potential New Therapeutic Approaches for BPs and Their Analogues

An emerging potential approach in the treatment of metastatic bone disease is the use of an N-BP in combination with other cytotoxic agents, which has been shown to result in synergistic effects in both cell and animal models assessing potential anti-cancer activity (6;65;66). Furthermore, there is also evidence that combination therapy with inhibitors of other enzymes of the mevalonate pathway can produce synergistic inhibition of prenylation and anti-tumor effects *in vitro*. For example, N-BPs such as ZOL act synergistically with FTIs to reduce growth and induce apoptosis in epidermoid cancer cells (67), and with statins to induce apoptosis in several myeloma cell lines (68). Moreover, a specific GGPPS inhibitor (GDPS) reduces the proliferation of K562 leukemia cells synergistically with both lovastatin and ZOL (47), suggesting that combination therapy of a PC with a statin or an N-BP may also prove useful.

In addition, studies using a mouse model suggest that a novel combination therapy of N-BPs and statins could be an effective treatment for the premature aging disease Hutchinson-Gilford progeria syndrome. This

treatment works by blocking the farnesylation and nuclear localization of a truncated form of farnesylated prelamin A, which causes the disease (69). Other potential non-skeletal therapeutic avenues include short-term treatment in diseases caused by protozoan parasites, such as trypanosomes, which are sensitive to growth-inhibition by N-BPs, as a result of inhibition of FPPS (70). In this regard, analogues with very low bone affinity would be expected to be more effective. Although it is unclear whether inhibition of RGGT could also affect trypanosomes, the observation that Rab-dependent endocytosis in *Trypanosoma brucei* is essential for viability in the mammalian host (71) supports this possibility.

Conclusion

Since the identification of FPPS as the molecular target of the N-BPs, structure-activity studies and crystallography have revealed the structural basis for the differences in potency of the N-BPs in clinical use. Strikingly, these studies also suggest that the potency of N-BPs towards FPPS is unlikely to be significantly improved, even through rational design of new compounds. However, there is still considerable scope for the further development of these drugs, since subtle variations of both bone affinity and FPPS inhibitory potency could produce compounds with the ideal duration of action and concentration in the bone microenvironment to match the clinical need. In addition, analogues that inhibit different targets in the mevalonate pathway, and which can be more easily modulated to generate active compounds with much lower bone affinities such as the PCs, offer potential for development as novel therapeutics for bone diseases.

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