

Bisphosphonates

From Bench to Bedside

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ABSTRACT: The discovery and development of the bisphosphonates (BPs) as a major class of drugs for the treatment of bone diseases has been a fascinating journey that is still not over. In clinical medicine, several BPs are established as the treatments of choice for various diseases of excessive bone resorption, including Paget's disease of bone, myeloma and bone metastases, and osteoporosis. Bisphosphonates are chemically stable analogues of inorganic pyrophosphate, and are resistant to breakdown by enzymatic hydrolysis. Bisphosphonates inhibit bone resorption by being selectively taken up and adsorbed to mineral surfaces in bone, where they interfere with the action of the bone-resorbing osteoclasts. Bisphosphonates are internalized by osteoclasts and interfere with specific biochemical processes. Bisphosphonates can be classified into at least two groups with different molecular modes of action. The simpler non-nitrogen-containing bisphosphonates (such as clodronate and etidronate) can be metabolically incorporated into nonhydrolyzable analogues of adenosine triphosphate (ATP) that may inhibit ATP-dependent intracellular enzymes. The more potent, nitrogen-containing bisphosphonates (such as pamidronate, alendronate, risedronate, ibandronate, and zoledronate) are not metabolized in this way but can inhibit enzymes of the mevalonate pathway, thereby preventing the biosynthesis of isoprenoid compounds that are essential for the posttranslational modification of small GTP-binding proteins (which are also GTPases) such as rab, rho, and rac. The inhibition of protein prenylation and the disruption of the function of these key regulatory proteins explain the loss of osteoclast activity and induction of apoptosis. The key target for bisphosphonates is farnesyl pyrophosphate synthase (FPPS) within osteoclasts, and the recently elucidated crystal structure of this enzyme reveals how BPs bind to and inhibit at the active site via their critical N atoms. In conclusion, bisphosphonates are now established as an important class of drugs for the treatment of many bone diseases, and their mode of action is being unraveled. As a result their full therapeutic potential is gradually being realized.

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INTRODUCTION

The bisphosphonates (BPs) are stable analogues of a naturally occurring pyrophosphate (PPi) compound. BPs were first synthesized in the 1800s,¹ but have only been used progressively in medicine since the 1960s.

Etidronate, the first BP to be used in humans in Paget's disease,² was synthesized just over 100 years ago.³ The early uses of BPs were industrial, mainly as corrosion inhibitors, also as complexing agents in the textile, fertilizer, and oil industries, as well as for many other industrial processes.⁴ Their use as "water softeners" was based on their ability to inhibit calcium carbonate precipitation, as do polyphosphates, and has been applied in the prevention of scaling in domestic and industrial water installations. There are many books and review articles available that describe the chemistry, pharmacology, and clinical applications of BPs.⁵⁻¹⁸

EARLY STUDIES ON PPI AND BPs

In the early 1960s, William Neuman and Herbert Fleisch¹⁹ were studying mechanisms of calcification induced by collagen, and showed that body fluids, such as plasma and urine, contained inhibitors of calcification. Since it had been known since the 1930s that trace amounts of polyphosphates were capable of acting as water softeners by inhibiting the crystallization of calcium salts, such as calcium carbonate, they proposed that compounds of this type might be natural regulators of calcification under physiological conditions. Fleisch and his colleagues showed that inorganic PPi, a naturally occurring polyphosphate and a known byproduct of many biosynthetic reactions in the body, was present in serum and urine and could prevent calcification by binding to newly forming crystals of HAP.^{20,21} It was therefore postulated that PPi might be the body's own "water softener" that normally prevents calcification of soft tissues, and regulates bone mineralization. The concentrations of PPi in body fluids would be expected to be regulated by hydrolytic enzymes.

At this time, I was completing my Ph.D. in the UK and was studying kidney stone formation and other disorders of calcification. It seemed possible that some of these pathologic disorders might be linked to disturbances in PPi metabolism.²² Prominent among these was the rare but fascinating inherited disorder, hypophosphatasia, in which lack of alkaline phosphatase is associated with mineralization defects of the skeleton. My studies showed that PPi levels were elevated in urine,²³ and indicated that alkaline phosphatase was probably

the key extracellular enzyme responsible for hydrolyzing PPI. We later showed elevated concentrations of PPI in plasma,²⁴ further supporting the notion that the activity of alkaline phosphatase regulates circulating amounts of PPI to below the critical levels that would otherwise prevent normal physiological calcification processes.

Attempts to exploit these concepts by using PPI and polyphosphates to inhibit ectopic calcification in blood vessels, skin, and kidneys in laboratory animals were successful only when the compounds were injected.²⁵ Orally administered PPI and polyphosphates were inactive, due to the hydrolysis of PPI in the gastrointestinal tract, probably by mucosal brush border phosphatases. During the search for more stable analogues of PPI that might also have the antimineralization properties of PPI but that would be resistant to hydrolysis, several different chemical classes were studied, including P-N-P and P-C-C-P compounds. The BPs (at that time called diphosphonates) characterized by P-C-P motifs were among these.²⁶ These early studies with BPs were the result of a very successful collaboration with Marion (Dave) Francis of the Procter and Gamble company and the group working in Switzerland.^{27,28}

Like PPI, BPs had high affinity for bone mineral²⁹ and were found to prevent the formation and aggregation of calcium phosphate crystals. BPs had high affinity for bone mineral and were found to prevent calcification both *in vitro* and *in vivo*, but, unlike PPI, were also able to prevent experimentally induced pathological calcification when given orally to rats *in vivo*.³⁰ This property of being active by mouth was key to their future use in man.

In these early studies BPs were shown to not only prevent the experimentally induced calcification of many soft tissues, including skin, kidneys, and blood vessels *in vivo*, but with some of the compounds, for example, etidronate to also inhibit mineralization of ectopic bone as well of normal calcified tissues such as bone and cartilage.³¹ BPs appear to prevent calcification by physicochemical mechanisms producing direct impairment of the calcification process by acting as crystal poisons after adsorption to mineral surfaces, rather than by effects on the deposition of matrix.

Perhaps the most important step toward the future use of BPs occurred when we found that BPs, like we had already shown for PPI,³² also had the novel property of being able to inhibit the dissolution of HAP crystals.³³ This led to studies to determine whether they might also inhibit bone resorption.

Many studies using a variety of experimental systems showed that BPs inhibit osteoclast-mediated bone resorption, not only in organ cultures of bone *in vitro*, but also both in normal animals and in those with experimentally increased resorption. The first experimental model studied was thyroparathyroidectomized rats treated with parathyroid hormone to stimulate bone resorption *in vivo*. BPs also suppress resorption induced by many other agents such as calcitriol, vitamin D, and retinoids. The effect on retinoid-induced hypercalcemia has been used to develop a powerful and rapid-screening assay for new compounds.^{34,35} In growing intact rats, the BPs block the removal of both bone and cartilage,

thus retarding the remodeling of the metaphysis, which becomes club-shaped and radiologically denser than normal.³⁶ This effect is the basis of the 'Schenk' model also used to compare the potency of new compounds. The inhibition of endogenous bone resorption can also be monitored by kinetic studies³⁷ using radio-calcium (⁴⁵Ca), and by using biochemical markers of bone resorption.

The BPs are also effective in preventing bone destruction in a number of animal models of human disease. One of the first to be studied was the sciatic nerve section as a model of immobilization osteoporosis,³⁸ as well as bone loss due to spinal cord section. Other commonly used models of osteoporosis include the prevention of bone loss associated with ovariectomy. Less commonly used models involve orchidectomy, lactation, low calcium diets, or the administration of agents such as heparin or corticosteroids. If not given in excess, BPs maintain or improve the biomechanical properties of bone both in normal animals and in experimental models of osteoporosis.³⁹

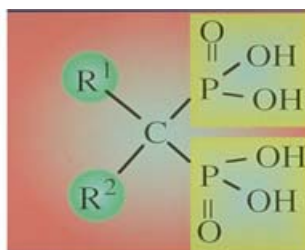
In general there is a good correlation between potency and structure-activity relationships *in vitro* and *in vivo*.⁴⁰ In the presence of BPs isolated osteoclasts form fewer and smaller erosion cavities on various mineralized matrices *in vitro*.⁴¹

THE PHARMACOLOGICAL DEVELOPMENT OF BPs

Once the potential clinical value of BPs had been appreciated, research efforts were devoted to the development of compounds with a more powerful antiresorptive activity, but without a corresponding ability to inhibit mineralization. With compounds such as etidronate there was only a 10- to 100-fold difference between doses that inhibit mineralization compared with doses that reduce bone resorption. Enhancing this window was readily achieved and many hundreds of BPs have been synthesized, and more than a dozen have been used in humans. With the development of BPs that were more potent inhibitors of bone resorption, these dose differences widened to several orders of magnitude, which meant that inhibition of skeletal mineralization ceased to be a major clinical concern. The gradation of potency evaluated in the animal models corresponded quite well with that found in humans, although the differences in potency are much smaller in humans.

Since BPs accumulate in bone, it is important to know what happens during long-term administration. It is reassuring from a clinical point of view that the inhibition of bone resorption reaches a new steady-state level, rather than becoming progressively lower, even when the compounds are given continuously.⁴² The level of suppression depends on the administered dose, and has also been observed in humans.⁴³ These results show that there is no progression of the antiresorptive effect with time and suggest that the bisphosphonate buried in the bone is inactive at least as long as it remains buried there. They

When R¹ is an OH group,
binding to bone is enhanced



Both phosphonate
groups act as a
"bone hook" and
are essential both
for binding to
hydroxyapatite
and biochemical
mechanism of
action

**R² Site determines anti-
resorptive potency
biochemically, including effects
on binding to hydroxyapatite**

FIGURE 1. The generic structure of bisphosphonates and their functional domains.

also show that, within the therapeutic dosage range, there is little risk of a continuous decrease in bone turnover in the long run, which might lead to an increase in bone fragility. An additional important pharmacological property of BPs is that the total dose administered is a major determinant of their effects. This has been well studied for ibandronate⁴⁴ and zoledronate.⁴⁵ In both cases the same inhibition of bone resorption has been documented whether the BP is given in small frequent (e.g., daily) doses compared with larger doses given less frequently. This is the basis for the development of intermittent dosing regimens in humans.

DEFINING STRUCTURE–ACTIVITY RELATIONSHIPS

The features of the bisphosphonate molecule necessary for biological activity were well defined in the early studies. The P-C-P moiety is responsible for the strong affinity of the BPs for binding to hydroxyapatite (HAP) and allows for a number of variations in structure based on substitution in the R1 and R2 positions on the carbon atom (FIG. 1). The ability of the BPs to bind to HAP crystals, and to prevent both crystal growth and dissolution, was enhanced when the R1 side chain (attached to the geminal carbon atom of the P-C-P group) was a hydroxyl group (as in etidronate) rather than a halogen atom such as chlorine (as in clodronate). The presence of a hydroxyl group at the R1 position increases the affinity for calcium (and thus bone mineral) due to the ability of BPs to chelate calcium ions by tridentate rather than bidentate binding.⁴⁶

The ability of BPs to inhibit bone resorption *in vitro* and *in vivo* also requires the P-C-P structure. Monophosphonates, for example, pentane monophosphonate, or P-C-C-P or P-N-P compounds, are ineffective as inhibitors of bone resorption. Furthermore, the antiresorptive effect cannot be accounted for simply by adsorption of BPs to bone mineral and prevention

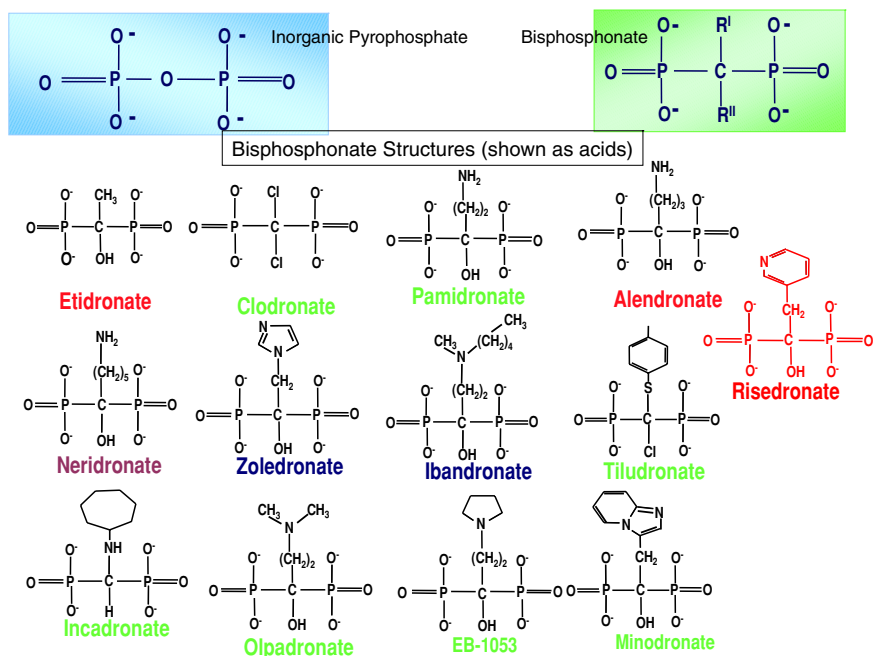


FIGURE 2. Structures of the bisphosphonates used in clinical studies and under clinical development.

of HAP dissolution. It became clear that BPs must inhibit bone resorption by cellular effects on osteoclasts, rather than simply by physicochemical mechanisms.

Following the successful clinical use of clodronate and etidronate in the 1970s and 1980s, more potent antiresorptive BPs were studied, which had different R2 side chains, but in which R1 was unaltered. In particular, BPs containing a basic primary nitrogen atom in an alkyl chain (as in pamidronate and alendronate) were found to be 10- to 100-fold more potent than etidronate and clodronate. After this in the 1980s, there was a phase in which synthesis of novel compounds took place specifically to determine their possible effects on calcium metabolism, with the result that compounds highly effective as inhibitors of bone resorption were identified and studied (FIG. 2).

These compounds, especially those that contain a tertiary nitrogen, such as ibandronate⁴⁷ and olpadronate,⁴⁸ were even more potent at inhibiting bone resorption. Among this generation of compounds that were synthesized to optimize their antiresorptive effects, the most potent antiresorptive BPs were those containing a nitrogen atom within a heterocyclic ring (as in risedronate⁴⁹ and zoledronate⁵⁰), which are up to 10,000-fold more potent than etidronate in some experimental systems.

The analysis of structure–activity relationships allowed the spatial features of the active pharmacophore to be defined in considerable detail, even before the molecular mechanism of action was fully elucidated. For maximal potency, the nitrogen atom in the R2 side chain must be a critical distance away from the P-C-P group, and in a specific spatial configuration.⁵¹ This was used successfully for predicting the features required in the chemical design of new and more active compounds.

Although the structure of the R2 side chain is the major determinant of antiresorptive potency, both phosphonate groups are also required for the drugs to be pharmacologically active. Alterations to one or both phosphonate groups reduces the affinity for bone mineral and this may be one reason why such bisphosphonate analogues are less active. For example, replacement of one of the phosphonate hydroxyl groups with a methyl group (to form a phosphonophosphinate) markedly reduces both bone affinity and antiresorptive potency. Methylation of both phosphonate groups to form a bisphosphinate leads to loss of bone affinity and loss of antiresorptive activity *in vivo*. However, bisphosphonate analogues (e.g., a phosphonophosphinate and a phosphonocarboxylate) with similar affinity for bone can have very different antiresorptive potencies. This suggests that the two phosphonate groups (or alternatively, the combination of a phosphonate and a carboxylate group) are required both for targeting the bone and for the molecular mechanism of antiresorptive action, presumably because BPs mimic naturally occurring PPI-containing compounds.

In summary, studies of the relationships between bisphosphonate structure and antiresorptive potency suggested that the ability of BPs to inhibit bone resorption depended on two separate properties of the bisphosphonate molecule. The two phosphonate groups, together with a hydroxyl group at the R1 position,⁵² impart high affinity for bone mineral and act as a “bone hook,” allowing rapid and efficient targeting of BPs to bone mineral surfaces (FIG. 3). Once localized within bone, the structure and three-dimensional conformation of the R2 side chain (as well as the phosphonate groups in the molecule) determined the biological activity of the molecule and influenced the ability of the drugs to interact with specific molecular targets. Our understanding of what these molecular targets might be has become much clearer as a result of recent work.

CLINICAL APPLICATIONS OF BPs

After it was shown that BPs inhibited experimentally induced calcification and bone resorption, their potential application to clinical disorders was obvious but it took many years for them to become well established. The most impressive clinical application of BPs was as inhibitors of bone resorption, often for diseases where no effective treatment existed previously. Thus BPs became the treatment of choice for a variety of bone diseases in which excessive

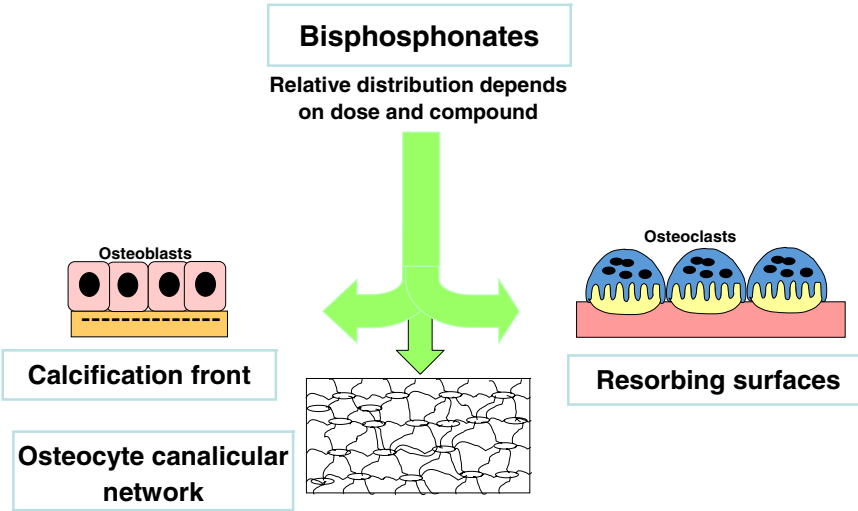


FIGURE 3. Uptake and distribution of BPs into different compartments of bone. This distribution may be affected by both the dose and the compound given.

osteoclast activity is an important pathological feature, including Paget's disease of bone, metastatic and osteolytic bone disease, and hypercalcemia of malignancy, as well as osteoporosis.

BPs and Inhibition of Calcification

Exploration of BPs as inhibitors of calcification showed some promise and early applications of etidronate included use in fibrodysplasia ossificans progressiva (FOP, formerly known as myositis ossificans) and in patients who had undergone total hip replacement surgery to prevent subsequent heterotopic ossification and to improve mobility.⁵³ Etidronate has also been used to prevent ectopic calcification and ossification, after spinal cord injury, and as topical applications in toothpastes to prevent dental calculus.

It should be emphasized that these effects required very high doses of etidronate, and that inhibition of skeletal mineralization is not a significant clinical problem when etidronate is used at the low doses recommended in the treatment of osteoporosis.

BPs for Radio-Nuclide Imaging of Bone

One of the earliest clinical uses of BPs was as agents for bone imaging, "bone scanning," for which they still remain outstandingly useful for detecting bone metastases and other bone lesions. The application of PPI and simple BPs as bone-scanning agents depends on their strong affinity for bone mineral,

particularly at sites of increased bone turnover, and their ability to be linked to a gamma-emitting technetium isotope.^{54,55}

BPs in Paget's Disease

Paget's disease was the first clinical disorder in which a dose-dependent inhibition of bone resorption could be demonstrated using BPs in human.^{56,57}

The central feature of Paget's disease is the osteoclast, since the pathological characteristics of the disease (such as bone pain, fractures, and skeletal deformities) are the result of increased numbers of osteoclasts and increased osteoclast activity. The pathogenesis of Paget's disease is gradually being elucidated. The reason why large multinucleate osteoclasts accumulate may be because they do not undergo apoptosis in the normal way. This may have a genetic basis⁵⁸ in many patients associated with mutations in the sequestosome 1 (SQSTM1) gene, which encodes for a scaffold protein in the NF-kappaB-signaling pathway. A viral etiology may also contribute.⁵⁹ If defective apoptosis of osteoclasts contributes to the pathogenesis of Paget's disease, the BPs may be viewed as bone-selective drugs that specifically induce apoptosis in the affected osteoclasts.

BPs have become the most important drugs used in the treatment of Paget's disease.⁶⁰ For many years pamidronate given by intravenous infusion was extensively used,⁶¹ but the newer and more potent BPs can produce even more profound suppression of disease activity than was possible with the BPs available in former years.^{62,63} The latest advance is with zoledronate,⁶⁴ which when given as a single 5-mg infusion produced a greater and longer-lasting suppression of excess bone turnover than even oral risedronate given at 30 mg/day over 2 months, hitherto one of the most effective treatments.

BPs in Oncology

Many cancers in humans are associated with hypercalcemia (raised blood calcium) and/or increased bone destruction. This may be due to the release from tumors of factors that increase bone resorption, such as parathyroid hormone-related protein (PTH-rP), or bone-resorbing cytokines such as interleukin 6.

BPs can prevent the increase in bone resorption associated with experimental tumors, particularly those that localize in or metastasize to bone.⁶⁵ In view of clinical results, it is interesting that BPs may not only reduce metastases in bone but reduce the overall tumor burden,⁶⁶ although in some models soft tissue tumor mass may increase. The reasons for changes in tumor burden induced by BPs are still uncertain, but are of considerable potential clinical significance. These effects may be due to changes in the release of growth factors, which are present in bone matrix and which may stimulate tumor cell growth during bone resorption.^{67,68} In addition, there may be direct effects

of BPs on tumor cells themselves, for example, by altering cell attachment and inducing apoptosis.⁶⁹ Many of these appear to be due to inhibition of the mevalonate pathway, and there is evidence that synergistic antitumor effects can be achieved in the presence of other chemotherapeutic agents.

BPs are remarkably effective in the treatment of bone problems associated with malignancy⁷⁰ and are now the drugs of choice.^{71–73} Clinical trials investigating the benefit of bisphosphonate therapy utilize a composite end point defined as a skeletal-related event (SRE) or bone event, which typically includes pathologic fracture, spinal cord compression, radiation, or surgery to bone, and hypercalcemia of malignancy. BPs significantly reduced the incidence of these events in myeloma⁷⁴ and in patients with breast cancer metastases,^{75,76} and in metastatic prostate cancer,⁷⁷ lung cancer, renal cell carcinoma, and other solid tumors. The goals of treatment for bone metastases are also to prevent disease-related skeletal complications, palliate pain, and maintain quality of life. Zoledronate,⁷⁸ pamidronate, clodronate, and ibandronate^{79,80} have demonstrated efficacy compared with placebo.

There is an important possibility that the survival of patients may be prolonged^{81–83} in some groups of patients. Recently, osteonecrosis of the jaw (ONJ)⁸⁴ has been identified as a potential complication of high-dose BP therapy in malignant diseases.

BPs in Osteoporosis

Osteoporosis is acknowledged to be a major health problem.⁸⁵ There have been impressive advances in understanding the epidemiology and pathogenesis of osteoporosis⁸⁶ and its associated fractures, and in the application of physical and biochemical methods to its diagnosis and evaluation. Up until the 1990s, there were few treatments for osteoporosis, but in the past few years, there have been remarkable advances in the therapeutic approaches to prevention and treatment of postmenopausal and other forms of osteoporosis.

As a drug class, the BPs have emerged as the leading effective treatments for postmenopausal and other forms of osteoporosis. Etidronate was the first of these,^{87–89} followed by alendronate,^{90–92} and then risedronate.^{93,94} All have been approved as therapies in many countries, and can increase bone mass and reduce fracture rates in the spine by 30–50%, and also other sites in postmenopausal women.⁹⁵ The reduction in fractures may be related not only to the increase in bone mass arising from the inhibition of bone resorption and reduced activation frequency of bone remodeling units, but also to enhanced osteon mineralization.⁹⁶ These BPs also prevent bone loss associated with glucocorticosteroid administration.^{97,98}

Pamidronate has proved remarkably effective in increasing bone in children with the inherited “brittle bone” disorder, osteogenesis imperfecta.^{99,100}

Among the newer BPs, ibandronate¹⁰¹ has been recently introduced as a once monthly tablet. In addition to formulations to be taken by mouth

weekly or monthly, new routes of administration are being studied, especially periodic (e.g., 3 monthly) injections with ibandronate, and once yearly treatment with zoledronate.¹⁰² This has the great attraction of delivering a defined dose without the variability associated with oral administration as well as avoiding potential gastrointestinal intolerance. If these approaches are accompanied by greater compliance and convenience, they may become popular methods of treatment.

CLINICAL PHARMACOLOGY AND USE OF BPs

The clinical pharmacology of BPs is characterized by low intestinal absorption (~1–4%), but highly selective localization and retention in bone. Significant side effects of BPs are minimal.^{103–105} Although there are more similarities than differences among individual compounds and each bisphosphonate is potentially capable of treating any of the disorders of bone resorption in which they are used, in practice different compounds have come to be favored for the treatment of different diseases. Currently, there are at least 10 BPs (etidronate, clodronate, tiludronate, pamidronate, alendronate, risedronate, zoledronate, and ibandronate, and also to a limited extent olpadronate and neridronate) that have been registered for various clinical applications in various countries. To a major extent, the diseases in which they are used reflect the history of their clinical development and the degree of commercial interest in and sponsorship of the relevant clinical trials. The one most used in Paget's disease was formerly pamidronate given parenterally, which is now being superseded by zoledronate. In cancer, i.v. pamidronate or clodronate given orally has been extensively used, but is being replaced by zoledronate, which is superior in trials. In osteoporosis, the major current drugs are risedronate and alendronate.

Other clinical issues under consideration with BPs include the choice of therapeutic regimen, for example, the use of intermittent dosing rather than continuous, intravenous versus oral therapy, the optimal duration of therapy, the combination with other drugs such as teraparotide, and their extended use in related indications, for example, glucocorticosteroid-associated osteoporosis, male osteoporosis, childhood osteopenic disorders, arthritis, and other disorders. There is therefore much that needs to be done to improve the way in which existing drugs can be used, as well as enable new drugs to be introduced.

UNDERSTANDING THE MECHANISMS OF ACTION OF BPs AT A CELLULAR LEVEL

The pronounced selectivity of BPs for bone rather than other tissues is the basis for their value in clinical practice. Their preferential uptake by and

adsorption to mineral surfaces in bone brings them into close contact with osteoclasts. During bone resorption, BPs are probably internalized by endocytosis, along with other products of resorption. Many studies have shown that BPs can affect osteoclast-mediated bone resorption in a variety of ways that include effects on osteoclast recruitment, differentiation, and resorptive activity, and may induce apoptosis.

Since mature, multinucleated osteoclasts are formed by the fusion of mononuclear precursors of hematopoietic origin, BPs could also inhibit bone resorption by preventing osteoclast formation, in addition to affecting mature osteoclasts. *In vitro*, BPs can inhibit in a dose-dependent manner the formation of osteoclast-like cells in long-term cultures of human bone marrow.¹⁰⁶ In organ culture also, some BPs can inhibit the generation of mature osteoclasts, possibly by preventing the fusion of osteoclast precursors.^{107,108}

It is likely that BPs are selectively internalized by osteoclasts rather than other cell types because of their accumulation in bone and the endocytic activity of osteoclasts. During the process of bone resorption, the subcellular space beneath the osteoclast is acidified by the action of vacuolar-type proton pumps in the ruffled border of the osteoclast membrane.¹⁰⁹ The acidic pH of this microenvironment causes dissolution of the HAP bone mineral, while the breakdown of the extracellular bone matrix is brought about by the action of proteolytic enzymes. Since BPs adsorb to bone mineral, especially at sites of bone resorption where the mineral is most exposed,^{110,111} osteoclasts are the cell type in bone most likely to be exposed to the highest concentrations of free, non-mineral-bound bisphosphonate, as a result of the release of the bisphosphonate from bone mineral in the low pH environment beneath osteoclasts. It has been estimated that pharmacological doses of alendronate that inhibit bone resorption *in vivo* could give rise to local concentrations as high as 1 mM alendronate in the resorption space beneath an osteoclast. This is much higher than the concentrations of BPs required to affect osteoclast morphology and cause osteoclast apoptosis *in vitro*.¹¹²

Another observation that remains unexplained is that BPs may act at nanomolar concentrations to stimulate osteoblasts to produce an osteoclast inhibitory factor.¹¹³⁻¹¹⁶

Despite reports that some BPs do not have toxic effects on osteoclasts,¹¹⁷ it is clear from many studies that BPs can reduce osteoclast number¹¹⁸ and can induce apoptotic cell death in osteoclasts.¹¹⁹ These effects occur both with the nitrogen-containing bisphosphonates (N-BPs), as well as the simpler BPs, such as clodronate.¹²⁰

In addition, apoptosis triggered by exposure to BPs is not restricted to osteoclasts, since macrophages, such as the murine cell line J774,^{121,122} and human myeloma cell lines^{123,124} also undergo apoptosis after treatment with several (N-BPs) *in vitro*.

UNDERSTANDING THE MECHANISMS OF ACTION OF BPs AT A BIOCHEMICAL LEVEL

Over the years there have been many efforts to explain how BPs work on cells, especially via inhibitory effects on enzymes, for example, by direct or indirect inhibition of the osteoclast proton-pumping H^+ ATPase,¹²⁵⁻¹²⁷ phosphatases, or lysosomal enzymes.^{128,129}

Because osteoclasts are highly endocytic, bisphosphonate present in the resorption space is likely to be internalized by endocytosis, and thereby affect osteoclasts directly.¹³⁰ The uptake of BPs by osteoclasts *in vivo* has been confirmed using radiolabeled¹³¹ and fluorescently labeled alendronate, which was internalized into intracellular vacuoles. Following cellular uptake, a characteristic morphological feature of bisphosphonate-treated osteoclasts is the lack of a ruffled border, the region of invaginated plasma membrane facing the resorption cavity. BPs also disrupt the cytoskeleton of the osteoclast.¹³² Early explanations for these effects invoked the inhibition of protein kinases or phosphatases that regulate cytoskeletal structure, such as protein tyrosine phosphatases.¹³³⁻¹³⁶ However, a more likely mechanism by which the cytoskeleton may be affected involves loss of function of small GTPases, such as Rho and Rac.

Since the early 1990s there has been a systematic effort to elucidate the molecular mechanisms of action of BPs. Our work in this area has been led by Michael Rogers,¹³⁷ and we have proposed that BPs can be classified into at least two major groups with different modes of action (FIG. 4). The first group comprises the non-nitrogen BPs that perhaps most closely resemble PPi, such as clodronate and etidronate, and these can be metabolically incorporated into nonhydrolyzable analogues of adenosine triphosphate (ATP). It is likely that intracellular accumulation of these metabolites within osteoclasts inhibits their function and may cause osteoclast cell death. In contrast, the second group contains the more potent, (N-BPs), such as alendronate, risedronate, and zoledronate. Members of this group interfere with other metabolic reactions, notably in the mevalonate biosynthetic pathway, and affect cellular activity and cell survival by interfering with protein prenylation and therefore the signaling functions of key regulatory proteins. These mechanisms are discussed in greater detail below.

The ATP BP Metabolite Mechanism

This work has its origins in the study of the inhibitory effects of BPs on the growth of the amebas of the slime mold *Dictyostelium discoideum*.^{138,139} In the first of what appear to be two major but distinct molecular mechanisms by which BPs affect osteoclasts, we found that some, but not all, BPs could be metabolically incorporated by the amebas into analogues of adenosine

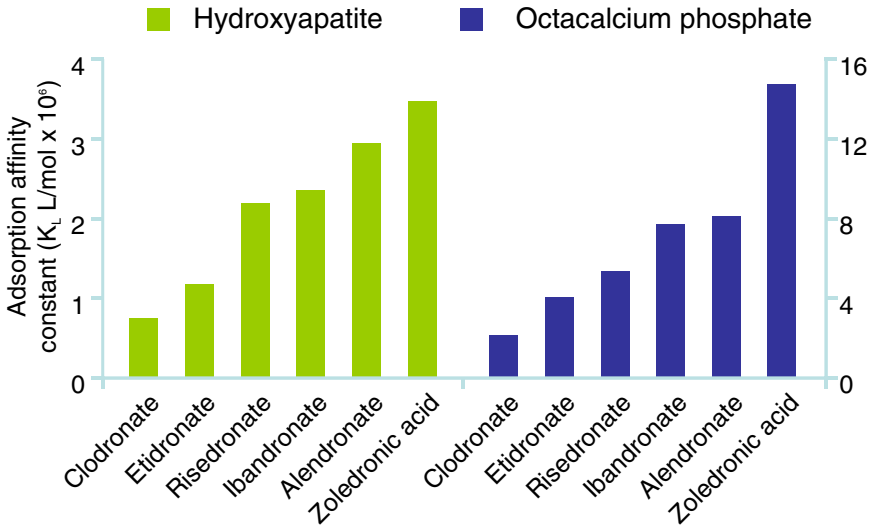


FIGURE 4. Differential binding of BPs to 2 calcium phosphate minerals found in bone, hydroxyapatite and octacalcium phosphate. There are unexpected differences between the BPs indicating that not only the P-C-P structure but also the R2 side chains contribute to mineral binding. Nancollas GH, et al. Bone 2006 in press. doi:10.1016/j.bone.2005.05.003

triphosphate (ATP or Appp).^{140,141} The resulting metabolites contained the P-C-P moiety in place of the β, γ -phosphate groups of ATP, thus resulting in nonhydrolyzable (AppCp) nucleotides.

The BPs that were metabolized by *Dictyostelium discoideum* all contained short R1 and R2 side chains, with the exception of tiludronate, and were relatively weak inhibitors of bone resorption. A similar classification into metabolizable and nonmetabolizable BPs was obtained with cell-free lysates from mammalian cells. We found that the incorporation of BPs into these AppCp nucleotide analogues is brought about by members of the family of aminoacyl-tRNA synthetases,¹⁴² which catalyze a reversible reaction in which an amino acid condenses with ATP to form an aminoacyladenylate, together with the release of PPI (reaction 1, shown below). Since this reaction is reversible, it appears that BPs with short R1 and R2 side chains (which most resemble PPI in structure) can replace PPI in the back reaction (reaction 2). This results in the condensation of a bisphosphonate (pCp) with an aminoacyladenylate (amino acid-AMP), to form an analogue of ATP (AppCp).

1. Enzyme + amino acid + ATP \rightleftharpoons amino-acyl-AMP + PPI
2. Amino-acyl-AMP + pCp \rightleftharpoons amino acid + AppCp

The aminoacyl-tRNA synthetases that can use a bisphosphonate in place of PPI all belong to the type II subclass of enzymes (e.g., Asn-, Asp-, Gly-, His-, Lys-, Phe-, Ser-aminoacyl-tRNA synthetases), which differ from the type

I subclass in the structure of the catalytic site. Thus, it appears that BPs with short side chains, but also rather surprisingly tiludronate, can replace PPI and be accommodated into the active site of type II aminoacyl-tRNA synthetases. In contrast, the more potent BPs that contain a nitrogen atom in the R2 side chain are not metabolized, presumably since the different, and in some cases bulkier, structure of the R2 side chain prevents these BPs from binding at the active site of these aminoacyl-tRNA synthetase enzymes.

Although the formation of AppCp-type bisphosphonate metabolites was first demonstrated in slime mold amoebas (which also produced diadenosine tetraphosphate metabolites, AppCpA) and with cell-free lysates,¹⁴³ it also occurs in intact mammalian cells *in vitro* (J774 macrophage-like cells and MG63 osteosarcoma cells), which can also metabolize clodronate to an analogue of ATP (AppCCl2p),¹⁴⁴ as confirmed by mass spectrometric analysis of cell lysates from clodronate-treated cells,¹⁴⁵ including purified rabbit osteoclasts *in vitro*.¹⁴⁶

The aminoacyl-tRNA synthetases are cytoplasmic enzymes, and the metabolism of BPs is dependent on cellular uptake.¹⁴⁷ As a result of the accumulation in the cell cytoplasm of these nonhydrolyzable AppCp analogues of ATP, they are likely to inhibit intracellular enzymes, thus having adverse effects on cell metabolism, function, and survival.

The AppCp-type metabolites of BPs are cytotoxic when internalized and cause similar changes in morphology to those observed in clodronate-treated cells, possibly by interference with mitochondrial ATP translocases.¹⁴⁸ Overall, this group of BPs therefore appears to act as prodrugs, being converted to active metabolites following intracellular uptake by osteoclasts *in vivo*.

The Mevalonate Pathway Mechanism

The potent, (N-BPs) are apparently not metabolized to AppCp-type metabolites as described above. A major step forward has been the demonstration that the (N-BPs) used as inhibitors of bone resorption all appear to be inhibitors of the mevalonate pathway. This is a biosynthetic pathway responsible for the production of cholesterol, other sterols, and isoprenoid lipids such as isopentenylidiphosphate (also known as isopentenylpyrophosphate IPP), as well as farnesylidiphosphate (FPP) and geranylgeranylidiphosphate (GGPP) (FIG. 5). FPP and GGPP are required for the posttranslational modification (prenylation) of small GTPases such as Ras, Rab, Rho, and Rac, which are prenylated at a cysteine residue in characteristic C-terminal motifs.¹⁴⁹ Small GTPases are important signaling proteins, which regulate a variety of cell processes important for osteoclast function, including cell morphology, cytoskeletal arrangement, membrane ruffling, trafficking of vesicles, and apoptosis.¹⁵⁰⁻¹⁵³ Prenylation is required for the correct function of these proteins, since the lipid prenyl group serves to anchor the proteins in cell membranes and may also participate in protein-protein interactions.¹⁵⁴

Molecular Mechanisms of Action of Bisphosphonates

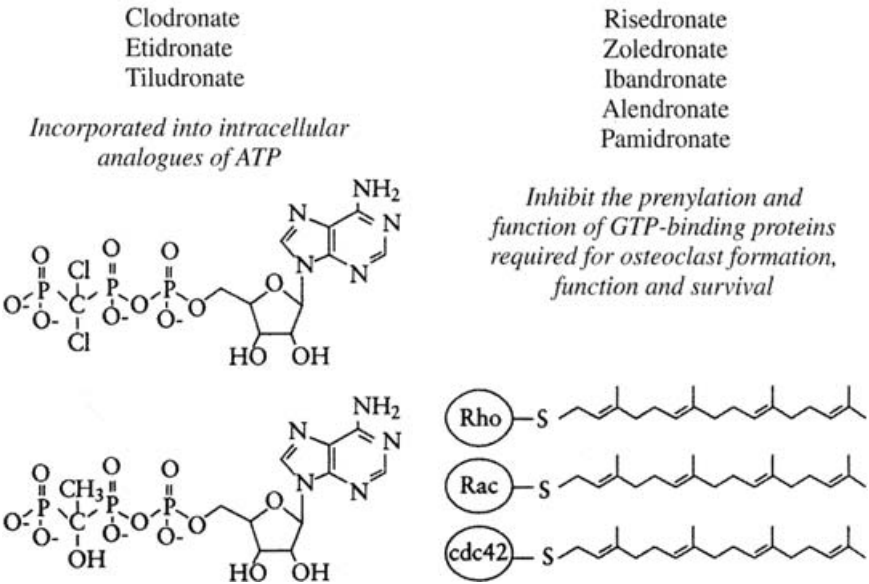


FIGURE 5. Bisphosphonates can be divided into two classes (those that do or do not contain nitrogen in the R2 side chain) according to their intracellular actions. Protein prenylation involves the transfer of a farnesyl or geranylgeranyl group onto cysteine residues near the C-terminus of small GTPases such as Rho, Rac, Rab and Ras.

There are now many observations that point to the importance of the mevalonate pathway for osteoclast function, and strengthen the proposal that the (N-BPs) inhibit osteoclastic bone resorption predominantly by inhibition of this pathway. These BPs inhibit the synthesis of mevalonate metabolites including FPP and GGPP, and thereby impair the prenylation of proteins,¹⁵⁵ and cause loss of function of small GTPases. There is a strong structure-activity relationship so that changes to the structure of the nitrogen-containing R2 side chain or to the phosphonate groups, which altered antiresorptive potency and also influenced the ability to inhibit protein prenylation to a corresponding degree.¹⁵⁶ An important verification of the critical importance of this pathway has come from showing that the addition of intermediates of the mevalonate pathway (such as FPP and GGPP) could overcome bisphosphonate-induced apoptosis and other events in many cell systems. A further prediction was that if inhibition of the mevalonate pathway could account for the antiresorptive effects of BPs, then the statin drugs should also inhibit bone resorption. Statins are inhibitors of HMG-CoA reductase, one of the first steps in the mevalonate pathway. They proved to be even more potent than BPs at inhibiting osteoclast formation and bone resorption *in vitro*,^{157,158} an effect that could also be

overcome by the addition of geranylgeraniol (which can be used for protein geranylgeranylation) but not farnesol (which is utilized for protein farnesylation). Hence, it appears that, although (N-BPs) can prevent both farnesylation and geranylgeranylation of proteins (probably by inhibiting enzymes required for synthesis of FPP and GGPP), loss of geranylgeranylated proteins in osteoclasts is of greater consequence than loss of farnesylated proteins. This is consistent with the known role of geranylgeranylated proteins, such as Rho, Rac, and Rab in processes that are fundamental to osteoclast formation and function (e.g., cytoskeletal rearrangement, membrane ruffling, and vesicular trafficking¹⁵⁹), and further work has confirmed this, particularly the importance of Rab proteins.

The comparison between BPs and statins is interesting. The statins are widely used as cholesterol-lowering drugs, through their ability to lower cholesterol biosynthesis by inhibiting HMG-CoA reductase. Despite several studies there is no substantial evidence that statins have effects on bone when used clinically, perhaps because they are selectively taken up by liver rather than bone, which is the converse of the case for BPs. This is therefore an excellent example of how drug specificity is achieved by highly selective tissue targeting (FIG. 6).

The exact enzymes of the mevalonate pathway that are inhibited by individual BPs have been partially elucidated. Several enzymes of the pathway utilize isoprenoid diphosphates as a substrate (IPP isomerase, FPP synthase, GGPP synthase, squalene synthase) and thus are likely to have similar substrate binding sites. Thus, if (N-BPs) act as substrate analogues of an isoprenoid diphosphate, it is possible that these BPs will inhibit more than one of the enzymes of the mevalonate pathway. Early studies revealed that incadronate and ibandronate, but not other BPs, are inhibitors of squalene synthase, an enzyme in the mevalonate pathway required for cholesterol biosynthesis.^{160,161} Inhibition of squalene synthase would not, however, lead to inhibition of protein prenylation.

However, it is now clear that farnesyl PPi synthase (FDPS or FPPS) is a major site of action of the N-BPs.¹⁶² FPPS catalyzes the successive condensation of isopentenyl PPi with dimethylallyl PPi and geranyl PPi (FIG. 7). There is a strong relationship among individual BPs between inhibition of bone resorption and inhibition of FPPS with the most potent BPs having IC₅₀s in the nM range.¹⁶³ Modeling studies provide a molecular rationale for BP binding to FPPS.¹⁶⁴ Our recent studies using protein crystallography, enzyme kinetics, and isothermal titration calorimetry have led to the first high-resolution X-ray structures of the human enzyme in complexes with risedronate and zoledronate.¹⁶⁵ These agents bind to the dimethylallyl/geranyl PPi ligand pocket and induce a conformational change. The interactions of the N-BPs cyclic nitrogen with Thr201 and Lys200 suggest that these inhibitors achieve potency by positioning their nitrogen in a proposed carbocation binding site.¹⁶⁶ This explains why the nitrogen moiety is so important to the potency of these BPs (FIG. 8). Kinetic analyses reveal that inhibition is competitive with geranyl

Selective Inhibition of the Mevalonate Pathway by Statins and Bisphosphonates is the Result of Selective Tissue Targeting

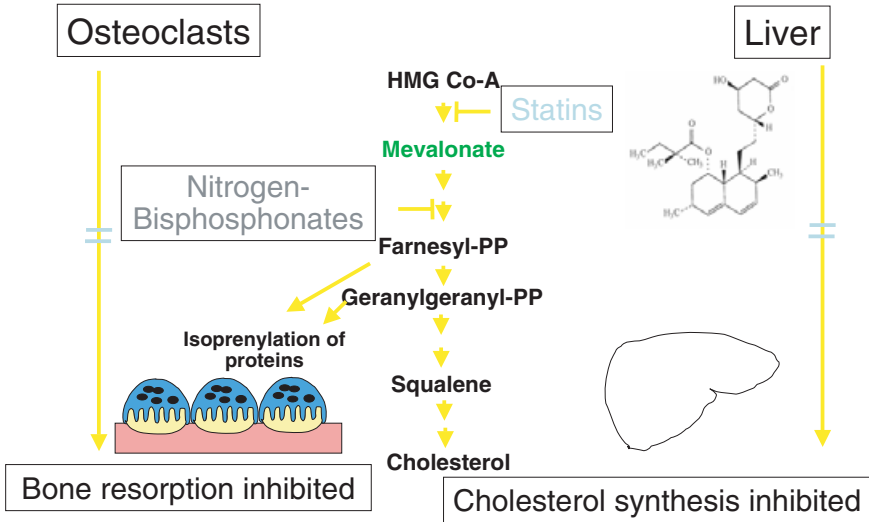


FIGURE 6. The differential effects of statins and BPs in the mevalonate pathway, showing how tissue selectivity of uptake determines their pharmacological specificity.

PPi and is of a slow, tight-binding character, indicating that isomerization of an initial enzyme-inhibitor complex occurs upon binding of the N-BP.

Taken together, these observations clearly indicate that BPs can be grouped into two classes: those that can be metabolized into nonhydrolyzable analogues of ATP (the least potent BPs) and those that are not metabolized but that can inhibit protein prenylation (the potent, N-BPs). The identification of two such classes may help to explain some of the other pharmacologic differences between the two classes.

CURRENT ISSUES AND NEW LEADS WITH BPs

Update on PPi

Since the early studies that indicated that PPi was a potential endogenous regulator of mineralization, there have been significant advances in understanding the metabolism of PPi and in identifying clinical disorders in which alterations in PPi may have a pathogenic role.

Much of the PPi in the extracellular compartment may be generated at the cell surface by the action of nucleoside triphosphate pyrophosphohydrolases

Human FPP synthase catalyses the synthesis of GPP and FPP

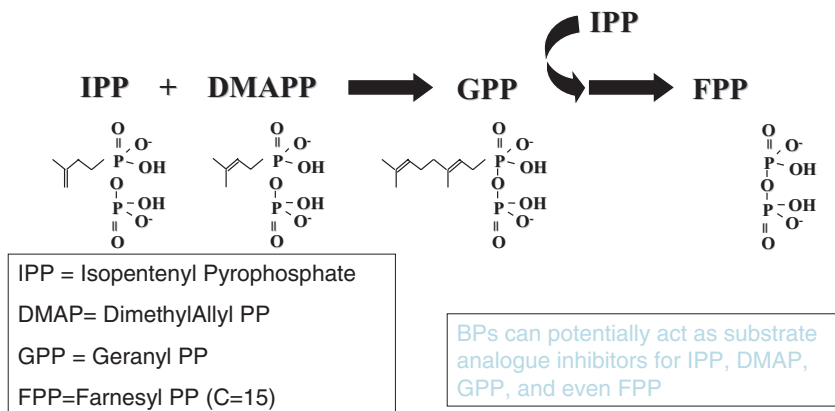


FIGURE 7. The enzyme reactions catalysed by Farnesyl Pyrophosphate Synthase. N-BPs act as substrate analogues for GPP.

(NTP-PPases), which liberate PPi from NTPs, such as ATP. The major enzyme involved in removing PPi is alkaline phosphatase or tissue nonspecific alkaline phosphatase (TNAP), as has been known for many years. TNAP is also located at cell surfaces and its tissue distribution is restricted, particularly to liver, cartilage, and bone. A third regulator of extracellular PPi has been postulated to be a membrane transporter of PPi called ANK, which is thought to extrude PPi from within cells.

Genetic mutations of all three of these regulatory proteins are associated with disturbances in PPi metabolism and disordered calcification.¹⁶⁷⁻¹⁶⁹ Skeletal mineralization is defective when PPi is high, e.g., in hypophosphatasia due to inactivating mutations in TNAP.^{170,171} Conversely, excessive mineralization and bone formation may occur when NTP-PPase (PC-1) is defective and PPi levels are low as in juvenile vascular calcification¹⁷² and another rare condition called ossification of the posterior longitudinal ligament (OPLL)¹⁷³ of the spine and osteoarthritis, which occurs particularly in Japanese populations.

Mutations of the ANK gene in mice produces a skeletal phenotype of progressive ankylosis and aberrant calcification,¹⁷⁴ while in humans mutations of ANKH are associated with familial chondrocalcinosis, a condition in which calcium PPi crystals deposit in articular cartilage and other sites.¹⁷⁵⁻¹⁷⁷ Mutations of the ANKH gene are also somewhat unexpectedly associated with craniometaphyseal dysplasia (CMD).^{178,179}

So far there is no evidence that BPs used clinically interfere with the endogenous metabolism of extracellular PPi.

Risedronate and Zoledronate located in GPP binding site of Farnesyl Pyrophosphate Synthase (FPPS or FDPS).

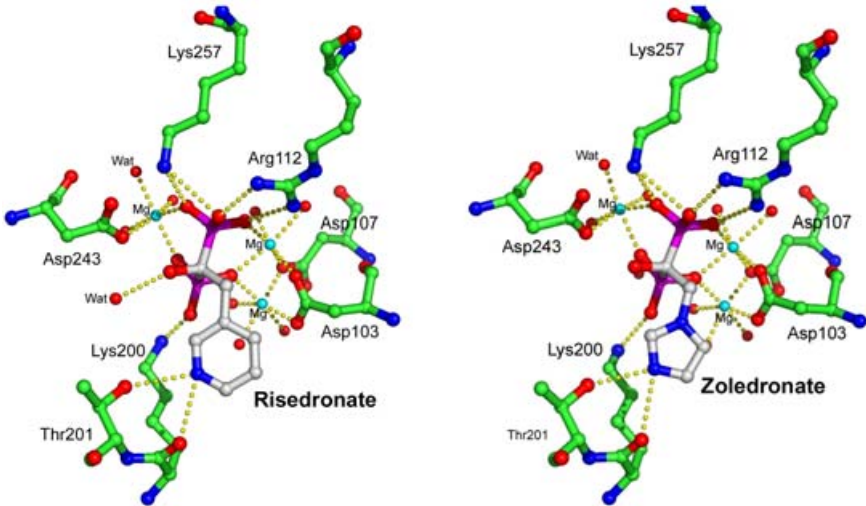


FIGURE 8. Risedronate and Zoledronate located in the GPP binding site of Farnesyl Pyrophosphate Synthase (FDPS or FPPS). See Kavanagh K., Guo K, Dunford J, Wu X, Knapp S., Ebetino FH, Rogers MJ, Russell RGG, Oppermann U. Human Farnesyl Diphosphate Synthase (FDPS): Crystal structure and molecular interactions with nitrogen-containing bisphosphonates. PNAS in press 2006 & J Bone Min Res Sa409 2005

The Effects of BP on Bone Architecture, Structure and Strength, and on Bone Healing and Fracture Repair

Many experimental and clinical studies show that BPs conserve bone architecture and strength.¹⁸⁰⁻¹⁸⁴ However, there have been concerns about whether the use of prolonged high doses of BPs may impair bone turnover to such an extent that bone strength is impaired. High doses in animals are associated with increased microdamage^{185,186} and even fractures.¹⁸⁷ It has been suggested that BPs might prevent naturally occurring microscopic cracks in bone from healing. There are isolated reports of adynamic bone associated with BP usage¹⁸⁸ but the long-term use of the BPs in the therapy of osteoporosis appears to be safe.¹⁸⁹ Case reports of induction of osteopetrosis-like lesions in children treated with excessive doses of pamidronate have been reported.¹⁹⁰

A question often asked is whether BPs inhibit fracture repair. By reducing bone turnover one might expect BPs to interfere with fracture healing. However, a recent long-term study in a beagle dog model that simulated fracture repair has demonstrated that ibandronate treatment did not adversely affect normal bone healing.¹⁹¹ Studies of repair processes after creating drill hole defects in dogs also showed no impairment with ibandronate.¹⁹²

Several other recent studies raise the intriguing possibility that BPs may enhance fracture repair and related processes.¹⁹³ In studies of the osseointegration of metal implants in OVX rats, treatment with ibandronate resulted in improved osseointegration, rather than impairment of the healing process.¹⁹⁴ Potential applications of BPs in orthopedics include protection against loosening of prostheses,¹⁹⁵ better integration of biomaterials and implants, improved healing in distraction osteogenesis,¹⁹⁶ and conserving bone architecture after osteonecrosis^{197,198} and in Perthes disease.¹⁹⁹

How do BPs Work? Explaining the Long Duration of Action. Recycling Within Bone

BPs are well accepted as the main class of antiresorptive agents and have many clinical applications. There are potentially important differences between clinically useful BPs regarding their potency and duration of action. Efficacy is closely related to affinity for bone mineral and ability to inhibit FPP synthase. Recent studies (see FIG. 9) have shown that there are marked differences among BPs in binding to HAP,²⁰⁰ may explain the variations in retention and persistence of effect observed in animal and clinical studies. In the case of zoledronic acid in particular, the remarkable magnitude of effect and prolonged duration of action can be explained in part by these new observations. In explaining the long duration of action, it has been proposed that there is continuous recycling of BP off and back onto the bone surface. This notion is supported by observations that BPs can be found in plasma and urine many months after dosing.

How do BPs Work? Actions on Osteocytes

In contrast to their ability to induce apoptosis in osteoclasts, which contributes to the inhibition of resorptive activity, some experimental studies suggest that BPs may protect osteocytes and osteoblasts from apoptosis induced by glucocorticoids.²⁰¹ Recent evidence suggests that the inhibition of osteocyte apoptosis by BPs is mediated through the opening of connexin43 hemichannels and activation of extracellular signal-regulated kinases.²⁰² The possibility that BPs used clinically may get access to osteocytes differentially depending on their mineral-binding affinities and inherent structural properties needs to be studied.

The “Acute Phase Response”

A well-recognized side effect of the (N-BPs) is to cause an acute phase response *in vivo*,^{203–205} which can lead to induction of fever and “flu”-like

Bisphosphonate Uptake and Detachment from Bone Surfaces.
Effect of Binding Affinity

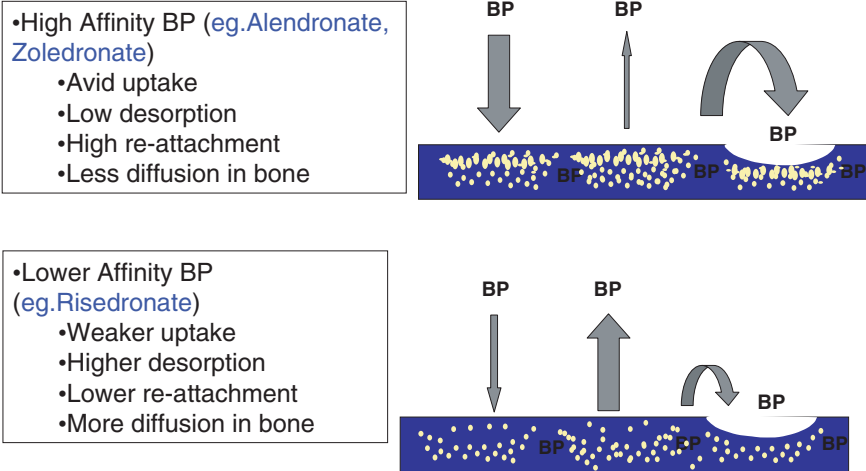


FIGURE 9. Effect of Binding Affinity of Bisphosphonates on their Uptake and Detachment from bone surfaces (Adapted from Nancollas GH, et al. Bone 2006 in press.)

symptoms in patients. These effects are transient and occur predominantly on first exposure to the drug, especially with i.v. administration. The mechanism has been attributed to release of pro-inflammatory cytokines, and the mechanism has been further unraveled by showing that it involves selective receptor-mediated activation of γ , Δ T cells leading to their proliferation and activation.²⁰⁶ The BP effect involves the mevalonate pathway *in vitro* and can be overcome by using statins.²⁰⁷

Nonskeletal Effects of BPs

There are numerous examples of BPs having effects on cells and tissues outside the skeleton. The effects on osteoclast precursors, tumor cells, macrophages, and γ Δ T cells are examples, and in all cases are probably explained by sufficient BPs entering cells to inhibit the mevalonate pathway.

A particularly interesting aspect of these nonskeletal effects is the observations made on protozoan parasites, the growth of which can be inhibited by BPs acting on FPPS.^{208,209} The therapeutic potential is enticing given the importance of these diseases. The range of affected protozoa include *Entamoeba*,²¹⁰ *Plasmodia*,²¹¹ *Trypanosomes*,²¹² *Toxoplasma*,²¹³ *Cryptosporidia*,²¹⁴ and *Leishmania* spp.²¹⁵

SUMMARY AND FUTURE PROSPECTS

It has taken over 30 years since the discovery of the profound effects of the BPs on calcium metabolism for them to become well established as clinically successful antiresorptive agents, and their availability has enabled new approaches to the therapy of bone diseases.

There have now been many years of mostly favorable experience with the use of BPs in diseases such as Paget's disease of bone, myeloma, and bone metastases. BPs represent an important class of drugs for the treatment of these bone diseases.

Their application in osteoporosis is more recent and was spurred on by the development of techniques to measure bone mass with precision, the increased awareness of osteoporosis as a major socioeconomic problem, and the willingness of the larger pharmaceutical companies to invest in clinical studies on the scale necessary to demonstrate their effects on fractures.

The difficulties of bringing these drugs to the market are illustrated by those that fall by the wayside, such as oral pamidronate and tiludronate. There are important lessons to be learned from the need to do good dose-response studies during phase II development and making appropriate choices of doses.

However, despite the enormous potential for developing "better" BPs based on current knowledge of their structure-activity properties, it is uncertain, given the high cost of development, that further agents will be developed unless they offer distinct advantages over currently available BPs.

Other clinical indications ripe for future study include the prevention of bone loss and erosions in rheumatoid arthritis, possible applications in other joint diseases, and the reduction of bone loss associated with periodontal disease, and loosening of joint prostheses.

The recent elucidation of the likely mode of action of BPs within cells opens up the possibility of exploiting the subtle and potentially important differences between classes of BPs and individual compounds.

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