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 Fleisch[19] 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 mineral[29] 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 studies[37] using radio-calcium (^{45}Ca), 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

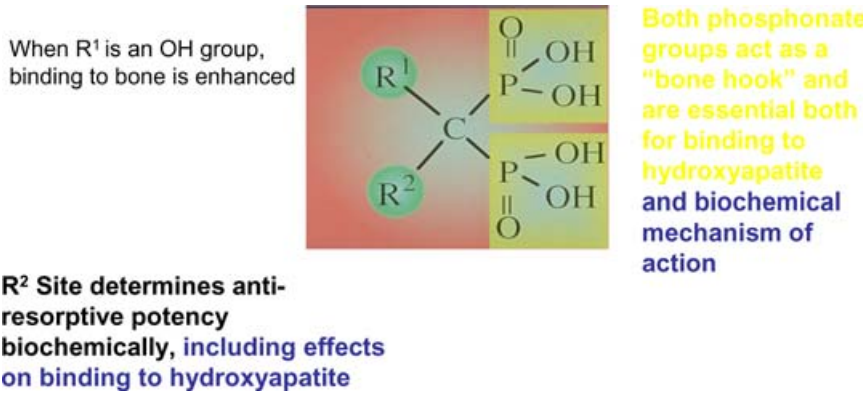

FIGURE 1. The generic structure of bisphosphonates and their functional domains.

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 ibandronate[44] 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

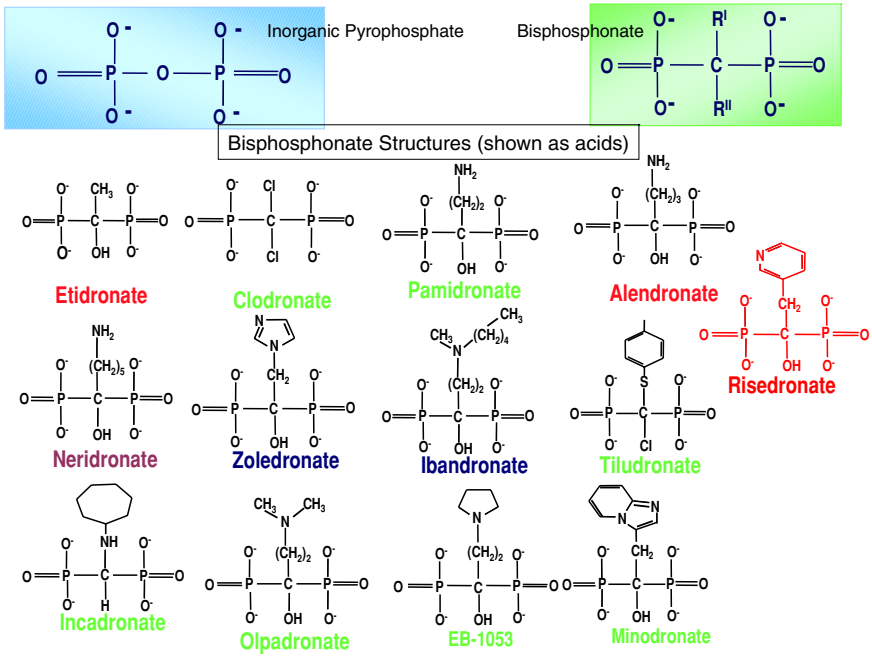

FIGURE 2. Structures of the bisphosphonates used in clinical studies and under clinical development.

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 ibandronate[47] 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 risedronate[49] and zoledronate[50]), 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

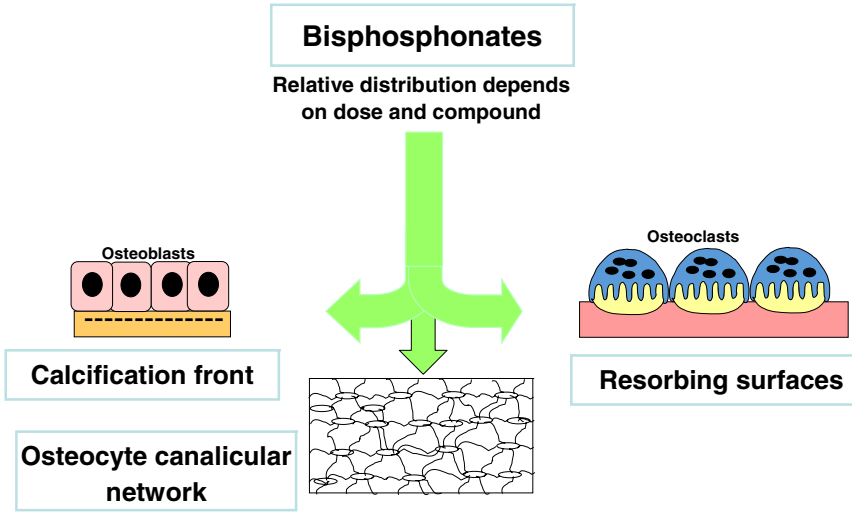

FIGURE 3. Uptake and distribution of BPs into different compartments of bone. This distribution may be affected by both the dose and the compound given.

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 fibrodysplasis ossicans 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 basis[58] 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 malignancy[70] 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 myeloma[74] 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 osteoporosis[86] 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, ibandronate[101] 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 teraparatide, 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 number[118] 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 radiolabeled[131] 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

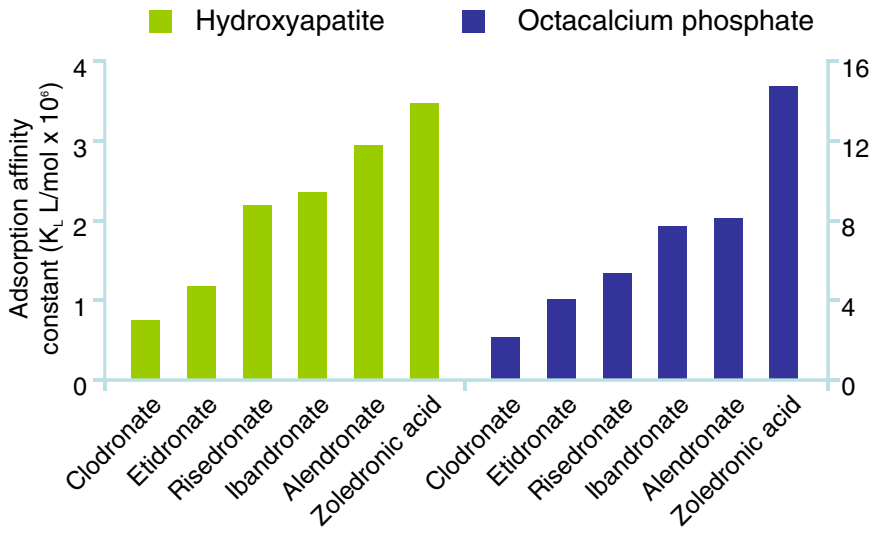

FIGURE 4. Differential binding of BPs to 2 calcium phosphate minerals found in bone, hydroxyapatite and octacalcium phosphate. There are unexpected differences between the BPs indicating that not only the P-C-P structure but also the R2 side chains contribute to mineral binding. Nancollas GH, et al. 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 ⇔ amino-acyl-AMP + PPi
2. Amino-acyl-AMP + pCp ⇔ 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 amebas (which also produced diadenosine tetraphosphate metabolites, AppCppA) 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 isopentenyldiphosphate (also known as isopentenylpyrophosphate IPP), as well as farnesyldiphosphate (FPP) and geranylgeranyldiphosphate (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]

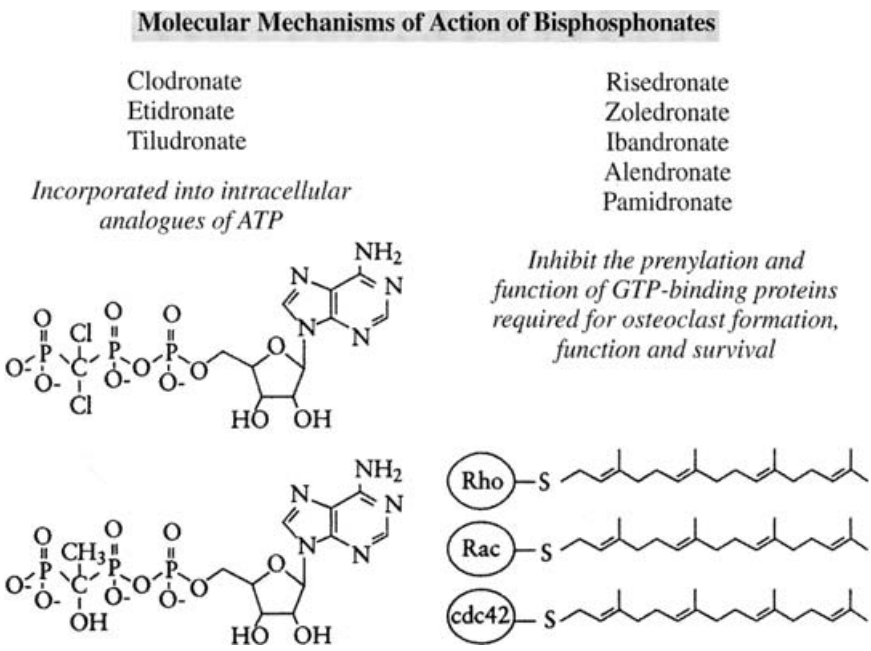

FIGURE 5. Bisphosphonates can be divided into two classes (those that do or do not contain nitrogen in the R2 side chain) according to their intracellular actions. Protein prenylation involves the transfer of a farnesyl or geranylgeranyl group onto cysteine residues near the C-terminus of small GTPases 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 trafficking[159]), 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

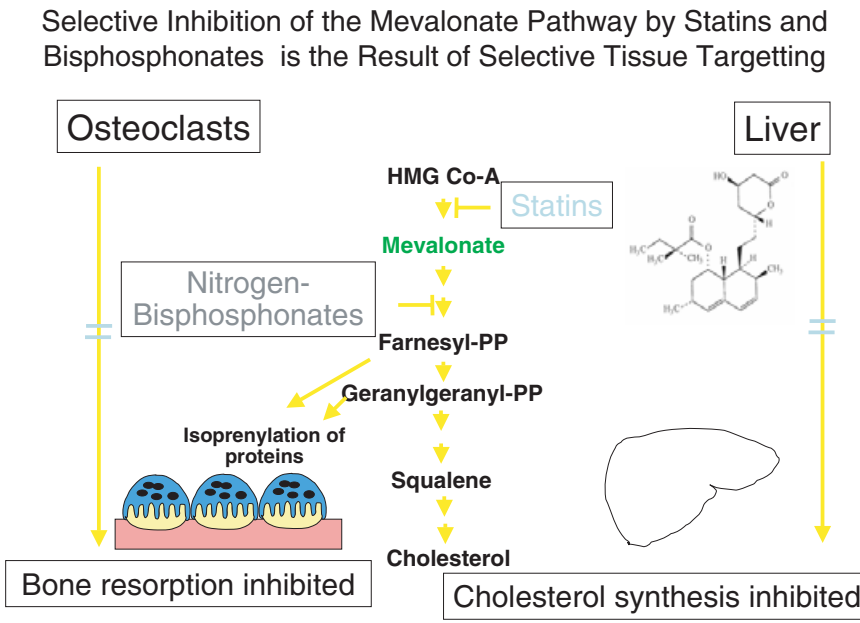

FIGURE 6. The differential effects of statins and BPs in the mevalonate pathway, showing how tissue selectivity of uptake determines their pharmacological specificity.

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

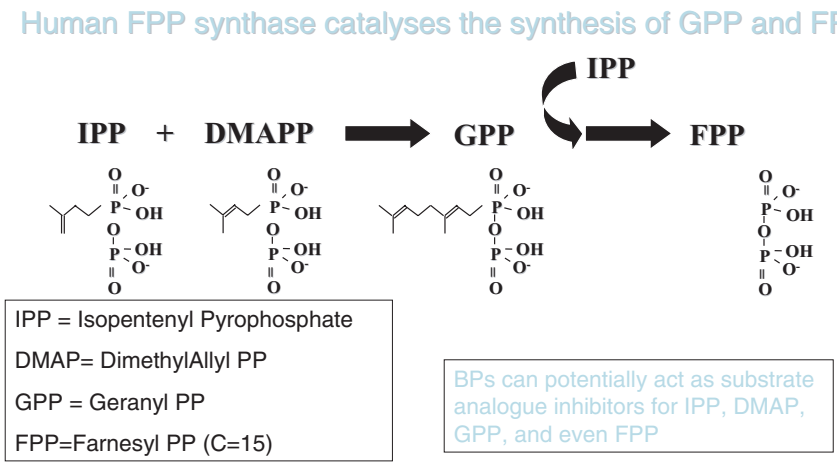

FIGURE 7. The enzyme reactions catalysed by Farnesyl Pyrophosphate Synthase. N-BPs act as substrate analogues for GPP.

(NTP-PPases), which liberate PPi from NTPs, such as ATP. The major enzyme involved in removing PPi is alkaline phosphospatase or tissue nonspecific alkaline phosphatase (TNAP), as has been known for many years. TNAP is also located at cell sufaces 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 calcification[172] 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.

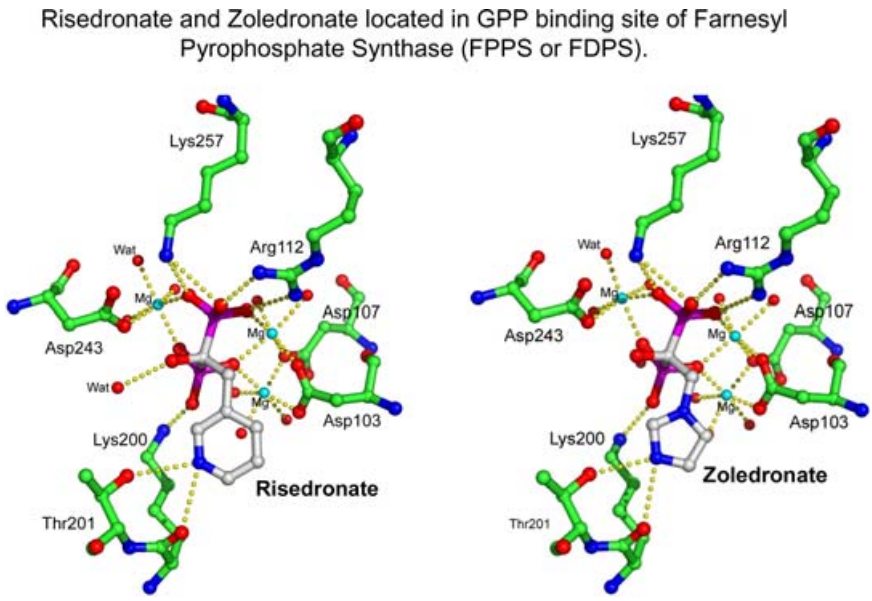

FIGURE 8. Risedronate and Zoledronate located in the GPP binding site of Farnesyl Pyrophosphate Synthase (FDPS or FPPS). See Kavanagh K., Guo K, Dunford J, Wu X, Knapp S., Ebetino FH, Rogers MJ, Russell RGG, Oppermann U. Human Farnesyl Diphosphate Synthase (FDPS): Crystal structure and molecular interactions 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 usage[188] but the long-term use of the BPs in the therapy of osteoporosis appears to be safe.[189] Case reports of induction of ostepetrosis-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 connexion43 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

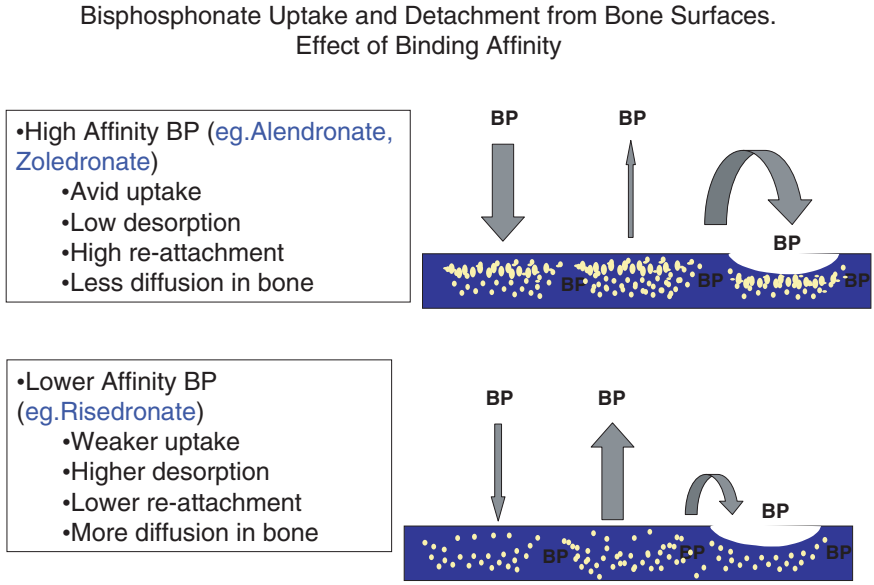

FIGURE 9. Effect of Binding Affinity of Bisphosphonates on their Uptake and Detachment from bone surfaces (Adapted from Nancollas GH, et al. Bone 2006 in press.)

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.

REFERENCES

1. MENSCHUTKIN, N. 1865. Ueber die Einwirkung des Chloracetyls auf phosphorige Saure. Ann. Chem. Pharm. **133:** 317–320.
2. SMITH, R., R.G.G. RUSSELL & M. BISHOP. 1971. Diphosphonates and Paget's disease of bone. Lancet **1:** 945–947.
3. VON BAEYER, H. & K.A. HOFMANN. 1897. Acetodiphosphorige Saure. Berichte Dtsch. Chem. Ges. **20:** 1973–1978.
4. BLOMEN, L.J.M.J. 1995. Discovery and history of the non-medical uses of bisphosphonates. Chapter 7. *In* Bisphosphonates on Bone. O. Bijvoet, *et al*. Eds.: 111–124. Elsevier Science B.V. Amsterdam.
5. RUSSELL, R.G. & M.J. ROGERS. 1999. Bisphosphonates: from the laboratory to the clinic and back again [review]. Bone **25:** 97–106.

6. BIJVOET, O., H. FLEISCH, R.E. CANFIELD & R.G.G. RUSSELL, Eds. 1995. Bisphosphonates on Bone. Elsevier Science B.V. Amsterdam.
7. FLEISCH, H. 2000. Bisphosphonates in Bone Disease. From the Laboratory to the Patient, 4th ed. Academic Press. New York.
8. FLEISCH, H. 1998. Bisphosphonates: mechanisms of action. Endocr. Rev. **19:** 80–100.
9. EBETINO, F.H., M.D. FRANCIS, M.J. ROGERS, *et al*. 1998. Etidronate. Mechanisms of action of etidronate and other bisphosphonates. Revs. Contemp. Pharma **9:** 233–243.
10. GREEN, J.R. 2004. Bisphosphonates: preclinical review. Oncologist. **9**(Suppl 4): 3–13.
11. RUSSELL, R.G.G. & M.J. ROGERS. 1997. Introduction to bisphosphonates and the clinical pharmacology of alendronate. Br. J. Rheumatol. **36**(S): 10–14.
12. JOHNSON, S. & F.N. JOHNSON, Eds. 1998. Etidronate in Osteoporosis, **Vol 9**. Rev in Contemp Pharm. Marius Press. Lancashire, UK.
13. GEDDES, A.D., S.M. D'SOUZA, F.H. EBETINO, *et al*. 1994. Bisphosphonates: structure-activity relationships and therapeutic implications. Bone Miner. Res. **8:** 265–306.
14. SIETSEMA, W.K., F.H. EBETINO, A.M. SALVAGNO, *et al*. 1989. Antiresorptive dose-response relationships across three generations of bisphosphonates. Drugs Exp. Clin. Res. **15:** 389–396.
15. ROGERS, M.J. 2004. From molds and macrophages to mevalonate: a decade of progress in understanding the molecular mode of action of bisphosphonates [review]. Calcif. Tissue Int. **75:** 451–461.
16. PAPAPOULOS, S.E. 1995. Pharmacodynamics of bisphosphonates in man. Chapter 15. *In* Bisphosphonates on Bone. O. Bijvoet, *et al*. Eds.: Elsevier Science B.V. Amsterdam.
17. RONDEAU, J.M., F. BITSCH, E. BOIRGIER, *et al*. 2006. Structural basis for the exceptional *in vivo* efficacy of bisphosphonate drugs. Chem. Med. Chem. **1:** 267–273.
18. MICHAELSON, M.D. & M.R. SMITH. 2005 Bisphosphonates for treatment and prevention of bone metastases [review]. J. Clin. Oncol. **23:** 8219–8224.
19. FLEISCH, H. & W.F. NEUMAN. 1961. Mechanisms of calcification: role of collagen, polyphosphates, and phosphatase. Am. J. Physiol. **200:** 1296–1300.
20. FLEISCH, H. & S. BISAZ. 1962. Isolation from urine of pyrophosphate, a calcification inhibitor. Am. J. Physiol. **203:** 671–675.
21. FLEISCH, H., R.G.G. RUSSELL & F. STRAUMANN. 1966. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. Nature **212:** 901–903.
22. RUSSELL, R.G.G. & A. HODGKINSON. 1966. The urinary excretion of inorganic pyrophosphate by normal subjects and patients with renal calculus. Clin. Sci. **31:** 51–62.
23. RUSSELL, R.G.G. 1965. Excretion of inorganic pyrophosphate in hypophosphatasia. Lancet **10:** 461–464.
24. RUSSELL, R.G.G., S. BISAZ, A. DONATH, *et al*. 1971. Inorganic pyrophosphate in plasma in normal persons and in patients with hypophosphatasia, osteogenesis imperfecta and other disorders of bone. J. Clin. Invest. **50:** 961–969.
25. SCHIBLER, D., R.G.G. RUSSELL & H. FLEISCH. 1968. Inhibition by pyrophosphate of aortic calcification induced by Vitamin D3 in rats. Clin. Sci. **35:** 363–372.

26. FLEISCH, H., R.G.G. RUSSELL, S. BISAZ, *et al*. 1968. The influence of pyrophosphate analogues (diphosphonates) on the precipitation and dissolution of calcium phosphate in vitro and in vivo. Calcif. Tissue Res. **2:** 10–10A.
27. FRANCIS, M.D., R.G.G. RUSSELL & H. FLEISCH. 1969. Diphosphonates inhibit formation of calcium phosphate crystals *in vitro* and pathological calcification *in vivo*. Science **165:** 1264–1266.
28. FLEISCH, H., R.G.G. RUSSELL & M.D. FRANCIS. 1969. Diphosphonates inhibit hydroxyapatite dissolution *in vitro* and bone resorption in tissue culture and *in vivo*. Science **165:** 1262–1264.
29. JUNG, A., S. BISAZ & H. FLEISCH. 1973. The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals. Calcif. Tissue Res. **11:** 269–280.
30. FLEISCH, H.A., R.G.G. RUSSELL, S. BISAZ, *et al*. 1970. The inhibitory effect of phosphonates on the formation of calcium phosphate crystals *in vitro* and on aortic and kidney calcification *in vivo*. Eur. J. Clin. Invest. **1:** 12–18.
31. SCHENK, R., W.A. MERZ, R. MUEHLBAUER, *et al*. 1973. Effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl2MDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. Calcif. Tissue Res. **11:** 196–214.
32. FLEISCH, H., J. MAERKI & R.G.G. RUSSELL. 1966. Effect of pyrophosphate on dissolution of hydroxyapatite and its possible importance in calcium homeostasis. Proc. Soc. Exp. Biol. **122:** 317–320.
33. RUSSELL, R.G.G., R.C. MUHLBAUER, S. BISAZ, *et al*. 1970. The influence of pyrophosphate, condensed phosphates, phosphonates and other phosphate compounds on the dissolution of hydroxyapatite in vitro and on bone resorption induced by parathyroid hormone in tissue culture and in thyroparathyroidectomised rats. Calcif. Tissue Res. **6:** 183–196.
34. TRECHSEL, U., A. STUTZER & H. FLEISCH. 1987. Hypercalcaemia induced with an arotinoid in thyroparathyroidectomised rats. A new model to study bone resorption in vivo. J. Clin. Invest. **80:** 1679–1686.
35. STUTZER, A., H. FLEISCH & U. TRECHSEL. 1988. Short and long term effects of a single dose of bisphosphonates on retinoid-induced bone resorption in thyroparathyroidectomised rats. Calcif. Tissue Int. **43:** 294–299.
36. SCHENK, R., P. EGGLI, H. FLEISCH, *et al*. 1986. Quantitative morphometric evaluation of the inhibitory activity of new aminobisphosphonates on bone resorption in the rat. Calcif. Tiss. Int. **38:** 342–349.
37. GASSER, A.B., D.B. MORGAN, H.A. FLEISCH, *et al*. 1972. The influence of two diphosphonates on calcium metabolism in the rat. Clin. Sci. **43:** 31–45.
38. MUEHLBAUER, R.C., R.G.G. RUSSELL, D.A. WILLIAMS, *et al*. 1971. The effects of diphosphonates, polyphosphates and calcitonin on "immobilisation osteoporosis" in rats. Eur. J. Clin. Invest. **1:** 336–344.
39. FERRETTI, J.L. 1995. Effects of bisphosphonates on bone biomechanics. Chapter 31. *In* Bisphosphonates on Bone. O. Bijvoet, *et al*. Eds.: Elsevier Science B.V. Amsterdam.
40. SHINODA, H., G. ADAMEK & R. FELIX, *et al*. 1983. Structure-activity relationships of various bisphosphonates. Calcif. Tissue Int. **35:** 87–99.
41. FLANAGAN, A.M. & T.J. CHAMBERS. 1989. Dichloromethylenebisphosphonate (Cl2MBP) inhibits bone resorption through injury to osteoclasts that resorb Cl2MBP-coated bone. Bone Miner. **6:** 33–43.

42. REITSMA, P.H., O.L.M. BIJVOET, H. VERLINDEN-OOMS, *et al*. 1980. Kinetic studies of bone and mineral metabolism during treatment with (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (APD) in rats. Calcif. Tissue Int. **32:** 145–157.
43. GARNERO, P., W.J. SHIH, E. GINEYTS, *et al*. 1994. Comparison of new biochemical markers of bone turnover in late postmenopausal osteoporotic women in response to alendronate treatment. J. Clin. Endocrinol. Metab. **79:** 1693–1700.
44. BAUSS, F. & R.G.G. RUSSELL. 2004. Ibandronate in osteoporosis: preclinical data and rationale for intermittent dosing. Osteoporos. Int. **15:** 423–433.
45. GASSER, J.A. & J.R. GREEN. 2002. Long-term protective effect of a single IV injection of zoledronic acid on cancellous bone structure and cortical bone in ovariectomized rats. Bone. **30**(Suppl.): 41S.
46. EBRAHIMPOUR, A. & M.D. FRANCIS. 1995. Bisphosphonate therapy in acute and chronic bone loss: physical chemical considerations in bisphosphonate-related therapies. *In* Bisphosphonate on bones. O. Bijvoet, *et al*. Eds.: Elsevier Science B.V Amsterdam.
47. MUEHLBAUER, R.C., F. BAUSS, R. SCHENK, *et al*. 1991. BM 21.0955, a potent new bisphosphonate to inhibit bone resorption. J. Bone Miner. Res. **6:** 1003–1011.
48. PAPAPOULOS, S.E., K. HOEKMAN, C.W.G.M. LOWIK, *et al*. 1988. Application of an *in vitro* model and a clinical protocol in the assessment of the potency of a new bisphosphonate. J. Bone Miner. Res. **4:** 775–781.
49. GOA, K.L. & J.A. BALFOUR. 1998. Risedronate. Drugs Aging **13:** 83–91.
50. GREEN, J.R., K. MUELLER & K.A. JAEGGI. 1994. Preclinical pharmacology of CGP 42′446, a new, potent, heterocyclic bisphosphonate compound. J. Bone Miner. Res. **9:** 745–751.
51. EBETINO, F.H., A.V. BAYLESS, J. AMBURGEY, *et al*. 1996. Elucidation of a pharmacore for the bisphosphonate mechanism of bone antiresorptive activity. Phosphorus Sulfur Silicon Relat. Elem. **109/110:** 217–220.
52. VAN BEEK, E., C. LOWIK, I. OUE, *et al*. 1996. Dissociation of binding and antiresorptive properties of hydroxybisphosphonates by substitution of the hydroxyl with an amino group. J. Bone Miner. Res. **11:** 1492–1499.
53. SMITH, R. 1995. Ectopic calcification and ossification. Chapter 31. *In* Bisphosphonate on Bones. O. Bijvoet, *et al*. Eds.: Elsevier Science B.V. Amsterdam.
54. FOGELMAN, I., R.G. BESSENT, J.F. TURNER, *et al*. 1978. The use of whole-body retention of Tc-99m diphosphonate in the diagnosis of metabolic bone disease. J. Nucl. Med. **19:** 270–275.
55. FRANCIS, M.D. & I. FOGELMAN. 1987. 99mTc- diphosphonate uptake mechanisms on bone. *In* Bone Scanning in Clinical Practice. I. Fogelman, Ed.: 1–6. Springer Verlag. London.
56. RUSSELL, R.G.G., R. SMITH, C. PRESTON, *et al*. 1974. Diphosphonates in Paget's disease. Lancet **1:** 894–898.
57. DELMAS, P.D. & P.J. MEUNIER. 1997. The management of Paget's disease of bone. N. Engl. J. Med. **336:** 558–566.
58. DAROSZEWSKA, A. & S.H. RALSTON. 2005. Genetics of Paget's disease of bone. Clin. Sci. (Lond). **109:** 257–263.
59. MEE, A.P. 1999. Paramyxoviruses and Paget's disease: affirmative view. Bone **24:** 19S–21S.
60. DOUGLAS, D.L., R.G.G. RUSSELL, C.J. PRESTON, *et al*. 1980. Effect of dichloromethylene diphosphonate in Paget's disease of bone and in hypercalcaemia due to primary hyperparathyroidism or malignant disease. Lancet **1:** 1043–1047.

61. COUKELL, A.J. & A. MARKHAM. 1998. Pamidronate. A review of its use in the management of osteolytic bone metastases, tumour-induced hypercalcaemia and Paget's disease of bone. Drugs Aging **12:** 149–168.
62. SINGER, F.R., T.L. CLEMENS & R.E. EUSEBIO. 1998. Risedronate, a highly effective oral agent in the treatment of patients with severe Paget's disease. J. Clin. Endocrinol Metab. **83:** 1906–1910.
63. SIRIS, E.S., A.A. CHINES, R.D. ALTMAN, *et al*. 1998. Risedronate in the treatment of Paget's disease of bone: an open label, multicenter study. J. Bone Miner. Res. **13:** 1032–1038.
64. REID, I.R., P. MILLER, K. LYLES, *et al*. 2005. Comparison of a single infusion of zoledronic acid with risedronate for Paget's disease. N. Engl. J. Med. **353:** 898–908.
65. MARTODAM, R.R., K.S. THORNTON, D.A. SICA, *et al*. 1983. The effects of dichloromethylene diphosphonate on hypercalcemia and other parameters of the humoral hypercalcemia of malignancy in the rat Leydig cell tumor. Calcif. Tissue Int. **35:** 512–519.
66. SASAKI, A., B.F. BOYCE, B. STORY, *et al*. 1995. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res. **55:** 3551–3557.
67. MUNDY, G.R. & T. YONEDA. 1995. Facilitation and suppression of bone metastasis. Clin. Orthop. Relat. Res. **312:** 34–44.
68. MUNDY, G.R. 1998. Bisphosphonates as anticancer drugs. N. Engl. J. Med. **339:** 398–400.
69. CLEZARDIN, P., F.H. EBETINO & P.G. FOURNIER. 2005. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity [review]. Cancer Res. **65:** 4971–4974.
70. ROSS, J.R., Y. SAUNDERS, P.M. EDMONDS, *et al*. 2004. A systematic review of the role of bisphosphonates in metastatic disease [review]. Health Technol. Assess. **8:** 1–176.
71. PATERSON, A.D., J.A. KANIS, E.C. CAMERON, *et al*. 1983. The use of dichloromethylene diphosphonate for the management of hypercalcaemia in multiple myeloma. Br. J. Haematol. **54:** 121–131.
72. RALSTON, S.H., S.J. GALLACHER, U. PATEL, *et al*. 1989. Comparison of three bisphosphonates in cancer-associated hypercalcaemia. Lancet **ii:** 1180–1182.
73. BODY, J.J. 2004. Hypercalcemia of malignancy [review]. Semin. Nephrol. **24:** 48–54.
74. SIROHI, B. & R. POWLES. 2004. Multiple myeloma [review]. Lancet **363:** 875–887.
75. PAVLAKIS, N., R. SCHMIDT & M. STOCKLER. 2005. Bisphosphonates for breast cancer. Cochrane Database Syst Rev. CD003474.
76. COLEMAN, R.E. 2005. Bisphosphonates in breast cancer [review]. Ann. Oncol. **16:** 687–695.
77. PARKER, C.C. 2005. The role of bisphosphonates in the treatment of prostate cancer [review]. BJU Int. **95:** 935–938.
78. SMITH, M.R. 2005. Zoledronic acid to prevent skeletal complications in cancer: corroborating the evidence. Cancer Treat. Rev. **31**(Suppl 3): 19–25.
79. BELL, R. 2005. Efficacy of ibandronate in metastatic bone disease: review of clinical data. Oncologist **10**(Suppl 1): 8–13.
80. BODY, J.J., I.J. DIEL, M. LICHINITZER, *et al*. 2004. Oral ibandronate reduces the risk of skeletal complications in breast cancer patients with metastatic bone disease: results from two randomised, placebo-controlled phase III studies. Br. J. Cancer **90:** 1133–1137.

81. McCloskey, E.V., I.C.M. MacLennan, M. Drayson, *et al*. 1998. A randomized trial of the effect of clodronate on skeletal morbidity in multiple myeloma. MRC working party on leukaemia in adults. Br. J. Haematol. **100:** 317–325.
82. Berenson, J.R., A. Lichtenstein, L. Porter, *et al*. 1996. Efficacy of pamidronate in reducing skeletal events in patients with advanced multiple myeloma. N. Engl. J. of Med. **334:** 488–493.
83. Diel, I.J., E-F. Solomayer, S.D. Costa, *et al*. 1998. Reduction in new metastases in breast cancer with adjuvant clodronate treatment. N. Engl. J. Med. **339:** 357–363.
84. Maerevoet, M., C. Martin & L. Duck. 2005. Osteonecrosis of the jaw and bisphosphonates. N. Engl. J. Med. **353:** 99–102.
85. 2004. Bone Health and Osteoporosis: A Report of the Surgeon General. U.S. Department of Health and Human Services, Office of the Surgeon General. Rockville, MD. www.surgeongeneral.gov/library.
86. Russell, R.G.G. 2003. Pathogenesis of osteoporosis. *In* Rheumatology, 3rd ed. M.C. Hochberg *et al*. Eds.: 2075–2147. publ. Mosby.
87. Watts, N.B., S.T. Harris, H.K. Genant, *et al*. 1990. Intermittent cyclical etidronate treatment of postmenopausal osteoporosis. N. Engl. J. Med. **323:** 73–79.
88. Storm, T., G. Thamsborg, T. Steiniche, *et al*. 1990. Effect of intermittent cyclical etidronate therapy on bone mass and fracture rate in women with postmenopausal osteoporosis. N. Engl. J. Med. **322:** 1265–1271.
89. van Staa, T.P., L. Abenhain & C. Cooper. 1998. Use of cyclical etidronate and prevention of non-vertebral fractures. Br. J. Rheumatol. **37:** 87–94.
90. Liberman, U.A., S.R. Weiss, J. Broll, *et al*. 1995. Effect of oral alendronate on bone mineral density and the incidence of fractures in postmenopausal osteoporosis. N. Engl. J. Med. **333:** 1437–1443.
91. Black, D.M., S.R. Cummings, D.B. Karpf, *et al*. 1996. Randomised trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture intervention trial research group. Lancet **348:** 1535–1541.
92. Bone, H.G., D. Hosking, J.-P. Devogelaer, *et al.* for the Alendronate Phase III Osteoporosis Treatment Study Group. 2004. Ten years' experience with alendronate for osteoporosis in postmenopausal women. N. Engl. J. Med. **350:** 1189–1199.
93. Reginster, J., H.W. Minne, O.H. Sorensen, *et al*. 2000. Randomized trial of the effects of risedronate on vertebral fractures in women with established postmenopausal osteoporosis. Vertebral Efficacy with Risedronate Therapy (VERT) Study Group. Osteoporos. Int. **11:** 83–91.
94. McClung, M.R., P. Geusens, P.D. Miller, *et al.* and Hip Intervention Program Study Group. 2001. Effect of risedronate on the risk of hip fracture in elderly women. N. Engl. J. Med. **344:** 333–340.
95. Boonen, S., R.F. Laan, I.P. Barton, *et al*. 2005. Effect of osteoporosis treatments on risk of non-vertebral fractures: review and meta-analysis of intention-to-treat studies. Osteoporos. Int. **16:** 1291–1298.
96. Chavassieux, P.M., M.E. Arlot, C. Reda, *et al*. 1997. Histomorphometric assessment of the long term effects of alendronate on bone quality and remodeling in patients with osteoporosis. J. Clin. Invest. **100:** 1475–1480.
97. Adachi, J.D., W.G. Bensen, J. Brown, *et al*. 1997. Intermittent etidronate therapy to prevent corticosteroid-induced osteoporosis. N. Engl. J. Med. **337:** 381–387.

98. SAAG, K.G., K. EMKEY, T.J. SCHNITZER, *et al*. 1998. Alendronate for the prevention and treatment of glucocorticoid-induced osteoporosis. N. Engl. J. Med. **339:** 292–229.
99. GLORIEUX, F.H., N.J. BISHOP, H. PLOTKIN, *et al*. 1998. Cyclic administration of pamidronate in children with severe osteogenesis imperfecta. N. Engl. J. Med. **33:** 947–952.
100. RAUCH, F. & F.H. GLORIEUX. 2005. Osteogenesis imperfecta, current and future medical treatment. Am. J. Med. Genet. C. Semin. Med. Genet. **139:** 31–37.
101. CHESNUT, C.H., A. SKAG, C. CHRISTIANSEN, *et al*. 2004. Effects of oral ibandronate administered daily or intermittently on fracture risk in postmenopausal osteoporosis. J. Bone Miner. Res. **19:** 1241–1249.
102. REID, I.R., J.P. BROWN, P. BURCKHARDT, *et al*. 2002. Intravenous zoledronic acid in postmenopausal women with low bone mineral density. N. Engl. J. Med. **346:** 653–661.
103. ADAMI, S. & N. ZAMBERLAN. 1996. Adverse effects of bisphosphonates. Drug Saf. **14:** 158–170.
104. VAN STAA, T.P. 1998. Post marketing survey with ICT-etidronate therapy. Rev. Contemp. Pharm. **9:** 277–286.
105. DEGROEN, P.C., D.F. LUBBE, L.J. HIRSCH, *et al*. 1996. Esophagitis associated with the use of alendronate. N. Engl. J. Med. **355:** 1016–1021.
106. HUGHES, D.E., B.R. MACDONALD, R.G.G. RUSSELL, *et al*. 1989. Inhibition of osteoclast-like cell formation by bisphosphonates in long-term cultures of human bone marrow. J. Clin. Invest. **83:** 1930–1935.
107. BOONEKAMP, P.M., L.J.A. VAN DER WEE-PALS, M.L.L. VAN WIJK-VAN LENNEP, *et al*. 1986. Two modes of action of bisphosphonates on osteoclastic resorption of mineralized matrix. Bone Miner. **1:** 27–39.
108. LÖWIK, C.W.G.M., G. VAN DER PLUIJM, L.J.A. VAN DER WEE-PALS, *et al*. 1988. Migration and phenotypic transformation of osteoclast precursors into mature osteoclasts: the effect of a bisphosphonate. J. Bone Miner. Res. **3:** 185–192.
109. SUDA, T., I. NAKAMURA, E. JIMI, *et al*. 1997. Regulation of osteoclast function. J. Bone Miner. Res. **12:** 869–879.
110. MASARACHIA, P., M. WEINREB, R. BALENA, *et al*. 1996. Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones. Bone **19:** 281–290.
111. AZUMA, Y., H. SATO, Y. OUE, *et al*. 1995. Alendronate distributed on bone surfaces inhibits osteoclastic bone resorption in vitro and in experimental hypercalcemia models. Bone **16:** 235–245.
112. SATO, M. & W. GRASSER. 1990. Effects of bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. J. Bone Miner. Res. **5:** 31–40.
113. NISKIKAWA, M., T. AKATSU, Y. KATAYAMA, *et al*. 1996. Bisphosphonates act on osteoblastic cells and inhibit osteoclast formation in mouse marrow cultures. Bone **18:** 9–14.
114. YU, X., J. SCHOLLER & N.T. FOGED. 1996. Interaction between effects of parathyroid hormone and bisphosphonate on regulation of osteoclast activity by the osteoblast-like cell line UMR-106. Bone **19:** 339–345.
115. SAHNI, M., H. GUENTHER, H. FLEISCH, *et al*. 1993. Bisphosphonates act on rat bone resorption through the mediation of osteoblasts. J. Clin. Invest. **91:** 2004–2011.

116. VITTE, C., H. FLEISCH & H.L. GUENTHER. 1996. Bisphosphonates induce osteoblasts to secrete an inhibitor of osteoclast-mediated resorption. Endocrinology **137:** 2324–2333.
117. ITO, M., M. CHOKKI, Y. OGINO, *et al*. 1998. Comparison of the cytotoxic effects of bisphosphnates *in vitro* and *in vivo*. Calcif. Tissue Int. **63:** 143–147.
118. BREUIL, V., F. COSMAN, L. STEIN, *et al*. 1998. Human osteoclast formation and activity *in vitro*: effects of alendronate. J. Bone Miner. Res. **13:** 1721–1729.
119. HUGHES, D.E., K.R. WRIGHT, H.L. UY, *et al*. 1995. Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. J. Bone Miner. Res. **10:** 1478–1487.
120. SELANDER, K.S., J. MÖNKKÖNEN, E.K. KARHUKORPI, *et al*. 1996. Characteristics of clodronate-induced apoptosis in osteoclasts and macrophages. Mol. Pharmacol. **50:** 1127–1138.
121. ROGERS, M.J., K.M. CHILTON, F. COXON, *et al*. 1996. Bisphosphonates induce apoptosis in mouse macrophage-like cells by a nitric-oxide-independent mechanism. J. Bone Miner. Res. **11:** 1482–1491.
122. COXON, F.P., H.L. BENFORD, R.G.G. RUSSELL, *et al*. 1998. Protein synthesis is required for caspase activation and induction of apoptosis by bisphosphonate drugs. Mol. Pharmacol. **54:** 631–638.
123. SHIPMAN, C.M., M.J. ROGERS, J.F. APPERLEY, *et al*. 1997. Bisphosphonates induce apoptosis in human myeloma cells; a novel anti-tumour activity. Br. J. Haematol. **98:** 665–672.
124. SHIPMAN, C.M., P.I. CROUCHER, R.G.G. RUSSELL, *et al*. 1998. The bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway. Cancer Res. **58:** 5294–5297.
125. DAVID, P., H. NGUYEN, A. BARBIER, *et al*. 1996. The bisphosphonate tiludronate is a potent inhibitor of the osteoclast vacuolar H^+ ATPase. J. Bone Miner. Res. **11:** 1498–1507.
126. CARANO, A., S.A. TEITELBAUM, J.D. KONSEK, *et al*. 1990. Bisphosphonates directly inhibit the bone resorption activity of isolated avian osteoclasts in vitro. J. Clin. Invest. **85:** 456–461.
127. ZIMOLO, Z., G. WESOLOWSKI & G.A. RODAN. 1995. Acid extrusion is induced by osteoclast attachment to bone. J. Clin. Invest. **96:** 2277–2283.
128. FELIX, R., R.G.G. RUSSELL & H. FLEISCH. 1976. The effect of several diphosphonates on acid phosphohydrolases and other lysosomal enzymes. Biochim. Biophys. Acta **429:** 429–438.
129. LERNER, U.H. & A. LARSSON. 1987. Effects of four bisphosphonates on bone resorption, lysosomal enzyme release, protein synthesis and mitotic activities in mouse calvarial bones *in vitro*. Bone **8:** 179–189.
130. SATO, M., W. GRASSER, N. ENDO, *et al*. 1991. Bisphosphonate action. Alendronate localisation in rat bone and effects on osteoclast ultrastructure. J. Clin. Invest. **88:** 2095–2105.
131. MURAKAMI, H., N. TAKAHASHI, T. SASAKI, *et al*. 1995. A possible mechanism of the specific action of bisphosphonates on osteoclasts: tiludronate preferentially affects polarized osteoclasts having ruffled borders. Bone **17:** 137–144.
132. SCHMIDT, A., S.J. RUTLEDGE, N. ENDO, *et al*. 1995. Alendronate inhibition of protein tyrosine phosphatase activity. Proc. Natl. Acad. Sci. USA **93:** 3068–3073.
133. ENDO, N., S.J. RUTLEDGE, E.E. OPAS, *et al*. 1996. Human protein tyrosine phosphatases: alternative splicing and inhibition by bisphosphonates. J. Bone Miner. Res. **11:** 535–543.

134. Olpas, E.E., S.J. Rutledge, E. Golub, *et al*. 1997. Alendronate inhibition of protein-tyrosine-phosphatase-Meg1. Biochem. Pharmacol. **54:** 721–727.
135. Murakami, H., N. Takahashi, S. Tanaka, *et al*. 1997. Tiludronate inhibits protein tyrosine phosphatase activity in osteoclasts. Bone **20:** 399–404.
136. Rogers, M.J. 2004. From molds and macrophages to mevalonate: a decade of progress in understanding the molecular mode of action of bisphosphonates [review]. Calcif. Tissue Int. **75:** 451–461.
137. Rogers, M.J., D.J. Watts, R.G.G. Russell, *et al*. 1994. Inhibitory effects of bisphosphonates on growth of amoebae of the cellular slime mould *Dictyostelium discoideum*. J. Bone Miner. Res. **9:** 1029–1039.
138. Rogers, M.J., X. Xiong, R.J. Brown, *et al*. 1995. Structure-activity relationships of new heterocycle-containing bisphosphonates as inhibitors of bone resorption and as inhibitors of growth of *Dictyostelium discoideum* amoebae. Mol. Pharmacol. **47:** 398–402.
139. Rogers, M.J., X. Ji, R.G.G. Russell, *et al*. 1994. Incorporation of bisphosphonates into adenine nucleotides by amoebae of the cellular slime mould *Dictyostelium discoideum*. Biochem. J. **303:** 303–311.
140. Rogers, M., R.G.G. Russell, G.M. Blackburn, *et al*. 1992. Metabolism of halogenated bisphosphonates by the cellular slime mould *Dictyostelium discoideum*. Biochem. Biophys. Res. Commun. **189:** 414–423.
141. Rogers, M.J., R.J. Brown, V. Hodkin, *et al*. 1996. Bisphosphonates are incorporated into adenine nucleotides by human aminoacyl-tRNA synthetase enzymes. Biochem. Biophys. Res. Commun. **224:** 863–869.
142. Pelorgeas, S., J.P. Martin & M. Satre. 1992. Cytotoxicity of dichloromethane diphosphonate and of 1-hydroxyethane-1,1-diphosphonate in the amoebae of the slime mould *Dictyostelium discoideum*. Biochem. Pharmacol. **44:** 2157–2163.
143. Frith, J.C., J. Mönkkönen, G.M. Blackburn, *et al*. 1996. Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5′-(β,γ-dichloromethylene) triphosphate, by mammalian cells *in vitro*. J. Bone Miner. Res. **12:** 1358–1367.
144. Auriola, S., J. Frith, M.J. Rogers, *et al*. 1997. Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography-electrospray mass spectrometry. J. Chrom. B. **704:** 187–195.
145. Frith, J.C., J. Monkkonen, S. Auriola, *et al*. 2001. The molecular mechanism of action of the antiresorptive and antiinflammatory drug clodronate: evidence for the formation in vivo of a metabolite that inhibits bone resorption and causes osteoclast and macrophage apoptosis. Arthritis Rheum. **44:** 2201–2210.
146. Rogers, M.J., X. Xiong, X. Ji, *et al*. 1997. Inhibition of growth of *Dictyostelium discoideum* amoebae by bisphosphonates is dependent on cellular uptake. Pharm. Res. **14:** 625–630.
147. Lehenkari, P.P., M. Kellinsalmi, J.P. Napankangas, *et al*. 2002. Further insight into mechanism of action of clodronate: inhibition of mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing metabolite. Mol. Pharmacol. **61:** 1255–1262.
148. Zhang, F.L. & P.J. Casey. 1996. Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. **65:** 241–269.
149. Ridley, A.J. & A. Hall. 1992. The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell **70:** 389–399.

150. RIDLEY, A.J., H.F. PATERSON, C.L. JOHNSTON, *et al*. 1992. The small GTP-binding protein, rac, regulates growth factor-induced membrane ruffling. Cell **70:** 401–410.
151. ZERIAL, M. & H. STENMARK. 1993. Rab GTPases in vesicular transport. Curr. Opin. Cell Biol. **5:** 613–620.
152. ZHANG, D., N. UDAGAWA, I. NAKAMURA, *et al*. 1995. The small GTP-binding protein, Rho p21, is involved in bone resorption by regulating cytoskeletal organization in osteoclasts. J. Cell Sci. **108:** 2285–2292.
153. MARSHALL, C. 1993. Protein prenylation: a mediator of protein-protein interactions. Science **259:** 1865–1866.
154. LUCKMAN, S.P., D.E. HUGHES, F.P. COXON, *et al*. 1998. Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J. Bone Miner. Res. **13:** 581–589.
155. LUCKMAN, S.P., F.P. COXON, F.H. EBETINO, *et al*. 1998. Heterocycle-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: evidence from structure-activity relationships in J774 macrophages. J. Bone Miner. Res. **13:** 1668–1678.
156. STAAL, A., J.C. FRITH, M.H. FRENCH, *et al*. 2003. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. J. Bone Miner. Res. **18:** 88–96.
157. FISHER, J.E., M.J. ROGERS, J.M. HALASY, *et al*. 1999. Mechanism of action of alendronate: geranylgeraniol, an intermediate of the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption and kinase activation in vitro. Proc. Natl. Acad. Sci. USA **96:** 133–138.
158. HALL, A. 1998. Rho GTPases and the actin cytoskeleton. Science **279:** 509–514.
159. AMIN, D., S.A. CORNELL, M.H. PERRONE, *et al*. 1996. 1-hydroxy-3-(methylpentylamino)-propylidene-1,1-bisphosphonic acid as a potent inhibitor of squalene synthase. Drug Res. **46:** 759–762.
160. AMIN, D., S.A. CORNELL, S.K. GUSTAFSON, *et al*. 1992. Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase and cholesterol biosynthesis. J. Lipid. Res. **33:** 1657–1663.
161. VAN BEEK, E., E. PIETERMAN, L. COHEN, *et al*. Nitrogen-containing bisphosphonates inhibit isopentenyl pyrophosphate isomerase/farnesyl pyrophosphate synthase activity with relative potencies corresponding to their antiresorptive potencies *in vitro* and *in vivo*. Biochem. Biophys. Res. Commun. **255:** 491–494.
162. DUNFORD, J.E., K. THOMPSON, F.P. COXON, *et al*. 2001. Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. J. Pharmacol. Exp. Ther. **296:** 235–242.
163. EBETINO, F.H., C.N. ROZÉ, C.E. MCKENNA, *et al*. 2005. Molecular interactions of nitrogen-containing bisphosphonates within farnesyl diphosphate synthase. J. Organomet. Chem. **690:** 2679–2687.
164. KAVANAGH, K., K. GUO, J. DUNFORD, *et al*. 2006. Human Farnesyl Diphosphate Synthase (FDPS): crystal structure and molecular interactions with nitrogen-containing bisphosphonates. Proc. Natl. Acad. Sci. USA In press.
165. MARTIN, M.B., W. ARNOLD, H.T. HEATH, III, *et al*. 1999. Nitrogen-containing bisphosphonates as carbocation transition state analogs for isoprenoid biosynthesis. Biochem. Biophys. Res. Commun. **263:** 754–758.

166. TERKELTAUB, R.A. 2001. Inorganic pyrophosphate generation and disposition in pathophysiology [review]. Am. J. Physiol. Cell Physiol. **281:** C1–C11.
167. ANDERSON, H.C., D. HARMEY, N.P. CAMACHO, *et al*. 2005. Sustained osteomalacia of long bones despite major improvement in other hypophosphatasia-related mineral deficits in tissue nonspecific alkaline phosphatase/nucleotide pyrophosphatase phosphodiesterase 1 double-deficient mice. Am. J. Pathol. **166:** 1711–1720.
168. TIMMS, A., Y. ZHANG, R.G.G. RUSSELL, *et al*. 2002. Genetic studies of disorders of calcium crystal deposition. Rheumatology (Oxford) **41:** 725–729.
169. MUMM, S., J. JONES, P. FINNEGAN, *et al*. 2001. Hypophosphatasia: molecular diagnosis of Rathbun's original case. J. Bone Miner. Res. **16:** 1724–1727.
170. FEDDE, K.N., L. BLAIR, J. SILVERSTEIN, *et al*. 1999. Alkaline phosphatase knockout mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. J. Bone Miner. Res. **14:** 2015–2026.
171. RUF, N., B. UHLENBERG, R. TERKELTAUB, *et al*. 2005. The mutational spectrum of ENPP1 as arising after the analysis of 23 unrelated patients with generalized arterial calcification of infancy (GACI). Hum. Mutat. **26:** 495–496.
172. NAKAMURA, I., S. IKEGAWA, A. OKAWA, *et al*. 1999. Association of the human NPPS gene with ossification of the posterior longitudinal ligament of the spine (OPLL). Hum. Genet. **104:** 492–497.
173. HO, A.M., M.D. JOHNSON & D.M. KINGSLEY. 2000. Role of the mouse ank gene in control of tissue calcification and arthritis. Science **289:** 265–270.
174. WILLIAMS, C.J., Y. ZHANG, A. TIMMS, *et al*. 2002. Autosomal dominant familial calcium pyrophosphate dihydrate deposition disease is caused by mutation in the transmembrane protein ANKH. Am. J. Hum. Genet. **71:** 985–991.
175. ZHANG, Y., K. JOHNSON, R.G. RUSSELL, *et al*. 2005. Association of sporadic chondrocalcinosis with a -4-basepair G-to-A transition in the 5'-untranslated region of ANKH that promotes enhanced expression of ANKH protein and excess generation of extracellular inorganic pyrophosphate. Arthritis Rheum. **52:** 1110–1117.
176. PENDLETON, A., M.D. JOHNSON, A. HUGHES, *et al*. 2002. Mutations in ANKH cause chondrocalcinosis. Am. J. Hum. Genet. **71:** 933–940.
177. REICHENBERGER, E., V. TIZIANI, S. WATANABE, *et al*. 2001. Autosomal dominant craniometaphyseal dysplasia is caused mutations in the transmembrane protein ANK. Am. J. Hum. Genet. **68:** 1321–1326.
178. NUERNBERG, P., H. THIELE, D. CHANDLER, *et al*. 2001. Heterozygous mutations in ANKH, the human ortholog of the mouse progressive ankylosis gene, result in craniometaphyseal dysplasia. Nat. Genet. **28:** 37–41.
179. BORAH, B., E.L. RITMAN, T.E. DUFRESNE, *et al*. 2005. The effect of risedronate on bone mineralization as measured by micro-computed tomography with synchrotron radiation: correlation to histomorphometric indices of turnover. Bone **37:** 1–9.
180. BORAH, B., T.E. DUFRESNE, P.A. CHMIELEWSKI, *et al*. 2002. Risedronate preserves trabecular architecture and increases bone strength in vertebra of ovariectomized minipigs as measured by three-dimensional microcomputed tomography. J. Bone Miner. Res. **17:** 1139–1147.
181. BORAH, B., T.E. DUFRESNE, P.A. CHMIELEWSKI, *et al*. 2004. Risedronate preserves bone architecture in postmenopausal women with osteoporosis as measured by three-dimensional microcomputed tomography. Bone **34:** 736–746.

182. LALLA, S., L.A. HOTHORN, N. HAAG, *et al*. 1998. Lifelong administration of high doses of ibandronate increases bone mass and maintains bone quality of lumbar vertebrae in rats. Osteoporos. Int. **8:** 97–103.
183. BAUSS, F., S. LALLA, R. ENDELE, *et al*. 2002. Effects of treatment with ibandronate on bone mass, architecture, biomechanical properties, and bone concentration of ibandronate in ovariectomized aged rats. J. Rheumatol. **29:** 2200–2208.
184. MASHIBA, T., S. MORI, D.B. BURR, *et al*. 2005. The effects of suppressed bone remodeling by bisphosphonates on microdamage accumulation and degree of mineralization in the cortical bone of dog rib. J. Bone Miner. Metab. **23**(Suppl): 36–42.
185. MASHIBA, T., T. HIRANO, C.H. TURNER, *et al*. 2000. Suppressed bone turnover by bisphosphonates increases microdamage accumulation and reduces some biomechanical properties in dog rib. J. Bone Miner. Res. **15:** 613–620.
186. FLORA, L., G.S. HASSING, G.G. CLOYD, *et al*. 1981. The long-term skeletal effects of EHDP in dogs. Metab. Bone Dis. Relat. Res. **3:** 289–300.
187. ODVINA, C.V., J.E. ZERWEKH, D.S. RAO, *et al*. 2005. Severely suppressed bone turnover: a potential complication of alendronate therapy. J. Clin. Endocrinol. Metab. **90:** 1294–1301.
188. OTT, S.M. 2005. Long-term safety of bisphosphonates. J. Clin. Endocrinol. Metab. **90:** 1897–1899.
189. WHYTE, M.P., D. WENKERT, K.L. CLEMENTS, *et al*. 2003. Bisphosphonate-induced osteopetrosis. N. Engl. J. Med. **349:** 457–463.
190. MONIER-FAUGERE, M.C., Z. GENG, E.P. PASCHALIS, *et al*. 1999. Intermittent and continuous administration of the bisphosphonate ibandronate in ovariohysterectomized beagle dogs: effects on bone morphometry and mineral properties. J. Bone Miner. Res. **14:** 1768–1778.
191. BAUSS, F., R.K. SCHENK, S. HORT, *et al*. 2004. New model for simulation of fracture repair in full-grown beagle dogs: model characterization and results from a long-term study with ibandronate. J. Pharmacol. Toxicol. Methods **50:** 25–34.
192. LITTLE, D.G., M. MCDONALD, R. BRANSFORD, *et al*. 2005. Manipulation of the anabolic and catabolic responses with OP-1 and zoledronic acid in a rat critical defect model. J. Bone Miner. Res. **20:** 2044–2052.
193. BOBYN, J.D., S.A. HACKING, J.J. KRYGIER, *et al*. 2005. Zoledronic acid causes enhancement of bone growth into porous implants. J. Bone Joint Surg. Br. **87:** 416–420.
194. KURTH, A.H., C. EBERHARDT, S. MÜLLER, *et al*. 2005. The bisphosphonate ibandronate improves implant integration in osteopenic ovariectomized rats. Bone **37:** 204–210.
195. WILKINSON, J.M., A.C. EAGLETON, I. STOCKLEY, *et al*. 2005. Effect of pamidronate on bone turnover and implant migration after total hip arthroplasty: a randomized trial. J. Orthop Res. **23:** 1–8.
196. LITTLE, D.G., N.C. SMITH, P.R. WILLIAMS, *et al*. 2003. Zoledronic acid prevents osteopenia and increases bone strength in a rabbit model of distraction osteogenesis. J. Bone Miner. Res. **18:** 1300–1307.
197. LITTLE, D.G., R.A. PEAT & A. MCEVOY. 2003. Zoledronic acid treatment results in retention of femoral head structure after traumatic osteonecrosis in young Wistar rats. J. Bone Miner. Res. **18:** 2016–2022.
198. LAI, K.A., W.J. SHEN, C.Y. YANG, *et al*. 2005. The use of alendronate to prevent early collapse of the femoral head in patients with nontraumatic osteonecrosis. A randomized clinical study. J. Bone Joint Surg. Am. **87:** 2155–2159.

199. LITTLE, D.G., M. MCDONALD, I.T. SHARPE, *et al*. 2005. Zoledronic acid improves femoral head sphericity in a rat model of Perthes disease. J. Orthop. Res. **23:** 862–868.
200. NANCOLLAS, G.H., R. TANG, R.J. PHIPPS, *et al*. 2005. Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. Bone [Epub ahead of print].
201. PLOTKIN, L.I., R.S. WEINSTEIN, A.M. PARFITT, *et al*. 1999. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. J. Clin. Invest. **104:** 1363–1374.
202. PLOTKIN, L.I., J.I. AGUIRRE, S. KOUSTENI, *et al*. 2005. Bisphosphonates and estrogens inhibit osteocyte apoptosis via distinct molecular mechanisms downstream of extracellular signal-regulated kinase activation. J. Biol. Chem. **280:** 7317–7325.
203. SAUTY, A., M. PECHERSTORFER, I. ZIMMERROTH, *et al*. 1996. Interleukin-6 and tumor necrosis factor alpha levels after bisphosphonate treatment *in vitro* and in patients with malignancy. Bone **18:** 133–139.
204. SCHWEITZER, D.H., M. OOSTENDORP-VAN DE RUIT, G. VAN DER PLUIJM, *et al*. 1995. Interleukin-6 and the acute phase response during treatment of patients with Paget's disease with the nitrogen-containing bisphosphonate dimethylamino-hydroxypropylidene bisphosphonate. J. Bone Miner. Res. **10:** 956–962.
205. ADAMI, S., A.K. BHALLA, R. DORIZZI, *et al*. 1987. The acute-phase response after bisphosphonate administration. Calcif. Tissue Int. **41:** 326–331.
206. SANDERS, J.M., S. GHOSH, J.M. CHAN, *et al*. 2004. Quantitative structure-activity relationships for gammadelta T cell activation by bisphosphonates. J. Med. Chem. **47:** 375–384.
207. THOMPSON, K. & M.J. ROGERS. 2004. Statins prevent bisphosphonate-induced gamma,delta-T-cell proliferation and activation *in vitro*. J. Bone Miner. Res. **19:** 278–288.
208. GABELLI, S.B., J.S. MCLELLAN, A. MONTALVETTI, *et al*. 2005. Structure and mechanism of the farnesyl diphosphate synthase from *Trypanosoma cruzi*: implications for drug design. Proteins **62:** 80–88 [Epub ahead of print].
209. SZABO, C.M., Y. MATSUMURA, S. FUKURA, *et al*. 2002. Inhibition of geranylgeranyl diphosphate synthase by bisphosphonates and diphosphates: a potential route to new bone antiresorption and antiparasitic agents. J. Med. Chem. **45:** 2185–2196.
210. GHOSH, S., J.M. CHAN, C.R. LEA, *et al*. 2004. Effects of bisphosphonates on the growth of Entamoeba histolytica and Plasmodium species in vitro and in vivo. J. Med. Chem. **47:** 175–187.
211. MARTIN, M.B., J.S. GRIMLEY, J.C. LEWIS, *et al*. 2001. Bisphosphonates inhibit the growth of *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondii*, and *Plasmodium falciparum*: a potential route to chemotherapy. J. Med. Chem. **44:** 909–916.
212. LING, Y., G. SAHOTA, S. ODEH, *et al*. 2005. Bisphosphonate inhibitors of Toxoplasma gondi growth: in vitro, QSAR, and in vivo investigations. J. Med. Chem. **48:** 3130–3140.
213. MORENO, B., B.N. BAILEY, S. LUO, *et al*. 2001. (31)P NMR of apicomplexans and the effects of risedronate on *Cryptosporidium parvum* growth. Biochem. Biophys. Res. Commun. **284:** 632–637.
214. RODRIGUEZ, N., B.N. BAILEY, M.B. MARTIN, *et al*. 2002. Radical cure of experimental cutaneous leishmaniasis by the bisphosphonate pamidronate. J. Infect. Dis. **186:** 138–140.