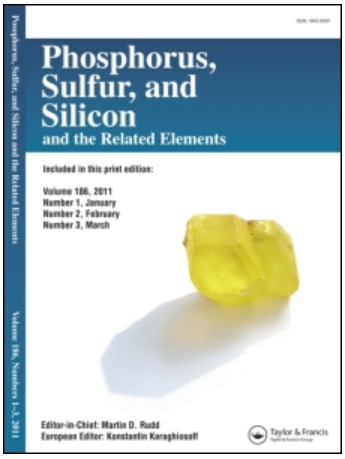

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The Bisphosphonate Odyssey. A Journey from Chemistry to the Clinic

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Bisphosphonates are chemically stable analogues of inorganic pyrophosphate, which are resistant to breakdown by enzymatic hydrolysis. Bisphosphonates bind to bone mineral and inhibit the resorption of living bone. The biological effects of bisphosphonates on calcium metabolism were originally ascribed to their physico-chemical effects to impede the dissolution of hydroxyapatite crystals. Although such effects may contribute to their overall action, their effects on cells are probably of greater importance, particularly for the more potent compounds. The marked structure-activity relationships observed among more complex compounds indicate that the pharmacophore required for maximal activity depends not only upon the bisphosphonate moiety but also on key additional features, especially nitrogen substitution in alkyl or heterocyclic side chains.

In clinical medicine, several bisphosphonates (eg. etidronate, clodronate, pamidronate, alendronate, tiludronate, risedronate & ibandronate) are established as effective treatments for diseases such as Paget's disease of bone, myeloma and bone metastases. In addition, etidronate and alendronate are approved in many countries for the prevention and treatment of osteoporosis. Both can increase bone mass and produce a reduction in fracture rates to approximately half of control rates at the spine, hip and other sites in post menopausal women. There are still considerable opportunities for extension of the use of bisphosphonates to other conditions, including steroid-associated osteoporosis, male osteoporosis, various types of arthritis, and to osteopenic disorders in childhood.

The clinical pharmacology of bisphosphonates is characterised by low intestinal absorption, but highly selective localisation and retention in bone. The close structural homology between the bisphosphonates and naturally occuring pyrophosphate-containing compounds now helps to explain their intracellular as well as their extracellular modes of action.

Bisphosphonates probably 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 cells known as osteoclasts. It is likely that bisphosphonates are internalised by osteoclasts and interfere with specific biochemical processes, and thereby induce programmed cell death or apoptosis. The molecular mechanisms by which these effects are brought about are becoming clearer. Our recent studies show that bisphosphonates can be classified into at least two groups with different modes of action. Bisphosphonates that most closely resemble pyrophosphate (such as clodronate and etidronate) can be metabolically incorporated into non-hydrolysable analogues of ATP that may inhibit ATP-dependent intracellular enzymes. The more potent, nitrogen-containing bisphosphonates (such as pamidronate, alendronate, risedronate and ibandronate) are not metabolised in this way but can inhibit enzymes of the mevalonate pathway, thereby preventing the biosynthesis of isoprenoid compounds that are essential for the post-translational modification of small GTP-binding proteins (which are

also GTPases) such as ras, rho and rac. The inhibition of protein prenylation and the disruption of the function of these key regulatory proteins explains the loss of osteoclast activity and induction of apoptosis. These different modes of action might account for subtle differences between compounds in terms of their clinical effects.

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 unravelled. As a result their full therapeutic potential is gradually being realised.

Keywords: Bone resorption; Osteoclast; Bisphosphonates; Protein prenylation; Bone Metastases; Myeloma; Osteoporosis

INTRODUCTION

The discovery and development of the bisphosphonates (BPs) as a major class of drugs for the treatment of bone diseases has been a fascinating saga that is not yet completed. As the title of this presentation implies, the journey started with chemistry, which led on to laboratory studies related to mechanisms of biological calcification and bone metabolism, and was followed later by the clinical exploitation of bisphosphonates as inhibitors of bone resorption. More recently there has a been return to laboratory studies that are helping to unravel how these drugs work at a cellular level[1].

There are several recent books and reviews available that describe the chemistry, pharmacology, and clinical applications of bisphosphonates[2 3 4 5 6 7 8 9 10 11 12 13].

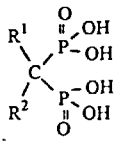
THE EARLY HISTORY

The bisphosphonates have been known to chemists since the middle of the 19th century, and the first synthesis dates back to 1865 in Germany[14]. Etidronate, the first bisphosphonate to be used in humans in Pagets disease[15], was synthesized just over 100 years ago[16]. The early uses of bisphosphonates 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[17]. 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. *It is only in the past three decades that the bisphosphonates have been* developed as drugs for use in various diseases of calcium metabolism.

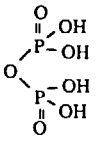
In the early 1960s, William Neuman and Herbert Fleisch[18] were studying mechanisms of calcification induced by collagen, and showed that body fluids such as plasma and urine contained inhibitors of calcification. Since it had been known since the 1930s that trace amounts of polyphosphates were capable 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 pyrophosphate, a naturally occurring polyphosphate and a known by-product of many biosynthetic reactions in the body, was present in serum and urine and could prevent calcification by binding to newly-forming crystals of hydroxyapatite [19] [20]. It was therefore postulated that pyrophosphate (PPi) might be the agent that normally prevents calcification of soft tissues, and regulates bone mineralization. It was also proposed that some pathologic disorders, such as the formation of kidney stones[21], might be linked to disturbances in PPi metabolism. The concentrations of pyrophosphate in body fluids would be expected to be regulated by hydrolytic enzymes.

At this time, I was completing my PhD in the UK and was studying the rare but fascinating inherited disorder, hypophosphatasia, in which lack of alkaline phosphatase is associated with mineralisation defects of the skeleton. This and later work showed that PPi levels were elevated in both plasma and urine [22] [23], and indicated that alkaline phosphatase was probably the key extracellular enzyme responsible for hydrolysing pyrophosphate. The activity of alkaline phosphatase would thereby maintain circulating amounts of PPi below the critical levels that would otherwise prevent normal physiological calcification processes.

Attempts to exploit these concepts by using pyrophosphate and polyphosphates to inhibit ectopic calcification in blood vessels, skin and kidneys, in laboratory animals were successful only when the compounds were injected[24]. Orally-administered pyrophosphate and polyphosphates were inactive, due to the hydrolysis of pyrophosphate in the gastrointestinal tract, probably by mucosal brush border phosphatases. During the search for more stable analogues of pyrophosphate that might also have the anti-mineralization properties of pyrophosphate but that would be resistant to hydrolysis, several different chemical classes were studied. The bisphosphonates (at that time called diphosphonates) were among these[25]. These early studies with bisphosphonates were the result of a very successful collaboration with Marion (Dave) Francis of the Procter and Gamble company and the group working in Switzerland[26] [27].



Bisphosphonic acid



Pyrophosphoric acid

Figure 1

Like pyrophosphate, bisphosphonates had high affinity for bone mineral [28] and were found to prevent the formation and aggregation of calcium phosphate crystals. Bisphosphonates had high affinity for bone mineral and were found to prevent calcification both *in vitro* and *in vivo*, but, unlike pyrophosphate, were also able to prevent pathological calcification when given orally to rats *in vivo*[29]. This property of being active by mouth was key to their future use in man.

Perhaps the most important step towards the future use of bisphosphonates occurred when we found that bisphosphonates, like we had already shown [30] for PPi, also had the novel property of being able to inhibit the dissolution of hydroxyapatite crystals [31]. This led to studies to determine whether they might also inhibit bone resorption.

THE PHARMACOLOGY OF BISPHOSPHONATES IN EXPERIMENTAL SYSTEMS *IN VITRO* AND *IN VIVO*.

Bisphosphonates as inhibitors of calcification

Bisphosphonates can prevent the experimentally induced calcification of many soft tissues, including skin, kidneys and blood vessels *in vivo*. They differ importantly from pyrophosphate in that they are active when administered orally, as well as when given parenterally. They also inhibit ectopic formation and mineralisation of bone, probably mainly by an impairment of the calcification process rather than by direct effects on the deposition of matrix. Bisphosphonates appear to prevent calcification by a physicochemical mechanism, acting as crystal poisons after adsorption to bone surfaces.

Many bisphosphonates can inhibit soft tissue mineralisation when injected at doses in the order of 1mg P/kg body weight. At comparable doses, many bisphosphonates can also impair the mineralization of normal calcified tissues such as bone and cartilage[32].

With compounds such as etidronate there is only a 10-100 fold difference between doses that inhibit mineralisation compared with doses that reduce bone resorption, whereas with the bisphosphonates that are more potent inhibitors of bone resorption, these dose differences widen to several orders of magnitude, which means that inhibition of skeletal mineralisation ceases to be a clinical concern.

Bisphosphonates as inhibitors of bone resorption *in vitro* and *in vivo*.

Many studies using a variety of experimental systems show that bisphosphonates 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 of these studied was thyroparathyroidectomized rats treated with parathyroid hormone to stimulate bone resorption *in vivo*. Bisphosphonates also suppress resorption induced by many other agents such as calcitriol, vitamin D, and retinoids. The effect on retinoid-induced hypercalcaemia has been used to develop a powerful and rapid screening assay for new compounds[33,34].

In general there is a good correlation between potency and structure-activity relationships *in vitro* and *in vivo*[35]. An inhibition of resorption was also found when the

behaviour of isolated osteoclasts was investigated on various mineralized matrices *in vitro*[36]. In the presence of bisphosphonates the osteoclasts form fewer erosion cavities and these are of smaller size, but the correlation with potency *in vivo* is less marked.

In growing intact rats, the bisphosphonates block the removal of both bone and cartilage, thus retarding the remodelling of the metaphysis which becomes club-shaped and radiologically denser than normal [37]. This effect is the basis of the 'Schenk' model used to compare the potency of new compounds.

The inhibition of endogenous bone resorption can also be monitored by kinetic studies [38] using radio-calcium (^{45}Ca), and by using biochemical markers of bone resorption.

Since bisphosphonates 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[39]. The level of suppression depends on the administered dose, and has also been observed in humans[40]. These results show that there is no progression of the anti-resorptive effect with time and suggest that the bisphosphonate buried in the bone is inactive at least as long as it remains buried there. They also show that, within the therapeutic dosage range, there is little risk of a continuous decrease in bone turnover in the long run, that might lead to an increase in bone fragility, as seen in osteopetrosis.

Bisphosphonates as inhibitors of bone resorption in animal models of human disease

The bisphosphonates are also effective in preventing bone destruction in a number of animal models of human disease, which include one of the first to be studied, sciatic nerve section as a model of immobilisation osteoporosis[41], 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 heparin or corticosteroids. If not given in excess, bisphosphonates maintain or improve the biomechanical properties of bone both in normal animals and in experimental models of osteoporosis[42].

Bisphosphonates as inhibitors of bone resorption in relation to cancers.

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

Bisphosphonates can prevent the increase in bone resorption associated with experimental tumors, particularly those that metastasise to bone [43]. In view of recent clinical results, it is interesting that bisphosphonates may not only reduce metastases in bone but reduce the overall tumor burden[44], although in some models soft tissue tumor mass may increase. The reasons for changes in tumor burden induced by bisphosphonates 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[45 46]. In addition there may be direct effects of bisphosphonates on tumour cells themselves, for example by altering cell attachment and inducing apoptosis.

CLINICAL APPLICATIONS OF BISPHOSPHONATES

After it was shown that bisphosphonates 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 bisphosphonates was as inhibitors of bone resorption, often for diseases where no effective treatment existed previously. Thus bisphosphonates became the treatment of choice for a variety

of bone diseases in which excessive 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.

Bisphosphonates and inhibition of calcification

Exploration of bisphosphonates as inhibitors of calcification showed some promise and early applications of etidronate included use in myositis ossificans and in patients who had undergone total hip replacement surgery to prevent subsequent heterotopic ossification and to improve mobility[47]. 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.

Bisphosphonates for radio-nucleide imaging of bone.

One of the earliest clinical uses of bisphosphonates was as agents for bone imaging, "bone scanning," for which they remain outstandingly useful for detecting bone metastases and other bone lesions. The application of pyrophosphate and simple bisphosphonates 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[48 49].

Bisphosphonates 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 bisphosphonates in man.[50 51].

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. Bisphosphonates have become the most important drugs used in the treatment of Paget's disease[52], and pamidronate given by intravenous infusion is probably the most extensively used[53]. The newer and more potent bisphosphonates such as risedronate can produce even more profound suppression of disease activity than was possible with the bisphosphonates available in former years[54 55].

These new observations described later on the mode of action of bisphosphonates to induce apoptosis are of particular interest in relation to Paget's disease because there is evidence that one reason why osteoclasts accumulate and are larger in Pagetic than normal bone is because they do not undergo apoptosis in the normal way. The reasons for this are not known but the proposed viral etiology of the disease has been implicated[56]. If defective apoptosis of osteoclasts contributes to the pathogenesis of Pagets disease, the bisphosphonates may be viewed as bone-selective drugs that specifically induce apoptosis in the affected osteoclasts.

Bisphosphonates as inhibitors of bone resorption in relation to cancers.

Bisphosphonates are remarkably effective in the treatment of bone problems associated with malignancy. They are now the drugs of choice for the treatment of hypercalcaemia[57. 58]. Many of the other skeletal complications of malignancy are reduced with bisphosphonate therapy, including the occurrence of bone pain, the need for radiotherapy, and fractures. The major studies have been in myeloma and in patients with breast cancer metastases, and there is also the important possibility that the survival of patients may be prolonged[59 60 61].

Bisphosphonates in osteoporosis

In recent years there has been a remarkably greater awareness of osteoporosis as a major health problem. Alongside the impressive advances in understanding the epidemiology and pathogenesis of osteoporosis and its associated fractures, and in the application of physical and biochemical methods to its diagnosis and evaluation, there have been significant advances in the therapeutic approaches to prevention and treatment of postmenopausal and other forms of osteoporosis.

Several bisphosphonates, notably etidronate and alendronate, are now well established as effective treatments for postmenopausal and other forms of osteoporosis[62 63 64 65 66]. Etidronate and alendronate, which are approved as therapies in many countries, can both increase bone mass and approximately half fracture rates at the spine, hip and other sites in post menopausal women. 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 remodelling units, but also to enhanced osteon mineralisation[67]. Both these compounds also prevent bone loss associated with glucocorticosteroid administration[68 69], while pamidronate has proved remarkably effective in increasing bone in children with the inherited 'brittle bone' disorder, osteogenesis imperfecta[70].

Among the new bisphosphonates, risedronate and ibandronate, are coming towards the end of Phase 3 evaluation. In addition to formulations to be taken by mouth, new routes of administration are being studied, especially periodic (eg 3 monthly) injections with ibandronate. 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 this approach is accompanied by greater compliance and convenience, it may become a popular method of treatment.

CLINICAL PHARMACOLOGY OF BISPHOSPHONATES

The clinical pharmacology of bisphosphonates is characterised by low intestinal absorption, but highly selective localisation and retention in bone. Significant side effects of bisphosphonates are minimal[71 72 73]. Although there are more similarities than differences between individual compounds and each bisphosphonate is potentially capable of treating any of the disorders of bone resorption in which they are used, in practise different compounds have come to be favoured for the treatment of different diseases. Currently there are seven bisphosphonates (etidronate, clodronate, tiludronate, pamidronate, alendronate, risedronate, and ibandronate) that have been registered for various clinical applications in various countries. To a major extent, the diseases in which they are used reflects the history of their clinical development and the degree of commercial sponsorship of the relevant clinical trials. Those most used in Paget's disease are pamidronate given parenterally, or etidronate and now risedronate (USA) given orally. In cancer are pamidronate given parenterally, or clodronate given orally, whereas in osteoporosis the major current drugs are still etidronate and alendronate.

Other clinical issues under consideration with bisphosphonates include the choice of therapeutic regimen, eg the use of intermittent dosing rather than continuous, intravenous versus oral therapy, the optimal duration of therapy, the combination with other drugs such as oestrogens, and their extended use in related indications eg 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 introducing new ones.

THE STRUCTURE ACTIVITY RELATIONSHIPS AMONG THE BISPHOSPHONATES USED IN BIOLOGICAL SYSTEMS

Once the potential clinical value of bisphosphonates 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. This was readily achieved and many hundreds of bisphosphonates have now been synthesized, and more than a dozen have been used in man. 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.

The features of the bisphosphonate molecule necessary for biological activity have been well defined. The P-C-P moiety is responsible for the strong affinity of the bisphosphonates for the skeleton and allows for a number of variations in structure based on substitution in the R_1 and R_2 positions on the carbon atom. The ability of the bisphosphonates to bind to the 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 bisphosphonates to chelate calcium ions by tridentate rather than bidentate binding[74].

The ability of bisphosphonates to inhibit bone resorption *in vitro* and *in vivo* also requires the P-C-P structure. Monophosphonates, e.g. pentane monophosphonate, or P-C-C-P or P-N-P compounds are ineffective as inhibitors of bone resorption. Furthermore, the anti-resorptive effect cannot be accounted for simply by adsorption of bisphosphonates to bone mineral and prevention of hydroxyapatite dissolution. It is now clear that bisphosphonates 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 anti-resorptive bisphosphonates were studied which had different R_2 side chains, but in which R_1 (and hence affinity for bone mineral) was unaltered. In particular, bisphosphonates containing a basic primary nitrogen atom in an alkyl chain (as in pamidronate and alendronate) were found to be 10-100 fold more potent than etidronate and clodronate. After this, 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.

These compounds, such as those that contain a tertiary nitrogen (such as ibandronate[75] and olpadronate[76]), are even more potent at inhibiting bone resorption. Amongst this new generation of compounds that were synthesised to optimise their anti-resorptive effects, the most potent anti-resorptive bisphosphonates were those containing a nitrogen atom within a heterocyclic ring (as in risedronate[77] and zoledronate[78]), which are up to 10,000 fold more potent than etidronate in some experimental systems.

The analysis of structure-activity relationships has allowed the spatial features of the active pharmacophore to be defined in considerable detail. For maximal potency, the nitrogen atom in the R_2 side chain must be a critical distance away from the P-C-P group, and in a specific spatial configuration[79]. This has been used successfully in predicting the features required in the chemical design of new and more active compounds.

Although the structure of the R_2 side chain is the major determinant of anti-resorptive 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 anti-resorptive potency. Methylation of both phosphonate groups to form a bisphosphinate leads to loss of bone affinity and loss of anti-

resorptive activity *in vivo*. However, bisphosphonate analogues (for example a phosphonophosphinate and a phosphonocarboxylate) with similar affinity for bone can have very different anti-resorptive potencies. This suggests that the two phosphonate groups (or alternatively, the combination of a phosphonate and a carboxylate group) are required both for targeting to bone and for the molecular mechanism of anti-resorptive action, presumably because bisphosphonates mimic naturally-occurring, pyrophosphate-containing, compounds.

In summary, studies of the relationships between bisphosphonate structure and anti-resorptive potency suggest that the ability of bisphosphonates to inhibit bone resorption is dependent on two separate properties of the bisphosphonate molecule. The two phosphonate groups, together with a hydroxyl group at the R_1 position[80], impart high affinity for bone mineral and act as a "bone hook", allowing rapid and efficient targeting of bisphosphonates to bone mineral surfaces. Once localised within bone, the structure and three dimensional conformation of the R_2 side chain (as well as the phosphonate groups in the molecule) determine the biological activity of the molecule and influence 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.

MECHANISMS OF ACTION OF BISPHOSPHONATES AS INHIBITORS OF BONE RESORPTION.

The pronounced selectivity of bisphosphonates for bone rather than other tissues is the basis for their value in clinical practise. Their preferential uptake by and adsorption to mineral surfaces in bone brings them into close contact with osteoclasts. During bone resorption bisphosphonates are probably internalised by endocytosis, along with other products of resorption. Many studies have shown that bisphosphonates can affect osteoclast-mediated bone resorption in a variety of ways that include effects on osteoclast recruitment, differentiation and resorptive activity. Given the structural similarities to pyrophosphate, it is likely that bisphosphonates internalized by osteoclasts interfere with one or more of the many biochemical processes that involve pyrophosphate-containing compounds. Bisphosphonates will therefore perturb cellular metabolism and eventually induce apoptosis.

Since mature, multinucleated osteoclasts are formed by the fusion of mononuclear precursors of haematopoietic origin, bisphosphonates could also inhibit bone resorption by preventing osteoclast formation, in addition to affecting mature osteoclasts. *In vitro*, bisphosphonates can inhibit dose-dependently the formation of osteoclast-like cells in long-term cultures of human bone marrow[81]. In organ culture also, some bisphosphonates can inhibit the generation of mature osteoclasts, possibly by preventing the fusion of osteoclast precursors [82 83]

Direct and indirect effects of bisphosphonates on osteoclasts in bone.

For the reasons already mentioned, it is likely that bisphosphonates are selectively internalised 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 [84]. The acidic pH of this microenvironment causes dissolution of the hydroxyapatite bone mineral, whilst the breakdown of the extracellular bone matrix is brought about by the action of proteolytic enzymes. Since bisphosphonates adsorb to bone mineral, especially at sites of bone resorption where the mineral is most exposed [85 86], 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 1mM alendronate in the resorption space beneath an osteoclast. This is much higher than the concentrations of bisphosphonates required to affect osteoclast morphology and cause osteoclast apoptosis *in vitro* [87].

There has also been much interest in the observations that bisphosphonates may act via the bone forming cells, osteoblasts, rather than on osteoclasts to inhibit bone resorption, particularly since these effects may occur after only very brief exposure to nanomolar concentrations of bisphosphonates[88], apparently by stimulating osteoblasts to produce an osteoclast-inhibitory factor [89 90]. The biochemical structure of this factor remains unknown, but it appears to be of low molecular weight (<10 kDa), and is present in the conditioned medium of bisphosphonate-treated osteoblasts [91]. The relevance of these effects to the overall biological actions of bisphosphonates *in vivo* remains uncertain.

Is apoptosis a key event in the action of bisphosphonates?

Despite reports that some bisphosphonates do not have toxic effects on osteoclasts [92], it is clear from many studies that bisphosphonates can reduce osteoclast number [93] and can induce apoptotic cell death in osteoclasts, as first shown by David Hughes *et al*[94] Apoptosis induced by bisphosphonates displays the features that distinguish this form of cell death from necrosis, namely the characteristic changes in morphology involving cell and nuclear condensation, chromatin condensation and nuclear fragmentation, as well as a complex sequence of biochemical events, including activation of proteolytic caspases and internucleosomal DNA cleavage following the activation of an endonuclease. These characteristic morphological features of apoptosis have been described in isolated murine osteoclasts *in vitro* and in osteoclasts in histological sections from bisphosphonate-treated mice. These effects occur both with the nitogen-containing bisphosphonates, as well as the simpler bisphosphonates, such as clodronate [95]. It is still unclear whether bisphosphonate-induced apoptosis is critical to the inhibitory effect of bisphosphonates on bone resorption.

In addition, apoptosis triggered by exposure to bisphosphonates is not restricted to osteoclasts, since macrophages (such as the murine cell line J774) [96 97] and human myeloma cell lines [98 99] also undergo apoptosis after treatment with several nitrogen-containing bisphosphonates *in vitro*. These cell types have proved very useful in defining the mechanisms in greater detail, and in pointing to other potentially important effects of bisphosphonates, for example as anti-tumour agents.

In summary, although it is possible that bisphosphonates inhibit bone resorption by causing osteoclast apoptosis, there is currently insufficient evidence to conclude that the inhibitory effect of bisphosphonates on bone resorption can be accounted for solely by an increase in apoptosis. It is perhaps more likely that bisphosphonates inhibit metabolic pathways that firstly affect osteoclast function (eg via disruption of the osteoclast cytoskeleton and ruffled border) and cause osteoclast cell death as a later effect. Differences probably also exist between the ability of different bisphosphonates to cause apoptosis, depending on their molecular mechanism of action.

BIOCHEMICAL BASIS FOR THE MECHANISMS OF ACTION OF BISPHOSPHONATES

Because osteoclasts are highly endocytic, bisphosphonate present in the resorption space is likely to be internalised by endocytosis, and thereby affect osteoclasts directly [100]. The uptake of bisphosphonates by osteoclasts *in vivo* has been confirmed using radiolabeled alendronate, which was internalised into intracellular vacuoles, and other subcellular compartments such as the

cytoplasm, mitochondria and nuclei[101]. 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. Bisphosphonates also disrupt the cytoskeleton of the osteoclast. In particular, alendronate and tiludronate disrupt the formation of actin rings in polarised, resorbing osteoclasts, an effect that appears to be dependent on cellular uptake (supporting the concept of an intracellular mechanism) since osteoclasts lacking ruffled borders (and hence unable to resorb bone and internalise the released bisphosphonate) were not affected[102]. Disruption of the cytoskeleton could be brought about indirectly by inhibition of protein kinases or phosphatases that regulate cytoskeletal structure. One of the more specific biochemical effects of bisphosphonates that has attracted attention is their ability to inhibit several protein tyrosine phosphatases, without affecting serine or threonine phosphatases [103 104 105]. These effects are most pronounced with alendronate and tiludronate[106]. However, a more likely mechanism by which the cytoskeleton may be affected involves loss of function of small GTPases such as Rho and Rac. Nevertheless, inhibitory effects on other enzymes, for example direct or indirect inhibition of the osteoclast proton pumping H^+ATPase [107 108 109], phosphatases, and lysosomal enzymes [110 111], could also contribute to the loss of resorptive capacity of osteoclasts following exposure to certain bisphosphonates.

There have been several recent studies of the mechanism of action of bisphosphonates. Our work in this area has been led by Michael Rogers, and we have proposed that bisphosphonates can be classified into at least two major groups with different modes of action. The first group comprises those bisphosphonates that perhaps most closely resemble pyrophosphate, such as clodronate and etidronate, and these can be metabolically incorporated into non-hydrolysable analogues of 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, nitrogen-containing bisphosphonates, such as alendronate and risedronate. Members of this group interfere with other metabolic reactions, notably in the mevalonate biosynthetic pathway, and may affect cellular activity and cell survival by interfering with protein prenylation and therefore the signalling functions of key regulatory proteins. These mechanisms are discussed in greater detail below.

THE INCORPORATION OF BISPHOSPHONATES INTO ADENINE NUCLEOTIDE TRIPHOSPHATE ANALOGS AS ONE OF THE MOLECULAR MECHANISMS OF ACTION OF THE SIMPLER BISPHOSPHONATES

This is the first of what appear to be two major but distinct molecular mechanisms by which bisphosphonates affect osteoclasts. This work has its origins in the study of the inhibitory effects of bisphosphonates on the growth of the amoebae of the slime mould *Dictyostelium discoideum* [112 113]. We found that some, but not all, bisphosphonates could be metabolically incorporated by the amoebae into analogues of adenosine triphosphate (ATP or Appp) [114 115]. The resulting metabolites contained the P-C-P moiety in place of the β,γ-phosphate groups of ATP, thus resulting in non-hydrolysable (AppCp) nucleotides.

The bisphosphonates that were metabolised by *Dictyostelium discoideum* all contain short R_1 and R_2 side chains, with the exception of tiludronate, and are relatively weak inhibitors of bone resorption. An identical classification into metabolisable and non-metabolisable bisphosphonates were obtained with cell-free lysates from mammalian cells. We showed that the incorporation of bisphosphonates into these AppCp nucleotide analogues is brought about by members of the family of aminoacyl-tRNA synthetases[116], which catalyse a reversible reaction in which an amino acid condenses with ATP to form an aminoacyladenylate, together with the release of pyrophosphate (PPi) (reaction 1, shown below). Since this reaction is reversible, it

appears that bisphosphonates with short R_1 and R_2 side chains (which most resemble pyrophosphate 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

We found that the aminoacyl-tRNA synthetases that can utilise a bisphosphonate in place of pyrophosphate 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 bisphosphonates with short side chains, but also rather surprisingly tiludronate, can replace pyrophosphate and be accommodated into the active site of Type II aminoacyl-tRNA synthetases. In contrast, the more potent bisphosphonates that contain a nitrogen in the R_2 side chain are not metabolised, presumably since the different and in some cases bulkier structure of the R_2 side chain prevents these bisphosphonates 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 mould amoebae (which also produced diadenosine tetraphosphate metabolites, AppCppA) and with cell-free lysates[117], it has recently been confirmed that intact mammalian cells *in vitro* (J774 macrophage-like cells and MG63 osteosarcoma cells) can also metabolise clodronate to an analogue of ATP (AppCCl2p) [118]. The identity of this metabolite has been confirmed by mass spectrometric analysis of cell lysates from clodronate-treated cells [119]. The high sensitivity of this technique has enabled the demonstration that both etidronate and tiludronate can also be metabolised by mammalian cells. These observations raise the likelihood that osteoclasts could also metabolise these bisphosphonates, and Julie Frith and her colleagues have now confirmed that clodronate is metabolised to AppCCl2p by purified rabbit osteoclasts *in vitro* [120].

The aminoacyl-tRNA synthetases are cytoplasmic enzymes, and the metabolism of bisphosphonates is dependent on cellular uptake [121]. As a result of the accumulation in the cell cytoplasm of these non-hydrolysable AppCp analogues of ATP, they are likely to inhibit many intracellular enzymes, thus having adverse effects on cell metabolism, function and survival. To examine this further, we have performed experiments in which J774 macrophage-like cells were loaded with the chemically-synthesised metabolite of clodronate (AppCCl2p) by using liposome-encapsulated AppCCl2p. This is internalised by the cells by phagocytosis. The AppCCl2p is then released into the cytoplasm following intracellular breakdown of the liposomes. Under these conditions, AppCCl2p was of similar potency in reducing cell viability as clodronate itself and caused similar changes in morphology to those observed in clodronate-treated cells. This confirms that AppCp-type metabolites of bisphosphonates are cytotoxic and suggests that some bisphosphonates act as prodrugs, being converted to active metabolites following intracellular uptake by osteoclasts *in vivo*. However, it remains to be shown exactly how these AppCp-type analogues interfere with cell metabolism.

THE CRITICAL ROLE OF MEVALONATE METABOLISM FOR OSTEOCLAST ACTIVITY, AND THE INTERFERENCE BY BISPHOSPHONATES OF PROTEIN PRENYLATION

The potent, nitrogen-containing bisphosphonates (such as alendronate and ibandronate) are not metabolised to AppCp-type metabolites as described above. They therefore appear to

have a different mechanism of action to that of the bisphosphonates that can be metabolised. A major step forward has been the demonstration that the nitrogen-containing bisphosphonates 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). FPP and GGPP are required for the post-translational modification (prenylation) of small GTPases such as Ras, Rho and Rac, which are prenylated at a cysteine residue in characteristic C-terminal motifs [122]. Small GTPases are important signalling proteins which regulate a variety of cell processes important for osteoclast function, including cell morphology, cytoskeletal arrangement, membrane ruffling, trafficking of vesicles and apoptosis [123 124 125 126]. 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 [127].

There are now many observations that point to the importance the mevalonate pathway for osteoclast function, and strengthen our proposal that the nitrogen-containing bisphosphonates inhibit osteoclastic bone resorption predominantly by inhibition of this pathway. These bisphosphonates inhibit the synthesis of mevalonate metabolites including FPP and GGPP, and thereby impair the prenylation of proteins, including Ras, and cause loss of function of these small GTPases. Steve Luckman as part of his PhD project studied J774 macrophages (which undergo bisphosphonate-induced apoptosis, like osteoclasts) and demonstrated that nitrogen-containing bisphosphonates, but not clodronate, inhibited the incorporation of [^{14}C]mevalonate into prenylated proteins, thus demonstrating that nitrogen-containing bisphosphonates inhibit enzymes in the mevalonate pathway[128]. Furthermore, there was a remarkably strong structure-activity relationship so that changes to the structure of the nitrogen-containing R_2 side chain or to the phosphonate groups which altered anti-resorptive potency also influenced the ability to inhibit protein prenylation to a corresponding degree [129]. Hence, potent anti-resorptive bisphosphonates are effective inhibitors of protein prenylation, whereas less potent anti-resorptive bisphosphonates are less effective inhibitors of protein prenylation. This was the first time that the structure-activity relationships of bisphosphonates could be correlated with a specific biochemical effect. Important verification of the key nature of this pathway has come from the demonstration that the addition of intermediates of the mevalonate pathway (such as FPP and GGPP) could prevent bisphosphonate-induced apoptosis in J774 macrophages. In bone organ cultures using calvaria, mevalonate could also reverse bisphosphonate-induced inhibition of osteoclast formation and bone resorption. This is clear evidence that the dominant mechanism of action of some bisphosphonates involves inhibition of the mevalonate pathway, rather than other mechanisms such as inhibition of protein tyrosine phosphatases. Finally, to confirm that inhibition of the mevalonate pathway could account for the anti-resorptive effects of bisphosphonates, we examined the effect of mevastatin, another inhibitor (unrelated to bisphosphonates) of the mevalonate pathway. Mevastatin, an inhibitor of HMG-CoA reductase, was even more potent than bisphosphonates at inhibiting bone resorption *in vitro* and, like bisphosphonates, caused apoptosis of osteoclasts and J774 macrophages *in vitro*. In addition, several characteristic features of bisphosphonate-induced apoptosis in J774 cells (e.g. the time of occurrence of apoptosis, and the dependence of caspase activation and apoptosis on protein synthesis) were strikingly similar to the features of mevastatin-induced apoptosis, supporting the notion that bisphosphonates cause apoptosis by preventing protein prenylation.

Fisher *et al* [130] have now shown that another statin, lovastatin, inhibits osteoclast formation in cocultures of osteoblasts and murine bone marrow, and inhibits bone resorption by isolated osteoclasts *in vitro*. This study also showed that the effects of alendronate and lovastatin could be overcome by the addition of geranylgeraniol (which can be used for protein geranylgeranylation) but not farnesol (which is utilised for protein farnesylation). Hence it appears that, although nitrogen-containing bisphosphonates 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[131]). Furthermore, unlike with alendronate, the effect of clodronate on osteoclast formation and bone resorption in calvariae *in vitro* could not be overcome by supplementation with mevalonate, reaffirming the hypothesis that clodronate and alendronate have different molecular mechanisms of action.

It is interesting to note that the statins are widely used as cholesterol-lowering drugs, and that they act at one of the earliest steps in cholesterol biosynthesis by inhibiting HMG CoA reductase. At present there is no evidence that statins have effects on bone when used clinically, perhaps because they are selectively targetted to liver rather than bone, which is the converse of the case for bisphosphonates. However in view of the fact that statins have now been shown to have anabolic effects on bone both in organ culture and in experimental animals, their effects on bone are likely to be subject to more intensive scrutiny in the future.

The exact enzymes of the mevalonate pathway that are inhibited by individual bisphosphonates have not yet been fully elucidated. However, incadronate and ibandronate are known to be inhibitors of squalene synthase, an enzyme in the mevalonate pathway required for cholesterol biosynthesis[132 133]. Alendronate and pamidronate are less potent inhibitors of squalene synthase but can also inhibit sterol biosynthesis, suggesting that these bisphosphonates may inhibit upstream enzymes of the mevalonate pathway other than squalene synthase. Several enzymes of the pathway utilise an isoprenoid diphosphate as a substrate (IPP isomerase, FPP synthase, GGPP synthase, squalene synthase) and thus are likely to have similar substrate binding sites. Thus, if nitrogen-containing bisphosphonates act as substrate analogues of an isoprenoid diphosphate, it is likely that these bisphosphonates will inhibit more than one of the enzymes of the mevalonate pathway. This could explain why alendronate and pamidronate can inhibit sterol biosynthesis without being potent inhibitors of squalene synthase. A recent report indicates that several bisphosphonates may inhibit at the level of the IPP isomerase or the FPP synthase, but do not significantly affect the prenyl transferases in brain-derived enzymes[134]. Thus the overall anti-resorptive potency of the nitrogen-containing bisphosphonates may ultimately depend on the number and the combination of the enzymes that are inhibited, as well as on the potencies (IC_{50}s) of each bisphosphonate as inhibitors of the each enzyme in the pathway. Further studies in this area will surely lead to the identification of the exact molecular targets of bisphosphonates and clarification of the structure-activity relationships of these compounds.

Taken together, these observations clearly indicate that bisphosphonates can be grouped into two classes; those that can be metabolised into non-hydrolysable analogues of ATP (the least potent bisphosphonates) and those that are not metabolised but that can inhibit protein prenylation (the potent, nitrogen-containing bisphosphonates). The identification of two such classes may help to explain some of the other pharmacologic differences between the two classes. For example, the ability of the nitrogen-containing bisphosphonates to cause an acute phase response *in vivo* [135 136 137], which can lead to induction of fever in patients, particularly after first exposure to pamidronate.

FUTURE PROSPECTS.

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

There have now been many years of mostly favourable experience with the use of bisphosphonates in diseases such as Paget's disease of bone, myeloma, and bone metastases. Bisphosphonates represent an important class of drugs for the treatment of these bone diseases. Their potential anti-tumour effects and apparent ability to prolong survival in patients with myeloma or breast cancer metastases certainly merit further study.

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

The difficulties of bringing these drugs to the market is illustrated by those that fall by the wayside, such as oral pamidronate and tiludronate. There are important lessons to be learnt from the need to do good dose-response studies during Phase 2 development and making appropriate choices of doses.

However, despite the enormous potential for developing 'better' bisphosphonates based on current knowledge of their structure-activity properties, it is unlikely, given the high cost of development, that further agents will be developed unless they offer distinct advantages over currently available bisphosphonates. For example, attempts to improve intestinal absorption, eg by better formulations or by creating pro-drugs, such as peptide derivatives, have not so far resulted in clinically significant successes.

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.

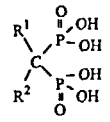
The recent elucidation of the likely mode of action of bisphosphonates within cells opens up the possibility of exploiting the subtle and potentially important differences between classes of bisphosphonates and individual compounds

TABLE I
Major Current and Potential Future Uses of Bisphosphonates

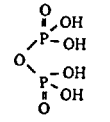
- Bone scanning agents (linked to technetium-99m)
- Inhibition of calcification
  - Heterotopic bone formation (Etidronate at high doses).
  - Dental Calculus.
- Reducing bone resorption
  - Paget's disease.
  - Hypercalcemia of malignancy.
  - Multiple myeloma.
  - Bone metastases, especially breast cancer.
  - Osteoporosis; Treatment of established postmenopausal osteoporosis.
  - Osteoporosis; Prevention of postmenopausal bone loss.
  - Glucocorticosteroid induced bone loss.
- Newer and potential clinical indications
  - Extended use in specific indications, eg. osteoporosis in men.
  - Use in children with osteogenesis imperfecta and other osteopenic disorders.

Use after cardiac or liver transplantation.
Wider use to prevent glucocorticosteroid induced bone loss in children and adult of both sexes and with a spectrum of underlying diseases.
Extended use in cancers to optimise anti-tumour effects and survival.
Prevention of bone loss and erosions in rheumatoid arthritis.
Possible applications in other joint diseases, such as osteoarthritis.
Reduction of bone loss associated with periodontal disease.
Prevention of loosening of joint prostheses.

FIGURES AND LEGENDS

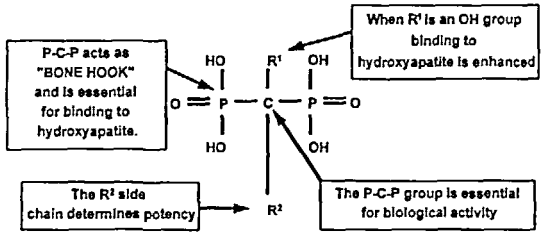


Bisphosphonic acid



Pyrophosphoric acid

Fig 1



BISPHOSPHONATE	R1	R2
*Etidronate**	*OH*	CH_3
Clodronate*	Cl	Cl
Pamidronate*	OH	$CH_2CH_2NH_2$
Alendronate*	OH	$(CH_2)_3NH_2$
Risedronate*	OH	CH_2-3-pyridine
Tiludronate*	H	CH_2-S-phenyl-Cl
Ibandronate*	OH	$CH_2CH_2N(CH_3)$ (pentyl)
Zoledronate	OH	CH_2-imidazole
YH529	OH	CH_2-2-imidazo-pyridinyl
Incadronate (YM175)	H	N-(cyclo-heptyl)
Olpadronate	OH	$CH_2CH_2N(CH_3)_2$
Neridronate	OH	$(CH_2)_5NH_2$
EB-1053	OH	CH_2-1-pyrrolidinyl

* indicates bisphosphonates already approved for one or more indications in one or more countries. Pamidronate is the most extensively used for Pagets Disease.

FIG. 2. Structure of bisphosphonates used in clinical studies and under clinical development.

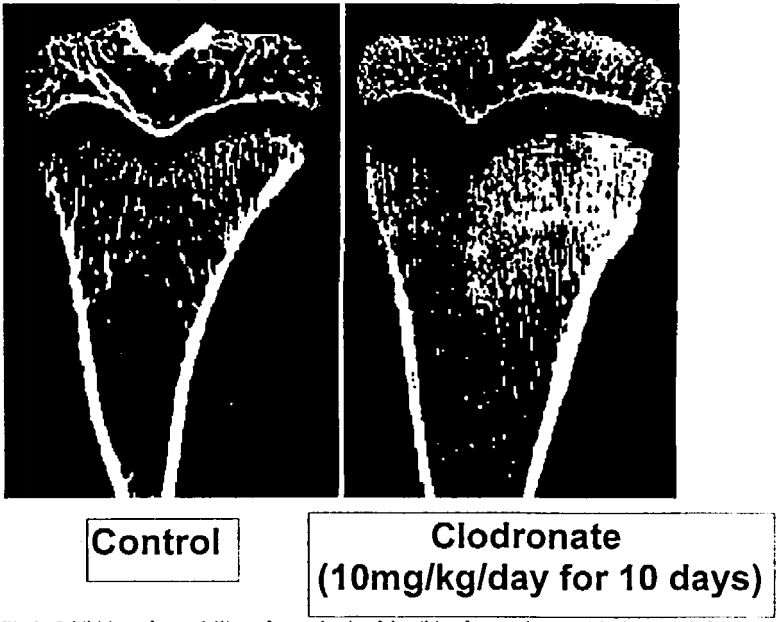


Fig 3. Inhibition of remodelling of metaphysis of the tibia of a growing rat. The increase in bone mass induced by bisphosphonates is used as a pharmacological assay to compare their potency ("Schenk" assay).

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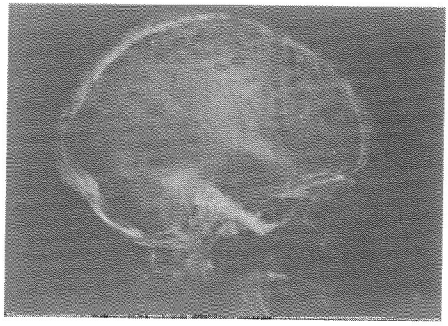

Fig. 4. Lytic lesions in the skull of a patient with multiple myeloma. Bisphosphonates inhibit bone resorption and skeletal complications in such patients.

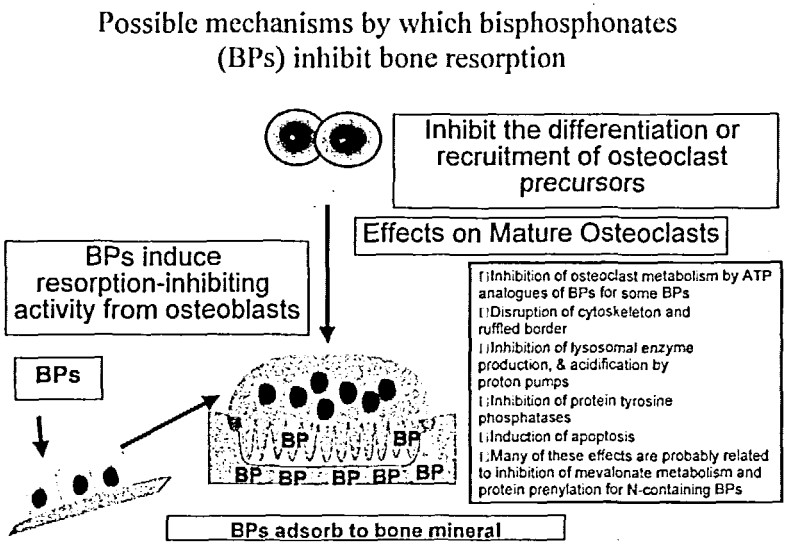

Fig. 5. The ways in which bisphosphonates may affect bone-resorbing osteoclasts. See text for details.

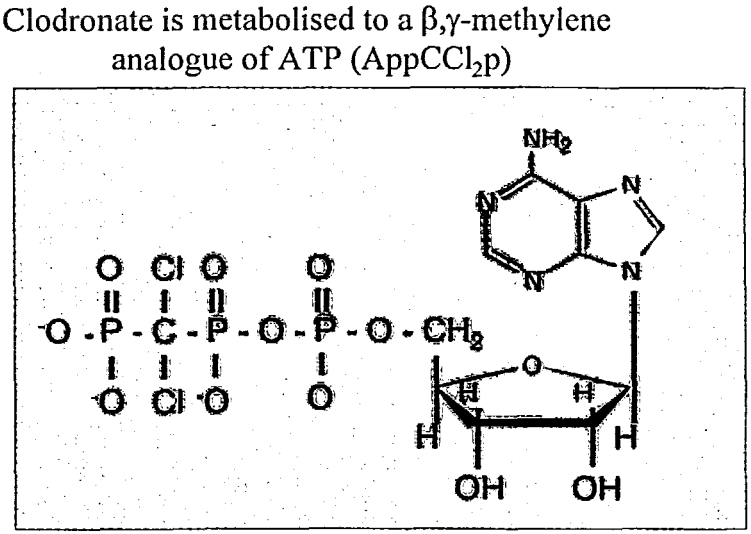

FIG 6

Bisphosphonates can be divided into two classes (those that do or do not contain nitrogen in the R_2 side chain) according to their intracellular actions. This illustrates the metabolites produced by the incorporation of clodronate into an analogue of ATP (for mechanisms see text).

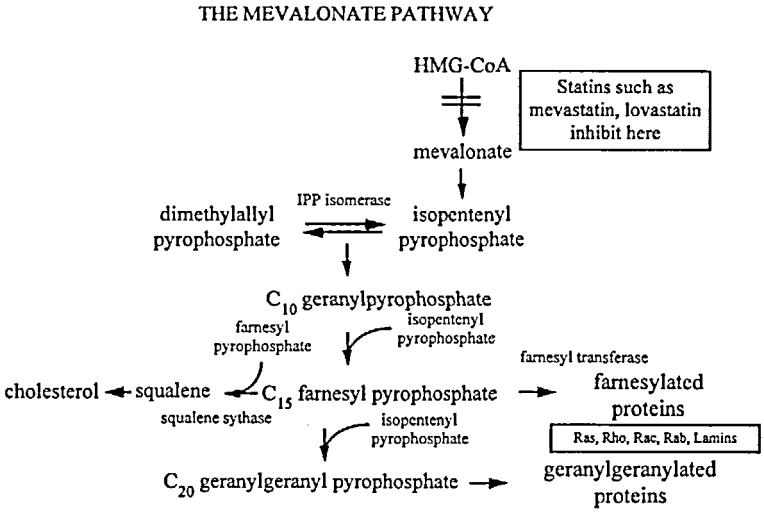

FIG 7. Schematic representation of the mevalonate pathway, leading to synthesis of cholesterol, farnesyl diphosphate and geranylgeranyl diphosphate. The latter are utilised as substrates for proten prenylation. The nitrogen-contaning bisphosphonates inhibit in this pathway.

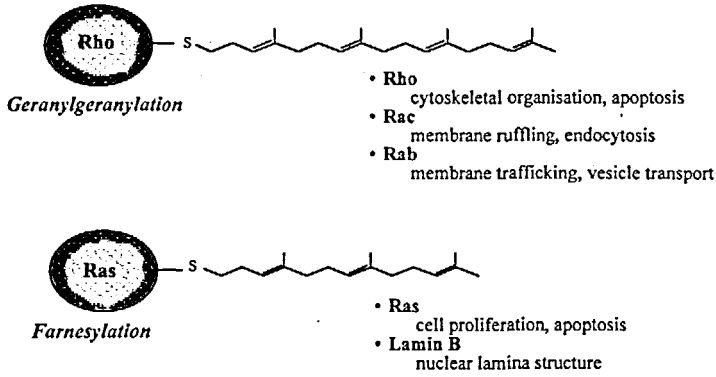

Fig. 8. Protein prenylation involves the transfer of a farnesyl or geranylgeranyl group onto cysteine residues near tthe C-terminus of small GTPases such as Rho, Rac, Rab and Ras. The functions of several prenylated proteins are shown.

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