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Intracellular Metabolism of Bisphosphonates

Impact on the Molecular Mechanism of Action and Side-Effects

Doctoral dissertation

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ABSTRACT

Bisphosphonates are a class of drugs developed for the treatment of metabolic bone diseases that are associated with increased bone resorption. Nitrogen-containing bisphosphonates (N-BPs), such as alendronate and zoledronate, inhibit enzymes of the intracellular mevalonate pathway, thereby preventing the modification of important signalling proteins with isoprenoid lipids. Loss of prenylated proteins causes loss of cell function and, consequently, indirect apoptotic cell death. The less potent non-nitrogen-containing bisphosphonates (non-N-BPs), such as clodronate, do not inhibit isoprenylation but are metabolised into a cytotoxic analog of adenosine triphosphate (ATP), which accumulates in the cell cytoplasm and evokes directly apoptosis. The non-N-BPs have also specific anti-inflammatory actions.

The purpose of this study was to clarify the intracellular metabolism of bisphosphonates and how this impacts the molecular mechanism of action and the potential side-effects. The specific aims were (a) to develop an analytical method for the quantitation of the adenine nucleotide-containing metabolites of bisphosphonates in cell extracts, (b) to study the cellular uptake of clodronate (a non-N-BP) and the kinetics of its metabolism into $AppCCl_2p$ in macrophages, (c) to investigate the cellular uptake and metabolism of clodronate and its derivatives in Caco-2 epithelial cells in order to explore the mechanism of gastrointestinal side-effects, and (d) to characterise the N-BP-induced accumulation of a new ATP analog (ApppI) and its role in the molecular mechanism of action of N-BPs in macrophages and osteoclasts.

The results from this work show that ion-pairing HPLC-ESI-MS is a sensitive method to study the metabolism of bisphosphonates. Furthermore, acetonitrile extraction of cells was very suitable for HPLC analysis. Results from cellular uptake and clodronate metabolism studies supported the view that the clodronate metabolite ($AppCCl_2p$) is responsible for the cellular actions, such as the anti-inflammatory properties and apoptosis, of this bisphosphonate, indicating that non-N-BPs act via AppCp-type metabolites. Additionally, clodronate can probably be metabolised into a cytotoxic $AppCCl_2p$ by any cell type capable of internalising the drug. However, the cytotoxic effect depends on the degree of uptake of clodronate, and thus, the amount of the metabolite. Therefore, when lipophilic derivatives of clodronate are developed to improve their bioavailibility, the possibility for a simultaneous increase in gastrointestinal side-effects should be taken into account. The results further indicate that in addition to causing indirect apoptosis by preventing protein prenylation, N-BP-induced inhibition of FPP synthase leads to the formation of a novel endogenous ATP analog (ApppI). This compound inhibits mitochondrial ADP/ATP translocase, which then evokes direct apoptosis in osteoclasts. This finding provides a new plausible mechanism for the action of N-BPs.

In conclusion, the present study demonstrates the significance of the metabolism of bisphosphonates and how this impacts their molecular mechanism of action and potential side-effects. This data could also be of particular interest for assessing new indications of BPs, such as for treatment of arthritis and cancer.

To Kati

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Hannu Mönkkönen

ABBREVIATIONS

AA	Adjuvant arthritis
ACN	Acetonitrile
AF-ALN	Fluorescently-labelled analog of alendronate
AIA	Antigen-induced arthritis
ANT	Adenine nucleotide translocase
AppCCl$_2$p	Adenosine 5'(β,γ-dichloromethylene) triphosphate
AppCp	Methyleneadenosine 5'-triphosphate
ApppI	Triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester
APR	Acute phase response
BMU	Bone metabolic unit
BP	Bisphosphonate
DMF	N,N-dimethylformamid
DMHA	Dimethylhexylamine
DSPG	Distearoylphosphatidylglycerol
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
FPLC	Fast protein liquid chromatography
FPP	Farnesyl pyrophosphate
FTase	Farnesyl transferase
GGPP	Geranylgeranyl pyrophosphate
GGTase	Geranylgeranyl transferase
GI	Gastrointestinal
GTP	Guanosine triphosphate
HMG-CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
HPLC	High pressure liquid chromatography
IL	Interleukin
IPP	Isopentenyl pyrophosphate
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N-BP	Nitrogen-containing bisphosphonate
NF-κB	Nuclear factor-κB
NMR	Nuclear magnetic resonance
NO	Nitric oxide
Non-N-BP	Non-nitrogen-containing bisphosphonate

PBS	Phosphate buffered saline
Pgp	P-glycoprotein
PT	Permeability transition
PTH	Parathyroid hormone
$t_{1/2}$	Half-life
TEA	Triethylamine
TNF	Tumor necrosis factor
TPA	Tripropylamine
TRAP	Tartrate resistant acid phosphatase
TRFIA	Time-resolved fluoroimmunoassay
tRNA	Transfer ribonucleic acid
VDAC	Voltage-dependent anion channel

LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by Roman numerals **I-IV**:

I   Hannu Mönkkönen, Päivi Moilanen, Jukka Mönkkönen, Julie C. Frith, Michael J. Rogers, Seppo Auriola: Analysis of an adenine nucleotide-containing metabolite of clodronate using ion pair high-performance liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr B 738: 395-403, 2000

II   Hannu Mönkkönen, Michael J. Rogers, Niina Makkonen, Sanna Niva, Seppo Auriola, Jukka Mönkkönen: The cellular uptake and metabolism of clodronate in RAW 264 macrophages. Pharm Res 18: 1550-1555, 2001

III   Hannu Mönkkönen, Soili Törmälehto, Kari Asunmaa, Riku Niemi, Seppo Auriola, Jouko Vepsäläinen, Jukka Mönkkönen: Cellular uptake and metabolism of clodronate and its derivatives in Caco-2 cells: A possible correlation with bisphosphonate-induced gastrointestinal side-effects. Eur J Pharm Sci 19: 23-29, 2003

IV   Hannu Mönkkönen, Seppo Auriola, Petri Lehenkari, Maarit Kellinsalmi, Ilmo E. Hassinen, Jouko Vepsäläinen, Jukka Mönkkönen: A new endogenous ATP analog (ApppI) inhibits the mitochondrial adenine nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-containing bisphosphonates. Br J Pharmacol. Submitted

CONTENTS

1   INTRODUCTION

Bisphosphonates (BPs) are a class of drugs developed over the past four decades for the treatment of metabolic bone diseases that are associated with increased bone resorption, such as Paget's disease (Roux and Dougados 1999), postmenopausal osteoporosis (Delmas 2002), and tumor-induced osteolysis (Coleman 2001). BPs are powerful inhibitors of bone resorption. The mechanism of action of BPs was originally ascribed to their ability to adsorb strongly to bone mineral and prevent the hydroxyapatite dissolution (Francis *et al.* 1969; Fleisch *et al.* 1969). However, it is now clear that BPs inhibit bone resorption by exerting cellular effects on osteoclasts, rather than by a pure physicochemical mechanism. The cellular and molecular mechanisms by which BPs inhibit bone resorption are just only beginning to be clarified.

BPs can be divided into two pharmacological classes which have distinct molecular mechanisms of action. Nitrogen-containing BPs (N-BPs) appear to inhibit at least one enzyme of the intracellular mevalonate pathway. The main target enzyme in mevalonate pathway is currently considered to be farnesyl pyrophosphate synthase (FPP synthase) (van Beek *et al.* 1999a; Bergstrom *et al.* 2000), and its inhibition prevents the modification of important signalling proteins with isoprenoid lipids. Loss of prenylated proteins causes loss of osteoclast function and, consequently, apoptotic cell death (Luckman *et al.* 1998; Benford *et al.* 1999; Frith *et al.* 2001). The less potent non-nitrogen-containing BPs (non-N-BPs) do not inhibit isoprenylation but are metabolised intracellularly by osteoclasts into cytotoxic analogs of adenosine triphosphate (ATP), which accumulate in the cell cytoplasm and cause direct apoptosis in osteoclasts as well as having anti-inflammatory effects on macrophages (Benford *et al.* 1999; Makkonen *et al.* 1999; Frith *et al.* 2001. Thus, non-N-BPs have both anti-resorptive and anti-inflammatory properties.

Earlier data strongly suggest that the metabolism of BPs, especially non-N-BPs, plays a major role in the molecular actions of BPs. This was clarified by determining the intracellular metabolism of BPs and how this can influence the molecular mechanism of action and the side-effects of these drugs. This data could be of particular interest for assessing the new indications of BPs, such as for treatment of arthritis and cancer. The following review of the literature provides a short summary of the effects of BPs on bone cells and the molecular mechanisms of action of BPs.

2 REVIEW OF LITERATURE

2.1 Bone

2.1.1 Overview of bone

The function of bone is to provide mechanical support for joints, tendons and ligaments, to protect vital organs from damage and to act as a reservoir for calcium and phosphate in the preservation of normal mineral homeostasis. Bone is composed of an organic matrix that is strengthened by the deposition of calcium salts. Type I collagen constitutes approximately 95% of the organic matrix; the remaining 5% is composed of proteoglycans, several noncollagenous proteins, and glycoproteins. Crystalline salts deposited in the organic matrix are primarily calcium and phosphate in the form of hydroxyapatite. Bone is composed of four different cell types. Osteoblasts, osteoclasts, bone lining cells which are present on bone surfaces, and finally osteocytes which permeate the mineralized interior. Osteoblasts are fully differentiated cells responsible for the production of the bone matrix, whereas osteoclasts resorb bone. After becoming entrapped inside the mineralized bone matrix, a fraction of osteoblasts differentiate into osteocytes; these cells are responsible for the maintenance of bone matrix. Bone lining cells are flat, elongated, inactive cells which cover bone surfaces that are undergoing neither bone formation nor resorption (Marks and Odgren 2002).

There are two types of bone in the adult skeleton; cortical bone, which makes up most of shafts (diaphysis) of the long bones (femur, tibia and humerus), and trabecular bone which makes up most of the vertebral bodies and the ends of the long bones. Cortical bone consists of an arranged series of concentric lamellae of collagen fibres surrounding a central canal that contains blood vessels. Trabecular bone has a similar structure, but here the lamellae run in a parallel direction to the bone surface, rather than concentrically as in cortical bone. Trabecular bone has a greater surface area than cortical bone and because of this, it is remodelled more rapidly. This means that the conditions associated with increased bone turnover tend to affect trabecular bone more quickly and more profoundly than cortical bone. Therefore, differences in the structural arrangements of the two bone types are related to their primary functions: cortical bone provides mechanical and protective functions and trabecular bone is responsible for metabolic functions (Marks and Odgren 2002).

2.1.2 Bone remodelling

The remodelling of bone consists of a strict coupling of bone resorption and formation that continues throughout life and is necessary not only for skeletal growth

but also to maintain normal bone structure. The process begins with the resorption of bone by osteoclasts followed by new bone formation by osteoblasts, with a positive balance occurring during growth and a negative balance with ageing. If the volume of bone resorbed exceeds the volume formed, the resulting bone loss eventually leads to bone fragility and osteoporotic fractures. Circulating hormones, such as parathyroid hormone (PTH) and gonadal steroids, exert modulating influences on bone remodelling, but crucial regulation is provided by many cytokines and growth factors that are the products of bone cells and the immune system (Martin and Sims 2005). Bone resorption and bone formation do not occur randomly throughout the skeleton, but are coupled together at about 10^6 discrete foci, the so-called 'bone multicellular units' or 'bone metabolic units' (BMUs) (Parfitt 2002). The progressive stages of the bone remodelling sequence within BMUs are shown schematically in Fig. 1.

Both osteoblasts and osteoclasts are derived from progenitors that reside in the bone marrow; osteoblasts belong to the mesenchymal lineage of the marrow stroma, and osteoclasts to the hematopoietic lineage (monocyte-macrophage lineage). Mature osteoclasts form a tight seal over the bone surface and resorb bone by secreting hydrochloric acid and proteolytic enzymes through the "ruffled border" into a space beneath the osteoclast. The formation of this ruffled border is critically dependent on the presence of *c-src*, a cell membrane associated signalling protein. The hydrochloric acid secreted by osteoclasts dissolves hydroxyapatite and allows proteolytic enzymes (mainly Cathepsin K and matrix metalloproteinases) to degrade collagen and other matrix proteins. During bone resorption, osteoclasts endocytose both organic and inorganic degradation products of bone matrix through the ruffled border membrane. These products are then transcytosed through the cell and finally secreted into the extra cellular fluid through functional secretory domain (Salo *et al*. 1997; Nesbitt and Horton 1997). After resorption is completed, osteoclasts undergo programmed cell death (apoptosis), in the so-called reversal phase which heralds the start of bone formation. Mature osteoblasts lay down bone matrix which is initially unmineralised (osteoid), but which subsequently becomes calcified after about 10 days to form mature bone. During bone formation, some osteoblasts become trapped within the matrix and differentiate into osteocytes, whereas others mature into flattened "lining cells" which cover the bone surface (Teitelbaum 2000; Nakamura *et al*. 2003; Riggs and Parfitt, 2005). Osteocytes connect with one another and with lining cells on the bone surface through an intricate network of cytoplasmic processes. Osteocytes appear to act as sensors of mechanical strain in the skeleton, and release signalling molecules such as prostaglandins and nitric oxide (NO), which can modulate the function of neighbouring bone cells (Nijweide *et al*. 2002). Secondary cell types such as monocytes/macrophages and endothelial cells also contribute to bone remodelling either through direct contact with osteogenic cells

or by the release of soluble factors (cytokines, growth factors) (Collin-Osdoby *et al.* 2001; Roodman GD 1999).
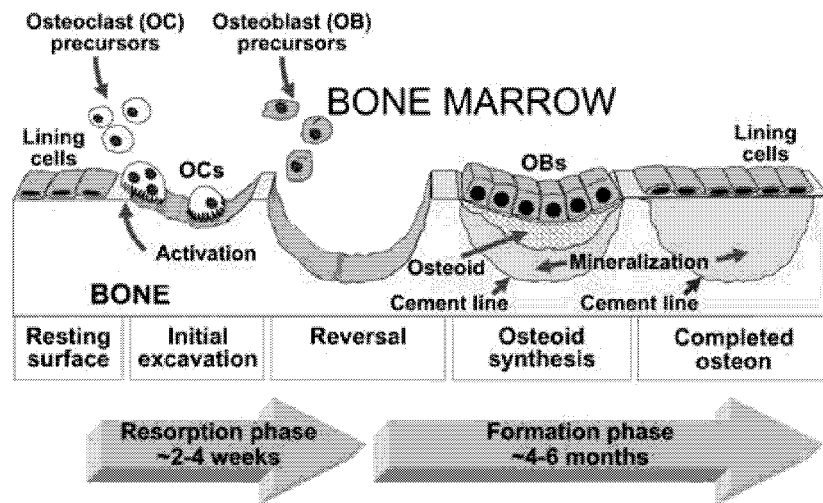
Fig. 1. At the beginning of the bone remodelling sequence, lining cells on the surface of bone become activated and retract. Osteoclasts are recruited to the active site from precursors in bone marrow or from circulating precursors, and these excavate a resorption cavity, the furthermost extension of which becomes bounded by a sclerotic border called the cement line. The resorption phase requires only about 2-4 weeks to become completed and is terminated when the osteoclasts disappear by apoptosis. After a brief reversal phase, the resorption cavity becomes lined by osteoblasts. The osteoblasts secrete osteoid which, after a lag period, mineralizes to form new bone. Over a period of some 4-6 months, the formation phase refills the resorption cavity with bone to form a completed osteon (Riggs and Parfitt, 2005).

2.2 Bisphosphonates

2.2.1 Chemical structure and targeting to bone

Bisphosphonates (BPs) are compounds with a chemical structure similar to that of inorganic pyrophosphate (PPi) (Fig. 2), an endogenous regulator of bone mineralization (Russell *et al.* 1970a). While PPi contains two phosphate groups linked by phosphoanhydride bonds (a P-O-P structure), BPs consist of two phosphonate groups linked by phosphoether bonds to a central (geminal) carbon atom (a P-C-P structure). The geminal carbon also has two side chains attached, R^1 and R^2, which allows a wide range of possible chemical structures. BPs can be divided into two classes in accordance

with their structure. In contrast to non-nitrogen-containing BPs (non-N-BPs), such as clodronate and etidronate, nitrogen-containing bisphosphonates (N-BPs), such as alendronate and ibandronate, contain a nitrogen atom in the R^2 side chain (Fig. 2). Unlike the unstable nature of the P-O-P bonds, the P-C-P structure is highly resistant to hydrolysis under acidic or alkaline conditions or by enzymatic breakdown by pyrophosphatases. This means that BPs are extremely stable against enzymatic or chemical degradation (Russell *et al.* 1970a).

Like PPi, BPs have a high affinity for bone mineral. The affinity for calcium (and thus bone mineral) can be increased further if one of the side chains (R^1) is a hydroxyl (-OH) or a primary amino (-NH$_2$) group, because this allows the formation of a tridentate conformation that is able to bind Ca^{2+} more effectively (Jung *et al.* 1973; van Beek *et al.* 1994). The R^1 side chain together with the two phosphonate groups are often referred to as the "bone hook" (Rogers *et al.* 1999). It was shown recently that even BPs that share a common P–C–P structure, with OH at R^1, can have significantly different kinetic binding affinities for hydroxyapatite with a rank order of highest to lowest for the BPs studied of zoledronate > alendronate > ibandronate = risedronate > etidronate. These differences must be attributed to differences in the R^2 side chain (Nancollas et al. in press). BPs are poorly absorbed in humans; their oral bioavailability is less than 3 %. The low bioavailability is thought to be attributable to the highly hydrophilic nature of BPs, which prevents the transcellular transport across intestinal epithelium and favors the paracellular route (Twiss *et al.* 1994; Lin 1996; Raiman *et al.* 2001). Following absorption, BPs are rapidly cleared from the circulation ($t_{1/2}$ one hour or less), about half being excreted in the urine and the other half deposited into bone ($t_{1/2}$ one year or more) (Mönkkönen *et al.* 1987, 1990; Mönkkönen 1988; Lin 1996). Studies with radiolabelled alendronate have shown that at pharmacological doses, BPs localise preferentially to osteoclast-covered bone surfaces at sites of bone resorption rather than at sites of bone formation (Sato *et al.* 1991; Masarachia *et al.* 1996). The pharmacokinetics and the tissue specific targeting of BPs to the bone mineral hydroxyapatite, especially to sites of osteoclast activity, probably explains why osteoclasts and also other bone cells in their immediate vicinity are especially affected by bisphosphonates.
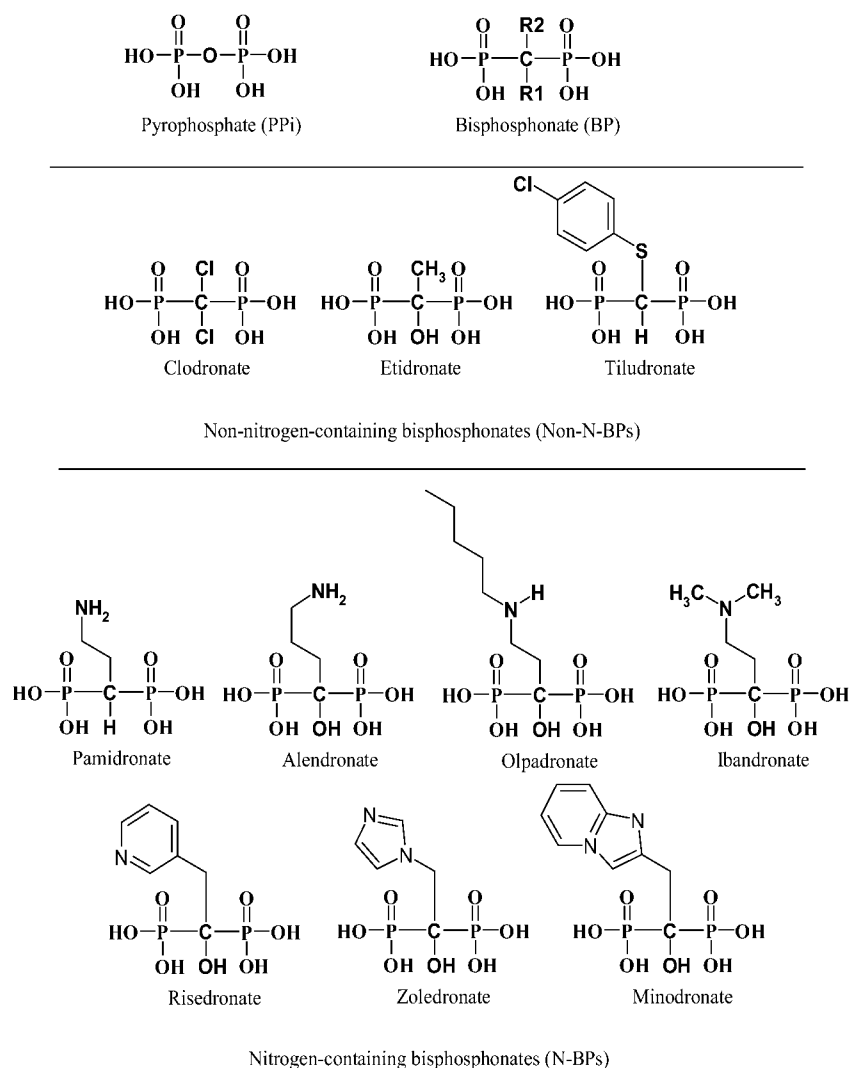
Fig. 2. The structure of pyrophosphate, a generic bisphosphonate, some non-nitrogen-containing, and nitrogen-containing bisphosphonates.

2.2.2 Cellular uptake of bisphosphonates

As mentioned above, BPs are rapidly deposited into the skeleton and localize preferentially on exposed mineral at bone resorption surfaces. During the process of bone resorption, BPs can be released from the bone surface in the acidic environment of the resorption lacuna beneath the osteoclast, since the ability to chelate Ca^{2+}, and hence binding to hydroxyapatite, is reduced at acidic pH (Rogers *et al.* 1999). Thus, BPs could achieve very high local concentrations in the osteoclast resorption lacuna after adsorption to bone, followed by their release during the resorption process (Fig. 3). For example, Sato *et al.* (1991) 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 (Sato *et al.* 1991). This, together with the fact that osteoclasts can internalise negatively-charged compounds via endocytosis (Salo *et al.* 1997; Nesbitt and Horton 1997; Stenbeck *et al.* 2000), indicates that osteoclasts are the cells in bone that are most likely to be exposed to BPs. The importance of release of BP from bone mineral prior to cellular uptake is supported by the finding that osteoclasts derived from *oc/oc* mice, which are unable to form ruffled borders and cannot resorb bone mineral, are not affected when cultured on bisphosphonate-coated bone (Murakami *et al.* 1995). Additionally, it has been recently demonstrated *in vitro* that osteoclasts internalise large amounts of BP due to their ability to release the BP from the bone surface during resorption and their high endocytic activity (Coxon *et al.* 2005). In this study, osteoclasts isolated from rabbits were cultured for 24 h on dentine slices pre-coated with fluorescently-labelled analog of alendronate (AF-ALN). Confocal microscopic analysis revealed that AF-ALN was avidly internalised by resorbing osteoclasts into intracellular vesicles throughout the cell. This pattern of uptake also occurred with AF-ALN in the absence of dentine. In comparison, non-resorbing but highly endocytic cell types, such as macrophages, avidly internalised AF-ALN from solution, but took up little from the surface of dentine, due to their inability to resorb dentine (Coxon *et al.* 2005).

Although BPs are likely to be membrane-impermeable due to their high negative charge, they can be internalised by cells. Osteoclasts *in vivo* have been shown to internalise radiolabeled BPs into numerous endocytic vacuoles, with the appearance of the bisphosphonate in other subcellular compartments, including the cytoplasm, mitochondria, and nuclei (Sato *et al.* 1991; Masarachia *et al.* 1996). Studies with slime mould amoebae have demonstrated that fluid-phase endocytosis is the likely route by which osteoclasts internalise BPs from the resorption lacunae (Rogers *et al.* 1997). In support of this theory, it was recently confirmed in osteoclasts and macrophages using AF-ALN and confocal microscopy that BPs are internalised initially by fluid-phase endocytosis, not by adsorptive or receptor-mediated endocytosis. Vesicular acidification is then essential for the BPs to exit the endocytic vesicles and enter the cytosol, presumably by causing protonation of the negatively-charged phosphonate groups to allow movement across the vesicular membrane (Thompson *et al.* 2005).
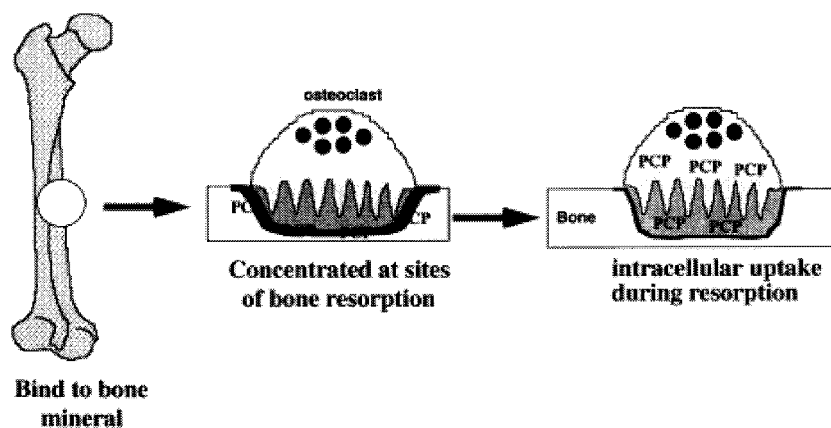
Fig. 3. The route by which bisphosphonate (PCP) is taken up by osteoclasts (Rogers *et al.* 1999, with permission from Elsevier).

2.2.3 Structure-activity relationships of bisphosphonates

It is 35 years ago when etidronate was found to be less potent than clodronate at inhibiting bone resorption both *in vitro* and *in vivo*, even though etidronate has a higher affinity for bone mineral owing to the presence of a hydroxyl group in the R^1 position (Russell *et al.* 1970b). Since the mechanism of action of BPs was unknown at that time, this discrepancy between anti-resorptive potency and affinity for Ca^{2+} led to the currently accepted view that BPs inhibit bone resorption by cellular effects on bone-resorbing osteoclasts, rather than by preventing hydroxyapatite crystal dissolution due to some physicochemical mechanism (Russell *et al.* 1970b).

After attaching to bone, the structure and three-dimensional conformation of the R2 side chain (as well as the phosphonate groups in the molecule) determine the biological activity of the molecule and influence the ability of the BPs to interact with specific molecular targets. BPs 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 those lacking the nitrogen atom (etidronate and clodronate) (Schenk *et al.* 1986; Shinoda *et al.* 1983), whereas derivatives of these compounds containing a tertiary nitrogen (such as ibandronate and olpadronate) were even more potent at inhibiting bone resorption (Papapoulos *et al.* 1989; Muhlbauer *et al.* 1991). BPs containing a hydroxyl group at the R^1 side chain and a tertiary nitrogen within a ring structure in the R^2 side chain appear to be the most potent anti-resorptive BPs discovered to date (Fig. 2). These heterocycle-containing BPs include risedronate (Sietsema *et al.* 1989), zoledronate (Green *et al.* 1994), and minodronate (Sasaki *et al.* 1998), which are up to 10 000 fold more potent than etidronate *in vivo* in bone resorption. In summary, the least potent anti-resorptive BPs include those that most closely resemble PPi, having R^1 and R^2 side chains of a simple chemical structure, such as clodronate and etidronate (non-nitrogen-containing

BPs, non-N-BPs). More potent anti-resorptive BPs have been developed by the insertion of a primary, secondary or tertiary nitrogen function in the R^2 side chain, generally referred to as nitrogen-containing bisphosphonates (N-BPs).

2.2.4 Clinical uses of bisphosphonates

BPs are widely used in the treatment of bone diseases that are characterized by increased bone resorption, such as Paget's disease (Roux and Dougados 1999), postmenopausal osteoporosis (Delmas 2002), and tumor-induced osteolysis (Coleman 2001).

In Paget's disease, which is a localised disorder of bone remodelling, abnormal osteoclasts (both in structure and function) exhibit excessive resorption activity, which results in abnormal architecture of bone, and thereby in bone deformities and weakness (Roux and Dougados 1999). In postmenopausal osteoporosis, increased osteoclast activity, which can be seen in early postmenopause, is caused by the loss of circulating estrogen (Heaney et al. 1978). At the site of bone metastases, interactions between metastatic cancer cells and bone cells lead to disruption of normal bone metabolism. This is especially seen in breast and prostate cancer. Increased osteoclast activity and uncontrolled bone resorption at the site of osteolytic lesions have been shown to be mediated via cancer-derived PTHrP and other osteoclastogenic cytokines (Coleman 2001).

2.3 Effects of bisphosphonates on bone cells

2.3.1 Osteoclasts

It is well established that BPs affect osteoclast mediated bone resorption. The direct effect of BP on mature osteoclasts may result in the induction of osteoclast apoptosis, since osteoclasts exhibiting apoptotic morphology have been detected following BP treatment in vitro (Hughes et al. 1995; Selander et al. 1996) and in vivo (Hughes et al. 1995). In addition to causing osteoclast apoptosis, BPs can evoke more subtle changes in osteoclast function that affect their ability to resorb bone. The most striking feature of osteoclasts treated with BPs in vitro and in vivo is the lack of a ruffled border that is essential for the resorption process. The ability of BPs to inhibit actin ring formation is also sufficient for the molecules to prevent bone resorption (Murakami et al. 1995; Sato and Grasser 1990).

In addition to affecting mature osteoclasts, it is possible that BPs could indirectly inhibit bone resorption by acting on mononuclear osteoclast precursors (Boonekamp et al. 1986; Löwik et al. 1988), thereby preventing osteoclast formation. Additionally,

Hughes *et al.* (1989) found that BPs are potent inhibitors of osteoclast-like cell formation in long-term human marrow cultures, and that this may be related to their ability to inhibit bone resorption *in vivo*. Recent studies have suggested that, as with mature osteoclasts, inhibition of FPP synthase and loss of prenylated proteins following release of bone-bound N-BP, is the major mechanism by which osteoclast formation is prevented *in vitro* (Fisher *et al.* 1999; van Beek *et al.* 1999b, 2002). The mechanisms for the inhibitory effects of non-N-BPs on osteoclast formation are unclear, but it may be related to the metabolism of these BPs. The detailed molecular basis by which BPs affect osteoclast-mediated bone resorption is described below.

2.3.2 Osteoblasts and osteocytes

Although the primary action of BPs is the inhibition of osteoclastic bone resorption, there is increasing evidence that these compounds also act on the osteoclasts indirectly through the bone-forming osteoblasts. One interesting finding was that low concentrations of BPs are able to stimulate osteoblasts to release a factor that subsequently inhibits osteoclast formation. This factor, which has a low molecular weight (<10 kD), appears to be present in the conditioned medium of BP-treated osteoblasts (Vitte *et al.* 1996; Niskikawa *et al.* 1996). When osteoclasts are co-cultured with osteoblast-like cells (which have been incubated in the presence of BP), an inhibition of bone resorption is observed (Sahni *et al.* 1993). Evidence from these studies implies that, for this mechanism to be effective *in vivo*, osteoblasts would need to be exposed to nanomolar concentrations of BPs. How this would be achieved is not clear. It is possible that BP could be released into the bone marrow during resorption, for example BP may be released from hydroxyapatite by the osteoclast. It appears that BPs may have also anabolic effects on osteoblasts. Recent studies indicate that bisphosphonates enhance proliferation and maturation of osteoblasts (Fromigue *et al.* 2002; Im *et al.* 2004; von Knoch *et al.* 2005). These observations are evidence in support of the theory that BPs have an anabolic effect on osteoblasts and subsequently promote bone formation. Nevertheless, BPs do not have an anabolic effect on bone *in vivo*, and thus, the importance of these effects in humans *in vivo* remains to be clarified.

There are some studies examining the effects of BPs on osteocytes. It has been shown that BPs prevent apoptosis of both osteoblasts and osteocytes induced by glucocorticoids. Thus, contrary to their pro-apoptotic effect on osteoclasts, BPs have an anti-apoptotic effect on osteoblasts and osteocytes. It has been demonstrated that BPs prevent apoptosis of osteoblasts and osteocytes via the opening of hemichannels formed by connexin (Cx) 43, leading to activation of Src and the extracellular signal-regulated kinases (ERKs) (Plotkin *et al.* 1999, 2002). These results suggest that the therapeutic efficacy of BPs in diseases such as glucocorticoid-induced osteoporosis may be due, in

part, to their ability to prevent osteocyte and osteoblast apoptosis (Plotkin *et al.* 1999; Weinstein *et al.* 2002).

2.4 Molecular mechanisms of action of bisphosphonates

2.4.1 Metabolism of non-N-bisphosphonates to ATP analogs

BPs were initially considered to be metabolically inert. However, Rogers *et al.* (1992, 1994) and others (Pelorgeas *et al.* 1992) found that clodronate and other non-N-BPs that closely resemble PPi in structure could be metabolised by the slime mold amoebae *Dictyostelium discoideum* into non-hydrolysable, adenine-containing analogs of adenosine triphosphate (ATP) (Fig. 4). The AppCp-type metabolites of non-N-BPs are formed by a back reaction catalysed by members of the family of type II class of aminoacyl-tRNA synthetases (Rogers *et al.* 1994, 1996), which play an essential role in protein synthesis. It appears that non-N-BPs with short side chains (such as clodronate, etidronate, and tiludronate) can replace PPi and be accommodated into the enzyme active site (Fig. 4). N-BPs with larger, bulkier R^2 side chains are not metabolised to ATP analogs (Auriola *et al.* 1997; Benford *et al.* 1999). In initial studies, the metabolites of non-N-BPs were detected by using anion-exchange FPLC (Rogers *et al.* 1992, 1994; Frith *et al.* 1997) or ^{31}P NMR spectroscopy (Pelorgeas *et al.* 1992). Later, a very sensitive analytical method was developed for detection of the metabolites of non-N-BPs by using an ion-pairing HPLC method that is compatible with negative ion electrospray ionization mass spectrometry (ESI-MS) (Auriola *et al.* 1997). It was confirmed that clodronate, etidronate and tiludronate are also metabolised *in vitro* by intact mammalian cells, such as highly endocytic macrophages (Auriola *et al.* 1997, Frith *et al.* 1997; Benford *et al.* 1999). Importantly, it has been demonstrated that osteoclasts can metabolise clodronate to AppCCl$_2$p also *in vivo*. The metabolite of clodronate was detected in extracts from osteoclasts purified from clodronate-treated rabbits using electrospray mass spectrometry (Frith *et al.* 2001). In conclusion, these qualitative results evidence the metabolism of non-N-BPs to ATP analogs. For evaluating the role of metabolism in the molecular mechanisms of action of BPs, there should also be available quantitative results for the metabolism of non-N-BPs in cells. Naturally, the analytical method for this purpose should first be developed.
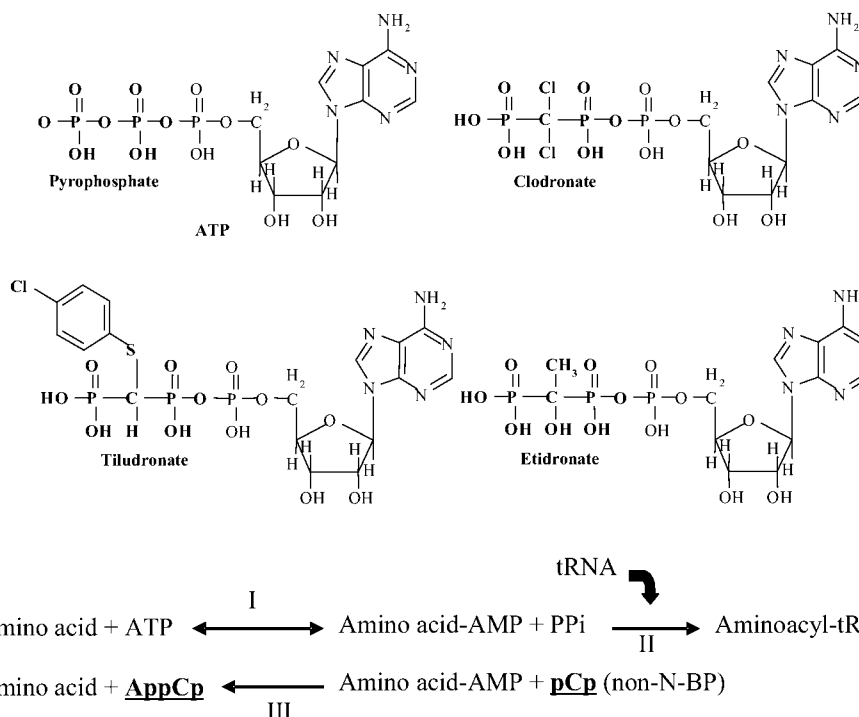
Fig. 4. The structure of ATP, and the AppCp-type metabolites of clodronate, etidronate and tiludronate. The formation of AppCp-type metabolites of non-N-BPs is catalysed by aminoacyl-tRNA synthetases. An amino acid condenses with ATP to form an aminoacyl-adenylate (amino acid-AMP), releasing pyrophosphate (PPi) in a reversible reaction (I). The aminoacyl-adenylate then condenses with a molecule of tRNA to form aminoacyl-tRNA (II). Since non-N-BPs (pCp) resemble PPi in structure, the reverse reaction of (I) can occur with pCp in place of PPi, to form an analog of ATP (AppCp) containing the non-N-BP (Rogers 2004).

2.4.1.1 ATP analogs induce osteoclast apoptosis

Owing to the nonhydrolyzable nature of the ATP analogs, their accumulation is likely to inhibit numerous intracellular metabolic enzymes and thus to have detrimental effects on cell function and survival. In agreement, Frith *et al.* (2001) found that treatment of osteoclasts with the clodronate metabolite (AppCCl$_2$p) *in vitro* causes a reduction in the number of osteoclasts, increases osteoclast apoptosis, and inhibits bone resorption to the same extent as treatment with clodronate. The detailed molecular basis by which AppCp-type metabolites of non-N-BPs cause apoptosis of osteoclasts was unknown until Lehenkari *et al.* (2002) discovered that AppCCl$_2$p inhibits mitochondrial oxygen consumption by a mechanism that involves competitive inhibition of the ADP/ATP translocase (ANT). By inhibiting the mitochondrial ANT, AppCCl$_2$p induces

mitochondrial membrane depolarisation, which may lead to the opening of the mitochondrial permeability transition (PT) pore and the release of cytochrome c and other apoptogenic proteins (Kroemer and Reed 2000), which then leads to caspase activation (Benford *et al.* 2001) and therefore to direct apoptotic cell death.

2.4.1.2 ATP analogs have an anti-inflammatory effect

In addition to the ability of non-N-BPs to inhibit bone resorption, these compounds (particularly clodronate) have been shown to have anti-inflammatory effects. While free BPs target bone and selectively affect osteoclasts (Sato *et al.* 1991; Coxon *et al.* 2005), BPs encapsulated in liposomes selectively target phagocytic cells such as macrophages *in vivo* (van Rooijen and Sanders 1994). Macrophages play a key role in inflammatory diseases like rheumatoid arthritis. In animal models of arthritis, liposome-encapsulated clodronate has been found to eliminate macrophages and reduce inflammation in both rat adjuvant arthritis (AA) (Kinne *et al.* 1995) and antigen-induced arthritis (AIA) (Richards *et al.* 1999). Barrera *et al.* (2002) showed that a single intra-articular injection of clodronate liposomes caused synovial macrophage depletion and was well tolerated in patients with rheumatoid arthritis. Intra-articular administration of low, noncytotoxic doses of liposomal clodronate had temporary anti-inflammatory and joint-sparing effects on AIA in rabbits. Liposomal clodronate did not prevent formation of joint erosions over the long term, but the loss of cartilage proteoglycans was halted (Ceponis *et al.* 2001).

Clodronate inhibits the lipopolysaccharide- (LPS) induced release of proinflammatory cytokines (IL-1β, IL-6, and TNFα) (Pennanen *et al.* 1995) and NO (Makkonen *et al.* 1996) from macrophages. The anti-inflammatory effect of clodronate on macrophages appears to be attributable to the AppCCl$_2$p metabolite, since it was found that chemically synthesised AppCCl$_2$p encapsulated in liposomes has the same potency for reducing macrophage cell viability (Frith *et al.* 1997) and similar inhibitory effects on cytokine release as clodronate itself (Makkonen *et al.* 1999). Additionally, both clodronate and its metabolite also inhibit the LPS-stimulated binding of nuclear factor-κB (NF-κB) to DNA in macrophages (Makkonen *et al.* 1999). The transcription factor NF-κB is an inducible protein that regulates gene expression in rheumatoid arthritis (Handel *et al.* 1995). Binding of NF-κB to DNA in the nucleus leads to increased production of pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) (Barnes and Karin 1997; Baldwin 1996). These results support the hypothesis that the metabolite of clodronate (AppCCl$_2$p) is responsible for the anti-inflammatory properties of this bisphosphonate.

2.4.2   N-bisphosphonates inhibit FPP synthase in the mevalonate pathway

N-BPs are not metabolised to ATP analogs (Auriola *et al.* 1997; Benford *et al.* 1999) and therefore appear to have a different mechanism of action to that of the non-N-BPs that can be metabolised. Amin *et al.* (1992) were the first to demonstrate that BPs could interfere with the mevalonate pathway, since they found that the N-BPs inhibited cholesterol biosynthesis in mouse J774 macrophages. By contrast, the non-N-BPs did not affect cholesterol synthesis (Amin *et al.* 1992). In addition to cholesterol, the mevalonate pathway is responsible for the production of isoprenoid lipids such as isopentenyl pyrophosphate (IPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Fig. 5). N-BPs interfere with the mevalonate pathway by inhibiting farnesyl pyrophosphate (FPP) synthase, an enzyme not inhibited by non-N-BPs (van Beek *et al.* 1999a; Bergstrom *et al.* 2000). Using molecular modelling, Martin *et al.* (1999) have shown that N-BPs inhibit FPP synthase by acting as isoprenoid pyrophosphate transition state analogs. The phosphonate groups of N-BPs appear to fit into the pyrophosphate binding site of the enzyme. Furthermore, the relative ability of N-BPs to inhibit FPP synthase appears to be dependent on the orientation of the nitrogen atom in the R^2 side chain relative to the phosphonate groups (Martin *et al.* 1999). The length and orientation of the bisphosphonate R^2 side chain may affect the interaction of the nitrogen in the side chain with amino acid residues in the active site cleft of FPP synthase, perhaps explaining why minor changes to the structure or conformation of the side chain also affect the ability of the N-BP to inhibit FPP synthase (Dunford *et al.* 2001).

2.4.3   Inhibition of FPP synthase prevents protein prenylation and the subsequent inhibition of osteoclast function

By inhibiting FPP synthase in the mevalonate pathway, N-BPs prevent the synthesis of FPP and GGPP (Fig. 5). These molecules are required for the posttranslational lipid modification (prenylation) of small GTP-binding signalling proteins (GTPases). The process of prenylation involves the transfer of the isoprenoid groups from FPP or GGPP onto a cysteine residue in characteristic COOH-terminal motifs of specific target proteins catalysed by farnesyl transferase (FTase) or geranylgeranyl transferase (GGTase) (Zhang and Casey 1996). 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 (Marshall 1993; Zhang and Casey 1996). Small GTPases are localised to membrane compartments, and it is here that GTP exchange is thought to occur. The loss of FPP and GGPP synthesis resulting from FPP synthase inhibition by N-BPs prevents the prenylation of small

GTPases (Fig. 5). Studies with macrophages provided the first direct evidence that N-BPs inhibit protein prenylation *in vitro*, whereas the non-N-BPs had no effect (Luckman *et al.* 1998; Benford *et al.* 1999). Importantly, Frith *et al.* (2001) showed that unlike non-N-BP compounds and their metabolites, N-BPs prevent protein prenylation in osteoclasts *in vivo*. Furthermore, there is a clear correlation between the ability of N-BPs to inhibit FPP synthase and protein prenylation *in vitro* and their abilities to prevent bone resorption *in vivo* (Dunford *et al.* 2001), strongly suggesting that FPP synthase is the major pharmacologic target of N-BPs in osteoclast *in vivo*.

N-BPs prevent the prenylation of small GTPases, such as Ras, Rho, Rac and Rab. These signalling proteins regulate a variety of cell processes important for osteoclast function, including cytoskeletal arrangement, membrane ruffling, trafficking of intracellular vesicles, and apoptosis (Coxon and Rogers 2003). By disturbing these cell processes, N-BPs cause the loss of the osteoclast ruffled border and disruption of the actin rings that are essential for bone resorption (Sato and Grasser 1990; Sato *et al.* 1991; Selander *et al.* 1994) (Fig. 5). Especially, Rab GTPases appear to play a critical role for the bone resorption prevented by N-BPs. Rab GTPases regulate vesicular trafficking in osteoclasts, thereby the loss of prenylation of Rab GTPases can affect the formation of ruffled border, trafficking of lysosomal enzymes, and transcytosis of degraded bone matrix. The regulated transport of vesicles is essential for bone resorption (Mulari *et al.* 2003; Salo *et al.* 1997; Nesbitt and Horton 1997) which appears to be disrupted in osteoclasts following N-BP treatment (Alakangas *et al.* 2002). Loss of prenylation of small GTPases, and disruption of downstream signalling pathways promoting cell survival is also the likely route by which N-BPs induce osteoclast apoptosis (Glantschnig *et al.* 2003) (Fig. 5). However, induction of osteoclast apoptosis does not completely to account for the inhibition of bone resorption caused by N-BPs, since preventing osteoclast apoptosis *in vitro* by treatment with caspase inhibitor, did not prevent N-BPs from inhibiting bone resorption. In contrast, the non-N-BPs failed to inhibit bone resorption when apoptosis was blocked. Therefore, it seems that apoptosis is required for inhibition of osteoclastic bone resorption by non-N-BPs but not by N-BPs (Halasy-Nagy *et al.* 2001; Alakangas *et al.* 2002). Hence, it appears that N-BPs inhibit bone resorption primarily by affecting the resorptive activity of osteoclasts, although osteoclast apoptosis may occur as a secondary effect.

The importance of prenylated proteins for osteoclast function has been confirmed by using specific inhibitors that prevent either protein farnesylation (FTI-277, an inhibitor of farnesyl transferase) or protein geranylgeranylation (GGTI-298, an inhibitor of geranylgeranyl transferase I) (Fig. 5). The inhibitory effect of N-BPs on bone resorption is likely to result largely from the loss of geranylgeranylated proteins rather than from the loss of farnesylated proteins in osteoclasts. Loss of farnesylated proteins in osteoclasts has little effect on the cytoskeleton or survival of osteoclasts or on bone

resorption. In contrast, loss of geranylgeranylated proteins causes disruption of actin rings, induces osteoclast apoptosis, and inhibits bone resorption (Coxon *et al.* 2000). Therefore, geranylgeranylated proteins, rather than farnesylated proteins, are fundamental to osteoclast function. In support of this hypothesis, addition of geranylgeraniol (a cell-permeable form of GGPP) rather than farnesol (a cell-permeable form of FPP) can overcome the inhibitory effect of the N-BPs on osteoclast formation and bone resorption (Fisher *et al.* 1999; van Beek *et al.* 1999b).
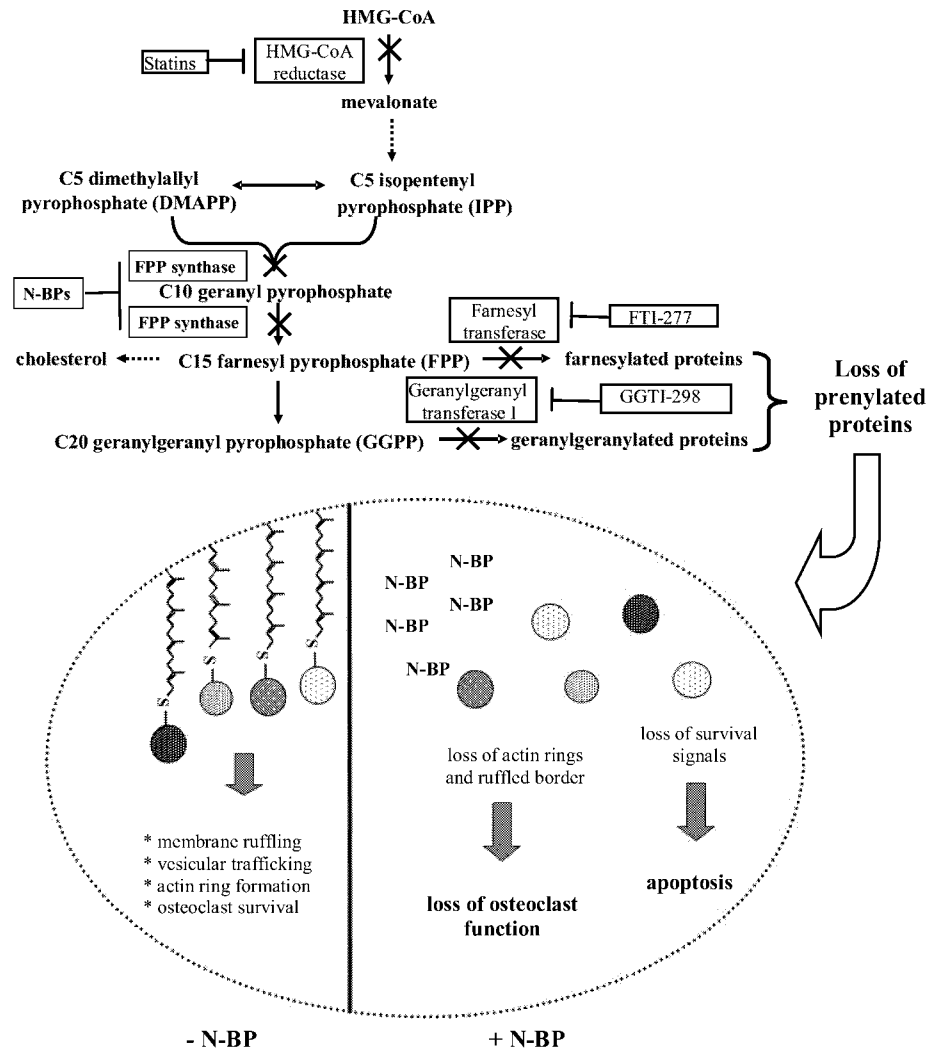
Fig. 5. N-BPs inhibit FPP synthase in the mevalonate pathway, thereby preventing the synthesis of FPP and GGPP required for protein prenylation. Loss of prenylated proteins, mainly geranylgeranylated ones, disrupts specialised features of the osteoclast required for bone resorption. Statins, FTI-277, and GGTI-298 also prevent protein prenylation, by inhibiting HMG-CoA reductase or farnesyl/geranylgeranyl transferases respectively (Rogers *et al.* 2000, with permission from John Wiley and Sons Inc).

2.4.4 Other mechanisms of action

As well as being incorporated into cytotoxic metabolites or inhibiting FPP synthase, some BPs appear to be weak inhibitors of the vacuolar-type ATP-dependent proton pump as well as blocking some hydrolytic enzymes, e.g., metalloproteases, phosphatases, and acid phosphohydrolases. Since bone resorption requires the acidic dissolution of bone mineral and proteolytic degradation of bone matrix proteins, these effects may also contribute to the overall inhibition of bone resorption. However, the effects on proteolytic enzymes and acidification do not account for the morphological changes of BP-treated osteoclasts, which are better explained by inhibition of FPP synthase and loss of prenylated small GTPases. BPs can also inhibit protein tyrosine phosphatases (PTPs), which are essential for both osteoclast formation and osteoclast resorptive activity. However, the lack of correlation between the ability of BPs to inhibit PTPases and their anti-resorptive potency indicates that inhibition of PTPs is not the major mechanism by which BPs inhibit bone resorption. Nevertheless, effects of BPs on PTPases and downstream signalling pathways in osteoclasts may contribute to the overall decreased ability of osteoclasts to resorb bone (Rogers 2004).

2.4.5 Summary

Bisphosphonates are currently the most important class of antiresorptive drugs used for the treatment of diseases with excess bone resorption. On the basis of their molecular mechanism of action, bisphosphonates can be divided into two pharmacological classes with distinct molecular mechanisms of action. Nitrogen-containing bisphosphonates (N-BPs) inhibit bone resorption and cause apoptosis of osteoclasts by preventing the modification of important signalling GTP-binding proteins with isoprenoid lipids. Non-nitrogen-containing bisphosphonates (non-N-BPs) do not inhibit protein isoprenylation but can be metabolically incorporated into ATP analogs that accumulate within osteoclasts, resulting in induction of osteoclast apoptosis by inhibiting the mitochondrial adenine nucleotide translocase (ANT).

2.5 Molecular basis for the major adverse effects of bisphosphonates

2.5.1 Acute phase response

An acute phase response (APR), typically related to an increase in the circulating levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) may develop in one in every three patients treated for the first time with N-BPs via the intravenous route. In addition to the increased levels of cytokines, APR is characterized by transient pyrexia.

Non-N-BPs do not induce APR (Adami *et al.* 1987; Schweitzer *et al.* 1995; Thiebaud *et al.* 1997). This is supported by the fact that clodronate and its metabolite have anti-inflammatory properties by inhibiting LPS-induced cytokine secretion, while alendronate (N-BP) increases cytokine secretion of macrophages *in vitro*, and thus, has pro-inflammatory effects (Makkonen *et al.* 1999). Additionally, clodronate (non-N-BP) has been found to inhibit the N-BP-induced inflammatory reaction *in vivo*, suggesting that combined administration of clodronate and N-BP could prevent the APR (Endo *et al.* 1999).

It was recently proposed that the molecular basis of APR is connected to the structural similarity of N-BPs and isoprenoid lipids, such as isopentenyl pyrophosphate (IPP). N-BPs could directly activate and stimulate proliferation of γ,δ-T cells by acting as non-peptide phosphoantigens, such as IPP, a compound known to be a γ,δ-T cell agonist (Tanaka *et al.* 1995; Thompson and Rogers 2004). It is also conceivable that stimulation of γ,δ-T cells by N-BPs is an indirect consequence of the accumulation and release of IPP upstream of FPP synthase. This hypothesis is supported by the findings that N-BP-treated tumor cells activate γ,δ-T cells due to the accumulation of isoprenoid lipids, such as IPP, in the mevalonate pathway (Gober *et al.* 2003). Additionally, the potency of N-BPs to inhibit FPP synthase is in accordance to their potency to activate γ,δ-T cells and N-BP-induced γ,δ-T cell proliferation and activation was prevented by statins, which inhibit HMG-CoA reductase upstream of FPP synthase and prevent the synthesis of IPP (Gober *et al.* 2003; Thompson and Rogers 2004). Hence, these observations indicate that the APR to N-BPs is caused by indirect activation of γ,δ-T cells as a consequence of inhibition of the mevalonate pathway and subsequent accumulation of isoprenoid lipids, such as IPP, in circulating blood mononuclear cells.

2.5.2 Gastrointestinal effects

Oral administration of bisphosphonates, especially N-BPs, is accompanied by esophageal and gastrointestinal disturbances, such as nausea, dyspepsia, vomiting, gastric pain and diarrhea and even severe esophageal erosions and ulcerations. Non-N-BPs can also induce gastrointestinal side-effects, however, these tend to be mostly minor (Fleisch 2000). It has been shown that concentrations of N-BPs, that are similar to the concentrations that could be achieved in the GI tract, inhibited the proliferation of and induced apoptosis in Caco-2 epithelial cells (which are widely used as an *in vitro* model of human intestinal epithelial permeability). These effects were likely due to the prevention of protein prenylation after the inhibition of FPP synthase (Suri *et al.* 2001). Unlike the N-BPs, non-N-BPs had only a minor effect on Caco-2 apoptosis. Non-N-BPs had no effect on the protein prenylation in Caco-2 cells, which explained why these BPs did not cause apoptosis of Caco-2 cells (Suri *et al.* 2001). However, non-N-BPs can be

metabolised intracellularly to AppCp-type analogs, which do appear to cause apoptosis in osteoclasts (Frith *et al.* 2001). The cytoxic effect depends on the degree of uptake of BP, and considerable differences exist between cells of high and low endocytic capacity. Therefore, one possibility is that owing to their low endocytic capacity, Caco-2 cells do not internalize non-N-BPs efficiently, and therefore do not accumulate sufficient intracellular concentrations of the metabolite to affect cell function (Suri et al. 2001). It is therefore possible, that a similar situation occurs in the GI epithelia cells in vivo. These cells are protected possibly due to the low uptake of the drug, which would explain the low frequency of gastrointestinal side-effects caused by non-N-BPs.

3   AIMS OF THE STUDY

The general aim of this study was to investigate the intracellular metabolism and molecular mechanisms of action of bisphosphonates. The specific aims were as follows:

1.   To develop a selective and sensitive analytical method for the quantitation of the adenine nucleotide-containing metabolites of BPs in cell extracts.

2.   To study the cellular uptake of clodronate (non-N-BP) and the kinetics of its metabolism into $AppCCl_2p$ in macrophages.

3.   To investigate the cellular uptake and metabolism of clodronate and its derivatives in Caco-2 epithelial cells as a model in which to study the mechanism of gastrointestinal side-effects.

4.   To characterise the N-BP-induced accumulation of a new ATP analog (ApppI) and its role in explaining the molecular mechanism of action of N-BPs in macrophages and osteoclasts.

4   MATERIALS AND METHODS

4.1   Chemicals (I-IV)

Unlabelled and ^{14}C-labelled clodronate were kindly provided by Leiras Pharmaceutical Co., (Turku, Finland), zoledronate by Novartis Pharma (Basel, Switzerland), alendronate by Merck Sharp and Dohme (Rahway, NJ, USA), ibandronate, and risedronate by Procter and Gamble Pharmaceuticals (Cincinnati, OH). Triamide-, dianhydride, and triPOM-clodronate were synthesised in the Department of Chemistry, University of Kuopio according to previously published procedures (Niemi *et al.* 1998, 1999; Ahlmark *et al.* 1999). The metabolite of clodronate (AppCCl₂p) was kindly provided by Prof. A. Azhayev (University of Kuopio, Finland) and Prof. G. M. Blackburn (University of Sheffield, UK). ApppI was synthesised in the Department of Chemistry as described in the original publication **(IV)**. Other chemicals were obtained from commercial suppliers and used as received.

4.2   Analysis of AppCCl₂p, IPP, and ApppI using HPLC-MS-ESI (I-IV)

4.2.1   HPLC conditions

On-line HPLC-ESI-MS measurements were carried out with a Rheos 4000 (Flux Instruments, Danderyd, Sweden), Ultimate (LC Packings, Netherlands) or Surveyor (Thermo Electron Corporation, San Jose, CA, USA) pump and a Rheodyne 7725 (Cotati, CA, USA), Famos (LC Packings) or Surveyor (Thermo Electron Corporation) autosampler with a 20-50 μl loop. The reversed phase column was a Genesis C₁₈ (50x2 mm) (Jones Chromatography, Lakewood, CO, USA), which was eluted with a mobile phase at a flow-rate of 100-200 μl/min.The eluents were 20 mM DMHA formate with the pH adjusted to 7.0 with formic acid and 50-80 % methanol containing 2 mM DMHA formate, pH 7.0. The HPLC gradient was from 0 % to 50-80 % methanol, while the buffer concentration was decreased from 20 mM to 2 mM. An appropriate injection volume, flow-rate of mobile phase, concentration of methanol, and timetable for the gradient were chosen to achieve a good retention time and ratio of signal/noise.

4.2.2   Measurement of AppCCl₂p, IPP, and ApppI using tandem mass spectrometry

After HPLC separation, negative ion mass spectra for AppCCl₂p, IPP, and ApppI were acquired using an LCQ or LTQ quadrupole ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Electron Corporation, San Jose,

CA, USA). The total eluent flow of 100-200 µl/min was directed to the ESI source. The samples were redissolved in 100-200 µl of 40 mM DMHA formate or water containing AppCp as internal standard. Negative ion ESI and selective reaction monitoring (SRM) was used for analysis of the compounds in the sample. The quantitation was based on fragment ions at m/z 225 and 227 obtained by MS/MS of the molecular ion of AppCCl$_2$p at m/z 572 and 574 (^{35}Cl & ^{37}Cl). The fragment ions of IPP, ApppI, and AppCp at m/z 159, 408, and 406 (respectively) were obtained by MS/MS of the deprotonated molecules at m/z 245, 574, and 504 (respectively). The standard curve was created by spiking extracts from untreated cells with synthesised AppCCl$_2$p, IPP or ApppI. The concentrations of the samples were determined using the peak areas of the SRM chromatograms and the standard curve.

4.2.3 Sample preparation

After treatment, the cells were scraped from the wells, centrifuged (220g, 5min) and washed in PBS. For the acetonitrile (ACN) extraction, ACN was added to cell pellets to precipitate the macromolecules; water was added within 2 min to extract the cellular content (300 µl of ACN and 200 µl of water) (Au *et al.* 1989). The soluble and precipitated fractions were separated by centrifugation (13000g, 1min). The soluble supernatant extract was transferred within 10 min to a fresh eppendorf tube. The ACN/water extract were dried down in a vacuum centrifuge and then stored at -20°C until mass spectrometry analysis.

4.3 Cell culture (I-IV)

The experiments were performed using human intestinal epithelial Caco-2 **(III)**, rat C6 glioma **(IV)**, murine RAW 264 **(I, II)** and J774 macrophage **(IV)** secondary cell lines and primary osteoclasts **(IV)**, which were grown as described in the original publications **(I-IV)**. Primary osteoclasts were isolated from 1- to 2-day old newborn rats after decapitation. Caco-2 cells originate from a human colon adenocarcinoma and in cell culture they spontaneously differentiate with the characteristics of human intestinal epithelia. Caco-2 cells are widely used as an *in vitro* model of human intestinal epithelial permeability (Hidalgo *et al.* 1989; Audus *et al.* 1990).

4.4 Liposome preparation (I, II, IV)

Liposome encapsulation was used to enhance the cellular uptake and to prevent enzymatic decomposition of the compounds **(I, II, IV)**. Negatively charged liposomes are more efficient for cellular drug delivery than neutral liposomes (Mönkkönen *et al.*

1994a), and thus, the negatively charged phospholipid DSPG was chosen for the liposome preparation. The liposomes contained phospholipid and cholesterol in a molar ratio of 2:1. The compounds were encapsulated in liposomes by reverse-phase evaporation, as previously described (Mönkkönen *et al.* 1994b). The concentrations of zoledronate, clodronate, ApppI, and AppCCl$_2$p were measured spectrophotometrically; the concentration of ^{14}C-labeled clodronate was measured in a liquid scintillation counter LKB-Wallac RackBeta (Wallac Co, Turku, Finland). The lipid content of the liposomes was determined by a phosphorus assay (Mönkkönen *et al.* 1994b), and the size distribution of liposomes was analysed by Nicomp Zeta Potential/Particle Sizer (model 380 XLS, NicompTM, Santa Barbara, CA). Nonloaded liposomes were used as a control.

4.5   Cellular uptake studies in RAW 264 macrophages (II) and Caco-2 cells (III)

For studies on the cellular uptake and metabolism of clodronate and the influence of clodronate metabolism on the intracellular ATP concentration, the RAW 264 cells were seeded into 6-well plates (Nunc, Roskilde, Denmark) at a density of 5×10^6 cells/well and allowed to adhere for 2 hours. The medium was changed just before the treatments. The cellular uptake of clodronate was measured using ^{14}C-labelled clodronate. After treatment with liposome-encapsulated or free ^{14}C-labelled clodronate, medium was collected and the macrophages were washed five times with PBS. The cells were then extracted with 1 ml 20 % SDS-buffer (1:1 milli-Q water/DMF, pH 4.6) and scraped off from the wells. The radioactivity of medium, washes, and cell extract were measured by liquid scintillation counting (Rackbeta 1218, LKB Wallac, Turku, Finland). The intracellular concentration of clodronate was determined by comparing the radioactivity of medium, washes, and cell extracts. The cellular volume was estimated as 0.408 mm^3 per million cells. This was determined by measuring the approximate diameter of macrophages (9.2 μm) using a light microscope with an eyepiece graticule.

For studies on the cellular uptake and metabolism of clodronate, Caco-2 cells were seeded into 6-well tissue culture plates (Costar, Cambridge, MA) at a density of 8×10^4 cells/well and grown in culture medium for 5 days. Medium was replaced with 2.5 ml of fresh solution every second day. ^{14}C-clodronate uptake and total protein content were determined from separate wells. Confluent cultures were treated with free ^{14}C-clodronate (uptake studies) or unlabelled clodronate (protein assay) in serum-free medium (pH 7.4). After treatment, the cells were rinsed five times with PBS. The cells were then scraped from the wells and extracted using acetonitrile (300 μl of ACN and 200 μl of water). To determine the intracellular amount of clodronate, the radioactivity of the medium, washes, and the cell extracts were analysed by 1450 MicroBeta Trilux LSC (Wallac Co., Turku, Finland). Prior to the protein assay, the soluble and

precipitated fractions were separated by centrifugation (13 000 g, 1 min). The soluble supernatant was removed within 10 minutes and the pellet was digested with 1 ml of 1 M NaOH at 60°C for 2 hours and analysed for total protein content by a modified Bradford procedure (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) (Sigma Diagnostics, St. Louis, MO) as the standard. Absorbances (λ = 595 nm) were measured by Automated Microplate Reader ELx800UV (Bio-Tek Instruments, Inc., Winooski, VT). The intracellular amount of clodronate was reported as molar amount of drug / mg protein.

4.6   Studies of clodronate metabolism in RAW 264 macrophages (II) and Caco-2 cells (III)

After treatment with free or liposome-encapsulated clodronate, the RAW 264 macrophage cells were scraped off the wells, counted using a Coulter counter, centrifuged (5 minutes, 220g), and washed in PBS. Extracts from the cells were prepared using acetonitrile and the molar amount of clodronate metabolite (AppCCl$_2$p) was determined in cell extracts by using HPLC-ESI-MS as described above. The intracellular concentration of AppCCl$_2$p was then estimated assuming a cellular volume of 0.408 mm^3 per million cells.

Metabolism studies in Caco-2 cells were performed by using clodronate, Ca-clodronate or the lipophilic derivatives of clodronate (triamide, dianhydride or triPOM-clodronate) (III: Table 1). Extracts from Caco-2 cells were prepared and the molar amount of AppCCl$_2$p was analysed in the same way as RAW 264 macrophages, except after extraction, the supernatants from three separate wells of Caco-2 cells were combined to establish a single sample. The pellet was digested with 1 ml of 1 M NaOH at 60°C for 2 hours and analysed for total protein content as described above. The intracellular amount of clodronate metabolite (AppCCl$_2$p) was reported as the molar amount of AppCCl$_2$p / mg protein.

4.7   Measurement of intracellular ATP concentration and cytokine release in RAW 264 macrophages (II)

After treatment with free or liposome-encapsulated clodronate in RAW 264 macrophages, the intracellular ATP concentration was measured with a Wallac Victor^2 1420 luminometer and Multilabel counter (Wallac Co, Turku, Finland) using a bioluminescent luciferin-luciferase assay and the estimated cellular volume of 0.408 mm^3 per million cells. All the assay reagents were purchased as a kit (ATP Monitoring Kit, Labsystems Oy, Helsinki, Finland) and prepared according to the manufacturer's instructions. Preliminary tests revealed that the presence of AppCCl$_2$p did not affect the

luciferase assay and thus the method was reliable for measuring ATP concentrations despite the intracellular accumulation of $AppCCl_2p$.

In the cytokine experiments, the cells were dispensed into 96-well plates (Nunc, Roskilde, Denmark), at the density of $2x10^5$ cells/well. After 2 hours, the nonadherent cells were removed and fresh serum-free medium was added to the wells before the treatments. The modulation of cytokine secretion by clodronate was assessed in RAW 264 cells as described previously (Pennanen *et al.* 1995a). RAW 264 cells were treated with liposome-encapsulated or free clodronate for 1-24 hours, then the cells were washed free of drugs and cytokine secretion was stimulated by addition of 10 µg/ml lipopolysaccharide (LPS) (E. coli, serotype 0127:B8, Sigma) for 24 hours. After LPS treatment, the cell free supernatants were collected and assayed for IL-6 and TNFα by time-resolved fluoroimmunoassay (TRFIA) (Pennanen *et al.* 1995b).

4.8   Proliferation and viability studies in Caco-2 cells (III)

In the proliferation experiments, Caco-2 cells were seeded at a density of $1x10^4$ cells in 96-well plates (Costar, Cambridge, MA). Cells were allowed to adhere overnight and the next day exposed to free clodronate or PBS (control). The cell growth was assayed 48 hours later using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) assay as previously described (Mosmann1983; Hansen *et al.* 1989).

In the viability experiments, Caco-2 cells were seeded at a density of $1x10^4$ cells in 96-well plates and grown in culture medium for 5-7 days. Medium was replaced with 100 µl of fresh solution every second day. Non-proliferating, confluent cultures were treated with free clodronate for 3 h, 6 h or 24 h. In the viability experiments of Ca-, triamide- and dianhydride-clodronate, the cells were exposed with appropriate compounds for 24 h. The viability of Caco-2 cells was determined by the MTT method (Mosmann1983; Hansen *et al.* 1989).

4.9   ApppI production (IV)

N-BP-induced ApppI production was studied in J774 macrophages, C6 glioma cells, and primary osteoclasts. More specifically, the macrophage cell line was used to determine the potency of different BPs to promote the production of ApppI, whereas the glioma cells were utilized for obtaining an NMR spectrum of biological ApppI. The primary osteoclasts were used due to their physiologically relevant role as cellular targets of BPs. The molar amount of ApppI was determined in macrophage and osteoclast cell extracts by HPLC negative ion electrospray ionization mass spectrometry (HPLC-ESI-MS) as described above.

For studies on ApppI production in J774 macrophages, the cells were seeded into 6-well plates (Nunc, Roskilde, Denmark) at a density of $3x10^6$ cells/well and left to adhere for 4 hours, then treated with different free BPs for 24 hours. After treatment, the sample preparation and extraction procedures were similar as in the studies of clodronate metabolism. The intracellular amount of ApppI was reported as a molar amount of ApppI / mg protein.

The ApppI sample for NMR analysis was obtained using C6 glioma cells. The cells were seeded into three 75 cm^2 flasks at a density of $1.5x10^7$ cells/flask and incubated for 3 days (to confluence) prior to N-BP treatment. Extracts from cells were prepared using acetonitrile. Extracts from 3 separate flasks were combined to establish a single sample. The ^1H NMR spectrum of ApppI was acquired after purification of the sample by anion exchange chromatography in a SAX column using ammonium acetate as an eluent.

Primary osteoclasts were isolated from 1- to 2-day old newborn rats after decapitation. Osteoclasts were cultured on bone slices which were prepared from frozen bovine cortical bone shafts. Bone was cut with a diamond saw into 200 μm thick slices, sterilized with sonication and given a brief rinse in 70% ethanol. Prior to culture, bovine bone slices were coated with N-BP. Control bone slices were rinsed with 0.9% saline. Subsequently the bovine slices were transferred into 24-well plates containing fresh 37°C medium. Procedures for osteoclast culture have been described previously in detail by Boyde *et al.* (1984) and Chambers *et al.* (1984). After 24 hours, the cultured cells were scraped off the bone slices and washed in PBS and extracted using acetonitrile.

4.10   ADP/ATP translocase assay (IV)

Rat liver mitochondria were isolated by standard differential centrifugation and suspended in 0.25 M sucrose/0.5 mM EDTA/5 mM Tris, pH 7.4. The ADP/ATP translocator activity was assayed in the "forward" direction as described by Paulson and Shug (1984). The reaction which took place at 0°C in 100 mM KCl, 40 mM Tris, 1 mM EDTA, pH 7.4, was initiated by the addition of [8-^{14}C] ATP and stopped after 10, 20, 30 or 60 s with 50 μM atractyloside. The mitochondria were then collected and washed in the same medium in the presence of 50 μM atractyloside, and solubilized in 0.2% sodium dodecylsulphate before the radioactivity was measured by liquid scintillation counting.

4.11   Osteoclast apoptosis assay (IV)

Osteoclasts were cultured similarly as for the apoptosis assay conducted in the studies of ApppI production described above, except that the attached osteoclasts were

not treated with bone-bound drug but with different concentrations of liposome-encapsulated compounds instead. After a 24h treatment period, the cells were fixed with 3% paraformaldehyde and 2% sucrose for 5 minutes, and stained for tartrate resistant acid phosphatase (TRAP) using a histochemical kit (Sigma-Aldrich). TRAP positive giant cells with three or more nuclei were counted as osteoclasts. In order to define the number of apoptotic osteoclasts, the nuclei were stained with Hoechst 33258. Osteoclasts were designated as being apoptotic if they showed strong staining for TRAP, a hallmark of cytoplasmic contraction, chromatin condensation and nuclear fragmentation, as previously described (Selander *et al*. 1996; Hughes *et al*. 1995).

4.12 Statistical analysis (II, IV)

The Mann-Whitney U-test was used to compare the effect of clodronate on intracellular ATP concentrations in RAW 264 macrophages **(II)**. The osteoclast apoptosis data were tested by using the Mann-Whitney U test with Bonferroni correction **(IV)**.

5 RESULTS AND DISCUSSION

5.1 Analysis of adenine nucleotide containing analogs (I)

Clodronate metabolite (AppCCl$_2$p), N-BP-induced ATP analog (ApppI) and isopentenyl pyrophosphate (IPP) were analysed in the acetonitrile-extracted cells by using an ion-pairing HPLC method that is compatible with negative ion electrospray ionization mass spectrometry (ESI-MS). The analytical method for these compounds was developed by using AppCCl$_2$p (I). The ATP analogs are very hydrophilic compounds and therefore the use of an ion-pair is necessary to retain these compounds in reversed-phase columns. Four different ion-pairing agents were tested; ammonia, triethylamine (TEA), tripropylamine (TPA), and dimethylhexylamine (DMHA). Our results showed that good retention times and high signal intensity were obtained by using DMHA (I: Fig. 2). Therefore, DMHA was chosen as the ion-pairing agent. Methyleneadenosine 5'-triphosphate (AppCp) was chosen as the internal standard. The compounds were eluted from the column in 50-80 % methanol and 2 mM DMHA, which has a favorable properties for the ionization process (I: Fig. 4). The analysis and extraction methods were reproducible in the quantitation of the ATP analogs in cell extracts.

The MS/MS spectra of AppCCl$_2$p, AppCp, IPP and ApppI are shown in Figs. 6A, B, C, and D, respectively. The quantitation of these compounds was based on fragment ions at m/z 225 and 227 (for AppCCl$_2$p), m/z 406 (for AppCp), m/z 159 (for IPP) and m/z 408 (for ApppI). The highest signal intensities were obtained by using these fragment ions.

Ion-pairing HPLC-ESI-MS is a sensitive method for the quantitation of the ATP analogs in cell extracts. Acetonitrile extraction is simple and very suitable for HPLC analysis. In conclusion, these techniques can be applied to study the metabolism of bisphosphonates *in vitro* and *in vivo*.
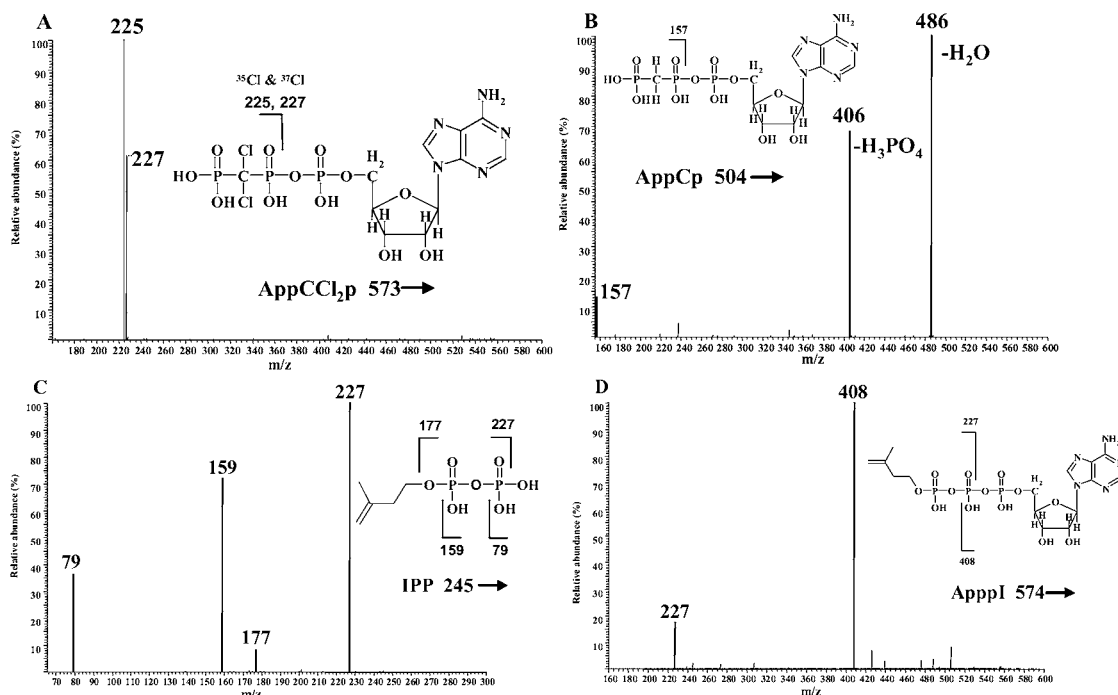
Fig. 6. The MS/MS spectra of AppCCl₂p (A), AppCp (B), IPP (C), and ApppI (D).

5.2 Cellular uptake and metabolism of clodronate to AppCCl₂p and its effect on some cellular functions *in vitro* (II, III)

5.2.1 Cellular uptake and metabolism of clodronate in macrophages (II)

Although BPs are likely to be membrane-impermeable due to their high hydrophilicity, they can be internalised by cells. Studies with slime mould amoebae have demonstrated that the cellular uptake of free BPs occurs by fluid-phase endocytosis (Rogers *et al.* 1997) and this is the likely route by which osteoclasts internalise BPs from the resorption lacunae (Salo *et al.* 1997; Nesbitt and Horton 1997; Stenbeck *et al.* 2000). In support of this, it has been recently confirmed in osteoclasts as well as in macrophages using a fluorescently-labelled analog of alendronate (AF-ALN) and confocal microscopy that BPs are internalised initially by fluid-phase endocytosis, and not by adsorptive or receptor-mediated endocytosis (Thompson *et al.* 2005). Unfortunately, the molecular size of AF-ALN is quite bulky due to the fluorecent-label used in this study, which can affect the cellular uptake mechanism of AF-ALN compared to the parent alendronate (ALN). Fluid-phase endocytosis (pinocytosis) is a passive nonspecific uptake of extracellular molecules into an endocytic vesicle, and the obtained intracellular concentration of a given molecule is proportional to the molecular concentration in the extracellular solution (Darnell et al. 1990).

In this study, both free and liposome-encapsulated clodronate were avidly taken up by macrophages. After 12 hours of exposure to 1 mM free clodronate, the intracellular concentration reached a plateau of about 1.3 mM (**II**: Fig. 2). This result supports the view that the cellular uptake of free BPs occurs via fluid-phase endocytosis, because the intracellular and extracellular concentrations of clodronate were approximately in equilibrium. Cellular uptake of liposomal drugs occurs by phagocytosis in macrophages, which as highly phagocytic cells avidly take up liposomal clodronate (Mönkkönen and Heath 1993; van Rooijen and Sanders 1994) (II: Fig. 2). In comparison to fluid-phase endocytosis, phagocytosis is more effective cellular uptake mechanism (Darnell et al. 1990), which allows higher intracellular concentrations of clodronate to be achieved as compared with extracellular medium. This was demonsrated by exposing the cells for 24 hours with $30\mu M$ of liposome-encapsulated clodronate, which resulted in 4 mM intracellular concentration of clodronate (**II**: Fig. 2).

BPs were initially considered to be metabolically inert. However, Rogers *et al.* (1992, 1994) and others (Pelorgeas *et al.* 1992) have shown that clodronate and other non-N-BPs that closely resemble PPi in structure can be metabolised by slime mold amoebae *Dictyostelium discoideum* into non-hydrolysable, adenine-containing analogs of adenosine triphosphate (ATP) (Fig. 4 in Review of Literature). It was demonstrated that clodronate is also metabolised *in vitro* by intact mammalian cells, such as macrophages (Auriola *et al.* 1997, Frith *et al.* 1997; Benford *et al.* 1999) and *in vivo* by osteoclasts (Frith *et al.* 2001). Although earlier data strongly indicated that clodronate and other non-N-BPs are metabolised into ATP analogs and these metabolites are responsible for the cellular effects of non-N-BPs (Frith *et al.* 1997; Makkonen *et al.* 1999), nothing was known on the time course and efficacy of non-N-BP metabolism in the cells. This question was addressed in this study by investigating the intracellular accumulation of the clodronate metabolite (AppCCl$_2$p) in RAW 264 macrophages. These cells internalised free and liposome-encapsulated clodronate very efficiently, which was then metabolised to AppCCl$_2$p (**II**: Fig. 2). The metabolite was observable already after 1 hour of exposure of the cells to liposomal or free clodronate. The concentration of AppCCl$_2$p gradually increased during the first 12 hours of clodronate treatment, then reached a plateau. The maximum concentration of AppCCl$_2$p, which accumulated intracellularly in macrophages, was about 0.8 mM after treatment with either liposomal or free clodronate (**II**: Fig. 2). Thus, the amount of clodronate converted into AppCCl$_2$p corresponded to 30 % and 55 % of total clodronate taken up by the cells as liposomal or free drug, respectively. The AppCCl$_2$p concentration did not increase in the cell after 12 hours of treatment, although the intracellular uptake of liposomal drug gradually increased up to 24 hours. This suggests that the enzyme reaction converting clodronate to AppCCl$_2$p (Fig. 4 in Review of Literature) may be a rate-limiting step. In conclusion, the high intracellular concentrations of clodronate

metabolite support the idea that the active form of clodronate is its intracellular AppCCl$_2$p metabolite.

5.2.2 Influence of clodronate metabolism on cytokine secretion and intracellular ATP concentration in macrophages (II)

It has been previously shown that clodronate inhibits the LPS-induced release of proinflammatory cytokines and NO from RAW 264 macrophages at non-cytotoxic concentrations (Pennanen *et al.* 1995; Makkonen *et al.* 1996). The metabolite of clodronate, AppCCl$_2$p, when delivered to macrophages within liposomes, had similar inhibitory effects on cytokine and NO release as clodronate itself (Makkonen et al., 1999). Thus, the ability of clodronate to prevent the release of proinflammatory cytokines from macrophages is likely to be the result of intracellular accumulation of AppCCl$_2$p. In support of this, the formation of AppCCl$_2$p occurred concomitantly with the inhibition of cytokine release (**II**: Fig. 4). Compared to free clodronate, exposure of the cells to liposome-encapsulated clodronate led to faster intracellular uptake of the drug (**II**: Fig. 2), and consequently, faster accumulation of the metabolite (**II**: Fig. 2) and faster inhibition of the cytokine secretion (**II**: Fig. 4), further supporting the hypothesis that the metabolite of clodronate is responsible for the anti-inflammatory properties of this bisphosphonate.

The effects of AppCCl$_2$p on cytokine secretion do not appear to be a consequence of the impairment of energy metabolism in the cells, because the intracellular ATP concentration did not drastically decrease in clodronate treated cells during the first 12 hours of exposure (**II**: Fig. 3), although the intracellular concentration of AppCCl$_2$p reached its peak level and cytokine secretion was already strongly affected by this time (**II**: Fig. 4). This suggests that the intracellular formation of AppCCl$_2$p does not affect the ability of the cells to synthesise ATP. The intracellular accumulation of AppCCl$_2$p appears eventually to cause a decrease in ATP levels after 24 hours (**II**: Fig. 3), perhaps as a consequence of cell death (Frith *et al.* 1997).

5.2.3 Cellular uptake and metabolism of clodronate and its derivatives in Caco-2 epithelial cells (III)

The gastrointestinal tract is the most common route for BPs administration. Oral administration of bisphosphonates, especially N-BPs, is accompanied by esophageal and gastrointestinal disturbances, such as nausea, dyspepsia, vomiting, gastric pain and diarrhea and even severe esophageal erosions and ulcerations (Fleisch 2000). It appears that apoptosis of epithelial cells and/or inhibition of proliferation of non-differentiated epithelial cells could contribute to the mechanism of esophagitis and ulceration in the

GI tract caused by N-BPs, since normal growth and restitution of cells in the gut mucosa would likely be affected. Actually, it has been shown that N-BPs inhibit the proliferation and cause apoptosis of Caco-2 epithelial cells, which is likely due to the prevention of protein prenylation by inhibiting FPP synthase (Suri *et al.* 2001).

Unlike the N-BPs, non-N-BPs do not cause any serious gastrointestinal side-effects (Fleisch 2000) and had no effect on the protein prenylation in Caco-2 cells and thus had only minor effects on Caco-2 apoptosis (Suri *et al.* 2001). However, non-N-BPs can be metabolised intracellularly to the cytotoxic AppCp-type analogs in osteoclasts (Frith *et al.* 2001) and macrophages (Auriola *et al.* 1997; Benford *et al.* 1999; Frith *et al.* 1997). Thus, one possibility is that Caco-2 cells do not internalise non-N-BP efficiently, and therefore do not accumulate sufficient intracellular concentrations of the metabolite that it would impair cellular functions (Suri *et al.* 2001). In order to study the reason for the low frequency of gastrointestinal side-effects caused by clodronate, it was of interest to explore its effects on the proliferation and viability, as well as its cellular uptake and metabolism in the Caco-2 cell model. The maximum amount of clodronate taken up by the cells was 4.2 and 11.5 nmol per milligrams protein, when the cells were treated with clinically relevant concentrations of 1 and 10 mM free clodronate, respectively, for 24 hours (**III**: Fig. 3). The uptake corresponded to only 0.04 % of extracellular drug after treatment with 1 mM clodronate for 24 hours. When macrophages were exposed to 1 mM clodronate for 24 hours, the uptake was 0.2 % of extracellular drug concentration, which is five times more than with Caco-2 cells. The clodronate metabolite could be detected in Caco-2 cell extracts after 3 hours of exposure with 1 mM clodronate (**III**: Fig. 3A), whereas the metabolite was present within 1 hour in macrophages (**II**: Fig. 2). The maximum amount of AppCCl$_2$p was 2.9 and 10.0 nmol/mg protein, when Caco-2 cells were exposed to 1 and 10 mM clodronate, respectively, for 24 hours (**III**: Fig. 3). Additionally, clodronate had little effect on the proliferation, or the viability of Caco-2 cells (**III**: Figs. 1 and 2). These results strongly suggest that owing to their low endocytic capacity, Caco-2 epithelial cells do not accumulate sufficient intracellular concentrations of metabolite to affect cell function. This may explain the low frequency of gastrointestinal side-effects caused by clodronate and other non-N-BPs.

BPs are poorly absorbed in humans, oral bioavailability being less than 3 %. The low bioavailability is likely to be attributable to the highly hydrophilic nature of bisphosphonates, which prevents the transcellular transport across intestinal epithelium and favors the paracellular route (Twiss *et al.* 1994; Lin 1996; Raiman *et al.* 2001). However, paracellular transport is hindered because of the tight junctions between epithelial cells, and the relatively small total area of pores compared to the area of the transcellular route. One strategy for improving oral absorption of these types of molecules would be to develop more lipophilic derivatives. It has been speculated that it is possible to change the intestinal absorption mechanism of bisphosphonates from

paracellular to a transcellular pathway by creating more lipophilic derivatives (Raiman *et al.* 2001). The capacities of clodronate, triamide-, dianhydride, and triPOM-clodronate to be metabolised to AppCCl$_2$p and their impact on cell viability were tested in Caco-2 cells. Dianhydride- and triPOM-clodronate were converted into the AppCCl$_2$p-metabolite in Caco-2 cells more than the parent compound clodronate (**III**: Fig. 5). Dianhydride-clodronate also affected the viability of cells more than clodronate (**III**: Fig. 4). Triamide-clodronate was not metabolised to AppCCl$_2$p, which is due to its high stability to enzymatic and chemical hydrolysis (**III**: Table 2, Niemi et al. 1998). Therefore no intact clodronate was released into cells to undergo further metabolism to AppCCl$_2$p. Instead, dianhydride- and triPOM-clodronate, compounds which are susceptible to chemical and enzymatic hydrolysis (**III**: Table 2, Niemi et al. 1999; Ahlmark et al. 1999), do eventually release the active parent drug (clodronate) and this is subsequently metabolised to AppCCl$_2$p (**III**: Fig. 5). The more efficient metabolism of these compounds to AppCCl$_2$p is possibly caused by the higher capacity of the cells to take up the lipophilic derivative than the parent drug. This can, however, lead also to increased toxicity of the derivatives as more intracellular clodronate would be available for metabolism to AppCCl$_2$p. Therefore, when lipophilic derivatives of clodronate are developed for better bioavailibility, the possibility of a simultaneous increase in gastrointestinal side-effects needs to be taken into account.

5.3   Identification of ApppI induced by N-BPs (IV)

It has been previously shown that N-BPs are not metabolised to ATP-analogs (Auriola *et al.* 1997; Benford *et al.* 1999). In this study it was observed that although N-BPs themselves are not metabolised into AppCp-type metabolites, cells treated with N-BPs do synthetise a new type of ATP analog. The IUPAC name for this molecule is triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester, abbreviated to 'ApppI'. It was detected in various cell lines *in vitro*, such as in osteoclasts, macrophages, and glioma cells. Mass spectrometry was used to analyse ApppI in N-BP treated cells and the structure of ApppI was ascertained by using nuclear magnetic resonance (NMR). The inhibition of FPP synthase by N-BPs leads to the accumulation of isopentenyl pyrophosphate (IPP), which is then converted to ApppI, presumably via aminoacyl-tRNA-synthetases (**IV**: Figs. 1 and 7), the same enzyme that catalyses the formation of AppCp-type metabolites from non-N-BPs (Rogers et al., 1996). Since IPP also resembles PPi in structure, the reverse reaction can occur with IPP in place of PPi, leading to the formation of ApppI. Thus, ApppI does not contain the actual N-BP-molecule in its structure, unlike the AppCp-type metabolites of non-N-BPs (**IV**: Fig. 7). Furthermore, non-N-BPs, such as clodronate, do not inhibit FPP synthase (van Beek *et al.* 1999a) and therefore do not induce ApppI formation (**IV**: Fig. 4).

5.4 ApppI production in macrophages (IV)

According to Dunford *et al.* (2001), the N-BPs inhibit FPP synthase in the following order of potency; zoledronate > risedronate > ibandronate > alendronate. Clodronate (non-N-BP) does not have any significant inhibitory effect on the FPP synthase activity. To determine the potency of BPs in the promotion of ApppI production of ApppI, J774 macrophages were treated with various BPs for 24 hours. After the drug treatment, the ApppI contents from the cell lysates were measured by using HPLC-ESI-MS. This experiment indicated that ApppI production correlated well with the capacity of N-BPs to inhibit FPP synthase (**IV**: Fig. 4). Other inhibitors of the mevalonate pathway, such as lovastatin (inhibitor of HMG-CoA reductase), FTI-227 (inhibitor of farnesyl transferase), and GGTI-298 (inhibitor of geranylgeranyl transferase I), did not evoke ApppI production in macrophages. However, lovastatin (which inhibits HMG-CoA reductase upstream of FPP synthase) decreased the IPP accumulation and the ApppI production induced by zoledronate ($p<0.001$) (**IV**: Fig. 6a). In addition, ApppI production correlated well with the increase in the IPP concentration in macrophages ($R = 0.93$) (**IV**: Fig. 6b). Taken together, these data strongly suggest that ApppI production is a unique effect of N-BPs and results from inhibition of FPP synthase in the mevalonate pathway and the subsequent accumulation of IPP (**IV**: Fig.1).

These results also support the role of IPP in acute phase response (APR) induced by N-BPs, because it has been suggested that stimulation of γ,δ-T cells by N-BPs is an indirect consequence of the accumulation and release of IPP upstream of FPP synthase and the potency of N-BPs to inhibit FPP synthase is in accordance to their potency to activate γ,δ-T cells, and thus to evoke an APR. Additionally, N-BP-induced γ,δ-T cell proliferation and activation was prevented by statins (Gober *et al.* 2003; Thompson and Rogers 2004). The role of ApppI in APR remains yet unclear.

5.5 The role of ApppI in the molecular mechanism of action of N-BPs (IV)

5.5.1 ApppI inhibits mitochondrial adenine nucleotide translocase (ANT) (IV)

It has been previously shown that the clodronate metabolite (AppCCl₂p) inhibits mitochondrial oxygen consumption by a mechanism that involves competitive inhibition of the ADP/ATP translocase (ANT) (Lehenkari *et al.* 2002). To determine whether ApppI could modify the action of ANT, ApppI was synthesised and its effect on ANT was tested in isolated rat liver mitochondria after treatment with 0-100 μM ApppI for 10-60 s. The dose-dependent inhibition of ADP/ATP translocation in rat liver mitochondria after treatment with ApppI is depicted in Fig. 9 (**IV**). Zoledronate at 100

µM concentration did not have any effect on ADP/ATP translocase (data not shown), indicating that N-BPs themselves do not affect this enzyme.

5.5.2 Inhibition of ANT by ApppI causes apoptosis in osteoclasts (IV)

Mitochondria are potent integrators and coordinators of apoptosis. Dissipation of the mitochondrial membrane potential, an increase in mitochondrial Ca^{2+} levels, extensive oxidation of mitochondrial NADPH, a decrease in cellular ATP levels and the burst of reactive oxygen species precede the opening of the permeability transition pore (PT), leading to the release of cytochrome c and other apoptogenic proteins (Kroemer and Reed 2000; Mayer and Oberbauer 2003). The main components of the PT pore are VDAC and ANT, which are positioned adjacent to each other in the outer mitochondrial membrane and inner mitochondrial membrane, respectively (Mayer and Oberbauer 2003). Inhibitors of ANT are known to influence the mitochondrial permeability transition pores and to evoke apoptosis. These inhibitors can be divided into two classes, PT pore-closing (e.g. bongkrekic acid) and PT pore-opening (e.g. atractyloside) compounds (Zamzami et $al.$ 1996; Chavez et $al.$ 1999). By inhibiting the mitochondrial ANT, AppCCl$_2$p causes mitochondrial membrane depolarisation (Lehenkari et $al.$ 2002), which may lead to opening the mitochondrial PT pores and the release of cytochrome c and other apoptogenic proteins (Kroemer and Reed 2000), which then leads to caspase activation (Benford et $al.$ 2001) and therefore direct apoptotic cell death.

Since ApppI also inhibits ANT translocation in the mitochondria (**IV**: Fig. 9), it could also cause apoptosis in a similar manner to AppCCl$_2$p. This was confirmed in the experiments where the apoptotic effects of zoledronate, ApppI, clodronate, or AppCCl$_2$p were examined in isolated rat osteoclasts. Compared with control cultures, all of the tested compounds significantly induced apoptosis (p<0.001) in isolated osteoclasts cultured on bone slices after 24 hr treatment (**IV**: Fig. 10). Liposome-encapsulated delivery was purposefully selected to ensure controlled and similar transportation of the compound into the target cells. Additionally, liposome-encapsulation is known to slow down the degradation of AppCCl$_2$p, and to enhance and control the cellular uptake of AppCCl$_2$p (Mönkkönen et $al.$ 1994a; Frith et $al.$ 2001), and presumably also that of ApppI. Therefore, it is notable that the concentrations of ApppI used in the ANT (0-100 µM) (**IV**: Fig. 9) and apoptosis assays (0.1 and 1 µM) (**IV**: Fig. 10) cannot be directly compared. The ANT assay was carried out by using free ApppI in a cell free system whereas the osteoclast apoptosis assay utilized liposome-encapsulated ApppI. It was anticipated that similar to the cellular uptake studies of liposomal clodronate in macrophages (**II**: Fig. 2), the intracellular concentration of

ApppI in the highly endocytic osteoclasts would also be much higher than the extracellular concentrations after liposomal ApppI treatment (0.1 and 1 μM).

The role of the ANT inhibition as the mechanisms of action of BPs is dependent on the intracellular concentration of ApppI or AppCCl$_2$p. The cytoplasmic concentration of AppCCl$_2$p can reach almost 1000 μM in clodronate-treated macrophages (**II**: Fig. 2). The intracellular concentration of AppCCl$_2$p was estimated assuming a cellular volume of 0.408 mm^3 per million cells. A similar calculation indicates that the cytoplasmic ApppI concentration can reach about 130 μM in the N-BP-treated macrophages. The concentration of 30 μM ApppI approximately halved ANT activity (47% inhibition) in isolated mitochondria (**IV**: Fig. 9). Therefore, treatment of osteoclasts with liposome-encapsulated ApppI (0.1 or 1 μM) should result in an intracellular concentration high enough to inhibit ANT activity in cells. These suggestions are also relevant with the *in vivo* situation, because osteoclasts may be exposed to the very high concentrations of BPs. It was shown previously that alendronate (N-BP), can reach about 1mM concentration in the resorption space beneath an osteoclast (Sato *et al*. 1991). The high concentration of BP achieved in the resorption lacunae and the high endocytic capacity of osteoclasts thus make it very likely that osteoclasts are also able to produce high concentrations of ApppI *in vivo*. The cellular uptake of N-BPs and their inhibitory activity on the mevalonate pathway are probably the critical points for ApppI production and thus for the inhibition of ANT also in osteoclasts.

Taken together, N-BP-induced inhibition of FPP synthase leads to formation of a novel endogenous ATP analog (ApppI), and this compound inhibits mitochondrial ANT, resulting in apoptosis. This finding also introduces a new interesting metabolic concept; according to current knowledge, there are no other drugs which are able to induce the synthesis of new molecules which are neither metabolites of the drug nor endogenous molecules naturally occurring in cells.

5.6   Implications and future aspects

In the last ten years, HPLC-mass spectrometry has emerged as a leading technology for measuring molecular structures of various classes of metabolites. In the present study, HPLC-mass spectrometry proved to be a very suitable tool to study the metabolism of bisphosphonates. It also enables detection of various other endogenous metabolites (such as sugar phosphates and nucleotides) by negative ion HPLC-mass spectrometry. Therefore, it should be possible to study the effects of BPs and other drugs on the mevalonate or additional biochemical pathways using a metabolomics approach. The general aim of metabolomics is to identify, measure and interpret the

complex time-related concentration and activity of endogenous metabolites in cells or tissues.

An overview of the cellular actions of BPs is presented in Fig. 7. Treatment of cells with drugs of either BP class, results in the intracellular production of cytotoxic ATP analogs. There are, however, BP-class related differences in how these ATP analogs are formed. Non-N-BPs are metabolised to AppCp-type analogs in such a way that the whole BP-molecule becomes a part of the newly formed, AppCp-type analog of ATP. N-BPs, in contrast, induce the formation of ApppI by first inhibiting the FPP synthase in the mevalonate pathway, which results in the accumulation of intracellular IPP. The IPP molecules are then converted to ApppI, presumably via aminoacyl-tRNA-synthetases.

These ATP analogs accumulate in the cell cytoplasm and directly cause apoptosis by inhibiting the mitochondrial adenine nucleotide translocase (ANT). The mitochondria-mediated apoptosis caused by AppCp-type metabolites of non-N-BPs is an essential for mediating the ability of non-BPs to inhibit osteoclast-mediated bone resorption. Unlike non-N-BPs, the osteoclast apoptosis is not necessary for antiresorptive effects of N-BPs. According to current knowledge, the major molecular mechanism of N-BPs causing inhibition of bone resorption seems to be the loss of prenylated proteins due to the inhibition of FPP synthase. However, the results of the present study suggest that ApppI may also contribute to the cellular actions of N-BPs. In order to further evaluate the pharmacological role of ApppI, detailed data on its formation is required, such as the time course of IPP/ApppI formation and IPP/ApppI production after a pulse or continuous treatment with N-BP. Naturally, also N-BP induced IPP/ApppI formation *in vivo* should be demonstrated in order to establish the biological significance of this molecule. Overall, these studies could clarify the roles of accumulation of IPP and ApppI molecules in the mechanism of action, both in beneficial and in adverse reactions, of N-BPs.

The metabolism of clodronate depends on cellular uptake of the drug, which was demonstrated by using different compounds (free drug vs. liposome-encapsulated drug) and cell models (macrophages vs. Caco-2 epithelial cells). Cellular uptake of N-BP might also be a critical point for the ApppI formation. According to our preliminary studies (unpublished data), several differences in ApppI production between different cancer cell lines have been found. In addition to cellular uptake, this could also be due to the activity of the mevalonate pathway in different cell types. These observations are interesting, as in addition to their antiresorptive properties, the most potent N-BPs, like zoledronate, also have anti-tumor effects *in vitro* (Green 2003; Neville-Webbe *et al.* 2005). It is possible that N-BP induced ApppI production may account for a part of the anti-tumor effects of N-BPs. In addition, recent data also suggests that especially the newer N-BPs have anti-angiogenic properties (Green 2003; Heymann *et al.* 2004). With

respect to the anti-cancer role of BPs and their ATP analogs, the ATP analogs may disrupt many other cellular functions, not only the mitochondrial ANT. ATP acts as a co-factor in a large number of biochemical processes, e.g. the action of protein kinases and P-glycoprotein (Pgp) in cells. Abnormal kinase activity has been implicated in the initiation and progression of a variety of human cancers (Noonberg and Benz 2000) and Pgp (also called multidrug-resistance protein) is of major interest as a contributor to resistance to chemotherapy in cancer (Kartner *et al*. 1985).

IPP

AMP

non-N-BP

AMP

AMP + non-N-BP

HMG-CoA → mevalonate → C5 isopentenyl diphosphate (IPP)

C5 dimethylallyl diphosphate (DMAPP)

FPP synthase

C10 geranyl diphosphate

FPP synthase

cholesterol ←······C15 farnesyl diphosphate → farnesylated proteins

C20 geranylgeranyl diphosphate → geranylgeranylated proteins

N-BPs

AppI + AMP

catalysed by aminoacyl-tRNA-synthetase ?

AppCp + AMP

catalysed by aminoacyl-tRNA-synthetase

Loss of prenylated proteins

Loss of survival signals

Loss of actin rings and ruffled border

Inhibition of mitochondrial ANT

Direct apoptosis in osteoclasts

Indirect apoptosis in osteoclasts

Inhibition of the release of proinflammatory cytokines and NO from macrophages

Loss of osteoclast function

Primary action for N-BPs to inhibit osteoclast-mediated bone resorption

Secondary effect of N-BPs on the inhibition of osteoclast-mediated bone resorption

Primary action for non-N-BPs to inhibit osteoclast-mediated bone resorption

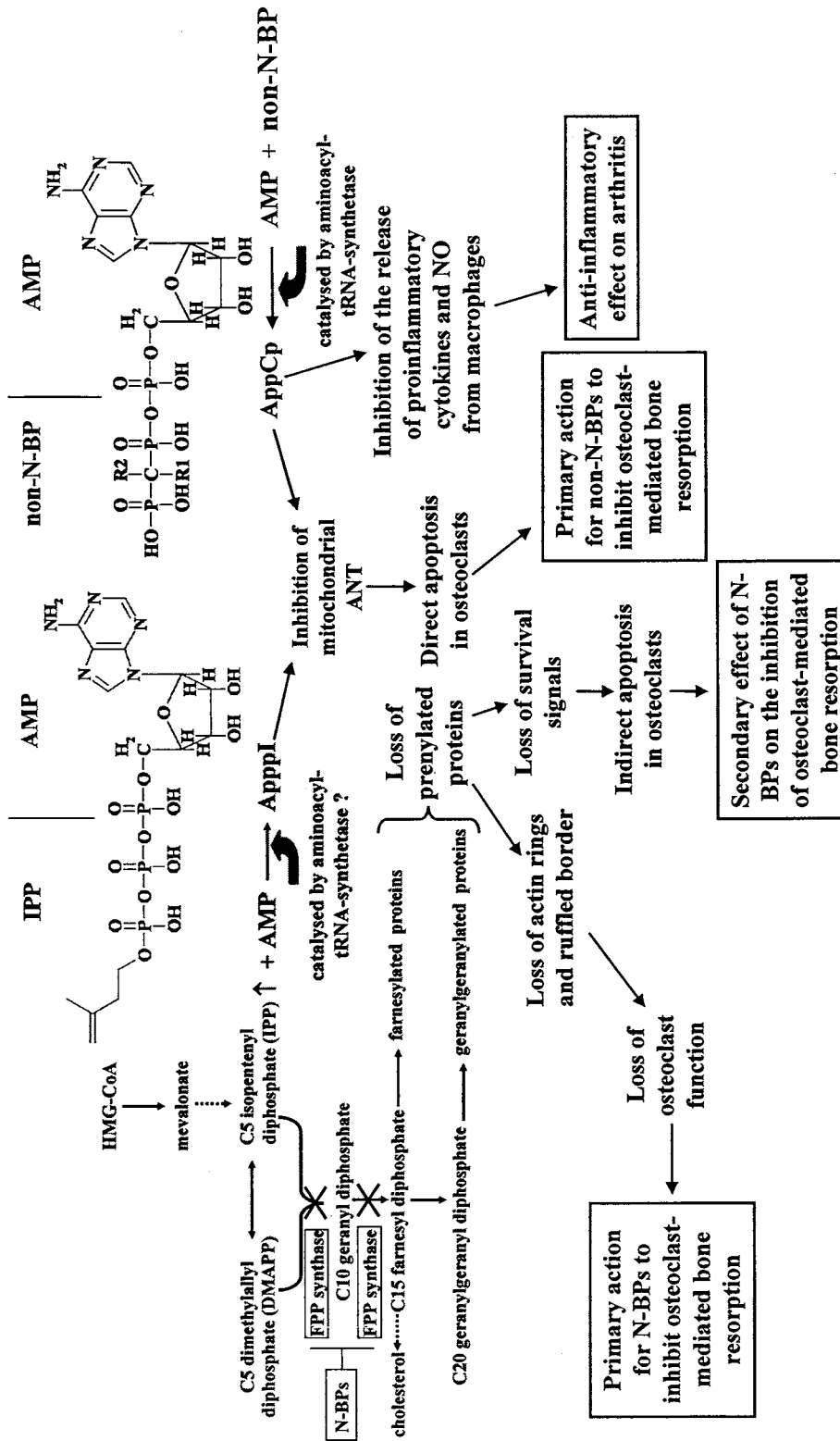Anti-inflammatory effect on arthritis

Fig. 7. An overview of the cellular actions of bisphosphonates. N-BPs have dual role in the molecular mechanisms of action; they prevent protein prenylation and induce production of cytotoxic ApppI by inhibiting FPP synthase in the mevalonate pathway. Non-N-BPs act via cytotoxic AppCp-type metabolites.

6   CONCLUSIONS

This study clarified the intracellular metabolism of bisphosphonates (BPs) and the role of this metabolism in the mechanisms of action and side-effects of BPs. The main conclusions of the present study are as follows:

1. Ion-pairing HPLC-ESI-MS is a sensitive method for quantitation of the ATP analogs in cell extracts. Acetonitrile extraction is simple and very suitable for HPLC analysis. These techniques can be applied to study the metabolism of bisphosphonates *in vitro* and *in vivo*.

2. Macrophages, as highly endocytic cells, avidly take up both free and liposome-encapsulated clodronate, which is then efficiently metabolised to AppCCl$_2$p and this metabolite can accumulate to very high intracellular concentrations.

3. A clodronate metabolite (AppCCl$_2$p) is responsible for the cellular actions, e.g. anti-inflammatory properties and apoptosis, of this bisphosphonate, indicating that non-N-BPs act through these AppCp-type metabolites.

4. Clodronate is likely to be metabolised into a cytotoxic compound, by any cell type capable of internalising the drug. However, the cytotoxic potential depends on the degree of uptake of clodronate, and thus, the amount of metabolite produced. Therefore, should new lipophilic derivatives of clodronate be developed to improve their bioavailibility, it is very possible that these novel drugs may cause a simultaneous increase in gastrointestinal side-effects.

5. In addition to causing indirect apoptosis by preventing protein prenylation, N-BP-induced inhibition of FPP synthase leads to the formation of a novel endogenous ATP analog (ApppI). This compound inhibits mitochondrial ANT, which then evokes direct apoptosis in osteoclasts. This finding provides a new plausible mechanism of action for N-BPs.

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