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Johanna Räikkönen

Bisphosphonate-Induced IPP/ApppI Accumulation in Cells

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JOHANNA RÄIKKÖNEN

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ABSTRACT

This study explored the molecular mechanisms of action of bisphosphonates (BPs). BPs are currently among the most used and effective class of drugs developed for the treatment of metabolic bone disorders that are associated with increased bone destruction, such as osteoporosis and tumor-induced osteolysis. Moreover, there is now extensive preclinical evidence available that BPs could have the ability to act directly on tumor cells of various origins. However, the underlying mechanisms are still unclear.

Non-nitrogen-containing BPs (non-N-BPs), such as clodronate, are metabolised into cytotoxic ATP analogs capable of inducing apoptosis in cells, while nitrogen-containing BPs (N-BPs), such as zoledronic acid, act by inhibiting the farnesyl pyrophosphate synthase (FPPS) enzyme of the mevalonate pathway causing a concomitant decrease in the formation of the isoprenoid lipids required for normal cellular function. Moreover, a new possible mechanism of action of N-BPs has been revealed, a finding which may account for the observed effects of N-BPs. N-BPs induce the formation of a novel endogenous ATP analog ApppI as a consequence of the inhibition of the FPPS enzyme, and the subsequent accumulation of isopentenyl pyrophosphate (IPP). ApppI is a pharmacologically active compound capable of directly triggering apoptosis.

This study aimed to characterize the role of the intracellular accumulation of IPP/ApppI in the mechanism of action of N-BPs. The detailed aims were: (1) to investigate BP-induced IPP accumulation and ATP analog formation in various cancer cell lines, (2) to obtain detailed data, such as time-course and dose-dependence, on N-BP-induced IPP/ApppI formation in cancer cells, (3) to explore N-BP-induced ApppI formation in rabbit osteoclasts, *in vivo*, (4) to identify the specific intermediates and enzymes in the mevalonate pathway involved in regulating the BP-induced IPP/ApppI formation in cancer cells.

This study provided the first conclusive evidence that the pro-apoptotic ApppI is formed in osteoclasts *in vivo*, the pharmacological target cells for BPs, even after a single clinically relevant dose of N-BP. This result is of considerable importance, as it establishes the biological significance of this molecule. Furthermore, it was shown for the first time, that BPs induce the accumulation of cytotoxic ATP analogs, ApppI and AppCCl$_2$p, in several types of cancer cells. The metabolites accumulated in cells in a time- and dose-dependent manner. However, the accumulation of these metabolites was remarkably cell line dependent suggesting that BPs may have distinct antitumor effects dependent upon the extent of accumulation of cytotoxic compounds within the cells. In addition to cancer cell type, the intracellular amounts of IPP and ApppI were shown to be dependent on the FPPS binding characteristics of the N-BP. N-BP-induced IPP/ApppI levels in cells can also be downregulated by the mevalonate pathway intermediates, isoprenoids. This represents a novel insight into the mechanism of action of isoprenoids on the regulation of the mevalonate pathway after inhibition by N-BPs, and supports the role of ApppI in mediating N-BP-induced apoptosis.

In conclusion, this study provided further evidence that BP-induced IPP and the subsequent ApppI accumulation are important metabolic events underlying the molecular mechanisms of action of BPs. Furthermore, the mass spectrometric IPP/ApppI analysis was demonstrated to be a useful and sensitive tool for investigating the intracellular action of BPs and other mevalonate pathway inhibitor candidates under development. The introduction of novel markers such as IPP and ApppI as surrogates of N-BP efficacy might be also useful both in preclinical and clinical research to better understand the anticancer activities of N-BPs.

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TIIVISTELMÄ

Tutkimus kohdistui bisfosfonaattien (BP) solutason vaikutusmekanismien selvittämiseen. BPt ovat tällä hetkellä käytetyimpiä ja tehokkaimpia lääkkeitä luun aineenvaihduntasairauksien, kuten osteoporoosin ja syövän aiheuttaman luun haurastumisen hoidossa. Bisfosfonaateilla on myös havaittu olevan suoria syövän kasvua estäviä vaikutuksia, mutta niiden vaikutusmekanismit ovat vielä varsin epäselviä.

Ei-typpeä sisältävät BPt (non-N-BPt), kuten klodronaatti, metaboloituvat solun sisällä ATP-analogeiksi, jotka aiheuttavat apoptoosin eli ohjelmoidun solukuoleman. Typpeä sisältävät BPt (N-BPt), kuten zoledronaatti, häiritsevät solun normaalitoiminnalle elintärkeiden isoprenoidilipidien muodostumista estämällä solunsisäisen mevalonaattitien entsyymin, farnesyylipyrofosfaattisyntaasin (FPPS), toimintaa. Viimeaikaisen tutkimuksen mukaan N-BP:lla on uusi, aiemmin tuntematon vaikutusmekanismi, joka voi selittää näiden bisfosfonaattien erittäin suuren tehon niin luun haurastumisen estossa kuin syöpäsoluvaikutuksissakin. N-BP:en aiheuttaman FPPS-entsyymitoiminnan estymisen seurauksena solujen sisälle kertyy isopentenyylipyrofosfaattia (IPP), josta muodostetaan uusi, aktiivinen yhdiste, ATP-analogi (ApppI). ApppI on farmakologisesti aktiivinen yhdiste, joka aiheuttaa kohdesolujen apoptoosin.

Tämän tutkimuksen tarkoituksena oli karakterisoida IPP/ApppI:n kertymisen roolia bisfosfonaattien solutason vaikutusmekanismina. Tavoitteena oli: (1) tutkia BP-indusoitua IPP ja ATP-analogien tuotantoa erilaisissa kasvatetuissa syöpäsolulinjoissa, (2) tuottaa yksityiskohtaisempaa tietoa, kuten annos- ja aikariippuvuutta, IPP/ApppI:n synnystä syöpäsoluissa, (3) tutkia ApppI:n muodostumista *in vivo*, kanin osteoklasteissa ja, (4) identifioida ne spesifiset mevalonaattitien välituotteet ja entsyymit, jotka vaikuttavat IPP/ApppI:n tuotannon säätelyyn syöpäsoluissa.

Tutkimuksessa osoitettiin ensimmäistä kertaa, että ApppI:a muodotuu bisfosfonaattien farmakologisissa kohdesoluissa, osteoklasteissa *in vivo*, jopa kliinisesti relevantilla N-BP-annoksella. Tulos todistaa, että ApppI on biologisesti merkittävä molekyyli. Tutkimus osoitti myös ensimmäistä kertaa, että BPt indusoivat solumyrkyllisten ATP-analogien, ApppI ja AppCCl$_2$p (klodronaatin metaboliitti), tuotannon erilaisissa syöpäsoluissa. Metaboliitit kertyivät soluihin aika- ja annosriippuvaisesti, mutta tuotannossa havaittiin olevan hyvin voimakkaita solulinjakohtaisia eroja. Tulokset viittaavat siihen, että bisfosfonaattien syöpävaikutukset saattavat vaihdella, johtuen eri syöpäsolujen kyvystä tuottaa solumyrkyllisiä metaboliitteja. Solulinjatyypin lisäksi solunsisäisten IPP- ja ApppI-määrien havaittiin riippuvan BP:n kyvystä sitoutua kohteeseensa, FPPS-entsyymiin. Tutkimuksessa osoitettiin myös, että solujen IPP/ApppI:n tuotantoa pystytään estämään mevalonaattitien välituotteilla, isoprenoideilla. Tulos esittää isoprenoideille uuden vaikutusmekanismin säädellä mevalonaattitietä bisfosfonaattikäsittelyn jälkeen, ja tukee ApppI-molekyylin roolia N-BP-indusoidusssa solukuolemassa.

Tutkimus tuotti lisää todisteita siitä, että N-BP-lääkkeiden aiheuttama IPP/ApppI:n tuotanto on bisfosfonaattien merkittävä vaikutusmekanismi. Tutkimus osoitti massaspektrometrisen IPP/ApppI-analyysin olevan herkkä ja hyödyllinen menetelmä, jolla voidaan tutkia bisfosfonaattien ja muiden kehitteillä olevien mevalonaattitien inhibiittorien solunsisäisiä vaikutuksia. Uusien markkereiden, kuten IPP- ja ApppI-molekyylien, käyttö bisfofonaattien tehokkuuden mittarina voi olla hyödyllistä sekä prekliinisissä että kliinisissä tutkimuksissa, joissa bisfosfonaattien syöpävaikutuksia arvioidaan.

Luokitus: QU 131, QU 57, QV 38, QZ 206
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Finally, I want to dedicate this thesis to my family and the loving memory of my father.

Kuopio, June 2011

Johanna Räikkönen

List of the original publications

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III Räikkönen J, Mönkkönen H, Auriola S, Mönkkönen J: Mevalonate pathway intermediates downregulate zoledronic acid- induced isopentenyl pyrophosphate and ATP analog formation in human breast cancer cells. *Biochemical Pharmacology* 79:777-783, 2010.

IV Räikkönen J, Taskinen M, Dunford JE, Mönkkönen H, Auriola S, Mönkkönen J: Correlation between time-dependent inhibition of human farnesyl pyrophosphate synthase and blockade of mevalonate pathway by nitrogen-containing bisphosphonates in cultured cells. *Biochemical and Biophysical Research Communications* 407:663-667, 2011.

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Abbreviations

AMP	Adenosine monophosphate
ANT	Adenine nucleotide translocase
APR	Acute phase reaction
AppCCl$_2$p	5′(-βγ-dichloromethylene) triphosphate
AppCp	Methyladenosine 5'-triphosphate
ApppD	Triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-2-enyl) ester
ApppI	Triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester
ATP	Adenosine triphosphate
BP	Bisphosphonate
BSA	Bovine serum albumin
CLOD	Clodronate
DMAPP	Dimethylallyl pyrophosphate
DPTI	Dual prenyltransferase inhibitor
ESI	Electrospray ionization
HAP	Hydroxyapatite
FOH	Farnesol
FPP	Farnesyl pyrophosphate
FPPS	Farnesyl pyrophosphate synthase
FTase	Farnesyl transferase
FTI	Farnesyl transferase inhibitor
GGOH	Geranylgeraniol
GGPP	Geranylgeranyl pyrophosphate
GGTase	Geranylgeranyl transferase
GGTI	Geranylgeranyl transferase inhibitor
GPP	Geranyl pyrophosphate
GTP	Guanosine triphosphate
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HPLC	High-performance liquid chromatography
IPP	Isopentenyl pyrophosphate

K_{isom}	Isomerization constant
LDL	Low-density lipoprotein
MDDase	Mevalonate diphosphate decarboxylase
MKase	Mevalonate kinase
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N-BP	Nitrogen-containing bisphosphonate
Non-N-BP	Non-nitrogen-containing bisphosphonate
NE58027	Piperidine hydroxy-ethyl-1,1-bisphosphonic acid
NE58043	3-pyrethane-1,1-bisphosphonic acid
NE11808	2-pyridylaminoethane-1,1-bisphosphonic acid
NE97220	N-(3-picoly)aminomethane-1,1-bisphosphonic acid
NE21650	2-aminophenyl-2-ethane-1-hydroxy-1,1-bisphosphonic acid
ONJ	Osteonecrosis of the jaw
PBS	Phosphate buffered saline
PMKase	Phosphomevalonate kinase
PPi	Inorganic pyrophosphate
RIS	Risedronate
TNF	Tumor necrosis factor
tRNA	Transfer ribonucleic acid
ZOL	Zoledronic acid

1 Introduction

Bisphosphonates (BPs) are currently among the most important and effective class of drugs developed for the treatment of metabolic bone disorders that are associated with increased osteoclast-mediated bone resorption, such as osteoporosis (Delmas 2002, Kanis et al. 2008) and Paget's disease (Delmas and Meunier 1997, Roux and Dougados 1999). Additionally, BPs are effective inhibitors of tumor-induced bone destruction and significantly reduce the incidence of skeletal complications in patients with bone metastases from several forms of cancer, including breast and prostate cancer (Coleman 2004). BPs have a high affinity for calcium and therefore target specifically bone mineral, where they are internalized by bone-destroying osteoclasts, eventually inhibiting their function (Fleisch 2000). BPs primarily affect the function of osteoclasts but there is recent preclinical evidence indicating that other neighboring cell types, such as macrophages, monocytes and cancer cells, could also be targets for these drugs (Clezardin 2011). Importantly, evidence has accumulated on the direct anticancer effects of BPs. BPs have been shown to induce tumor cell apoptosis, to modulate cells in the immune system and to inhibit tumor angiogenesis. All these recent findings suggest that the target cells for bisphosphonates as well as their molecular mechanism of action may be more diverse and complex than realized so far.

Only in recent years, have major advances been made in understanding the cellular and molecular mechanisms of action of BPs. BPs can be classified into two structural subgroups based on differences in their chemical structure and mechanism of action. The low-potency BPs lacking a nitrogen group (non-N-BPs) are metabolised to cytotoxic ATP analogs (Rogers et al. 1992, Auriola et al. 1997, Frith et al. 1997, Frith et al 2001) that accumulate intracellularly and subsequently trigger direct apoptosis (Lehenkari et al. 2002). In contrast, the more potent nitrogen-containing BPs (N-BPs) act by inhibiting farnesyl pyrophosphate synthase (FPPS) enzyme at a branch point in the mevalonate pathway (van Beek et al. 1999a, van Beek et al. 1999b, Bergstrom et al. 2000). The mevalonate pathway is the metabolic pathway involved in the synthesis of a number of essential cellular compounds, the most prominent being cholesterol (Goldstein and Brown 1990). In osteoclasts, however, two other products, farnesyl and geranylgeranyl pyrophosphate, are essential to attach small important signaling proteins, such as GTPases, to the cell membrane (Luckman et al. 1998b, Coxon et al. 2000). By inhibiting FPPS, N-BPs disrupt the signaling functions of GTPases, and consequently induce a series of changes leading to decreased activity and indirect cell death via apoptosis not only in osteoclasts but also in other cell types (Luckman et al. 1998a, Luckman et al. 1998b, Shipman et al.1998, Benford et al. 1999, Reszka et al. 1999, Coxon et al. 2000, Coxon et al. 2004). This mechanism of action is now thought to be of prime importance in mediating the cytotoxic effects of N-BPs. However, recent research has revealed a new possible mechanism of action for N-BPs, establishing that the potent N-BPs induce formation of a new type of ATP analog, ApppI (Mönkkönen et al. 2006). ApppI formation results from the inhibition of FPPS in the mevalonate pathway and subsequent accumulation of an early intermediate, isopentenyl pyrophosphate (IPP). ApppI formation from IPP is produced, presumably, in the same metabolic pathway as the ATP analog of non-N-BP, but it does not contain a BP in its structure and thus is not a metabolite of the drug. ApppI is a pharmacologically active compound capable of triggering osteoclast apoptosis in a similar manner to the ATP analogs of non-N-BPs. Therefore, these two classes of BPs may have more in common than previously thought.

N-BP-induced IPP and ApppI are both active compounds, the former activating an immune response (Gober et al. 2003) and the latter triggering apoptosis in osteoclasts *in vitro* (Mönkkönen et al. 2006). However, very little is known about their accumulation in cells. This study characterized the effects of BPs on the intracellular mevalonate pathway, and the consequent accumulation of IPP

and ApppI. The intention was to gather more detailed data on N-BP-induced IPP/ApppI formation in cancer cell cultures and in an animal model. In addition, the regulation of N-BP-induced IPP accumulation and the consequent ApppI formation by the mevalonate pathway products was characterized. The following review of the literature presents a brief description of the biochemistry of the mevalonate pathway, together with current knowledge about this pathway as an anticancer target. The literature review focuses on BPs and provides an overview of their pharmacology, e.g. evaluating N-BPs as potent mevalonate pathway inhibitors. Additionally, new insights into the intracellular molecular mechanism of action of BPs, and the potential of BPs to function as anticancer agents are described.

2 Review of the literature

2.1 MEVALONATE PATHWAY

The mevalonate pathway is an important metabolic pathway synthetizing essential bioactive molecules that are vital for many cellular functions, ranging from cholesterol synthesis to growth control (Goldstein and Brown 1990). Furthermore, the wide spectrum of diseases resulting from defects in the mevalonate pathway illustrates the importance of this pathway (Tozawa et al. 1999, Zhang et al. 2002, Ohashi et al. 2003, Prietsch et al. 2003, Seabra et al. 2002).

2.1.1 Biochemistry and regulation

The first product of the pathway, mevalonate, is a precursor for isoprenoid intermediates which are subsequently incorporated into various end-products (Figure 1), e.g. *sterol isoprenoids* such as cholesterol, not only utilized in cell membranes but also some being converted into steroid hormones, bile acids, and vitamin D; and *nonsterol isoprenoids* such as haem A and ubiquinone, which take part in electron transport; dolichol, required for glycoprotein synthesis; and farnesylated and geranylgeranylated proteins, which are membrane associated proteins involved in intracellular signaling (Goldstein and Brown 1990, Hinson et al. 1997). Cholesterol, the bulk end-product, is either derived from squalene or can be obtained by uptake of plasma low-density lipoprotein (LDL). The cell must balance these internal and external sources in order to sustain mevalonate synthesis while avoiding overaccumulation of potentially toxic sterols (Goldstein and Brown 1990).

The principal regulatory point in the mevalonate pathway is 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), one of the most highly regulated enzymes in the body (Goldstein and Brown 1990). In normal cells, HMGR activity is controlled by several feedback-regulation mechanisms by sterol and nonsterol products of the pathway, and also at the level of transcription, translation, protein stability, phosphorylation and thiol status of the cell (Goldstein and Brown 1990, Nakanishi et al. 1988, Osborne et al. 1988). A full reduction in enzyme activity requires both a sterol that can be derived from LDL and a nonsterol metabolite that must be synthesized from mevalonate. These products suppress HMGR by at least two mechanisms; by decreasing the transcription of the gene and by enhancing the degradation of the enzyme. Conversely, HMGR activity in tumor cells is elevated and dysregulated (Mo and Elson 2004). Tumor cells are resistant to sterol-mediated feedback regulation, and are several-fold more sensitive than normal cells to nonsterol-mediated post-transcriptional downregulation.

After HMGR, mevalonate is converted to isopentenyl pyrophosphate (IPP) by a cascade of three enzymes, mevalonate kinase (MKase), phosphomevalonate kinase (PMKase), and mevalonate diphosphate decarboxylase (MDDase) (Hinson et al. 1997). At least, the activity of MKase is regulated via feedback inhibition by isoprenoid intermediates geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Hinson et al. 1997, Dorsey and Porter 1968). All the isoprenoid intermediates contain a polyisoprene structure derived from repeated polymerizations of IPP (Brown and Goldstein 1980). In the initial reaction, IPP is coupled with its isomer dimethylallyl pyrophosphate (DMAPP) to form a 10-carbon intermediate, GGP. This compound then combines with another molecule of IPP to produce the key 15-carbon intermediate FPP, which further reacts with IPP to form the 20-carbon intermediate GGPP. The productions of FPP and GGPP are catalyzed by FPP and GGPP synthases. FPP is a major branch-point precursor for several products of the mevalonate pathway, such as squalene, cholesterol, and also GGPP. Thus, the FPPS enzyme occupies an important position in the pathway.

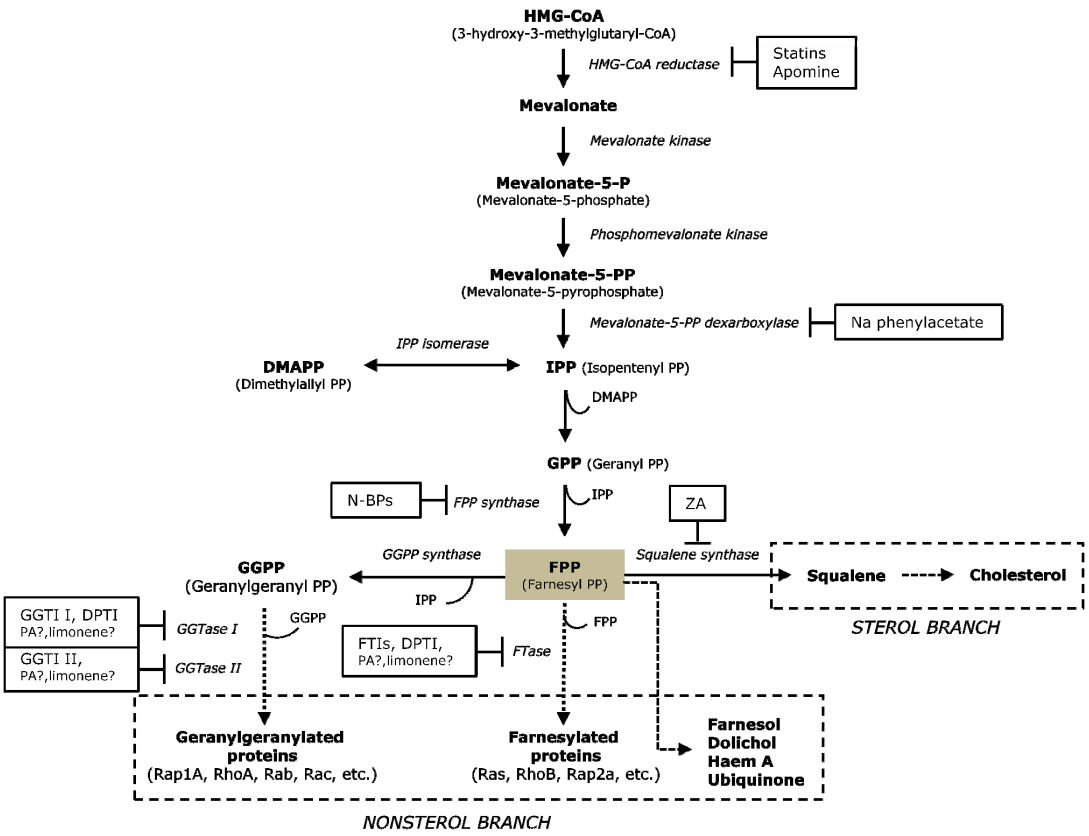


Figure 1. The mevalonate pathway. Mevalonate is converted to farnesyl pyrophosphate (FPP), which can be used for the synthesis of sterol or nonsterol isoprenoids, products essential for cell survival and proliferation. Agents targeting the pathway inhibit either the activities providing mevalonate derived intermediates, such as statin drugs and phenylacetate, or activities downstream from FPP, such as nitrogen-containing bisphosphonates (N-BPs), prenyltransferase inhibitors, and zaragozic acid (ZA). Importantly, in normal cells, cholesterol and isoprenoid products suppress HMG-CoA reductase via post-translational downregulation. Conversely, tumor cells are resistant to cholesterol-mediated suppression, although they remain sensitive to isoprenoid-mediated suppression. FTIs = farnesyl transferase inhibitors; GGTIs = geranylgeranyl transferase inhibitors; DPTIs = dual prenyltransferase inhibitors; FTase = farnesyl transferase; GGTase = geranylgeranyl transferase; PA = perillyl alcohol

Post-translational modifications of small GTP-binding proteins

Both FPP and GGPP are substrates for protein prenyltransferase enzymes carrying out post-translational modification and activation of small important GTP-binding proteins (GTPases) (Zhang and Casey 1996). The post-translational modifications of GTPases are essential to ensure their targeted localization and anchorage on the inner side of the cell membrane and subsequent signal activation and biological function. In this activation step, the farnesyl or geranylgeranyl lipidic residues are coupled to the protein, resulting in the formation of a farnesylated or geranylgeranylated protein (Figure 2). This type of protein activation is referred to as (iso)prenylation. In order to undergo prenylation, proteins must contain a carboxy-terminal CaaX

motif. C denotes cysteine, A represents an aliphatic amino acid and X refers to leucine for geranylgeranyltransferase I (GGTase I) and to any other amino acid other than leucine for farnesyltransferase (FTase). Protein GGTase II catalyzes the geranylgeranylation of Rab proteins that terminate in CC or CXC sequences. FTase catalyzes farnesylation of proteins, such as Ras, RhoB, HDJ2, and lamins A and B, whereas geranylgeranyltransferase I and II (GGPTases) ensure the geranylgeranylation of proteins, such as Rab, Rap1A, RhoA, Rac1 and CDC42 (Zhang and Casey 1996, Swanson and Hohl 2006). It is important to note that some Ras isoforms (K-Ras and N-Ras) can alternately be geranylgeranylated when farnesylation is inhibited (Whyte et al. 1997). Following prenylation, the -aaX residues are cleaved by an endoprotease and the carboxyl group of the modified cysteine is methylated by a specific methyltransferase. In addition, Rab proteins ending with the CXC motif are methyl esterified. For some Ras proteins, a further modification, termed palmitoylation, occurs. Finally, the protein is ready to be translocated onto the cellular membranes to receive extra- and intracellular signals.

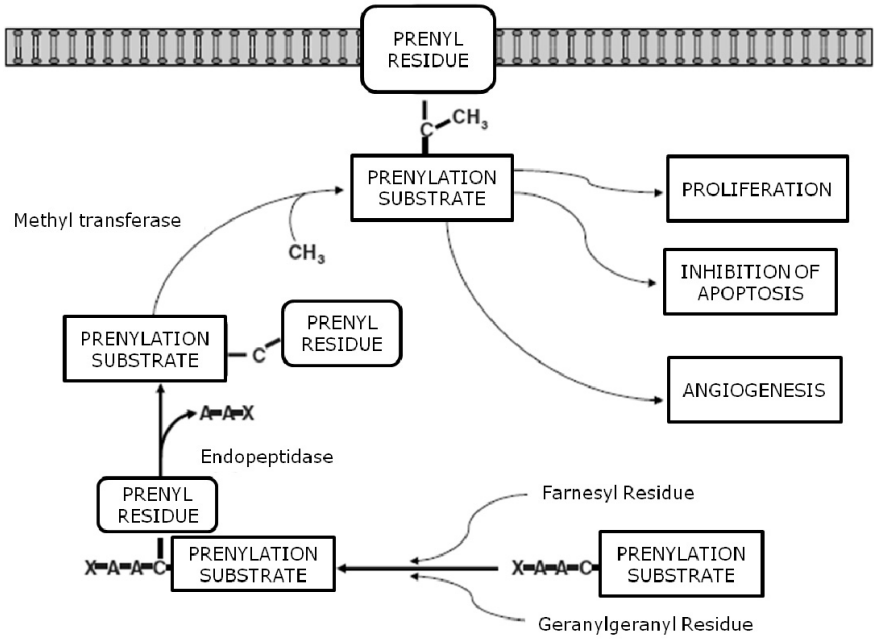


Figure 2. Prenylation mechanisms of intracellular proteins. After prenylation, the protein is ready to be translocated to the inner side of the cellular membranes in order to receive signals mediated by external and internal factors. Modified from Caraglia et al. 2006.

2.1.2 Mevalonate pathway as a therapeutic target in cancer

The mevalonate pathway is an attractive target for many areas of therapeutic research, such as autoimmune disorders, atherosclerosis, and Alzheimer disease, through inhibition of prenylation of small GTPases (Buhaescu and Izzedine 2007). Importantly, manipulation of this pathway results in alteration of malignant cell growth and survival in cell culture and animal models, with promising potential for application in human cancers (Caraglia et al. 2005, Swanson and Hohl 2006). However, the mevalonate pathway is very complex, and it needs to be better understood before it can be successfully manipulated to achieve a therapeutic anticancer effect.

There are several pharmacological options for interfering with the mevalonate pathway in cancer cells (Figure 1 and Table 1). Most research has been focused on statins and nitrogen-

containing bisphosphonates (N-BPs). Statins inhibit the rate-limiting step at the apex of the mevalonate pathway, the conversion of HMG-CoA to mevalonate by HMGR, while N-BPs inhibit FPP synthase, the other key enzyme in the pathway. In addition to the HMGR inhibitors (e.g. statins and apomine) and FPP synthase inhibitors (N-BPs), there are FTase inhibitors (FTIs), GGTase inhibitors (GGTIs), dual prenyltransferase inhibitors (DPTIs), monoterpenes (e.g. perillyl alcohol and limonene), and MDDase inhibitors (e.g. phenylacetate), which have an influence on isoprene metabolism (Mo and Elson 2004, Swanson and Hohl 2006). These agents induce cell cycle arrest and initiate apoptosis and differentiation, effects attributed to modulation of cell signaling pathways either by modulating gene expression, or by suppressing the prenylation of signaling proteins and growth factor receptors (Table 1).

Table 1. The compounds targeting the mevalonate pathway and their observed anticancer effects

Inhibitor	Inhibitory point	Effect on cancer cells	References
Statins	HMGR	e.g. Induction of the cell cycle arrest and apoptosis, inhibition of protein prenylation, cholesterol reduction	van de Donk et al. 2003, Lee et al. 1998, Kusama et al. 2002, Wong et al. 2002, Denoyelle et al. 2003a, Li et al. 2003, Graaf et al. 2004, Cho et al. 2008
Apomine	HMGR	Reduction of the levels of HMGR, induction of apoptosis	Flach et al. 2000, Roitelman et al. 2004, Edwards et al. 2007
N-BPs	FPPS	e.g. Induction of the cell cycle arrest and apoptosis, inhibition of protein prenylation	Jagdev et al. 2001, Shipman et al. 1998, Evdokiou et al. 2003, Wakchoure et al. 2006, Brown et al. 2009, Guenther et al. 2010, Wasko et al. 2011
Sodium phenylacetate	MDDase	Growth inhibition, inhibition of protein prenylation	Hudgins et al. 1995, Harrison et al. 1998
Zaragozic acid	Squalene synthase	Cholesterol reduction	Li et al. 2003, Brusselmans et al. 2007
FTIs	FTase	Growth inhibition, inhibition of protein farnesylation	Kohl et al. 1994, Bolick et al. 2003, Kurimoto et al. 2003, Venet et al. 2003, Virtanen et al. 2010
GGTIs I	GGTase I	Cell cycle arrest, inhibition of cancer cell invasion and migration	van de Donk et al. 2003, Sun et al. 1999, Coxon et al. 2000, Kusama et al. 2003, Sun et al. 2003, Virtanen et al. 2010
GGTIs II	GGTase II	Disruption of Rab dependent functions	Wasko et al. 2011, Coxon et al. 2001, Sane et al. 2010, Lawson et al. 2008
DPTIs	FTase+GGTase I	Growth inhibition, inhibition of K-Ras prenylation	Lerner et al. 1997, Bolick et al. 2003, Lobell et al. 2002
Limonene, perillyl alcohol	FTase, GGTase I and GGTase II(?)	Growth inhibition, suppression of the synthesis of small GTPases and HMGR	Crowell et al. 1994, Ren et al. 1997, Hardcastle et al. 1999, Holstein and Hohl 2003, Peffley and Gayen 2003, Yuri et al. 2004

HMGR = 3-hydroxy-3-methylglutaryl-coenzyme A reductase; N-BPs = nitrogen-containing bisphosphonates; FPPS = farnesyl pyrophosphate synthase; MDDase = mevalonate diphosphate dekarboxylase; FTIs = farnesyl transferase inhibitors; FTase = farnesyl transferase; GGTIs I and II = geranylgeranyl transferase inhibitors I and II; GGTase I and II = geranylgeranyl transferase I and II; DPTIs = dual prenyltransferase inhibitors

Much attention has been paid to the induction of Ras-mediated apoptosis because Ras mutations in cells are often associated with malignancy (Caraglia et al. 2005, Swanson and Hohl 2006, Konstantinopoulos et al. 2007), and this makes membrane localized Ras a rational target for antitumor therapy. For example, the FTase inhibitors are of particular interest since the oncogenic transformation activity of Ras dependent on its farnesylation (Qian et al. 1997, Sinensky 2000). However, K-Ras is resistant to treatment with FTI alone and therefore treatments with both FTI and GGTI are necessary to inhibit K-Ras prenylation, and to inhibit growth in a number of cell lines (Lerner et al. 1997). The major challenge in the case of GGTI/FTI combinations is toxicity, which could be overcome by the development of DPIs, which are better tolerated. N-BPs and statins also inhibit prenylation of Ras and other proteins, and this effect can be inhibited by the addition of the free nonsterol mevalonate pathway intermediates. Farnesol (FOH) and especially geranylgeraniol (GGOH), are capable of salvaging protein prenylation and, thus they can overcome statin and N-BP-induced apoptosis in many cell types (Goffinet et al. 2006, Jagdev et al. 2001, Benford et al. 1999, Shipman et al. 1998, van de Donk et al. 2003).

It is usually difficult to inhibit efficiently one enzyme of the pathway completely with a single inhibitory drug, which potentially result in toxic side effects (Swanson and Hohl 2006). Therefore, the inhibition of more than one enzyme in the metabolic pathway e.g. by combined drug treatment might, be more effective than inhibition of only one individual enzyme. Mevalonate pathway inhibitors in combinations and combined with cytotoxic drugs represent a promising approach to enhance the anticancer efficiency of these agents. There is good evidence that mevalonate pathway inhibitors might improve the therapeutic outcome of conventional (i.e. DNA damaging) and non-conventional (such as antibodies) anticancer drugs (Konstantinopoulos et al. 2007, Fritz 2009). This synergistic effect with other agents would appear to depend not only on the choice of agents and the cancer type, but also on the sequencing of these compounds (Swanson and Hohl 2006). The mechanism for sequence-dependent synergy is not entirely clear.

2.2 BISPHOSPHONATES

2.2.1 Background to the pharmacological development

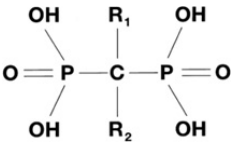
BPs have been known to chemists since the middle of 19th century and these compounds were used in industry as antiscaling agents because of their property to inhibit the precipitation of calcium carbonate (Quimby 1947). Fleisch and Bisaz reported in 1962 that inorganic pyrophosphate (PPi), a known by-product of many biosynthetic reactions in the body, was present in serum and urine and prevented ectopic calcification. However, PPi had no effect on bone resorption, possibly because its rapid hydrolysis. This prompted the search for analogs of pyrophosphate which would display a similar physicochemical activity, but which would resist enzymatic hydrolysis and metabolism. The bisphosphonates (BPs) fulfilled these conditions. The observation that BPs were able to inhibit the dissolution of hydroxyapatite (HAP) crystals (Fleisch et al. 1969) led to studies demonstrating that they also inhibited bone resorption in many different experimental models (See review in Fleisch 2000). This discovery was a breakthrough leading to the rapid development of the BPs in medicine for diseases with increased bone resorption and with bone loss.

2.2.2 Chemical features of BPs

All BPs have the same generic structure (Table 2). BPs are stable, water-soluble, synthetic analogs of PPi in which the oxygen bridge has been replaced by a carbon (P-C-P) with various side chains (Fleisch 2002). In contrast to the P-O-P binding of PPi, the P-C-P backbone structure is stable towards heat and most chemical reagents, and completely resistant to enzymatic degradation. The P-C-P motif of the BP structure is required for the chelation of Ca^{2+} ions by these compounds and

hence is the basis for their bone-targeting property. In general, BPs can chelate divalent metal ions such as Ca^{2+}, Mg^{2+}, and Fe^{2+} in a bidentate manner through the oxygen atoms of the phosphate groups, but if the R_1 chain is a hydroxyl, then the affinity of BPs for cations is even greater, allowing tridentate coordination (Rogers et al. 2000). In fact, the two phosphonate groups, together with R_1 side chain, are often described as a "bone hook", because of their role in targeting efficiently to bone mineral surfaces (Russell et al. 2008).

Table 2. The most widely used BPs in the clinic and their chemical structures. The binding of BPs to HAP depends on the P-C-P group and the R_1 side chain. The biological activity is dependent on the R_2 side chain. The BPs are grouped into first-, second-, and third-generation drugs according to their side chains, and their potency for inhibiting bone resorption in rats.



The generic structure of BP

	BP	R_1	R_2	Potency	Application
1st generation	Etidronate*	OH	CH_3	1 x	oral
	Clodronate**	Cl	Cl	10 x	oral/i.v.
2nd generation	Tiludronate*	H	CH_2-S-phenyl-Cl	10 x	oral/i.v.
	Pamidronate**	OH	$CH_2CH_2NH_2$	100 x	i.v.
	Alendronate*	OH	$(CH2)_3NH_2$	100-1000 x	oral
	Ibandronate**	OH	$CH_2CH_2N(CH_3)$(pentyl)	1000 -10 000 x	oral/i.v.
3rd generation	Risedronate*	OH	CH_2-3-pyridine	1000 -10 000 x	oral
	Zoledronic acid**	OH	CH_2-imidazole	>10 000 x	i.v.

* BPs approved for non-malignant conditions
** BPs approved for use in malignancy for one or more indications

The backbone structure of BPs allows for number of possible chemical variations by changing the two lateral side chains (R_1 and R_2 positions) attached to the carbon atom (Rogers et al. 2000). The R_1 and R_2 side-chains are responsible for the wide range of activity observed among the BPs (Table 2). R_1 substituents such as a hydroxyl (OH) or amino (NH_2) group enhance binding to bone mineral, conferring on these BPs ability to chelate calcium ions more effectively, while varying the R_2 substituents results in differences in their antiresorptive potency (Shinoda et al. 1983, van Beek et al. 1994, van Beek et al. 1996). Increasing the number of carbon atoms in the side-chain (i.e. the length) will initially increase and then decrease the effect on bone resorption. The increased antiresorptive potency observed with different R_2 groups is linked to their ability to affect biochemical activity, and is thought to also be connected to their ability to bind to HAP (Fleisch 2002, Rogers et al. 2000, Nancollas et al. 2006).

One of the aims of BP research has been to develop compounds with more powerful antiresorptive activity, without any more potent inhibition of mineralization. This has proven to be

possible. Over the past 40 years, several generations of BPs with increasing antiresorptive potency have been developed (Table 2, Fleisch 2000). The early compounds (first-generation) contained simple substituents (H, OH, Cl, CH_3) and lacked a nitrogen atom (they are referred to as non-nitrogen-containing BPs). Subsequently, the more recently developed (second- and third-generation) compounds are more potent and complex and consist of a hydroxyl group together with nitrogen-containing aliphatic side chains or heterocyclic rings (referred as nitrogen-containing BPs). The most active compound described so far, zoledronic acid, is up to 10 000 – fold more potent than etidronate, the first BP used in humans. As shown in Table 2, further substitutions have enabled synthesis of a series of biologically active BPs, each of which has its own distinct chemical, physicochemical and biological characteristics. Therefore, this variability in effect makes it impossible to extrapolate from data for one compound to others with respect to either its use or its actions.

2.2.3 Pharmacokinetics of bisphosphonates

Pharmacokinetically, bisphosphonates represent a unique class of drugs, since it is probably the only class of drugs being developed to be orally administered, although they have very poor bioavailability, generally < 2 % (Lin 1996, Porras et al. 1999). The low intestinal absorption of BPs is generally attributed to their poor lipophilicity, which hampers transcellular transport, and their high negative charge which hinders their paracellular transport. Furthermore, absorption is impaired by the presence of food, calcium, iron, coffee, tea, and orange juice in the gut. However, adsorption occurs very rapidly, probably by passive diffusion through a paracellular pathway (Boulenc et al. 1993, Twiss et al. 1994). Once in the blood, BPs are quickly cleared from plasma, with about 50 % deposited in bone and 50 % excreted in urine (Mönkkönen et al. 1987, Mönkkönen 1988, Mönkkönen et al. 1990, Lin 1996). Consequently, extra-osseous cells are exposed to BPs for only short periods, explaining their bone-specific effects and their low toxicity. BPs are bound to albumin in the blood, which determines their half-life and the kinetics of their elimination via the kidneys (Lin 1996).

BPs bind to Ca^{2+}-containing bone mineral surfaces at sites of active bone remodelling, particularly areas undergoing osteoclastic resorption (Sato et al. 1991, Masarachia et al. 1996). The skeletal retention of BPs is very long, for some BPs it can be over 10 years (Lin 1996, Fleisch 2000). This creates the potential for BPs to have a very long half life in their target tissue, and the potential for a prolonged drug effect. Once the bone is destroyed during bone resorption, BPs are released in high concentrations into the acidic environment (Sato et al. 1991) of the resorption lacunae under the osteoclasts and are taken up by these cells in endocytic vacuoles, probably by fluid-phase endocytosis, and perhaps as complexes with calcium (Thompson et al. 2006). The mechanisms involved in the release of BPs from intracellular vacuoles, such as endosomes or lysosomes, into cytoplasm are not clear, although they seem to require acidification of vacuoles (Thompson et al. 2006). Acidification of endocytic vesicles is required in order for BPs to enter the cytosol, by reducing the negative charge on the phosphonate groups of BPs and thereby allowing either diffusion or transport of BPs across the vesicular membrane. Osteoclasts are able to take up large amounts of BP due to their ability to release the BP from the bone surface during resorption, while non-resorbing cells take up only small amounts of BP that become available due to natural desorption from the bone surface (Coxon et al. 2005, Coxon et al. 2008). BPs inhibit bone resorption by reducing the recruitment and activity of osteoclasts and increasing their apoptosis through recently identified molecular mechanisms (Rogers et al. 2011, Chapter 2.3).

2.2.4 Clinical use

BPs are widely used in the treatment of skeletal diseases associated with high osteoclast activity and accelerated bone turnover, such as Paget's disease (Delmas and Meunier 1997, Roux and Dougados

1999), and postmenopausal osteoporosis (Delmas 2002, Kanis et al. 2008). Furthermore, BPs are effective inhibitors of tumor-induced osteolysis and have been shown to modify the progression of skeletal metastasis in several forms of cancer, especially breast cancer and myeloma (Ross et al. 2004). The most important clinical effect of BPs is the inhibition of bone resorption. Currently, there are at least 8 BPs (Table 2) that are in active therapeutic use and have been registered for clinical applications in many countries.

Paget's disease and osteoporosis

Paget's disease is a localized and progressive disorder of bone, characterized by increased bone remodeling, and abnormal bone structure (Papapoulos 1997). BPs improve the morphology of bone, decrease the number of osteoclasts, and reduce bone pain (Delmas and Meunier 1997, Roux and Dougados 1999). BP therapy is considered to be first-line therapy for the treatment of osteoporosis (Gueldner et al. 2007). Osteoporosis is defined as a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone, which leads to an enhanced fragility of the skeleton. The cause of osteoporosis in postmenopausal women is the lack of estrogen (Delmas 2002, Kanis et al. 2008). BPs act in various types of osteoporosis by decreasing turnover and thus bone loss, and by increasing the degree of mineralization of bone.

Tumor-induced osteolysis

Metastatic bone disease is a major cause of morbidity in cancer patients (Coleman 2004). Bone metastases most commonly result from breast, prostate and lung carcinomas. Multiple myeloma also causes a considerable skeletal morbidity (Kanis and McCloskey 2000). The basis for the use of BPs in tumor-related bone disease is their effect on prevention of hypercalcemia and hypercalciuria (Ross et al. 2004). Tumor-induced osteolysis is a condition in which tumors destroy bone mainly through the production or the induction of bone resorbing cytokines. Localized bone resorption can lead to fractures, pain, and hypercalcemia. BPs reduce both the local destruction of bone near the invading tumor cells, as well as resorption induced by systemically circulating factors. BPs very often diminish bone pain, leading to an improvement in the quality of life. There are some results suggesting that BP's can also diminish the formation of bone metastases in patients who have not yet developed them (Ross et al. 2004).

Side-effects

Generally, BPs are well tolerated, rarely inducing any clinically significant side effects (Adami and Zamberlan 1996, Pazianas and Abrahamsen 2011). The most serious side-effect is renal failure after intravenous administration, which can be avoided by slow intravenous infusion in plenty of fluid. Oral BPs, especially N-BPs, may cause mild gastrointestinal disturbances, and some of them can occasionally cause oesophagitis. Intravenous N-BPs can cause a transient acute phase reaction (APR) with fever, bone and muscle pain that lessens or even disappears after subsequent treatment periods. However, recent reports have described osteonecrosis of the jaw (ONJ) as a potentially serious complication of the long-term use of intravenous pamidronate or zoledronic acid. In this condition, the bone tissue in the jaw fails to heal often after minor trauma, such as a tooth extraction, leaving the bone exposed.

2.3 MOLECULAR MECHANISMS OF ACTION OF BISPHOSPHONATES

2.3.1 Intracellular metabolism of non-N-bisphosphonates to ATP analogs

A breakthrough in understanding the mode of action of the non-nitrogen-containing bisphosphonates (non-N-BPs), such as clodronate, etidronate and tilundronate, on osteoclasts was the discovery that clodronate and other non-N-BPs that closely resemble PPi could be metabolised intracellularly by *Dictyostelium* amoebae to non-hydrolyzable, methylene-containing (AppCp-type) analogs of ATP (Rogers et al. 1992, Pelorgeas et al. 1992). Identification of these ATP analogs was based on FPLC, UV absorbance, and ^{31}P-NMR analysis. Later, a much more sensitive analytical method was developed for the detection of AppCp-type metabolites of non-N-BPs, using ion pairing HPLC electrospray ionization mass spectrometry (ESI-MS) (Auriola et al. 1997). The method was suitable for quantitation of the metabolites at high nanomolar concentrations (Auriola et al. 1997, Mönkkönen et al. 2000). Using this method, it was confirmed that also mammalian cells could metabolise clodronate, etidronate and tilundronate to ATP analogs *in vitro* (Benford et al. 1999, Auriola et al. 1997, Frith et al. 1997, Mönkkönen et al. 2000, Mönkkönen et al. 2001). Importantly, it has been demonstrated that osteoclasts can metabolise clodronate to AppCCl$_2$p also *in vivo* (Frith et al. 2001).

The incorporation of non-N-BPs into ATP analogs is most likely achieved by the reversal of aminoacyl-tRNA synthetase reactions normally involved in activating amino acids during protein synthesis (Rogers et al. 1994, Rogers et al. 1996). Apparently, non-N-BPs resemble PPi sufficiently to be accommodated into the enzyme active site in place of PPi (Rogers et al. 1994). This allows a back-reaction involving the condensation of a BP with adenosine monophosphate (AMP) to form a non-hydrolyzable AppCp-type nucleotide.

The ATP analog of clodronate (AppCCl$_2$p) has been shown to inhibit the mitochondrial adenine nucleotide translocase (ANT), and prevent translocation of ATP across inner mitochondrial membranes (Lehenkari et al. 2002). This is believed to affect mitochondrial permeability triggering caspase activation, which is thought to be an irreversible step on the road to apoptotic cell death (Benford et al. 2001). Induction of osteoclast apoptosis following the intracellular accumulation of such a metabolite, which may reach an intracellular concentration as high as 1 mM (Mönkkönen et al. 2001), appears to be the major mode of action of these BPs (Hughes et al. 1995, Selander et al. 1996, Frith et al. 2001, Benford et al. 2001, Halasy-Nagy et al. 2001, Sutherland et al. 2009).

2.3.2 N-bisphosphonates inhibit FPP synthase in the mevalonate pathway

Newer nitrogen-containing bisphosphonates (N-BPs), such as zoledronic acid (ZOL) and risedronate (RIS), have an unique mechanism of action and greater clinical activity than non-N-BPs. The first clue that helped in the clarification of the mechanism of action of N-BPs appeared in a study of Amin et al. (1992) where novel squalene inhibitors were examined. In that study, N-BPs incadronate, ibandronate, pamidronate, and alendronate inhibited the mevalonate pathway, whereas non-N-BPs clodronate and etidronate were inactive. While incadronate and ibandronate inhibited squalene synthase, pamidronate and alendronate were inactive, yet nonetheless inhibited cholesterol biosynthesis. This suggested the presence of a target upstream in the mevalonate pathway. Several years later the major target was identified as farnesyl pyrophosphate synthase (FPPS) (van Beek et al. 1999a, van Beek et al. 1999b, Bergstrom et al. 2000, Dunford et al. 2001), although some N-BPs were also found to be much weaker inhibitors of other enzymes in the mevalonate pathway, including IPP isomerase (Thompson et al. 2002), squalene synthase (Amin et al. 1992), and GGPP synthase (Szabo et al. 2002b). As many enzymes in the cell utilize phosphate/pyrophosphate compounds, it is not surprising that a BP can have several minor targets. However, more detailed studies demonstrated that N-BPs can cause strong inhibition at nanomolar concentrations only at FPPS, and that there is a significant relationship between the degree of

inhibition of FPPS and the antiresorptive potencies of N-BPs *in vivo* (Dunford et al. 2001, Dunford et al. 2008). Importantly, minor variations in the structure and conformation of the R_2 side chain were shown to lead to major differences in antiresorptive potency (Dunford et al. 2001, Luckman et al. 1998a). These studies have indicated that the ability to inhibit FPPS is an important determinant of the antiresorptive potency of N-BPs. The potency differences among N-BPs can be rationally explained by the observed and predicted binding interactions at the enzyme active site (Kavanagh et al. 2006, Rondeau et al. 2006, Chapter 2.3.3).

2.3.3 Mechanism of inhibition of FPP synthase by N-bisphosphonates

The exact mechanism of inhibition of FPPS is only now becoming clear. Recently, the structure of human FPPS has been elucidated, which has aided in understanding how N-BPs work (Kavanagh et al. 2006, Rondeau et al. 2006). These studies have indicated that the inhibition of FPPS by N-BPs is not a classical enzyme inhibition but is more complicated with the characteristics of "slow-tight binding" inhibition (Figure 3). It has been postulated that N-BPs inhibit the enzyme by a mechanism that involves time-dependent isomerization of the enzyme, which on the molecular scale of events takes a relatively long time to occur (Dunford et al. 2008). Crystallographic studies have revealed that the FPPS enzyme has an open structure when there is nothing bound in either of the two isoprenoid lipid-binding pockets (the GPP/DMAPP pocket) in the enzyme active site (Kavanagh et al. 2006, Rondeau et al. 2006). When N-BP binds its two phosphonate residues via magnesium ions to an aspartic-rich region of the FPPS enzyme, the active site of the enzyme closes in around the inhibitor and a lid is formed over the active site. The nitrogen in the R_2 side-chain of the potent N-BP is known to be critical for this effect and is properly oriented for hydrogen bonding with the amino acid residues in the active site of the FPPS. The conformational change in the enzyme upon binding of the N-BP is followed by a second more complex conformational change after the binding of the second substrate, IPP, to form a tightly bound inhibition complex that provides further stabilization of the FPPS and N-BP complex. Therefore, IPP is able to shield the N-BP in the active site from the substrate (DMAPP or GPP) that would compete with the N-BP, removing it from the enzyme and thus reversing the inhibition (Rondeau et al. 2006). In this way, prolonged inactivation of the mevalonate pathway can be achieved. The associated conformational change can be measured as an isomerization constant, K_{isom}, and this further magnifies the binding differences of the BPs with the most potent compounds, such as ZOL and RIS, displaying nearly irreversible binding characteristics (Dunford et al. 2008). The exceptional potency of N-BPs to inhibit FPPS is important because it is likely that only small amounts of internalized N-BPs enter the cytosol to become available for inhibition of FPPS (Thompson et al. 2006).

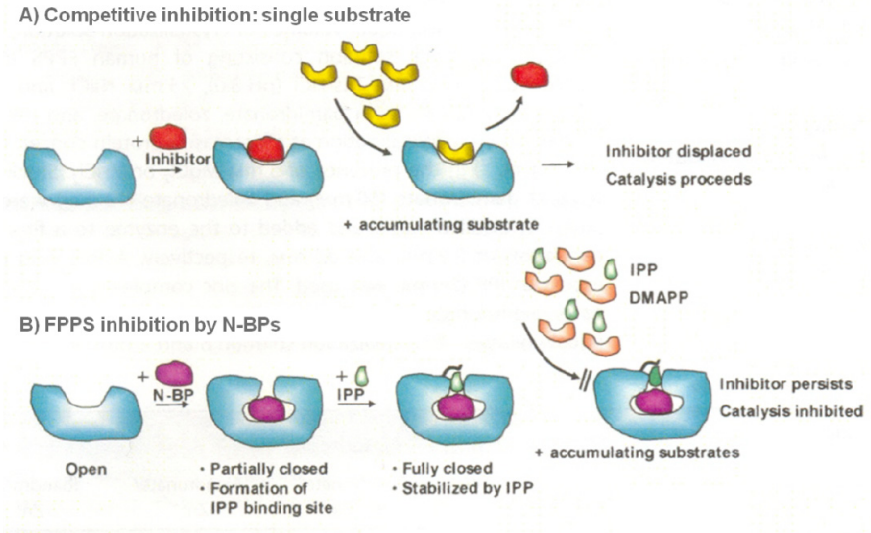


Figure 3. Illustration depicting the high *in vivo* efficacy of FPPS inhibition by N-BPS. A) In a competitive inhibition mechanism as with most enzymes, the accumulation of substrate eventually displaces inhibitor, leading to resumption of catalytic activity. B) With FPPS, the accumulating substrate (IPP) binds to and further stabilizes the FPPS-N-BP complex, thereby preventing the other substrate (DMAPP) from accessing and displacing the N-BP inhibitor. Suppression of catalysis is thus efficient and sustainable. Modified from Rondeau et al. 2006.

2.3.4 Inhibition of protein prenylation

By inhibiting FPPS enzyme in the mevalonate pathway, N-BPs block the synthesis of isoprenoids, FPP and its downstream metabolite GGPP, which are required for the prenylation of GTPases such as Ras, Rho, Rac and Rab (Zhang and Casey 1996, Sinensky 2000). GTPases regulate a variety of cellular processes which are important for osteoclast function, i.e. ensuring correct cell morphology, integrin signaling, membrane ruffling, transport of endosomes, and apoptosis (Rogers et al. 2000). Prenylation of GTPases is necessary for their correct subcellular localization and hence their biological function. At the molecular level, the inhibition of protein prenylation by N-BPs can be demonstrated by measuring the incorporation of [^{14}C]mevalonate into farnesylated and geranylgeranylated proteins (Benford et al. 1999, Luckman et al. 1998a, Luckman et al. 1998b). More recently, a western blot technique for the detection of the unprenylated form of the small GTPase Rap1A has become extensively utilized, since unprenylated Rap1A acts as a surrogate marker for inhibition of FPPS and accumulates in cells exposed to N-BPs (Frith et al. 2001, Reszka et al. 2001). However, there is no direct correlation between the level of unprenylated Rap1A and the extent of apoptosis (Mitrofan et al. 2010).

The inhibition of protein prenylation is assumed to be one of the principal mechanisms by which N-BPs exert their effects. By inhibiting FPPS, N-BPs cause a depletion of prenylated proteins and an accumulation of unprenylated proteins within the cell, and consequently induce a series of changes leading to decreased activity and indirect cell death by apoptosis not only in osteoclasts, but also in other cell types (Benford et al. 1999, Shipman et al. 1998, Coxon et al. 2000, Luckman et al. 1998a, Luckman et al. 1998b, Reszka et al. 1999, Coxon et al. 2004). In osteoclasts, N-BPs induce changes in the cytoskeleton, such as loss of the ruffled border and disruption of actin rings, leading to inactivation of these cells (Rogers et al. 2000). Eventually osteoclasts may undergo apoptosis, but this does not appear to be mandatory for inhibition of resorption (Halasy-Nagy et al. 2001). In tumor cells, N-BPs activate apoptosis, and inhibit tumor cell proliferation, adhesion, migration, and invasion *in vitro* (Clezardin 2011). Most of these antitumor effects are due to inhibition of protein

prenylation because the effects of N-BPs can be largely overcome by replenishing cells with isoprenoid substrates, GGOH and FOH, which store geranylgeranylation and farnesylation, respectively. Overall, inhibition of geranylgeranylated proteins seems more important than inhibition of farnesylated proteins. It has been demonstrated that loss of farnesylated proteins in osteoclasts has little biological consequences, whereas loss of geranylgeranylated proteins causes disruption of actin rings, inhibits bone resorption and stimulates apoptosis (Coxon et al. 2000), emphasizing the fundamental importance of geranylgeranylated small GTPases rather than farnesylated proteins in osteoclasts. Furthermore, in a number of studies, the effects of N-BPs on osteoclasts, and other cell types have been overcome *in vitro* by bypassing the mevalonate pathway and replenishing cells with a substrate that can be used for protein geranylgeranylation (Goffinet et al. 2006, Jagdev et al. 2001, Halasy-Nagy et al. 2001, Reszka et al. 1999, Coxon et al. 2004, Fisher et al. 1999, van beek et al. 1999c, van Beek et al. 2002, van Beek et al. 2003, Sonnemann et al. 2007). Additionally, a later study demonstrated that the effect of N-BPs *in vitro* could be mimicked by GGTI-298, a specific inhibitor of protein geranylgeranylation (Coxon et al. 2000, Benford et al. 2001). It is unclear whether the importance of geranylgeranylated proteins is cell-type specific, or whether the lack of reversal of BP effects by isoprenoid substrates is specific to certain BPs. Overall, the consequences of blockade of protein prenylation may be more complex than simply loss of function, since a recent study has suggested that the effect of N-BPs on cells may actually be due to accumulation of unprenylated GTPases in their active state, GTP-bound form (Dunford et al. 2006). Therefore, it is possible that N-BPs may exert their effects through inappropriate and sustained stimulation, rather than inhibition, of GTPase-mediated downstream signaling pathways.

2.3.5 Accumulation of IPP and the formation of a cytotoxic ATP analog ApppI

In addition to depletion of the biosynthesis of FPP and GGPP, the inhibition of FPPS by N-BPs results in the accumulation of the early metabolites of the mevalonate pathway, IPP (Mönkkönen et al. 2006) and its isomer DMAPP (Jauhiainen et al. 2009) (Figure 4). IPP becomes conjugated to adenosine- 5′-monophosphate (AMP) to form a novel ATP analog ApppI [triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester] in cells, such as macrophages and osteoclasts (Mönkkönen et al. 2006, Mönkkönen et al. 2007). Simultaneously, the accumulation of isomeric DMAPP appears to lead to the formation of ApppD [triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-2-enyl) ester] (Jauhiainen et al. 2009). However, for the sake of clarity, IPP/ApppI will be used to describe both IPP/DMAPP and ApppI/ApppD formation throughout the text. ApppI formation from IPP is believed to be catalyzed by the aminoacyl-tRNA synthetases (Mönkkönen et al. 2006), the same enzymes that metabolise non-N-BPs into AppCp-type analogs of ATP (Rogers et al. 1996). Instead of BP, ApppI contains IPP in its structure (Figure 4). This represents a novel and a very interesting metabolic concept, since there are no other drugs known to produce molecules in the cells, which are not naturally present there, but are not metabolites of the drug either. The order of potency of N-BPs to induce IPP/ApppI production (Mönkkönen et al. 2006) has been shown to be identical to their order of inhibitor potency of FPPS (Dunford et al. 2001). Importantly, the amount of IPP/ApppI in cells has been shown to correlate with N-BP-induced apoptosis *in vitro* (Mitrofan et al. 2009). Like the AppCp-type metabolite of non-N-BPs, ApppI is able to inhibit the mitochondrial ANT in a cell free system, which is an additional potential mechanism for BP-induced apoptosis (Mönkkönen et al. 2006). Thus, although distinct molecular mechanisms are involved, the non-N-BPs and N-BPs both induce the formation of an ATP analog capable of promoting apoptosis (Figure 4).

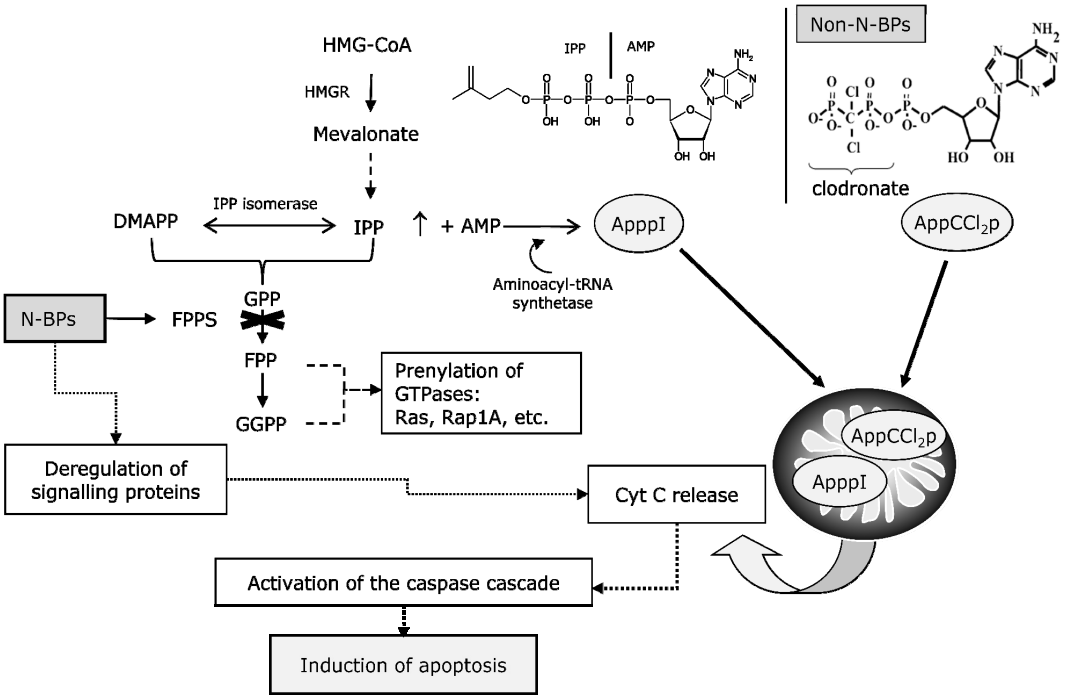


Figure 4. N-BPs inhibit specifically the activity of FPPS in the mevalonate pathway, thereby preventing the prenylation of GTPases that are essential for cell function and survival. In addition, inhibition of FPPS leads to the accumulation of IPP, which is incorporated into an ATP analog ApppI that can induce apoptosis in a similar manner to the AppCp-type metabolite of non-N-BPs by inhibiting the mitochondrial function.

2.3.6 The immunomodulatory effects of N-bisphosphonates

A particular subset of human gamma-delta T-cells in peripheral blood, Vγ9Vδ2 T-cells, play an important role in immune system surveillance and defence. N-BPs have been reported to stimulate the expansion of Vγ9Vδ2 T-cells through inhibition of FPPS and subsequent accumulation IPP and DMAPP (Kunzmann et al. 1999, Kunzmann et al. 2000). These metabolites are known to be ligands for a receptor on the Vγ9Vδ2 T-cells (TCR) (Morita et al. 1995, Tanaka et al. 1995, Gober et al. 2003). The exact mechanisms through which Vγ9Vδ2 T-cells become activated by IPP/DMAPP are unclear, but are most likely γδ TCR-mediated and require cell-to-cell contact with antigen-presenting cells (such as macrophages, dendritic cells, and B-cells). It has been suggested that, F1-ATP synthase, which is a mitochondrial enzyme that is translocated to the cell surface, could behave like an antigen-presentation molecule for the Vγ9Vδ2 TCR (Moogerjee-Basu et al. 2010). IPP/DMAPP could possibly bind to F1-ATP synthase in order to be recognized by the Vγ9Vδ2 TCR. Following activation by IPP/DMAPP, Vγ9Vδ2 T-cells produce and release pro-inflammatory cytokines such as tumor necrosis factor-*alpha* (TNF-α), chemokines, and growth factors. Additionally, very recent evidence has shown that IPP may also be secreted, promoting Vγ9Vδ2 T-cell activation (Benzaid et al. 2011). By contrast, ApppI appears to have little stimulatory activity on human Vγ9Vδ2 T-cells, but it could represent an inactive storage form of phosphoantigen which would require conversion to IPP to activate γδ T-cells (Vantourout et al. 2009). The cause of the acute phase reaction (APR), a common adverse effect after intravenous administration, to N-BPs may be a result of the accumulation of IPP/DMAPP in monocytes (Roelofs et al. 2009, Thompson et al. 2010). Statins are able to prevent N-BP-induced IPP/DMAPP accumulation, and thus may potentially diminish or even prevent the APR to N-BPs (Thompson and Rogers 2004).

2.4 POTENTIAL NEW INDICATIONS FOR BPS

2.4.1 Antitumor actions of BPs

BPs have been demonstrated to prevent skeletal related events in patients with advanced malignancies involving bone across tumor types: multiple myeloma, prostate cancer, breast cancer, lung cancer, renal cancer and other solid tumors (Woodward and Coleman 2010). In addition to the effects of BPs on bone, there is extensive evidence from preclinical research accumulating that BPs, particularly N-BPs, exert antitumor activity without the involvement of osteoclasts by interacting with endothelial cells, tumor cells and tumor-associated macrophages, and by stimulating Vγ9Vδ2 T-cells (Clezardin 2011, Stresing et al. 2007, Roelofs et al. 2010). BPs exert their anticancer actions either by direct effects on tumor cells, especially when administered with cytotoxic agents, or indirectly by affecting bone resorption, thereby reducing tumor-cell migration to the bone, or possibly both mechanisms (Figure 5). Although the antitumor effects of BPs are beginning to be elucidated, the relative contributions of direct and indirect effects of BPs on tumors are still debated.

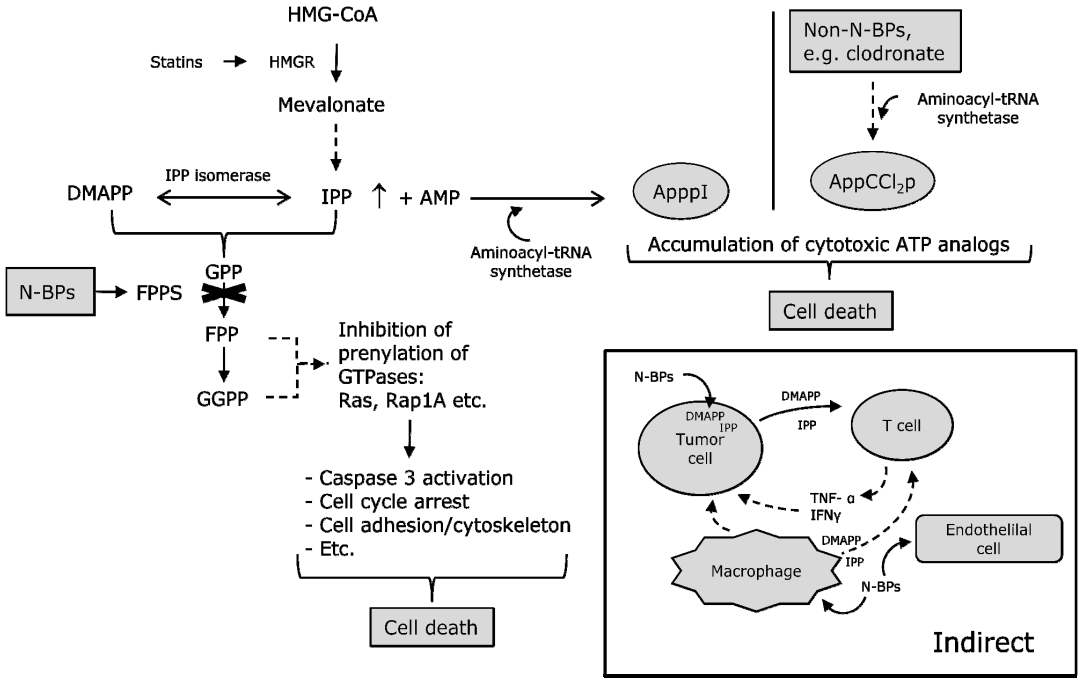


Figure 5. Direct and indirect effects of BPs on bone tumor cells. N-BPs inhibit the formation of FPP and GGPP, which are required for the prenylation of small GTPases that regulate a variety of important cell processes. Concurrently, N-BPs evoke cell death through the accumulation of cytotoxic ATP analog, ApppI. They also exert indirect effects by inhibiting angiogenesis, and activating a cytotoxic T cell mediated immune response through the IPP/DMAPP produced by tumor cells and tumor-associated macrophages. The non-N-BPs have been shown to have cytotoxic and antiproliferative effects on macrophages and tumor cells through the accumulation of cytotoxic analogs of ATP.

Indirect effects

As inhibitors of bone resorption, BPs reduce tumor-induced osteolysis and local release of cytokines and growth factors providing the bone with a less favorable microenvironment for tumor cell migration, adhesion and invasion, proliferation and survival (Fromigue et al. 2003, Denoyelle et al. 2003b, Santini et al. 2003). More recently, antiangiogenic effects of N-BPs have been discovered. The process of angiogenesis, the growth of new blood vessels from pre-existing vessels, is paramount for cancer cell growth and spread (Clezardin 2011). N-BPs inhibit angiogenesis by interfering with endothelial cell migration, proliferation and tube formation (Wood et al. 2002, Fournier et al. 2002, Bezzi et al. 2003, Santini et al. 2003, Santini et al. 2007, Stresing et al. 2011). Furthermore, it has been shown that N-BPs may have direct antiangiogenic effects on intra-tumor endothelial cells by inhibiting Rho geranylgeranylation (Hashimoto et al. 2007).

Another suggested indirect antitumor effects of N-BPs include immunomodulatory activities (Clezardin and Massaia 2010). N-BPs have the ability to target cancer cells by generating of a potentially cytotoxic γδ T-cell mediated immune response. Thus, N-BPs could be considered as immunotherapy agents. Activation of γδ T-cells requires cell-to-cell contacts with tumor cells and peripheral blood mononuclear cells, like monocytes and macrophages (Green et al. 2004). The accumulation of IPP/DMAPP in cancer cells after N-BP-stimulation is possibly the crucial step in the activation and proliferation of γδ T-cells (Morita et al. 1995, Tanaka et al. 1995, Gober et al. 2003). As a response to this activation, γδ T-cells secrete cytokines (Kunzmann et al. 2000, Wilhelm et al. 2003) enhancing antitumor activity perhaps by inhibiting tumor cell growth, blocking angiogenesis, or by stimulation of macrophages (Boehm et al. 1995). Vγ9Vδ2 T-cells activated by N-BPs, in particular of zoledronic acid, have been shown to exert a potent antitumor effect *in vitro* (Mattarollo et al. 2007, Watanabe et al. 2006, Muraro et al. 2007, Marten et al. 2007). Furthermore, *in vitro* expanded Vγ9Vδ2 T-cells maintained their antitumor activity *in vivo* upon adoptive transfer into immunodeficient mice transplanted with human tumor cells when this therapy was combined with N-BP treatment (Kabelitz et al. 2004, Sato et al. 2005). In contrast, non-N-BPs have failed to show this stimulatory effect on γδ T-cells.

Direct effects

There is extensive data to suggest that BPs are able to directly affect tumor cells, with zoledronic acid being particularly potent in this respect. However, BPs do not appear to have any direct effect on non-malignant cells (Senaratne et al. 2000). Several *in vitro* studies have shown that N-BPs inhibit adhesion of tumor cells to extracellular matrix proteins, impairing the process of tumor-cell invasion and metastasis (Denoyelle et al. 2003b, van der Pluijm et al. 1996, Boissier et al. 1997, Boissier et al. 2000, Virtanen et al. 2002), whereas non-N-BPs have little or no effect. One of the primary mechanisms responsible for the direct antitumor activity of BPs is induction of cell apoptosis. However, the exact mechanisms and mediators of N-BP-induced apoptosis are currently unknown.

Both N-BPs and non-N-BPs inhibit proliferation and induce apoptosis, *in vitro*, of a variety of human tumor cell types, including those causing breast (Jagdev et al. 2001, Senaratne et al. 2000, Senaratne et al. 2002, Senaratne and Colston 2002, Fromigue et al. 2000, Hiraga et al. 2001) prostate (Sonnemann et al. 2007, Lee et al. 2001, Oades et al. 2003), osteosarcoma (Mackie et al. 2001, Kubista et al. 2006), and myeloma (Shipman et al. 1998, Shipman et al. 1997). N-BP-induced apoptosis has been shown to be mediated through mitochondrial pathways associated with cytochrome c release (Senaratne and Colston 2002,) and caspase activation (Senaratne et al. 2000, Senaratne and Colston 2002, Fromigue et al. 2000, Hiraga et al. 2001, Oades et al. 2003, Dunford et al. 2006, Sonnemann et al. 2007). Furthermore, induction of apoptosis has been confirmed by identification of the morphological changes, including cellular shrinkage, chromatin condensation, and DNA

fragmentation (Shipman et al. 1998, Shipman et al. 1997, Senaratne et al. 2000, Mackie et al. 2001, Senaratne and Colston 2002, Sonnemann et al. 2007). Interestingly, the very recent study of Mitrofan et al. (2010) has identified mechanisms for zoledronic acid-induced apoptosis revealing the interplay between mitochondria and lysosomes. That study suggested that lysosomal break down with cathepsin release could be a consequence of mitochondrial damage, and thus lysosomes could be playing an additional/amplification role in zoledronic acid- induced apoptosis.

Overall, the potency of direct antitumor effects of N-BPs *in vitro* generally reflects the antiresorptive potency *in vivo,* indicating that underlying inhibitory mechanisms act primarily through the blockade of the mevalonate pathway (Boissier et al. 1997, Boissier et al. 2000). In addition, many of the N-BP-induced antitumor effects can be largely overcome by replenishing cells with isoprenoid substrates (FOH or GGOH) required for protein prenylation, evidence for the involvement of this pathway in the mechanisms of action (Stresing et al. 2007). The sensitivity of different cell types to N-BPs most likely depends largely on their ability to internalize sufficient amounts of N-BP to inhibit FPPS. Table 3 gives an overview of preclinical studies demonstrating that the inhibition of the mevalonate pathway is the fundamental molecular mechanism behind many of the observed direct anticancer effects of N-BPs.

Table 3. Preclinical evidence for anticancer properties of BPs, related to the inhibition of mevalonate pathway

BP	Cancer model	Effect(s)	Reference
Incad	*In vitro*, myeloma	Apoptosis, inhibition of proliferation. Effects reversed by GGOH and FOH.	Shipman et al. 1998
Zol	*In vitro*, breast	Apoptosis. Effects reversed by GGOH.	Jagdev et al. 2001
Alend	*In vitro*, prostate	Inhibition of invasion and migration. Effects reversed by GGOH and FOH.	Virtanen et al. 2002
Zol	*In vitro*, breast	Apoptosis. Effects reversed by FOH and partially GGOH.	Senaratne et al. 2002
Zol	*In vitro*, breast	Inhibition of invasion. Effects reversed by GGOH.	Denoyelle et al. 2003
Zol	*In vitro*, osteosarcoma	Apoptosis and cell cycle arrest. Effects reversed by GGOH.	Evdokiou et al. 2003
Zol	*In vitro*, pancreatic	Growth inhibition and apoptosis.	Tassone et al. 2003
Incad	*In vitro*, myeloma	Apoptosis, cell cycle arrest. Effects reversed by GGOH and FOH.	Iguchi et al. 2003
Zol	*In vitro*, prostate	Apoptosis. Effects reversed by FOH.	Oades et al. 2003
Zol	*In vitro*, prostate	Apoptosis and inhibition of invasion. Effects suppressed by GGOH.	Coxon et al. 2004
Pam	*In vitro*, hepatoma	Growth inhibition and apoptosis.	Wada et al. 2006
Zol	*In vitro*, prostate	Inhibition of proliferation. Effects reversed by GGOH.	Goffinet et al. 2006
Zol	*In vitro*, prostate	Apoptosis. Effects reversed by GGOH.	Sonnemann et al. 2007
Zol	*In vitro*, osteosarcoma	Apoptosis, cell cycle arrest.	Kubista et al. 2006
Ris, Zol	*In vitro*/*vivo*, mesothelioma	Inhibition of growth and protein prenylation. Effects reversed by GGOH in vitro.	Wakchoure et al. 2006
Zol	*In vitro*, breast	Inhibition of protein prenylation. Effects reversed by FOH.	Brown et al. 2009
Zol	*In vivo*, myeloma	Apoptosis, inhibition of protein prenylation.	Guenther et al. 2010
Zol	*In vitro,* lymphoma	Apoptosis. Effects suppressed by GGOH.	Mitrofan et al. 2010
Zol	*In vitro*, prostate	Induction of autophagy by depleting cellular GGPP.	Wasko et al. 2011

Combination treatments

Cancer patients often receive BPs at the same time as other antitumor agents. Preclinical studies have demonstrated that combining N-BPs with a variety of standard chemotherapy agents, such as doxorubicin and paclitaxel, results in additive or even synergistic antitumor effects against a range of tumor cell lines, as demonstrated by a reduction of tumor cell invasion, inhibition of growth and induction of apoptosis (Jagdev et al. 2001, Tassone et al. 2000, Witters et al. 2003, Neville-Webbe et al. 2005, Ullen et al. 2005, Woodward et al. 2005, Ottewell et al. 2008, Clyburn et al. 2010). This synergistic activity seems to be sensitive to the sequence and schedule of the dosing of these compounds. Furthermore, there are several *in vitro* studies providing evidence for additive or synergistic apoptotic effects of N-BPs in combination with molecularly targeted agents such as FTIs and GGTIs, and the aromatase inhibitor, letrozole (Caraglia et al. 2004, Lau et al. 2011, Neville-Webbe et al. 2010). These observations may be of clinical relevance, particularly in breast cancer patients receiving these drugs in an adjuvant setting.

Clinical evidence

The clinical relevance of the antitumor effects of BPs observed *in vitro* and *in vivo* is not yet clear, although attempts have been made to mimic the human situation of metastatic disease using specially designed animal models of bone metastases (Ottewell et al. 2006). One major shortcoming of most animal studies is the use of high BP dosages which greatly exceed the clinical dosing regimens given to patients with bone metastases. However, recent findings indicate that a continuous or frequent low-dose therapy with BPs might affect tumor cells directly, by prolonging the exposure time of bone-residing tumor cells to the drug, whereas BP therapy with longer dosing intervals only leads to the inhibition of bone resorption in animals (Daubine et al. 2007). Based on the potential anticancer properties observed in preclinical research, several clinical trials have been initiated to test the efficacies of BPs in various oncology settings (Woodward and Coleman 2010). Now, there is clinical evidence that the addition of zoledronic acid to endocrine therapy in the adjuvant setting can improve disease-free survival in premenopausal women with estrogen-responsive early breast cancer, indicating that BPs exert clinically-significant antitumor effects (Gnant et al. 2009). Furthermore, oral BP use has been also recently associated with significantly lower invasive breast cancer incidence in postmenopausal women (Chlebowski et al. 2010), again suggesting that BPs may have inhibitory effects on breast cancer. As the other trials start to report over the next few years, many profound questions relating to the potential antitumor effects of BPs as s clinical combination therapy will hopefully be answered.

2.4.2 Other applications

Targeting BPs to cells other than osteoclasts, monocytes and macrophages will be likely restricted by their rapid targeting to bone surfaces and hence their low bioavailability. Therefore, development of new BP analogs with a lower affinity for bone mineral compared with the parent N-BPs could increase drug accessibility to other cells and offer therefore the prospect of new classes of FPPS inhibitors which may be of clinical use as antitumor or possess even more diverse therapeutic applications. Furthermore, changes to the structure of N-BPs might give rise to compounds capable of inhibiting other enzymes in the mevalonate pathway, such as Rab GGTase (Coxon et al. 2001, Coxon et al. 2005, Roelofs et al. 2006, Fournier et al. 2008, Baron et al. 2009, McKenna et al. 2010). In addition to using BP analogues, the activity of BPs may be enhanced by using novel formulations, such as liposomes (Zeisberger et al. 2006, Shmeeda et al. 2010, Marra et al. 2011), nanoparticles (Salzano et al. 2011) or cyclodextrins (Daubine et al. 2009). These formulation methods could increase the availability of BPs in extra-bone tissues and improve the plasma half-lives of the drugs. Furthermore, BPs with high affinity to bone mineral, are ideal targeting agents for delivery to bone

after systemic administration of other therapeutic agents, such as proteins (Bansal et al. 2005), antibiotics (Tanaka et al. 2010), and anti-inflammatory drugs (Hirabayashi et al. 2002).

BPs could well have specific anti-inflammatory and analgesic effects in rheumatic diseases by inhibiting the production of pro-inflammatory cytokines, nitric oxide and prostaglandins (Pennanen et al. 1995, Makkonen et al. 1996, Le Goff et al. 2010). BPs have also been shown to be efficacious against certain parasites (Martin et al. 2001, Martin et al. 2002, Yardley et al. 2002, Ling et al. 2005), and thus numerous new BPs have been synthesized and tested to determine the structure-activity relationships for the inhibition of parasitic FPP and GGP synthases (Szabo et al. 2002b, Ling et al. 2005, Szabo et al. 2002a, Sanders et al. 2003). Furthermore, there seems to be a potential for use of N-BPs as antiviral drugs. N-BPs have been shown to decrease viral activity through prenylation dependent (Ishikawa et al. 2007) and independent pathways (Masaike et al. 2010).

3 Aims of the study

The general objective of this study was to characterize bisphosphonate-induced IPP/ApppI accumulation in cancer cell cultures and in an animal model. The specific aims were as follows:

1. To investigate BP-induced IPP accumulation and ATP analog formation in various cancer cell lines, and to evaluate the role of apoptotic ATP analogs in the anticancer properties of BPs.

2. To obtain detailed data, such as time-course and dose-dependence, on N-BP-induced IPP/ApppI formation in cultured cancer cells.

3. To explore N-BP-induced ApppI formation *in vivo* in osteoclasts, the pharmacological target cells for BPs.

4. To identify the specific intermediates and enzymes of the mevalonate pathway involved in regulating the BP-induced IPP/ApppI formation in cancer cells.

4 General experimental procedures

4.1 REAGENTS

All BPs used in the experiments are described in Table 4. The stock solutions of BPs were prepared in phosphate-buffered saline (PBS; pH 7.4; Gibco, Paisley, UK) and filter-sterilized before use. AppppI and AppCCl$_2$p were synthesized as previously described (Mönkkönen et al. 2006). IPP, DMAPP, AppCp, BSA and sodium orthovanadate were from Sigma Chemical Co. (St. Louis, MO, USA). Sodium fluoride was from Riedel-de-Haën (Seelze, Germany). HPLC-grade methanol was from J.T. Baker (Deventer, The Netherlands) and dimethylhexylamine (DMHA) was obtained from Sigma Aldrich, (Milwaukee, WI, USA). The cell culture reagents were from Biowhittaker (Cambrex Bio Science, Verviers, Belgium) and Invitrogen (Paisley, UK). Plastics were supplied by Nunc (Roskilde, Denmark) and Costar (High Wycombe, UK). All the reagents used were of analytical grade or better.

Table 4. The BPs used in the experiments

BP	Chemical name	Supplier	Used in Chapter
Zoledronic acid (ZOL) and [^{14}C]ZOL [a]	2-(imidazol-1-yl)-hydroxy-ethylidene-1,1-bisphosphonic acid, disodium salt, 4.75 hydrate	Novartis Pharma AG (Basel, Switzerland)	5-8
Risedronate (RIS)	2-(3-pyridinyl)1-hydroxyethylidene-bisphosphonic acid	Procter & Gamble Pharmaceuticals (Cincinnati, OH, USA)	8
Clodronate (CLOD) and [^{14}C]CLOD [a]	dichloromethylene-1,1-bisphosphonate	Schering Oy (Bayer Schering Pharma AG, Berlin, Germany)	5-7
NE58027	piperidine hydroxy-ethyl-1,1-bisphosphonic acid	Procter & Gamble Pharmaceuticals	8
NE58043	3-pyrethane-1,1-bisphosphonic acid	Procter & Gamble Pharmaceuticals	8
NE11808	2-pyridylaminoethane-1,1-bisphosphonic acid	Procter & Gamble Pharmaceuticals	8
NE97220	N-(3-picoly)aminomethane-1,1-bisphosphonic acid	Procter & Gamble Pharmaceuticals	8
NE21650	2-aminophenyl-2-ethane-1-hydroxy-1,1-bisphosphonic acid	Procter & Gamble Pharmaceuticals	8

[a] Used in Chapter 5 and 6

4.2 CELL LINES AND CULTURE

The experiments were performed using the cell lines described in Table 5. All cell lines were maintained in 75-cm^2 flasks at 37 °C in a 5 % CO_2 atmosphere. All other cells, except MCF-10A, were cultured in RPMI-1640 media with L-glutamine, supplemented with 10 % of fetal calf serum, 100

U/ml penicillin and 100 μg/ml streptomycin. MCF-10A cells were cultured in DMEM:F12 (1:1) (Invitrogen, Paisley, UK), supplemented with 5 % of horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 100 ng/ml Cholera toxin (Calbiochem, Darmstadt, Germany), 20 ng/ml EGF (Sigma, St. Louis, MO), 0.1 μM hydrocortisol (Sigma) and 10 μg/ml insulin (Sigma). Cells were harvested using trypsin, except for MDA-MB-436 cells which were harvested by scraping.

Table 5. The cell lines used in the experiments

Cell line	Description	Supplier
MCF-7	Human estrogen-dependent breast adenocarcinoma	European Collection of Animal Cell Cultures (Salisbury,UK)
MDA-MB-436 [a]	Human estrogen-independent breast adenocarcinoma	American Type Culture Collection (Manassas, VA)
MG-63 [a]	Human osteosarcoma	European Collection of Animal Cell Cultures
NCI-H929 [a]	Human myeloma	European Collection of Animal Cell Cultures
RPMI-8226 [a]	Human myeloma	European Collection of Animal Cell Cultures
PC-3 [a]	Human androgen-independent prostate cancer cell line	American Type Culture Collection
MCF-10A [a]	Human non-malignant breast cell line	American Type Culture Collection

[a] Used only in Chapter 5

4.3 PREPARATION OF CELL LYSATES FOR MASS SPECTROMETRIC DETECTION

Sample collection was performed by carefully scraping the cultured cells off from the 6-well culture plates and washing them in ice-cold PBS. The analytes were extracted from the cell samples by adding 300 μl of ice-cold acetonitrile and either 200 μl of water (Chapters 5 and 6) or water (200 μl) containing sodium orthovanadate and sodium fluoride as phosphatase inhibitors (Chapters 7 and 8). The soluble (analyte) and precipitated (protein) fractions were separated by centrifugation (13 000g, 2 min, + 4°C). The supernatant extract was transferred to a new tube and evaporated using vacuum centrifugation. Dried samples were stored at -70 °C until mass spectrometric analysis. Protein content determinations were performed as previously described (Mönkkönen et al. 2003).

4.4 MASS SPECTROMETRY ANALYSIS

For analysis, the evaporated cell extracts were redissolved in 150 μl or 200 μl (*in vivo* samples in Chapter 6) of water containing 5 μM internal standard (AppCp) to compensate for the variability in ionization. In Chapters 5 and 6, phosphatase inhibitors, sodium fluoride (0.25 mM) and sodium orthovanadate (0.25 mM), were added to prevent degradation of IPP and ApppI. The molar amounts of IPP, ApppI, and AppCCl$_2$p in cell extracts were determined as previously described (Mönkkönen et al. 2000 and 2007) by a high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) system in negative ion mode. Detection was performed either by a Finnigan LTQ quadrupole ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA) (Chapters 5 – 7), or by an Agilent triple quadrupole mass spectrometer (Agilent 6410 Triple Quad LC/MS, Agilent Technologies, Santa Clara, CA, USA) (Chapter 8), both equipped with an electrospray ionization source. Selected reaction monitoring (SRM) was used for analysis of the compounds in the sample and quantitation was based on the fragment ions

characteristic of each molecule. The following transitions were monitored: m/z 245 → 159 for IPP, m/z 574 → 408 for ApppI, m/z 572 → 225 and m/z 574 → 227 for $AppCCl_2p$ (^{35}Cl & ^{37}Cl) and, m/z 504 → 406 for internal standard. The pattern of fragmentation for each sample was compared with that of the authentic standard. Standards were constructed by adding known amounts of synthetic IPP, ApppI, or $AppCCl_2p$ to extracts from untreated cells. The concentrations of the samples were determined using the peak areas of the SRM chromatograms and the standard curve. Quantitation was done either with LCquan 2.0 software (Thermo Finnigan) (Chapters 5-7), or Agilent Technologies MassHunter Work Station Software for Triple Quad Version B.01.03 (Agilent, Waldbronn, Germany) (Chapter 8), using the standard curve and the transitions mentioned above.

4.5 CELLULAR UPTAKE (CHAPTERS 5 AND 6)

For studies on the cellular uptake of BPs, the cells were treated with 25 μM [^{14}C]ZOL or 500 μM [^{14}C]CLOD. After treatment, medium was recovered, and the wells were rinsed five times with PBS solution. All wash-solutions were collected. Finally, the cells were carefully scraped off from the wells (two wells were pooled together) and extracted with acetonitrile (300 μl) and water (200 μl). The soluble and precipitated protein fractions were separated by centrifugation (13000g, 1 min). Cell precipitates were digested with 1 ml of 1 M sodium hydroxide at 60 °C for 2 hours. The protein content determinations were carried out as previously described (Mönkkönen et al. 2003). The soluble ACN/water extracts were evaporated in a vacuum centrifuge. For the radioactivity measurements, the evaporated samples were redissolved in 120 μl of Milli-Q water. Radioactivity in the cell medium, washes and cell extracts was determined by using liquid scintillation counting (Wallac Microbeta™ TriLux) after mixing with OptiPhase HiSase3 scintillation cocktail (Wallac).

*5 Bisphosphonate–induced ATP analog formation and its effect on inhibition of cancer cell growth **

Abstract. Recent studies have demonstrated that BPs inhibit growth, attachment and invasion of cancer cells in culture and promote apoptosis. The mechanisms responsible for the observed anticancer effects of BPs are beginning to be elucidated. The aim of this study was to investigate BP-induced ATP analog formation and its effect on cancer cell growth. Seven different cell lines were used to evaluate ZOL-induced IPP/ApppI accumulation and inhibition of protein prenylation, and clodronate metabolism to analog of ATP. The inhibition of cell growth by BPs were studied in three selected cancer cell lines with diverse potency to produce ATP analogs. As a result, marked differences in ZOL-induced IPP/ApppI formation and clodronate metabolism between the cancer cell lines were observed. However, these differences were not due to differences in uptake of BPs into the cells. Thus, the production of cytotoxic ATP analogs in cancer cells after BP treatment is likely to depend on the activity of enzymes, such as FPPS or aminoacyl-tRNA synthetase, responsible for the ATP analog formation. Additionally, the potency of clodronate to inhibit cancer cell growth corresponds to ATP analog formation.

*Adapted with permission of Wolter Kluwer Health from: Mönkkönen H, Kuokkanen J, Holen I, Evans A, Lefley DV, Jauhiainen M, Auriola S, Mönkkönen J: Bisphosphonate–induced ATP analog formation and its effect on inhibition of cancer cell growth. Anti-cancer drugs 19:391-399, 2008.

5.1 INTRODUCTION

The molecular mechanisms whereby BPs inhibit bone resorption are well established, whereas the mechanisms underlying anticancer activity of these compounds remain to be clarified. As BPs are used to treat tumor-induced bone disease in multiple myeloma, breast and prostate cancer we investigated the role of apoptotic ATP analogs induced by BPs in anticancer properties of these drugs. Thus, IPP/ApppI accumulation induced by ZOL, protein prenylation and clodronate (CLOD) metabolism to $AppCCl_2p$ were explored in various cancer cell lines. This was also expected to clarify the formation mechanism of ApppI from IPP, and the role of aminoacyl-tRNA-synthetase in the reaction. The cellular uptake of ZOL and CLOD in selected cancer cell lines was investigated in order to determine whether there is a correlation between drug uptake and subsequent ATP analog accumulation. Additionally, the inhibition of cell growth by ZOL and CLOD in selected cancer cell lines was studied in order to elucidate the role of ATP analogs in cancer cell growth. This is the first study to show that BPs induce the accumulation of ATP analogs, ApppI and $AppCC_2p$ in cancer cells.

5.2 EXPERIMENTAL

The reagents and cell lines used in this study are described in Chapter 4.1 and Chapter 4.2, respectively.

5.2.1 IPP/ApppI production and metabolism of clodronate

For studies on the ZOL-induced IPP/ApppI production and CLOD metabolism to $AppCCl_2p$, the MCF-7, MDA-MB-436, PC-3, MG-63, MCF-10A, RPMI-8226, and NCI-H929 cells were seeded into 6-well culture plates at 1 x 10^6 cells/well and left to adhere for 2 h, then treated with 25 μM ZOL or 500 μM CLOD for 24 h. After treatment, the extracts from cells were prepared as described in Chapter 4.3. Analysis of IPP/ApppI and $AppCCl_2p$ by HPLC-ESI-MS was performed as described in Chapter 4.4.

5.2.2 Cellular uptake

MCF-7, RPMI-8226 and MDA-MB-436 cells were seeded in 6-well culture plates at 1 x 10^6 cells/well and allowed to adhere for 2 h, then treated with 25 μM ^{14}C-ZOL or 500 μM ^{14}C-CLOD for 24 h. The cell extract preparations and radioactivity determinations were performed as described in Chapter 4.5. The cellular uptake percentage of BP was counted by comparing the radioactivity of cell extracts (ACN/water and digested protein fractions) to the total radioactivity (cell extracts, medium and washes).

5.2.3 Western blot analysis

The effect of ZOL on protein prenylation in different cancer cells was analyzed by western blotting using a specific antibody that only recognizes the unprenylated form of Rap1A. One million MCF-7, MDA-MB-436, PC-3, MG-63, MCF-10A, RPMI-8226, and NCI-H929 cells were treated with 25μM ZOL on the 6-well tissue plate for 24 h and then the cells were lysed in 500 μl of cell precipitation buffer (mammalian cell lysis kit, Sigma Chemical Co., St.Louis, MO, USA). An equal amount of protein from each sample was electrophoresed on 10 % SDS- polyacrylamide gel followed by transfer onto nitrocellulose membrane (Millipore Corporation, IPVH00010, Boston, Massachusetts, USA). Nonspecific antibody binding was blocked by incubating the membrane in 5 % non-fat milk in PBS with 0.1 % Tween prior to incubation with the primary antibody goat polyclonal anti-Rap1A (Santa Cruz, SC-1482 clone C-17) used at a dilution of 1/200 overnight at 4 °C. The secondary

antibody was HRP-conjugated anti-goat (Autogen Bioclear, ABN021HRP, Wiltshire, UK) used at a concentration of 1/50,000. Membranes were developed using Supersignal (Pierce, 34075, Tattenhall, UK). After visualizing the chemiluminescent signal, the membrane was then stripped in PBS pH 2, neutralised in PBS pH 7.5 then incubated in mouse monoclonal anti-actin antibody (Autogen Bioclear, ABJ1275, Wiltshire, UK), followed by a 1/30,000 dilution of anti-mouse HRP conjugate (Amersham, Buckinghamshire, UK). The blots were again visualized with Supersignal. Densitometric analysis of blots were carried out using Quantity One software (Bio-Rad, Hemel Hempstead, UK).

5.2.4 Inhibition of cell growth

MCF-7, RPMI-8226 and MDA-MB-436 cells were seeded into 96-well tissue culture plates at a density of 5000, 7500 and 2500 cells/well, respectively. The cells were incubated overnight and exposed to 1-100 μM ZOL or 10-2000 μM CLOD. The cell growth was assayed 72 h later using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) assay as previously described (Mossmann 1983, Mönkkönen et al. 1994). The cell culture medium was replaced with 100 μl of serum-free culture medium and 0.5 mg/ml MTT (Sigma) followed by incubation for a further 1 h at 37°C. The formazan precipitate was dissolved by addition of 100 μl of SDS-DMF buffer (20 % w/v sodium dodecyl sulphate in a solution of 50 % N,N-dimethyl formamide, pH 4,7) and incubation for overnight at 37 °C. Absorbances (Abs) were measured at 560 nm using automated microplate reader (Dynex Technologies Inc., Chantilly, VA). The percentage of viable cells was calculated using the following formula:

$$\frac{\left(Abs_{sample} - Abs_{blank}\right) \times 100\%}{Abs_{control} - Abs_{blank}}$$

where Abs_{sample} = Absorbance of the cells treated with drug
Abs_{blank} = Absorbance of solutions used
$Abs_{control}$ = Absorbance of the cells treated with 1 % of PBS (ZOL) or 2 % of PBS (CLOD)

5.2.5 Statistical analysis

For cellular uptake and inhibition of cell growth studies one-way ANOVA with Tukey's multiple comparison tests were used to assess significant differences in drug uptake or IC_{50} values between different cell lines.

5.3 RESULTS

5.3.1 IPP/ApppI production varies between different cancer cell types

Prior to this study, there were no reports of ApppI production by tumor cells. Both IPP and ApppI production varied between cell lines following drug treatment (Figure 6). IPP levels in cancer cell lines were 511 (MCF-7), 374 (RPMI-8226), 174 (MG-63), 87 (MDA-MB-436), 71 (PC-3), 7 (NCI-H929), and 2 pmol (MCF-10A) (Figure 6A). Capabilities of ZOL to induce ApppI production in these cells were 177 (MCF-7), 30 (MG-63), 11 (PC-3), 3 (RPMI-8226), and ~0.03 pmol (MDA-MB-436) (Figure 6C). MCF-10A and NCI-H929 cells did not produce detectable levels of ApppI after ZOL treatment.

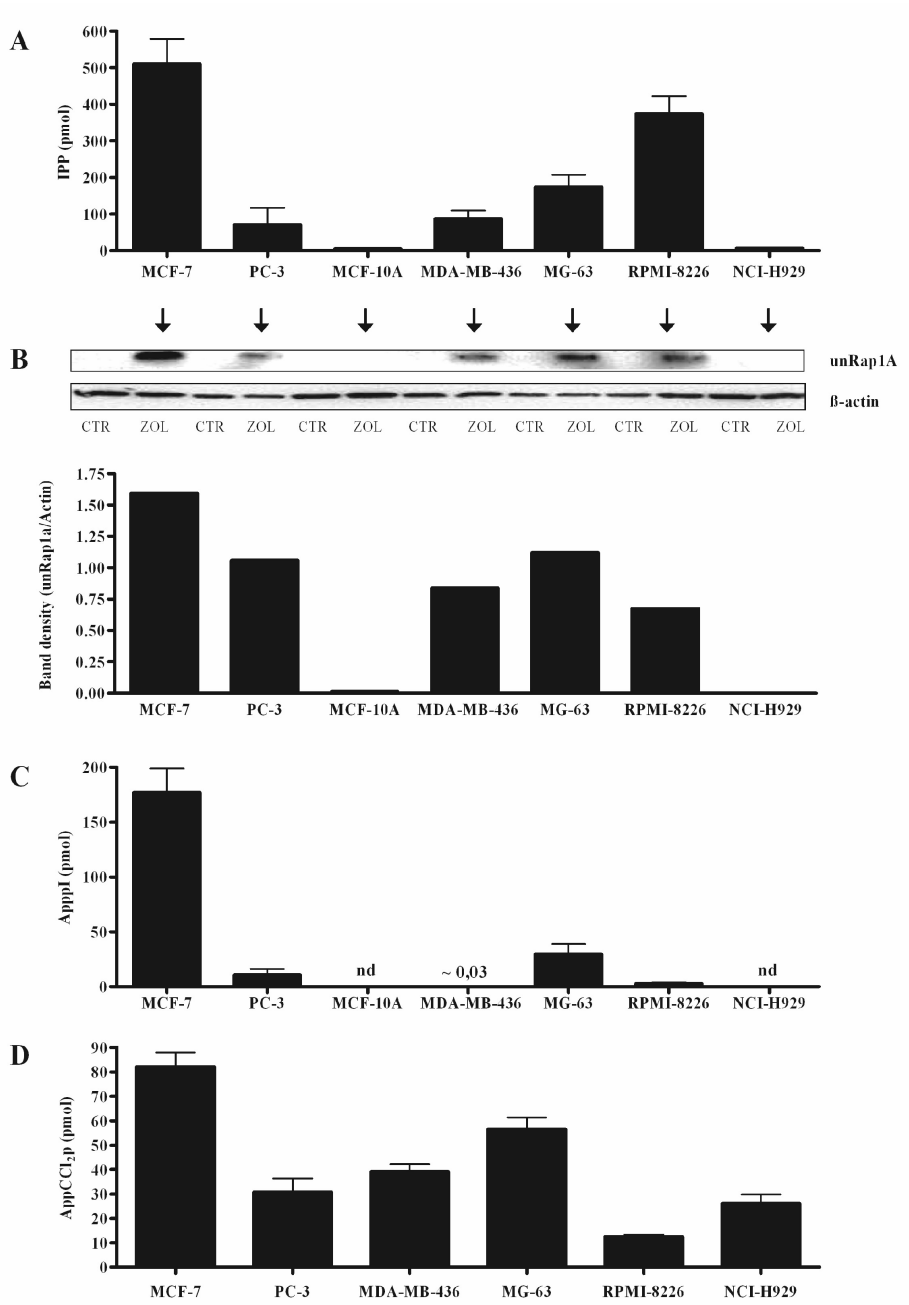


Figure 6. Zoledronic acid-induced IPP/ApppI formation, inhibition of Rap1A prenylation and clodronate metabolism to $AppCCl_2p$ in different cancer cell lines. IPP and ATP analogs were analyzed by HPLC-ESI-MS and Rap1A prenylation by Western blotting for unprenylated Rap1A. IPP accumulation (A), unprenylated Rap1A and β-actin (B), ApppI production (C), clodronate metabolism to $AppCCl_2p$ (D) in cancer cells after treatment with 25 µM zoledronic acid or 500 µM clodronate for 24 h (mean±SEM, n=6-9). nd = not detected.

5.3.2 IPP production correlates with the capacity of ZOL to inhibit Rap1A prenylation, but does not correlate with cellular uptake of the drug

The differences between IPP and ApppI production detected in the various cell lines in the initial experiments may be due to differential uptake of ZOL by the cells. The levels of cellular uptake of ZOL (Figure 7A) did not correlate with IPP accumulation in MCF-7, MDA-MB-436 and RPMI-8226 cells (Figure 6A). This finding raised the question of whether there is any connection between IPP accumulation induced by ZOL and the activity of FPPS. Therefore, the inhibition of Rap1A prenylation after ZOL treatment in the cells was explored. The measurement of accumulation of unprenylated Rap1A was used for estimating the activity of FPPS in different cancer cell lines. This experiment indicated that IPP production (Figure 6A) correlated with the capacity of ZOL to inhibit Rap1A prenylation (Figure 6B).

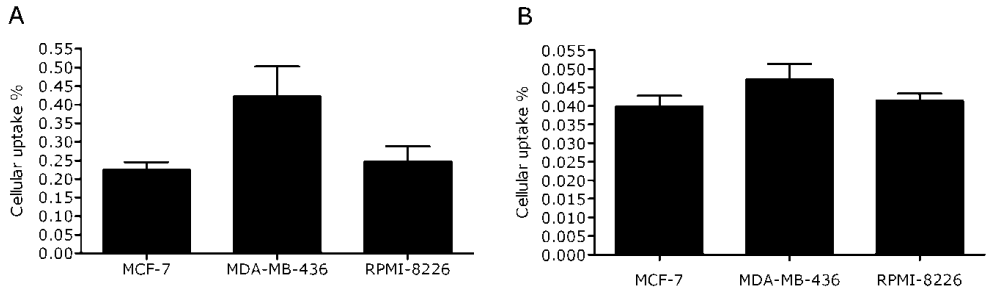


Figure 7. The differences in cellular uptake of zoledronic acid or clodronate between cell lines are not significant. The cells were treated with ^{14}C-labelled drugs, 25 µM zoledronic acid (A) and 500 µM clodronate (B), for 24 h (mean±SEM, n=6). $p>0.05$ using Tukey's multiple comparision test.

5.3.3 Clodronate metabolism correlates with the efficiency of IPP to ApppI conversion

It has been previously shown that CLOD decreases the ApppI production, and reciprocally, risedronate decreases the metabolism of CLOD to its AppCCl$_2$p-metabolite after the co-treatment in J774 macrophages (Mönkkönen et al. 2006). Therefore, it was hypothesized that aminoacyl-tRNA-synthetases may also be involved in ApppI production induced by N-BPs. The metabolism of CLOD to AppCCl$_2$p in different cells were 82 (MCF-7), 57 (MG-63), 39 (MDA-MB-436), 31 (PC-3), 26 (NCI-H929), and 13 pmol (RPMI-8226) (Figure 6D). There were no significant differences in the CLOD uptake between cell lines ($p>0.05$) (Figure 7B). Therefore, as was the case for ZOL-induced IPP accumulation, the differences in CLOD metabolism were not due to different levels of uptake of BP between the cell lines. The efficiency of ApppI production from IPP in different cells followed the order of potency: MCF-7 > MG-63 > PC-3 > RPMI-8226. This order correlated with the potency of CLOD metabolism to AppCCl$_2$p-metabolite ($R^2 = 0.9461$) (Figure 8). These results support the hypothesis that the N-BP-induced ApppI production is catalyzed by aminoacyl-tRNA-synthetases, similarly to the metabolism of non-N-BPs to the AppCp-metabolite. As non-N-BPs (pCp) and IPP resemble pyrophosphate in structure, the reverse reaction can also take place but with pCp or IPP replacing pyrophosphate, to form an AppCp-metabolite or ApppI, respectively (Figure 9).

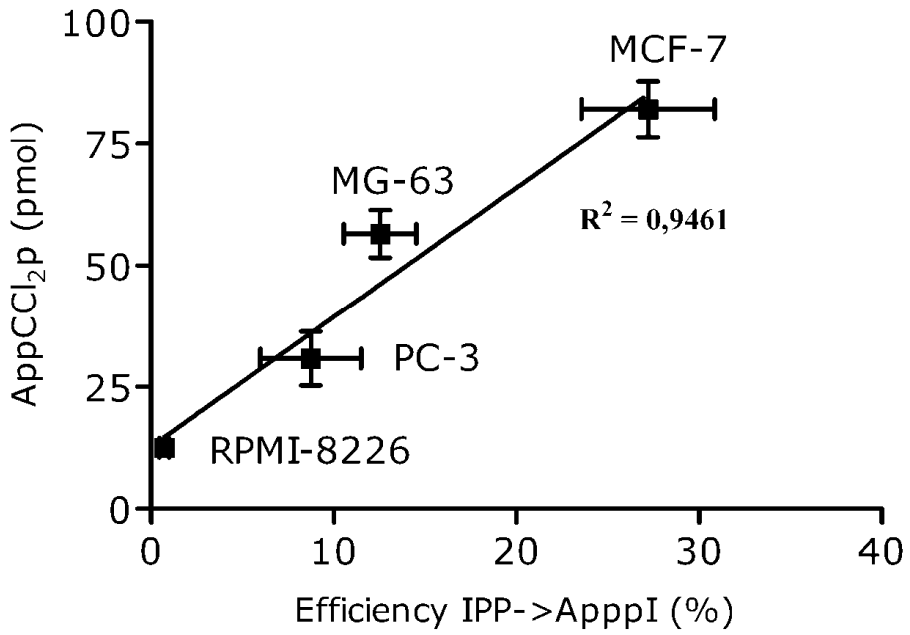


Figure 8. Clodronate metabolism correlates with the efficacy of ApppI formation from IPP.

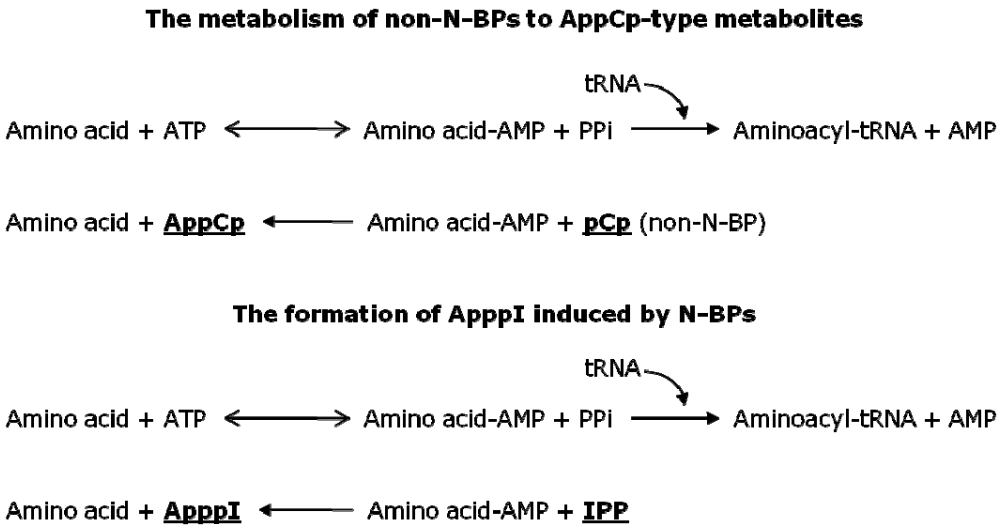


Figure 9. The metabolism of non-N-BP to AppCp-metabolite and the formation of ApppI induced by N-BPs is catalyzed by aminoacyl-tRNA-synthetases.

5.3.4 Potency of clodronate to inhibit cancer cell growth corresponds to ATP analog formation

In order to compare the BP-induced ATP analog formation and its effect on cell growth inhibition, cell growth was studied in MCF-7, MDA-MB-436 and RPMI-8226 cells following exposure to ZOL or CLOD. These cell lines were chosen for this experiment because of their diverse potency to produce ATP analogs (Figures 6C-D). MCF-7 and MDA-MB-436 breast cancer cells were more sensitive for the effect of ZOL on cell growth inhibition than RPMI-8226 myeloma cells (Figure 10A). IC_{50} values (50 % inhibition of cell growth) of ZOL for MCF-7, MDA-MB-436 and RPMI-8226 cells were 7.0, 6.0 and 18.1 μM, respectively. There were no significant differences in IC_{50} values between MCF-7 and MDA-MB-436 cells ($p>0.05$) (Figure 10C). Instead, IC_{50} value of RPMI-8226 cells was significantly higher compared to breast cancer cells ($p<0.001$) (Figure 10C). The inhibition of cell growth by CLOD was rather similar between the cell lines (Figure 10B). IC_{50} values of CLOD for MCF-7, MDA-MB-436 and RPMI-8226 cells were 893, 1035 and 1179 μM, respectively. A significant difference in IC_{50} values between MCF-7 and RPMI-8226 cells ($p<0.01$) was observed (Figure 10D). These observations show that the metabolism of CLOD into $AppCCl_2p$ (Figure 6D) corresponds better than ZOL-induced ApppI formation (Figure 6C) to the potency of BP to inhibit cancer cell growth (Figure 10).

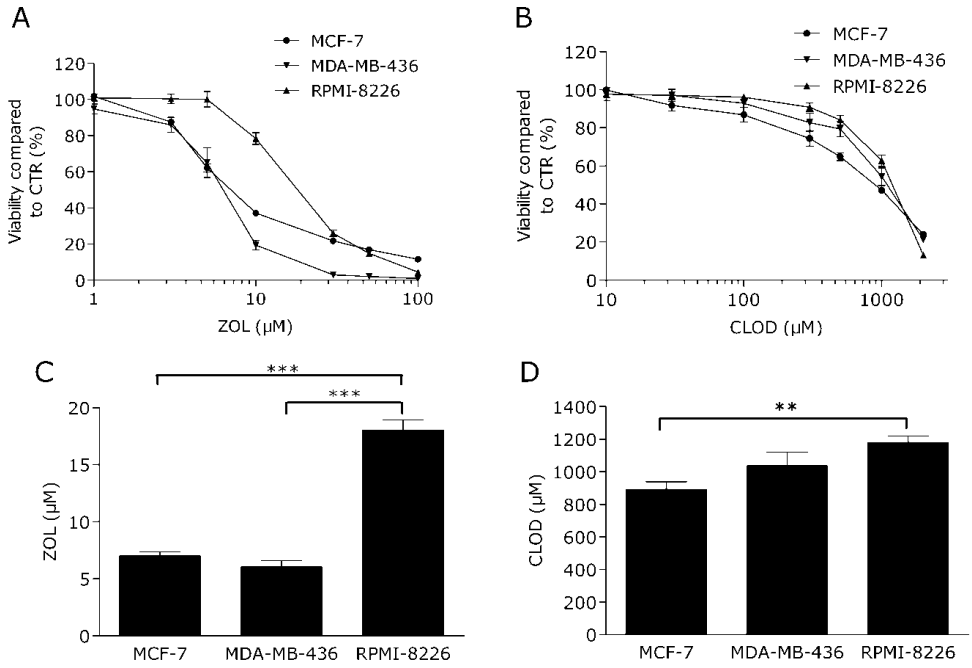


Figure 10. Inhibition of cell growth by zoledronic acid and clodronate. The cells were treated with 1-100 μM zoledronic acid or 10-2000 μM clodronate for 72 h. Cell growth was determined by MTT assay. The effect of zoledronic acid (A) and clodronate (B) on cell growth inhibition. IC_{50} values of zoledronic acid (C) and clodronate (D) (mean±SEM, n=12). **, $p<0.01$; ***, $p<0.001$ using Tukey's multiple comparision test.

5.4 DISCUSSION

One of the primary mechanisms responsible for the direct anticancer activity of BPs is inhibition of cancer cell growth. The mechanism by which BPs inhibit cancer cell growth might be through the production of cytotoxic ATP analogs. Here, we investigated the ATP analog formation in various cancer cell lines. Interestingly, we observed that ZOL-induced IPP/ApppI accumulation and metabolism of CLOD into $AppCCl_2p$ were remarkably cell line dependent. In order to clarify the reasons for variation of BP-induced IPP and ATP analog accumulation between different cancer cell lines, drug uptake studies were carried out. For this purpose, three cancer cell lines that differed significantly from each other in their BP-induced IPP and ATP analog expression were selected. No significant differences, however, in BP uptake between selected cell lines were observed ($p>0.05$). Therefore, these data demonstrated that IPP and ApppI accumulation are independent from the cellular uptake of BP. Regarding BP uptake and ATP analog formation in cancer cells *in vivo*, it is more likely that BPs as bone-targeting drugs can reach cancer cells on bone surface rather than in soft tissues. As osteoclasts can become exposed to very high concentrations of BPs *in vivo* (Sato et al. 1991), it is possible that the tumor cells on bone surface may be exposed to relatively high concentrations of BPs and be capable of the production of cytotoxic ATP analogs.

As the cellular uptake of ZOL did not correspond to IPP accumulation, this raised the question of whether there is any connection between ZOL-induced IPP accumulation and the activity of FPPS? The results of Western blot analysis evidenced that the unprenylated form of Rap1A could be only detected in cell lines that produced relatively high levels of IPP. This demonstrated that IPP production correlated with the capacity of ZOL to inhibit Rap1A prenylation (i.e. FPPS). Taken together, the data from studies of prenylation and drug uptake strongly suggest that the efficiency of ZOL to inhibit protein prenylation is dependent on the activity of FPPS in cancer cells. In addition, the measurement of IPP levels by mass spectrometry provides a very sensitive new technique to study the FPPS inhibition by N-BPs.

Previously, it has been shown that ApppI production correlates with the increase in the IPP concentration (Mönkkönen et al. 2006). ApppI production from IPP, however, did not correspond to IPP accumulation between different cancer cells. Therefore, the efficiency of ApppI production from IPP seems to vary depending on to cell type. We hypothesized that the novel ATP analog ApppI is produced by the same metabolic pathway as the metabolite of non-N-BP. The hypothesis was tested by comparing the N-BP-induced ApppI formation from IPP to formation of ATP analog of a non-N-BP in the same cancer cell lines. We found that the efficiency of ApppI production from IPP correlated with CLOD metabolism in the investigated cell lines. However, MDA-MB-436 cell line was an outlier because ZOL-induced IPP accumulation and metabolism of CLOD into $AppCCl_2p$ were relatively high in this cell line. Thus, this data suggests that the aminoacyl-tRNA-synthetase family, which catalyzes the formation of AppCp-type metabolites from non-N-BPs (Rogers et al. 1996), may also be involved in the ApppI production induced by N-BPs.

It has been suggested that AppCp-type metabolites are mainly responsible for the effect of non-N-BPs (Russell et al. 2008). However, the relationship between N-BP-induced ApppI accumulation and cellular effect is unknown. One main strategy in cancer therapy is to inhibit cancer cell growth. BP-induced ATP analog formation and its relation to the inhibition of cell growth were tested in selected cancer cell lines. The effect of CLOD on cell growth inhibition corresponded to the potency of CLOD metabolism to $AppCCl_2p$. This finding supports the hypothesis that AppCp-type metabolites are responsible for the effects of non-N-BPs (Russell et al. 2008). In contrast, ZOL-induced ApppI formation does not correspond to the effect of drug on cell growth inhibition. In addition to cytotoxic ApppI production (Mönkkönen et al. 2006, Mönkkönen et al. 2007), the inhibition of FPPS by N-BPs, such as ZOL, cause a lack of essential prenylated proteins (Russell et al. 2008). As N-BPs have a more complex mechanism of action compared to non-N-BPs, a direct

connection between ApppI formation and cellular effects may not be found. It is possible that the N-BP-induced IPP accumulation prior to ApppI formation plays a role in cell function, for example activating cytotoxic T cell mediated immune response (Chapter 2.3.5 and Chapter 2.4.1.1). As the present data proved the considerable variation of IPP accumulation between different cancer cells after ZOL treatment, it suggests that N-BPs may have differences in their capabilities to induce cancer cell killing.

In conclusion, the results of this study suggest that BPs may have diverse anticancer effects. This was seen as significant variation between IPP/ApppI formation and clodronate metabolism to $AppCCl_2p$. The differences may be related to different activity in the enzymes responsible for IPP accumulation and ATP analog formation, such as FPPS or aminoacyl-tRNA synthetase. Therefore, BP-induced cytotoxic ATP analog production in different cancer cells is likely to depend on the activity of the appropriate pathways involved in BP metabolism. Taken together, the ability of the cancer cells to metabolize the cytotoxic compounds varies, implying a potential new mechanism contributing to the specificity of BPs against different cancer cell types.

*6 Zoledronic acid-induced IPP/ApppI formation in vivo and in vitro**

Abstract. In order to further evaluate a pharmacological role of ApppI, the aim of the current study was to obtain more detailed data on ZOL-induced IPP/ApppI formation *in vivo* and *in vitro*. Additionally, ZOL-induced ApppI formation from IPP was compared to the metabolism of clodronate. As bone-resorbing osteoclasts are the main cellular target for BP action, IPP/ApppI formation and accumulation were assessed in rabbit osteoclasts following *in vivo* administration of ZOL. The formation of ApppI from IPP was compared to the metabolism of clodronate in MCF-7 cells *in vitro*. As a result, IPP/ApppI were formed in osteoclasts *in vivo*, after a single, clinically relevant dose of ZOL. Furthermore, exposure of MCF-7 cells *in vitro* to ZOL at varying times and concentrations induced time- and dose-dependent accumulation of IPP/ApppI. One hour pulse treatment was sufficient to induce IPP accumulation and subsequent ApppI formation, or the metabolism of clodronate into $AppCCl_2p$. Moreover, IPP/ApppI formation profile *in vivo* resembled the *in vitro* pulse profile, both demonstrating that ApppI formation results from accumulation of IPP as a result of FPP synthase inhibition. As a conclusion, this study provides the first conclusive evidence that pro-apoptotic ApppI is a biologically significant molecule, and demonstrates that IPP/ApppI analysis is a sensitive tool for investigating pathways involved in BP action.

*Adapted with permission of John Wiley and Sons from: Räikkönen J, Crockett JC, Rogers MJ, Mönkkönen H, Auriola S, Mönkkönen J: Zoledronic acid induces formation of a pro-apoptotic ATP analogue and isopentenyl pyrophosphate in osteoclasts *in vivo* and in MCF-7 cells *in vitro*. Br J Pharmacol 157:427-35, 2009.

6.1 INTRODUCTION

Previously, N-BP-induced ApppI formation has been detected in macrophages, osteoclasts, and various cancer cells *in vitro* (Mönkkönen et al. 2006, Chapter 5), but also *in vivo* in peritoneal macrophages after local injection of a high dose of ZOL (Mönkkönen et al. 2007). However, in order to establish the biological significance of ApppI, the primary aim of this study was to elucidate whether ApppI could be detected in osteoclasts, the pharmacological target cell for BPs, after *in vivo* administration of ZOL at a clinically relevant dose. An additional aim was to further evaluate the pharmacological role of ApppI by obtaining detailed data on its formation *in vitro*. MCF-7 cells, instead of osteoclasts, were selected as an *in vitro* cell model, because earlier we have used this cell line to evaluate the role of pro-apoptotic ATP analogs in the anticancer properties of BPs, and observed abundant BP-induced IPP/ApppI or AppCCl$_2$p accumulation in these cells (Chapter 5). We studied the dependence of IPP/ApppI formation on ZOL concentration and the time-course of IPP/ApppI formation after pulse and continuous treatment with ZOL. Furthermore, since pro-apoptotic effects of ApppI seem to be similar to those of other ANT-inhibiting ATP analogs, the ZOL-induced ApppI formation from IPP was compared to the metabolism of clodronate to its ATP analog, AppCCl$_2$p.

6.2 EXPERIMENTAL

The reagents used in this study are described in Chapter 4.1.

6.2.1 IPP/ApppI accumulation in osteoclasts *in vivo*

All animal care and experimental procedures complied with the NIH Guide for the Care and Use of Laboratory Animals. Rabbit osteoclasts were used as they have successfully been used to establish the formation of an AppCp-type metabolite (AppCCl$_2$p) of the non-N-BP CLOD *in vivo* (Frith et al. 2001). Three-day-old rabbits were injected subcutaneously with 1 mg/kg or 100 μg/kg ZOL in PBS, or an equivalent volume of PBS alone. Each experiment involved one treated rabbit and one control rabbit. The osteoclasts were isolated 24 or 48 h after injection using a previously well-characterized method (Frith et al. 2001, Staal et al. 2003). Briefly, the rabbits were euthanized with halothane and long bones from each rabbit were removed and cleaned and then minced in 25 ml of serum-free α-MEM with L-glutamine (Life Technologies, Paisley, UK). The osteoclasts were released from the bone fragments by vortexing. The cell pellet was washed, resuspended in 1 ml of α-MEM containing 3.3 μg/ml mouse anti-$\alpha_v\beta_3$ (23c6) antibody (Serotec AbD, Oxford, UK), and incubated at 37 °C for 20 min. Next, the cells were washed twice in 1xPBS containing 0.1% (w/v) of bovine serum albumin (BSA). For separation of osteoclasts, the cell pellet was resuspended in 0.1 % BSA in 1xPBS containing 2 x10^7 anti-mouse IgG conjugated magnetic Dynal beads (Invitrogen, Paisley, UK), and incubated rotating at 4 °C for 30 min. Vitronectin receptor (VNR)-positive (osteoclast) and VNR-negative (non-osteoclast) cells were separated using a magnetic particle concentrator (Dynal). The erythrocytes within the non-osteoclast-fraction were then lysed in BD Pharm™ Lyse (BD Biosciences, Pharmingen, San Diego, USA). As in earlier studies (Frith et al. 2001), the purity of the osteoclast fraction was >90 % multinucleated, VNR-positive cells. Finally, the osteoclast cells (with Dynal beads attached) and non-osteoclast cells were extracted with ice-cold acetonitrile (400 μl) and water (200 μl), and centrifuged at 14 000*g* for 2 min at 4 °C. The supernatants were transferred to fresh tubes and dried and stored at -70 °C until mass spectrometric analysis. Analysis of IPP/ApppI was performed as described in Chapter 4.4.

6.2.2 IPP/ApppI and AppCCl$_2$p accumulation *in vitro*

MCF-7cells were seeded in 6–well plates at 1 x10^6 cells/well overnight. Non-adherent cells were then removed and medium was replaced with treatment medium containing ZOL or CLOD, or an equivalent volume of sterile PBS. For dose-response assay the cells were treated with 1, 10, 25, 50 or 100 μM of ZOL for 24 h. For time course experiments, two different treatment protocols were used: pulse and continuous treatment. For pulse treatment, the cells were exposed to BP for 1 h only, after which the drug was removed and replaced by fresh medium, and the samples were collected at 0, 1, 3, 6, 12, 18, 24, or 48 h. For continuous treatment, the cells were treated continuously with BP for 1, 3, 6, 12, 18, 24, or 48 h. Sample collection, cell extract preparations and protein content determinations were carried out as described in Chapter 4.3. Analysis of IPP/ApppI and AppCCl$_2$p by HPLC-ESI-MS was performed as described in Chapter 4.4. Results shown are representative of at least 3 independent experiments (mean±SEM).

6.2.3 Cell viability

The viability of MCF-7 cells in dose-response assay and after pulse treatment with ZOL was determined by the MTT test (Mönkkönen et al. 2003).

6.2.4 Cellular uptake of BPs in vitro

Differences in cellular uptake of BPs could be a critical factor affecting formation of the ATP analogs. Therefore, in order to compare the kinetic profiles of drug uptake to that of ATP analog formation in cells, the cellular uptake of [^{14}C]-labeled BPs was assessed. The cells were treated with 25 μM [^{14}C]ZOL or 500 μM [^{14}C]CLOD according to the two different time-course treatment protocols described in Chapter 6.2.2, except that the 18 h time point was excluded. The cell extract preparations and the radioactivity determinations were performed as described in Chapter 4.5. The level of drug uptake was quantified as molar amount of drug per mg protein. Results shown are representative of at least 2 independent experiments (mean±SEM).

6.2.5 Statistical Analysis

For dose-response and pulse treatment studies one-way ANOVA with Tukey's multiple comparison tests were used to assess significant differences in the cell viability or IPP/ApppI formation.

6.3 RESULTS

6.3.1 ZOL induces IPP/ApppI formation in osteoclasts *in vivo*

In the present study, IPP and ApppI could be monitored by mass spectrometric detection in osteoclasts following clinically relevant dose of ZOL *in vivo*. Analysis was carried out on lysates from osteoclasts that had been isolated and purified from neonatal rabbits by immunomagnetic bead separation *ex vivo* following subcutaneous injection with 1 mg/kg ZOL (for 24 and 48 h), or 100 μg/kg ZOL (for 24 h). The formation was identified by a highly sensitive HPLC-ESI-MS technique (Auriola et al. 1997) in cell extracts by monitoring the daughter fragment ions in the SRM mode. Typical SRM chromatograms of osteoclast extract isolated from untreated rabbit, osteoclast extract isolated from ZOL-treated rabbit, and IPP and ApppI standards are shown in Figure 11. Major components were not detected in the chromatograms of osteoclast extracts generated from PBS injected rabbit (Figures 11A-B). The peaks that eluted at 6.47 min (IPP) and 6.49 min (ApppI) were detected in SMR chromatograms of osteoclast extract from ZOL-treated rabbit (Figures 11C-D). Comparable peaks were present in the chromatograms of osteoclast lysates from untreated rabbits, with added synthesized IPP and ApppI (Figures 11E-F).

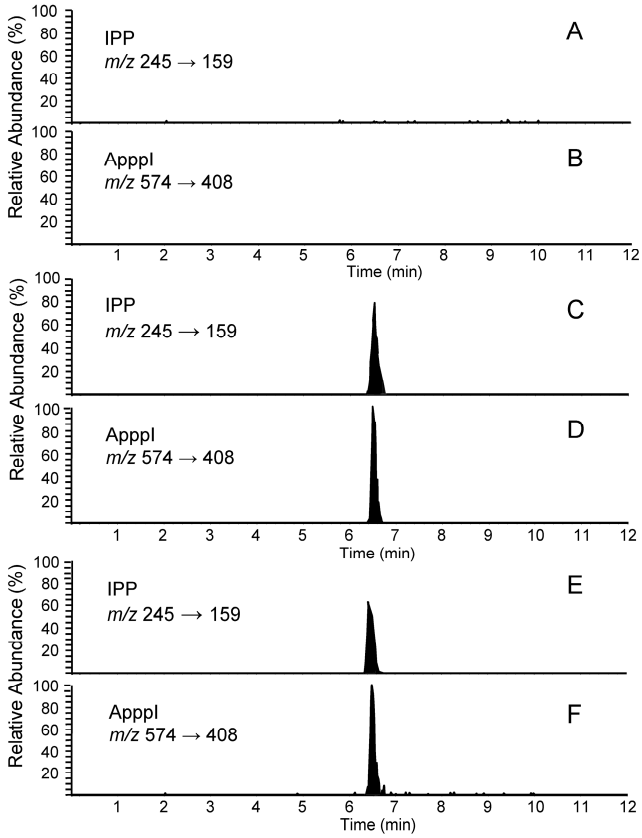


Figure 11. Identification of IPP and ApppI in rabbit osteoclasts *in vivo*. Vitronectin receptor-positive osteoclasts were isolated by immunomagnetic bead separation from rabbit bone marrow 24 h after single injection with 100 µg/kg ZOL. Acetonitrile cell extracts were then analysed by HPLC-ESI-MS. Selective reaction monitoring chromatogram of osteoclast extract isolated from a saline injected rabbit (A, B), extract isolated from ZOL-treated rabbit (C, D), untreated rabbit osteoclast extract with added 37 nmol/mg protein IPP (E) and 11 nmol/mg protein ApppI (F). The detection limits for IPP and ApppI are 2 pmol/mg protein and 0.2 pmol/mg protein respectively. No IPP or ApppI were detected in the chromatograms of osteoclast extracts generated from saline injected rabbits. The chromatograms are drawn on the same scale.

Identical patterns of fragmentation obtained with authentic IPP and ApppI were identified from lysates of osteoclasts isolated from ZOL-treated rabbits (Figure 12). The molecular ions of IPP (m/z 245) and ApppI (m/z 574) were fragmented, by CID, to form ions of m/z 79, 159, 177 and 227 (IPP) (Figures 12A-B), m/z 227 and 408 (ApppI) (Figures 12B-C). For internal standard, two major fragment ions, m/z 486 and m/z 406 were observed (data not shown). The quantitation of IPP and ApppI was based on fragment ions, m/z 245 → 159 for IPP, m/z 574 → 408 for ApppI. The molar amounts of IPP and ApppI in each osteoclast fraction are shown in Figure 13. The maximum amount of IPP/ApppI could be detected in osteoclast extracts isolated after 24 h of single injection with high dose (1 mg/kg) of ZOL, corresponding to 360 pmol/mg protein of IPP and 100 pmol/mg protein of ApppI, followed by two and a half-fold decrease of IPP and four-fold decrease of ApppI at 48 h post-injection. After 24 h, 21.7 % of the IPP was converted to ApppI. Additionally, the same pattern was seen after treatment with a clinically relevant dose (100µg/kg) of ZOL, corresponding to

21.5 % conversion of IPP to ApppI. A minor amount of IPP/ApppI could also be detected in the non-osteoclast (VNR-negative) fractions (data not shown), probably due to contamination of this fraction with osteoclasts. These data confirm that ZOL induces IPP/ApppI formation in osteoclasts *in vivo* after injection of a single clinical dose, 100 µg/kg, roughly equivalent to the 4 mg dose given to patients.

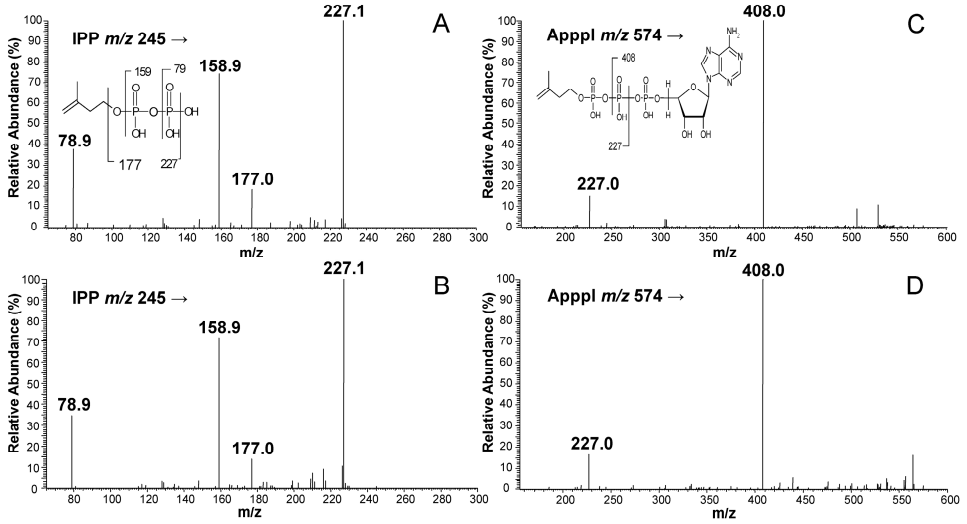


Figure 12. Mass spectrometric identification of IPP and ApppI. MS/MS spectra of IPP from the peak of Figure 11C (A) and Figure 11E (B), MS/MS spectra of ApppI from the peak Figure 11D (C) and Figure 11F (D). m/z = mass-to-charge ratio.

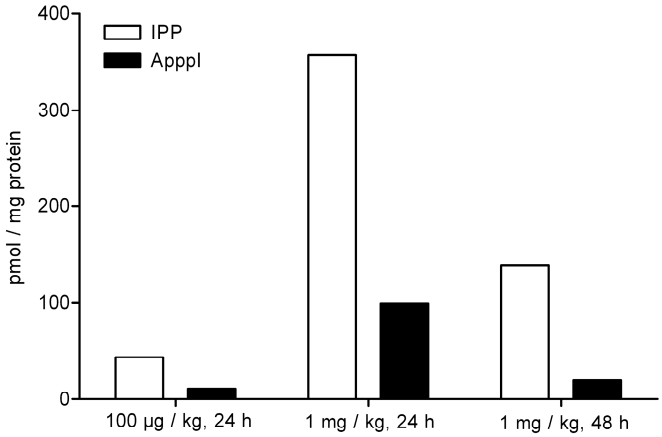


Figure 13. IPP/ApppI concentrations (pmol/mg protein) in each osteoclast fraction from ZOL-treated rabbits assessed by mass spectrometry. Animals were injected subcutaneously with 100 µg/kg or 1 mg/kg ZOL in PBS. The osteoclasts were isolated by immunomagnetic bead separation from rabbit bone marrow 24 or 48 h after injection.

6.3.2 ZOL induces dose-dependent IPP/ApppI formation *in vitro*

To investigate the dependence of ZOL concentration on IPP/ApppI formation, MCF-7 cells were incubated with variable concentrations of ZOL. At 1 - 50 μM concentration, ZOL induced dose-dependent IPP accumulation and ApppI formation in cells (Figure 14A). However, treatment of cells with 25 μM ZOL for 24 h resulted in a statistically non-significant increase in IPP/ApppI formation compared with the value after incubation with 10 μM ZOL. Additionally, ZOL at a high concentration of 100 μM did not induce more IPP/ApppI compared with 50 μM ZOL. Thus, the maximum inhibition of FPPS and subsequent IPP/ApppI accumulation in cells was achieved with 50 μM of ZOL. However, both concentrations, 50 and 100 μM, significantly ($p < 0.001$) reduced the viability of MCF-7 cells by 17 % and 28 %, respectively, when measured by the MTT test (Figure 14B). Thus, 25 μM ZOL was chosen for further time-course studies.

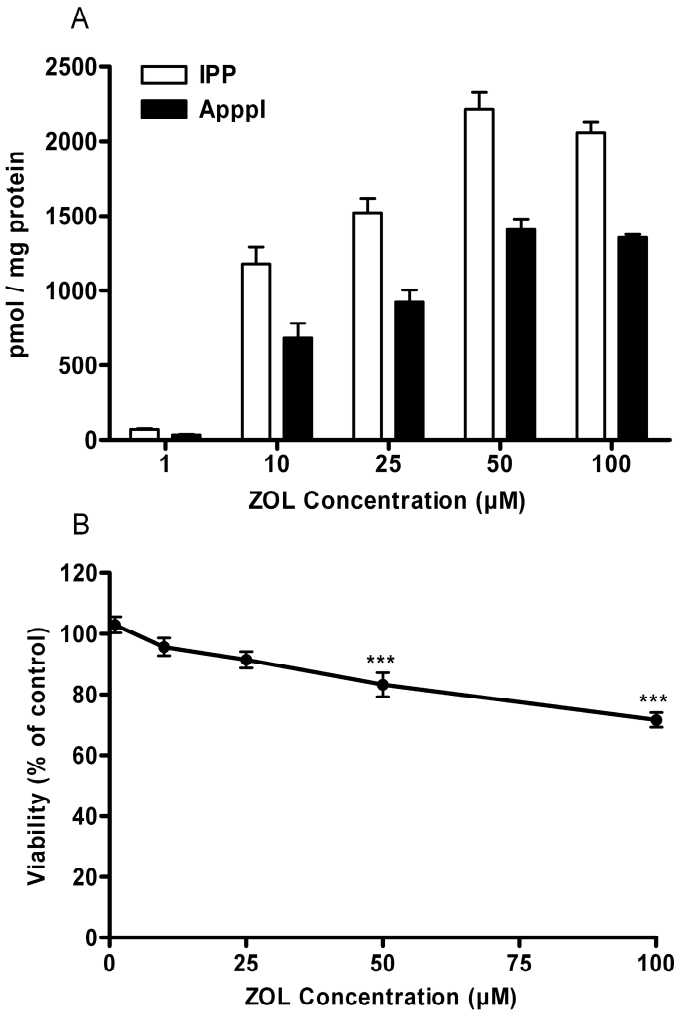


Figure 14. Effect of 1-100 μM ZOL on IPP/ApppI formation (A), and the cell viability (B) of confluent MCF-7 cells after 24 h, assessed by mass spectrometry and MTT test, respectively. (mean±SEM, n=9).***, $p < 0.001$ compared with control.

6.3.3 IPP/ApppI and AppCCl$_2$p formation, and cellular uptake of BPs *in vitro* after pulse treatment

ZOL and CLOD were used to compare formation kinetics of IPP/ApppI and AppCCl$_2$p, respectively. Pulse exposure to BP for 1 h either 25 μM ZOL or 500 μM CLOD, was sufficient to induce IPP/ApppI, or AppCCl$_2$p accumulation, respectively, in MCF-7 breast cancer cells during observation period of 0-48 h (Figures 15A-B). ApppI levels correlated well with the increase in the IPP levels in cells, confirming that ApppI is a result of IPP accumulation in cells after FPPS inhibition. ApppI formation from IPP could be detected without delay after drug removal (0 h), although it was close to the detection limit (0.2 pmol/mg protein). The amount of IPP and ApppI increased gradually up to 12 h, and reached the maximum level of IPP (49.2 pmol/mg protein) and ApppI (8.7 pmol/mg protein), then decreased until the end of the experiment (Figure 15A). The decrease in levels of IPP/ApppI after 12 h of drug removal was not a consequence of cell death since treatment did not affect the viability of the cells, assessed by MTT assay (data not shown). Contrary to the formation of IPP/ApppI, the metabolism of CLOD into AppCCl$_2$p in cells reached the maximum level (21 pmol/mg protein) immediately after drug removal, followed by a considerable decrease of metabolite at 1 h after treatment (Figure 15B). However, the amount of the metabolite increased again up to 12 h (reached the peak value of 21 pmol/mg protein), and then decreased gradually until the end of the experiment.

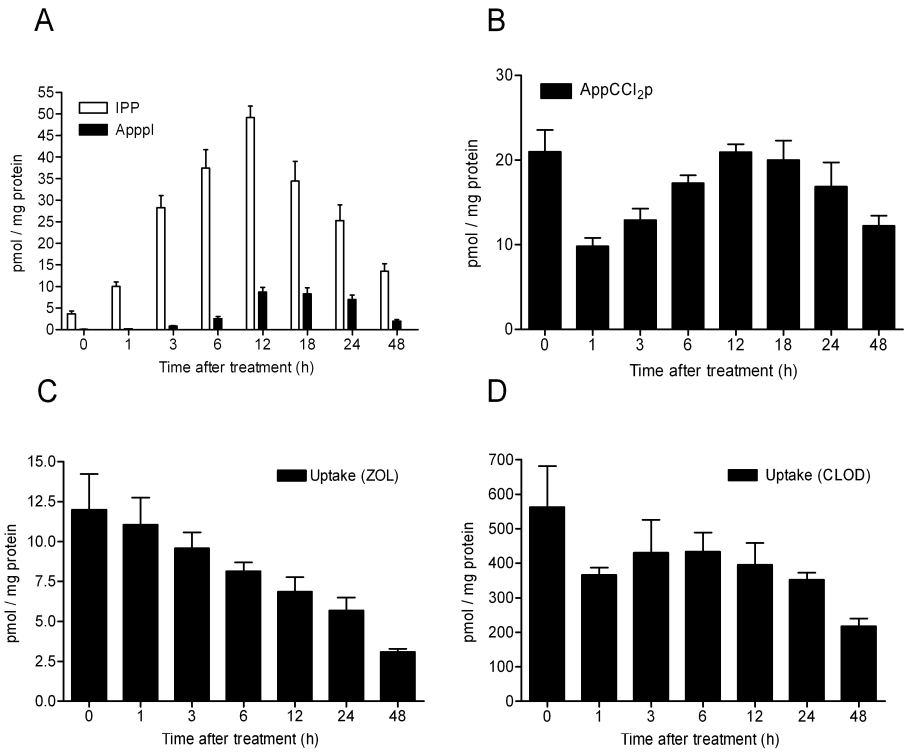


Figure 15. ZOL-induced IPP/ApppI formation (A), accumulation of AppCCl$_2$p (B), the cellular uptake of ZOL (C) and CLOD (D), in confluent MCF-7 cells after a pulse exposure to BPs. The MCF-7 cells were treated with 25 μM ZOL or 500 μM CLOD for 1 h, and the samples were collected 0 - 48 h after drug removal. The intracellular concentration of BPs was determined from the radioactivity measurements and the molar amounts of IPP/ApppI or AppCCl$_2$p were assessed by mass spectrometry (mean±SEM, n=4-8).

The maximum amount of [^{14}C]-labeled BP (12 pmol/mg protein of ZOL or 562 pmol/mg protein of CLOD) was detected in cell extracts immediately after drug removal (Figures 15C-D). After this, the amount of ZOL in cells decreased constantly until the end of observation time at 48 h (Figure 15C). The cellular uptake of CLOD after pulse treatment differed substantially from the cellular uptake of ZOL. This was illustrated by the kinetic profile of the CLOD uptake (Figure 15D), which was closely similar to the profile seen in CLOD metabolism (Figure 15B), though the differences between the time points of 1-48 h were not significant. In both cases of BP uptake, ZOL and CLOD were still found in cells 48 h after treatment.

6.3.4 IPP/ApppI and AppCCl$_2$p formation, and cellular uptake of BPs *in vitro* after continuous treatment

For continuous drug treatment, the cells were treated with ZOL or CLOD for 1-48 h. IPP/ApppI and AppCCl$_2$p were detectable 1 h after removal of BP, although the observed amount of ApppI was around detection limit and thus not visible in the figure (Figures 16A-B). The amount of IPP/ApppI, and AppCCl$_2$p increased gradually and time-dependently during the treatment. Thus, the highest intracellular concentrations were achieved at 48 h of BP exposure, corresponding to 1624 pmo/mg protein of IPP, 472 pmol/mg protein of ApppI, and 758 pmol/mg protein of AppCCl$_2$p, in cells.

The amount of [^{14}C]-labeled BPs taken up by the cells increased gradually during 1-48 h of continuous exposure (Figures 16C-D). Therefore, the highest intracellular amounts of ZOL (170 pmol/mg protein) and CLOD (1946 pmol/mg protein) were detected at 48 h, after treatment with ZOL and CLOD, respectively.

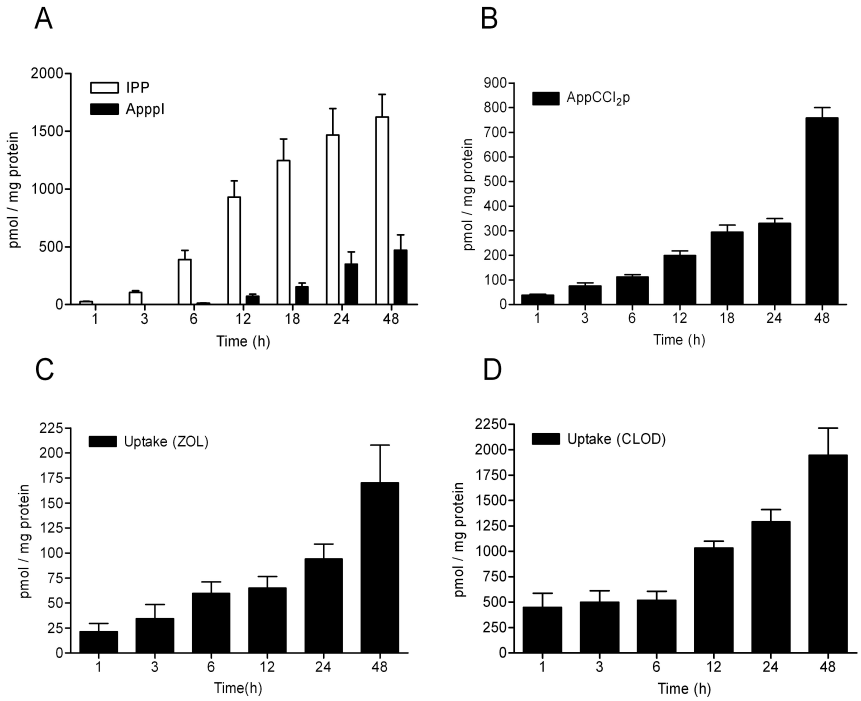


Figure 16. ZOL-induced IPP/ApppI formation (A), accumulation of AppCCl$_2$p (B), the cellular uptake of ZOL (C) and CLOD (D), in confluent MCF-7 cells. The cells were treated with 25 µM ZOL or 500 µM CLOD continuously for 1-48 h. The intracellular concentration of BPs was determined from the radioactivity measurements and the molar amounts of IPP/ApppI or AppCCl$_2$p were assessed by mass spectrometry (mean±SEM, n=4-8).

6.4 DISCUSSION

In order to further evaluate the pharmacological role of ApppI, the aim of the current study was to obtain more detailed data on IPP/ApppI formation *in vivo* and *in vitro*. We discovered that IPP/ApppI was formed in rabbit osteoclasts following a single dose of ZOL *in vivo*. Previously, ApppI has been identified in peritoneal macrophages *in vivo* after local injection of a high dose of ZOL (Mönkkönen *et al*., 2007), but this is the first published study to show the presence of pro-apoptotic ApppI in osteoclasts, the pharmacological target cell for BPs, *in vivo*. Importantly, ApppI formation was detected when ZOL was given at the dose of 100 ug/kg, roughly equivalent to a 4 mg single dose given to patients (Daubine et al. 2007). Although bone turnover and metabolism are different in humans and rabbits, the result indicated that ApppI was formed in osteoclasts *in vivo* after a clinically relevant dose of ZOL. IPP/ApppI accumulation in osteoclasts was lower at 48 h post-injection by comparison with that at 24 h post-injection, perhaps due to possible changes in the activity of the metabolizing enzymes in the mevalonate pathway, or to apoptosis of ApppI-containing osteoclasts. In addition, minor amounts of IPP/ApppI were also detected in non-osteoclast cell fractions (data not shown), which could have been a contamination from osteoclasts and thus not a true effect of ZOL on non-osteoclast cells. This hypothesis is also supported by earlier *in vivo* data demonstrating that N-BPs have a very low or no detectable effect on protein prenylation in non-osteoclast bone cells when administered *in vivo* at doses that cause a robust inhibition of protein prenylation in osteoclasts (Frith et al. 2001, Coxon et al. 2005). However, on the basis of this study, the possibility that cell types other than osteoclasts in the bone microenvironment can internalize BPs and consequently accumulate IPP/ApppI cannot be excluded.

As shown earlier (Chapter 5), MCF-7 cells produced high amount of IPP/ApppI, and were thus an appropriate model to characterize the kinetics of ApppI formation from IPP reliably. Time-course studies on the continuous treatment with 25 μM ZOL (concentration was chosen on the grounds of dose-dependence studies) or 500 μM CLOD demonstrate that both BPs and IPP/ApppI or $AppCCl_2p$ accumulate in cells in a time-dependent manner. These results together with the data obtained after *in vivo* treatment (with 1 mg/kg ZOL) illustrate that after 24 h, approximately 20 % of IPP was converted to ApppI in cells. Interestingly, at the same time point, a similar amount (26 %) of internalized CLOD was metabolized to $AppCCl_2p$, suggesting that the same metabolizing enzymes with same capacity might be responsible for the ApppI formation from IPP, and for CLOD metabolism. In addition, time-course results revealed that one-hour pulse treatment with BP was sufficient to cause IPP accumulation and subsequent ApppI or $AppCCl_2p$ formation in cells during the entire observation time. However, the accumulation profile of $AppCCl_2p$ after a 1 h exposure with CLOD differed from that seen with ZOL-induced IPP/ApppI accumulation. In comparison to IPP/ApppI, the formation of CLOD metabolite attained the maximum intracellular amount immediately after drug removal, but then unexpectedly decreased to the next observation time point. Differences between the profiles were to some extent expected, since formation of $AppCCl_2p$ from CLOD via aminoacyl-tRNA synthetases is more straightforward and more rapid than that of ApppI, which is formed after ZOL-induced FPPS inhibition and subsequent accumulation of IPP in the mevalonate pathway. Since metabolism of CLOD depends on cellular uptake, drug uptake was thought to account for the abrupt reduction in accumulation profile of $AppCCl_2p$. Although the CLOD uptake profile was found to be similar to that of the metabolite accumulation profile, it could not reliably account for the observation, and thus warrants further studies.

Similar formation profiles of IPP/ApppI were observed both *in vivo,* after a single injection of 1 mg/kg ZOL, and *in vitro* after a pulse exposure. In these studies, IPP levels were observed to decrease significantly after 24 h of treatment, although cellular uptake studies *in vitro* showed that there were still considerable amounts of ZOL present in cells 48 h after the initial exposure. This

was a rather surprising result, as IPP binds and further stabilizes the inhibited FPPS-N-BP complex in a closed conformation, leading to sustained inhibition of the enzyme (Chapter 2.3.3). However, the results in this study suggest that FPPS might be partly restored even in the presence of intracellular ZOL. Previously, similar results have been obtained in a cholesterol biosynthesis study where 4 h treatment of PC-3 cells with 20 μM ZOL completely inhibited cholesterol biosynthesis, but as early as 24 h later, about 10 % of the biosynthesis was rescued, reaching 90 % after 48 h (Goffinet et al. 2006). It is conceivable that the expression of FPPS is elevated through increased protein synthesis, or that feedback inhibition by IPP on upstream enzymes leads to decreased flux through the mevalonate pathway (and hence decreased IPP synthesis). There are no known alternative pathways for the production of FPP from IPP in mammalian cells (Edwards and Ericsson 1999). However, some studies have postulated that IPP could be recycled back to HMG-CoA and eventually to acetyl-CoA by a sequence of reactions (Edmond and Popjak 1974, Brown and Goldstein 1980).

In this study, we obtained more detailed information of ZOL-induced IPP/ApppI formation *in vivo* and *in vitro* and provide the first conclusive evidence that pro-apoptotic ApppI is formed in osteoclasts *in vivo*, even after a single clinically-relevant dose of ZOL. This result is of considerable importance since it establishes the biological significance of this molecule. Both *in vivo* and *in vitro* data further support that ApppI formation results from the accumulation of IPP after FPPS inhibition. However, both studies reveal that some possible changes in the activity of the metabolizing enzymes in the mevalonate pathway might occur fairly shortly after drug exposure. This observation needs to be studied further, since it could indicate that in order to achieve more efficacious treatment, such as in cancer therapy, continuous or frequent pulse treatment with BPs is needed. This hypothesis is supported by previous *in vivo* findings demonstrating that treatment of animals with a single clinical dose did not reduce the tumor burden, whereas a low dose of ZOL administered to animals on a daily or weekly intermittent schedule not only inhibited bone destruction, but also exhibited antitumor effects (Daubine et al. 2007, Stresing et al. 2007).

IPP/ApppI analysis is shown to be a useful and sensitive tool for investigating the pathways involved in BP action. In addition, identification of the molecular mechanism of action of ApppI may help in further understanding the differences and similarities between N-BPs and non-N-BPs in general. Furthermore, ApppI studies have revealed the potential of N-BPs to affect a wide range of cellular processes via the inhibition of FPPS, and may also help to explain the observed preclinical and clinical activity of these compounds. For example, further studies to clarify the relationship between IPP/ApppI, cancer cell apoptosis and γδ T-cell activation are in progress.

*7 Mevalonate pathway isoprenoids downregulate ZOL-induced IPP/ApppI formation in breast cancer cells **

Abstract. Accumulation of unprenylated proteins in the cells after FPPS inhibition is believed to largely account for the cytotoxic effects of ZOL. FPPS inhibition leads also to the accumulation IPP/ApppI, but the role of this mechanism in the cytotoxic action of N-BPs is less clear. As treatment with mevalonate pathway isoprenoids has been shown to overcome N-BP-induced apoptosis via rescuing protein prenylation, the objective of this study was to determine their mechanism of action on ZOL-induced IPP/ApppI accumulation. Interestingly, the results revealed that ZOL-induced IPP/ApppI accumulation in MCF-7 breast cancer cells were decreased by farnesol, and almost completely blocked by geranylgeraniol and geranylpyrophosphate. The functionality of the regulatory enzymes of IPP and ApppI, IPP isomerase and aminoacyl-tRNA-synthase, respectively, or protein levels of FPPS were not affected by the treatments. However, the protein levels of HMGR and unprenylated Rap1A were observed to be strongly downregulated by geranylgeraniol and geranylpyrophosphate. This study presents a novel insight into the mechanism of action of isoprenoids on the regulation of mevalonate pathway after FPPS inhibition. The data implies that in addition to the previously reported effects on rescuing protein prenylation, isoprenoids can preserve cancer cell activity by inhibiting the accumulation of IPP/ApppI via HMGR downregulation. This suggests that IPP/ApppI formation is a significant mechanism in the anticancer action of ZOL.

*Adapted with permission of Elsevier Inc. from: Räikkönen J, Mönkkönen H, Auriola S, Mönkkönen J: Mevalonate pathway intermediates downregulate zoledronic acid- induced isopentenyl pyrophosphate and ATP analog formation in human breast cancer cells. Biochem Pharmacol 79:777-783, 2010.

7.1 INTRODUCTION

Inhibition of the mevalonate pathway has been proposed as being the fundamental molecular mechanism of many of the observed anticancer effects of statins and N-BPs both *in vitro* and *in vivo* (Buhaescu and Izzedine 2007). It has been demonstrated that free nonsterol mevalonate pathway intermediates, farnesol (FOH) and especially geranylgeraniol (GGOH), are capable of salvaging protein prenylation and, thus overcome statin and N-BP-induced apoptosis in many cell types (Table 1 and Table 3). In addition, we have evidence that GGOH may possess the capacity to inhibit ZOL-induced IPP/ApppI formation in cancer cells (Mitrofan et al. 2009), but the mechanism underlying this effect is not known. As IPP is capable of activating an immune response (Chapter 2.3.5 and Chapter 2.4.1.1) and ApppI has been shown to be an apoptotic molecule (Mönkkönen et al. 2006), it was felt important to define whether the salvage properties of the isoprenoids involve regulation of IPP/ApppI levels in cells. Therefore, we determined which intermediates and enzymes of the mevalonate pathway are involved in regulating the ZOL-induced IPP/ApppI formation in cancer cells. The results suggest that in addition to the previously reported effects on rescuing protein prenylation, isoprenoids can influence cell activity by downregulating IPP/ApppI levels in cells. This data provides further evidence that IPP/ApppI formation is a significant mechanism in the anticancer action of N-BPs.

7.2 EXPERIMENTAL

7.2.1 Reagents

Mevalonic acid lactone (MVL, which is readily converted to mevalonate, MVA), farnesol (FOH), geranylgeraniol (GGOH), geranylgeranylpyrophosphate (GGPP), and lovastatin (LOV) were from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of GGOH, FOH and MVL were prepared in pure ethanol and diluted in culture medium just before use. GGPP was dried to remove solvent, and resuspended in culture medium immediately before use. All the other reagents used in this study are described in Chapter 4.1.

7.2.2 IPP/DMAPP, ApppI and AppCCl$_2$p detection

The MCF-7 cells were seeded in 6–well plates at 1 x10^6 cells/well. On the next morning, the medium was replaced with the treatment medium containing the following drugs alone or in combinations: 5 μM LOV, 25-100 μM ZOL, 500 μM CLOD, 25/50 μM FOH with or without 25 μM ZOL or 500 μM CLOD, 25/50 μM GGOH with or without 25 μM ZOL or 500 μM CLOD, 100 μM GGPP with 25 μM ZOL, 500/1000 μM MVA with or without 25 μM ZOL, or vehicle (CTR). After 24 h treatment, the cell extract preparations and protein content determinations were carried out as described in Chapter 4.3. Analysis of IPP/ApppI and AppCCl$_2$p by HPLC-ESI-MS was performed as described in Chapter 4.4. **T**he relative amounts of IPP and DMAPP isomers in the treated cell samples were calculated using a protocol described earlier (Jauhiainen et al. 2009). This method utilizes the peak intensity ratios of two characteristic fragment ions, *m*/*z* 177 and *m*/*z* 159, of IPP and DMAPP received from MS^2 monitoring. The results shown are representative of at least 4 independent experiments (mean±SEM).

7.2.3 Cell viability

The influence of the drugs alone and in combinations on cell viability was determined by MTT test (Mönkkönen et al. 2003).

7.2.4 Western blot analysis

The MCF-7 cells were seeded at 2 x10^6 cells per 25-cm^2 flask. On the next morning, the cells were treated for 24 h with drugs alone or in combinations: 5 μM LOV (positive control), 25 μM ZOL, 25/50 μM FOH with or without ZOL, 25/50 μM GGOH with or without ZOL, 100 μM GGPP with ZOL, or vehicle (CTR). After treatment, the cells were washed twice with ice-cold phosphate-buffered saline (PBS), and lysed in cell precipitation buffer (mammalian cell lysis kit, Sigma (MCL-1)). The protein content was quantified by using Bio-Rad DC Protein Assay Kit (Bio-Rad laboratories, Hercules, CA) using BSA as a standard. An equal amount of protein from each sample was separated on 10 - 12 % polyacrylamide-SDS gel and transferred to polyvinylidene fluoride membrane (GE Healthcare). The membranes were blocked for 1 h in 5 % non-fat milk in PBS with 0.1 % Tween at room temperature and incubated overnight at + 4 °C with either goat polyclonal anti-Rap1A (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-HMG-CoA reductase (1:1000 dilution, Upstate, Dundee, UK), rabbit polyclonal anti-FDPS (1:200 dilution, Abgent, San Diego, CA, USA), or mouse monoclonal anti-β-actin (1:4000 dilution, Autogen Bioclear, Wiltshire, UK) primary antibodies. After washing, the membranes were incubated for 1.5 h with horseradish-peroxidase-conjugated donkey anti-goat (Autogen Bioclear, Wiltshire, UK), goat anti-rabbit (Pierce Biotechnology, Rockford, USA) or sheep anti-mouse (Amersham, Buckinghamshire, UK) secondary antibodies. An enhanced chemiluminescence (ECL) system (Amersham Biosciences) was used for detection and Image Quant RT ECL (GE Healthcare) for blot scanning. Scanning densitometry of protein bands was determined by pixel intensity using NIH Image J software (v1.4.3, available at http://rsb.info.nih.gov/ij/index.html) and normalized against that of β-actin and control (HMGR and FPPS bands) or β-actin and ZOL (unprenylated Rap1A bands). Results of the densitometrical analyses shown are combined data of at least three independent experiments. The experiments were performed in triplicate.

7.2.5 Statistical analysis

The data are presented as means ± SEM. One-way ANOVA with Tukey's multiple comparison or Bonferroni's comparison test were used to analyze the significant differences in the cell viability or IPP/ApppI levels. $p < 0.05$ was considered statistically significant.

7.3 RESULTS

7.3.1 FOH and GGOH decrease ZOL-induced IPP/ApppI levels in MCF-7 cells

MCF-7 cells were incubated for 24 h with exogenous FOH and GGOH, which are metabolized to their phosphate forms in the cells (Crick et al. 1997), together with increasing concentrations of ZOL, for 24 h. As seen in Figure 17, IPP accumulation and consequent ApppI formation in cells were evenly and significantly ($p>0.001$) decreased by both intermediates at all tested ZOL concentrations. No significant differences in the levels IPP/ApppI between 50 and 100 μM ZOL were observed. At concentrations of 25, 50 and 100 μM, ZOL significantly ($p>0.001$) reduced cell viability by 18, 22 and 31 %, respectively, when measured by the MTT test (data not shown). The maximum inhibitory effects induced by both isoprenoids were observed with 25 μM ZOL, and thus this dose was chosen for further studies.

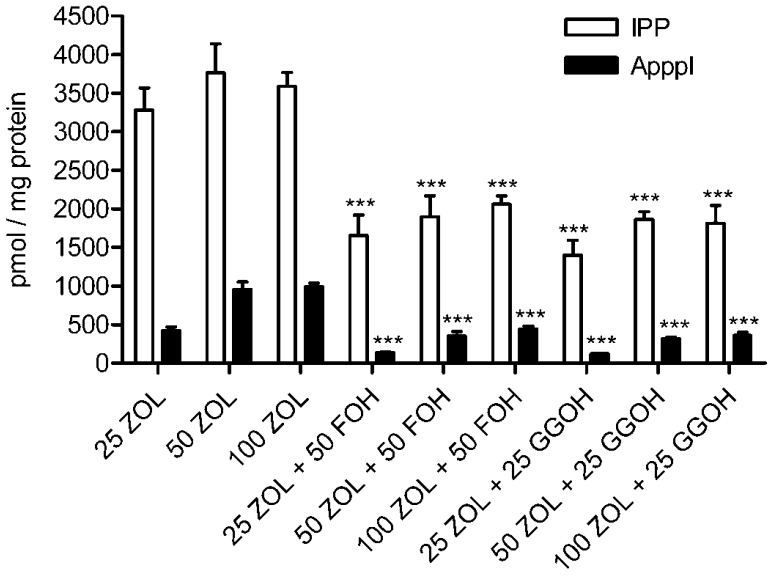


Figure 17. FOH and GGOH inhibit ZOL-induced IPP/ApppI accumulation in cells**.** The amount of IPP/ApppI in MCF-7 cells after 24 h treatment with 25 - 100 μM zoledronic acid (25-100 ZOL) with or without 50 μM farnesol (50 FOH) or 25 μM geranylgeraniol (25 GGOH). The molar amounts of the compounds were determined by the HPLC-ESI-MS method (mean ±SEM, n=6-9). ***, P<0.001 compared to ZOL treatment using one-way ANOVA with Tukey's Multiple Comparison test.

7.3.2 Isoprenoids do not interact with regulatory enzymes of IPP or ApppI synthesis

As both of the isomers, IPP and DMAPP, are essential in the synthesis of isoprenoids in the mevalonate pathway, the effect of exogenous isoprenoids on the activity of the catalyzing enzyme, IPP isomerase, was studied. MCF-7 cells were incubated for 24 h with 25 and 50 μM FOH or GGOH concurrently with 25 μM ZOL, or 25 μM ZOL alone. In our previous studies, only the mixture of the isomeric molecules has been analyzed, but recently we have described a method which is capable of quantifying the relative amount of both isomers, IPP and DMAPP, in cell extracts (Jauhiainen et al. 2009). Using this procedure, we discovered that there were no differences in the isomerization capacity of the enzyme between the treatments (Figure 18A). The ratio of IPP and DMAPP in all cell extracts was approximately 1:4. The same treatment protocol was tested, except that ZOL was replaced with non-N-BP clodronate, to clarify the functionality of the aminoacyl-tRNA-synthase enzyme, known to catalyze ATP-analogs from non-N-BPS (Rogers et al. 1996), and which is proposed to catalyze also the formation of ApppI from IPP. The cells were observed to metabolize CLOD into AppCCl$_2$p regardless of isoprenoid treatments, and therefore the functionality of the aminoacyl-tRNA-synthase was not affected (Figure 18B).

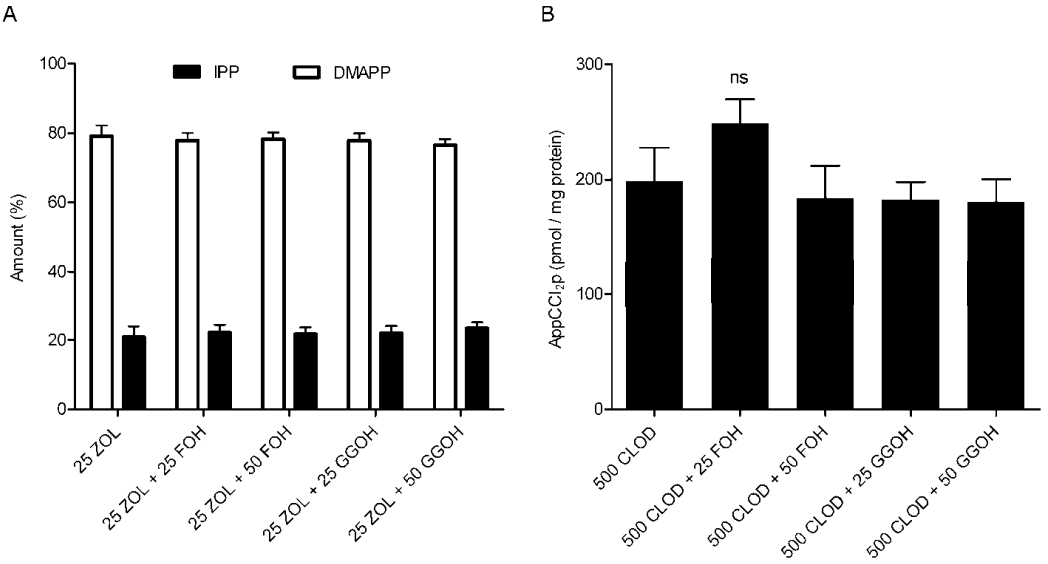


Figure 18. Isoprenoids do not interfere with enzyme activities of IPP isomerase and aminoacyl-tRNA synthetase. The relative amounts of IPP and DMAPP isomers catalyzed by IPP isomerase (A) and the metabolism of clodronate to $AppCCl_2p$ catalyzed by aminoacyl-tRNA synthetase (B) in MCF-7 cells. The cells were incubated for 24 h treatment with 25 μM zoledronic acid (25 ZOL) or 500 μM clodronate (500 CLOD), with or without 25/50 μM FOH (25/50 FOH) or 25/50 μM GGOH (25/50 GGOH). The molar amount of $AppCCl_2p$ was determined by the HPLC-ESI-MS method (mean ±SEM, n=6). The relative amounts of the isomeric compounds, IPP and DMAPP, in the cell extracts were determined with a previously developed quantitative method (Jauhiainen et al. 2009), which is based on HPLC-ESI-MS separation (mean±SEM, n=10). ns = non significant using Tukey's Multiple Comparison Test.

7.3.3 GGOH and GGPP inhibit ZOL-induced IPP/ApppI accumulation by decreasing protein levels of HMGR in cells

To further examine the effects of isoprenoid compounds on the regulation of mevalonate pathway, we studied the dependence of the isoprenoid dose on inhibition of ZOL-induced IPP/ ApppI accumulation in MCF-7 cells. Co-treatment of ZOL at the doses of 25 and 50 μM FOH or 25 and 50 μM GGOH resulted in a significant ($p>0.001$) and dose-dependent decrease in the levels of IPP/ApppI compared to treatment with ZOL alone (Figure 19A). GGOH was more effective than FOH in reversing the effects of ZOL, inducing almost complete prevention. However, the viability of the cells decreased to 68 % compared to control (Figure 19A). In contrast to GGOH, co-treatment of ZOL with 100 μM GGPP, the analogue of GGOH, did not affect cell viability (viability 96 % compared to control), but inhibited IPP/ApppI accumulation in cells in the same manner as GGOH. There was no evidence of IPP/ApppI found in the cell samples treated with LOV, GGOH, FOH or vehicle (CTR) (Figure 19A).

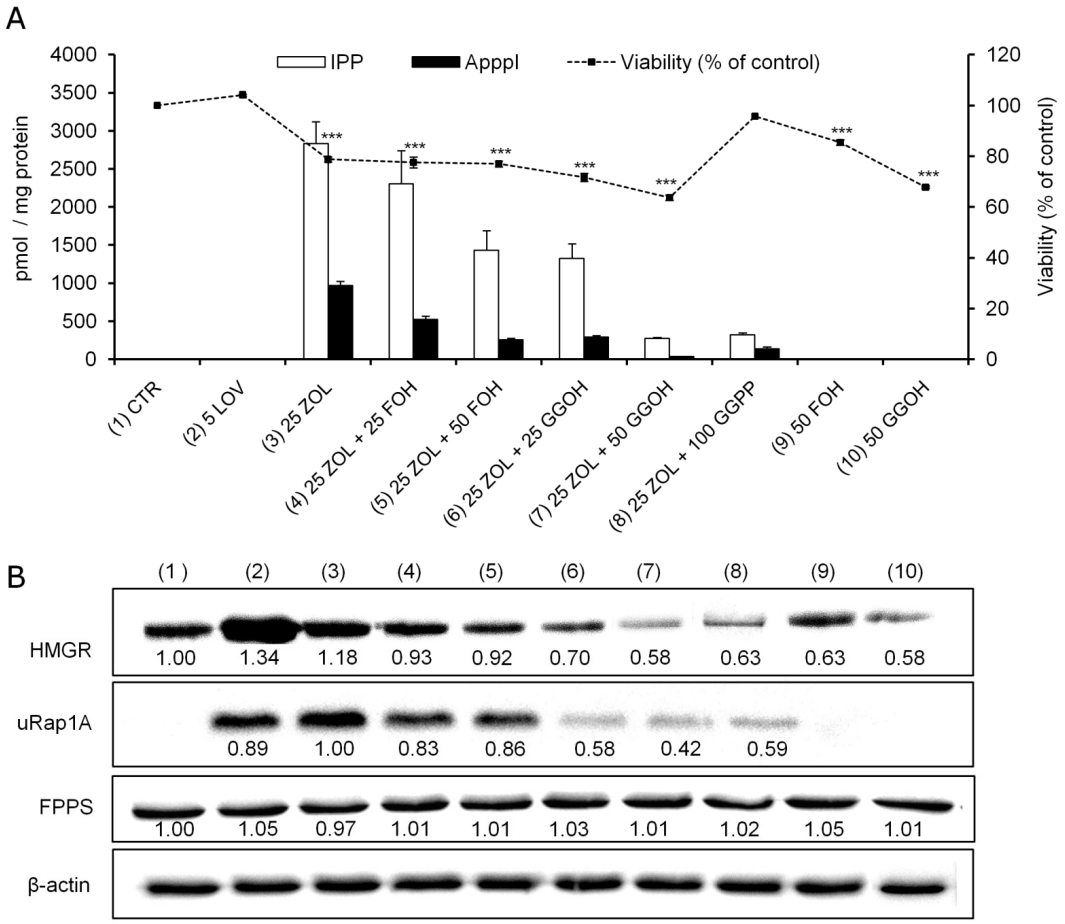


Figure 19. GGOH and GGPP suppress ZOL-induced IPP/ApppI accumulation by downregulating HMGR protein levels in cells. Figure A represents HPLC-ESI-MS data of IPP/ApppI accumulation, and cell viability. Cell viability was determined by the MTT assay. Figure B represents the effect of the isoprenoids on the regulation of the mevalonate pathway proteins in MCF-7 cells. Total levels of HMG-Co-A reductase (HMGR), β-actin (internal control), FPP synthase (FPPS) and unprenylated Rap1A (uRap1A) were determined by Western blotting in cell lysates. The cells were incubated for 24 h with drugs, 5 μM lovastatin (5 LOV); 25 μM zoledronic acid (25 ZOL); 25/50 μM farnesol (25/50 FOH) with ZOL; 25/50 μM geranylgeraniol (25/50 GGOH) with ZOL; 100 μM geranylgeranylpyrophosphate (100 GGPP with ZOL); 50 μM farnesol (50 FOH); 50 μM geranylgeraniol (50 GGOH), or with vehicle (CTR) (mean±SEM, n=6-12). *P<0.05; *** P<0.001 denotes values significantly different from treatment with ZOL alone using Tukey's Multiple Comparison Test. The blots were quantified by Image J software. The bands were normalized to β-actin and control (HMGR and FPPS) or β-actin and ZOL (uRap1A).

Next, we investigated the effects of co-treatments at the protein level on FPPS, HMGR and unprenylated Rap1A (uRap1A) in Western blot analysis. Membranes were probed with β-actin antibody to ensure that an equal amount of protein was loaded onto the gel. As a positive control, cells were treated with 5 μM LOV, which is known to upregulate HMGR protein levels and cause uRap1A accumulation (Goldstein and Brown 1990, Edwards et al. 1992, Ownby and Hohl 2003). In Figure 19B, representative Western blots of FPPS, HMGR and uRap1A protein from the treated cells are shown. The treatments did not alter the protein level of FPPS, the target enzyme for ZOL.

Furthermore, accumulation of uRap1A correlated with the extent of ZOL-induced IPP/ApppI formation. As in the case of IPP/ApppI formation (Figure 19A), GGOH and GGPP were again more potent at restoring Rap1A protein prenylation than FOH. No accumulation of uRap1A was observed in the presence of vehicle, GGOH or FOH alone. The most interesting result was obtained when HMGR protein expression levels were monitored. GGOH and GGPP resulted in a significant reduction in the amount of HMGR enzyme present in the treated cells (Figure 19B).This result was not a consequence of the reduced cell viability by GGPP (Figure 19A). Furthermore, the protein level of HMGR was increased by LOV and ZOL, and slightly decreased by FOH. These results indicate that the observed inhibition of IPP/ApppI accumulation (Figure 19A) by isoprenoids is a consequence of HMGR enzyme downregulation (Figure 19B).

7.3.4 Mevalonate can partly restore ZOL-induced IPP/ApppI accumulation during isoprenoid co-treatment

To confirm whether the decrease in the levels of IPP/ApppI induced by isoprenoids was a result of the interruption of HMGR function and a consequent lack of precursor for IPP, MVA was added to the culture medium. As shown in Figures 20A and 20B, the inhibitory effect of isoprenoids on ZOL-induced IPP/ApppI levels could be partially prevented by the addition of 500 μM MVA. MVA at a higher concentration (1 mM) reduced IPP/ApppI accumulation, as well as cell viability compared to the 500 μM dose (data not shown). These results further suggest that the regulation of the IPP/ApppI accumulation is mediated by HMGR enzyme.

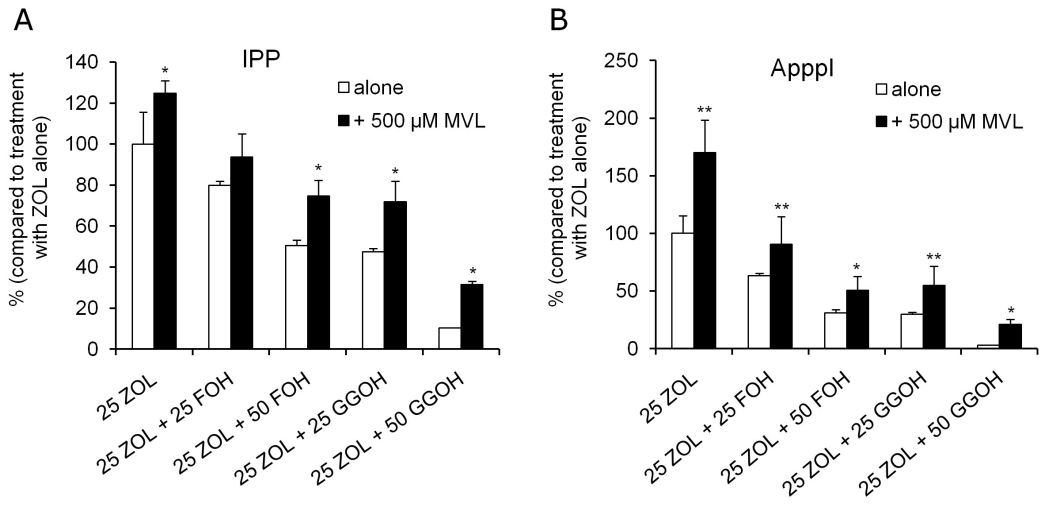


Figure 20. Mevalonate partially overcomes isoprenoid-induced inhibition on IPP/ApppI accumulation. The effect of mevalonate lactone (MVL) on accumulation of IPP (A) and the formation of ApppI (B) in MCF-7 cells. The cells were incubated for 24 h with drugs: 25 μM zoledronic acid (25 ZOL) with or without 500 μM MVL; 25 μM ZOL plus 25/50 μM farnesol (25/50 FOH) with or without 500 μM MVL; 25 μM ZOL plus 25/50 μM geranylgeraniol (25/50 GGOH) with or without 500 μM MVL. The molar amounts of IPP/ApppI were determined by the HPLC-ESI-MS method (mean±SEM, n=6). *, $P<0.05$; **, $P<0.01$ compared to treatment without MVL using one-way ANOVA with Bonferroni's comparison test.

7.4 DISCUSSION

Evidence from previous studies indicate that the inhibition of the mevalonate pathway play a central role in the anticancer effects of N-BPs (Table 3). These studies have demonstrated that interruption of protein prenylation is a major contributor to the anticancer action of N-BPs, whereas the role and the significance of the additional mechanism of action, accumulation of IPP/ApppI, is less clear.

Previously, it has been shown that the inhibitory action of N-BPs on cell function could be prevented by isoprenoids (Table 3). Until now, this has been attributed to their action on the mevalonate pathway downstream from FPPS by rescuing protein prenylation, but here we show that they could simultaneously act also upstream from FPPS by inhibiting N-BP-induced IPP/ApppI accumulation. The results illustrate that co-treatment with cell permeable isoprenoids, GGOH and FOH, significantly reduced expression of IPP/ApppI accumulation in human estrogen-dependent cancer cells, regardless of the ZOL dose. This is a very interesting observation, as it is known that the interaction of ZOL with the FPPS enzyme produces a very stable complex (Chapter 2.3.3), and thus, IPP accumulation upstream of FPPS should stay constant during the treatment. Interestingly, 50 μM GGOH and 100 μM GGPP almost completely blocked IPP accumulation, and the consequent ApppI formation in cells, whereas FOH only partially attenuated these effects of ZOL. The results are consistent with the appearance of unprenylated form of the small GTPase Rap1A that was seen to correlate with the level of accumulating IPP/ApppI in cells. One possible explanation for the failure of FOH to rescue protein prenylation could be that only a small amount of FPP formed from FOH is metabolized further to GGPP (Crick et al. 1997). Conversion of FPP to GGPP requires IPP, which is available after FPPS activity is blocked by ZOL. This could be sufficient to allow FOH-induced partial recovery of Rap1A prenylation. Alternatively, the cells may convert FOH to FPP for the farnesylation of proteins that are normally geranylgeranylated (Gibbs and Oliff 1997).

All the isoprenoids of the mevalonate pathway are synthesized by a condensation reaction of two simple precursors: IPP and DMAPP. Furthermore, both of the isomers are required for the activity of N-BP in cells: IPP for binding and stabilizing FPPS enzyme-N-BP complex (Chapter 2.3.3), and both isomers for conjugation reaction to AMP to form ATP analogs, ApppI and ApppD (Chapter 2.3.5). Recently, we developed a new method for quantification of these isomers in cell extracts (Jauhiainen et al. 2009). Using this method, we demonstrate that DMAPP is the predominant isomeric form detected in MCF-7 cells, which is consistent with our previous finding that the ratio of IPP:DMAPP is 1:4 after ZOL treatment (Jauhiainen et al. 2009). The ratio of the isomers in cell samples was found to be stable irrespective of the treatments, evidencing that isoprenoids do not affect the enzyme activity of IPP isomerase. Similarly, no differences in the formation of $AppCCl_2p$ from a non-N-BP clodronate were detected between the treatments, indicating that aminoacyl-tRNA synthase converting of IPP/DMAPP into ApppI/ApppD, is not modified by FOH or GGOH. Furthermore, no changes were observed in the FPPS levels analyzed by Western blot, indicating FPPS enzyme levels are not affected by the isoprenoids.

Since our results revealed that isoprenoids affected neither the expression levels of FPPS nor inhibited the function of IPP isomerase or aminoacyl-tRNA synthetase enzymes, mechanisms upstream from FPPS were investigated. HMGR is the rate-limiting enzyme of isoprenoid synthesis from mevalonate, and its activity has been reported to be regulated at many levels (Chapter 2.1.1). Our findings indicate that the inhibition of ZOL-induced IPP/ApppI accumulation results from HMGR downregulation, since both GGOH and GGPP clearly decrease the amount of HMGR enzyme in cells, presumably by increasing its rate of degradation. In addition, the ability of MVA, a precursor for IPP, to partially overcome the isoprenoid-induced inhibition on IPP/ApppI accumulation suggests that the regulation could be mediated by HMGR, which catalyzes MVA formation. The effect of MVA on restoring IPP/ApppI levels was not very potent, perhaps due to

the mevalonate-mediated HMGR control. This suggestion is supported by previous studies showing that MVA at higher concentrations can regulate the translation and degradation of HMGR (Nakanishi et al. 1988, Goldstein and Brown 1990). Our results do not solve the puzzle of whether the active form for HMGR regulation is the isoprenoid alcohol or the corresponding pyrophosphate or even some consequent products. The study of Correll *et al.* (1994) suggests that isoprenoid alcohol is the active component or at least is having a more direct role in enzyme degradation. The results of that study indicated that FPP-induced, but not FOH-induced, degradation of HMGR was blocked by the addition of sodium fluoride, a general phosphatase inhibitor, in permeabilized cells. In the present study, only a slight reduction on the protein levels of HMGR could be detected with FOH. However, its lack of effect on enzyme degradation is in agreement with our findings obtained from the MS analysis, where FOH was not as effective at inhibiting IPP/ApppI accumulation as GGOH or GGPP. We have previously shown that the level of IPP/ApppI correlates well with ZOL-induced cancer cell death *in vitro* (Mitrofan et al. 2009). The outcome of the present study is consistent with observations that MCF-7 cells are rescued from ZOL-induced apoptosis by GGOH, but not by FOH or MVA (Jagdev et al. 2001). In addition, previous studies indicate that geranylgeranylated proteins rather than farnesylated proteins are required for suppression of apoptosis in cancer cells, and thus seem to be largely responsible for the antitumor effects of N-BPs, at least *in vitro* (Chapter 2.3.4 and Table 3)**.**

Taken together, this study strongly suggests that isoprenoids, GGOH or GGPP, inhibit ZOL-induced IPP/ApppI accumulation via downregulation of HMGR in the mevalonate pathway. This represents a novel insight into the mechanism of action of isoprenoids on the regulation of the mevalonate pathway after FPPS inhibition. The data implies that additionally to the previously reported effects on rescuing protein prenylation, isoprenoids can preserve cell activity by inhibiting the accumulation of IPP/ApppI in cells. This knowledge of the different mechanisms of action involved is relevant for understanding the anticancer action of N-BPs, and furthermore for expanding application of BPs in cancer treatment.

8 In vitro correlation between time-dependent inhibition of FPPS and blockade of mevalonate pathway by N-BPs

Abstract. Previous cell-free data has reported that N-BPs inhibit FPPS by time-dependent manner as a result of the conformational change. This associated conformational change can be measured as an isomerization constant (K_{isom}) and reflects the binding differences of the N-BPs to FPPS. In the present study, we tested the biological relevance of the calculated K_{isom} values of ZOL, RIS and five experimental N-BP analogs in the cell culture model. We used IPP/ApppI formation as a surrogate marker for blocking of FPPS in the mevalonate pathway. As a result, a correlation between the time-dependent inhibition of FPPS and IPP/ApppI formation by N-BPs was observed. This outcome indicates that the time-dependent inhibition of FPPS enzyme is a biologically significant mechanism and further supports the use of the K_{isom} calculations for evaluation of the overall potency of the novel FPPS inhibitors. Additionally, our data illustrates that IPP/ApppI analysis is a useful method to monitor the intracellular action of drugs and drug candidates based on FPPS inhibition.

*Adapted with permission of Elsevier from: Räikkönen J, Taskinen M, Dunford JE, Mönkkönen H, Auriola S, Mönkkönen J: Correlation between time-dependent inhibition of human farnesyl pyrophosphate synthase and blockade of mevalonate pathway by nitrogen-containing bisphosphonates in cultured cells. Biochem Biophys Res Commun 407:663-667, 2011.

8.1 INTRODUCTION

The molecular mechanism of FPPS inhibition has only become apparent in the past few years. Recent publications have been established that the inhibition of FPPS by N-BPs has a time-dependent component, as a result of an isomerization of the enzyme–inhibitor complex binding (Kavanagh et al. 2006, Rondeau et al. 2006, Dunford et al. 2008, Chapter 2.3.3). Briefly, after N-BP binding the accumulating substrate IPP binds to FPPS and further stabilizes the FPPS-N-BP-complex in the fully closed conformation producing a very efficient and sustainable complex. The associated conformational change can be measured as an isomerization constant (K_{isom}) and has been used to indicate the reversibility of the isomerization of the FPPS-inhibitor complex (*i.e.* time-dependent inhibition). It implies that the compounds with higher K_{isom} will inhibit the enzyme for longer and be less reversible. It has been shown that the ability of a N-BP to hold the enzyme in the isomerized state plays an important role in the overall potency of the drug (Dunford et al. 2008). However, previous studies have been conducted in cell-free systems. Therefore, the aim of this study was to define the biological relevancy of the calculated K_{isom} values of different N-BPs in the cultured cells by using N-BP-induced IPP/ApppI production to monitor FPPS blockade of the mevalonate pathway. As a result, we discovered a significant *in vitro* relationship between the time-dependent inhibition of FPPS by N-BPs, zoledronic acid (ZOL), risedronate (RIS) and five experimental N-BP analogs, and the inhibition of the mevalonate pathway.

8.2 EXPERIMENTAL

All the reagents used in this study are described in Chapter 4.1.

8.2.1 IPP/DMAPP and ApppI detection

The MCF-7 cells were seeded in 6–well plates at 2 x10^6 cells/well. Two different treatment protocols were used: pulse and continuous treatment. For pulse treatment, the cells were synchronized by serum starvation in order to minimize the variability in cell cycle between the samples. Five hours after seeding, the cells were washed once with starving medium containing RPMI-1620 medium + 1 % P/S + 0.1 % bovine serum albumin. After 18 h of incubation in starving medium, normal culture medium with 30 μM N-BP or an equivalent volume of sterile PBS was added to the cells for 3 h. The samples were collected at 0, 1, 3, 6, 12, 24, 48 or 72 h after drug removal. For continuous treatment, the cells were left to adhere overnight. Non-adherent cells were then removed and cells were exposed for 24 h to 30 μM N-BP, or an equivalent volume of sterile PBS. NE58062 was excluded from the continuous treatment study because of the lack of compound. At the time point, the cell extract preparations and protein content determinations were carried out as described in Chapter 4.3. Analysis of IPP/ApppI by HPLC-ESI-MS was performed as described in Chapter 4.4. The relative amount of IPP and DMAPP isomers in the treated cell samples were calculated using a protocol described earlier (Jauhiainen et al. 2009). This method utilizes the peak intensity ratios of two characteristic fragment ions, *m*/*z* 177 and *m*/*z* 159, of IPP and DMAPP obtained from MS^2 monitoring. The results shown are representative of at least 3 independent experiments (mean±SEM).

8.2.2 Cell viability assay

The influence of the N-BP concentration and incubation time on cell viability was evaluated by the CellTiter-Blue™ Assay (Promega, USA) as described in the manufacturer's manual. MCF-7 cells were seeded into a 96-well microplate at 1.25 x10^4 cells/well. Next day, the cells were treated with 10-100 μM of N-BP, or an equivalent volume of sterile PBS (control), for 3 or 24 h, after which the

CellTiter-Blue™ reagent (20 μl/well) was added directly to each well at 37 °C for 1 h. The fluorescence intensity was measured using Wallac Victor 1420 Multilabel counter (Perkin Elmer) with 560/590 nm excitation/emission wavelengths. Experiments were run in sextuplicate and repeated twice.

8.2.3 Statistics

The data are expressed as the mean ± standard error of the mean. The relationship between IPP/ApppI and isomerization constant was examined by linear regression and correlations were calculated using Spearman's correlation. All statistical analyses were performed with GraphPad Prism. A p-value less than 0.05 was considered statistically significant.

8.3 RESULTS AND DISCUSSION

Previous analyses based on molecular modeling and experiments with cell-free systems have shown that N-BPs inhibit FPPS by a mechanism that involves time-dependent isomerization of the enzyme where the enzyme undergoes a structural rearrangement on inhibitor binding (Kavanagh et al. 2006, Rondeau et al. 2006, Dunford et al. 2008, Chapter 2.3.3). Herein we study the correlation of the time-dependent inhibition of FPPS enzyme and blockade of mevalonate pathway by N-BPs in cultured human cells.

We have previously shown that IPP and ApppI production correlates well with the potency of N-BPs to inhibit FPPS activity in cells (Mönkkönen et al. 2006, Chapter 5). In the present study, therefore, we used IPP/ApppI formation as a surrogate marker for FPPS inhibition and applied the obtained data to research the relevance of K_{isom} values at the cellular level. We explored IPP/ApppI formation profiles induced by two potent N-BPs in clinical use (ZOL and RIS), two experimental N-BPs (NE97220 and NE58062), and three experimental analogs of RIS (NE58043, NE58027 and NE21650) in human breast cancer cells. In addition, the influence of the drugs on cell viability was determined. All N-BPs induced IPP/ApppI accumulation in cells after continuous and pulse drug exposure (Figure 21, Figures 22A-B). Prior to this, there was no IPP/ApppI formation data of the NE-compounds used in this study, available. Importantly, in the case of pulse treatment both IPP and ApppI could be detected immediately after drug removal (0 h) and even 3 days following the treatment (Figure 21). None of the drug concentrations (10-100 μM) or incubation times (3 or 24 h) tested influenced significantly on cell viability when compared to untreated cells (control) (data not shown). As a whole, ZOL and RIS, with the highest K_{isom} (Dunford et al. 2008), produced the highest amount of IPP and ApppI over time (Figure 21). After continuous drug treatment, the potency of N-BP to induce IPP/ApppI was ZOL > RIS, NE58043 and NE97220 > NE58027 > NE12650 (Figure 22A). In addition to inhibiting FPPS, NE21650 has been shown to be a weak inhibitor of IPP isomerase (Thompson et al. 2002), the enzyme proximal to FPPS in the mevalonate pathway converting IPP to DMAPP (Hahn et al. 1996). This IPP isomerase inhibition was clearly seen in the IPP/DMAPP ratio (Figure 23). In NE21650 treated cells IPP was not converted to DMAPP, whereas with other N-BPs, the ratio of IPP and DMAPP isomers in cells was approximately 1:4, as shown in the previous studies (Jauhiainen et al. 2009, Chapter 7). By contrast, pulse exposure to NE21650 for 3 h was insufficient to inhibit the IPP isomerase during 3 day observation time (data not shown).

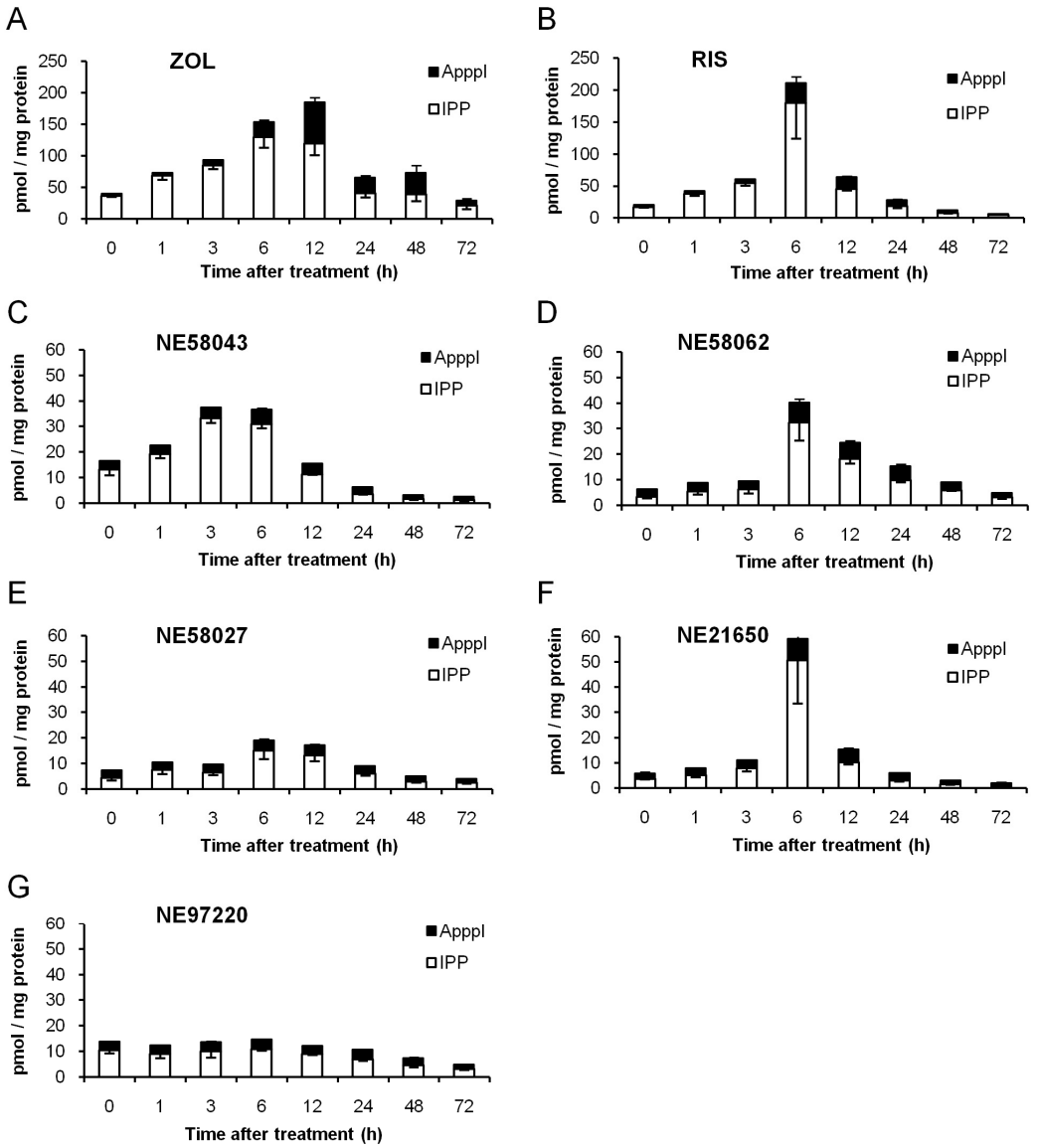


Figure 21. IPP/ApppI formation in cells after a pulse exposure to different N-BPs. After 18 h of serum-starvation, the MCF-7 cells were treated with 30 μM of zoledronate (ZOL) (A), risedronate (RIS) (B), NE58043 (C), NE58062 (D), NE58027 (E), NE21650 (F), or NE97220 (G) for 3 h, and the samples were collected 0-72 h after drug removal. IPP and ApppI formation were determined in cell extracts by mass spectrometry (mean±SEM, n=6). Graphs of ZOL and RIS are presented with the different scale than the N-BP analogues.

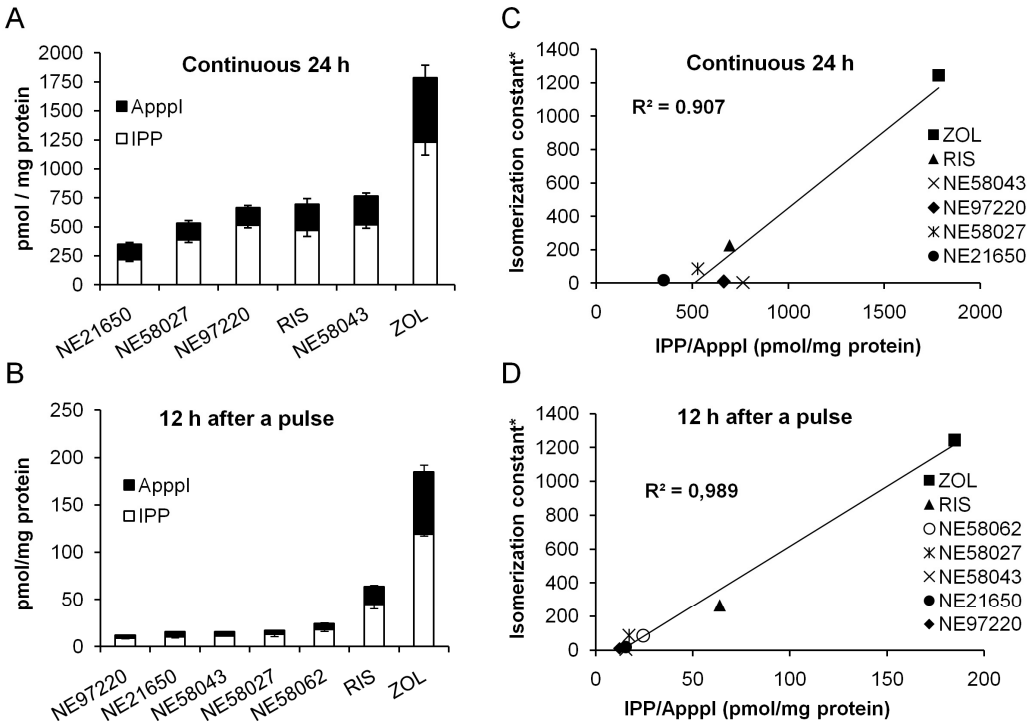


Figure 22. The relationship between N-BP-induced IPP/ApppI formation and the time-dependent inhibition of FPPS. IPP/ApppI formation in MCF-7 cells after continuous drug treatment (A) and pulse treatment (B), the relationship between isomerization constant and the amount of IPP/ApppI obtained from continuous exposure data (C) and pulse exposure data (D). IPP/ApppI were determined by mass spectrometry either in 24 h 30 μM N-BP-treated cell extracts (A) or in pulse N-BP-treated cell extracts collected 12 h after exposure (B). For pulse treatments, 18 h of serum-starvation was used, after which the cells were treated with 30 μM of drug for 3 h (mean±SEM, n=6). *Isomerization constant determined by Dunford et al. (2008).

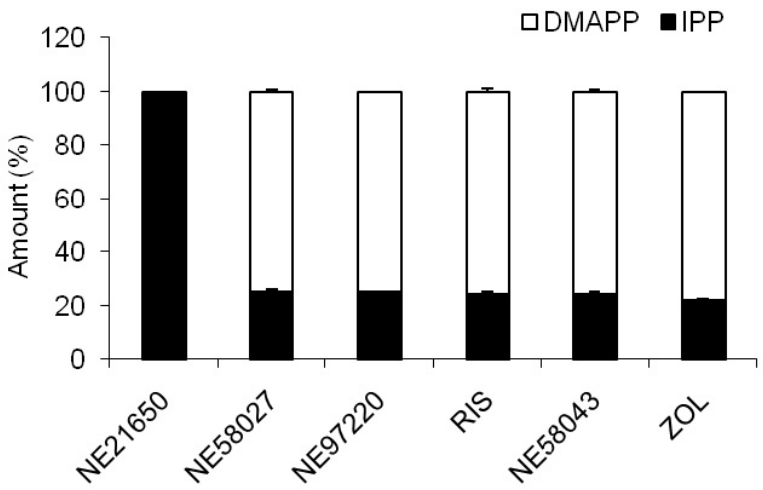


Figure 23. The relative intracellular amount of IPP and DMAPP isomers catalyzed by IPP isomerase. The MCF-7 cells were exposed to 30 μM of N-BP continuously for 24 h (mean±SEM, n=6). The relative amounts of isomeric compounds, IPP and DMAPP, were determined with a previously developed quantitative method (Jauhiainen et al. 2009), which is based on mass spectrometric separation.

In order to elucidate the relationship between isomerization and inhibition of FPPS by N-BPs in cells, the K_{isom} values were plotted against the levels of IPP and ApppI (Figure 22). Since the ATP analog ApppI is a result of the condensation of AMP with IPP (Mönkkönen et al. 2006), the total number of accumulated IPP and ApppI was used in data analyses. The time point of 12 h after drug removal was selected to represent pulse treatment data in Figure 22. This time point was chosen, in order to detect the reversibility of the enzyme isomerization. For example, the half-life for the reversal of the enzyme-RIS isomerized complex has been calculated to be about 5 h (Dunford et al. 2008). Data obtained from both pulse and continuous drug treatments showed significant correlations between IPP/ApppI production and K_{isom} (Table 6, Figure 22). Similar correlations were found when the relationship of either IPP or ApppI alone with K_{isom} were examined (data not shown). In both experiments, NE58043 was an outlier. It appeared to be more potent in inducing IPP/ApppI than its K_{isom} value would suggest. This is in accordance with the observation in the study of Dunford et al. (2008), where NE58043 inhibits FPPS better than the parent compound RIS, but ultimately loses out to RIS in the ability to hold the enzyme-inhibitor in the isomerized state. In the continuous treatment, inhibitor is present for 24 h until sample collection. Thus, it is likely that the stability of the isomerization is constant and therefore the reversal of the enzyme-N-BP isomerized complex cannot be observed. This phenomenon can also be detected during 0-6 h after pulse exposure (Table 6). Instead, after 6 h of pulse treatment, the correlation between IPP/ApppI and K_{isom} was shown to be good. Therefore, the data from pulse treatments reflects more likely the real feature of N-BPs, since the inhibitor is present only momentarily and the stability of the complex can be detected over time. Thus, the time-dependent inhibition of FPPS by different N-BPs can be seen in the kinetics of formation of IPP and ApppI after the pulse treatment (Figure 21). Compounds NE58043, NE21650 and NE97220, with lower K_{isom}, induced fast IPP/ApppI accumulation and the peak value of IPP/ApppI is reached by 6 h. On the contrary, ZOL, RIS, NE58062 and NE58027, compounds with higher K_{isom}, induced maximal IPP/ApppI after 6-12 h of treatment. In general, all the NE-compounds have relatively low K_{isom} values, and they induced

similar amounts of IPP and ApppI in the cells. Therefore, the enzyme inhibition potency of these agents was demonstrated to be substantially weaker relative to the other N-BPs.

Table 6. The relationship between N-BP-induced IPP/ApppI formation and isomerization constant shows a significant correlation. The relationship between IPP/ApppI and K_{isom} was calculated from pulse and continuous treatment data (shown in Figures 21-22) using Spearman's correlation (r^2). The values of isomerization constant (K_{isom}) have previously been established by Dunford et al. (2008). $p<0.05$ was considered statistically significant.

Treatment	**Time point (h)**	**r^2**	***p* Value**
Pulse	0	0.82	< 0.01
Pulse	1	0.84	< 0.01
Pulse	3	0.74	< 0.05
Pulse	6	0.33	> 0.05
Pulse	12	0.99	< 0.0001
Pulse	24	0.96	< 0.0001
Pulse	48	0.99	< 0.0001
Pulse	72	0.99	< 0.0001
Continuous	24	0.91	< 0.01

In summary, the data indicate that the time-dependent inhibition of FPPS by N-BPs is a biologically relevant mechanism evidenced by the strong correlation found between the K_{isom} values and IPP/ApppI formation by N-BPs. This outcome further supports the use of the K_{isom} calculations for evaluation of the overall potency of the novel FPPS inhibitors. Additionally, the data illustrates that even though the interaction of FPPS with N-BPs is highly complex, IPP/ApppI detection offers a simple way to monitor the intracellular action of bisphosphonate and non-bisphosphonate FPPS inhibitor candidates under development.

9 General Discussion and Future Prospects

Despite their widespread clinical use, the exact biochemical mechanism of action of BPs in metabolic bone diseases was until recently largely unknown. The study of Mönkkönen et al. (2006) revealed a novel possible mechanism of action of N-BPs, a finding which may partially account for the antiresorptive and anticancer effects of N-BPs. In addition to preventing protein prenylation, N-BPs induce the formation of a novel endogenous ATP analog, ApppI, as a consequence of the inhibition of FPPS, and the subsequent accumulation of IPP. As observed for AppCCl$_2$p, the corresponding metabolite from the non-N-BP clodronate, ApppI is a pharmacologically active compound capable of inducing direct apoptosis in osteoclasts. The current study characterized the role of the intracellular accumulation of IPP/ApppI in the mechanism of action of N-BPs by providing more detailed data on IPP/ApppI formation both *in vivo* and *in vitro*.

ApppI in vivo

This study provided the first conclusive evidence that ApppI is formed in osteoclasts *in vivo*, the pharmacological target cells for BPs, after a single administration of ZOL at a clinically relevant dose. ZOL-induced IPP/ApppI accumulation by authentic osteoclasts *in vivo* has not been proven until now, and therefore this data established that the pro-apoptotic ApppI is a biologically significant molecule in the action of N-BPs. Additionally, minor amounts of IPP/ApppI were also detected in non-osteoclast cell fractions. Thus, it is possible that other neighboring cell types in the bone microenvironment can internalize BPs and consequently accumulate IPP/ApppI.

General characteristics of ApppI formation

All the data from time-course experiments confirmed that ApppI formation results from the accumulation of IPP after the mevalonate pathway is blocked by N-BPs, and thus suggest that IPP/ApppI formation can be used as a surrogate marker for N-BP-induced inhibition of FPPS. Exposure of cells *in vitro* to N-BPs at varying times (1-72 h) and concentrations (1-100 μM) induced a time- and dose-dependent accumulation of IPP and consequent ApppI production. The earlier hypothesis that ApppI formation from IPP may be catalyzed by the same metabolic pathway (i.e. aminoacyl-tRNA-synthetases) as the ATP analogs of non-N-BPs (Mönkkönen et al. 2006) was further confirmed in this study, demonstrating that the efficiency of ApppI production from IPP correlated with clodronate metabolism. In addition to this observed correlation, *in vitro* results together with the data obtained after *in vivo* treatment illustrated that approximately 20 % of IPP was being converted to ApppI in cells, and 26 % of internalized clodronate was metabolized to AppCCl$_2$p, suggesting that the same metabolizing enzymes with same capacity might be responsible for the ApppI formation from IPP, and for clodronate metabolism. However, to prove unequivocally that the aminoacyl-tRNA-synthetase family was involved in the production, experiments with purified enzymes will need to be performed. Confirming the role of the aminoacyl-tRNA-synthetase in ApppI formation in cells also requires further studies, for example through siRNA down-regulation of the enzymes thought to be responsible for their synthesis.

IPP and ATP analog formation in cancer cells

The N-BP-induced inhibition of FPPS enzyme of the mevalonate pathway seems to account for their anticancer effects observed *in vitro* and for activation of γδ T-cells (Stresing et al. 2007). Apoptosis and growth inhibition have generally been suggested to be the major mechanisms underlying the

direct anticancer activity of BPs. The mechanism by which BPs inhibit cancer cell growth might be mediated through the production of cytotoxic ATP analogs, ApppI and $AppCCl_2p$. We demonstrated for the first time that BPs can induce the accumulation of ATP analogs, ApppI and $AppCCl_2p$, in cancer cells. However, ZOL-induced IPP/ApppI accumulation and clodronate metabolism to $AppCCl_2p$ were clearly cell line dependent. Although, the amounts of the metabolites were independent from the cellular uptake of BP, this does not exclude the possibility that some differences in the release mechanisms, and consequent intracellular distribution of BPs may still be involved, which could potentially alter the efficacy of BP in cells. However, most likely, the differences between the cell lines are related to different activities of the intracellular enzymes, such as FPPS and/or aminoacyl-tRNA synthetases, since IPP production was seen to correlate with the capacity of ZOL to inhibit protein prenylation (a surrogate marker for FPPS inhibition), and the efficiency of ApppI production from IPP correlated with the rate of clodronate metabolism. To support this hypothesis, Ory and colleagues (2008) have demonstrated resistance to ZOL-induced apoptosis through higher expression of FPPS. There is also new evidence that the cells with low IPP/ApppI may have low HMGR expression, an enzyme upstream of FPPS (Benzaid et al. 2011).

Taken together, the data strongly suggests that BPs may have diverse antitumor effects according to the extent of accumulation of pro-apoptotic ATP analogs within the cells. This was evidenced by the present study together with the study of Mitrofan et al. (2009) demonstrating that ZOL-induced IPP/ApppI accumulation as well as inhibition of protein prenylation correlated with the capacity of different cancer cells to undergo apoptosis, since the cells with low levels of IPP/ApppI and unprenylated Rap1A proteins were resistant to ZOL-induced apoptosis. Our study also showed that the effect of clodronate on different cancer cell growth inhibition corresponded to the potency of clodronate metabolism to $AppCCl_2p$. Furthermore, as IPP could be recognised by γδ T-cells as tumor antigens (Morita et al. 1995, Tanaka et al. 1995, Gober et al. 2003), this further implies that regardless of the tumor location and circulating concentrations of BPs, some cancer cells might not be able to trigger immune response because of low IPP induction after stimulation with N-BP. An N-BP-based immunotherapeutic approach may therefore only be successful in certain types of cancer which have a high IPP induction capacity. This hypothesis is supported by a very recent study demonstrating that Vγ9Vδ2 T-cell cytotoxicity correlates with ZOL-induced IPP/ApppI accumulation in breast cancer cells *in vitro* and *in vivo* (Benzaid et al. 2011). N-BP-based immunotherapy may play a major role in the control of cancer and thus, future studies should be directed to identify the best treatment strategies for different types of cancer.

Overall, the IPP/ApppI formation profiles obtained from the time-course experiments demonstrated that the levels of IPP/ApppI in cancer cells decreased rather shortly after pulse treatment with N-BP. This might suggest that in order to achieve more efficacious treatment in cancer therapy, continuous or frequent pulse treatment with BPs would be needed. This hypothesis is supported by previous *in vivo* findings demonstrating that treatment of animals with a single clinical dose did not reduce the tumor burden, whereas a low dose of ZOL administered to animals on a daily or weekly intermittent schedule not only inhibited bone destruction, but also exhibited antitumor effects (Daubine *et al*., 2007; Stresing *et al.*, 2007). However, many studies have shown conflicting results regarding the optimal dosing regimen for BPs to inhibit the formation of bone metastases. Also, whether clinically relevant BP doses are sufficient to affect tumor growth is a hotly debated issue.

Regulation of IPP/ApppI accumulation

In addition to cancer cell type, the kinetic profiles and levels of intracellular IPP and ApppI were shown to be dependent on the concentration and dosing interval of the N-BP used, and also the on the FPPS binding characteristics of the drug. Furthermore, ZOL-induced IPP/ApppI levels in cells can be downregulated by the mevalonate pathway isoprenoids (Chapter 7, Mitrofan et al. 2009,

Mitrofan et al. 2010). Co-treatment with GGOH, FOH, and especially GGPP significantly reduced the levels of IPP/ApppI in breast cancer cells, regardless of the ZOL dose. This represents a novel insight into the mechanism of action of isoprenoids in the regulation of mevalonate pathway after FPPS inhibition. The results suggest that in addition to the previously reported effects on rescuing protein prenylation, isoprenoids can salvage cell activity by downregulating IPP/ApppI levels in cells, presumably by inducing HMGR degradation. This result supports the role of ApppI in mediating ZOL-induced apoptosis, which is further supported by the study of Mitrofan et al. (2010) demonstrating that the accumulation of unprenylated proteins is not an absolute prerequisite for ZOL-induced apoptosis. However, it still remains unclear why the addition of FOH is less effective than adding GGOH or GGPP in reversing the effects of the drugs. Perhaps, FPP may be routed towards cholesterol production, resulting in decreased FPP pools available for prenylation and regulation of the mevalonate pathway (Figure 1). In the future experiments, a squalene synthase inhibitor should be added together with FPP to the cell cultures in order to eliminate the excess use of FPP for cholesterol synthesis.

IPP/ApppI analysis as a tool for investigating the intracellular action of the mevalonate pathway inhibitors

The present study has illustrated that mass spectrometric IPP/ApppI analysis is a useful and sensitive tool for investigating the intracellular action of BPs and other mevalonate pathway inhibitor candidates under development. The development of new BP analogs with a lower affinity for bone mineral could increase drug accessibility to soft tissues and therefore offer the prospect of developing new classes of FPPS inhibitors which may be of clinical use as antitumor drugs or may even possess more diverse therapeutic applications (Chapter 2.4.2).

Identification of IPP/ApppI formation as a true molecular mechanism of action of N-BPs has provided further understanding of the differences and similarities between N-BPs and non-N-BPs in general. Furthermore, IPP/ApppI studies have revealed the potency of N-BPs to affect a wide range of cellular processes via the inhibition of FPPS, and may also help to explain the observed preclinical and clinical activity of these compounds.

Future challenges

This study provided further evidence that BP-induced IPP and consequent ApppI accumulation are important metabolic events underlying the molecular mechanisms of action of BPs. However, one challenge for future research will be to determine how much the IPP/ApppI accumulation accounts for the observed effects of N-BPs. Since the isoprenoids of the mevalonate pathway can salvage protein prenylation, but concurrently inhibit IPP/ApppI accumulation, they cannot be used to explore the cellular effect of IPP/ApppI. Thus in future studies, liposome-encapsulated IPP and ApppI could be used for controlled cellular delivery. However, in this kind of experiment, it would be important to determine the intracellular concentration of IPP/ApppI after N-BP treatment in order to achieve more reliable results.

10 Conclusions

This study characterized the role of the intracellular accumulation of IPP/ApppI in the mechanism of action of nitrogen-containing bisphosphonates (N-BPs) and provided more detailed data on IPP/ApppI formation both in cultured cancer cells, *in vitro,* and in an animal model, *in vivo*. The main conclusions of the present study are as follows:

1. Single administration of zoledronic acid at a clinically relevant dose induces ApppI formation in osteoclasts *in vivo*, the pharmacological target cells for BPs. This finding confirmes the hypothesis that ApppI is a biologically significant molecule in the action of N-BPs.

2. BPs induce the accumulation of IPP and pro-apoptotic ATP analogs, ApppI and $AppCCl_2p$, in different cancer cells. However, the accumulation of these metabolites is remarkably cell line dependent, and this phenomenon is likely attributable to the activity of the intracellular enzymes responsible for metabolite formation. This provides a potential new mechanism contributing to the specificity of BPs against different cancer cell types suggesting that BPs may have wide ranging antitumor effects according to the ability of the cancer cells to metabolize the cytotoxic compounds.

3. Clodronate metabolism to $AppCCl_2p$ corresponds better than zoledronic acid-induced ApppI formation to the potency of BP to inhibit cancer cell growth. As N-BPs have a more complex mechanism of action compared to non-N-BPs, it is possible that no direct connection between the level of ApppI formation and the observed cellular effects may be found.

4. IPP and consequently ApppI accumulate in cells in a time and dose-dependent manner, and even a one-hour pulse treatment with N-BP is sufficient to induce IPP/ApppI formation. Furthermore, the efficiency of N-BP-induced IPP/ApppI accumulation seems to be dependent on the cell type, enzyme activities of the FPP synthase and aminoacyl-tRNA synthetases, and also on the FPP synthase binding characteristics of the drug.

5. N-BP-induced IPP/ApppI accumulation in cells can be inhibited by the mevalonate pathway isoprenoids, presumably via downregulation of HMG-CoA reductase. This suggests that in addition to rescuing protein prenylation, isoprenoids can salvage cell activity by inhibiting IPP/ApppI levels in cells, and thus these results support the hypothesis that ApppI has a significant role in N-BP-induced apoptosis.

6. IPP/ApppI analysis is a useful and sensitive tool for investigating the intracellular action of BPs and other mevalonate pathway inhibitor candidates under development. The use of IPP/ApppI accumulation as a surrogate marker of N-BP efficacy might have applications both in preclinical models and in clinical trials attempting to clarify the anticancer activities of N-BPs.

10 References

Adami S and Zamberlan N: Adverse effects of bisphosphonates. A comparative review. Drug Saf. 14:158-170, 1996

Amin D, Cornell SA, Gustafson SK, Needle SJ, Ullrich JW, Bilder GE, Perrone MH: Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase and cholesterol biosynthesis. J.Lipid Res. 33:1657-1663, 1992

Auriola S, Frith J, Rogers MJ, Koivuniemi A, Mönkkönen J: Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography-electrospray mass spectrometry. J.Chromatogr.B Biomed.Sci.Appl. 704:187-195, 1997

Bansal G, Wright JEI, Kucharski C, Uludag H: A dendritic tetra(bisphosphonic acid) for improved targeting of proteins to bone. Angew.Chem.Int.Ed. 44:3710-3714, 2005

Baron RA, Tavare R, Figueiredo AC, Blazewska KM, Kashemirov BA, McKenna CE, Ebetino FH, Taylor A, Rogers MJ, Coxon FP, Seabra MC: Phosphonocarboxylates inhibit the second geranylgeranyl addition by Rab geranylgeranyl transferase. J.Biol.Chem. 284:6861-6868, 2009

Benford HL, Frith JC, Auriola S, Mönkkönen J, Rogers MJ: Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates: biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. Mol.Pharmacol. 56:131-140, 1999

Benford HL, McGowan NW, Helfrich MH, Nuttall ME, Rogers MJ: Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts in vitro. Bone 28:465-473, 2001

Benzaid I, Mönkkönen H, Stresing V, Bonnelye E, Green J, Mönkkönen J, Touraine JL, Clezardin P: High Phosphoantigen Levels in Bisphosphonate-Treated Human Breast Tumors Promote V{gamma}9V{delta}2 T-Cell Chemotaxis and Cytotoxicity In Vivo. Cancer Res. 71:4562-4572, 2011

Bergstrom JD, Bostedor RG, Masarachia PJ, Reszka AA, R-odan G: Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. Arch.Biochem.Biophys. 373:231-241, 2000

Bezzi M, Hasmim M, Bieler G, Dormond O, Ruegg C: Zoledronate sensitizes endothelial cells to tumor necrosis factor-induced programmed cell death: evidence for the suppression of sustained activation of focal adhesion kinase and protein kinase B/Akt. J.Biol.Chem. 278:43603-43614, 2003

Boehm U, Klamp T, Groot M, Howard JC: Cellular responses to interferon-gamma. Annu.Rev.Immunol. 15:749-795, 1997

Boissier S, Ferreras M, Peyruchaud O, Magnetto S, Ebetino FH, Colombel M, Delmas P, Delaisse JM, Clezardin P: Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Res. 60:2949-2954, 2000

Boissier S, Magnetto S, Frappart L, Cuzin B, Ebetino FH, Delmas PD, Clezardin P: Bisphosphonates inhibit prostate and breast carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. Cancer Res. 57:3890-3894, 1997

Bolick SC, Landowski TH, Boulware D, Oshiro MM, Ohkanda J, Hamilton AD, Sebti SM, Dalton WS: The farnesyl transferase inhibitor, FTI-277, inhibits growth and induces apoptosis in drug-resistant myeloma tumor cells. Leukemia 17:451-457, 2003

Boulenc X, Marti E, Joyeux H, Roques C, Berger Y, Fabre G: Importance of the paracellular pathway for the transport of a new bisphosphonate using the human CACO-2 monolayers model. Biochem.Pharmacol. 46:1591-1600, 1993

Brown HK, Ottewell PD, Coleman RE, Holen I: The kinetochore protein Cenp-F is a potential novel target for zoledronic acid in breast cancer cells. J.Cell.Mol.Med. 15:501-513, 2009

Brown MS and Goldstein JL: Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J.Lipid Res. 21:505-517, 1980

Brusselmans K, Timmermans L, Van de Sande T, Van Veldhoven PP, Guan G, Shechter I, Claessens F, Verhoeven G, Swinnen JV: Squalene synthase, a determinant of Raft-associated cholesterol and modulator of cancer cell proliferation. J.Biol.Chem. 282:18777-18785, 2007

Buhaescu I and Izzedine H: Mevalonate pathway: a review of clinical and therapeutical implications. Clin.Biochem. 40:575-584, 2007

Caraglia M, Budillon A, Tagliaferri P, Marra M, Abbruzzese A, Caponigro F: Isoprenylation of intracellular proteins as a new target for the therapy of human neoplasms: preclinical and clinical implications. Curr.Drug Targets 6:301-323, 2005

Caraglia M, D'Alessandro AM, Marra M, Giuberti G, Vitale G, Viscomi C, Colao A, Prete SD, Tagliaferri P, Tassone P, Budillon A, Venuta S, Abbruzzese A: The farnesyl transferase inhibitor R115777 (Zarnestra) synergistically enhances growth inhibition and apoptosis induced on epidermoid cancer cells by Zoledronic acid (Zometa) and Pamidronate. Oncogene 23:6900-6913, 2004

Chlebowski RT, Chen Z, Cauley JA, Anderson G, Rodabough RJ, McTiernan A, Lane DS, Manson JE, Snetselaar L, Yasmeen S, O'Sullivan MJ, Safford M, Hendrix SL, Wallace RB: Oral bisphosphonate use and breast cancer incidence in postmenopausal women. J.Clin.Oncol. 28:3582-3590, 2010

Cho SJ, Kim JS, Kim JM, Lee JY, Jung HC, Song IS: Simvastatin induces apoptosis in human colon cancer cells and in tumor xenografts, and attenuates colitis-associated colon cancer in mice. Int.J.Cancer 123:951-957, 2008

Clezardin P: Bisphosphonates' antitumor activity: an unravelled side of a multifaceted drug class. Bone 48:71-79, 2011

Clezardin P and Massaia M: Nitrogen-containing bisphosphonates and cancer immunotherapy. Curr.Pharm.Des. 16:3007-2014, 2010

Clyburn RD, Reid P, Evans CA, Lefley DV, Holen I: Increased anti-tumour effects of doxorubicin and zoledronic acid in prostate cancer cells in vitro: supporting the benefits of combination therapy. Cancer Chemother.Pharmacol. 65:969-978, 2010

Coleman RE: Bisphosphonates: Clinical experience. Oncologist 9:14-27, 2004

Correll CC, Ng L, Edwards PA: Identification of farnesol as the non-sterol derivative of mevalonic acid required for the accelerated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J.Biol.Chem. 269:17390-3, 1994

Coxon FP, Ebetino FH, Mules EH, Seabra MC, McKenna CE, Rogers MJ: Phosphonocarboxylate inhibitors of Rab geranylgeranyl transferase disrupt the prenylation and membrane localization of Rab proteins in osteoclasts in vitro and in vivo. Bone 37:349-358, 2005

Coxon FP, Helfrich MH, Larijani B, Muzylak M, Dunford JE, Marshall D, McKinnon AD, Nesbitt SA, Horton MA, Seabra MC, Ebetino FH, Rogers MJ: Identification of a novel phosphonocarboxylate inhibitor of Rab geranylgeranyl transferase that specifically prevents Rab prenylation in osteoclasts and macrophages. J.Biol.Chem. 276:48213-48222, 2001

Coxon FP, Helfrich MH, Van't Hof R, Sebti S, Ralston SH, Hamilton A, Rogers MJ: Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. J.Bone Miner.Res. 15:1467-1476, 2000

Coxon FP, Thompson K, Roelofs AJ, Ebetino FH, Rogers MJ: Visualizing mineral binding and uptake of bisphosphonate by osteoclasts and non-resorbing cells. Bone 42:848-860, 2008

Coxon JP, Oades GM, Kirby RS, Colston KW: Zoledronic acid induces apoptosis and inhibits adhesion to mineralized matrix in prostate cancer cells via inhibition of protein prenylation. BJU Int. 94:164-170, 2004

Crick DC, Andres DA, Waechter CJ: Novel salvage pathway utilizing farnesol and geranylgeraniol for protein isoprenylation. Biochem.Biophys.Res.Commun. 237:483-7, 1997

Crowell PL, Ren Z, Lin S, Vedejs E, Gould MN: Structure-activity relationships among monoterpene inhibitors of protein isoprenylation and cell proliferation. Biochem.Pharmacol. 47:1405-1415, 1994

Daubine F, Cortial D, Ladam G, Atmani H, Haikel Y, Voegel JC, Clezardin P, Benkirane-Jessel N: Nanostructured polyelectrolyte multilayer drug delivery systems for bone metastasis prevention. Biomaterials 30:6367-6373, 2009

Daubine F, Le Gall C, Gasser J, Green J, Clezardin P: Antitumor effects of clinical dosing regimens of bisphosphonates in experimental breast cancer bone metastasis. J.Natl.Cancer Inst. 99:322-330, 2007

Delmas PD: Treatment of postmenopausal osteoporosis. Lancet 359:2018-2026, 2002

Delmas PD and Meunier PJ: The management of Paget's disease of bone. N.Engl.J.Med. 336:558-566, 1997

Denoyelle C, Albanese P, Uzan G, Hong L, Vannier JP, Soria J, Soria C: Molecular mechanism of the anti-cancer activity of cerivastatin, an inhibitor of HMG-CoA reductase, on aggressive human breast cancer cells. Cell.Signal. 15:327-338, 2003a

Denoyelle C, Hong L, Vannier JP, Soria J, Soria C: New insights into the actions of bisphosphonate zoledronic acid in breast cancer cells by dual RhoA-dependent and -independent effects. Br.J.Cancer 88:1631-1640, 2003b

Dorsey JK and Porter JW: The inhibition of mevalonic kinase by geranyl and farnesyl pyrophosphates. J.Biol.Chem. 243:4667-4670, 1968

Dunford JE, Kwaasi AA, Rogers MJ, Barnett BL, Ebetino FH, Russell RG, Oppermann U, Kavanagh KL: Structure-activity relationships among the nitrogen containing bisphosphonates in clinical use and other analogues: time-dependent inhibition of human farnesyl pyrophosphate synthase. J.Med.Chem. 51:2187-2195, 2008

Dunford JE, Rogers MJ, Ebetino FH, Phipps RJ, Coxon FP: Inhibition of protein prenylation by bisphosphonates causes sustained activation of Rac, Cdc42, and Rho GTPases. J.Bone Miner.Res. 21:684-694, 2006

Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, Ebetino FH, Rogers MJ: Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. J.Pharmacol.Exp.Ther. 296:235-242, 2001

Edmond J and Popjak G: Transfer of carbon atoms from mevalonate to n-fatty acids. J.Biol.Chem. 249:66-71, 1974

Edwards PA and Ericsson J: Sterols and isoprenoids: Signaling molecules derived from the cholesterol biosynthetic pathway. Annu.Rev.Biochem. 68:157-185, 1999

Edwards CM, Mueller G, Roelofs AJ, Chantry A, Perry M, Russell RG, Van Camp B, Guyon-Gellin Y, Niesor EJ, Bentzen CL, Vanderkerken K, Croucher PI: Apomine, an inhibitor of HMG-CoA-reductase, promotes apoptosis of myeloma cells in vitro and is associated with a modulation of myeloma in vivo. Int.J.Cancer 120:1657-1663, 2007

Evdokiou A, Labrinidis A, Bouralexis S, Hay S, Findlay DM: Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis. Bone 33:216-228, 2003

Fisher JE, Rogers MJ, Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, Wesolowski G, Russell RG, Rodan GA, Reszka AA: Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation in vitro. Proc.Natl.Acad.Sci.U.S.A. 96:133-138, 1999

Flach J, Antoni I, Villemin P, Bentzen CL, Niesor EJ: The mevalonate/isoprenoid pathway inhibitor apomine (SR-45023A) is antiproliferative and induces apoptosis similar to farnesol. Biochem.Biophys.Res.Commun. 270:240-246, 2000

Fleisch H: Bisphosphonates in Bone Disease. From the Laboratory to the Patient. Academic Press, San Diego, 2000

Fleisch H: Development of bisphosphonates. Breast Cancer Res. 4:30-34, 2002

Fleisch H and Bisaz S: Isolation from urine of pyrophosphate, a calcification inhibitor. Am.J.Physiol. 203:671-675, 1962

Fleisch H, Russell RG, Francis MD: Diphosphonates inhibit hydroxyapatite dissolution in vitro and bone resorption in tissue culture and in vivo. Science 165:1262-1264, 1969

Fournier P, Boissier S, Filleur S, Guglielmi J, Cabon F, Colombel M, Clezardin P: Bisphosphonates inhibit angiogenesis in vitro and testosterone-stimulated vascular regrowth in the ventral prostate in castrated rats. Cancer Res. 62:6538-6544, 2002

Fournier PG, Daubine F, Lundy MW, Rogers MJ, Ebetino HF,Clezardin P: Lowering bone mineral affinity of bisphosphonates as a therapeutic strategy to optimize skeletal tumor growth inhibition in vivo. Cancer Res. 68:8945-8953, 2008

Frith JC, Mönkkönen J, Auriola S, Mönkkönen H, Rogers MJ: The molecular mechanism of action of the antiresorptive and antiinflammatory drug clodronate: evidence for the formation in vivo of a metabolite that inhibits bone resorption and causes osteoclast and macrophage apoptosis. Arthritis Rheum. 44:2201-2210, 2001

Frith JC, Mönkkönen J, Blackburn GM, Russell RG, Rogers MJ: Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5'-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells in vitro. J.Bone Miner.Res. 12:1358-1367, 1997

Fritz G: Targeting the mevalonate pathway for improved anticancer therapy. Curr.Cancer.Drug Targets 9:626-638, 2009

Fromigue O, Kheddoumi N, Body JJ: Bisphosphonates antagonise bone growth factors' effects on human breast cancer cells survival. Br.J.Cancer 89:178-184, 2003

Fromigue O, Lagneaux L, Body JJ: Bisphosphonates induce breast cancer cell death in vitro. J.Bone Miner.Res. 15:2211-2221, 2000

Gibbs JB and Oliff A: The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. Annu.Rev.Pharmacol.Toxicol. 37:143-66, 1997

Gnant M, Mlineritsch B, Schippinger W, Luschin-Ebengreuth G, Postlberger S, Menzel C, Jakesz R, Seifert M, Hubalek M, Bjelic-Radisic V, Samonigg H, Tausch C, Eidtmann H, Steger G, Kwasny W, Dubsky P, Fridrik M, Fitzal F, Stierer M, Rucklinger E, Greil R, ABCSG-12 Trial Investigators, Marth C: Endocrine therapy plus zoledronic acid in premenopausal breast cancer. N.Engl.J.Med. 360:679-691, 2009

Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, De Libero G: Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J.Exp.Med. 197:163-168, 2003

Goffinet M, Thoulouzan M, Pradines A, Lajoie-Mazenc I, Weinbaum C, Faye JC, Seronie-Vivien S: Zoledronic acid treatment impairs protein geranyl-geranylation for biological effects in prostatic cells. BMC Cancer 6:60, 2006

Goldstein JL and Brown MS: Regulation of the mevalonate pathway. Nature 343:425-430, 1990

Graaf MR, Beiderbeck AB, Egberts AC, Richel DJ, Guchelaar HJ: The risk of cancer in users of statins. J.Clin.Oncol. 22:2388-2394, 2004

Green AE, Lissina A, Hutchinson SL, Hewitt RE, Temple B, James D, Boulter JM, Price DA, Sewell AK: Recognition of nonpeptide antigens by human V gamma 9V delta 2 T cells requires contact with cells of human origin. Clin.Exp.Immunol. 136:472–482, 2004

Gueldner, SH, Grabo TN, Newman ED, Cooper DR: Osteoporosis: Clinical Guidelines for Prevention, Diagnosis, and Management. pp. 47-48, Springer Publishing Company, New York, 2007

Guenther A, Gordon S, Tiemann M, Burger R, Bakker F, Green JR, Baum W, Roelofs AJ, Rogers MJ, Gramatzki M: The bisphosphonate zoledronic acid has antimyeloma activity in vivo by inhibition of protein prenylation. Int.J.Cancer 126:239-246, 2010

Hahn FM, Xuan JW, Chambers AF, Poulter CD: Human isopentenyl diphosphate: dimethylallyl diphosphate isomerase: overproduction, purification, and characterization. Arch.Biochem.Biophys. 332:30-34, 1996

Halasy-Nagy JM, Rodan GA, Reszka AA: Inhibition of bone resorption by alendronate and risedronate does not require osteoclast apoptosis. Bone 29:553-559, 2001

Hardcastle IR, Rowlands MG, Barber AM, Grimshaw RM, Mohan MK, Nutley BP, Jarman M: Inhibition of protein prenylation by metabolites of limonene. Biochem.Pharmacol. 57:801-809, 1999

Harrison LE, Wojciechowicz DC, Brennan MF, Paty PB: Phenylacetate inhibits isoprenoid biosynthesis and suppresses growth of human pancreatic carcinoma. Surgery 124:541-550, 1998

Hashimoto K, Morishige K, Sawada K, Tahara M, Shimizu S, Ogata S, Sakata M, Tasaka K, Kimura T: Alendronate suppress tumor angiogenesis by inhibiting Rho activation of endothelial cells. Biochem. Biophys.Res.Commun. 354:478-484, 2007

Hinson DD, Chambliss KL, Toth MJ, Tanaka RD, Gibson KM: Post-translational regulation of mevalonate kinase by intermediates of the cholesterol and nonsterol isoprene biosynthetic pathways. J.Lipid Res. 38:2216-2223, 1997

Hiraga T, Williams PJ, Mundy GR, Yoneda T: The bisphosphonate ibandronate promotes apoptosis in MDA-MB-231 human breast cancer cells in bone metastases. Cancer Res. 61:4418-4424, 2001

Hirabayashi H, Sawamoto T, Fujisaki J, Tokunaga Y, Kimura S, Hata T: Dose-dependent pharmacokinetics and disposition of bisphosphonic prodrug of diclofenac based on osteotropic drug delivery system (ODDS). Biopharm.Drug Dispos. 23:307-315, 2002

Holstein SA and Hohl RJ: Monoterpene regulation of Ras and Ras-related protein expression. J.Lipid Res. 44:1209-1215, 2003

Hudgins WR, Shack S, Myers CE, Samid D: Cytostatic activity of phenylacetate and derivatives against tumor cells. Correlation with lipophilicity and inhibition of protein prenylation. Biochem.Pharmacol. 50:1273-1279, 1995

Hughes DE, Wright KR, Uy HL, Sasaki A, Yoneda T, Roodman GD, Mundy GR, Boyce BF. J.Bone Miner.Res. 10:1478-1487, 1995

Iguchi T, Miyakawa Y, Yamamoto K, Kizaki M, Ikeda Y: Nitrogen-containing bisphosphonates induce S-phase cell cycle arrest and apoptosis of myeloma cells by activating MAPK pathway and inhibiting mevalonate pathway. Cell.Signal. 15:719-727, 2003

Ishikawa C, Matsuda T, Okudaira T, Tomita M, Kawakami H, Tanaka Y, Masuda M, Ohshiro K, Ohta T, Mori N: Bisphosphonate incadronate inhibits growth of human T-cell leukaemia virus type I-infected T-cell lines and primary adult T-cell leukaemia cells by interfering with the mevalonate pathway. Br.J.Haematol. 136:424-432, 2007

Jagdev SP, Coleman RE, Shipman CM, Rostami-H A, Croucher PI: The bisphosphonate, zoledronic acid, induces apoptosis of breast cancer cells: evidence for synergy with paclitaxel. Br.J.Cancer 84:1126-1134, 2001

Jauhiainen M, Mönkkönen H, Räikkönen J, Mönkkönen J, Auriola S: Analysis of endogenous ATP analogs and mevalonate pathway metabolites in cancer cell cultures using liquid chromatography-electrospray ionization mass spectrometry. J.Chromatogr.B.Analyt Technol.Biomed.Life.Sci. 877:2967-2975, 2009

Kabelitz D, Wesch D, Pitters E, Zoller M: Characterization of tumor reactivity of human V gamma 9V delta 2 gamma delta T cells in vitro and in SCID mice in vivo. J.Immunol. 173:6767-6776, 2004

Kanis JA, Burlet N, Cooper C, Delmas PD, Reginster JY, Borgstrom F, Rizzoli R, European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO): European guidance for the diagnosis and management of osteoporosis in postmenopausal women. Osteoporos.Int. 19:399-428, 2008

Kanis JA and McCloskey EV: Bisphosphonates in multiple myeloma. Cancer 88:3022-3032, 2000

Kavanagh KL, Guo K, Dunford JE, Wu X, Knapp S, Ebetino FH, Rogers MJ, Russell RG, Oppermann U: The molecular mechanism of nitrogen-containing bisphosphonates as antiosteoporosis drugs. Proc.Natl.Acad.Sci.U.S.A. 103:7829-7834, 2006

Kohl NE, Wilson FR, Mosser SD, Giuliani E, deSolms SJ, Conner MW, Anthony NJ, Holtz WJ, Gomez RP, Lee TJ: Protein farnesyltransferase inhibitors block the growth of ras-dependent tumors in nude mice. Proc.Natl.Acad.Sci.U.S.A. 91:9141-9145, 1994

Konstantinopoulos PA, Karamouzis MV, Papavassiliou AG: Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. Nat.Rev.Drug Discov. 6:541-555, 2007

Kubista B, Trieb K, Sevelda F, Toma C, Arrich F, Heffeter P, Elbling L, Sutterluty H, Scotlandi K, Kotz R, Micksche M, Berger W: Anticancer effects of zoledronic acid against human osteosarcoma cells. J.Orthop.Res. 24:1145-1152, 2006

Kunzmann V, Bauer E, Wilhelm M: Gamma/delta T-cell stimulation by pamidronate. N.Engl.J.Med. 340:737-738, 1999

Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M: Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood 96:384-392, 2000

Kurimoto M, Hirashima Y, Hamada H, Kamiyama H, Nagai S, Hayashi N, Endo S: In vitro and in vivo growth inhibition of human malignant astrocytoma cells by the farnesyltransferase inhibitor B1620. J.Neurooncol. 61:103-112, 2003

Kusama T, Mukai M, Iwasaki T, Tatsuta M, Matsumoto Y, Akedo H, Inoue M, Nakamura H: 3-Hydroxy-3-Methylglutaryl-Coenzyme a Reductase Inhibitors Reduce Human Pancreatic Cancer Cell Invasion and Metastasis. Gastroenterology 122:308-317, 2002

Kusama T, Mukai M, Tatsuta M, Matsumoto Y, Nakamura H, Inoue M: Selective inhibition of cancer cell invasion by a geranylgeranyltransferase-I inhibitor. Clin.Exp.Metastasis 20:561-567, 2003

Lau CP, Huang L, Tsui SK, Ng PK, Leung PY, Kumta SM: Pamidronate, farnesyl transferase, and geranylgeranyl transferase-I inhibitors affects cell proliferation, apoptosis, and OPG/RANKL mRNA expression in stromal cells of giant cell tumor of bone. J.Orthop.Res. 29:403-413, 2011

Lawson MA, Coulton L, Ebetino FH, Vanderkerken K, Croucher PI: Geranylgeranyl transferase type II inhibition prevents myeloma bone disease. Biochem.Biophys.Res.Commun. 377:453-457, 2008

Le Goff B, Berthelot JM, Maugars Y, Romas E: Alternative use of bisphosphonate therapy for rheumatic disease. Curr.Pharm.Des. 16:3045-3052, 2010

Lee MV, Fong EM, Singer FR, Guenette RS: Bisphosphonate treatment inhibits the growth of prostate cancer cells. Cancer Res. 61:2602-2608, 2001

Lee SJ, Ha MJ, Lee J, Nguyen P, Choi YH, Pirnia F, Kang WK, Wang XF, Kim SJ, Trepel JB: Inhibition of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase pathway induces p53-independent transcriptional regulation of p21(WAF1/CIP1) in human prostate carcinoma cells. J.Biol.Chem. 273:10618-10623, 1998

Lehenkari PP, Kellinsalmi M, Napankangas JP, Ylitalo KV, Mönkkönen J, Rogers MJ, Azhayev A, Väänänen HK, Hassinen IE: Further insight into mechanism of action of clodronate: inhibition of mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing metabolite. Mol.Pharmacol. 61:1255-1262, 2002

Lerner EC, Zhang TT, Knowles DB, Qian Y, Hamilton AD, Sebti SM: Inhibition of the prenylation of K-Ras, but not H- or N-Ras, is highly resistant to CAAX peptidomimetics and requires both a farnesyltransferase and a geranylgeranyltransferase I inhibitor in human tumor cell lines. Oncogene 15:1283-1288, 1997

Li HY, Appelbaum FR, Willman CL, Zager RA, Banker DE: Cholesterol-modulating agents kill acute myeloid leukemia cells and sensitize them to therapeutics by blocking adaptive cholesterol responses. Blood 101:3628-3634, 2003

Lin JH: Bisphosphonates: a review of their pharmacokinetic properties. Bone 18:75-85, 1996

Ling Y, Sahota G, Odeh S, Chan JM, Araujo FG, Moreno SN, Oldfield E: Bisphosphonate inhibitors of Toxoplasma gondi growth: in vitro, QSAR, and in vivo investigations. J.Med.Chem. 48:3130-3140, 2005

Lobell RB, Liu D, Buser CA, Davide JP, DePuy E, Hamilton K, Koblan KS, Lee Y, Mosser S, Motzel SL, Abbruzzese JL, Fuchs CS, Rowinsky EK, Rubin EH, Sharma S, Deutsch PJ, Mazina KE, Morrison BW, Wildonger L, Yao SL, Kohl NE: Preclinical and clinical pharmacodynamic assessment of L-778,123, a dual inhibitor of farnesyl:protein transferase and geranylgeranyl:protein transferase type-I. Mol.Cancer.Ther. 1:747-758, 2002

Luckman SP, Coxon FP, Ebetino FH, Russell RG, Rogers MJ: Heterocycle-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: evidence from structure-activity relationships in J774 macrophages. J.Bone Miner.Res. 13:1668-1678, 1998a

Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G, Rogers MJ: Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J.Bone Miner.Res. 13:581-589, 1998b

Mackie PS, Fisher JL, Zhou H, Choong PF: Bisphosphonates regulate cell growth and gene expression in the UMR 106-01 clonal rat osteosarcoma cell line. Br.J.Cancer 84:951-958, 2001

Makkonen N, Hirvonen M-R, Teräväinen T, Savolainen K, Mönkkönen J: Different effects of three bisphosphonates on nitric oxide production by RAW 264 macrophage-like cells in vitro. J.Pharmacol.Exp.Ther. 277:1097-1102, 1996

Marra M, Salzano G, Leonetti C, Tassone P, Scarsella M, Zappavigna S, Calimeri T, Franco R, Liguori G, Cigliana G, Ascani R, La Rotonda MI, Abbruzzese A, Tagliaferri P, Caraglia M, De Rosa G: Nanotechnologies to use bisphosphonates as potent anticancer agents: the effects of zoledronic acid encapsulated into liposomes. Nanomedicine, in press, 2011

Marten A, Lilienfeld-Toal M, Buchler MW, Schmidt J: Zoledronic acid has direct antiproliferative and antimetastatic effect on pancreatic carcinoma cells and acts as an antigen for delta2 gamma/delta T cells. J.Immunother. 30:370-377, 2007

Martin MB, Grimley JS, Lewis JC, Heath HT 3r, Bailey BN, Kendrick H, Yardley V, Caldera A, Lira R, Urbina JA, Moreno SN, Docampo R, Croft SL, Oldfield E: Bisphosphonates inhibit the growth of Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondii, and Plasmodium falciparum: a potential route to chemotherapy. J.Med.Chem. 44:909-916, 2001

Martin MB, Sanders JM, Kendrick H, de Luca-Fradley K, Lewis JC, Grimley JS, Van Brussel EM, Olsen JR, Meints GA, Burzynska A, Kafarski P, Croft SL, Oldfield E: Activity of bisphosphonates against Trypanosoma brucei rhodesiense. J.Med.Chem. 45:2904-2914, 2002

Masaike Y, Takagi T, Hirota M, Yamada J, Ishihara S, Yung TM, Inoue T, Sawa C, Sagara H, Sakamoto S, Kabe Y, Takahashi Y, Yamaguchi Y, Handa H: Identification of dynamin-2-mediated endocytosis as a new target of osteoporosis drugs, bisphosphonates. Mol.Pharmacol. 77:262-269, 2010

Masarachia P, Weinreb M, Balena R, Rodan GA: Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones. Bone 19:281-290, 1996

Mattarollo SR, Kenna T, Nieda M, Nicol AJ: Chemotherapy and zoledronate sensitize solid tumour cells to Vgamma9Vdelta2 T cell cytotoxicity. Cancer Immunol.Immunother. 56:1285-1297, 2007

McKenna CE, Kashemirov BA, Blazewska KM, Mallard-Favier I, Stewart CA, Rojas J, Lundy MW, Ebetino FH, Baron RA, Dunford JE, Kirsten ML, Seabra MC, Bala JL, Marma MS, Rogers MJ, Coxon FP: Synthesis, chiral high performance liquid chromatographic resolution and enantiospecific activity of a potent new geranylgeranyl transferase inhibitor, 2-hydroxy-3-imidazo[1,2-a]pyridin-3-yl-2-phosphonopropionic acid. J.Med.Chem. 53:3454-3464, 2010

Mitrofan L Pelkonen J, Mönkkönen J: The level of ATP analogue and isopentenyl pyrophosphate correlates with zoledronic acid -induced apoptosis in cancer cells in vitro, Bone 45:1153-1160, 2009

Mitrofan LM, Castells FB, Pelkonen J, Mönkkönen J: Lysosomal-mitochondrial axis in zoledronic acid-induced apoptosis in human follicular lymphoma cells. J.Biol.Chem. 285: 1967-1979, 2010

Mo H and Elson CE: Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. Exp.Biol.Med. 229:567-585, 2004

Mo H and Elson CE: Isoprenoids and novel inhibitors of mevalonate pathway activities. Nutritional Oncology, 2nd ed., pp. 629-643. Academic Press, San Diego, CA, 2006

Mookerjee-Basu J, Vantourout P, Martinez LO, Perret B, Collet X, Périgaud C, Peyrottes S, Champagne E. F1-adenosine triphosphatase displays properties characteristic of an antigen presentation molecule for Vgamma9Vdelta2 T cells. J.Immunol. 184:6920-6928, 2010

Morita CT, Beckman EM, Bukowski JF, Tanaka Y, Band H, Bloom BR, Golan DE, Brenner MB: Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. Immunity 3:495-507, 1995

Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63, 1983

Muraro M, Mereuta OM, Carraro F, Madon E, Fagioli F: Osteosarcoma cell line growth inhibition by zoledronate-stimulated effector cells. Cell.Immunol. 249:63-72, 2007

Mönkkönen H, Auriola S, Lehenkari P, Kellinsalmi M, Hassinen IE, Vepsalainen J, Mönkkönen J: 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. 147:437-445, 2006

Mönkkönen H, Moilanen P, Mönkkönen J, Frith JC, Rogers MJ, Auriola S: Analysis of an adenine nucleotide-containing metabolite of clodronate using ion pair high-performance liquid chromatography-electrospray ionisation mass spectrometry. J.Chromatogr.B Biomed.Sci.Appl. 738:395-403, 2000

Mönkkönen H, Ottewell PD, Kuokkanen J, Mönkkönen J, Auriola S, Holen I: Zoledronic acid-induced IPP/ApppI production in vivo. Life Sci. 81:1066-1070, 2007

Mönkkönen H, Rogers MJ, Makkonen N, Niva S, Auriola S, Mönkkönen J: The cellular uptake and metabolism of clodronate in RAW 264 macrophages. Pharm.Res. 18:1550-1555, 2001

Mönkkönen J, Ylitalo P, Elo HA, Airaksinen MM: Distribution of [^{14}C]clodronate (dichloromethylene bisphosphonate) disodium in mice. Toxicol.Appl.Pharmacol. 89:287-292, 1987

Mönkkönen J: A one year follow-up study of the distribution of ^{14}C-clodronate in mice and rats. Pharmacol.Toxicol. 62:51-53, 1988

Mönkkönen J, Koponen H-M, Ylitalo P: Comparison of the distribution of three bisphosphonates in mice. Pharmacol.Toxicol. 66:294-298, 1990

Mönkkönen J, Taskinen M, Auriola SOK, Urtti A: Growth inhibition of macrophage-like and other cell types by liposome-encapsulated, calcium-bound, and free bisphosphonates in vitro. J.Drug Target 2: 299-308, 1994

Nakanishi M, Goldstein JL, Brown MS: Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. J.Biol.Chem. 263:8929-8937, 1988

Nancollas GH, Tang R, Phipps RJ, Henneman Z, Gulde S, Wu W, Mangood A, Russell RG, Ebetino FH: Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. Bone 38:617-627, 2006

Neville-Webbe HL, Coleman RE, Holen I: Combined effects of the bisphosphonate, zoledronic acid and the aromatase inhibitor letrozole on breast cancer cells in vitro: evidence of synergistic interaction. Br.J.Cancer 102:1010-1017, 2010

Neville-Webbe HL, Rostami-Hodjegan A, Evans CA, Coleman RE, Holen I: Sequence- and schedule-dependent enhancement of zoledronic acid induced apoptosis by doxorubicin in breast and prostate cancer cells. Int.J.Cancer 113:364-371, 2005

Oades GM, Senaratne SG, Clarke IA, Kirby RS, Colston KW: Nitrogen containing bisphosphonates induce apoptosis and inhibit the mevalonate pathway, impairing Ras membrane localization in prostate cancer cells. J.Urol. 170:246-252, 2003

Ohashi K, Osuga J, Tozawa R, Kitamine T, Yagyu H, Sekiya M, Tomita S, Okazaki H, Tamura Y, Yahagi N, Iizuka Y, Harada K, Gotoda T, Shimano H, Yamada N, Ishibashi S: Early embryonic lethality caused by targeted disruption of the 3-hydroxy-3-methylglutaryl-CoA reductase gene. J.Biol.Chem. 278:42936-42941, 2003

Ory B, Moriceau G, Trichet V, Blanchard F, Berreur M, Rédini F,Rogers M, Heymann D: Farnesyl diphosphate synthase is involved in the resistance to zoledronic acid of osteosarcoma cells. J.Cell Mol.Med. 12:928-941, 2008

Osborne TF, Gil G, Goldstein JL, Brown MS: Operator constitutive mutation of 3-hydroxy-3-methylglutaryl coenzyme A reductase promoter abolishes protein binding to sterol regulatory element. J.Biol.Chem. 263:3380-3387, 1988

Ottewell PD, Coleman RE, Holen I: From genetic abnormality to metastases: murine models of breast cancer and their use in the development of anticancer therapies. Breast Cancer Res.Treat. 96:101-113, 2006

Ottewell PD, Deux B, Mönkkönen H, Cross S, Coleman RE, Clezardin P, Holen I: Differential effect of doxorubicin and zoledronic acid on intraosseous versus extraosseous breast tumor growth in vivo. Clin.Cancer Res. 14:4658-4666, 2008

Ottewell PD, Woodward JK, Lefley DV, Evans CA, Coleman RE, Holen I: Anticancer mechanisms of doxorubicin and zoledronic acid in breast cancer tumor growth in bone. Mol.Cancer.Ther. 8:2821-2832, 2009

Papapoulos SE: Paget's disease of bone: clinical, pathogenetic and therapeutic aspects. Baillieres Clin.Endocrinol.Metab. 11:117-143, 1997

Pazianas M and Abrahamsen B: Safety of bisphosphonates. Bone 49:103-110, 2011

Peffley DM and Gayen AK: Plant-derived monoterpenes suppress hamster kidney cell 3-hydroxy-3-methylglutaryl coenzyme a reductase synthesis at the post-transcriptional level. J.Nutr. 133:38-44, 2003

Pelorgeas S, Martin JB, Satre M: Cytotoxicity of dichloromethane diphosphonate and of 1-hydroxyethane-1,1-diphosphonate in the amoebae of the slime mould Dictyostelium discoideum. A 31P NMR study. Biochem.Pharmacol. 44:2157-2163, 1992

Pennanen N, Lapinjoki S, Urtti A, Mönkkönen J:Effect of liposomal and free bisphosphonates on the IL-1β, IL-6, and TNFα, secretion from RAW 264 cells in vitro. Pharm.Res. 12:916-922, 1995

Porras AG, Holland SD, Gertz BJ: Pharmacokinetics of alendronate. Clin.Pharmacokinet. 36:315-328, 1999

Prietsch V, Mayatepek E, Krastel H, Haas D, Zundel D, Waterham HR, Wanders RJ, Gibson KM, Hoffmann GF: Mevalonate kinase deficiency: enlarging the clinical and biochemical spectrum. Pediatrics 111:258-261, 2003

Qian Y, Sebti SM, Hamilton AD: Farnesyltransferase as a target for anticancer drug design. Biopolymers 43:25-41, 1997

Quimby OT: The chemistry of sodium phosphates. Chem.Rev. 40:141-179, 1947

Ren Z, Elson CE, Gould MN: Inhibition of type I and type II geranylgeranyl-protein transferases by the monoterpene perillyl alcohol in NIH3T3 cells. Biochem.Pharmacol. 54:113-120, 1997

Reszka AA, Halasy-Nagy J, Rodan GA: Nitrogen-bisphosphonates block retinoblastoma phosphorylation and cell growth by inhibiting the cholesterol biosynthetic pathway in a keratinocyte model for esophageal irritation. Mol.Pharmacol. 59:193-202, 2001

Reszka AA, Halasy-Nagy JM, Masarachia PJ, Rodan GA: Bisphosphonates act directly on the osteoclast to induce caspase cleavage of mst1 kinase during apoptosis. A link between inhibition of the mevalonate pathway and regulation of an apoptosis-promoting kinase. J.Biol.Chem. 274:34967-34973, 1999

Roelofs AJ, Hulley PA, Meijer A, Ebetino FH, Russell RG, Shipman CM: Selective inhibition of Rab prenylation by phosphocarboxylate analogue of risedronate induces apoptosis, but not S-phase arrest, in human myeloma cells. Int.J.Cancer 119:1254-1261, 2006

Roelofs AJ, Jauhiainen M, Mönkkönen H, Rogers MJ, Mönkkönen J, Thompson K: Peripheral blood monocytes are responsible for gammadelta T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. Br.J.Haematol. 144:245-250, 2009

Roelofs AJ, Thompson K, Ebetino FH, Rogers MJ, Coxon FP: Bisphosphonates: molecular mechanisms of action and effects on bone cells, monocytes and macrophages. Curr.Pharm.Des. 16:2950-2960, 2010

Rogers MJ, Brown RJ, Hodkin V, Blackburn GM, Russell RG, Watts DJ: Bisphosphonates are incorporated into adenine nucleotides by human aminoacyl-tRNA synthetase enzymes. Biochem.Biophys.Res.Commun. 224:863-869, 1996

Rogers MJ, Crockett JC, Coxon FP, Mönkkönen J: Biochemical and molecular mechanisms of action of bisphosphonates. Bone 49:34-41, 2011

Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Mönkkönen J, Frith JC: Cellular and molecular mechanisms of action of bisphosphonates. Cancer 88:2961-2978, 2000

Rogers MJ, Ji X, Russell RG, Blackburn GM, Williamson MP, Bayless AV, Ebetino FH, Watts DJ: Incorporation of bisphosphonates into adenine nucleotides by amoebae of the cellular slime mould Dictyostelium discoideum. Biochem.J. 303 (Pt 1):303-311, 1994

Rogers MJ, Russell RG, Blackburn GM, Williamson MP, Watts DJ: Metabolism of halogenated bisphosphonates by the cellular slime mould Dictyostelium discoideum. Biochem.Biophys.Res.Commun. 189:414-423, 1992

Roitelman J, Masson D, Avner R, Ammon-Zufferey C, Perez A, Guyon-Gellin Y, Bentzen CL, Niesor EJ: Apomine, a novel hypocholesterolemic agent, accelerates degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and stimulates low density lipoprotein receptor activity. J.Biol.Chem. 279:6465-6473, 2004

Rondeau JM, Bitsch F, Bourgier E, Geiser M, Hemmig R, Kroemer M, Lehmann S, Ramage P, Rieffel S, Strauss A, Green JR, Jahnke W: Structural basis for the exceptional in vivo efficacy of bisphosphonate drugs. ChemMedChem 1:267-273, 2006

Ross JR, Saunders Y, Edmonds PM, Patel S, Wonderling D, Normand C, Broadley K: A systematic review of the role of bisphosphonates in metastatic disease. Health Technol.Assess. 8:1-176, 2004

Roux C and Dougados M: Treatment of patients with Paget's disease of bone. Drugs 58:823-830, 1999

Russell RG, Watts NB, Ebetino FH, Rogers MJ: Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos.Int. 19:733-759, 2008

Salzano G, Marra M, Porru M, Zappavigna S, Abbruzzese A, La Rotonda MI, Leonetti C, Caraglia M, De Rosa G: Self-assembly nanoparticles for the delivery of bisphosphonates into tumors. Int.J.Pharm. 403:292-297, 2011

Sanders JM, Gomez AO, Mao J, Meints GA, Van Brussel EM, Burzynska A, Kafarski P, Gonzalez-Pacanowska D, Oldfield E: 3-D QSAR investigations of the inhibition of Leishmania major farnesyl pyrophosphate synthase by bisphosphonates. J.Med.Chem. 46:5171-5183, 2003

Sane KM, Mynderse M, Lalonde DT, Dean IS, Wojtkowiak JW, Fouad F, Borch RF, Reiners JJ,Jr, Gibbs RA, Mattingly RR: A novel geranylgeranyl transferase inhibitor in combination with lovastatin inhibits proliferation and induces autophagy in STS-26T MPNST cells. J.Pharmacol.Exp.Ther. 333:23-33, 2010

Santini D, Gentilucci UV, Vincenzi B, Picardi A, Vasaturo F, La Cesa A, Onori N, Scarpa S, Tonini G: The antineoplastic role of bisphosphonates: from basic research to clinical evidence. Annals of Oncology 14:1468-1476, 2003

Santini D, Vincenzi B, Galluzzo S, Battistoni F, Rocci L, Venditti O, Schiavon G, Angeletti S, Uzzalli F, Caraglia M, Dicuonzo G, Tonini G: Repeated intermittent low-dose therapy with zoledronic acid induces an early, sustained, and long-lasting decrease of peripheral vascular endothelial growth factor levels in cancer patients. Clin.Cancer.Res. 13:4482–4486, 2007

Sato K, Kimura S, Segawa H, Yokota A, Matsumoto S, Kuroda J, Nogawa M, Yuasa T, Kiyono Y, Wada H, Maekawa T: Cytotoxic effects of gammadelta T cells expanded ex vivo by a third generation bisphosphonate for cancer immunotherapy. Int.J.Cancer 116:94-99, 2005

Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, Golub E, Rodan GA: Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. J.Clin.Invest. 88:2095-2105, 1991

Seabra MC, Mules EH, Hume AN: Rab GTPases, intracellular traffic and disease. Trends Mol.Med. 8:23-30, 2002

Selander KS, Mönkkönen J, Karhukorpi E-K, Härkönen P, Hannuniemi R, Väänänen K. Mol.Pharmacol. 50:1127-1138, 1996

Senaratne SG and Colston KW: Direct effects of bisphosphonates on breast cancer cells. Breast Cancer Res. 4:18-23, 2002

Senaratne SG, Mansi JL, Colston KW: The bisphosphonate zoledronic acid impairs Ras membrane localisation and induces cytochrome c release in breast cancer cells. Br.J.Cancer 86:1479-1486, 2002

Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW: Bisphosphonates induce apoptosis in human breast cancer cell lines. Br.J.Cancer 82:1459-1468, 2000

Shinoda H, Adamek G, Felix R, Fleisch H, Schenk R, Hagan P: Structure-activity relationships of various bisphosphonates. Calcif.Tissue Int. 35:87-99, 1983

Shipman CM, Croucher PI, Russell RG, Helfrich MH, Rogers MJ: The bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway. Cancer Res. 58:5294-5297, 1998

Shipman CM, Rogers MJ, Apperley JF, Russell RG, Croucher PI: Bisphosphonates induce apoptosis in human myeloma cell lines: a novel anti-tumour activity. Br.J.Haematol. 98:665-672, 1997

Shmeeda H, Amitay Y, Gorin J, Tzemach D, Mak L, Ogorka J, Kumar S, Zhang JA, Gabizon A: Delivery of zoledronic acid encapsulated in folate-targeted liposome results in potent in vitro cytotoxic activity on tumor cells. J.Control.Release 146:76-83, 2010

Sinensky M: Recent advances in the study of prenylated proteins. Biochim.Biophys.Acta 1484:93-106, 2000

Sonnemann J, Bumbul B, Beck JF: Synergistic activity of the histone deacetylase inhibitor suberoylanilide hydroxamic acid and the bisphosphonate zoledronic acid against prostate cancer cells in vitro. Mol.Cancer.Ther. 6:2976-2984, 2007

Stresing V, Daubine F, Benzaid I, Mönkkönen H, Clezardin P: Bisphosphonates in cancer therapy. Cancer Lett. 257:16-35, 2007

Stresing V, Fournier PG, Bellahcene A, Benzaid I, Mönkkönen H, Colombel M, Ebetino FH, Castronovo V, Clezardin P: Nitrogen-containing bisphosphonates can inhibit angiogenesis in vivo without the involvement of farnesyl pyrophosphate synthase. Bone 48:259-266, 2011

Sun J, Ohkanda J, Coppola D, Yin H, Kothare M, Busciglio B, Hamilton AD, Sebti SM: Geranylgeranyltransferase I inhibitor GGTI-2154 induces breast carcinoma apoptosis and tumor regression in H-Ras transgenic mice. Cancer Res. 63:8922-8929, 2003

Sun J, Qian Y, Chen Z, Marfurt J, Hamilton AD, Sebti SM: The geranylgeranyltransferase I inhibitor GGTI-298 induces hypophosphorylation of retinoblastoma and partner switching of cyclin-dependent kinase inhibitors. A potential mechanism for GGTI-298 antitumor activity. J.Biol.Chem. 274:6930-6934, 1999

Sutherland KA, Rogers HL, Tosh D, Rogers MJ: RANKL increases the level of Mcl-1 in osteoclasts and reduces bisphosphonate-induced osteoclast apoptosis in vitro. Arthritis Res.Ther. 11:R58, 2009

Swanson KM and Hohl RJ: Anti-cancer therapy: targeting the mevalonate pathway. Curr.Cancer.Drug Targets 6:15-37, 2006

Szabo CM, Martin MB, Oldfield E: An investigation of bone resorption and Dictyostelium discoideum growth inhibition by bisphosphonate drugs. J.Med.Chem. 45:2894-2903, 2002a

Szabo CM, Matsumura Y, Fukura S, Martin MB, Sanders JM, Sengupta S, Cieslak JA, Loftus TC, Lea CR, Lee HJ, Koohang A, Coates RM, Sagami H, Oldfield E: Inhibition of geranylgeranyl diphosphate synthase by bisphosphonates and diphosphates: a potential route to new bone antiresorption and antiparasitic agents. J.Med.Chem. 45:2185-2196, 2002b

Tanaka KS, Dietrich E, Ciblat S, Métayer C, Arhin FF, Sarmiento I, Moeck G, Parr TR Jr, Far AR: Synthesis an in vitro evaluation of bisphosphonated glycopeptide prodrugs for the treatment of osteomyelitis. Bioorg.Med.Chem.Lett. 20:1355-1359, 2010

Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR: Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. Nature 375:155-158, 1995

Tassone P, Forciniti S, Galea E, Morrone G, Turco MC, Martinelli V, Tagliaferri P, Venuta S: Growth inhibition and synergistic induction of apoptosis by zoledronate and dexamethasone in human myeloma cell lines. Leukemia 14:841-844, 2000

Tassone P, Tagliaferri P, Viscomi C, Palmieri C, Caraglia M, D'Alessandro A, Galea E, Goel A, Abbruzzese A, Boland CR, Venuta S: Zoledronic acid induces antiproliferative and apoptotic effects in human pancreatic cancer cells in vitro. Br.J.Cancer 88:1971-1978, 2003

Thompson K, Dunford JE, Ebetino FH, Rogers MJ: Identification of a bisphosphonate that inhibits isopentenyl diphosphate isomerase and farnesyl diphosphate synthase. Biochem.Biophys.Res.Commun. 290:869-873, 2002

Thompson K, Roelofs AJ, Jauhiainen M, Mönkkönen H, Mönkkönen J, Rogers MJ: Activation of gammadelta T Cells by Bisphosphonates. Adv.Exp.Med.Biol. 658:11-20, 2010

Thompson K and Rogers MJ: Statins prevent bisphosphonate-induced gamma,delta-T-cell proliferation and activation in vitro. J.Bone Miner.Res. 19:278-288, 2004

Thompson K, Rogers MJ, Coxon FP, Crockett JC: Cytosolic entry of bisphosphonate drugs requires acidification of vesicles after fluid-phase endocytosis. Mol.Pharmacol. 69:1624-1632, 2006

Tozawa R, Ishibashi S, Osuga J, Yagyu H, Oka T, Chen Z, Ohashi K, Perrey S, Shionoiri F, Yahagi N, Harada K, Gotoda T, Yazaki Y, Yamada N: Embryonic lethality and defective neural tube closure in mice lacking squalene synthase. J.Biol.Chem. 274:30843-30848, 1999

Twiss IM, de Water R, den Hartigh J, Sparidans R, Ramp-Koopmanschap W, Brill H, Wijdeveld M, Vermeij P: Cytotoxic effects of pamidronate on monolayers of human intestinal epithelial (Caco-2) cells and its epithelial transport. J.Pharm.Sci. 83:699-703, 1994

Ullen A, Lennartsson L, Harmenberg U, Hjelm-Eriksson M, Kalkner KM, Lennernas B, Nilsson S: Additive/synergistic antitumoral effects on prostate cancer cells in vitro following treatment with a combination of docetaxel and zoledronic acid. Acta Oncol. 44:644-650, 2005

van Beek E, Hoekstra M, van de Ruit M, Lowik C, Papapoulos S: Structural requirements for bisphosphonate actions in vitro. J.Bone Miner.Res. 9:1875-1882, 1994

van Beek E, Lowik C, Que I, Papapoulos S: Dissociation of binding and antiresorptive properties of hydroxybisphosphonates by substitution of the hydroxyl with an amino group. J.Bone Miner.Res. 11:1492-1497, 1996

van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S: Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. Biochem.Biophys.Res.Commun. 264:108-111, 1999a

van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S: Nitrogen-containing bisphosphonates inhibit isopentenyl pyrophosphate isomerase/farnesyl pyrophosphate synthase activity with relative potencies corresponding to their antiresorptive potencies in vitro and in vivo. Biochem.Biophys.Res.Commun. 255:491-494, 1999b

van beek E, Lowik C, van der Pluijm G, Papapoulos S: The role of geranylgeranylation in bone resorption and its suppression by bisphosphonates in fetal bone explants in vitro: A clue to the mechanism of action of nitrogen-containing bisphosphonates. J.Bone Miner.Res. 14:722-729, 1999c

van Beek ER, Cohen LH, Leroy IM, Ebetino FH, Lowik CW, Papapoulos SE: Differentiating the mechanisms of antiresorptive action of nitrogen containing bisphosphonates. Bone 33:805-811, 2003

van Beek ER, Lowik CW, Papapoulos SE: Bisphosphonates suppress bone resorption by a direct effect on early osteoclast precursors without affecting the osteoclastogenic capacity of osteogenic cells: the role of protein geranylgeranylation in the action of nitrogen-containing bisphosphonates on osteoclast precursors. Bone 30:64-70, 2002

van de Donk NW, Schotte D, Kamphuis MM, van Marion AM, van Kessel B, Bloem AC, Lokhorst HM: Protein geranylgeranylation is critical for the regulation of survival and proliferation of lymphoma tumor cells. Clin.Cancer Res. 9:5735-5748, 2003

van der Pluijm G, Vloedgraven H, van Beek E, van der Wee-Pals L, Lowik C, Papapoulos S: Bisphosphonates inhibit the adhesion of breast cancer cells to bone matrices in vitro. J.Clin.Invest. 98:698-705, 1996

Vantourout P, Mookerjee-Basu J, Rolland C, Pont F, Martin H, Davrinche C, Martinez LO, Perret B, Collet X, Perigaud C, Peyrottes S, Champagne E: Specific requirements for Vgamma9Vdelta2 T cell stimulation by a natural adenylated phosphoantigen. J.Immunol. 183:3848-3857, 2009

Venet M, End D, Angibaud P: Farnesyl protein transferase inhibitor ZARNESTRA R115777 - history of a discovery. Curr.Top.Med.Chem. 3:1095-1102, 2003

Virtanen SS, Väänänen HK, Härkönen PL, Lakkakorpi PT: Alendronate inhibits invasion of PC-3 prostate cancer cells by affecting the mevalonate pathway. Cancer Res. 62:2708-2714, 2002

Virtanen SS, Sandholm J, Yegutkin G, Väänänen HK, Härkönen PL: Inhibition of GGTase I and FTase disrupts cytoskeleton organization of human PC-3 prostate cancer cells. Cell.Biol.Int. 34:815-826, 2010

Wada A, Fukui K, Sawai Y, Imanaka K, Kiso S, Tamura S, Shimomura I, Hayashi N: Pamidronate induced anti-proliferative, apoptotic, and anti-migratory effects in hepatocellular carcinoma. J.Hepatol. 44:142-150, 2006

Wakchoure S, Merrell MA, Aldrich W, Millender-Swain T, Harris KW, Triozzi P, Selander KS: Bisphosphonates inhibit the growth of mesothelioma cells in vitro and in vivo. Clin.Cancer Res. 12:2862-2868, 2006

Wasko BM, Dudakovic A, Hohl RJ: Bisphosphonates Induce Autophagy by Depleting Geranylgeranyl Diphosphate. J.Pharmacol.Exp.Ther. 337:540-546, 2011

Watanabe N, Narita M, Yokoyama A, Sekiguchi A, Saito A, Tochiki N, Furukawa T, Toba K, Aizawa Y, Takahashi M: Type I IFN-mediated enhancement of anti-leukemic cytotoxicity of gammadelta T cells expanded from peripheral blood cells by stimulation with zoledronate. Cytotherapy 8:118-129, 2006

Whyte DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, Bishop WR, Pai JK: K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J.Biol.Chem. 272:14459-14464, 1997

Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, Tony HP: Gammadelta T cells for immune therapy of patients with lymphoid malignancies. Blood 102:200-206, 2003

Witters LM, Crispino J, Fraterrigo T, Green J, Lipton A: Effect of the combination of docetaxel, zoledronic acid, and a COX-2 inhibitor on the growth of human breast cancer cell lines. Am.J.Clin.Oncol. 26:S92-7, 2003

Wong WW, Dimitroulakos J, Minden MD, Penn LZ: HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia 16:508-519, 2002

Wood J, Bonjean K, Ruetz S, Bellahcene A, Devy L, Foidart JM, Castronovo V, Green JR: Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. J.Pharmacol.Exp.Ther. 302:1055-1061, 2002

Woodward EJ and Coleman RE: Prevention and treatment of bone metastases. Curr.Pharm.Des. 16:2998-3006, 2010

Woodward JK, Neville-Webbe HL, Coleman RE, Holen I: Combined effects of zoledronic acid and doxorubicin on breast cancer cell invasion in vitro. Anticancer Drugs 16:845-854, 2005

Yardley V, Khan AA, Martin MB, Slifer TR, Araujo FG, Moreno SN, Docampo R, Croft SL, Oldfield E: In vivo activities of farnesyl pyrophosphate synthase inhibitors against Leishmania donovani and Toxoplasma gondii. Antimicrob.Agents Chemother. 46:929-931, 2002

Yuri T, Danbara N, Tsujita-Kyutoku M, Kiyozuka Y, Senzaki H, Shikata N, Kanzaki H, Tsubura A: Perillyl alcohol inhibits human breast cancer cell growth in vitro and in vivo. Breast Cancer Res.Treat. 84:251-260, 2004

Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjallman AH, Ballmer-Hofer K, Schwendener RA: Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. Br.J.Cancer 95:272-281, 2006

Zhang FL and Casey PJ: Protein prenylation: molecular mechanisms and functional consequences. Annu.Rev.Biochem. 65:241-269, 1996

Zhang Q, Zhen L, Li W, Novak EK, Collinson LM, Jang EK, Haslam RJ, Elliott RW, Swank RT: Cell-specific abnormal prenylation of Rab proteins in platelets and melanocytes of the gunmetal mouse. Br.J.Haematol. 117:414-423, 2002

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Bisphosphonate-Induced IPP/ApppI Accumulation in Cells

Bisphosphonates are currently among the most important and effective class of drugs developed for the treatment of metabolic bone diseases, such as osteoporosis and tumor-induced osteolysis. Despite their widespread clinical use, the exact biochemical mechanisms of action of bisphosphonates are not completely known. This thesis provides both *in vitro* and *in vivo* evidence that bisphosphonate-induced IPP accumulation and the subsequent ApppI formation are important metabolic events underlying the molecular mechanisms of action of bisphosphonates.

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