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Johanna Räikkönen, Hannu Mönkkönen, Seppo Auriola, Jukka Mönkkönen

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4 **Mevalonate pathway intermediates downregulate zoledronic acid- induced**
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6 **isopentenyl pyrophosphate and ATP analog formation in human breast cancer**
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14 Johanna Räikkönen ^{a, c}, Hannu Mönkkönen ^a, Seppo Auriola ^b, and Jukka Mönkkönen ^{a,}
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16 ^c

17
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19
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21
22 ^a Department of Pharmaceutics, University of Kuopio, P.O. Box 1627, FIN-70211
23
24 Kuopio, Finland

25
26
27 ^b Department of Pharmaceutical Chemistry, University of Kuopio, P.O. Box 1627, FIN-
28
29 70211 Kuopio, Finland

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31
32
33 ^c Biocenter Kuopio, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland
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40 **Running Title:** Isoprenoids inhibit ZOL- induced IPP/Apppl formation
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46 **Corresponding author:**
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49
50 Johanna Räikkönen, Department of Pharmaceutics, University of Kuopio,
51
52 Yliopistonranta 1B, P.O. Box 1627, FIN-70211 Kuopio, Finland
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56 Mobile: +358-40-3553466, Fax. +358-17-162252, E-mail: Johanna.Raikkonen@uku.fi
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21 **Abbreviations:** AppCCl₂p, 5'(β,γ -dichloromethylene) triphosphate; Apppl, triphosphoric
22 acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester; ApppD, triphosphoric acid 1-
23 adenosin-5'-yl ester 3-(3-methylbut-2-enyl) ester; BP, bisphosphonate; CLOD;
24 clodronate; DMAPP, dimethylallyl pyrophosphate; ESI, electrospray ionization; FOH,
25 farnesol; FPP, farnesyl pyrophosphate; GGOH, geranylgeraniol; GGPP, geranyl
26 geranylpyrophosphate; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IPP,
27 isopentenyl pyrophosphate; MS, mass spectrometry; MVP, mevalonate pathway; ZOL,
28 zoledronic acid
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Abstract

Increasing evidence is accumulating that zoledronic acid (ZOL), a nitrogen-containing bisphosphonate (N-BP), is able to affect tumor cells by inhibiting the enzyme farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway (MVP). The consequent accumulation of unprenylated proteins is believed to largely account for the cytotoxic effects of ZOL. FPPS inhibition leads also to the accumulation of isopentenyl pyrophosphate (IPP) and the apoptotic ATP analog, Apppl, but the role of this mechanism in the cytotoxic action of bisphosphonates is less clear. Since treatment with MVP intermediates has been shown to overcome N-BP-induced apoptosis via rescuing protein prenylation, our aim here was to determine their mechanism of action on ZOL-induced IPP/Apppl accumulation.

Interestingly, the results revealed that ZOL-induced IPP/Apppl accumulation in MCF-7 cells were decreased by farnesol, and almost completely blocked by geranylgeraniol and geranylpyrophosphate. The functionality of the regulatory enzymes of IPP and Apppl, IPP isomerase and aminoacyl-tRNA-synthase, respectively, or protein levels of FPPS were not affected by the treatments. However, the protein levels of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) and unprenylated Rap1A were observed to be strongly downregulated by geranylgeraniol and geranylpyrophosphate.

This study represents a novel insight into the mechanism of action of MVP intermediates on the regulation of MVP after FPPS inhibition. The data implies that in addition to the previously reported effects on rescuing protein prenylation, MVP intermediates can preserve cell activity by inhibiting the accumulation of IPP/Apppl via

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4 HMGR downregulation. This supports the hypothesis that IPP/Apppl formation is a
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6 significant mechanism in the anticancer action of ZOL.
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11 **Key words:** Bisphosphonate-induced ATP analog, isopentenyl pyrophosphate,
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13 isoprenoid, mevalonate pathway, 3-hydroxy-3-methylglutaryl-coenzyme A, zoledronic
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1. Introduction

Mevalonate pathway (MVP) provides cells with isoprenoids that are fundamental for cell growth and survival [1], and this pathway is a target for antiresorptive nitrogen-containing bisphosphonates (N-BPs), such as zoledronic acid (ZOL) [2]. These effective inhibitors of bone resorption are successfully and widely used pharmaceutical agents for treatment of diseases characterized by excessive bone loss [3-5]. Furthermore, extensive evidence from preclinical research have demonstrated that N-BPs exhibit antitumor activity in a variety of cancer models [6-10]. Importantly, there is now clinical evidence that the addition of ZOL to endocrine therapy can improve disease-free survival in premenopausal women with estrogen-responsive early breast cancer, indicating that bisphosphonates exert clinical antitumor effects [11]. Inhibition of the MVP has been proposed as being the fundamental molecular mechanism of many of the observed anticancer effects of N-BPs both *in vitro* and *in vivo* [12].

The therapeutical efficacy of N-BPs can be traced to their specificity to bind and inhibit farnesyl pyrophosphate synthase (FPPS) in the MVP [13, 14]. Blockade of the enzymatic activity of FPPS by N-BP depletes the formation of essential MVP intermediate isoprenoids, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which are needed for post-translational prenylation of variety of GTPases, such as Rap, Rho and Ras [15, 16]. Disruption of the lipid modification of these proteins induces a series of changes leading to altered cell activity and indirect apoptosis, and has been suggested to underlie the cytotoxic effects of N-BPs.

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4 However, in addition to the loss of prenylated proteins, inhibition of FPPS by N-
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6 BPs in the MVP causes intracellular accumulation of isopentenyl pyrophosphate (IPP)
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8 and consequently induces the biosynthesis of a novel pro-apoptotic ATP analog Apppl
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10 (triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester), *in vitro* [17-20]
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12 and *in vivo* [19, 21]. Recently we observed that in addition to IPP/Apppl formation, N-
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14 BPs induce isomeric dimethylallyl pyrophosphate (DMAPP) accumulation and
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16 consequent ApppD (triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-2-enyl)
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18 ester) production [22]. However, for the sake of clarity, IPP/Apppl is used to describe
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20 both IPP/DMAPP and Apppl/ApppD formation throughout this text. Formation of Apppl
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22 from IPP is most probably catalyzed by the same metabolic pathway (i.e. aminoacyl-
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24 tRNA-synthetases) [23] as the nonhydrolyzable AppCp-type ATP analogs of non-
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26 nitrogen containing bisphosphonates (non-N-BPs), such as clodronate [18]. Similar to
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28 AppCCl₂p (i.e., a metabolite of clodronate) [24], Apppl interferes with mitochondrial
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30 function and induces apoptosis in osteoclasts [17]. Moreover, we have demonstrated
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32 that the amount of IPP/Apppl correlates with ZOL-induced FPPS inhibition [17, 18] and
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34 most importantly, cancer cell death *in vitro* [20]. Therefore, N-BPs evoke apoptosis by
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36 two mechanisms, indirectly *via* the inhibition of protein isoprenylation, and also directly
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38 *via* inhibition of mitochondrial adenine nucleotide translocase (ANT) by ATP analog,
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40 Apppl.
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50 In addition to FPPS, the intracellular concentration of isoprenoids is controlled by
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52 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) enzyme, which is tightly
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54 regulated in order to yield sufficient IPP and DMAPP to satisfy cellular requirements for
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56 all the mevalonate end-products [1]. HMGR is controlled by negative feedback
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4 regulation of transcription by sterol and nonsterol products of the pathway [1, 25, 26].
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6 These products suppress HMGR by at least two mechanisms; by decreasing
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8 transcription of the gene and by enhancing degradation of the enzyme [1]. Statins and
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10 N-BPs can block the enzymatic activity of HMGR and FFPS, respectively, and
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12 consequently prevent protein prenylation and induce apoptosis [12]. In this respect,
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14 nonsterol isoprenoids have been shown to play an important role. Several studies
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16 indicate that free nonsterol MVP intermediates, farnesol (FOH) and especially
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18 geranylgeraniol (GGOH), are capable of salvaging protein isoprenylation and, thus
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20 overcome statin and N-BP-induced apoptosis in many cell types [7,8,15,16,27,28]. In
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22 addition, we have evidence that GGOH may possess the capacity to inhibit ZOL-
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24 induced IPP/Apppl formation in tumor cells [20], but the mechanism underlying this
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26 effect is not known. Since IPP is capable of activating an immune response [29, 30] and
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28 Apppl has been shown to be an apoptotic molecule [17], it was felt important to define
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30 whether the salvage properties of the isoprenoids involve regulation of IPP/Apppl levels
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32 in cells. Therefore, we determined which MPV intermediates and enzymes are involved
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34 in regulating the ZOL-induced IPP/Apppl formation in cancer cells. The results suggest
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36 that in addition to the previously reported effects on rescuing protein prenylation, MVP
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38 intermediates can influence cell activity by downregulating IPP/Apppl levels in cells.
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40 This data provides further evidence that IPP/Apppl formation is a significant mechanism
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42 in the anticancer action of N-BPs.
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2. Materials and Methods

2.1 Reagents

Zoledronic acid [2-(imidazol-1-yl)-hydroxy-ethylidene-1,1-bisphosphonic acid, disodium salt, 4.75 hydrate] were kindly provided by Novartis Pharma AG (Basel, Switzerland) and clodronate (dichloromethylene-1,1-bisphosphonate) by Schering Oy (Bayer Schering Pharma AG, Berlin, Germany). Stock solutions of zoledronic acid (ZOL) and clodronate (CLOD) were prepared in phosphate-buffered saline (PBS; pH 7.4; Gibco, U.K.) and filter-sterilized before use. Mevalonic acid lactone (MVL, which is readily converted to mevalonate, MVA), farnesol (FOH), geranylgeraniol (GGOH), geranylgeranylpyrophosphate (GGPP), lovastatin (LOV), isopentenyl pyrophosphate (IPP), 3,3-dimethylallylpyrophosphate (DMAPP), methyladenosine 5'-triphosphate (AppCp), bovine serum albumin (BSA) and sodium orthovanadate 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. Sodium fluoride was from Riedel-de-Haën (Seelze, Germany). HPLC-grade acetonitrile was from J.T. Baker (Deventer, The Netherlands). Apppl [triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester] and AppCCl₂p [5'(β,γ -dichloromethylene) triphosphate] were synthesized as previously described [17]. Cell culture reagents were from BioWhittaker (Cambrex Bio Science, Verviers, Belgium) and plastics from Nunc (Roskilde, Denmark).

2.2. Cell culture

The experiments were performed using the human estrogen-dependent breast cancer cell line, MCF-7, obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in 75-cm³ flasks at + 37 °C in RPMI-1640 medium supplemented with 10 % fetal bovine serum and 100 IU/ml penicillin-streptomycin in a 5 % CO₂ atmosphere. Cells were harvested using 0.05 % trypsin-EDTA.

2.3. Preparation of cell lysates for IPP/Apppl and AppCCI₂p detection

The cells were seeded in 6-well plates at a density of 1 x10⁶ per 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, 50 μM FOH, 50 μM GGOH, 25/50 μM FOH with 25 μM ZOL or 500 μM CLOD, 25/50 μM GGOH with 25 μM ZOL or 500 μM CLOD, 100 μM GGPP with 25 μM ZOL, 500/1000 μM MVA with or without 25 μM ZOL, 25/50 μM FOH plus 25 μM ZOL with 500/1000 μM MVA, 25/50 μM GGOH plus 25 μM ZOL with 500/1000 μM MVA, or vehicle (CTR). After 24 h treatment, the cells were washed once with ice-cold phosphate-buffered saline (PBS), and carefully scraped off from the wells. The analytes were extracted from the cell samples by adding ice-cold acetonitrile (300 μl) and water (200 μl) containing sodium orthovanadate and sodium fluoride as phosphate inhibitors. The precipitated proteins were separated by centrifugation (13 000g, 2 min, + 4°C). The supernatant extract was transferred to a new tube and evaporated using vacuum centrifugation. Samples were stored at -70 °C until mass spectrometric analysis. Protein content determinations were done as previously described [31]. The influence of the drugs

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4 alone and in combination on cell viability was determined by MTT (3-[4,5-
5 dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) test [31].
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10 **2.4. Mass spectrometry analysis**

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14 The quantitative analysis of IPP/Apppl, and AppCCl₂p in cell extracts was
15 performed as previously described [21] by using a HPLC electrospray ionization mass
16 spectrometry (HPLC-ESI-MS) system in negative ion mode. The evaporated cell
17 extracts were redissolved in 150 µl of water containing 1 µM internal standard (AppCp)
18 to compensate for variabilities in ionization. Selected reaction monitoring (SRM) was
19 used for analysis of the compounds in the sample and quantitation was based on the
20 characteristic fragment ions of each molecule. The following transitions were monitored:
21 m/z 245 → 159 and m/z 245 → 177 for IPP/DMAPP, m/z 574 → 408 for Apppl, m/z
22 572 → 225 and m/z 574 → 227 for AppCCl₂p (³⁵Cl & ³⁷Cl) and, m/z 504 → 406 for
23 AppCp. The pattern of fragmentation for each sample was compared with that of the
24 authentic standard. Standards were constructed by spiking extracts from untreated cells
25 with synthesized IPP, Apppl, or AppCCl₂p. Quantitation of the molecules was done with
26 LCquan 2.0 software (Thermo Finnigan), using the standard curve and the transitions
27 mentioned above. The relative amount of IPP and DMAPP isomers in the treated cell
28 samples were calculated using a protocol described earlier [22]. This method utilizes the
29 peak intensity ratios of two characteristic fragment ions, m/z 177 and m/z 159, of IPP
30 and DMAPP received from MS² monitoring. The results shown are representative of at
31 least 4 independent experiments (mean±SEM).
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2.5. Western blot analysis

The MCF-7 cells were seeded at a density of 2×10^6 per 25-cm³ flask. On the next morning, the cells were treated for 24 h with drugs alone or in combinations: 5 μ M LOV (positive control), 25 μ M ZOL, 50 μ M FOH, 50 μ M GGOH, 25/50 μ M FOH with 25 μ M ZOL, 25/50 μ M GGOH with 25 μ M ZOL, 100 μ M GGPP with ZOL, or vehicle (CTR). After treatment, the cells were washed twice with ice-cold phosphate-buffered saline, and lysed in cell precipitation buffer (mammalian cell lysis kit, Sigma Chemical Co., St. Louis, MO, USA). 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 20 (Sigma-Aldrich, Steinheim, Germany) 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 antibody (1:4000 dilution, Autogen Bioclear, Wiltshire, UK) 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

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4 protein bands was determined by pixel intensity using NIH Image J software (v1.4.3,
5 available at <http://rsb.info.nih.gov/ij/index.html>) and normalized against that of β -actin
6 and control (HMGR and FPPS bands) or β -actin and ZOL (unprenylated Rap1A bands).
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9 Results of the densitometrical analyses shown are combined data of at least three
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12 independent experiments. The experiments were performed in triplicate.
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16 17 18 **2.6. Statistical analysis**

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21 The data are presented as means \pm SEM. One-way ANOVA with Tukey's
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23 multiple comparison or Bonferroni's comparison test were used to analyze the
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25 significant differences in the cell viability or IPP/Apppl levels. $p < 0.05$ was considered
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27 statistically significant.
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3. Results

3.1. FOH and GGOH decrease ZOL-induced IPP/Apppl levels in MCF-7 cells

Since the addition of intermediates of the MVP have been demonstrated to overcome N-BP-induced apoptosis and other events in many cell systems [12], the first objective of this study was to determine their influence on the levels of IPP/Apppl induced by ZOL in breast cancer cells. Therefore, MCF-7 cells were incubated for 24 h with exogenous FOH (50 μ M) and GGOH (25 μ M), which are metabolized to their phosphate forms in the cells [32], together with increasing concentrations of ZOL, for 24 h. As seen in Figure 1, IPP accumulation and consequent Apppl formation in cells was evenly and significantly ($p > 0.001$) decreased by both intermediates at all tested ZOL concentrations. No significant differences in the levels IPP/Apppl between 50 and 100 μ M ZOL were observed. At concentrations of 25, 50 and 100 μ M, 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 intermediates were observed with 25 μ M ZOL, and thus this dose was chosen for further studies.

3.2. Isoprenoids do not interact with regulatory enzymes of IPP or Apppl synthesis

Since both of the isomers, IPP and DMAPP, are essential in the synthesis of isoprenoids in the MVP, 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 μ M FOH or GGOH concurrently with 25 μ M ZOL, or 25 μ M ZOL alone.

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4 In our previous studies, only the mixture of the isomeric molecules has been analyzed,
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6 but recently we have described a method which is capable of quantifying the relative
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8 amount of both isomers, IPP and DMAPP, in cell extracts [22]. Using this procedure, we
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10 discovered that there were no differences in the isomerization capacity of the enzyme
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12 between the treatments (Fig. 2A). The ratio of IPP and DMAPP in all cell extracts was
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14 approximately 1:4. The same treatment protocol was tested, except that ZOL was
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16 replaced with non-N-BP clodronate (500 μ M), to clarify the functionality of the
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18 aminoacyl-tRNA-synthase enzyme, known to catalyze ATP-analogs from non-N-BPS
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20 [23], and which is proposed to catalyze also the formation of Apppl from IPP. The cells
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22 were observed to metabolize clodronate into AppCCl₂p regardless of isoprenoid
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24 treatments, and therefore the functionality of the aminoacyl-tRNA-synthase was not
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26 affected (Fig. 2B).
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36 **3.3. GGOH and GGPP inhibit ZOL-induced IPP/Apppl accumulation by decreasing** 37 38 **levels of HMG-CoA reductase protein in cells**

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41 To further examine the effects of isoprenoid compounds on the regulation of
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43 MVP, we studied the dependence of the isoprenoid dose on inhibition of ZOL-induced
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45 IPP/ Apppl accumulation in MCF-7 cells. Co-treatment of ZOL with the doses of 25 and
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47 50 μ M FOH or 25 and 50 μ M GGOH resulted in a significant ($p>0.001$) and dose-
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49 dependent decrease in the levels of IPP/Apppl compared to treatment with ZOL alone
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51 (Fig. 3A). GGOH was more effective than FOH in reversing the effects of ZOL, inducing
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53 almost complete prevention. However, the viability of the cells decreased to 68 %
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55 compared to control (Fig. 3A). In contrast to GGOH, co-treatment of ZOL with 100 μ M
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4 GGPP, the analogue of GGOH, did not affect cell viability (viability 96 % compared to
5 control), but inhibited IPP/Apppl accumulation in cells in the same manner as GGOH.
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7 Therefore, decreased cell viability induced by GGOH does not explain for the observed
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9 downregulation in ZOL-induced IPP/Apppl levels. There was no evidence of IPP/Apppl
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11 found in the cell samples treated with LOV, FOH, GGOH or vehicle (CTR) (Fig. 3A).
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17 Next, we investigated the effects of co-treatments at the protein level on FPPS,
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19 HMGR and unprenylated Rap1A (uRap1A) in Western blot analysis. Membranes were
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21 probed with β -actin antibody to ensure that an equal amount of protein was loaded onto
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23 the gel. As a positive control, cells were treated with 5 μ M LOV, which is known to
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25 upregulate HMGR protein levels and cause uRap1A accumulation [1, 33, 34]. In Figure
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27 3 B, representative Western blots of FPPS, HMGR and uRap1A protein from the treated
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29 cells are shown. The treatments did not alter the protein level of FPPS, the target
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31 enzyme for ZOL. Furthermore, accumulation of uRap1A correlated with the extent of
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33 ZOL-induced IPP/ Apppl formation. In general, Rap1A is a surrogate marker for
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35 inhibition of FPPS, since the uRap1A accumulates in cells exposed to N-BPs. As in the
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37 case of IPP/Apppl formation (Fig. 3A), GGOH and GGPP were again more potent at
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39 restoring Rap1A protein prenylation than FOH. No accumulation of uRap1A was
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41 observed in the presence of vehicle, GGOH or FOH alone. The most interesting result
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43 was obtained when HMGR protein expression levels were monitored. GGOH and
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45 GGPP resulted in a significant reduction in the amount of HMGR enzyme present in the
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47 treated cells (Fig. 3B). This result was not a consequence of the reduced cell viability by
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49 GGPP (Fig. 3A). Furthermore, the protein level of HMGR was increased by LOV and
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51 ZOL, and slightly decreased by FOH. These results indicate that the observed inhibition
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4 of IPP/Apppl accumulation (Fig. 3A) by MVP intermediates is a consequence of HMGR
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6 enzyme downregulation (Fig. 3B).
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10 11 **3.4. Mevalonate can partly restore ZOL-induced IPP/Apppl accumulation during** 12 13 **isoprenoid co-treatment** 14

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17 To confirm whether the decrease in the levels of IPP/Apppl induced by MVP
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19 intermediates was a result of the interruption of HMGR function and a consequent lack
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21 of precursor for IPP, MVA was added to the culture medium. As shown in Figures 4A
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23 and 4B, the inhibitory effect of isoprenoids on ZOL-induced IPP/Apppl levels could be
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25 partially prevented by the addition of 500 μ M MVA. MVA at a higher concentration (1
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27 mM) reduced IPP/Apppl accumulation, as well as cell viability compared to the 500 μ M
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29 dose (data not shown). There was no evidence of IPP/Apppl found in the cell samples
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31 treated with MVA alone (data not shown). These results further suggest that the
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33 regulation of the IPP/Apppl accumulation is mediated by HMGR enzyme.
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4. Discussion

Evidence from previous studies indicated that the inhibition of the MVP played a central role in the anticancer effects of N-BPs [12, 35]. After FPPS inhibition by N-BPs, the pathway is blocked and accumulation of IPP metabolites upstream occurs in conjugation with downstream inhibition of protein prenylation, due to lack of FPP and GGPP. IPP is further metabolized endogenously into an ATP analogue Apppl, which is able to induce apoptosis in cells by inhibiting mitochondrial adenine nucleotide translocase (ANT), causing also a loss of the mitochondrial membrane potential [17, 20]. Several studies have demonstrated that the interruption of the protein prenylation is a major contributor to the anticancer action of N-BPs [7, 36, 37], whereas the role and the significance of the additional mechanism of action, accumulation of IPP/Apppl, is less clear.

Previously, it has been shown that the inhibitory action of N-BPs on cell function could be prevented by MVP intermediates [15, 16, 27, 38]. Until now, this has been attributed to their action on MVP 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/Apppl accumulation. The results illustrate that co-treatment with cell permeable isoprenoids, GGOH and FOH, significantly reduced expression of IPP/Apppl 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 [14, 39], and thus, IPP accumulation upstream of FPPS should stay constant during the treatment. Interestingly, 50 μ M GGOH and 100 μ M GGPP almost completely blocked

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4 IPP accumulation, and the consequent Apppl formation in cells, whereas FOH only
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6 partially attenuated these effects of ZOL. The results are consistent with the
7
8 appearance of unprenylated form of the small GTPase Rap1A that was seen to
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10 correlate with the level of accumulating IPP/Apppl in cells. One possible explanation for
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12 the failure of FOH to rescue protein prenylation could be that only a small amount of
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14 FPP formed from FOH is metabolized further to GGPP [32]. Conversion of FPP to
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16 GGPP requires IPP, which is available after FPPS activity is blocked by ZOL. This could
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18 be sufficient to allow FOH-induced partial recovery of Rap1A prenylation. Alternatively,
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20 the cells may convert FOH to FPP for the farnesylation of proteins that are normally
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22 geranylgeranylated [40].
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30 All the isoprenoids of the MVP are synthesized by a condensation reaction of two
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32 simple precursors: IPP and DMAPP. Furthermore, both of the isomers are required for
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34 the activity of N-BP in cells: IPP for binding and stabilizing FPPS enzyme-N-BP complex
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36 [14, 39], and both isomers for conjugation reaction to AMP to form ATP analogs, Apppl
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38 and ApppD [17, 22]. The reversible reaction involved in the transformation of the
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40 relatively unreactive IPP into the relatively reactive isomer DMAPP is catalyzed by IPP
41
42 isomerase enzyme [41,42]. Recently, we developed a new method for quantification of
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44 these isomers in cell extracts [22]. Using this method, we demonstrate that DMAPP is
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46 the predominant isomeric form detected in MCF-7 cells, which is consistent with our
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48 previous finding that the ratio of IPP:DMAPP is 1:4 after ZOL treatment [22]. The ratio of
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50 the isomers in cell samples was found to be stable irrespective of the treatments,
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52 evidencing that MVP intermediates do not affect the activity of IPP isomerase. Similarly,
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54 no differences in the formation of AppCCI₂p from a non-N-BP clodronate was detected
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4 between the treatments, indicating that aminoacyl-tRNA synthase converting of
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6 IPP/DMAPP into Apppl/ApppD, is not modified by FOH or GGOH. Furthermore, no
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8 changes were observed in the FPPS levels analyzed by Western blot, indicating FPPS
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10 enzyme levels are not affected by the isoprenoids.
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14 Since our results revealed that isoprenoids affected neither the expression levels
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16 of FPPS nor inhibited the function of IPP isomerase or aminoacyl-tRNA synthetase
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18 enzymes, mechanisms upstream from FPPS were investigated. HMGR is the rate-
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20 limiting enzyme of isoprenoid synthesis from mevalonate, and its activity has been
21
22 reported to be regulated at many levels [1]. There is marked upregulation of HMGR
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24 protein expression after inhibition of this enzyme by statins [25, 43], whereas protein
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26 levels are decreased by the sterol and nonsterol products of the pathway [1]. Indeed,
27
28 our findings indicate that the inhibition of ZOL-induced IPP/Apppl accumulation results
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30 from HMGR downregulation, since both GGOH and GGPP clearly decrease the amount
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32 of HMGR enzyme in cells, presumably by increasing its rate of degradation. In addition,
33
34 the ability of MVA, a precursor for IPP, to partially overcome the isoprenoid-induced
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36 inhibition on IPP/Apppl accumulation suggests that the regulation could be mediated by
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38 HMGR, which catalyzes MVA formation. The effect of MVA on restoring IPP/Apppl
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40 levels was not very potent, perhaps due to the mevalonate-mediated HMGR control.
41
42 This suggestion is supported by previous studies showing that MVA at higher
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44 concentrations can regulate the translation and degradation of HMGR [1, 25]. Our
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46 results do not solve the puzzle of whether the active form for HMGR regulation is the
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48 isoprenoid alcohol or the corresponding pyrophosphate or even some consequent
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50 products. The study of Correll *et al.* [44] suggests that isoprenoid alcohol is the active
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4 component or at least is having a more direct role in enzyme degradation. The results of
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6 that study indicated that FPP-induced, but not FOH-induced, degradation of HMGR was
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8 blocked by the addition of sodium fluoride, a general phosphatase inhibitor, in
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10 permeabilized cells. In the present study, only a slight reduction on the protein levels of
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12 HMGR could be detected with FOH. However, its lack of effect on enzyme degradation
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14 is in agreement with our findings obtained from the MS analysis, where FOH was not as
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16 effective at inhibiting IPP/Apppl accumulation as GGOH or GGPP. We have previously
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18 shown that the level of IPP/Apppl correlates well with ZOL-induced cancer cell death *in*
19
20 *vitro* [20]. The outcome of the present study is consistent with observations that MCF-7
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22 cells are rescued from ZOL-induced apoptosis by GGOH, but not by FOH or MVA [8]. In
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24 addition, previous studies indicate that geranylgeranylated proteins rather than
25
26 farnesylated proteins are required for suppression of apoptosis in cancer cells, and thus
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28 seem to be largely responsible for the antitumor effects of N-BPs, at least *in vitro* [10,
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39 Taken together, this study strongly suggests that MVP intermediates, GGOH or
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41 GGPP, inhibit ZOL-induced IPP/Apppl accumulation via downregulation of HMG-CoA
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43 reductase. This represents a novel insight into the mechanism of action of isoprenoids
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45 on the regulation of MVP after FPPS inhibition. The data implies that in addition to the
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47 previously reported effects on rescuing protein prenylation, isoprenoids can preserve
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49 cell activity by inhibiting the accumulation of IPP/Apppl in cells. This knowledge of the
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51 different mechanisms of action involved is relevant for understanding the anticancer
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53 action of N-BPs, and furthermore for expanding application of BPs in cancer treatment.
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4 **References:**
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7

8 [1] Goldstein JL, Brown MS. Regulation of the mevalonate pathway. *Nature*
9 1990;343:425-30.
10

11
12
13
14 [2] Amin D, Cornell SA, Gustafson SK, Needle SJ, Ullrich JW, Bilder GE, et al.
15
16 Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase
17 and cholesterol biosynthesis. *J Lipid Res* 1992;33:1657-63.
18
19

20
21
22 [3] Delmas PD. Treatment of postmenopausal osteoporosis. *Lancet* 2002;359:2018-26.
23
24

25
26 [4] Diel IJ, Solomayer EF, Bastert G. Bisphosphonates and the prevention of
27 metastasis: first evidences from preclinical and clinical studies. *Cancer* 2000;88:3080-8.
28
29

30
31
32 [5] Roux C, Dougados M. Treatment of patients with Paget's disease of bone. *Drugs*
33 1999;58:823-30.
34
35

36
37
38 [6] Coleman RE. The role of bisphosphonates in breast cancer. *Breast* 2004;13 Suppl
39 1:S19-28.
40
41

42
43
44 [7] Goffinet M, Thoulouzan M, Pradines A, Lajoie-Mazenc I, Weinbaum C, Faye JC, et
45 al. Zoledronic acid treatment impairs protein geranyl-geranylation for biological effects in
46 prostatic cells. *BMC Cancer* 2006;6:60.
47
48
49

50
51
52
53 [8] Jagdev SP, Coleman RE, Shipman CM, Rostami-H A, Croucher PI. The
54 bisphosphonate, zoledronic acid, induces apoptosis of breast cancer cells: evidence for
55 synergy with paclitaxel. *Br J Cancer* 2001;84:1126-34.
56
57
58
59
60
61
62
63
64
65

1
2
3
4 [9] Shipman CM, Rogers MJ, Apperley JF, Russell RG, Croucher PI. Bisphosphonates
5
6 induce apoptosis in human myeloma cell lines: a novel anti-tumour activity. Br J
7
8 Haematol. 1997;98:665-72.
9

10
11
12 [10] Kubista B, Trieb K, Sevela F, Toma C, Arrich F, Heffeter P, et al. Anticancer
13
14 effects of zoledronic acid against human osteosarcoma cells. J Orthop Res
15
16
17 2006;24:1145-52.
18
19

20
21 [11] Gnant M, Mlineritsch B, Schippinger W, Luschin-Ebengreuth G, Postlberger S,
22
23 Menzel C, et al. Endocrine therapy plus zoledronic acid in premenopausal breast
24
25 cancer. N Engl J Med 2009;360:679-91.
26
27

28
29 [12] Buhaescu I, Izzedine H. Mevalonate pathway: a review of clinical and therapeutical
30
31 implications. Clin Biochem 2007;40:575-84.
32
33

34
35 [13] van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S. Farnesyl
36
37 pyrophosphate synthase is the molecular target of nitrogen-containing
38
39 bisphosphonates. Biochem Biophys Res Commun 1999;264:108-11.
40
41
42

43
44 [14] Rondeau JM, Bitsch F, Bourgier E, Geiser M, Hemmig R, Kroemer M, et al.
45
46 Structural basis for the exceptional in vivo efficacy of bisphosphonate drugs.
47
48 ChemMedChem 2006;1:267-73.
49
50

51
52 [15] Benford HL, Frith JC, Auriola S, Mönkkönen J, Rogers MJ. Farnesol and
53
54 geranylgeraniol prevent activation of caspases by aminobisphosphonates: biochemical
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 evidence for two distinct pharmacological classes of bisphosphonate drugs.

5
6 Mol.Pharmacol. 1999;56:131-40.
7
8
9

10 [16] Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G, Rogers MJ. Nitrogen-
11 containing bisphosphonates inhibit the mevalonate pathway and prevent post-
12 translational prenylation of GTP-binding proteins, including Ras. J Bone Miner Res
13 1998;13:581-9.
14
15
16
17
18
19
20

21 [17] Mönkkönen H, Auriola S, Lehenkari P, Kellinsalmi M, Hassinen IE, Vepsäläinen J,
22 et al. A new endogenous ATP analog (Apppl) inhibits the mitochondrial adenine
23 nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-
24 containing bisphosphonates. Br J Pharmacol 2006;147:437-45.
25
26
27
28
29
30
31

32 [18] Mönkkönen H, Kuokkanen J, Holen I, Evans A, Lefley D, Jauhiainen M, et al.
33 Bisphosphonate-induced ATP analog formation and its effect on inhibition of cancer cell
34 growth. Anticancer Drugs 2008;19:391-9.
35
36
37
38
39
40

41 [19] Räikkönen J, Crockett JC, Rogers MJ, Mönkkönen H, Auriola S, Mönkkönen J.
42 Zoledronic acid induces formation of a pro-apoptotic ATP analogue and isopentenyl
43 pyrophosphate in osteoclasts *in vivo* and in MCF-7 cells *in vitro*. Br J Pharmacol
44 2009;157:427-35.
45
46
47
48
49
50

51 [20] Mitrofan L, Pelkonen J, Mönkkönen J. The level of ATP analogue and isopentenyl
52 pyrophosphate correlates with zoledronic acid -induced apoptosis in cancer cells *in*
53 *vitro*. Bone 2009, doi:10.1016/j.bone.2009.08.010.
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 [21] Mönkkönen H, Ottewell PD, Kuokkanen J, Mönkkönen J, Auriola S, Holen I.

5
6 Zoledronic acid-induced IPP/Apppl production *in vivo*. Life Sci 2007;81:1066-70.
7
8
9

10 [22] Jauhiainen M, Mönkkönen H, Räikkönen J, Mönkkönen J, Auriola S. Analysis of
11 endogenous ATP analogs and mevalonate pathway metabolites in cancer cell cultures
12 using liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr
13 B Analyt Technol Biomed Life Sci 2009;877:2967-75.
14
15
16
17
18
19
20

21 [23] Rogers MJ, Brown RJ, Hodkin V, Blackburn GM, Russell RG, Watts DJ.

22
23 Bisphosphonates are incorporated into adenine nucleotides by human aminoacyl-tRNA
24 synthetase enzymes. Biochem Biophys Res Commun 1996;224:863-9.
25
26
27
28
29

30 [24] Lehenkari PP, Kellinsalmi M, Napankangas JP, Ylitalo KV, Mönkkönen J, Rogers
31 MJ, et al. Further insight into mechanism of action of clodronate: inhibition of
32 mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing
33 metabolite. Mol Pharmacol 2002;61:1255-62.
34
35
36
37
38
39
40

41 [25] Nakanishi M, Goldstein JL, Brown MS. Multivalent control of 3-hydroxy-3-
42 methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of
43 mRNA and accelerates degradation of enzyme. J Biol Chem 1988;263:8929-37.
44
45
46
47
48

49 [26] Osborne TF, Gil G, Goldstein JL, Brown MS. Operator constitutive mutation of 3-
50 hydroxy-3-methylglutaryl coenzyme A reductase promoter abolishes protein binding to
51 sterol regulatory element. J Biol Chem 1988;263:3380-7.
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 [27] Shipman CM, Croucher PI, Russell RG, Helfrich MH, Rogers MJ. The
5
6 bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells *in vitro*
7
8 by inhibiting the mevalonate pathway. *Cancer Res* 1998;58:5294-7.
9

10
11
12 [28] van de Donk NW, Schotte D, Kamphuis MM, van Marion AM, van Kessel B, Bloem
13
14 AC, et al. Protein geranylgeranylation is critical for the regulation of survival and
15
16 proliferation of lymphoma tumor cells. *Clin Cancer Res* 2003;9:5735-48.
17
18

19
20
21 [29] Gober HJ, Kistowska M, Angman L, Jenö P, Mori L, De Libero G. Human T cell
22
23 receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor
24
25 cells. *J Exp Med* 2003;197:163-8.
26
27

28
29
30 [30] Roelofs AJ, Jauhainen M, Mönkkönen H, Rogers MJ, Mönkkönen J, Thompson K.
31
32 Peripheral blood monocytes are responsible for gammadelta T cell activation induced
33
34 by zoledronic acid through accumulation of IPP/DMAPP. *Br J Haematol* 2009;144:245-
35
36 50.
37
38

39
40
41 [31] Mönkkönen H, Tormalehto S, Asunmaa K, Niemi R, Auriola S, Vepsäläinen J, et al.
42
43 Cellular uptake and metabolism of clodronate and its derivatives in Caco-2 cells: a
44
45 possible correlation with bisphosphonate-induced gastrointestinal side-effects. *Eur J*
46
47 *Pharm Sci* 2003;19:23-9.
48
49

50
51
52 [32] Crick DC, Andres DA, Waechter CJ. Novel salvage pathway utilizing farnesol and
53
54 geranylgeraniol for protein isoprenylation. *Biochem Biophys Res Commun*
55
56 1997;237:483-7.
57
58

1
2
3
4 [33] Edwards PA, Ashby MN, Spear DH, Marrero PF, Joly A, Popjak G. Polyisoprenoid
5
6 synthesis and metabolism. *Biochem Soc Trans* 1992;20:475-9.
7
8

9
10 [34] Ownby SE, Hohl RJ. Isoprenoid alcohols restore protein isoprenylation in a time-
11
12 dependent manner independent of protein synthesis. *Lipids* 2003;38:751-9.
13
14

15
16 [35] Brown JE, Neville-Webbe H, Coleman RE. The role of bisphosphonates in breast
17
18 and prostate cancers. *Endocr Relat Cancer* 2004;11:207-24.
19
20

21
22 [36] Senaratne SG, Mansi JL, Colston KW. The bisphosphonate zoledronic acid impairs
23
24 Ras membrane localisation and induces cytochrome c release in breast cancer cells. *Br*
25
26 *J Cancer* 2002;86:1479-86.
27
28

29
30 [37] Oades GM, Senaratne SG, Clarke IA, Kirby RS, Colston KW. Nitrogen containing
31
32 bisphosphonates induce apoptosis and inhibit the mevalonate pathway, impairing Ras
33
34 membrane localization in prostate cancer cells. *J Urol* 2003;170:246-52.
35
36
37

38
39 [38] van Beek E, Lowik C, van der Pluijm G, Papapoulos S. The role of
40
41 geranylgeranylation in bone resorption and its suppression by bisphosphonates in fetal
42
43 bone explants in vitro: A clue to the mechanism of action of nitrogen-containing
44
45 bisphosphonates. *J Bone Miner Res* 1999;14:722-9.
46
47
48

49
50 [39] Dunford JE, Kwaasi AA, Rogers MJ, Barnett BL, Ebetino FH, Russell RG, et al.
51
52 Structure-activity relationships among the nitrogen containing bisphosphonates in
53
54 clinical use and other analogues: time-dependent inhibition of human farnesyl
55
56 pyrophosphate synthase. *J Med Chem* 2008;51:2187-95.
57
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60
61
62
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1
2
3
4 [40] Gibbs JB, Oliff A. The potential of farnesyltransferase inhibitors as cancer
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chemotherapeutics. *Annu Rev Pharmacol Toxicol* 1997;37:143-66.

[41] Hahn FM, Xuan JW, Chambers AF, Poulter CD. Human isopentenyl diphosphate:
dimethylallyl diphosphate isomerase: overproduction, purification, and characterization.
Arch Biochem Biophys 1996;332:30-4.

[42] Zheng W, Sun F, Bartlam M, Li X, Li R, Rao Z. The crystal structure of human
isopentenyl diphosphate isomerase at 1.7 Å resolution reveals its catalytic mechanism
in isoprenoid biosynthesis. *J Mol Biol* 2007;366:1447-58.

[43] Brown MS, Faust JR, Goldstein JL, Kaneko I, Endo A. Induction of 3-hydroxy-3-
methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with
compactin (ML-236B), a competitive inhibitor of the reductase. *J Biol Chem*
1978;253:1121-8.

[44] 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* 1994;269:17390-3.

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4 **Figure legends:**
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9 **Fig. 1. FOH and GGOH inhibit ZOL-induced IPP/Apppl accumulation in cells.** The
10 amount of IPP/Apppl in MCF-7 cells after 24 h treatment with 25 - 100 μ M zoledronic
11 acid (25-100 ZOL) with or without 50 μ M farnesol (50 FOH) or 25 μ M geranylgeraniol
12 (25 GGOH). The molar amounts of the compounds were determined in acetonitrile
13 extracts with the HPLC-ESI-MS method (mean \pm SEM, n=6-9). ***, P<0.001 compared
14 to ZOL treatment using one-way ANOVA with Tukey's Multiple Comparison test.
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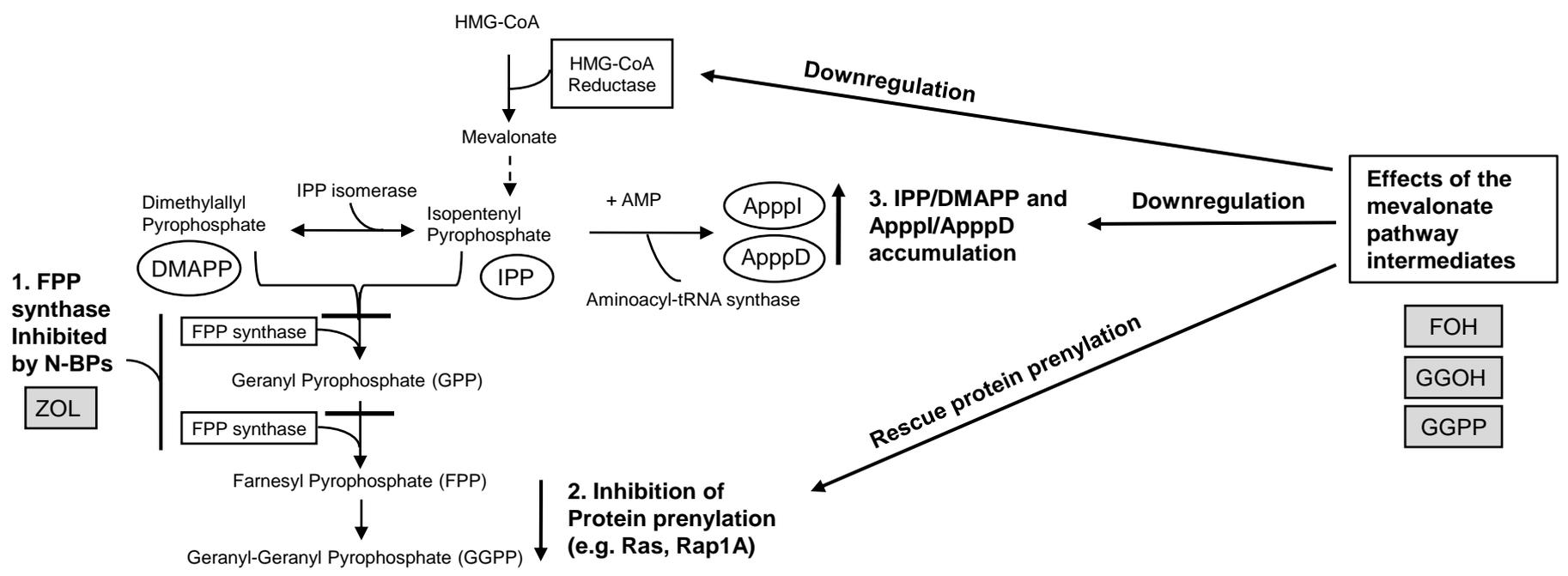
23
24 **Fig. 2. MVP intermediates do not interfere with enzyme activities of IPP isomerase**
25 **and aminoacyl-tRNA synthetase.** The relative amounts of IPP and DMAPP isomers
26 catalyzed by IPP isomerase (A) and the metabolism of clodronate to AppCCl₂p
27 catalyzed by aminoacyl-tRNA synthetase (B) in MCF-7 cells. The cells were incubated
28 for 24 h treatment with bisphosphonate, 25 μ M zoledronic acid (25 ZOL) or 500 μ M
29 clodronate (500 CLOD), with or without 25/50 μ M FOH (25/50 FOH) or 25/50 μ M GGOH
30 (25/50 GGOH). The molar amount of AppCCl₂p was determined in acetonitrile extracts
31 with the HPLC-ESI-MS method (mean \pm SEM, n=6). The relative amounts of the
32 isomeric compounds, IPP and DMAPP, in the acetonitrile cell extracts were determined
33 with a previously developed quantitative method [22], which is based on HPLC-ESI-MS
34 separation (mean \pm SEM, n=10). ns = non significant using Tukey's Multiple Comparison
35 Test.
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4 **Fig. 3. GGOH and GGPP suppress ZOL-induced IPP/Apppl accumulation by**
5 **downregulating HMGR protein levels in cells.** (A) represents HPLC-ESI-MS data of
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7 IPP/Apppl accumulation, and cell viability. Cell viability was determined by the MTT
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9 assay. (B) represents the effect of the isoprenoids on the regulation of the MVP proteins
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11 in MCF-7 cells. Total levels of HMG-Co-A reductase (HMGR), β -actin (internal control),
12
13 FPP synthase (FPPS) and unprenylated Rap1A (uRap1A) were determined by Western
14
15 blotting in cell lysates. The cells were incubated for 24 h with drugs, 5 μ M lovastatin (5
16
17 LOV); 25 μ M zoledronic acid (25 ZOL); 25/50 μ M farnesol (25/50 FOH) with ZOL; 25/50
18
19 μ M geranylgeraniol (25/50 GGOH) with ZOL; 100 μ M geranylpyrophosphate (100
20
21 GGPP) with ZOL; 50 μ M farnesol (50 FOH); 50 μ M geranylgeraniol (50 GGOH) or with
22
23 vehicle (CTR) (mean \pm SEM, n=6-12). *P<0.05; *** P<0.001 denotes values significantly
24
25 different from CTR using Tukey's Multiple Comparison Test. The blots were quantified
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27 by Image J software. The bands were normalized to β -actin and control (HMGR and
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29 FPPS) or β -actin and ZOL (uRap1A).
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39 **Fig. 4. Mevalonate partially overcomes isoprenoid-induced inhibition on**
40 **IPP/Apppl accumulation.** The effect of mevalonate lactone (MVL) on accumulation of
41
42 IPP (A) and the formation of Apppl (B) in MCF-7 cells. The cells were incubated for 24 h
43
44 with drugs: 25 μ M zoledronic acid (25 ZOL) with or without 500 μ M MVL; 25 μ M ZOL
45
46 plus 25/50 μ M farnesol (25/50 FOH) with or without 500 μ M MVL; 25 μ M ZOL plus
47
48 25/50 μ M geranylgeraniol (25/50 GGOH) with or without 500 μ M MVL. The molar
49
50 amounts of IPP/Apppl were determined in acetonitrile cell extracts by HPLC-ESI-MS
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52 (mean \pm SEM, n=6). *, P<0.05; **, P<0.01 compared to treatment without MVL using one-
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54 way ANOVA with Bonferroni's comparison test.
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Graphical abstract

Regulation of the mevalonate pathway after FPP synthase inhibition by using selected intermediates



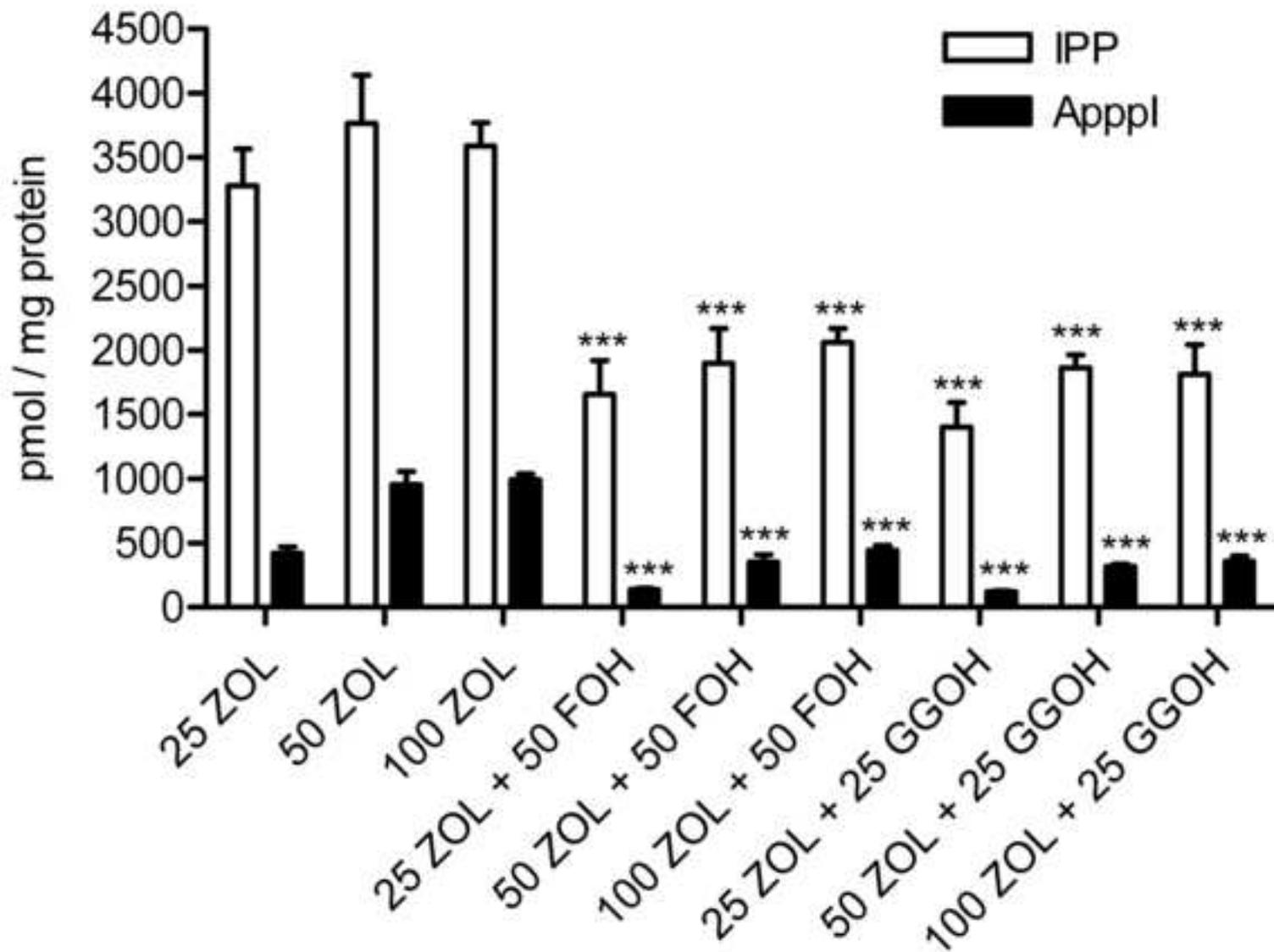


Fig1.

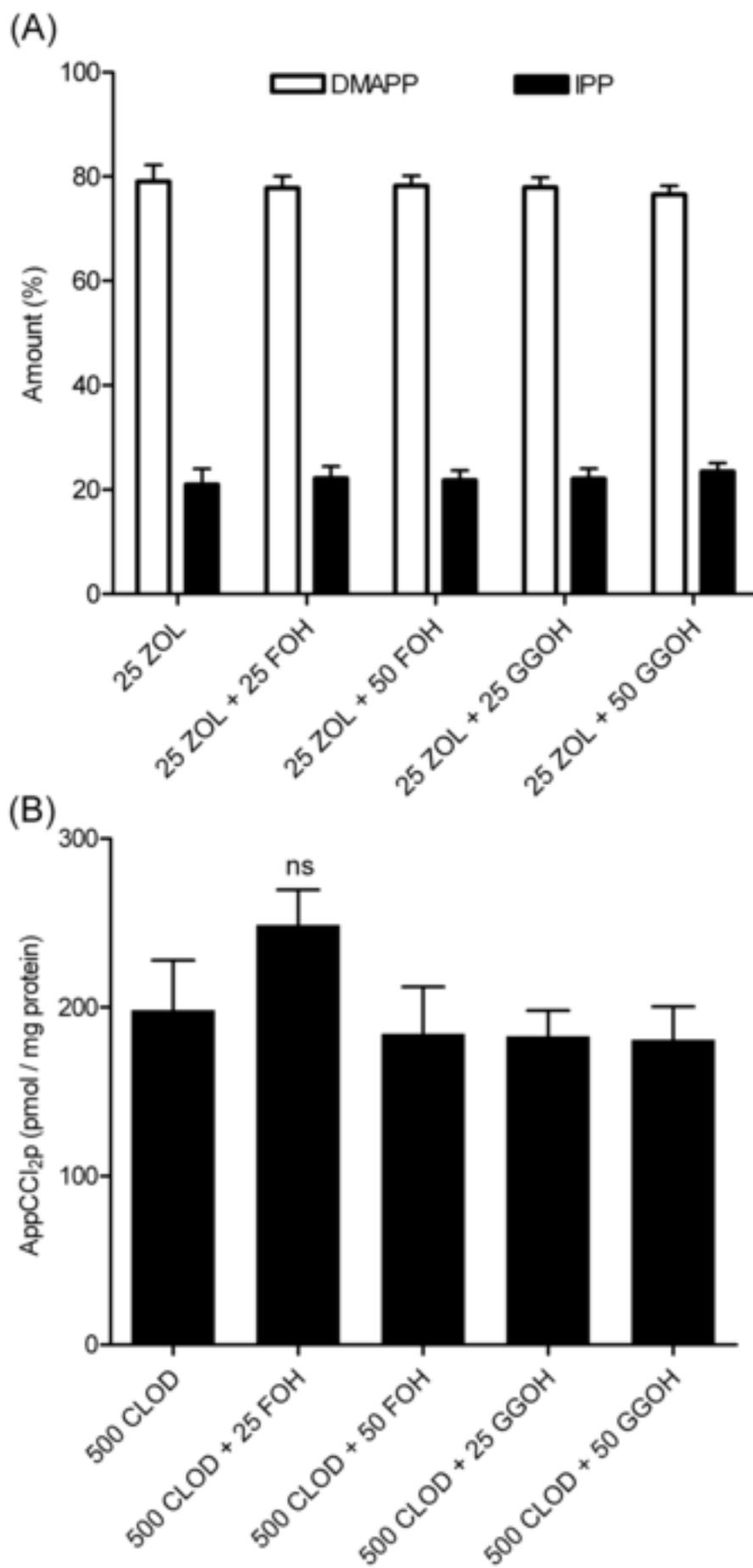


Fig2.

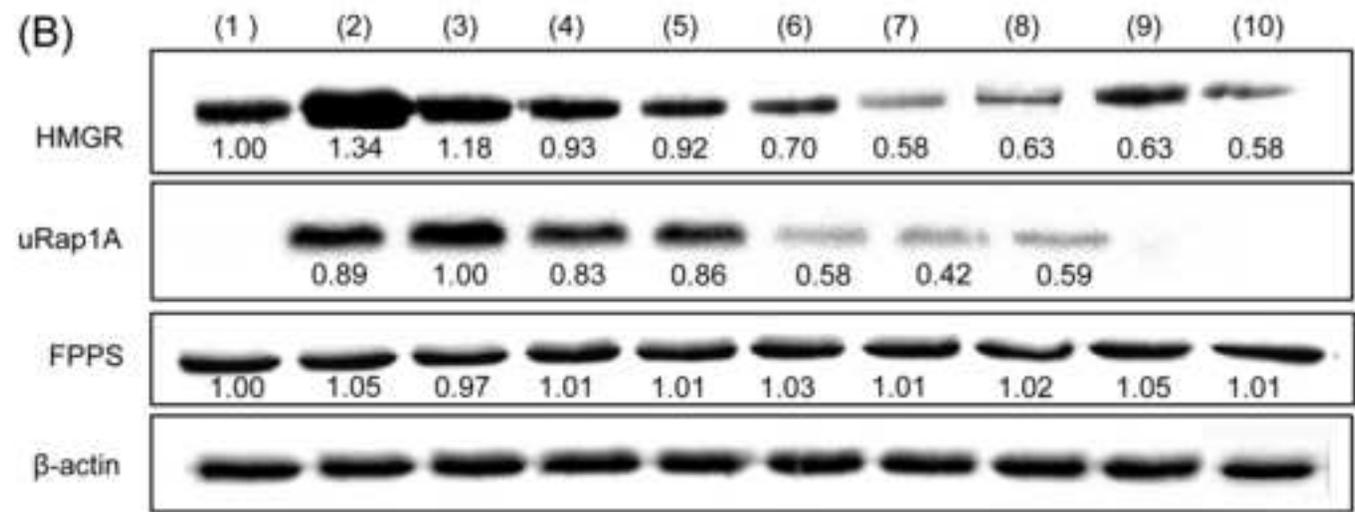
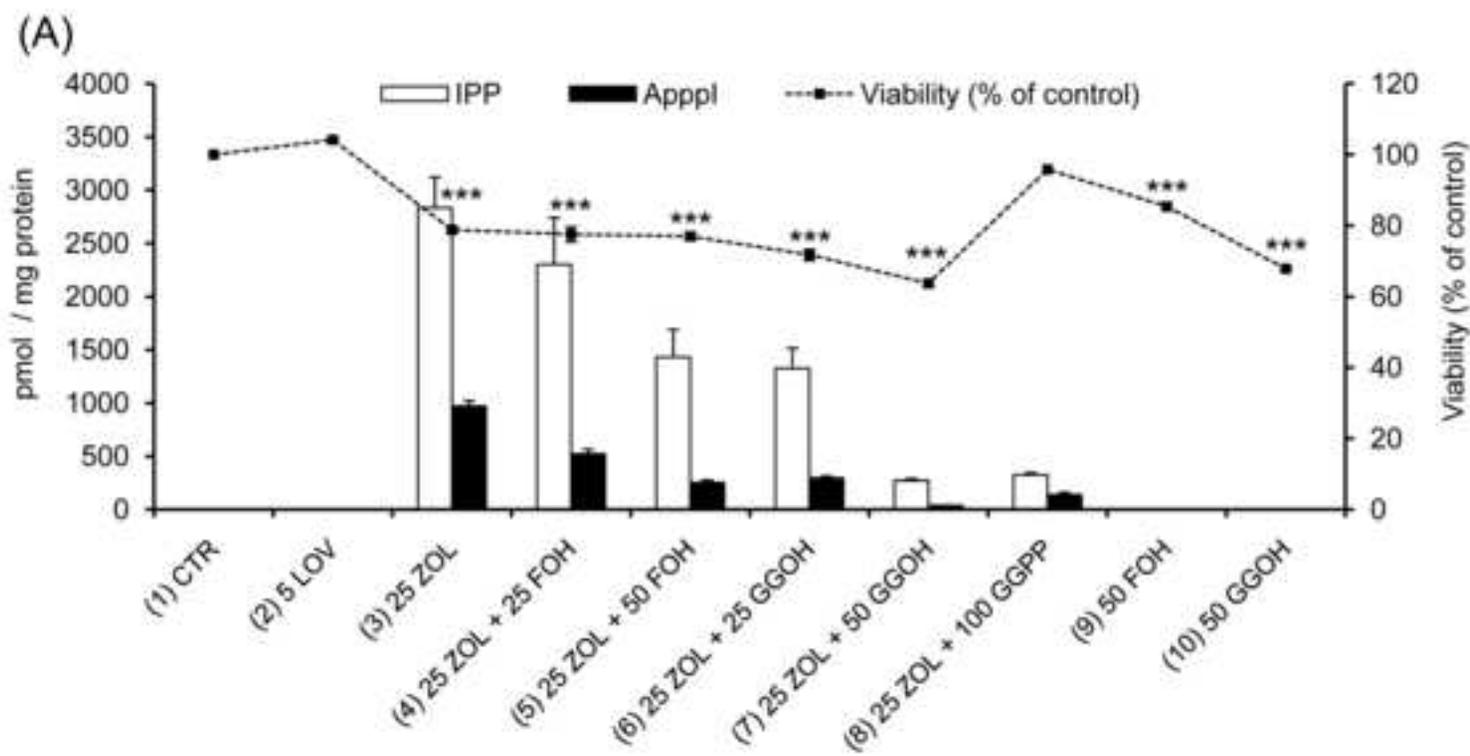


Fig3.

Script

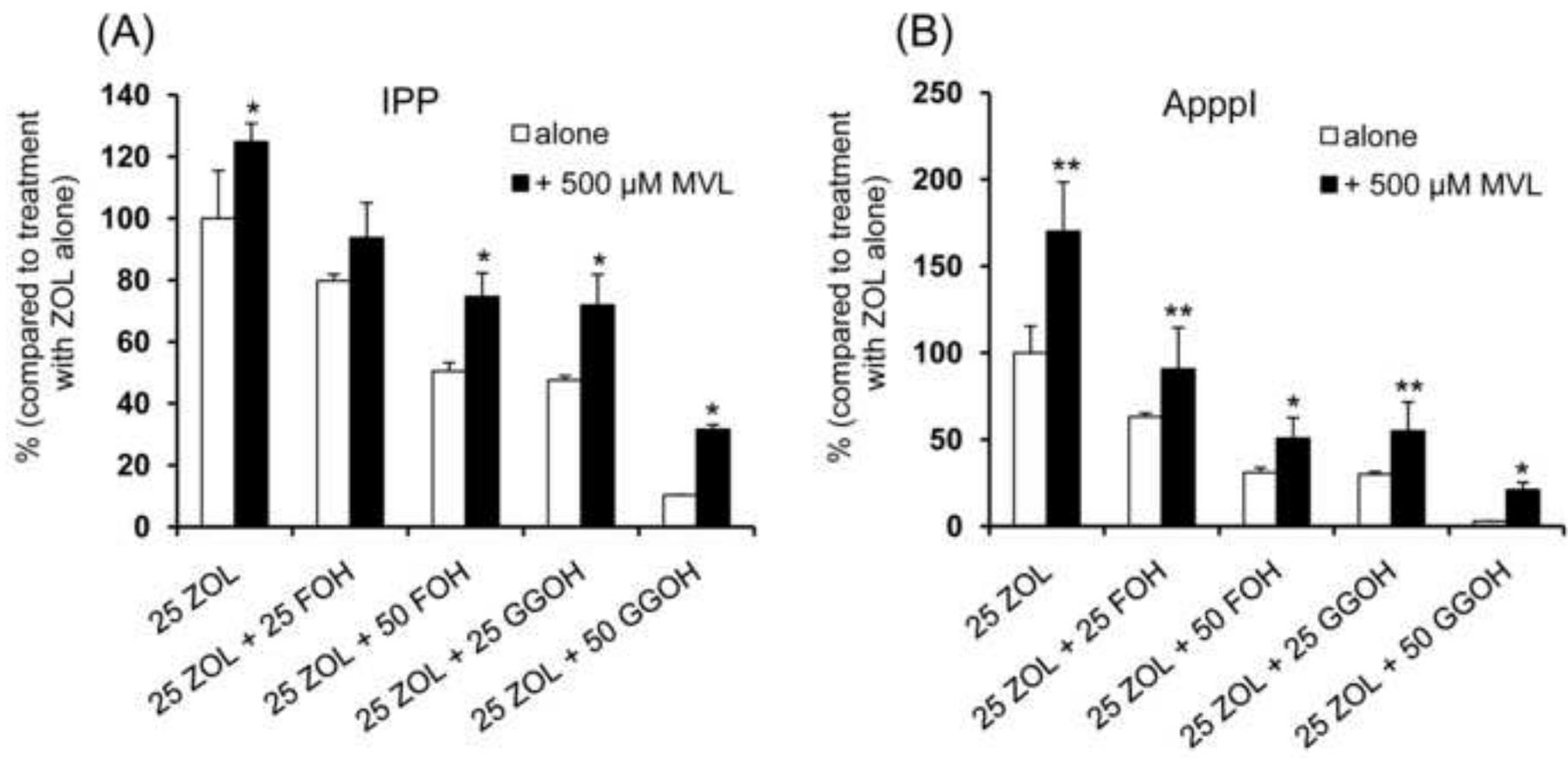


Fig4.