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Mevalonate pathway intermediates downregulate zoledronic acid-induced isopentenyl pyrophosphate and ATP analog formation in human breast cancer cells

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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, ApppI, 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/ApppI accumulation.

Interestingly, the results revealed that ZOL-induced IPP/ApppI 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 ApppI, 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/ApppI via HMGR downregulation. This supports the hypothesis that IPP/ApppI formation is a significant mechanism in the anticancer action of ZOL.

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 has 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

Abbreviations: AppCCl$_2$p, 5′(β,γ-dichloromethylene) triphosphate; ApppI, triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester; ApppD, triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-2-enyl) ester; BP, bisphosphonate; CLOD, clodronate; DMAPP, dimethylallyl pyrophosphate; ESI, electrospray ionization; FOH, farnesol; FPP, farnesyl pyrophosphate; GGOH, geranylgeraniol; GGPP, geranyl geranylpyrophosphate; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IPP, isopentenyl pyrophosphate; MS, mass spectrometry; MVP, mevalonate pathway; ZOL, zoledronic acid.

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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.

However, in addition to the loss of prenylated proteins, inhibition of FPPS by N-BPs in the MVP causes intracellular accumulation of isopentenyl pyrophosphate (IPP) and consequently induces the biosynthesis of a novel pro-apoptotic ATP analog ApppI (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester), *in vitro* [17–20] and *in vivo* [19,21]. Recently we observed that in addition to IPP/ApppI formation, N-BPs induce isomeric dimethylallyl pyrophosphate (DMAPP) accumulation and consequent ApppD (triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-2-enyl) ester) production [22]. However, for the sake of clarity, IPP/ApppI is used to describe both IPP/DMAPP and ApppI/ApppD formation throughout this text. Formation of ApppI from IPP is most probably catalyzed by the same metabolic pathway (i.e. aminoacyl-tRNA-synthetases) [23] as the nonhydrolyzable AppCp-type ATP-analogs of non-nitrogen-containing bisphosphonates (non-N-BPs), such as clodronate [18]. Similar to $AppCCl_2p$ (i.e. a metabolite of clodronate) [24], ApppI interferes with mitochondrial function and induces apoptosis in osteoclasts [17]. Moreover, we have demonstrated that the amount of IPP/ApppI correlates with ZOL-induced FPPS inhibition [17,18] and most importantly, cancer cell death *in vitro* [20]. Therefore, N-BPs evoke apoptosis by two mechanisms, indirectly *via* the inhibition of protein isoprenylation, and also directly *via* inhibition of mitochondrial adenine nucleotide translocase (ANT) by ATP analog, ApppI.

In addition to FPPS, the intracellular concentration of isoprenoids is controlled by 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) enzyme, which is tightly regulated in order to yield sufficient IPP and DMAPP to satisfy cellular requirements for all the mevalonate end-products [1]. HMGR is controlled by negative feedback regulation of transcription by sterol and nonsterol products of the pathway [1,25,26]. These products suppress HMGR by at least two mechanisms; by decreasing transcription of the gene and by enhancing degradation of the enzyme [1]. Statins and N-BPs can block the enzymatic activity of HMGR and FFPS, respectively, and consequently prevent protein prenylation and induce apoptosis [12]. In this respect, nonsterol isoprenoids have been shown to play an important role. Several studies indicate that free nonsterol MVP intermediates, farnesol (FOH) and especially geranylgeraniol (GGOH), are capable of salvaging protein isoprenylation and, thus overcome statin and N-BP-induced apoptosis in many cell types [7,8,15,16,27,28]. In addition, we have evidence that GGOH may possess the capacity to inhibit ZOL-induced IPP/ApppI formation in tumor cells [20], but the mechanism underlying this effect is not known. Since IPP is capable of activating an immune response [29,30] and ApppI has been shown to be an apoptotic molecule [17], 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 MPV intermediates and enzymes 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, MVP intermediates 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.

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 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). ApppI [triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester] and $AppCCl_2p$ [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-cm^3 flasks at +37 °C in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 IU/ml penicillin-streptomycin in a 5% CO_2 atmosphere. Cells were harvested using 0.05% trypsin-EDTA.

2.3. Preparation of cell lysates for IPP/ApppI and $AppCCl_2p$ detection

The cells were seeded in 6-well plates at a density of 1×10^6 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 (13,000 × *g*, 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 alone and in combination on cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) test [31].

2.4. Mass spectrometry analysis

The quantitative analysis of IPP/ApppI, and $AppCCl_2p$ in cell extracts was performed as previously described [21] by using a HPLC electrospray ionization mass spectrometry (HPLC-ESI-MS) system in negative ion mode. The evaporated cell extracts were redissolved in 150 μl of water containing 1 μM internal standard (AppCp) to compensate for variabilities in ionization. Selected reaction monitoring (SRM) was used for analysis of the compounds in the sample and quantitation was based on the characteristic fragment ions of each molecule. The following transitions were monitored: m/z 245 → 159 and m/z 245 → 177 for IPP/DMAPP, m/z 574 → 408 for ApppI, m/z 572 → 225 and m/z 574 → 227 for $AppCCl_2p$ (^{35}Cl and ^{37}Cl) and, m/z 504 → 406 for AppCp. The pattern of fragmentation for each sample was compared with that of the authentic standard. Standards were constructed by spiking extracts from untreated cells with synthesized IPP, ApppI, or $AppCCl_2p$. Quantitation of the molecules was done with LCquan 2.0 software (Thermo Finnigan), using the standard curve and the transitions mentioned above. The relative amount of IPP and DMAPP isomers in the treated cell samples were calculated using a protocol described earlier [22]. 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).

2.5. Western blot analysis

The MCF-7 cells were seeded at a density of 2×10^6 per 25-cm^3 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, 50 μM FOH, 50 μM GGOH, 25/50 μM FOH with 25 μM ZOL, 25/50 μM GGOH with 25 μM ZOL, 100 μM 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 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.

2.6. Statistical analysis

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

3. Results

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

Since the addition of intermediates of the MVP has 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/ApppI induced by ZOL in breast cancer cells. Therefore, MCF-7 cells were incubated for 24 h with exogenous FOH (50 μM) and GGOH (25 μM), which are metabolized to their phosphate forms in the cells [32], together with increasing concentrations of ZOL, for 24 h. As seen in Fig. 1, 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 intermediates were observed with 25 μM ZOL, and thus this dose was chosen for further studies.

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

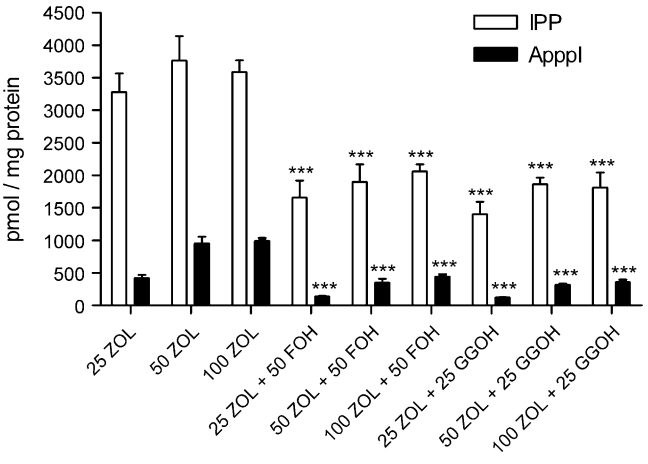

Fig. 1. FOH and GGOH inhibit ZOL-induced IPP/ApppI accumulation in cells. The amount of IPP/ApppI in MCF-7 cells after 24 h treatment with 25–100 μM zoledronic acid (25–100 ZOL) with or without 50 μM farnesol (50 FOH) or 25 μM geranylgeraniol (25 GGOH). The molar amounts of the compounds were determined in acetonitrile extracts with 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.

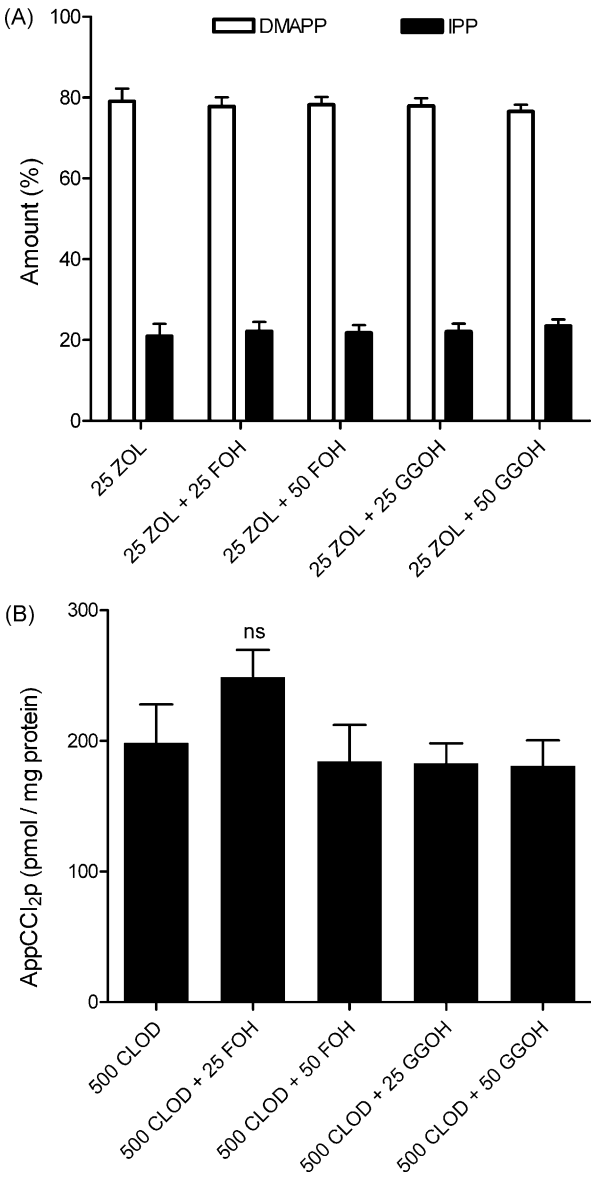

Fig. 2. MVP intermediates do not interfere with enzyme activities of IPP isomerase and aminoacyl-tRNA synthetase. The relative amounts of IPP and DMAPP isomers catalyzed by IPP isomerase (A) and the metabolism of clodronate to $AppCCl_2p$ catalyzed by aminoacyl-tRNA synthetase (B) in MCF-7 cells. The cells were incubated for 24 h treatment with bisphosphonate, 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 in acetonitrile extracts with the HPLC-ESI-MS method (mean ± SEM, $n = 6$). The relative amounts of the isomeric compounds, IPP and DMAPP, in the acetonitrile cell extracts were determined with a previously developed quantitative method [22], which is based on HPLC-ESI-MS separation (mean ± SEM, $n = 10$). ns = non significant using Tukey's Multiple Comparison Test.

amount of both isomers, IPP and DMAPP, in cell extracts [22]. Using this procedure, we discovered that there were no differences in the isomerization capacity of the enzyme between the treatments (Fig. 2A). 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 (500 μM), to clarify the functionality of the aminoacyl-tRNA-synthase enzyme, known to catalyze ATP-analogs from non-N-BPS [23], and which is proposed to catalyze also the formation of ApppI from IPP. The cells were observed to metabolize clodronate into $AppCCl_2p$ regardless of isoprenoid treatments, and therefore the functionality of the aminoacyl-tRNA-synthase was not affected (Fig. 2B).

3.3. GGOH and GGPP inhibit ZOL-induced IPP/ApppI accumulation by decreasing levels of HMG-CoA reductase protein in cells

To further examine the effects of isoprenoid compounds on the regulation of MVP, we studied the dependence of the isoprenoid dose on inhibition of ZOL-induced IPP/ApppI accumulation in MCF-7 cells. Co-treatment of ZOL with 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 (Fig. 3A). 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 (Fig. 3A). 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. Therefore, decreased cell viability induced by GGOH does not explain for the observed downregulation in ZOL-induced IPP/ApppI levels. There was no evidence of IPP/ApppI found in the cell samples treated with LOV, FOH, GGOH or vehicle (CTR) (Fig. 3A).

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 [1,33,34]. In Fig. 3B, 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. In general, Rap1A is a surrogate marker for inhibition of FPPS, since the uRap1A accumulates in cells exposed to N-BPs. As in the case of IPP/ApppI formation (Fig. 3A), 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 (Fig. 3B). This result was not a consequence of the reduced cell viability by GGPP (Fig. 3A). 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 (Fig. 3A) by MVP intermediates is a consequence of HMGR enzyme downregulation (Fig. 3B).

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 MVP intermediates 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 Fig. 4A and B, 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). There was no evidence of IPP/ApppI found in the cell samples treated with MVA alone (data not shown). These results further suggest that the regulation of the IPP/ApppI accumulation is mediated by HMGR enzyme.

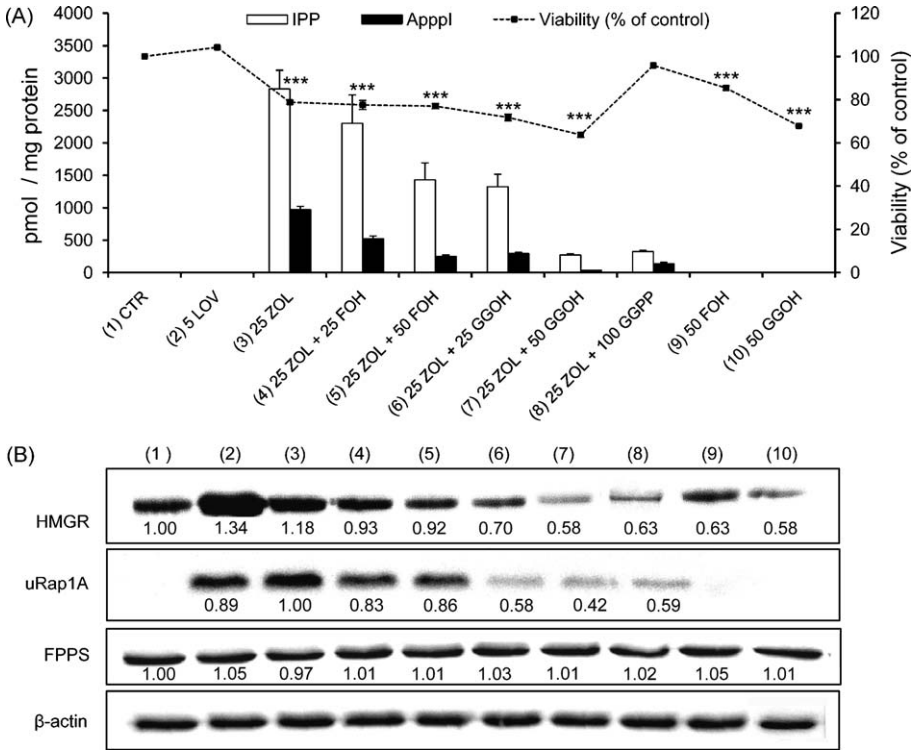

Fig. 3. GGOH and GGPP suppress ZOL-induced IPP/ApppI accumulation by downregulating HMGR protein levels in cells. Fig. (A) represents HPLC-ESI-MS data of IPP/ApppI accumulation, and cell viability. Cell viability was determined by the MTT assay. Fig. (B) represents the effect of the isoprenoids on the regulation of the MVP 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 geranylpyrophosphate (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 CTR 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).

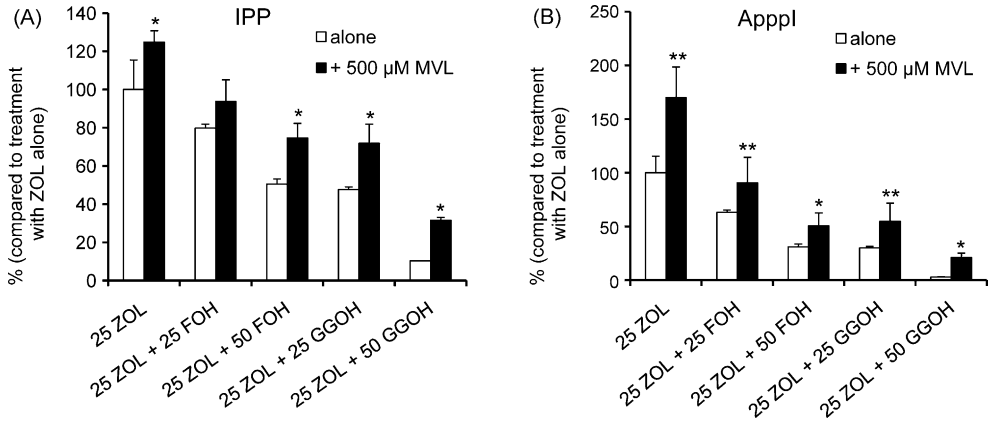

Fig. 4. Mevalonate partially overcomes isoprenoid-induced inhibition on IPP/ApppI accumulation. The effect of mevalonate lactone (MVL) on accumulation of IPP (A) and the formation of ApppI (B) in MCF-7 cells. The cells were incubated for 24 h with drugs: 25 μM zoledronic acid (25 ZOL) with or without 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 in acetonitrile cell extracts by HPLC-ESI-MS (mean ± SEM, $n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$ compared to treatment without MVL using one-way ANOVA with Bonferroni's comparison test.

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 ApppI, which is able to induce apoptosis in cells by inhibiting mitochondrial 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/ApppI, are 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/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 [14,39], 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 [32]. 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 [40].

All the isoprenoids of the MVP 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 [14,39], and both isomers for conjugation reaction to AMP to form ATP-analogs, ApppI and ApppD [17,22]. The reversible reaction involved in the transformation of the relatively unreactive IPP into the relatively reactive isomer DMAPP is catalyzed by IPP isomerase enzyme [41,42]. Recently, we developed a new method for quantification of these isomers in cell extracts [22]. 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 [22]. The ratio of the isomers in cell samples was found to be stable irrespective of the treatments, evidencing that MVP intermediates do not affect the 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 that FPPS enzyme levels are not affected by the isoprenoids.

Since our results revealed that isoprenoids neither affected 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 [1]. There is marked upregulation of HMGR protein expression after inhibition of this enzyme by statins [25,43], whereas protein levels are decreased by the sterol and nonsterol products of the pathway [1]. Indeed, 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 [1,25]. 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. [44] 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* [20]. 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 [8]. 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* [10,27,28].

Taken together, this study strongly suggests that MVP intermediates, GGOH or GGPP, inhibit ZOL-induced IPP/ApppI accumulation via downregulation of HMG-CoA reductase. This represents a novel insight into the mechanism of action of isoprenoids on the regulation of MVP after FPPS inhibition. The data implies that in addition 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.

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