Bisphosphonates Induce Autophagy by Depleting Geranylgeranyl Diphosphate

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Title Page.
Running Title Page.

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d) FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GGDPS, geranylgeranyl diphosphate synthase; FDPS, farnesyl diphosphate synthase; DGBP, digeranyl bisphosphonate

e) Cellular and Molecular
Abstract.

Multiple studies have implicated the depletion of isoprenoid biosynthetic pathway intermediates in induction of autophagy. However, the exact mechanism by which isoprenoid biosynthesis inhibitors induce autophagy has not been well established. We hypothesized that inhibition of farnesyl diphosphate synthase (FDPS) and geranylgeranyl diphosphate synthase (GGDPS) by bisphosphonates would induce autophagy by depleting cellular geranylgeranyl diphosphate (GGPP) and impairing protein geranylgeranylation. We show herein that an inhibitor of FDPS (zoledronate) and an inhibitor of GGDPS (digeranyl bisphosphonate, DGBP) induce autophagy in PC3 prostate cancer and MDA-MB-231 breast cancer cells as measured by accumulation of the autophagic marker LC3-II. Treatment of cells with lysosomal protease inhibitors (E-64d and pepstatin A) in combination with zoledronate or digeranyl bisphosphonate further enhances the formation of LC3-II, indicating that these compounds induce autophagic flux. Importantly, the addition of exogenous GGPP prevented the accumulation of LC3-II and impairment of Rab6 (a GGTase II substrate) geranylgeranylation by isoprenoid pathway inhibitors (lovastatin, zoledronate, and DGBP). However, exogenous GGPP did not restore isoprenoid pathway inhibitor-induced impairment of Rap1a (a GGTase I substrate) geranylgeranylation. In addition, specific inhibitors of farnesyl transferase and geranylgeranyl transferase I are unable to induce autophagy in our system. Furthermore, the addition of bafilomycin A1 (an inhibitor of autophagy processing) enhanced the anti-proliferative effects of digeranyl bisphosphonate. These results are the first to demonstrate that bisphosphonates induce autophagy. Our study suggests that induction of autophagy in PC3 cells with these
agents is likely dependent upon impairment of geranylgeranylation of GGTase II substrates.
Introduction.

The isoprenoid biosynthetic pathway (Fig. 1) is responsible for the production of a wide array of compounds with diverse biological functions. Small molecule inhibitors of this pathway have yielded clinical success. The statins (e.g. lovastatin) are commonly prescribed for hypercholesterolemia and inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) (Endo et al., 1976), which is the rate-limiting step of cholesterol biosynthesis (Siperstein and Fagan, 1966). The nitrogenous bisphosphonates (e.g. zoledronate) target farnesyl diphosphate synthase (FDPS) (van Beek et al., 1999) and are used for bone-related disorders such as osteoporosis.

While statins and nitrogenous bisphosphonates are used for distinct clinical disorders, they have common effects within cells, including depletion of pathway intermediates farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). FPP is at the major branch point of the pathway, being used in the synthesis of cholesterol or GGPP as well as other molecules. Farnesyl and geranylgeranyl moieties can be post-translationally adducted onto select proteins in processes termed farnesylation and geranylgeranylation (collectively referred to as prenylation), respectively (Zhang and Casey, 1996). Farnesylation is catalyzed by farnesyl transferase (FTase), while geranylgeranylation can be catalyzed by either geranylgeranyl transferase (GGTase) I or GGTase II (also referred to as Rab GGTase), depending upon the nature of the acceptor protein. For proteins that are farnesylated or geranylgeranylated, such as small GTPases of the Ras and Rho family, prenylation is essential for proper localization and function (Swanson and Hohl, 2006).
Macroautophagy (hereafter referred to as autophagy) is a cellular process which degrades damaged cytoplasmic organelles as well as long-lived, misfolded, or aggregated proteins (Glick et al., 2010). During autophagy, a target substrate is first encapsulated in a double membrane vesicle known as an autophagosome. Autophagosomes can then fuse with lysosomes to form autolysosomes, where the contents are degraded. This process ultimately allows for the recycling of amino acids and other degraded products and is upregulated in response to cellular stresses such as starvation.

Inhibitors of the isoprenoid biosynthetic pathway have been linked to autophagy. Studies have shown that statins are capable of inducing autophagy in A204 human rhabdomyosarcoma cells (Araki and Motojima, 2008). More recently, statins have been shown to induce autophagy in PC3 prostate cancer cells, and the induction of autophagy was prevented by the addition of the geranylgeraniol (GGOH), the alcohol form of GGPP (Parikh et al., 2010). It remains uncertain whether this prevention is due to restoration of isoprenoid levels or protein prenylation. In addition, a novel GGTase I/II inhibitor when combined with a statin induced autophagy in the STS-26T malignant peripheral nerve sheath tumor cell line (Sane et al., 2010), suggesting that the impairment of prenylation can induce autophagy. However, this drug combination does not allow for the distinction of whether the impairment of GGTase I or GGTase II substrate geranylgeranylation is responsible for autophagic induction. Further complicating the interrelationship of prenylation and autophagy, farnesyl transferase inhibitors have been shown to induce autophagy in Panc-1 pancreatic cancer and U2OS osteosarcoma cells (Pan et al., 2008). In addition, an inhibitor of isoprenylcysteine carboxyl methyltransferase, an enzyme required in later steps of prenylation processing, induced autophagy in PC3 and HepG2.
cells (Wang et al., 2008; Wang et al., 2010). A yeast deletion collection was treated with nitrogenous bisphosphonates, which identified ATG4, ATG11, ATG14 and ATG16 (all autophagy related genes) hemizygous strains as having increased sensitivity to nitrogenous bisphosphonates (Bivi et al., 2009).

Our collaborators have synthesized digeranyl bisphosphonate (DGBP) and we have shown it to specifically inhibit geranylgeranyl diphosphate synthase (GGDPS) (Shull et al., 2006; Wiemer et al., 2007). This compound specifically impairs protein geranylgeranylation via depletion of GGPP in various cell types. Furthermore, GGPP depletion results in the inhibition of cancer cell migration (Dudakovic et al., 2010) and induction of apoptosis (Dudakovic et al., 2008). We hypothesize and provide evidence herein to support that depletion of GGPP by bisphosphonate inhibitors of FDPS (i.e. zoledronate) or GGDPS (i.e. DGBP) results in the induction of autophagy.
Methods.

Cell culture. PC3, MDA-MB-231, MDA-MB-468, and HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in Ham’s F-12 (PC3), MEM (MDA-MB-231 and HepG2), and Leibovitz’s L-15 (MDA-MB-468) medium supplemented with 10% fetal bovine serum at 5% CO2 at 37°C.

Materials. Lovastatin, mevalonate, FPP, GGPP, pepstatin A, E-64d, bafilomycin A1, and GGTI-2133 were purchased from Sigma (St. Louis, MO). Zoledronate was obtained from Novartis (East Hanover, NJ). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and FTI-277 were purchased from Calbiochem (San Diego, CA). Anti pan-Ras was obtained from InterBiotechnology (Tokyo, Japan). Rap1a and αTub antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). LC3-II antibody was obtained from Abgent (San Diego, CA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit were from GE Healthcare, while anti-goat was from Santa Cruz Biotechnology, Inc. The enhanced chemiluminescence (ECL) detection kit was obtained from GE Healthcare (Buckinghamshire, UK).

Preparation of Cell Lysates. Cells were plated in T25 flasks and allowed to reach 50% confluence. Old media was then replaced with fresh media and relevant compounds added. All compounds were added simultaneously in experiments that required multiple agents in the same T25 flask. At the end of each experiment (24 or 48 hours), media was removed and cells were washed twice in phosphate-buffered saline. Cells were collected by the trypsin method.

Cell lysis. Cells were lysed in radioimmunoprecipitation buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100)
supplemented with protease inhibitor cocktail (Sigma, 1X), sodium vanadate (1 mM), sodium fluoride (25 mM), and phenylmethylsulphonyl fluoride (1 mM). Lysates were transferred to a 1.5 ml tube, vortexed several times over 30 minutes, and passed through a 27-gauge needle. Lysates were then centrifuged and supernatant transferred to a fresh 1.5 ml tube. All steps were performed at 4°C.

**Triton X-114 separation.** Based on the method of Bordier (Bordier, 1981). For separation of prenylated and unprenylated Rab6, cells were lysed in ice cold TX114 lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-114). Cell lysate was passed through a 27 gauge needle and cleared by centrifugation at 12,000 x g for 15 minutes at 4°C, after which the supernatant was transferred to a new tube and incubated at 37°C for 10 minutes and then spun down at room temperature at 12,000 x g for 2 minutes. The aqueous (upper) phase was transferred to a new tube, and the lower detergent phase was diluted into buffer without Triton X-114 prior to electrophoresis.

**Western Blot Analysis.** Protein concentrations were determined by the bicinchoninic acid method. Proteins were resolved on 12 or 15% gels and transferred to polyvinylidene difluoride membranes by electrophoresis. After blocking in 5% non-fat dry milk for 45 minutes, primary and secondary antibodies were added sequentially for 1 hour at 37°C and proteins visualized using an ECL detection kit.

**MTT Assay.** 8 x 10^4 cells per well were allowed to adhere in 24-well plates overnight. Cells were treated with relevant compounds and incubated 45 hours. MTT was added and cells additionally incubated. Three hours later, MTT stop solution (HCl, triton X-100, and isopropyl alcohol) was added to all of the wells and then incubated with gentle
agitation overnight at 37°C. Absorbance was measured at 540 nm with a reference wavelength at 650 nm.

**DNA Synthesis Assay.** Cells (2,500 in 200 µl media) were plated in 96-well plates and allowed to adhere overnight. Cells were then treated with compounds as indicated in the figure legends. After 44 hours, 20 µl of [³H]-thymidine (0.1385 TBq/mmol; 3.75 Ci/mmol in media) was added to each well. At 48 hours, cells were filtered through glass microfiber paper using a Brandel (Gaithersburg, MD) cell harvester. [³H]-Thymidine incorporated into cellular DNA was quantified by scintillation counting.

**Statistical Analysis.** The unpaired two-tailed t-test was used to calculate statistical significance. P<0.01 was set as the level of significance.
Results.

*Isoprenoid biosynthetic pathway inhibitors interfere with protein prenylation in a concentration-dependent manner in PC3 cells.* To determine the potency with which isoprenoid pathway inhibitors interfere with protein prenylation, PC3 cells were with several concentrations of lovastatin, zoledronate, and DGBP for 24 hours (Fig. 2A). The Ras antibody utilized in these experiments recognizes the modified (farnesylated) and the unmodified (non-farnesylated) form of the protein. The unmodified form of Ras is the slower migrating, upper-band, on the western blot Ras panel. In contrast, the antibody used to detect Rap1a only detects the unmodified form of this protein, which is normally geranylgeranylated by GGTase I. Therefore, the appearance of a band on the western blot Rap1a panel indicates impairment of its geranylgeranylation. Detection of alpha tubulin (αTub), a house keeping gene, was used as a loading control for all western blotting experiments. Lovastatin and zoledronate interfere with farnesylation and geranylgeranylation of proteins as indicated by the appearance of the unmodified forms of Ras and Rap1a. DGBP interferes with protein geranylgeranylation without disturbing protein farnesylation. Maximal impairment of Rap1a geranylgeranylation occurs at 0.5 to 1 µM lovastatin, 50 to 100 µM zoledronate, and 10 to 25 µM DGBP. Notably, these concentrations of lovastatin and zoledronate did not maximally impair protein farnesylation. The concentration that maximally impairs protein geranylgeranylation for each inhibitor (1 µM lovastatin, 100 µM zoledronate, and 25 µM DGBP) was used for subsequent experiments.

*Isoprenoid biosynthetic pathway inhibitors reduce MTT activity and DNA synthesis concentration-dependently in PC3 cells.* In order to assess viability of cells in the
presence of isoprenoid biosynthetic pathway inhibitors, MTT assay was performed at 48 hours (Fig. 2B). Additionally, cell proliferation was assessed by \( ^3 \)H-thymidine incorporation assay (Fig. 2C). Concentration-dependent reduction in MTT activity and inhibition of DNA synthesis (\(^3\)H-thymidine incorporation assay) are observed with all three inhibitors. Lovastatin is most potent while zoledronate is least potent at decreasing MTT activity and DNA synthesis.

**Bisphosphonates induce LC3-II accumulation in PC3 cells.** To establish if bisphosphonates induce LC3-II accumulation, PC3 cells were treated with lovastatin, zoledronate, and DGBP for 24 and 48 hours (Fig. 3A). As previously described, statins have been shown to induce autophagy in PC3 cells (Parikh et al., 2010). Thus, lovastatin is used as a positive control for induction of autophagy. To assess LC3-II protein levels, an antibody was used that specifically detects the LC3-II form of LC3 is used in our studies. The appearance of the LC3-II band on the western blot is an established method for the detection of autophagy (Glick et al., 2010; Mizushima and Yoshimori, 2007). LC3-II accumulation is not apparent at 24 hours with the use of isoprenoid pathway inhibitors. In contrast, LC3-II accumulation is observed at 48 hours with the use of the positive control (lovastatin) and bisphosphonates (zoledronate and DGBP). The appearance of LC3-II is detectable at 10 \( \mu \)M DGBP and 50 \( \mu \)M zoledronate, and this effect is concentration-responsive with respect to both drugs. Protein prenylation status was also assessed as described previously (Fig 3A). In addition, we utilized Rab6 to evaluate the status of proteins geranylgeranylated by GGTase II (Fig. 3B). In order to assess Rab6’s prenylation status, cells were lysed in Triton X-114, which can undergo a phase separation above 20°C allowing for separation of amphiphilic (detergent phase)
from hydrophilic (aqueous phase) proteins (Bordier, 1981). The detergent-rich fraction retains prenylated proteins, while unprenylated proteins are found in the aqueous phase (Ren et al., 1997); thus impairment of Rab6 geranylgeranylation is noted by the appearance of a band in the aqueous fraction of the western blots for Rab6. At 24 and 48 hours, the inhibition of Rab6 processing is noted with all isoprenoid pathway inhibitors.

**Bisphosphonate-induced LC3-II accumulation is dependent on GGPP depletion in PC3 cells.** In order to determine if the effects of bisphosphonates on LC3-II accumulation are dependent on the depletion of specific molecules within the isoprenoid pathway, inhibitors were co-administered with exogenous isoprenoid pathway intermediates for 48 hours (Fig. 3C). The addition of mevalonate and GGPP, but not FPP completely prevents the effects of lovastatin on the induction of autophagy as measured by LC3-II levels. FPP addition alone does not prevent lovastatin’s effects because of the lack of isopentenyl diphosphate (IPP) to generate GGPP from FPP (Fig. 1). Similarly, GGPP, but not FPP, completely prevents LC3-II accumulation by zoledronate. GGPP also entirely prevents the effects of DGBP on LC3-II accumulation. Notably, isoprenoid pathway inhibitor-induced impairment of Rap1a (a GGTase I substrate) geranylgeranylation was not prevented by GGPP addition under the conditions tested; while isoprenoid pathway inhibitor-induced impairment of Rab6 (a GGTase II substrate) geranylgeranylation was completely prevented by GGPP addition (Fig. 3D).

**Bisphosphonates induce autophagic flux in PC3 cells.** The accumulation of LC3-II can be caused by induction of autophagy as well as by inhibition of autophagosomal processing (Mizushima and Yoshimori, 2007). In order to confirm that GGPP depletion by bisphosphonates genuinely induces autophagy, experiments were performed to
evaluate autophagic flux (Fig. 4). Lysosomal protease inhibitors (pepstatin A and E-64d) were employed to prevent the degradation of LC3-II, allowing for analysis of autophagic flux. As shown previously, lovastatin, zoledronate, and DGBP increase accumulation of LC3-II. Dual administration of protease inhibitors with each of the isoprenoid pathway inhibitors further enhances the accumulation of LC3-II suggesting that bisphosphonates (zoledronate and DGBP) induce autophagy as opposed to interfering with autophagosomal processing. Of note, the lysosome inhibitors also increase LC3-II formation when compared to control by blocking basal autophagosomal degradation.

**Bisphosphonates induce LC3-II accumulation in MDA-MB-231 but not in MDA-MB-468 and HepG2 cells.** Experiments were performed with the breast cancer cell lines MDA-MB-231 and MDA-MB-468 and the hepatocellular carcinoma HepG2 cell line to determine whether autophagic effects in PC3 cells were cell line-specific (Fig. 5). Similar to PC3 cells, LC3-II accumulation is induced by each of the isoprenoid biosynthetic pathway inhibitors in MDA-MB-231 cells. In contrast, none of the inhibitors used result in detectable LC3-II formation in MDA-MB-468 or HepG2 cells. Of note, higher concentrations of zoledronate and DGBP were also unable to induce LC3-II accumulation in these two cell lines (data not shown).

**Inhibition of either FTase or GGTase I does not induce LC3-II accumulation in PC3 cells.** To determine whether direct impairment of protein farnesylation by FTase or protein geranylgeranylation by GGTase I induce LC3-II accumulation, inhibitors of these two enzymes were utilized (Fig. 6). As in previous experiments, lovastatin (positive control) induces the accumulation of LC3-II. At 48 hours, GGTI-2133 impairs geranylgeranylation of Rap1a (GGTase I substrate), but not farnesylation of Ras (FTase
substrate). In contrast, an inhibitor of FTase, FTI-277, interferes with farnesylation, but not geranylgeranylation. At longer exposure times, 10 µM FTI-277 results in some detectable impairment of Rap1a geranylgeranylation (data not shown), suggesting some promiscuous activity of this compound. Treatment of cells with GGTI-2133 and FTI-277 do not result in the accumulation of LC3-II, despite the effective inhibition of their respective target enzymes.

Inhibition of autophagy enhances DGBP-induced reduction in MTT activity and DNA synthesis in PC3 cells. In order to assess the role of autophagy inhibition with zoledronate and DGBP, combinational studies were performed with bafilomycin A1 using MTT assay and ³H-thymidine incorporation at 48 hours (Fig. 7). Bafilomycin A1 is an inhibitor of fusion between autophagosomes and lysosomes. The combination of zoledronate and bafilomycin A1 did not significantly decrease MTT activity (Fig. 7A), or DNA synthesis (Fig. 7B) when compared to single agents. In contrast, the combination of DGBP and bafilomycin A1 significantly decreased MTT activity (Fig. 7A) and DNA synthesis (Fig. 7B) when compared to each individual agent.
Discussion.

It is well established that statins and nitrogenous bisphosphonates deplete isoprenoid pathway intermediates (Wiener et al., 2009). Many of the cellular effects of these agents have been attributed to the depletion of GGPP (Coxon et al., 2004; Xia et al., 2001). Our prior work further explored cellular consequences of GGPP depletion through the utilization of a novel bisphosphonate that directly inhibits GGDPS (Dudakovic et al., 2008; Dudakovic et al., 2010). Other recent work has suggested that statins can induce autophagy (Parikh et al., 2010). In this study, we explore the possibility that more specific depletion of GGPP by bisphosphonates would induce autophagy in PC3 prostate cancer cells.

We have shown for the first time that bisphosphonate inhibitors of FDPS (i.e. zoledronate) and GGDPS (i.e. DGBP) results in the induction of autophagy as measured by LC3-II formation. The addition of exogenous GGGP completely prevents induction of LC3-II formation by bisphosphonates, suggesting that depletion of GGPP is the primary mechanism by which zoledronate and DGBP induce autophagy. Furthermore, GGPP did not prevent isoprenoid pathway inhibitor impairment of Rap1a (a GGTase I substrate) geranylgeranylation, but did prevent impairment of Rab6 (a GGTase II substrate) geranylgeranylation. This suggests that impairment of GGTase II substrates may be responsible for the increase in LC3-II resulting from GGPP depletion.

The accumulation of LC3-II can result from induction of autophagy or impaired basal autophagic processing (Mizushima and Yoshimori, 2007). The addition of lysosomal inhibitors was used to establish whether accumulation of LC3-II was a result of autophagy induction or decreased autophagic flux by bisphosphonate drugs. Similar to
the previously reported results with statins (Parikh et al., 2010), our results suggest that LC3-II accumulation was due to induction of autophagy since the lysosomal inhibitors further increased LC3-II protein levels.

To determine if induction of autophagy by bisphosphonates was not specific to the PC3 prostate cancer cell line, we evaluated three additional cancer cell lines. As is the case with PC3 cells, lovastatin as well as bisphosphonates (zoledronate and DGBP) induce autophagy in MDA-MB-231 breast cancer cells as measured by LC3-II accumulation. However, in HepG2 cells and MDA-MB-468 cells, LC3-II accumulation is not observed in the presence of lovastatin or bisphosphonates (zoledronate and DGBP). It has been previously reported that statins do not induce autophagy in HepG2 cells (Araki and Motojima, 2008). These results are not due to a lack of inducible autophagy, as both HepG2 and MDA-MB-468 have been reported to be capable of autophagic induction (Cheng et al., 2010; Wang et al., 2010).

We sought to determine if direct impairment of protein geranylgeranylation would induce autophagy in PC3 cells. As demonstrated by our studies, GGTI-2133 (GGTase I inhibitor) is not able to induce LC3-II accumulation despite effective impairment of target protein geranylgeranylation. This suggests that bisphosphonate-induced autophagy is due to the depletion of GGPP, but not due the impairment of geranylgeranylation of proteins by GGTase I. It is possible that the impairment of geranylgeranylation of GGTase II substrates facilitates the activation of autophagy. Our data with Rab6 and GGPP add backs correlates with this hypothesis. However, we cannot rule out the potential that disruption of other processes dependent upon GGPP is responsible. Other studies have shown a novel GGTI when combined with a statin induced autophagy in
STS-26T MPNST cells (Sane et al., 2010). These other results may be a consequence of this novel GGTI inhibiting both GGTase I and II. A recent study using the STS-26T human malignant peripheral nerve sheath tumor cell line found that the combination of an FTI and lovastatin (which also resulted in inhibition of Rab geranylgeranylation) caused the formation of LC-II but did not increase autophagic flux, which the authors suggest resulted in an abortive autophagic program and non-apoptotic cell death (Wojtkowiak et al., 2011). We did not detect apoptosis as measured by PARP cleavage in PC3 cells (data not shown); however, we did not directly measure cell death to determine if non-apoptotic cell death occurred. Specific impairment of geranylgeranylation of GGTase II substrates may be a mechanism by which GGPP depletion causes LC3-II accumulation. However, the lack of commercially available reagents does not allow for direct examination of this hypothesis at this time. Previous studies have shown that farnesyl transferase inhibitors can induce autophagy (Pan et al., 2008). Our results did not show accumulation of LC3-II due to FTI-277 treatment in PC3 cells. Pan et al. speculate that the inhibition of Rheb farnesylation by FTIs is responsible for autophagy induction (Pan et al., 2009). The difference between our and their data is likely attributable to differences in cell lines, as our own data show results dependent upon cell line usage. Furthermore, we speculate that PC3 cells may be dysfunctional in the Rheb-mTOR arm of the autophagic pathway because in addition to a lack of FTI-induced autophagy, we also did not detect LC3-II accumulation upon treatment with rapamycin (data not shown). Rapamycin is an mTOR inhibitor that can induce autophagy.

Nitrogenous bisphosphonates are currently used for treatment of bone-related metastatic cancers (Licata, 2005). The inhibition of autophagy is under intense
evaluation with respect to anti-cancer applications (Kondo et al., 2005). Therefore, zoledronate and DGBP were combined with bafilomycin A1, an inhibitor of autophagic function, to assess whether inhibition of autophagy would enhance the anti-proliferative effects of bisphosphonates. The addition of bafilomycin A1 with DGBP significantly decreased MTT activity and inhibited DNA synthesis greater than single agents alone. At the concentrations tested, bafilomycin A1 did not significantly decrease MTT activity or DNA synthesis when combined with zoledronate. While the reason for this difference is unclear, it is possible that DGBP specific effects, such as FPP accumulation, contribute to this difference. This suggests that the combination of inhibitors of autophagy with GGDPS inhibitors should be further explored as a possible therapeutic strategy.
Authorship Contributions.

Participated in research design: Dudakovic, Wasko, and Hohl.

Conducted experiments: Dudakovic and Wasko.

Contributed new reagents or analytic tools: Hohl.

Performed data analysis: Dudakovic, Wasko, and Hohl.

Wrote or contributed to the writing of the manuscript: Dudakovic, Wasko, and Hohl.

Other: Hohl acquired funding for this research.
References.


mechanisms and displays synergy with inhibition of other isoprenoid biosynthetic enzymes. *J Pharmacol Exp Ther* **324**:1028-1036.


Footnotes.

*These authors contributed equally to this manuscript.

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Legends for figures.

Figure 1. The isoprenoid biosynthetic pathway. GPP, FPP, and GGPP (structures shown) are key intermediates in the isoprenoid biosynthetic pathway. Relevant isoprenoid biosynthetic pathway enzymes (HMGCR, FDPS, GGDP, FTase, GGTase I, and GGTase II) and inhibitors (lovastatin, zoledronate, digeranyl bisphosphonate (DGBP), FTI-277, and GGTI-2133) are shown in the diagram.

Figure 2. Isoprenoid pathway inhibitors interfere with protein prenylation, decrease MTT activity, and decrease DNA synthesis in PC3 cells. A. Isoprenoid pathway inhibitors interfere with protein prenylation. Cells were treated with several concentrations of isoprenoid pathway inhibitors (lovastatin, zoledronate, and DGBP) for 24 hours. Cell lysis was followed by western blotting to assess protein prenylation. B. Isoprenoid pathway inhibitors decrease MTT activity. C. Isoprenoid pathway inhibitors decrease DNA synthesis. MTT and DNA synthesis assays were performed with indicated concentrations of lovastatin, zoledronate, and DGBP for 48 hours (mean +/- SE, n = 3).

Figure 3. Isoprenoid pathway inhibitor-induced LC3-II accumulation and impairment of GGTase-II protein geranylgeranylation, but not impairment of GGTase I geranylgeranylation is prevented by GGPP addition in PC3 cells. A. Isoprenoid pathway inhibitors (lovastatin, zoledronate, and DGBP) impair protein geranylgeranylation by GGTase I and induce LC3-II accumulation. B. Isoprenoid pathway inhibitors impair protein geranylgeranylation by GGTase II. Cells were treated with isoprenoid pathway inhibitors at indicated concentrations for 24 and 48 hours (A and B). Cell lysis was
followed by western blotting to assess protein levels. **C.** GGPP addition prevents the accumulation of LC3-II, but does not prevent impairment of Rap1a geranylgeranylation by isoprenoid pathway inhibitors. **D.** GGPP prevents the impairment of Rab6 prenylation by isoprenoid pathway inhibitors. Cells were treated with isoprenoid pathway inhibitors (1 µM lovastatin, 100 µM zoledronate, and 25 µM DGBP) in the presence or absence of exogenous isoprenoid pathway intermediates (500 µM mevalonate, 20 µM FPP, and 20 µM GGPP) for 48 hours (**C** and **D**). Protein fractionation was followed by western blotting to assess protein levels. A=aqueous fraction (unprenylated), D=detergent fraction (prenylated).

**Figure 4.** Bisphosphonates induce autophagic flux in PC3 cells. Cells were treated with isoprenoid pathway inhibitors (1 µM lovastatin, 100 µM zoledronate, and 25 µM DGBP) in the presence or absence of lysosomal protease inhibitors (10 µg/ml Pepstatin A and 10 µg/ml E64-d) for 48 hours. Cell lysis was followed by western blotting to assess LC3-II protein levels.

**Figure 5.** Bisphosphonates induce LC3-II accumulation in MDA-MB-231 but not in MDA-MB-468 and HepG2 cells. Cells were treated with isoprenoid pathway inhibitors (lovastatin, zoledronate, and DGBP) as shown in the figure for 48 hours. Cell lysis was followed by western blotting to assess LC3-II protein levels.

**Figure 6.** Inhibition of either FTase or GGTase I does not induce LC3-II accumulation in PC3 cells. Cells were treated with isoprenoid pathway inhibitors (lovastatin, GGTI-2133, and FTI-277) as shown in the figure for 48 hours. Cell lysis was followed by western blotting to assess LC3-II protein levels.
Figure 7. Inhibition of autophagy enhances DGBP-induced reduction in MTT activity and DNA synthesis in PC3 cells.  

A. MTT assay.  

B. DNA synthesis assay.  

PC3 cells were treated with 50 μM zoledronate and 25 μM DGBP in the presence or absence of 5 nM bafilomycin A1 for 48 hours (mean +/- SE, n = 3, ns = not significant, p<0.01**).
Figure 3

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LC3-II
Ras
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LC3-II
αTub
Rap1a

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Rab6
αTub

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LC3-II (long exposure)

LC3-II (short exposure)

αTub
Figure 5

LC3-II
Ras
Rap1a
αTub

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- Lov  | Zol  | DGBP |
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- 1    | 50   | 25   |
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Figure 6

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LC3-II
Ras
Rap1a
αTub

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