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Geranylgeranyl diphosphate synthase as a novel cancer therapeutic target

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GERANYLGERANYL DIPHOSPHATE SYNTHASE AS A NOVEL CANCER THERAPEUTIC TARGET

by

Amel Dudakovic

An Abstract

Of a thesis submitted in partial fulfillment
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Thesis Supervisor: Professor Raymond J. Hohl

ABSTRACT

The isoprenoid biosynthetic pathway is targeted in the treatment of several diseases, including hypercholesteremia and bone related disorders. Farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are isoprenoid biosynthetic pathway intermediates that are utilized during post-translational modification of proteins termed farnesylation and geranylgeranylation, respectively, together known as prenylation. The Ras and Rho GTPase family members are examples of proteins that are prenylated. Prenylation is essential for proper membrane localization and function of these small GTPases. Activating mutations or over-expression of these proteins promote oncogenic events, such as increased proliferation and migration.

Studies have demonstrated that farnesyl transferase inhibitors and geranylgeranyl transferase inhibitors possess anti-cancer effects in humans and animal models of cancer, respectively. An alternative way to impair protein prenylation is through the depletion of FPP and GGPP. Statins and nitrogenous bisphosphonates (NBPs) deplete FPP and GGPP leading to impaired protein prenylation by inhibiting HMG-CoA Reductase (HMGCR) and FPP synthase (FDPS), respectively. These drugs have been shown to induce apoptosis, inhibit cancer cell migration, and induce cell cycle arrest. The anti-cancer effects of statins and NBPs can be prevented by GGPP addition, suggesting that GGPP depletion may be the mechanism by which these agents interfere with cancer cell survival.

We and our collaborators have developed bisphosphonate inhibitors of GGPP synthase (GGDPS), an enzyme that produces GGPP from the substrates FPP and isopentenyl pyrophosphate.

The goal of this research was to identify novel GGDPS inhibitors and to assess the effects of specific inhibition of GGDPS on cancer cell survival and function. Two aromatic bisphosphonates were identified as potent inhibitors of GGDPS in enzyme and cellular assays. Apoptosis hallmarks such as PARP cleavage and DNA fragmentation demonstrated that GGDPS inhibition induces apoptosis in K562 chronic myeloid leukemia cells through GGPP depletion and FPP accumulation. Isobologram analysis and enhanced impairment of protein geranylgeranylation showed that GGDPS inhibition is synergistic with the inhibition of HMGCR. Migration assays, transwell assay and large scale digital cell analysis system microscopy, demonstrated that GGDPS inhibition interferes with MDA-MB-231 breast cancer cell migration. Increased LC3-II expression showed that FDPS and GGDPS inhibition induces autophagy in PC3 prostate and MDA-MB-231 breast cancer cells. Inhibition of autophagy enhances the toxic effects of GGDPS inhibition as measured by MTT assay. Propidium iodine staining of DNA and immunostaining of cell cycle proteins such as p27 did not show significant effects of GGDPS inhibition on cell cycle progression. Importantly, exogenous addition of GGPP prevented most of the effects observed with GGDPS inhibition, suggesting specific inhibition of GGDPS by our bisphosphonate inhibitors. The data obtained herein suggest that GGDPS can be targeted to interfere with the progression of cancer cells.

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Graduate College
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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To my loving wife Amela

A good word is like a good tree whose root is firmly fixed and whose top is in the sky

The Holy Quran, 14.24

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We and our collaborators have developed bisphosphonate inhibitors of GGPP synthase (GGDPS), an enzyme that produces GGPP from the substrates FPP and isopentenyl pyrophosphate.

The goal of this research was to identify novel GGDPS inhibitors and to assess the effects of specific inhibition of GGDPS on cancer cell survival and function. Two aromatic bisphosphonates were identified as potent inhibitors of GGDPS in enzyme and cellular assays. PARP and Caspase-3 cleavage and increased annexin V and PI staining demonstrated that GGDPS inhibition induces apoptosis in K562 chronic myeloid leukemia cells through GGPP depletion and FPP accumulation. Isobologram analysis and enhanced impairment of protein geranylgeranylation showed that GGDPS inhibition is synergistic with the inhibition of HMGCR. Migration assays, transwell assay and large scale digital cell analysis system microscopy, demonstrated that GGDPS inhibition interferes with MDA-MB-231 breast cancer cell migration. Increased LC3-II expression showed that FDPS and GGDPS inhibition induces autophagy in PC3 prostate and MDA-MB-231 breast cancer cells. Inhibition of autophagy enhances the toxic effects of GGDPS inhibition as measured by MTT assay. Propidium iodine staining of DNA and immunostaining of cell cycle proteins such as p27 did not show significant effects of GGDPS inhibition on cell cycle progression. Importantly, exogenous addition of GGPP prevented most of the effects observed with GGDPS inhibition, suggesting specific inhibition of GGDPS by our bisphosphonate inhibitors. The data obtained herein suggest that GGDPS can be targeted to interfere with the progression of cancer cells.

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LIST OF ABBREVIATIONS

IPP	isopentenyl diphosphate
DMAPP	dimethylallyl diphosphate
HMGCR	HMG-CoA reductase
GPP	geranyl diphosphate
FPP	farnesyl diphosphate
FDPS	farnesyl diphosphate synthase
GGPP	geranylgeranyl diphosphate
GGDPS	geranylgeranyl diphosphate synthase
SQS	squalene synthase
SQSIs	squalene synthase inhibitors
FOH	farnesol
GGOH	geranylgeraniol
FTase	farnesyl transferase
GGTase I	geranylgeranyl transferases I
GGTase II	geranylgeranyl transferases II
GDI	guanine nucleotide dissociation inhibitors
GEFs	guanine nucleotide exchange factors
GAPs	GTPase-activating proteins
LDL	low-density lipoprotein
NBPs	nitrogenous bisphosphonates
FTIs	Farnesyl transferase inhibitors
GGTIs	geranylgeranyl transferase inhibitors
DGBP	digeranyl bisphosphonate
IPTG	isopropyl β-D-1-thiogalactopyranoside
SDS	sodium dodecyl sulfate

BCA	bicinchoninic acid
PVDF	polyvinylidene fluoride
ECL	enhanced chemiluminescent
IC_{50}	inhibitory concentration 50
PBS	phosphate buffered saline
PARP	poly(ADP-ribose) polymerase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labeling
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PC	phosphatidylcholine
PCR	polymerase chain reaction
CI_{50}	combination index 50
ED_{50}	effective dose 50
FBS	fetal bovine serum
LPA	lysophosphatidic acid
LSDCAS	large scale digital cell analysis system

CHAPTER I: INTRODUCTION

The Isoprenoid Biosynthetic Pathway

The isoprenoid biosynthetic pathway (Figure 1) is responsible for the production of more than 23,000 compounds and to date is found in all living organisms (1). Isoprenoids and their derivatives are involved in many functions that are critical for life (e.g., regulation of gene expression, electron transport, signal transduction, photosynthesis, and reproduction) (2). Isoprenoids are derived from the five carbon building unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (3). IPP and DMAPP are referred to as the simple isoprenoids, because they contain a single five-carbon unit.

A series of enzymes is responsible for the production of isoprenoid diphosphates within the isoprenoid biosynthetic pathway (2). The first step of isoprenoid biosynthesis is catalyzed by HMG-CoA reductase (HMGCR) (4). In this rate-limiting step 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) is reduced to mevalonate with the oxidation of NADPH. Mevalonate is converted to mevalonate diphosphate through two enzyme reactions catalyzed by mevalonate kinase and phosphomevalonate kinase sequentially (5, 6). IPP is formed with the decarboxylation of mevalonate diphosphate by diphosphomevalonate decarboxylase (7, 8). IPP is then converted to DMAPP by IPP isomerase in a reversible reaction (9). The five-carbon compounds IPP and DMAPP are condensed to form the ten-carbon geranyl diphosphate (GPP). The addition of IPP to GPP leads to the production of the fifteen-carbon farnesyl diphosphate (FPP). Farnesyl diphosphate synthase (FDPS) catalyzes both these reactions that result in the production of the intermediate GPP and the product FPP in humans, while plants utilize a discrete

enzyme for the synthesis of GPP (2, 10). FPP lies at the major branch point within the isoprenoid biosynthetic pathway. The addition of IPP to FPP by geranylgeranyl diphosphate synthase (GGDPS) leads to the production of the twenty-carbon all trans geranylgeranyl diphosphate (GGPP) (11, 11, 12). Alternatively, the addition of IPP to FPP by dehydrodolichyl diphosphate synthase results in the GGPP isomer (2*Z*,6*E*,10*E*-GGPP) that is utilized for dolichol synthesis (13). Dolichol molecules are essential mediators of protein glycosylation (14). Squalene synthase (SQS) condenses two FPP molecules to make squalene (15). Squalene is used by animal cells in the *de novo* production of cholesterol (2). In addition, it is believed that FPP is used in the production of ubiquinone, an antioxidant that is involved in the electron transport chain (16). FPP and GGPP can be reversibly converted to their alcohol forms farnesol (FOH) and geranylgeraniol (GGOH), respectively (17). In plants, FPP and GGPP are utilized for the production of sesquiterpenes and diterpenes, respectively (18, 19).

Protein Prenylation

The 15 (FPP) and 20 (GGPP) carbon isoprene moieties are post-translationally incorporated into proteins in processes termed farnesylation and geranylgeranylation, respectively (20). Together these processes that are known as protein prenylation, termed farnesylation and geranylgeranylation, play a critical role in the membrane localization and function of proteins (21). FPP and GGPP are covalently attached to cysteines at the C-termini of proteins (20). Farnesylation is catalyzed by farnesyl transferase (FTase), while geranylgeranylation is catalyzed by geranylgeranyl transferases I (GGTase I) and II (GGTase II, also known as Rab GGTase) (22-24). The sequence of the last few amino acids at the C-terminus determines the type of prenylation

(20). Proteins that have a -CAAX box undergo farnesylation or geranylgeranylation by GGTase I, while proteins with a -CC or -CXC C-terminus will be geranylgeranylated by GGTase II (C=cysteine; A=aliphatic amino acid; X=any amino acid) (20). Once the FPP or GGPP moieties are added to the protein, the three C-terminal amino acids (-AAX) are cleaved from the protein by Ras-converting enzyme 1 (25). Lastly, the new C-terminus is methylated by isoprenylcysteine carboxyl methyltransferase (26). The process is slightly different for proteins that are geranylgeranylated by GGTase II. As mentioned above, GGTase II substrates have two cysteines at their C-termini. Both of these cysteines at the C-terminus (-CC or –CXC) are geranylgeranylated by GGTase II (27). In addition, Rab escort protein (REP) is responsible for the presentation of the unprenylated substrate to GGTase II (28). Finally, GGTase II substrates differ in that the –CXC proteins are methylated at the C-terminus whereas the –CC substrates are not (29).

Prenylated Proteins

According to recent estimates, about 300 proteins are believed to undergo prenylation (31). Various types of proteins with distinct functions undergo farnesylation or geranylgeranylation. Nuclear lamins A and B, heterotrimeric G protein subunits, rhodopsin kinase, and kinetochores CENP-E and F are some of the better known proteins that are either farnesylated or geranylgeranylated (3, 31). The small G proteins referred to as the Ras superfamily GTPases make up the largest family of proteins that undergo prenylation. The Ras superfamily GTPases are proteins with molecular masses of 20-40 kDa (32). These small GTPases are structurally subdivided into five families: Rho, Ras, Rab, Ran, and Sar1/Arf. Known as molecular switches, small GTPases cycle between GTP-bound active and GDP-bound inactive states (33). The replacement of GDP with

GTP by an upstream signal changes the conformation of these proteins, allowing Ras superfamily GTPases to interact with and activate downstream effectors (32). The intrinsic GTPase activity of small GTPases returns the proteins from the GTP-bound active to the GDP-bound inactive form and thus completes one cycle of activation and signaling by these proteins (34). Under resting conditions, most of the Ras superfamily members are in the inactive state (bound to GDP) (3). Three classes of proteins control the activity of Ras-related small GTPases under normal conditions. Guanine nucleotide dissociation inhibitors (GDIs) bind to the C-termini of small GTPases and prevent their activation (35). GDIs can interfere with the activation of small GTPases through three different mechanisms: i) by preventing the isoprenoid moiety from interacting with membranes, ii) by inhibiting dissociation of GDP from small GTPases, and iii) by interacting with the GTP-bound form of small GTPases to prevent interaction with downstream targets (36). Since the exchange of GDP with GTP is extremely slow in cells, a set of proteins have adapted to aid in the activation of small GTPase. Guanine nucleotide exchange factors (GEFs) interact with small GTPases and facilitate the exchange of GDP for GTP leading to the activation of these proteins (37). Finally, GTPase-activating proteins (GAPs) interact with the active form of small GTPases to increase the hydrolysis rate of GTP to return small GTPases to their inactive state (38).

Functions of Small GTPases

Over 100 members in the Ras superfamily of small GTPases have been identified (32). As mentioned earlier, the Ras superfamily GTPases are divided into five major sub-families (Ras, Rho, Rab, Ran, and Sar1/Arf). The Ras GTPases, which includes H-, K-, and N-Ras, are critical mediators of cell proliferation and diffentiation (39). Rho

proteins, which includes RhoA, Rac1, and Cdc42, play a role in actin cytoskeleton reorganization, cell shape, and cell movement (40). The Rab subfamily of small GTPases, which is made up of more than 60 members, are localized to various vesicle compartments and are important mediators of vesicle transport (41). Another set of small GTPase proteins that play a role in vesicular transport are Sar1 and Arf proteins. These Ras-superfamily members are responsible for the recruitment of coat proteins (COP1, COP2) during vesicle budding of donor membranes (32). Finally, the Ran proteins are involved in the transport of macromolecules between the nucleus and cytoplasm and in the microtubule organization during cell division (42, 43).

Small GTPases and Cancer

In the late 1970s, v-H-Ras and v-K-Ras were identified as oncogenes in avian sarcoma viruses (44, 45). Subsequent work revealed their oncogenic functions in humans (46). Additional studies showed that mutational activation of these oncogenes increases proliferation and transformation (47). Many of the small GTPases that belong to the Ras superfamily have been implicated in cancer. It is estimated that constitutive active mutations of Ras genes contribute to aberrant signaling in 20-30% of human cancers (48). Mutations of Ras family members (H-Ras, K-Ras, N-Ras) have been reported in all types of cancers, including thyroid, melanoma, pancreatic, non-small-cell lung, colorectal, bladder, and renal cancers (49). All of these mutations interfere with the GTPase activity of Ras and thus "lock" the proteins in their active GTP-bound form (48). In addition, Ras signaling can be altered by mutations or over-expression of proteins that lie upstream or downstream of Ras. Epidermal growth factor receptor is an important upstream activator of Ras. Mutation or over-expression of this receptor tyrosine kinase activates signaling

by Ras in glioblastoma, breast, and ovarian cancers (50, 51). Alteration of proteins downstream of Ras, such as mutation of BRAF, amplification of p110 or AKT2, or deletions of PTEN, are associated with the development of various cancers (49).

Members of the Rho sub-family of small GTPases have also been implicated with cancer. Thus far, constitutively active mutations of Rho proteins have not been documented in cancer (52). Rearrangement and mutation of the 5'-untranslated region of RhoH and over-expression of RhoA, RhoC, Rac1, Rac2, and Cdc42 have been documented in various cancers (e.g., breast, colon, bladder) (52-54). Over-expression of these small GTPases correlates with aggressive histological features and poor clinical outcomes (55-58). In addition to the over-expression of Rho small GTPases, many of the regulatory proteins that control these small GTPases are altered in cancer (49). p190RHOGAP and DLC2, which are Rho GAPs, have been shown to be deleted or under-expressed in some cancers (59, 60). LARG and BCR, which are Rho GEFs, contribute to the development of acute myeloid leukemia when fused to MLL and ABL, respectively (61, 62). Finally, Rho GDIs have been shown to be both over-expressed or under-expressed in different types of cancers (63, 64).

Over-expression of Rab and Arf subfamilies of small GTPases has also been documented in cancer. Several Arf family members (ARL5, SARA1, and SARA2) are over-expressed in liver cancers (65). The level of their expression may be important in that breast cancer cells exhibit a greater invasive phenotype with the over-expression of ARF6 (66). Rab25 over-expression, due to increased DNA copy number, is associated with decreased disease-free and overall survival of patients with breast and ovarian

cancer (67). Finally, many of other Rab proteins (e.g., Rab7, Rab10) are over-expressed in several types of human cancers (49).

Hallmarks of Cancer

In 2000, Hanahan and Weinberg published a review that summarized the six hallmarks of cancer: i) self-sufficiency in growth signals, ii) insensitivity to anti-growth signals, iii) evasion of apoptosis, iv) limitless replicative potential, v) sustained angiogenesis, and vi) tissue invasion and metastasis (68). In order to grow and replicate, cells need growth-promoting stimuli. Cancer cells have developed ways in which continuous growth signals can be generated by their cellular machinery. Cancer cells may generate their own growth factors for autocrine stimulation, express higher levels of receptors making them more sensitive to normal levels of growth factors, exhibit ligand-independent activation of receptors, and/or have altered downstream components of signal transduction that bypass the activation of upstream receptors (68, 69).

To survive and replicate, cancer cells have to evade the anti-proliferative signals that maintain tissue homeostasis. Cancer cells can acquire the ability to evade cell cycle arrest and terminal differentiation by various mechanisms to proliferate uncontrollably (70-72). Similar to the evasion of anti-growth signals, cancers cells must evade apoptosis, or programmed cell death, in order to expand their population (68). Cancer cells can evade apoptosis through the inactivation of tumor suppressors, such as p53, or through the activation of anti-apoptotic signals, such as the PI3/Akt pathway (73, 74). It is well established that normal cells have a limited doubling number. It is believed that this limitation is due to shortening of chromosomal telomeres (68). Unlike normal cells,

cancer cells increase their doubling number through the expression of telomere maintenance enzymes and activation of other DNA repair mechanisms (75, 76).

In order to support their proliferation and maintenance *in vivo*, tumors have developed ways to stimulate angiogenesis to increase vasculature and deliver oxygen and nutrients to their rapidly growing cells (77). Cancer cells are able to stimulate the expression of inducers (vascular endothelial growth factor (VEGF)) and decrease the expression of inhibitors (thrombospondin-1) of angiogenesis in the local environment to promote the development of new blood vessels (78).

Finally, cancer cells have developed the ability to invade and metastasize to distant areas of the human body. It is estimated that 90% of all cancer related deaths are a result of metastatic cancer (79). Alterations in the function of E-cadherin, a suppressor of invasion and metastasis, and increased expression and activation of matrix-degrading proteases allows cancer cells to escape the local tumor environment and travel to distant sites in the body (68, 80, 81).

Treatment of Disease with Isoprenoid Pathway Inhibitors

In 1976, Endo and colleagues isolated mevastatin, the first reported inhibitor of HMGCR (82). Their work fueled the search for isoprenoid biosynthetic pathway inhibitors in the treatment of human disease. Subsequent studies demonstrated that inhibition of HMGCR by mevastatin inhibited cholesterol synthesis in tissue culture cells (83). Ultimately, it was shown that mevastatin reduced plasma cholesterol in animals and humans (84, 85). Statins (e.g., mevastatin, lovastatin, simvastatin, and atorvastatin) disrupt cholesterol production in the liver, which increases the expression of low-density lipoprotein (LDL) receptors on the cell surface (86). The LDL receptors then take up

LDL particles from the bloodstream leading to the decrease of LDL cholesterol in the blood (87). Therefore, statins are used in the treatment of hypercholesteremia with an estimated eleven million Americans taking these drugs annually (88). The decrease in blood cholesterol due to statins drastically decreases cardiovascular morbidity and mortality (89, 90). The successful use of statins in the battle against cardiovascular disease has been compared to antibiotics in the treatment of bacterial infections (91). In addition to cardiovascular benefits, statins are associated with beneficial effects in the treatment of various other disease including osteoporosis, cancer, Alzheimer's disease, coagulation, and thrombosis (2). The effects of statins on cancer in pre-clinical and clinical trials are further discussed later in this chapter.

Nitrogenous bisphosphonates (NBPs) are another class of isoprenoid biosynthetic pathway inhibitors that are used in the treatment of human disease. NBPs (e.g., zoledronate, risedronate) are extensively used in the treatment of diseases associated with increased activity of osteoclasts (cells responsible for bone resorption), such as Paget's disease, osteoporosis, and metastatic bone disease (92). Once in the body, NBPs are rapidly cleared from the blood and bind to mineral surfaces of active bone remodeling, especially areas that are undergoing osteoclastic resorption (93). The high concentration of NBPs at sites of osteoclastic resorption allows for fluid-phase endocytosis into osteoclasts (94). Once inside the cells, NBPs bind to and inhibit the function of FDPS at nano-molar concentration (95). The inhibition of FDPS by NBPs prevents the synthesis of FPP and GGPP, which interferes with the prenylation of small GTPases (93). The disruption of protein prenylation is believed to affect the function and viability of

osteoclasts (96). Therefore, it is widely believed that the positive effects of NBPs on the bone stem from the inhibition of FDPS and induction of apoptosis of osteoclasts (92, 97).

In addition to statins and NBPs, other isoprenoid biosynthetic pathway inhibitors are being explored in the treatment of disease. Squalene synthase inhibitors (SQSIs) are being explored for hypercholesterolemia therapy. One such agent, lapaquistat, was not pursued in advanced clinical trials due to hepatotoxicity (98). Farnesyl transferase (FTase) inhibitors (FTIs) block protein farnesylation(99). FTIs inhibit growth of numerous cancer cell lines *in vitro* and show anti-cancer benefit in animal models (100-102). Numerous clinical studies have been performed with FTIs inhibitors. For the most part, FTIs are considered are failure in the treatment of solid tumors. In a phase III double-blind placebo-controlled study, treatment of refractory advanced colorectal cancer patients with FTI R115777 did not translate into a statistically significant increase in progression-free survival (103). Similarly, the combination of FTI R115777 and gemcitabine did not prolong overall survival of advanced pancreatic cancer patients when compared with single-agent gemcitabine in a phase III clinical trial (104). However, FTIs are showing some promise in human clinical trials for hematologic malignancies. In a phase II study, tipifarnib prolonged survival in some patients with refractory or relapsed acute myeloid leukemia (105). Similarly, treatment of previously untreated elderly acute myeloid leukemia patients with tipifarnib resulted in complete remission in 22 patients (14%), partial remission or hematologic improvement occurred in 15 patients, with an overall response rate of 23% in a phase II study(106).

Similar to FTIs, geranylgeranyl transferase (GGTase) inhibitors (GGTIs) interfere with protein geranylgeranylation by inhibiting GGTase I. As in the case with FTIs,

GGTIs are being tested as anti-cancer agents (17). GGTIs have been shown to induce cell cycle arrest and inhibit cancer cell invasion (107, 108). GGTIs also decreased tumor volumes in xenograft and transgenic animal models of cancer (109).

Hypothesis

As mentioned earlier, inhibition of protein prenylation by FTIs and GGTIs has shown success in the treatment of cancer *in vitro* and *in vivo*. In addition to direct inhibition of farnesylation (FTIs) and geranylgeranylation (GGTIs), protein prenylation can be inhibited through the depletion of isoprenoid pathway intermediates. The depletion of FPP and GGPP, the moiety donors for protein prenylation, interferes with farnesylation and geranylgeranylation (17). Statins and NBPs inhibit the isoprenoid biosynthetic pathway upstream of FPP and GGPP and thus deplete these two isoprenoid biosynthetic pathway intermediates that are needed for protein prenylation. Both of these clinically used agents (statins and NBPs) have been shown to interfere with protein farnesylation and geranylgeranylation (110, 111).

Numerous clinical studies have been performed to determine the effects of statin use on cancer. Follow-up studies showed that overall cancer incidence (all types of cancers) was decreased in statin users when compared to non-statin users in a population based study of more than 300,000 patients (112). A control study was performed to determine the association between lipid-modifying agents and cancer (113). It was demonstrated that statin use decreased the overall incidence of cancer by 28% when compared to bile acid sequestrants, agents that interfere with cholesterol uptake in the digestive tract. This study also demonstrated that statins were associated with reduced incidence of specific types of cancer, including cancers of the lung, skin, bladder, uterus,

colon, kidney, and breast. Clinical studies have also looked at the effects of statins and incidence of specific types of cancers. Below are examples of four such studies. A large case-control study of US veterans demonstrated that the use of statins for six months or longer associated with a risk reduction of lung cancer of 55% (114). The protective effects of statins increased with duration of statin therapy. Another case-controlled study demonstrated that men with any recorded statin use had a 65% reduction in risk of prostate cancer compared with nonusers (115). The use hydrophobic statins (i.e., simvastatin, lovastatin, and fluvastatin) was associated with an 18% lower breast cancer incidence in a clinical study that examined data of 156,351 postmenopausal women (116). In a population-based control study, the use of statins for at least five years was associated with a 47% relative reduction in the risk of colorectal cancer after adjustment for other known risk factors (117).

The role of statins in the treatment of cancer has been extensively studied in tissue culture and *in vivo*. Statins have been shown to induce cell cycle arrest by interfering with cell G1 to S phase transition, up-regulating cell cycle inhibitors, and down-regulating cyclin-dependent kinases (118, 119). Studies have also demonstrated that statins induce apoptosis in cancer cells and tissues (119-121). Interestingly, low concentrations of statins promote angiogenesis, while higher concentrations of these drugs interfere with the formation of new blood vessels (122). Statins also inhibit the migration and invasion of various cancer cells (123-125). Finally, synergistic interactions have been observed when statins were combined with chemotherapeutic agents and with NBPs (126-128).

Similar to statins, numerous studies have evaluated NBPs as possible anti-cancer agents. NBPs have been shown to possess anti-cancer properties such as induction of apoptosis and inhibition of cancer cell migration and invasion (129-131). In addition to the synergistic interaction with statins, NBPs potentiate chemotherapeutic agents such as doxorubicin and paclitaxel (132, 133).

Add-back experiments, which consist of adding exogenous isoprenoid biosynthetic pathway intermediates, have been performed to clarify the mechanism by which statins and NBPs interfere with cancer cell proliferation. Generally, the anti-cancer effects of statins and NBPs are due the depletion of cellular GGPP and inhibition of protein geranylgeranylation (17, 21, 134). For example, the blocking of protein geranylgeranylation (through depletion of GGPP) is required for statin-induced apoptosis in acute myeloid leukemia cells (121). Similarly, the addition of GGPP, but not FPP, prevented apoptosis induced by a NBPs (135). The addition of GGPP, but not FPP, prevented the effects of statins and NBPs on cancer cell migration and invasion (123, 136). When statins potentiate the anti-cancer effects of some chemotherapeutics (cisplatin and 5-fluorouracil), the addition of GGPP, but not FPP, prevented the synergy that was observed with the drug combinations (137). Likewise, the addition of GGPP prevented synergy that was observed with the combination of zoledronate (NBP) and doxorubicin (132).

A review of recent literature suggests that GGPP depletion is the mechanism by which isoprenoid biosynthetic inhibitors (statins and NBPs) interfere with cancer cell progression. Add-back experiments with isoprenoid biosynthetic intermediates illustrate the importance of GGPP to cancer cell survival and function. However, the development

of agents that specifically deplete GGPP and do not deplete upstream isoprenoid biosynthetic intermediates, such as FPP, will allow for a more specific analysis of cancer cell dependence on GGPP. To this end, our laboratories have developed specific bisphosphonate inhibitors of GGDPS (138-142). Our lead compound, (DGBP) (structure shown in Figure 1), impairs protein geranylgeranylation, but not protein farnesylation (138, 139). DGBP depletes GGPP but not FPP in cultured cells as well some mammalian tissues (139, 143).

With the development of specific GGDPS inhibitors, such as DGBP, we have the tools to assess the importance of GGPP depletion on cancer cell survival and function. Our approach is novel in that previous work with statins and NBPs has relied on the depletion of multiple isoprenoid biosynthetic intermediates, while our work will demonstrate the importance of GGPP to cancer cells through the direct inhibition of GGDPS and thus depletion of cellular GGPP. The development of DGBP and other GGDPS inhibitors allows for depletion of GGPP but not FPP leading to the impairment of geranylgeranylation, but not farnesylation. *The overall hypothesis of this work is to determine if GGDPS inhibition has anti-cancer effects.* The goals of the research described herein will be to assess whether GGDPS inhibition interferes with the various aspects of cancer cell survival and function. In addition, the continuous synthesis of potential GGDPS inhibitors with our collaborators will lead to the identification of novel and perhaps more potent inhibitors of GGDPS.

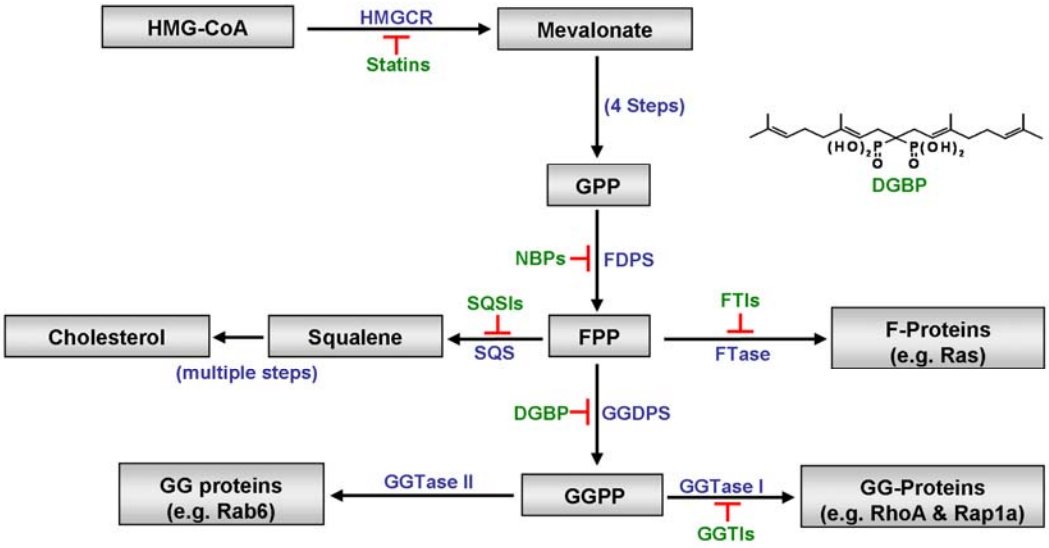


Figure 1. The human isoprenoid pathway. The diagram shows key enzymes (HMGCR, FDPS, and GGDPS) and intermediates (FPP and GGPP). In addition, the diagram illustrates the action site of drugs used in the clinic (statins and NBPs), drugs that are undergoing clinical trials (FTIs and SQSIs), and drugs that are in laboratory stages of development (GGTIs and DGBP).

CHAPTER II: IDENTIFICATION OF NOVEL GGDPS INHIBITORS

Abstract

Geminal bisphosphonates display varied biological activity depending on the nature of the substituents on the central carbon atom. For example, the nitrogenous bisphosphonates zoledronate and risedronate inhibit farnesyl diphosphate synthase while digeranyl bisphosphonate inhibits geranylgeranyl diphosphate synthase. We now have synthesized isoprenoid bisphosphonates where an aromatic ring has replaced one of the isoprenoid olefins in an isoprenoid bisphosphonate and investigated the degree to which these new compounds impair protein geranylgeranylation in enzyme assay and in intact cells. We show that two of these new compounds are potent *in vitro* and *in vivo* inhibitors of geranylgeranyl diphosphate synthase.

Introduction

Interest in geminal bisphosphonates (Figure 2, structure 1) as structural analogs of pyrophosphates (Figure 2, structure 2) is well established (Figure 1) (144). These compounds are formed by replacement of the P–O–P linkage with the P–C–P bond, but the bisphosphonates are more stable to metabolism and introduction of the methylene linker allows additional structural modifications that would otherwise not been possible in the pyrophosphate structure. Geminal bisphosphonates have demonstrated utility in a variety of applications. Historically, they have been used as chelating agents and water softeners (145, 146). More recently, bisphosphonates have found application in the clinic as drugs for treatment of bone related disease. For example, zoledronate (Figure 2, structure 3) has been used in the treatment of osteoporosis as well as multiple myeloma and a variety of other cancers, and risedronate (Figure 2, structure 4) is used for treatment

of osteoporosis (144, 147). Bisphosphonates ability to inhibit growth of malignant cells has also been documented (21).

Our laboratories have reported the synthesis of mono- and dialkyl isoprenoid bisphosphonates that selectively inhibit geranylgeranyl diphosphate synthase (GGDPS) (138, 140, 141). As described in the introduction, GGDPS catalyzes conversion of FPP to GGPP which is the required precursor for protein geranylgeranylation.

There are several reasons to include an aromatic moiety in the structure of potential GGDPS inhibitors. First, many of the most potent inhibitors of farnesyl diphosphate synthase (FDPS) are nitrogenous bisphosphonates with an aromatic substructure, including zoledronate and risedronate. It is not yet known whether introduction of an aromatic moiety will enhance or diminish specificity for GGDPS inhibition in an isoprenoid bisphosphonate. It will be interesting to determine if the aromatic compounds retain their activity as inhibitors of GGDPS despite their more sterically demanding profile. Second, there is evidence that the high charge to mass ratio in salts of bisphosphonic acids at physiological pH can limit their transversing of the cell membrane (141). Aromatic bisphosphonates are more lipophilic than the parent compound digeranyl bisphosphonate (DGBP), which may result in more facile drug delivery to the cell and relieve the need for use of a prodrug (148). Third, bisphosphonates that contain isoprenoid olefin isomers display differing biological activity, but the potential isomerization or transposition of an alkene *in vivo* would be eliminated with an aromatic substructure. Finally, the larger π system of an aromatic ring may lead to more favorable stacking interactions in the active site of GGDPS (e.g., with

tyrosine 205 or phenylalanine 175) (149). For these reasons, we have pursued the chemical synthesis and biological evaluation of aromatic-isoprenoid bisphosphonates.

Materials and Methods

Chemical synthesis. The chemical synthesis of aromatic bisphosphonates was performed by Rocky Barney (Dr. Wiemer's laboratory, Chemistry Department, University of Iowa) and is discussed in our publication (150).

Cell culture. K562 human-derived chronic myelogenous leukemia cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS at 37 °C and 5% CO_2.

GGDPS in vitro assay. The GGDPS *in vitro* assay was performed as described previously (139). Briefly, plasmids containing GST-tagged recombinant human GGDPS were expressed in BL21 gold bacteria by induction with IPTG. Proteins were purified by batch centrifugation with glutathione agarose. The GGDPS reaction mixtures contained 20 μM FPP and 40 μM ^{14}C-IPP in 20 μL buffer (50 mM imidazole pH 7.5, 0.5 mM $MgCl_2$, 0.5 mM $ZnCl_2$). Following a 10-min pre-incubation with the indicated compounds, reactions were initiated by simultaneous addition of ^{14}C-IPP and FPP. Reactions proceeded for 1 h at 37 °C, and then the longer isoprenoids were extracted with 1 ml saturated butanol and the extracts were washed twice with 300 μL saturated water. The amount of radioactivity in the butanol extracts were detected by liquid scintillation counting.

Western blotting. The K562 cells were diluted to a final concentration of 5×10^5 cells/ml. After 5 ml of cell suspension was added to 6-well plates in the presence

of compounds, the plates were incubated for 48 h. Cells were harvested by centrifugation and lysed (2% SDS in 66 mM Tris) by passing cells several times through a 27 gauge needle. Lysates were cleared by centrifugation and protein concentration determined by the bicinchoninic acid (BCA) assay. Proteins were resolved by electrophoretic fractionation on 12% and 15% gels and transferred to polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in 5% non-fat milk for 40 min at 37 °C. Primary and secondary antibodies were added sequentially for 1 h at 37 °C and proteins were visualized with enhanced chemiluminescence (ECL) reagents. Anti pan-Ras antibody was obtained from InterBiotechnology (Tokyo, Japan). The Rap1a (sc-1482), Rab6 (sc-310), and α-tubulin (sc-8025) antibodies were obtained from Santa Cruz biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit were obtained from GE Healthcare (Buckinghamshire, UK), and horseradish peroxidase-conjugated anti-goat was obtained from Santa Cruz Biotechnology, Inc.

Add-back experiments. Isoprenoid pathway intermediates (mevalonate, FPP, or GGPP) were added at indicated concentrations simultaneously with isoprenoid pathway inhibitors (lovastatin, compound 14, or compound 21). Lovastatin, mevalonate, FPP, and GGPP were purchased from Sigma (St. Louis, MO).

DNA synthesis assay. 200 μl of K562 cells were incubated in 96-well plates and treated with compounds as described previously (139). The 48 h experiments required 2×10^5 cells/ml while 72 h experiments required 1×10^5 cells/ml. After 44 or 68 h, 20 μL of [^{3}H]thymidine (0.1385 TBq/mmol; 3.75 Ci/mmol in media) was added to each well. At 48 or 72 h, cells were filtered through glass microfiber paper using a Brandel

(Gaithersburg, MD) cell harvester. [^{3}H]Thymidine incorporated into cellular DNA was quantified by scintillation counting.

Results

Synthesis of aromatic bisphosphonates. As described in the materials and methods sections, compound synthesis was performed by Rocky Barney (Dr. Wiemer's laboratory, Chemistry Department, University of Iowa). The synthesis of aromatic bisphosphonates will not be discussed in this thesis. Synthesis of these compounds can be viewed in our publication (150). The compounds that were synthesized as potential GGDPS are shown below (Figure 3). Three compounds were mono alkyl bisphosphonates (**28**, **12**, **19**) while four compounds were dialkyl bisphosphonates (**24**, **14**, **21**, **27**). Mono alkyl aromatic bisphosphonates have one functional group at the central carbon while dialkyl aromatic bisphosphonates have two (see Figures 2 and 3). DGBP (not an aromatic bisphosphonate), our positive control and lead GGDPS inhibitor, is also shown in the same figure.

Aromatic bisphosphonates inhibit GGDPS in vitro. Compounds were first screened *in vitro* against recombinantly purified human GGDPS enzyme (Figure 4A). DGBP was used as a positive control because it was previously shown to inhibit GGDPS (139). At 10 μM concentrations, the dialkyl bisphosphonate compounds **24**, **14**, **21**, and **27** all displayed various degrees of GGDPS inhibition while the mono alkyl compounds **12**, **19**, and **28** displayed little or no activity. Concentration–response curves were then generated to further characterize the compounds active in the initial screen. Compounds **14** and **21** were found to display potent inhibitory activity with IC_{50} values (the

concentration at which the enzyme is inhibited to 50% maximal activity) extrapolated to be 250 nM and 800 nM, respectively (Figure 4B).

Aromatic bisphosphonates inhibit protein geranylgeranylation but not farnesylation in K562 cells. This set of compounds then was tested against the K562 human myelogenous leukemia cell line for ability to impair protein prenylation. Western blots were performed and prenylation status of a panel of proteins was determined (Figure 5). Because the antibodies available to monitor protein prenylation have different specificities for modified and unmodified protein forms, these analyses must be interpreted with special care. The Ras protein is farnesylated and reduction of farnesylation is made evident by the appearance of a more slowly migrating, unmodified band on the gel. In contrast, Rap1a is geranylgeranylated in a reaction catalyzed by the enzyme geranylgeranyl transferase I (GGTase I) and the antibody used here detects only the unmodified form of the protein; thus accumulation of a detectable band represents impairment of geranylgeranylation.

At 48 h, lovastatin (Figure 5A, lane 2), an inhibitor of HMGCR, depletes mevalonate resulting in a reduction of protein farnesylation and geranylgeranylation. Compounds **14** and **21** diminish geranylgeranylation of Rap1a, while farnesylation of Ras appeared unaffected at this level of detection (Figure 5A). These compounds also decrease geranylgeranylation of Rab6 (data not shown), a GGTase II substrate. Other novel aromatic bisphosphonates (compounds **28**, **24**, **12**, **19**, **27**) do not interfere with protein farnesylation and geranylgeranylation.

Cellular concentration–response assays were also performed with both compounds **14** and **21** (Figure 5B). Reduction of Rap1a geranylgeranylation is apparent

at concentrations as low as 12.5 μM with aromatic bisphosphonate **14**, while both compounds appeared to display maximal effects at 50 μM.

Inhibition of protein geranylgeranylation by novel bisphosphonates is prevented by GGPP in K562 cells. To define further the specificity of the compounds for GGDPS in cells, 'add-back' experiments were performed wherein intermediates of the isoprenoid biosynthetic pathway were added in combination with the lead compounds (Figure 6). Lovastatin was again used as a positive control, where the reduction of protein farnesylation and geranylgeranylation is prevented by addition of mevalonate. Furthermore, FPP addition prevents lovastatin-induced reduction of farnesylation, but not geranylgeranylation, and the converse is true for GGPP. For bisphosphonates **14** and **21**, no effects are noticed from the addition of mevalonate or FPP, while the addition of GGPP prevents the impairment of Rap1a and Rab6 geranylgeranylation (Rab6 data not shown).

Novel bisphosphonates inhibit cell proliferation of K562 cells. Cell viability was determined in response to compound treatment by determination of the amount of DNA synthesis with a ^{3}H-thymidine incorporation assay (Figure 7). Bisphosphonates **14** and **21** inhibit DNA synthesis in concentration and time dependent manner, with the *meta* isomer compound **14** being the more potent compound.

Discussion

The aromatic bisphosphonates were evaluated for activity in both enzyme and various whole cell assays. Our laboratory had previously identified numerous mono- and dialkyl bisphosphonates as inhibitors of GGDPS, and many of these compounds had also been shown to impair protein geranylgeranylation in intact cell assays (140). Based on

our previous work, we hypothesized that the aromatic bisphosphonates synthesized would inhibit GGDPS. Dialkyl bisphosphonates (**24**, **14**, **21**, **27**) inhibited GGDPS *in vitro* while mono alkyl compounds (**12**, **19**, and **28**) did not. Compounds **14** and **21** are most potent aromatic bisphosphonate inhibitors of GGDPS with IC_{50} values of 250 nM and 800 nM, respectively. As a comparison, the published IC_{50} value of DGBP is 200 nM (139). The structure of GGDPS has been solved when complexed with DGBP, which bound to the 'inhibitor' binding site in a 'V-shaped' conformation occupying portions of both the FPP and GGPP binding sites (151). Based on the structure of the most potent compounds identified herein, it would be anticipated that these molecules bind GGDPS in a similar manner.

This set of compounds then was tested against the K562 human myelogenous leukemia cell line for impairment of protein prenylation. Western blots were performed and prenylation status of a panel of proteins was determined. Similar to lovastatin, compounds **14** and **21** interfered with geranylgeranylation of Rap1a and Rab6 (Rab6 data was not shown). Unlike lovastatin, compounds **14** and **21** did not impair farnesylation of Ras. The *in vitro* data correlated well with the intact cell data, as the two potent *in vitro* GGDPS inhibitors were the only compounds to significantly impair protein geranylgeranylation. Further experiments demonstrated that compound **14** was slightly more potent than compound **21** at impairing protein geranylgeranylation. Compounds **24, 14, 21,** and **27,** all had similar enzyme inhibitory effects on GGDPS under initial screening conditions while only compounds **14** and **21** impaired Rap1a modification in intact cells at 50 μM. This implies that intracellular levels of dialkyl compounds **14** and **21** were higher than those of mono alkyl compounds **24** and **27**, which may be a

consequence of greater cellular influx or diminished efflux. Were the former to be the case, then a prodrug approach might further enhance the potency of these molecules by increasing cellular entry (141).

Cell viability was determined in response to compound treatment by determination of the DNA synthesis with a ^{3}H-thymidine incorporation assay. Bisphosphonates **14** and **21** inhibited DNA synthesis in concentration and time dependent manners, with the *meta* isomer **14** being the more potent compound. These results show that the degree of impairment of geranylgeranylation correlates with cytotoxicity.

Although there has been some pursuit of multi-enzyme (i.e., both FDPS and GGDPS) inhibitors within the mevalonate pathway as potential anti-cancer agents (152), single target specificity remains the norm for molecular intervention. Compounds with the ability to inhibit a single enzyme are very useful tools to study the interrelationships of this complex system, and may have use in anti-cancer applications in the clinic (17). The aromatic bisphosphonates reported here, and especially compounds 14 and 21, demonstrate selective inhibition of GGDPS over FDPS, and thus expand the list of tools available for manipulation of isoprenoid biosynthesis.

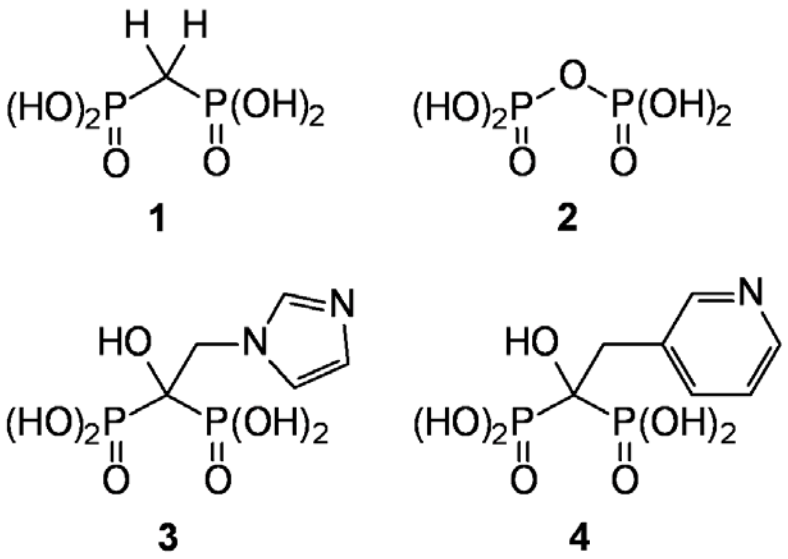


Figure 2. Structures of geminal bisphosphonate (1), pyrophosphate (2) and nitrogenous bisphosphonates (3 and 4).

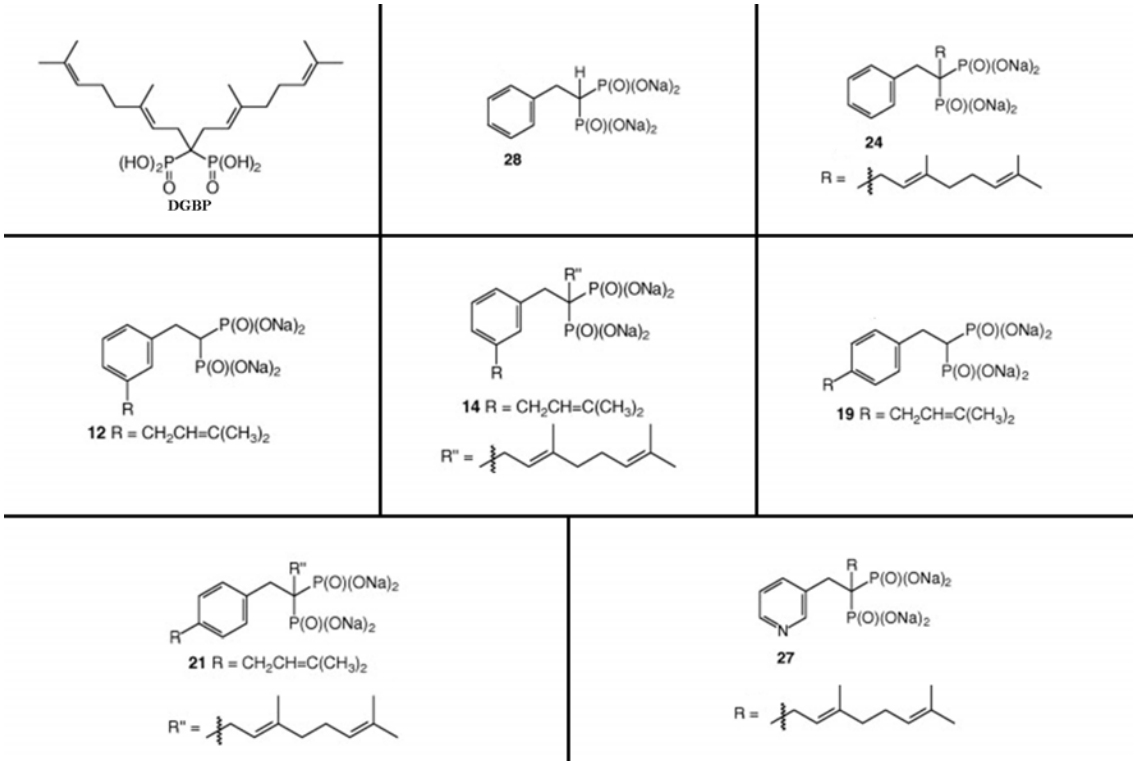


Figure 3. Structures of DGBP and aromatic bisphosphonates that have been designed as GGDPS inhibitors.

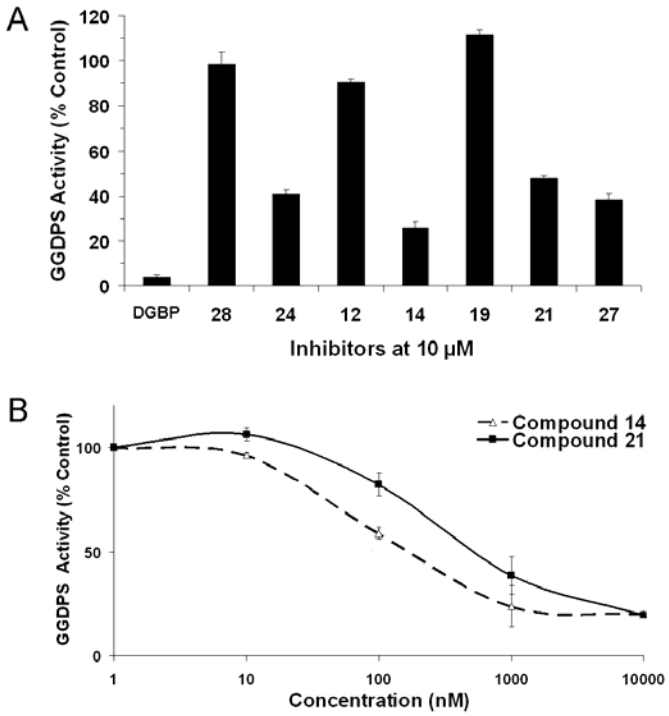
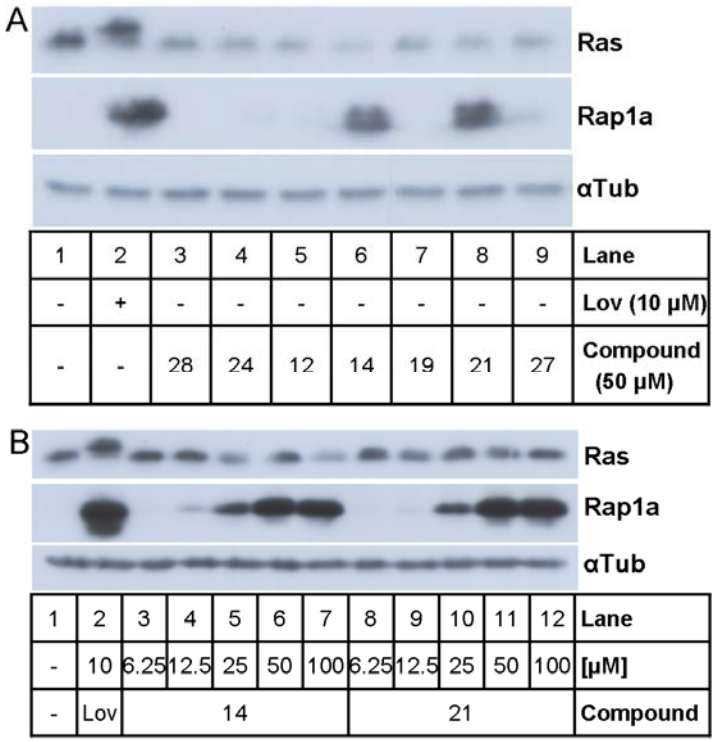


Figure 4. Inhibition of GGDPS *in vitro* by novel aromatic bisphosphonates. (A) Screen at 10 µM of each compound (mean ± SD, $n = 2$). (B) Concentration response of compounds 14 and 21 (mean ± SD, $n = 2$).



1	2	3	4	5	6	7	8	9	Lane
-	+	-	-	-	-	-	-	-	Lov (10 μM)
-	-	28	24	12	14	19	21	27	Compound (50 μM)

1	2	3	4	5	6	7	8	9	10	11	12	Lane
-	10	6.25	12.5	25	50	100	6.25	12.5	25	50	100	[μM]
-	Lov	14					21					Compound

Figure 5. Impairment of protein prenylation in intact cells by novel aromatic bisphosphonates in K562 cells. (A) Compound screen. (B) Concentration response of compounds 14 and 21. K562 cells were treated with lovastatin (lov) and compounds as indicated for 48 h.

Figure 6. Impairment of protein geranylgeranylation by compounds 14 and 21 is prevented by exogenous GGPP in K562 cells. Cells were treated with lovastatin (10 μM) and novel bisphosphonates (14 and 21 at 50 μM) in the presence or absence of exogenous mevalonate (Mev, 500 μM), FPP (F, 20 μM), and GGPP (GG, 20 μM) for 48 h.

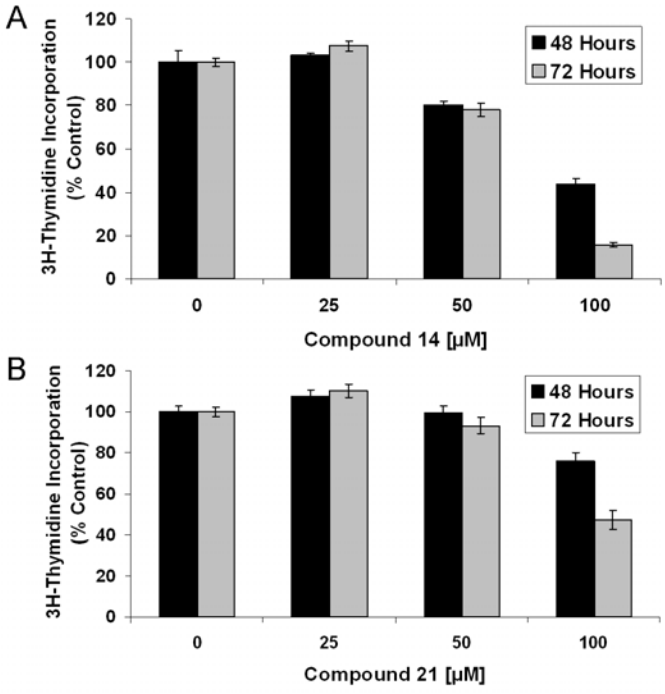


Figure 7. Compounds 14 and 21 inhibit proliferation of K562 cells. Cell proliferation as a percentage of untreated cells at 48 and 72 h was evaluated by [^{3}H]thymidine incorporation (mean ± S.E., n = 4). (A) Compound 14. (B) Compound 21.

CHAPTER III: GGDPS INHIBITION INDUCES APOPTOSIS AND DISPLAYS SYNERGY WITH THE INHIBTION OF OTHER ISOPRENOID BIOSYNTHETIC PATHWAY ENZYMES

Abstract

Inhibitors of isoprenoid synthesis are widely used for treatment of human diseases, including hypercholesterolemia and osteoporosis, and they have the potential to be useful for treatment of cancer. Statin drugs inhibit the enzyme HMG-CoA reductase, whereas nitrogenous bisphosphonates have more recently been shown to inhibit farnesyl diphosphate synthase. In addition, our laboratory has recently developed several potent and specific bisphosphonate inhibitors of geranylgeranyl diphosphate synthase, including digeranyl bisphosphonate. We show that inhibition of geranylgeranyl diphosphate synthase induces apoptosis in human-derived K562 chronic myeloid leukemia cells. This induction of apoptosis is in part dependent upon both geranylgeranyl diphosphate depletion and accumulation of farnesyl diphosphate. Combinations of either lovastatin or zoledronate with digeranyl bisphosphonate synergistically inhibit growth and induce apoptosis. These combinations also potently impair protein geranylgeranylation. These results support the potential for combinations of multiple inhibitors of isoprene biosynthesis to inhibit cancer cell growth or metastasis at clinically achievable concentrations.

Introduction

The isoprenoid biosynthetic pathway (Figure 1) is one of the most targeted biochemical pathways in human disease with millions of people currently taking statins or bisphosphonates. The statins, including lovastatin, have been used for years to treat

hypercholesterolemia (153). The statins inhibit HMG-CoA reductase (HMGCR), depleting cells of downstream isoprenoids, including farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) (154). The isoprene moieties from FPP and GGPP are also post-translationally incorporated into several proteins, including many members of the Ras family of small GTPases, which control cell growth and proliferation (32), and the Rho family of GTPases, which are important mediators of cell migration (155). Because prenylation is necessary for the proper localization and therefore activation of small GTPases, statins have been investigated as potential agents for use in cancer chemotherapy (21). Studies have demonstrated that statins are able to induce apoptosis, or programmed cell death, in cancer cells and tissues (119-121).

The nitrogenous bisphosphonates (NBPs), including zoledronate, are diphosphate analogs used clinically to treat bone disorders, including osteoporosis and metastatic bone disease (144). These drugs in the laboratory also induce apoptosis and have direct growth inhibitory effects on malignant cells (156). Like statins, the clinically used NBPs deplete cells of isoprenoid diphosphates, resulting in the impairment of posttranslational protein prenylation (157). In particular, the NBPs inhibit farnesyl diphosphate synthase (FDPS) (95). However, their cellular effects, including induction of apoptosis, may be largely a result of downstream GGPP depletion (21, 129).

Based on this rationale, we developed a series of isoprenoid-containing bisphosphonates that specifically inhibit geranylgeranyl diphosphate synthase (GGDPS) (138-140, 142, 150). We have shown that several of these compounds, including digeranyl bisphosphonate (DGBP), can potently inhibit GGDPS *in vitro* (139). We have

also shown that inhibition of cellular GGDPS leads to depletion of GGPP but not depletion of FPP in cells and human tissues (139, 143).

Several synergistic interactions between inhibitors of isoprenoid biosynthesis and chemotherapeutic agents have been observed (158). For example, statins are synergistic with cytosine arabinoside and paclitaxel (126, 127). NBPs are synergistic with farnesyl transferase inhibitors, paclitaxel, and imatinib (133, 159, 160). It is noteworthy that the combination of statins and NBPs is also synergistic (128).

Although HMGCR and FDPS have now been extensively studied as therapeutic targets, GGDPS has not. Our recent advances in the design of potent, specific, and cell-permeable GGDPS inhibitors allow the consequences of GGDPS inhibition to be investigated. In this study, we show for the first time that inhibition of GGDPS inhibits growth and induces apoptosis through at least two mechanisms, GGPP depletion and more surprisingly, the accumulation of FPP. It is notable that inhibition of GGDPS by DGBP is synergistic with either HMG-CoA reductase inhibition by lovastatin or FDPS inhibition by zoledronate.

Materials and Methods

Cell culture. K562 leukemia, HepG2 hepatocellular carcinoma, and MDA-MB-231 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA), and cultured in RPMI 1640 (K562) or MEM (HepG2 and MDA-MB-231) media. For Western blots and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays, 2.5×10^6 total cells were incubated for times and concentrations indicated (K562 cells). Annexin V and propidium iodide (PI) analysis required 0.5×10^6 total cells (K562 cells). Real-time PCR experiments required 5×10^6

total cells (K562 cells). For MTT assay, HepG2 and MDA-MB-231 cells were plated and allowed to reach ~60% confluence before drugs were added.

DNA synthesis assay. K562 cells were incubated in 96-well plates and treated with compounds as described elsewhere (138). After 22 h, 20 µl of [^{3}H]thymidine (0.1385 TBq/mmol; 3.75 Ci/mmol in media) was added to each well. At 24 h, cells were filtered through glass microfiber paper using a Brandel (Gaithersburg, MD) cell harvester. [^{3}H]Thymidine incorporated into cellular DNA was quantified by scintillation counting.

Annexin V and PI analysis. Annexin V and PI analyses were adapted from the technical data sheet for fluorescein isothiocyanate annexin V (556419; BD Biosciences PharMingen, San Diego, CA), with slight modifications. Treated cells were transferred to 1.5-ml microcentrifuge tubes, they were centrifuged at 1000 rpm for 10 min, and then the supernatant was aspirated. Cells were resuspended in 500 µl of buffer (10 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$,5mM KCl, and 1.8 mM $CaCl_2$, pH 7.4), and they were transferred to polystyrene test tubes. Five microliters of FITC annexin V was added, and the cells were vortexed and then incubated 15 min at room temperature (RT). Ten microliters of 50 µg/ml PI solution (Sigma-Aldrich, St. Louis, MO) was added to the cell suspension, and the mixture vortexed. Samples were analyzed using FACS-can (BD Biosciences, Franklin Lakes, NJ).

MTT assay. Cells were plated in 24-well plates. HepG2 and MDA-MB-231 cells were allowed to adhere overnight while K562 cells treated right away. Cells were treated with indicated compounds and incubated for 45 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. Three hours later, MTT stop solution

(HCl, triton X-100, and isopropyl alcohol) was added to all the wells and incubated with gentle agitation overnight at 37°C. Absorbance was measured at 540 nm with reference wavelength at 650 nm.

Western blotting. Protein concentrations were determined using the BCA method (Pierce Chemical, Rockford, IL). All proteins except poly(ADP-ribose) polymerase (PARP) were resolved by electrophoresis on a 12% gel, and they were transferred to a PVDF membrane. PARP was resolved using 7.5% gels. Primary and secondary antibodies were added sequentially for 45 min, and proteins were visualized using an ECL detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Anti pan-Ras was obtained from InterBiotechnology (Tokyo, Japan). Rap1a (sc-1482), Rab6 (sc-310), PARP (sc-7150), and α-tubulin (sc-8035) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Caspase-3 (9662) was obtained from Cell Signaling Technology Inc. (Danvers, MA). Horseradish peroxidase-conjugated anti-mouse secondary was obtained from GE Healthcare, and Horseradish peroxidase-conjugated anti-goat was obtained from Santa Cruz Biotechnology, Inc. After visualization, proteins were quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

TUNEL assay. Promega (Madison, WI) TUNEL assay kit (G3250) was performed according to the manufacturer's protocol. Cells were washed twice in PBS, resuspended in 0.5 ml of PBS, and fixed by the addition of 5 ml of methanol-free formaldehyde for 20 min on ice. Cells were washed twice, 5 ml of 70% ice-cold ethanol was added, and cells were stored overnight at –20°C. Washing in PBS was repeated, and cells were resuspended in 1 ml of PBS. The contents were centrifuged, and the cell pellet was resuspended in 80 µl of equilibrium buffer and incubated at RT for 5 min. The

contents were centrifuged, and cell pellet was resuspended with 50 µl of recombinant terminal deoxynucleotidyl transferase incubation buffer, incubated at 37°C for 1 h in the dark. Reactions were terminated by addition of 1 ml of 20 mM EDTA. Cells were washed twice in 1 ml of 0.1% Triton X-100 solution in PBS containing 5 mg/ml bovine serum albumin, resuspended in 0.5 ml of 5 µg/ml PI solution containing 250 µg of DNase-free RNase A, incubated at RT for 30 min, and finally analyzed by flow cytometry.

FPP and GGPP quantification. FPP and GGPP levels were determined as described previously (161). In brief, isoprenoid diphosphates were extracted, and they were used as substrates for incorporation into fluorescent CAAX peptides by farnesyltransferase or geranylgeranyl transferase. Prenylated fluorescent peptides were quantified by fluorescence detection.

Quantification of mRNA. Treated cells were lysed with Invitrogen (Carlsbad, CA) TRIzol reagent, and total RNA were extracted accordingly. An ABI reaction kit (Applied Biosystems, Foster City, CA) was used to synthesize the cDNA for each condition by reverse transcription. Primers for specific genes were mixed with SYBR Green intercalating dye, and they were added to cDNA. PCR was allowed to proceed for 40 cycles. Data were analyzed using ABI SDS 2.3 software (Applied Biosystems), normalized to 18S ribosomal RNA, and quantities were determined using the relative standard curve method as described by the manufacturer. Primers were designed using PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA). The PCR product was designed to be 200 to 400 base pairs, and primers were checked for specificity with Basic Local Alignment and Search Tool. The following primers were used: HMGCR (5′-

ACAGGCTTGAATGAAGCTTTGCCC-3′, 5′-GACATGCAGCCAAAGCAGCACATA-3′), FDPS (5′-CTTCCTGCAGAGTTCCTATCAGAC-3′,5′-TCTCCAGCAGGATCTTCTTGGCAT-3′), and SQS (5′-ACTTCCCAACGATCTCCCTTGAGT-3′,5′-TCCAAACCTCTTGAGGCCAGAACT-3′).

Isobologram and combination index analysis. Isobolograms were generated using Calcusyn software (Biosoft, Cambridge, UK). Combination index (CI) values were calculated according to the method of Chou and Talalay as described in the software manual (162). For each drug, 48-h IC_{50} values were determined by thymidine incorporation or MTT assay. Concentration-response curves were generated for each drug alone and in combination with other drug(s).

Statistical analysis. Unpaired two-tailed *t* tests were used to calculate statistical significance. Unless otherwise indicated, comparisons were done relative to the control. All columns in bar graphs represent the mean of the indicated number of replicates. Error bars on graphs represent S.E. An α level of 0.05 was selected as the level of significance.

Results

Inhibition of GGDPS inhibits growth and induces apoptosis in K562 cells. To determine whether GGDPS inhibition impairs growth of K562 human chronic myelogenous leukemia cells, cells were treated with DGBP for 48 h. The amount of cellular DNA synthesis was assessed by measuring levels of [^{3}H]thymidine incorporation (Figure 8A). DGBP inhibits growth of these cells, with an IC_{50} value of 55 μM. To determine whether this effect is due to cell death, cells were treated with DGBP, and total cell death was measured using annexin V and PI staining. DGBP induces a

concentration- and time-dependent increase in the number of apoptotic and necrotic cells (Figure 8B). This increase is observed in both the early apoptosis fraction (annexin V^+/PI^-) and the late apoptosis and necrosis fractions (annexin V^+/PI^+) (data not shown).

Inhibition of GGDPS leads to induction of the caspase cascade in K562 cells. To confirm the flow cytometric results, which showed that DGBP induces apoptosis, cells were treated with DGBP, and they were assessed for cleavage of PARP and caspase-3 by Western blot analysis. PARP is a DNA repair enzyme that is cleaved during apoptosis by executioner caspases such as activated caspase-3 (163). Caspases are cysteinyl aspartate-specific proteases that are activated by cleavage during early apoptosis (164). Etoposide (50 μM), which is a well-characterized activator of apoptosis, was used as a positive control. DGBP, like etoposide, induces time-dependent cleavage of PARP as demonstrated by the appearance of an 85-kDa band (Figure 8C). PARP cleavage is first observed at 24 h, and the effect is more pronounced after 72 h, analogous to what is observed with etoposide. DGBP treatment also leads to the cleavage and activation of caspase-3 as indicated by the appearance of a 17-kDa cleavage product. As expected, etoposide treatment results in caspase-3 cleavage. These results indicate that DGBP activates the apoptosis machinery, leading to caspase-3 activation, PARP cleavage, and eventual cell death.

Geranylgeraniol supplementation prevents DGBP-induced impairment of protein geranylgeranylation in K562 cells. To determine whether product supplementation prevented DGBP-mediated impairment of protein geranylgeranylation, cells were treated with GGOH, which is phosphorylated to form GGPP (165). Cells were treated for 48 h with DGBP in the presence or absence of GGOH, and then analyzed for impairment of

Rap1a and Rab6 geranylgeranylation by Western blot analysis (Figure 9). To maximize inhibition of prenylation and to limit apoptotic effects, DGBP was used at a concentration of 40 μM for this experiment. Both Rap1a and Rab6 are exclusively geranylgeranylated; Rap1a is a substrate for geranylgeranyl transferase (GGTase) I, whereas Rab6 is a substrate for GGTase II. It should be noted that the antibody for Rap1a is for the C terminus, and as such, it detects only the unmodified form. The antibodies for Rab6 and Ras detect both modified and unmodified forms.

As positive controls, cells were treated with lovastatin and zoledronate, which deplete cells of FPP and GGPP, and therefore limit farnesylation and geranylgeranylation. Cells were also treated with FOH (lane 2) and GGOH (lane 3), neither of which limits prenylation. Like GGOH, FOH is phosphorylated to form FPP. As expected, 20 μM lovastatin limits Ras farnesylation and Rap1a and Rab6 geranylgeranylation (lane 5). Lovastatin-induced limitation of Ras farnesylation is prevented by addition of 1 mM mevalonate (lane 6) or 10 μM FOH (lane 7), but not 10 μM GGOH (lane 8). Lovastatin-induced limitation of geranylgeranylation is prevented by addition of mevalonate (lane 6) or GGOH (lane 8), but not FOH (lane 7). Mevalonate alone does not affect prenylation (data not shown). Concentrations of 120 μM zoledronate limits farnesylation and geranylgeranylation (lane 10), and the effect on each is prevented by addition of 10 μM FOH and 10 μM GGOH, respectively (lanes 11 and 12). Treatment with 40 μM DGBP does not affect farnesylation, but it inhibits geranylgeranylation of both the GGTase I substrate Rap1a and GGTase II substrate Rab6 (lane 14), an effect that is completely reversed by addition of 10 μM GGOH (lane 15).

Unlike HMG-CoA reductase inhibition, apoptosis induced by GGDPS inhibition is not fully reversed by product supplementation in K562 cells. To determine whether product supplementation prevented the apoptosis induced by inhibition of GGDPS as indicated by PARP and caspase-3 cleavage, cells were treated with 100 μM DGBP for 72 h (Figure 10A). As controls, cells were treated with lovastatin, zoledronate, or GGTI-298, a peptidomimetic inhibitor of geranylgeranyl transferase I. It was unexpected that the addition of 10 μM exogenous GGOH did not prevent 100 μM DGBP from inducing apoptotic effects (lanes 5 and 9). In fact, addition of 10 μM GGOH to 100 μM DGBP enhances the cleavage of both PARP and caspase-3, whereas GGOH had no effects on PARP and caspase-3 on its own (lane 7). This contrasts to 50 μM lovastatin-induced PARP and caspase-3 cleavage, which is completely prevented by the addition of 1 mM mevalonate (lanes 3 and 8). Treatment with 250 μM zoledronate (lane 4) results in minor amounts of cleavage, whereas 20 μM GGTI-298 does not induce any PARP and caspase-3 cleavage (lane 6). We performed a TUNEL assay to detect DNA fragmentation that results from the apoptotic signaling cascade, using the same conditions (Figure 10B). As with PARP and caspase-3 cleavage, 50 μM lovastatin induces DNA fragmentation (column 3) that is prevented by the addition of 1 mM mevalonate (column 8). On the other hand, 10 μM GGOH (column 9) is not able to prevent DNA fragmentation resulting from 100 μM DGBP (column 5). GGOH (10 μM) (column 7) and 20 μM GGTI (column 6) do not lead to DNA fragmentation. Zoledronate (250 μM) (column 4) induces DNA fragmentation but not as well as DGBP or lovastatin. Mevalonate alone has no effect on cleavage of PARP or caspase-3 and DNA fragmentation (data not shown).

Inhibition of GGDPS leads to apoptosis through two different mechanisms in K562 cells. To further explore the effects seen by combinations of DGBP and GGOH, cells were treated with low and high concentrations of DGBP, and levels of intracellular FPP and GGPP were measured (Figure 11). Treatment with DGBP concentration-dependently decreases GGPP, whereas it increases FPP. GGOH partially reverses both the increase in FPP and decrease in GGPP.

Previous studies have shown that farnesol and geranylgeraniol induce apoptosis (166). Isoprenoid alcohols, but not diphosphates, inhibit choline phosphotransferase leading to depletion of phosphatidylcholine (PC) (167, 168). Cells were treated with low (25 μM) or high (100 μM) DGBP in the presence or absence of GGOH (10 μM) or PC (100 μM) (Figure 12). GGOH (10 μM) prevents apoptotic effects caused by low (25 μM) DGBP (Figure 12, A and B). Although 10 μM GGOH completely prevents staining, 100 μM PC doers not significantly impair annexin V and PI staining (Figure 12A, columns 4–6) and PARP cleavage (Figure 12B, lanes 4–6) that is caused by 25 μM DGBP. However, PC partially impairs the apoptotic effects caused by high (100 μM) DGBP (Figure 12, A and C). Addition of 100 μM PC prevents some annexin V and PI staining (Figure 12A, columns 7 and 9) and most PARP cleavage (Figure 12C, lanes 4 and 6) caused by 100 μM DGBP. In contrast, the addition of 10 μM GGOH to 100 μM DGBP leads to enhanced annexin V and PI staining (Figure 12A, columns 7 and 8) and PARP cleavage (Figure 12C, lanes 4 and 5). Etoposide-induced apoptotic effects are not significantly altered by the addition of PC (Figure 12, A–C, lanes/columns 2 and 3). Annexin V and PI staining and PARP cleavage are not affected by 10 μM GGOH or 100 μM PC (data not shown).

Another GGDPS inhibitor (compound 14) induces apoptosis in K562 cells. In order to determine whether induction of apoptosis through the inhibition of GGDPS is drug specific (due to DGBP specifically), we utilized compound 14 (see chapter two of this thesis) to quantify apoptosis by Annexin V and PI analysis (Figure 13). As expected, the positive control etoposide (50 μM) induces apoptosis as measured by Annexin V and PI staining. The addition of 100 μM of compound 14 did not induce apoptosis at 48 hours while significant cell death due to this compound is observed at 72 hours. Co-incubation with 20 μM GGPP completely prevents the effects of compound 14 on induction of apoptosis as measured by Annexin V and PI staining.

Inhibition of GGDPS alters steady-state mRNA levels of isoprenoid biosynthetic enzymes in K562 cells. Because some of the effects of DGBP seemed to result from accumulation of FPP, whose downstream sterol products are known to regulate the isoprenoid biosynthetic machinery through transcriptional events (169), we hypothesized treatment with DGBP would result in decreased mRNA expression of HMGCR, FDPS, and SQS. Cells were treated with DGBP, and then they were analyzed for altered expression of enzymes involved in isoprenoid biosynthesis via quantitative real-time PCR (Table 1). As characterized previously, treatment with either lovastatin or zaragozic acid results in increased mRNA expression of HMGCR, FDPS, and SQS. In agreement with our hypothesis, treatment of K562 cells with DGBP results in decreased expression of HMGCR, FDPS, and SQS mRNA.

DGBP is synergistic with lovastatin and zoledronate in K562 cells. To further examine the consequences of GGDPS inhibition in the context of other drugs that impair protein geranylgeranylation, cells were evaluated for synergistic interactions between

DGBP and either lovastatin, zoledronate, or GGTI-298. For these studies, concentration-response curves for each compound were constructed using [^{3}H]thymidine incorporation at the 48-h time point. All drugs were tested alone or in combination with constant ratios of DGBP according to the method of Chou and Talalay (162). The data were analyzed in two ways. First, isobologram analyses were performed (Figure 14). A general isobologram with a line of additivity and regions of synergy and antagonism is shown (Figure 14A). Lovastatin and DGBP are strongly synergistic (CI_{50} = 0.03) for inhibition of K562 cell growth (Figure 14B). Zoledronate is also synergistic with DGBP (CI_{50} = 0.84) (Figure 14C), but not to the extent of the synergy observed between DGBP and lovastatin. GGTI-298 is antagonistic to DGBP (CI_{50} = 1.64) (Figure 14D). Although isobologram analysis can determine whether a combination of compounds is synergistic and if so approximate the magnitude of the synergistic interaction, it does not *per se* quantify this at the experimental concentrations. Therefore, the CI values for each experimental combination of DGBP with lovastatin or zoledronate were calculated (Table 2). At all of the experimental concentrations tested, lovastatin and DGBP display stronger synergy than the combination of zoledronate and lovastatin (CI_{50} = 0.64). This result is observed in RPMI-8226 and U937 cells as well (data not shown). To further support the synergy observed in [^{3}H]thymidine incorporation assays, CI analysis was performed after annexin V and PI staining on cells treated with combinations of lovastatin and DGBP (Table 3). Similar to the [^{3}H]thymidine incorporation assays, the annexin V and PI staining assays revealed a synergistic interaction toward total cell death (CI_{50} = 0.01). More specifically, these assays also demonstrated a synergistic interaction occurs both for cells in early (CI_{50} = 0.76) and late apoptosis (CI_{50} = 0.50).

DGBP potentiates lovastatin-induced impairment of protein geranylgeranylation in K562 cells. Because both lovastatin and DGBP impair protein geranylgeranylation through different but overlapping mechanisms, combinations of the two compounds were tested for the ability to impair protein geranylgeranylation (Figure 15). Cells were treated with lovastatin and DGBP alone or in combination. To observe synergistic effects, it was necessary to use lower concentrations of lovastatin and DGBP. Lovastatin and DGBP exhibited submaximal impairment of geranylgeranylation at concentrations below 10 and 20 μM, respectively. Concentrations of 1.1 μM lovastatin and 2.2 μM DGBP have minimal effects, whereas the combination of these concentrations is sufficient to impair geranylgeranylation (lanes 3, 7, and 11). This effect is also pronounced at concentrations of 3.3 μM lovastatin and 6.6 μM DGBP (lanes 4, 8, and 12). It is noteworthy that the combination of lovastatin and DGBP has an antagonistic effect on impairment of Ras farnesylation. Although lovastatin causes the accumulation of an unmodified upper Ras band (lanes 4 and 5), DGBP does not (lanes 8 and 9). When combined, DGBP prevents the inhibition of Ras farnesylation induced by lovastatin (lanes 12 and 13). Quantification of the Rap1a bands and Rab6 upper bands further supports the synergistic interaction of lovastatin and DGBP toward impairment of geranylgeranylation.

Combination of DGBP and lovastatin enhances depletion of GGPP in K562 cells. Because both DGBP and lovastatin deplete GGPP through different but overlapping mechanisms, combinations of the two compounds were tested for the ability to deplete cellular GGPP (Figure 16). As previously shown, both of the compounds decrease intracellular levels of GGPP. Interestingly, the combination of the two drugs enhances GGPP

depletion. The enhanced depletion of GGPP appears to be additive and not synergistic with the drug combination.

Lovastatin and DGBP are synergistic in adherent cancer cells (HepG2 and MDA-MB-231). To determine whether the synergy between lovastatin and DGBP is specific to suspension cells (K562), combination studies were performed in two adherent cell lines, HepG2 (hepatocellular carcinoma cell line) and MDA-MB-231 (breast cancer cells). Drugs were added alone or in combination and cellular viability was assessed by the MTT assay after 48 hour. The resulting data was again analyzed by the isobologram method (Figure 17). Similar to K562 cells, the combination of lovastatin and DGBP is extremely synergistic in HepG2 (Figure 17A) and MDA-MB-231 (Figure 17B) cells.

Lovastatin is synergistic with compound 14 in K562 cells. In order to determine whether the synergy between a HMGCR inhibitor (lovastatin) and a GGDPS inhibitor (DGBP) is drug specific, compound 14 (see chapter two of this thesis) was utilized to assess for synergy with lovastatin. Drugs were added alone or in combination and cell viability was assessed by the MTT assay after 48 hour. The resulting data was again analyzed by the isobologram method. Similar to the synergy observed with lovastatin and DGBP, the combination of lovastatin and compound 14 is extremely synergistic in K562 cells (Figure 18).

Discussion

We have now shown for the first time that inhibition of GGDPS leads to growth inhibition and induces apoptosis. This demonstrates that like many of its counterparts in the mevalonate pathway, including HMG-CoA reductase (170), FDPS (156), and protein prenyltransferases (171), inhibition of GGDPS may be a viable therapeutic strategy.

Inhibition of GGDPS has two consequences, depletion of cellular GGPP and an accumulation of FPP. At any concentration of DGBP, the impairment of protein geranylgeranylation can be prevented by addition of exogenous GGOH. At low amounts of GGDPS inhibition, apoptotic consequences are prevented by exogenous GGOH. This agrees with previous studies that demonstrated that GGOH reverses apoptosis induced by the clinical NBPs (172). At greater GGDPS inhibition, apoptotic effects are not prevented by exogenous GGOH, rather they are enhanced. It is possible that increased cellular FPP (and FOH) levels caused by GGDPS inhibition in the presence of exogenous GGOH are high enough to inhibit cellular cholinephosphotransferase (168). Our data support this conclusion because apoptosis induced by high concentrations of DGBP can be partially prevented by addition of exogenous PC. It is possible that PC cannot fully prevent apoptosis because it does not prevent the impairment of protein geranylgeranylation caused by GGDPS inhibition. We propose a model that demonstrates the mechanisms by which GGDPS inhibition induces apoptosis (Figure 19).

In addition to inhibition of PC synthesis, there are at least three other well characterized consequences of FPP or FOH accumulation that are relevant to regulation of isoprenoid biosynthesis. First, FPP is converted to squalene and eventually cholesterol, which when elevated decreases transcription of the enzymes responsible for its synthesis (169). Our results clearly show decreased mRNA levels of three of these enzymes, HMGCR, FDPS, and SQS associated with FPP accumulation caused by GGDPS inhibition. The fact that these changes were seen in media containing serum is consistent with the requirement of endogenous sterol synthesis to regulate gene expression. However, further studies of GGDPS in serum-free media may reveal more

dramatic changes. Second, FPP and FOH have been shown conclusively to increase HMGCR degradation in pulse-chase experiments (173). Third, both FPP and FOH have been shown to inhibit the mevalonate kinase reaction (174).

We have also shown for the first time that inhibition of GGDPS, in combination with inhibition of either HMGCR or FDPS, results in synergistic inhibition of cell growth and induction of apoptosis. It is possible that both the FPP accumulation and GGPP depletion caused by GGDPS inhibition serve to enhance lovastatin-induced apoptosis. In contrast, lovastatin and zoledronate both similarly deplete FPP and GGPP and lack the apoptotic effects caused by FPP accumulation. This line of reasoning also explains why the combination of zoledronate and DGBP is more synergistic than the combination of zoledronate and lovastatin.

The data presented herein suggest that inhibition of GGDPS may be a good anti-cancer therapeutic strategy either alone on in combination with other isoprenoid pathway inhibitors. DGBP may be useful alone or in combination with statins for inhibition of cancer cell growth or other cellular processes that are dependent upon geranylgeranylation. Ongoing studies will further evaluate effects of GGDPS inhibition on cancer and bone disorders.

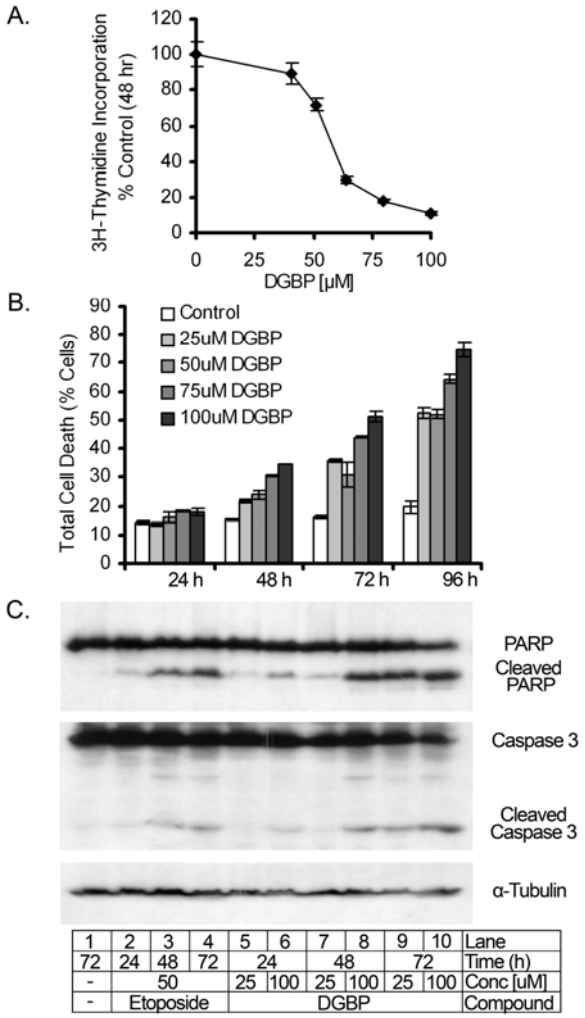


Figure 8. GGDPS inhibition induces growth inhibition and apoptosis in K562 cells. Cells were seeded and treated with different concentrations of DGBP. (A) Growth as a percentage of untreated control cells at 48 h was evaluated by [^{3}H]thymidine incorporation (mean ± S.E.; n = 4). (B) Total apoptosis at varying times was determined by annexin V and PI staining (mean ± S.E.; n = 2). (C) Induction of PARP and caspase-3 cleavage at varying times was determined by Western blot analysis.

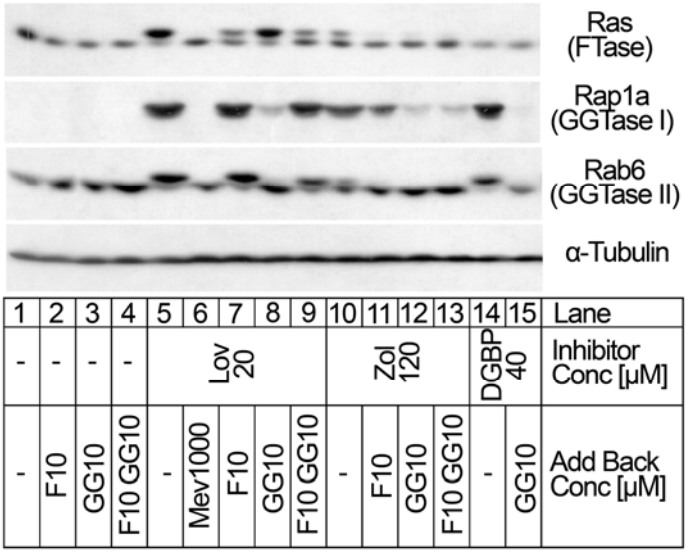


Figure 9. Impairment of geranylgeranylation by DGBP is prevented by addition of exogenous GGOH in K562 cells. Cells were seeded and treated with DGBP or other inhibitors of isoprenoid synthesis alone or with selective add backs of the reaction product(s). Inhibition of protein prenylation is shown for K562 cells treated with DGBP or other inhibitors of isoprenoid synthesis for 48 h as determined by Western blot analysis.

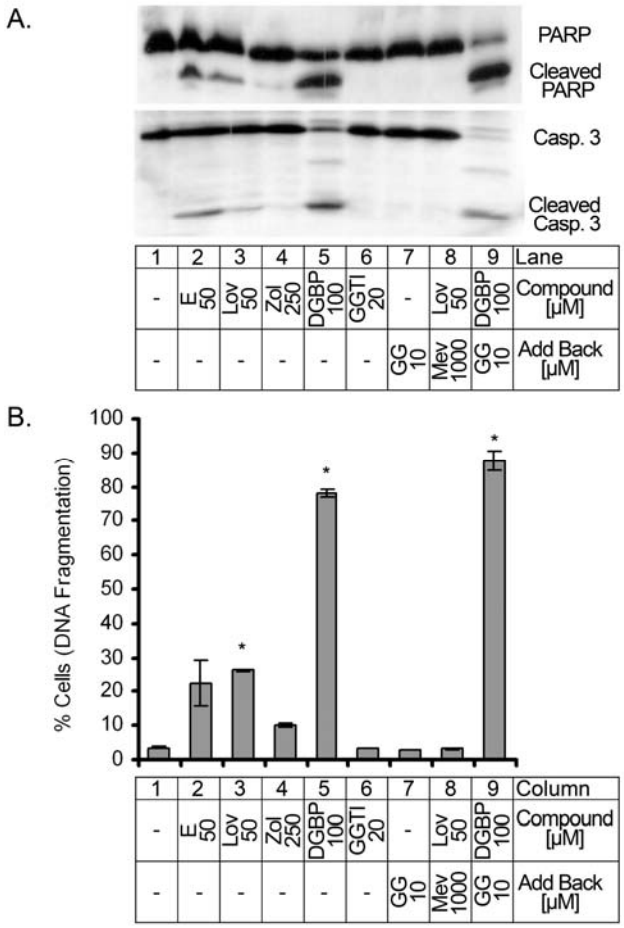


Figure 10. Apoptosis induced by high concentrations of DGBP is not prevented by exogenous GGOH in K562 cells. Cells were seeded and treated with DGBP or other inhibitors of isoprenoid synthesis alone or with selective add backs of the reaction product for 72 h. (A) Induction of PARP and caspase-3 cleavage as determined by Western blot analysis. One representative Western blot is shown (n = 3). (B) DNA fragmentation as determined by TUNEL assay (mean ± S.E.; n = 2; *, $p < 0.05$ relative to control).

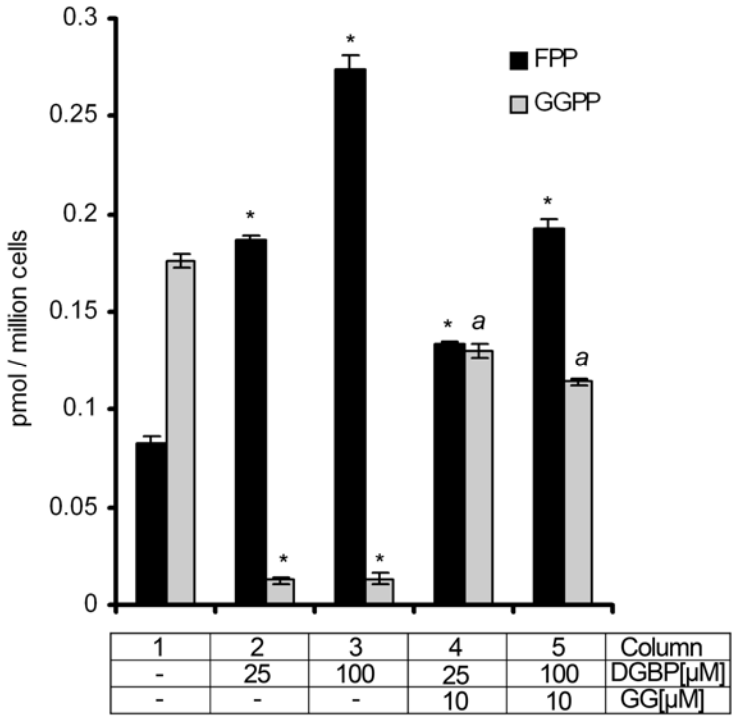


Figure 11. Inhibition of GGDPS leads to GGPP depletion and FPP accumulation. Cells were treated for 48 h with varying concentrations of DGBP in the presence or absence of exogenous GGOH. Total amounts of intracellular FPP and GGPP were measured as described. Amounts were expressed relative to the number of cells at the end of the incubation (mean ± S.E.; n = 2; *, $p < 0.05$ relative to control levels of each isoprenoid; *a,* $p < 0.05$ relative to DGBP treatment).

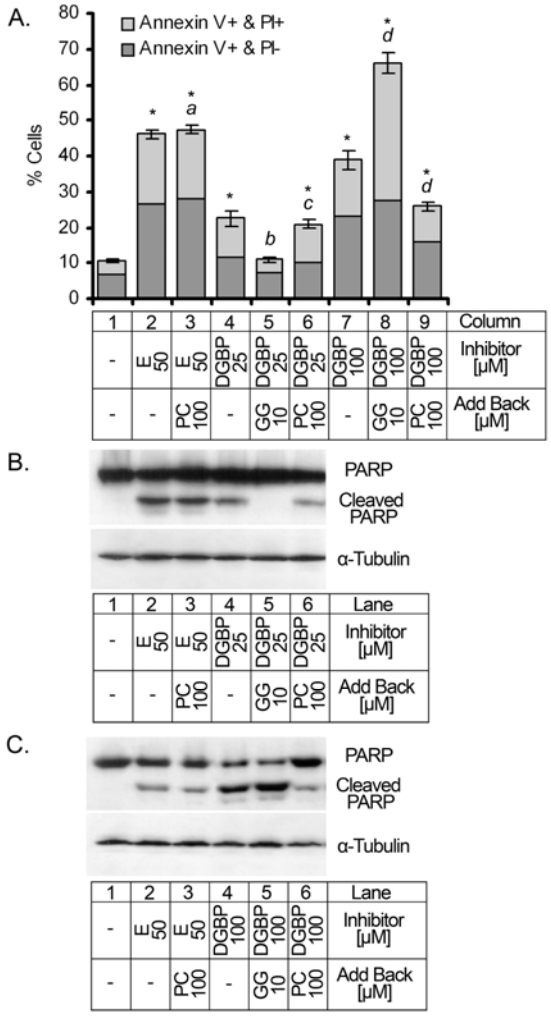


Figure 12. The mechanism of DGBP-induced apoptosis is concentration-dependent in K562 cells. Cells were treated with different concentrations of DGBP in the presence or absence of exogenous GGOH or PC for 72 h. (A) Total apoptosis as determined by annexin V and PI staining (mean ± S.E.; n = 4; *, $p < 0.05$ relative to control; *a*, $p = 0.58$ relative to 50 μM etoposide, *b*, $p < 0.05$ relative to 25 μM DGBP, *c*, $p = 0.37$ relative to 25 μM DGBP, and *d*, $p < 0.05$ relative to 100 μM DGBP). Total apoptosis is expressed as the sum of early apoptosis (annexin V^+/PI^-) and late apoptosis/necrosis (annexin V^+/PI^+) fractions. (B) Western blot analysis in cells treated with low (25 μM) DGBP concentration. (C) Western blot analysis in cells treated with high (100 μM) DGBP concentration.

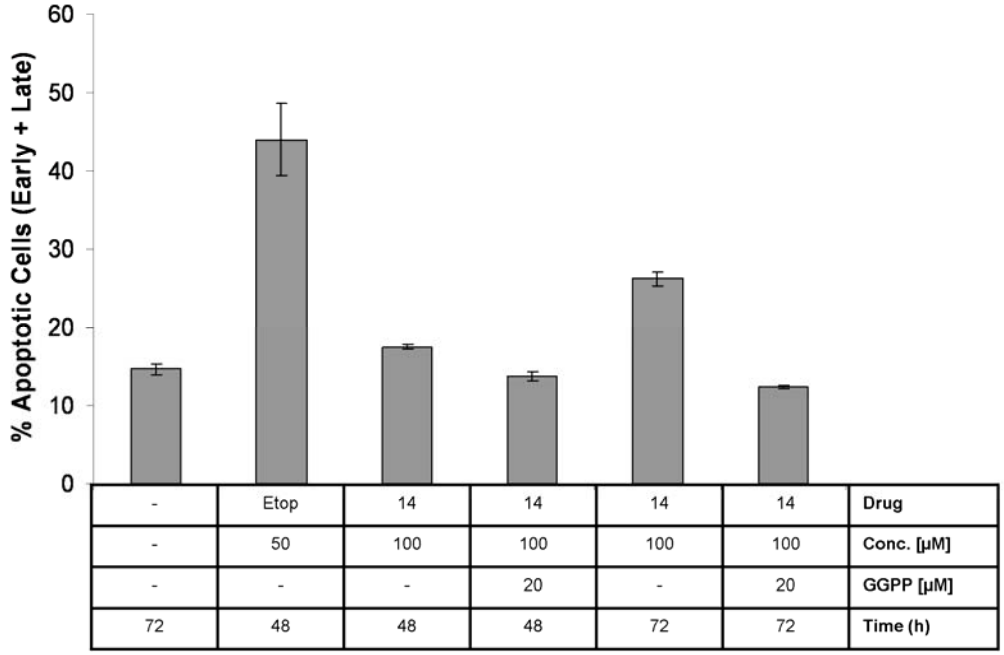


Figure 13. Compound 14 induces apoptosis in K562 cells. Cells were treated with indicated compounds (etoposide, compound 14, and GGPP) at indicated times and concentrations. Annexin V/PI analysis was performed to assess for the induction of apoptosis (mean ± S.E.; n = 3). Early and late apoptotic cells were combined (y-axis).

	Lovastatin [20 μM]	Zaragozic Acid [5 μM]	DGBP [10 μM]
HMGCR	1.98 +/- 0.21	1.92 +/- 0.15	0.15 +/- 0.04
FDPS	1.87 +/- 0.21	1.66 +/- 0.14	0.28 +/- 0.06
SQS	1.52 +/- 0.06	1.87 +/- 0.34#	0.12 +/- 0.06

Table 1. Differential effects of lovastatin, zaragozic acid, and DGBP on expression of HMGCR, FDPS, and SQS mRNA in K562 cells. Cells were treated with compounds for 48 h and mRNA levels were determined by real-time PCR. Data are mean ± S.E. (n = 4). All treatments are significantly different from control (except #).

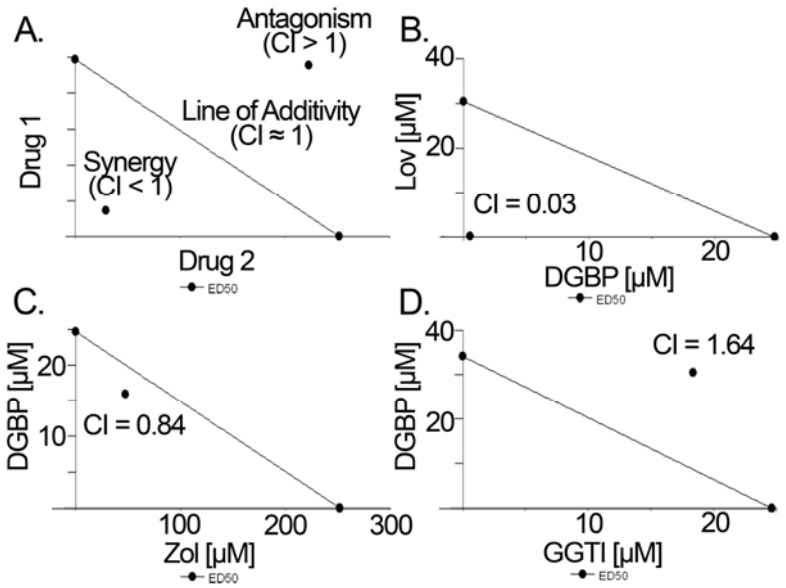


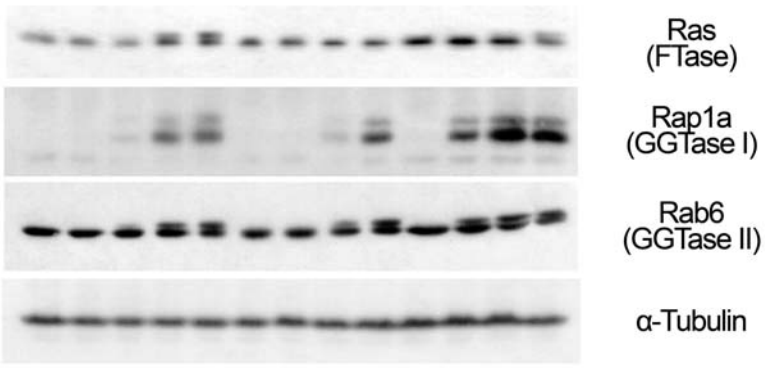
Figure 14. GGDPS inhibition is synergetic with HMGCR and FDPS inhibition, but it is antagonistic to GGTase inhibition in K562 cells. Cells were treated with varying concentrations of inhibitors alone or in indicated combinations at constant ratios for 48 h. Cell growth was assessed by [^{3}H]thymidine incorporation. Isobologram analysis was conducted as described. Isobolograms shown were generated from cells treated with five different concentrations of each drug or combination (n = 4). (A) Idealized isobologram analysis displays line of additivity, and regions of synergy and antagonism. (B) Experimental isobologram analysis of DGBP in combination with lovastatin. (C) Isobologram of DGBP and zoledronate. (D) Isobologram of DGBP and GGTI-298.

Combination Index (CI) for Experimental Values											
Lov [μM]	DGBP [μM]	CI	DGBP [μM]	GGTI [μM]	CI	DGBP [μM]	Zol [μM]	CI	Lov [μM]	Zol [μM]	CI
20	41	0.25	20	12	1.57	41	123	0.79	20	123	0.65
26	51	0.22	26	15	2.00	51	154	0.52	26	154	0.75
32	64	0.27	32	19	1.90	64	192	0.49	32	192	0.86
40	80	0.34	40	24	1.68	80	240	0.62	40	240	0.88

Table 2. Experimental combination indices for the [^{3}H]thymidine incorporation assay in K562 cells. Cells were treated with DGBP in combination with various inhibitors of isoprenoid biosynthesis for 48 h (n = 4).

Combination Index (CI) for Experimental Values				
Lov [μM]	DGBP [μM]	Early Apoptosis	Late Apoptosis	Total Cell Death
6.25	6.25	0.15	0.01	0.05
12.5	12.5	0.28	0.03	0.11
25	25	0.45	0.15	0.30
50	50	0.46	0.18	0.28

Table 3. Experimental combination indices for Annexin V and PI staining in K562 cells. Cells were treated with combinations of DGBP and lovastatin for 72 h (n = 2).



1	2	3	4	5	6	7	8	9	10	11	12	13	Lane
-	0.4	1.1	3.3	10	-	-	-	-	0.4	1.1	3.3	10	Lov[μM]
-	-	-	-	-	0.8	2.2	6.6	20	0.8	2.2	6.6	20	DGBP[μM]
0	2	6	33	41	1	1	9	41	2	46	100	82	Rap1a Inh (%)
0	0	15	62	65	2	2	34	82	0	78	100	61	Rab6 Inh (%)

Figure 15. Combinations of DGBP and lovastatin synergistically impair protein geranylgeranylation in K562 cells. Cells were treated with DGBP or lovastatin at various submaximal concentrations alone or in combination for 48 h. Inhibition of protein prenylation is shown as determined by Western blot analysis. Ras is exclusively farnesylated in these cells, Rap1a is geranylgeranylated by GGTase I, and Rab6 is geranylgeranylated by GGTase II (see text for full explanation). Unmodified bands were quantified using ImageJ as described. Rap1a and Rab6 inhibition values were expressed as a percentage of the maximal obtained inhibition (% max).

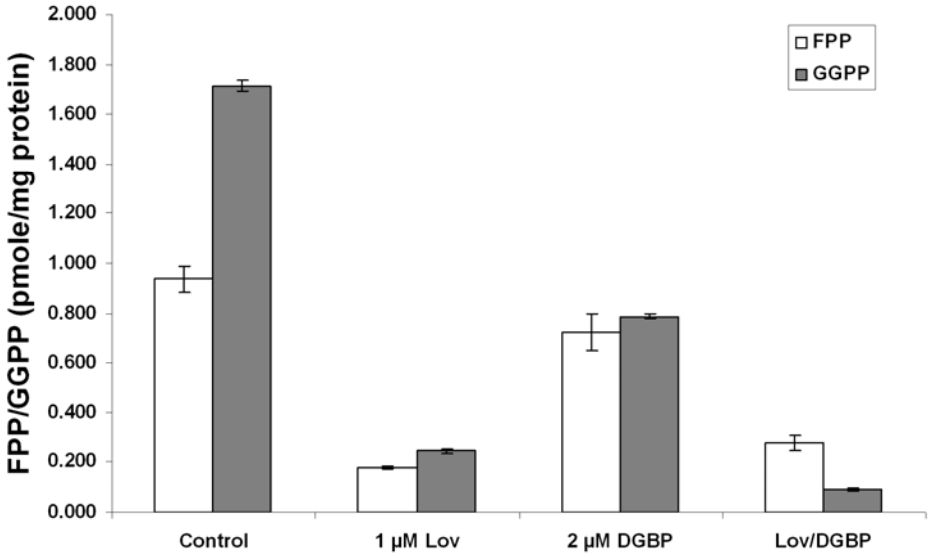


Figure 16. Combination of DGBP and lovastatin enhances depletion of GGPP in K562 cells. Cells were treated for 24 h with compounds as described. Total amounts of intracellular FPP and GGPP were measured as described. Amounts were expressed relative to the protein concentration at the end of the incubation (mean ± S.E.; n = 2).

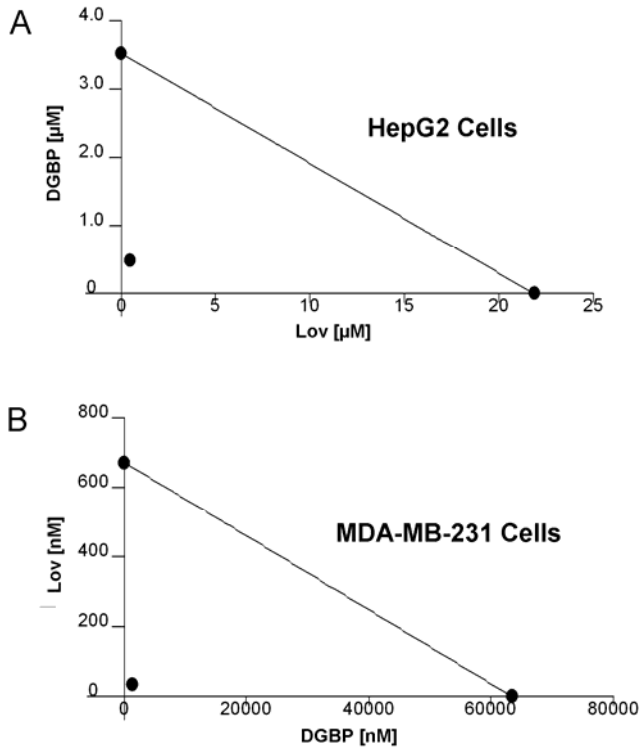


Figure 17. GGDPS inhibition is synergetic with HMGCR inhibition in HepG2 and MDA-MB-231 cells. Cells were treated with varying concentrations of inhibitors alone or in indicated combinations at constant ratios for 48 h. Cell viability was assessed by MTT assay. Isobologram analysis was conducted as described. The isobolograms shown were generated from cells treated with five different concentrations of each drug or combination (ED_{30}, n = 3). (A) Isobologram of lovastatin and DGBP in HepG2 cells. (B) Isobologram of lovastatin and DGBP in MDA-MB-231 cells.

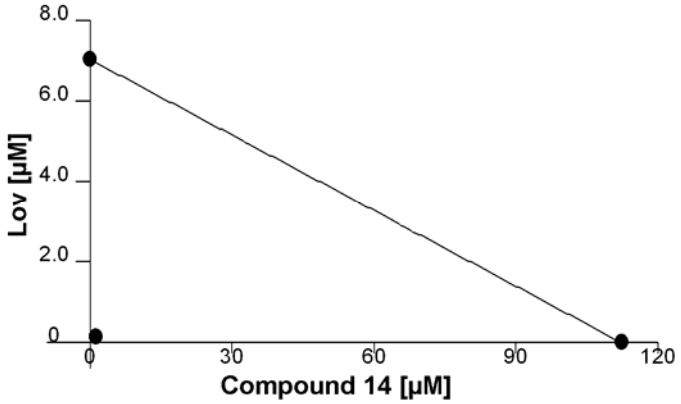


Figure 18. Lovastatin is synergistic with compound 14 in K562 cells. Cells were treated with varying concentrations of inhibitors alone or in indicated combinations at constant ratios for 48 h. Cell viability was assessed by MTT assay. Isobologram analysis was conducted as described. Isobologram shown was generated from cells treated with five different concentrations of each drug or combination (ED_{25}, n = 3).

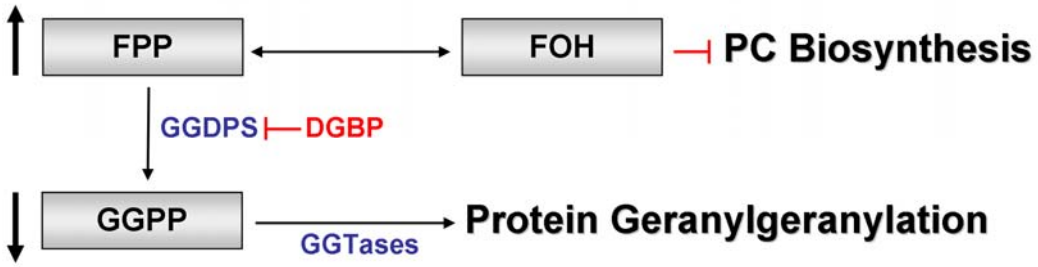


Figure 19. Proposed mechanism by which GGDPS inhibition induces apoptosis. GGDPS inhibition induces apoptosis by: 1) depleting cellular GGPP and thus inhibiting protein geranylgeranylation and 2) increasing FPP that is converted to FOH, which has been reported to interfere with phosphatidylcholine (PC) biosynthesis.

CHAPTER IV: GGDPS INHIBITION INTERFERES WITH BREAST CANCER CELL MIGRATION

Abstract

The isoprenoid pathway is highly targeted in the treatment of disease. Statins and nitrogenous bisphosphonates are used for the treatment of hypercholesteremia and bone diseases, respectively. Geranylgeranyl diphosphate synthase is an isoprenoid pathway enzyme responsible for the synthesis of geranylgeranyl diphosphate, a critical isoprenoid pathway intermediate utilized during geranylgeranylation, a post-translation modification of proteins. Rho family GTPases, which undergo protein geranylgeranylation, are key mediators of cell migration. Geranylgeranylation is essential for proper membrane localization and function of these small GTPases. Over-expression of Rho family members is associated with more aggressive disease and poor prognosis for breast cancer patients. The goal of this study was to determine whether geranylgeranyl diphosphate synthase inhibition, and therefore geranylgeranyl diphosphate depletion, interferes with breast cancer cell migration. Digeranyl bisphosphonate, a specific geranylgeranyl diphosphate synthase inhibitor, was used in this study. Digeranyl bisphosphonate depletes geranylgeranyl diphosphate and impairs protein geranylgeranylation in MDA-MB-231 cells. Similar to GGTI-286, a GGTase I inhibitor, digeranyl bisphosphate significantly inhibits migration of MDA-MB-231 cells as measured by transwell assay. Similarly, digeranyl bisphosphonate and compound 14, an aromatic bisphosphonate inhibitor of geranylgeranyl diphosphate synthase, reduce motility of MDA-MB-231 cells in a time-dependent manner as measured by large scale digital cell analysis system microscopy. Digeranyl bisphosphonate is mildly toxic and does not induce apoptosis at

relevant concentrations in MDA-MB-231 cells. Treatment of MDA-MB-231 cells with digeranyl bisphosphonate decreases membrane localization of RhoA while increasing its cytosolic localization. In addition, digeranyl bisphosphonate increases RhoA GTP binding in MDA-MB-231 cells. The specificity of geranylgeranyl diphosphonate synthase inhibition by digeranyl bisphosphonate is confirmed by exogenous addition of geranylgeranyl diphosphate. Geranylgeranyl diphosphate addition prevents the effects of digeranyl bisphosphonate on migration, RhoA localization, and GTP binding to RhoA in MDA-MB-231 cells. These studies suggest that geranylgeranyl diphosphate synthase inhibitors are an approach to interfere with cancer cell migration.

Introduction

The isoprenoid pathway (Figure 1) is frequently targeted for the treatment of disease. Statins such as lovastatin, which inhibit HMG-CoA reductase (HMGCR), are used clinically for the treatment of hypercholesteremia (2, 153). Nitrogenous bisphosphonates (NBPs) are a second class of isoprenoid pathway inhibitors utilized in the clinic. It has been demonstrated that clinically used NBPs inhibit farnesyl diphosphate synthase (FDPS) (95). NBPs such as zoledronate are widely used in the treatment of bone disorders such as osteoporosis and metastatic bone disease (144).

Farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are fifteen and twenty carbon isoprenoid pathway intermediates, respectively. FPP, which is at the major branch point in the isoprenoid pathway, serves as a precursor for many pathway intermediates, including GGPP. The isoprene moieties from FPP and GGPP are post-translationally incorporated into many proteins during prenylation, farnesylation and geranylgeranylation (20). Members of the Ras and Rho families of small GTPases are

examples of proteins that undergo farnesylation and geranylgeranylation, respectively (175). Ras proteins play a role in cell growth and proliferation while Rho proteins are important mediators of cell motility (176). Mutations or over-expression of these small GTPases can promote oncogenic events such as increased proliferation and migration (52, 177). It is believed that farnesylation and geranylgeranylation are essential for proper membrane localization and thus function of small GTPases (21).

Direct impairment of protein prenylation by farnesyltransferase inhibitors (FTIs) and geranylgeranyl transferase inhibitors (GGTIs) has anti-cancer properties in cellular and animal models (21). Impairment of protein farnesylation and geranylgeranylation can also be accomplished by the depletion of cellular FPP and GGPP, respectively. Due to their upstream site of action, statins and NBPs deplete cellular FPP and GGPP and thus impair protein prenylation. Statins and NBPs have been shown to induce apoptosis, inhibit cancer cell migration, and induce cell cycle arrest (21). Anti-cancer effects of statins and NBPs can be prevented by the addition of exogenous GGPP (121, 136, 178), suggesting that GGPP depletion may be the mechanism by which statins and NBPs interfere with cancer progression.

Our laboratory and collaborators have developed bisphosphonate inhibitors of geranylgeranyl diphosphate synthase (GGDPS) (138-140, 150). GGDPS is the enzyme responsible for the production of GGPP from FPP and isopentenyl diphosphate, an upstream isoprenoid pathway intermediate. Our lead compound, digeranyl bisphosphonate (DGBP), specifically inhibits GGDPS resulting in disruption of geranylgeranylation but not farnesylation of proteins (139). Methods developed in our

laboratory show that GGDPS inhibition depletes GGPP while increasing FPP levels in cultured cells and some mammalian tissues (143, 179).

Breast cancer is one of the most prevalent malignancies in the world. Tissues such as axillary lymph nodes, lungs, spinal cord, brain, and bones are major metastasis targets for breast cancer cells (180). Several Rho family members, which are geranylgeranylated, have been implicated as important for breast cancer progression (Table 4). It has been shown that RhoA is over-expressed in breast cancer and its expression correlates with more advanced breast carcinoma (55). In addition, increased expression of RhoC was found in small breast carcinoma (56, 57). Finally, malignant breast cancer tissues express higher levels of Rac1 when compared to benign tissues (181).

Previous studies have shown that direct impairment of protein geranylgeranylation by GGTIs interferes with migration of some cancer cells (107). In addition, exogenous addition of GGPP prevents inhibition of migration induced by statins (136). Finally, dual inhibition of FDPS and GGPDS has been demonstrated to inhibit migration of breast cancer cells (152). In this study, we show that sole inhibition of GGDPS, and therefore GGPP depletion, interferes with migration of highly invasive breast cancer cells.

Materials and Methods

Cell culture. MDA-MB-231 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in minimum essential medium supplemented with 10% FBS, penicillin and streptomycin, L-glutamine, amphotericin B, and sodium pyruvate at 5% CO_2 and 37°C.

Preparation of cell lysates. At the end of each experiment, media was removed and cells were washed twice in phosphate buffered saline. Cells were lysed with a cell scraper after the addition of radioimmunoprecipitation buffer supplemented with protease inhibitor cocktail, sodium vanadate, sodium fluoride, and phenylmethylsulphonyl fluoride. Lysates were transferred to a 1.5 ml tube, vortexed several times over 30 min, and passed through a 27-gage needle. Lysates were then centrifuged and supernatant transferred to a fresh 1.5 ml tube. All steps were performed at 4°C.

Western blotting. Protein concentrations were determined by the BCA method. Proteins were resolved on 7.5 or 12% gels and transferred to PVDF membranes by electrophoresis. Primary and secondary antibodies were added sequentially for 45 min and proteins visualized using an ECL detection kit from GE Healthcare (Buckinghamshire, UK). Anti pan-Ras was obtained from InterBiotechnology (Tokyo, Japan). Rap1a, PARP, and αTub antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). RhoA antibody was obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated anti-mouse and anti-goat were from GE Healthcare while anti-goat was from Santa Cruz Biotechnology, Inc.

FPP and GGPP quantification. FPP and GGPP levels were determined as described previously (161). Briefly, FPP and GGPP were extracted and used as substrates for incorporation into fluorescent CAAX peptides by farnesyl transferase and geranylgeranyl transferase. Prenylated fluorescent peptides were quantified by fluorescent detection. FPP and GGPP levels were normalized to protein levels measured by BCA assay.

Transwell migration assay. 5×10^4 cells were allowed to adhere in 8 μm transwell inserts from Corning Inc. (Corning, NY, USA) for 24 h. 10% FBS media was replaced with 200 μl of serum-free media containing GGTI-286, DGBP, and GGPP. 24 h later, 600 μl of 10% FBS media containing GGTI-286, DGBP, and GGPP was added to the bottom chamber. Cells were allowed to migrate to the other side of the transwell for 24 h. Non-migrated cells (top of the transwell) were gently removed with a cotton swab. Migrated cells were stained with Diff-Quik® solutions from Dade Behring Inc. (Newark, DE, USA). Membranes were cut from the inserts and mounted on slides for imaging. Three random digital images were taken for each membrane and migrated cells counted. Three transwell inserts (nine images total) were performed for each experimental condition. Student's t-test was performed for statistical analysis.

Large scale digital cell analysis system (LSDCAS) microscopy. 2×10^4 cells were allowed to adhere in 6-well plates for 24 h. Cells were treated with DGBP and GGPP and LSDCAS microscopy performed. Student's t-test was performed for statistical analysis. The *in vitro* estimation of cell motility for these studies was accomplished using the LSDCAS a live cell imaging Core Research Facility at the University of Iowa. LSDCAS automatically generates image sequences derived from selected microscope fields that each contain hundreds to thousands of individual image frames. Multi-well tissue culture plates allow for the analysis of multiple treatment groups in a single experiment. LSDCAS was designed to provide non-perturbing live cell imaging capabilities, and uses inverted phase-contrast microscopy to image cells growing under standard cell culture conditions. Details of the overall architecture of LSDCAS are described elsewhere (182). For this study, cell speed determinations were obtained using

the software package *casMotility,* a part of LSDCAS. The input image data consists of a set of time-lapse image sequences from multiple microscope fields per experimental sample. The analysis methods used provide individual cell spatial trajectories as a function of time. To determine these individual cell trajectories, first image segmentation methods were used to determine borders around cells and cell clusters in each frame of each image sequence (183). Then, feature analysis and a decision-tree statistical classifier system was used to extract single cell borders. A feature of the resulting cell borders, the border centroid, was then used to track cell motion frame-to-frame. Previous work has shown that the Euclidean distance traveled by centroids in manually segmented cell borders (i.e., the border displacement) exhibits a linear time-series response in the short time frame (184). Thus, single cell speed estimates were obtained through linear regression analysis of these border displacement curves. Given the large amount of cell border data in these data sets, statistical analysis can provide a description of the total distribution of cell speeds in the sample population, as well as a temporal description of the change in mean cell motility as a function of experiment time. In a typical experiment, 20 microscope fields are acquired for each sample, at 20 min interframe intervals, for three days. Thus, for each microscope field, about 215 frames are acquired (4300 frames / sample). When the image data was processed by the *casMotility* software, about 43,000 cell borders associated with about 200 single cells were analyzed in the sample to estimate changes in cell speed as a function of experimental time over the three-day time period.

MTT assay. 1×10^5 cells were allowed to adhere in 24-well plates overnight. Cells were treated with several DGBP concentrations and incubated 45 h. 3-(4,5-

Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and cells incubated. Three hours later, MTT stop solution (HCl, triton X-100, and isopropyl alcohol) was added to all the wells and incubated with gentle agitation overnight at 37°C. Absorbance was measured at 540 nm with reference wavelength at 650 nm.

Separation of membrane and cytosolic proteins. Cells were allowed to adhere in T75 flasks overnight. Cells were treated with lovastatin, DGBP, and GGPP and incubated for 24 h. Membrane and cytosolic fractions were isolated according to the kit (Mem-PER Eukaryotic membrane Protein Extraction Reagent Kit, #89826) from Pierce Biotechnology (Rockford, IL, USA). Once separated, membrane and cytosolic fractions were diluted five fold to minimize lane distortion during gel electrophoresis. 20 µl of each fraction was resolved by 12% gels. Western blotting was performed as described.

RhoA GTP-binding assay. Cells were allowed to adhere in T75 flasks overnight. Cells were treated in serum-free media with DGBP and GGPP for 24 h. A positive control flask was stimulated with the indicated lysophosphatidic acid (LPA) concentration for the last 20 min of the incubation period. Cells were lysed in magnesium buffer and the GTP-loading assay was performed according to the kit (Rho Assay Reagent, #14-383) from Millipore™ (Temecula, CA, USA). BCA assay was used to normalize protein concentrations for the GTP binding assay and whole cell lysate. The GTP-binding assay products and the whole cell lysates were resolved by 12% gels. Western blotting was performed as described.

Results

DGBP impairs protein geranylgeranylation in MDA-MB-231 cells. To establish the degree to which DGBP interferes with prenylation, MDA-MB-231 cells were treated

with lovastatin, an inhibitor of HMGCR, and DGBP for 24 hours (Figure 20A). The Ras antibody recognizes both farnesylated and non-farnesylated forms of this farnesylated protein. Impairment of protein farnesylation is indicated by the appearance of an upper, slower migrating, Ras band. In contrast, the Rap1a antibody only detects the non-geranylgeranylated form of Rap1a, an exclusively geranylgeranylated protein. An expected finding is that lovastatin impairs both farnesylation (Ras) and geranylgeranylation (Rap1a) of proteins. This is evident in that there is the appearance of unmodified Ras and Rap1a band. DGBP impairs Rap1a geranylgeranylation but not Ras farnesylation. The impairment of Rap1a geranylgeranylation by DGBP occurs in a concentration-dependent manner. Maximal inhibition of Rap1a geranylgeranylation occurs between 10 and 25 μM DGBP. As such, 25 μM DGBP will be used in all of the following studies in MDA-MB-231 breast cancer cells.

GGPP prevents DGBP-induced impairment of protein geranylgeranylation in MDA-MB-231 cells. In order to confirm that the impairment of Rap1a geranylgeranylation by DGBP is a consequence of GGDPS inhibition, experiments were performed that restored intermediates of the isoprenoid pathway (Figure 20B). In cells treated with lovastatin, co-incubation with mevalonate prevents impairment of farnesylation (Ras) and geranylgeranylation (Rap1a). FPP prevents impairment of farnesylation (Ras) and GGPP prevents impairment of geranylgeranylation (Rap1a) by lovastatin. Mevalonate and FPP do not prevent the impairment of protein geranylgeranylation (Rap1a) by DGBP. GGPP completely prevents DGBP-induced impairment of protein geranylgeranylation (Rap1a). For control purposes, mevalonate, FPP, and GGPP alone do not affect protein prenylation. In summary, DGBP-induced

impairment of protein geranylgeranylation is completely prevented by exogenous addition of GGPP in MDA-MB-231 cells.

DGBP increases FPP and depletes GGPP levels in MDA-MB-231 cells. To determine the effect of DGBP on FPP and GGPP levels, MDA-MB-231 cells were treated with DGBP for 24 hours (Figure 21). Lovastatin was used as a positive control. Lovastatin markedly decreases intra-cellular levels of both FPP and GGPP. DGBP decreases intra-cellular GGPP levels in a concentration-dependent manner. In contrast, there is a concentration-dependent increase in FPP levels with both 1 and 5 μM DGBP. Interestingly, higher concentrations of DGBP (25 μM) do not result in a further increase in FPP levels though GGPP levels are further decreased. In conclusion, DGBP increases FPP while it depletes intra-cellular GGPP levels in MDA-MB-231 breast cancer cells.

DGBP inhibits migration of MDA-MB-231 cells. In order to determine if DGBP interferes with cell migration, transwell migration assays were performed with MDA-MB-231 breast cancer cells (Figure 22). As expected, inhibition of GGTase I with 10 μM GGTI-286 decreases migration of MDA-MB-231 cells by more than 50%. The addition of 25 μM DGBP reduces MDA-MB-231 cell migration by a comparable amount. The addition of 30 μM GGPP prevents the effects of DGBP on cell migration. There is no significant difference between untreated control cells and cells that are treated with 25 μM DGBP in the presence of 30 μM GGPP. The height of the bars in the figure quantifies the relative numbers of cells that have migrated.

LSDCAS microscopy was used as an alternate method to analyze cell migration. Cells were treated with DGBP in the presence or absence of GGPP and cell migration was assessed by LSDCAS microscopy (Figure 23A). Control cells maintain a constant

migration speed of approximately 15 μm/hour for 71 hours. Conversely, cells treated with 25 μM DGBP display impaired migration to approximately 73% control cells shortly after treatment. Interestingly, there is an even greater reduction in mean cell speed (38% of control) with longer exposure to DGBP. The rate of migration of DGBP-treated cells decreases to 6 μm/hour at the end of the 71 hour experiment. Similar to transwell assays, the addition of 20 μM GGPP prevents DGBP-induced reduction in cell migration. The LSDCAS microscopy data was also evaluated by normalized histogram analysis (Figure 23B). Cells treated with DGBP are significantly slower than untreated cells. The addition GGPP to DGBP restores cell migration significantly when compared to DGBP treated cells.

In order to determine whether the effects of GGDPS inhibition on cancer cell migration is drug specific (due to DGBP), the same LSDCAS microscopy experiment was performed with compound 14, an aromatic bisphosphonate inhibitor of GGDPS (Figure 24). Similar to DGBP, compound 14 interferes with the migration of MDA-MB-231 breast cancer cells as measured by LSDCAS microscopy. Additionally, the supplementation of GGPP prevents the effects of compound 14 on breast cancer cell migration.

In summary, we have demonstrated that GGDPS inhibition interferes with MDA-MB-231 cell migration as measured by two different assays, transwells and LSDCAS microscopy. In both cases, the addition of exogenous GGPP restores cell migration to control levels.

DGBP is not toxic at relevant concentrations to MDA-MB-231 cells. A concern is that DGBP may be toxic and induce apoptosis at the concentrations that inhibit MDA-

MB-231 cell migration. Therefore, MTT assay and assessment of poly (ADP-ribose) polymerase (PARP) cleavage were performed in cells treated with DGBP for 48 hours (Figure 25). PARP is a DNA repair enzyme that is cleaved during apoptosis (163). DGBP decreases MTT activity in a concentration-dependent manner, although MTT activity is reduced to 72% at 100 μM DGBP (Figure 25A). There is minimal toxicity (88% MTT activity) at 25 μM DGBP, which was used to distort FPP/GGPP levels, alter Rap1a processing, and inhibit cell migration. There is no evidence of PARP cleavage due to DGBP (Figure 25B). The PARP antibody used detects the full-length as well as the cleaved form of PARP (cleaved form shown). The addition of etoposide, a well-characterized activator of apoptosis, was used as a positive control and its addition induces apoptosis as indicated by the appearance of the cleaved form of PARP. For control purposes, GGPP alone or in combination with etoposide has no effect on PARP cleavage. In summary, our data shows that 25 μM DGBP was not toxic and does not lead to induction of apoptosis at 48 hours in MDA-MB-231 cells.

DGBP interferes with the localization of RhoA in MDA-MB-231 cells. To ascertain whether DGBP interferes with the localization of small GTPases to the membrane, MDA-MB-231 cells were treated with lovastatin and DGBP in the presence or absence of GGPP for 24 hours (Figure 26A). Ras and RhoA proteins are localized to the membrane fraction in untreated and GGPP treated cells. The addition of lovastatin reduces Ras and RhoA in the membrane fraction while increasing their levels in the cytosolic fraction. During lovastatin treatment, the addition of GGPP completely restores the localization of RhoA to the membrane fraction. GGPP addition also partially restores Ras membrane localization after lovastatin treatment. Exposure of cells to DGBP

decreases membrane and increases cytosolic RhoA without interfering with membrane localization of Ras. The addition of GGPP completely prevents the effects of DGBP on RhoA localization. In summary, lovastatin interferes with the localization of farnesylated (Ras) and geranylgeranylated (RhoA) proteins while DGBP only interferes with the localization of a geranylgeranylated (RhoA) protein. The effects of lovastatin and DGBP on RhoA localization are reversed by the addition of exogenous GGPP, confirming the role of GGPP depletion on RhoA localization.

DGBP increases GTP binding to RhoA in MDA-MB-231 cells. GTP loading experiments were performed to assess GTP binding to RhoA in the presence of DGBP (Figure 26B). Addition of LPA, a positive control for RhoA activation, increases GTP binding to RhoA. Treatment of cells with DGBP increases GTP binding to RhoA, similar to LPA. Interestingly, DGBP also increases total RhoA expression. The addition of GGPP to DGBP-treated cells restores RhoA protein levels and GTP binding to RhoA to control levels. GGPP alone has no effect on RhoA expression or GTP binding to RhoA. In summary, DGBP increases the RhoA expression and GTP binding to RhoA and these effects are prevented by exogenous addition of GGPP.

Discussion

Previous work in our laboratory has demonstrated that DGBP impairs protein geranylgeranylation but not farnesylation in suspension cells such as K562 leukemia cells and RPMI-8226 myeloma cells (138, 139). In this report, we show that DGBP impairs geranylgeranylation without interfering with the farnesylation of proteins (measured by monitoring Ras farnesylation) in adherent MDA-MB-231 breast cancer cells. Similar to our previous studies, impairment of protein geranylgeranylation by DGBP was readily

prevented by exogenous addition of GGPP in MDA-MB-231 cells. Our laboratory has previously developed assays that measure FPP and GGPP levels in cell cultures and animal tissues (143, 161). Our previous work has shown that DGBP reduces GGPP and increases FPP levels in suspension cells (179). Similar results were obtained in MDA-MB-231 cells: DGBP decreased GGPP while it increased FPP levels. Of note, GGPP levels decreased while FPP levels reached a plateau with increasing concentrations of DGBP in MDA-MB-231 cells. Together with GGPP add-back Western blotting experiments, intra-cellular FPP and GGPP clearly show that DGBP impaired protein geranylgeranylation through the inhibition of GGDPS in MDA-MB-231 cells.

Previous studies have shown that direct impairment of protein geranylgeranylation by GGTIs interfered with cancer cell invasion (107). In addition, studies have shown that statins interfered with cancer cell migration and invasion. More importantly, it has been shown that statins inhibit cancer cell migration by the depletion of cellular GGPP (136). Therefore, we rationalized that inhibition of GGDPS, and therefore direct depletion of GGPP, would inhibit migration of the highly invasive MDA-MB-231 breast cancer cells. In order to quantify migration, transwell migration assays were performed. DGBP inhibited MDA-MB-231 cancer cells migration at a concentration that depleted cellular GGPP and maximally impaired protein geranylgeranylation. Importantly, co-administration of GGPP to DGBP-treated cells restored MDA-MB-231 cell migration to control levels. Transwell assays are routinely used for assessing cell motility but they are tedious, rely on manual methods, and are inconsistent at times. Therefore, we also quantified cell motility with LSDCAS microscopy. As observed with transwell assay, DGBP also inhibited migration of MDA-

MB-231 cells as measured by LSDCAS microscopy. Similar to transwell assay, the addition of GGPP prevented DGBP-induced inhibition of cell migration in LSDCAS microscopy. Interestingly, cell speed continued to decrease over the duration of the experiment. This suggests the DGBP effect may result from ongoing activity such as the gradual shift of geranylgeranylated proteins to unmodified forms based on protein half-life and turnover. These results also suggest that MDA-MB-231 cells did not develop resistance to DGBP over the duration of the experiment. Also, compound 14 was able to interfere with MDA-MB-231 motility and its effects were prevented by exogenous GGPP as measured by LSDCAS microscopy.

It is important to point out two major differences between transwell assay and LSDCAS microscopy. First of all, transwell assay quantifies the number of cells that have passed through the membrane over a period of time. There is no way of knowing when the cells are crossing the membrane and how fast the cells are moving. So, different migration times may result in drastically different results. On the other hand, LSDCAS microscopy measures the speed by which the cells are moving at any point in time. LSDCAS excludes subjective cell counting and allows for the determination of average cell speed at various time intervals. Also, it is difficult to control for cell proliferation and cell death in a transwell assay. Therefore, some of the migration results can be attributed to other cellular processes caused by the drug under investigation. Conversely, LSDCAS microscopy monitors each cell and is not influenced by cell death or proliferation. Therefore, it was important to assess MDA-MB-231 cell migration in the presence of DGBP by two different migration assays, transwell and LSDCAS microscopy. Importantly, DGBP concentration that was used in migration studies was

not very toxic and did not induce apoptosis in MDA-MB-231 cells at 48 hours. As mentioned above, these toxicity studies are essential when analyzing transwell assays.

In order to elucidate the mechanism by which DGBP inhibited cell migration, we performed experiments that assessed localization and GTP binding of RhoA, a geranylgeranylated protein. In order to function properly, small GTPases such as RhoA must be localized properly and be able to bind and hydrolyze GTP. Previous studies have shown that statins and GGTIs interfere with the localization of small GTPases to the membrane (109, 185). Similar to lovastatin, DGBP decreased membrane and increased cytosolic fraction of RhoA, suggesting a mis-localization of this small GTPase. Unlike lovastatin, DGBP did not lead to mis-localization of Ras, a farnesylated protein. The addition of exogenous GGPP prevented mis-localization of RhoA due to lovastatin and DGBP. Somewhat surprising was the finding that GGPP addition restored some of the mis-localized Ras by lovastatin to the membrane fraction. A possible explanation for this is alternative prenylation of Ras. Ras, more specifically K-Ras, has been shown to be geranylgeranylated when cells were treated with FTIs (186). Alternatively, the addition of exogenous GGPP may partially restore endogenous FPP levels and allow for some protein farnesylation to occur even in the presence of lovastatin. Results in our laboratory show that exogenous addition of GGPP during lovastatin treatment can prevent some of the effects of lovastatin on FPP depletion and impairment of protein farnesylation (unpublished data). When we assessed for RhoA activity, DGBP increased GTP binding to RhoA. Treatment of cells with DGBP also resulted in higher expression of total RhoA. It is unclear if increased expression of RhoA was due to increased RhoA transcription/translation or decreased RhoA degradation. Importantly, GGPP addition

completely prevented the effects of DGBP on RhoA expression and binding of GTP to RhoA. Increase in GTP-bound RhoA due to DGBP may suggest increased signaling by RhoA, which would be inconsistent with reduced migration and motility of cells treated with DGBP. However, a recent study has shown that geranylgeranylation but not GTP loading determines Rho migratory function (185). Waiczies and colleagues demonstrated that statins interfered with the activation of ROCK1, a key downstream target of RhoA that play an essential role in the migration of cells, by mislocalizing RhoA from the membrane to the cytosol. To summarize, increase in GTP binding may occur during the treatment of cells with isoprenoid pathway inhibitors but the mis-localization of small GTPases predicts a failure to activate proper downstream signaling.

In conclusion, we show for the first time that sole inhibition of GGDPS, and resulting depletion of intra-cellular GGPP, inhibits MDA-MB-231 breast cancer cell migration. We believe that mis-localization of small geranylgeranylated GTPases such as RhoA is the primary mechanism by which DGBP interferes with migration of MDA-MB-231 cells. Future studies should be designed to monitor downstream signaling of small GTPases. For example, ROCK1 activity should be monitored in the presence of DGBP. In addition, farnesylated forms of Rho family members such as RhoA, Rac1, and Cdc42 (all geranylgeranylated proteins) could be introduced into cells treated with DGBP to look at the individual roles of these members in motility and migration of normal and transformed cells.

Rho Protein	Deregulation	Tumor Type
RhoA	Over-expression	Colon, breast, lung, and others
RhoC	Over-expression	Breast and pancreatic
Rac1	Over-expression	Breast
Rac1B	Alternative splicing	Breast and colon
Rac2	Over-expression	Head and neck
Cdc42	Over-expression	Breast

Table 4. Abberant regulation of Rho proteins in cancer. Several Rho family members are over-expressed in several cancer types, most motably breast cancer. This table has been adapted from Sahai and Marshall with modifications (52).

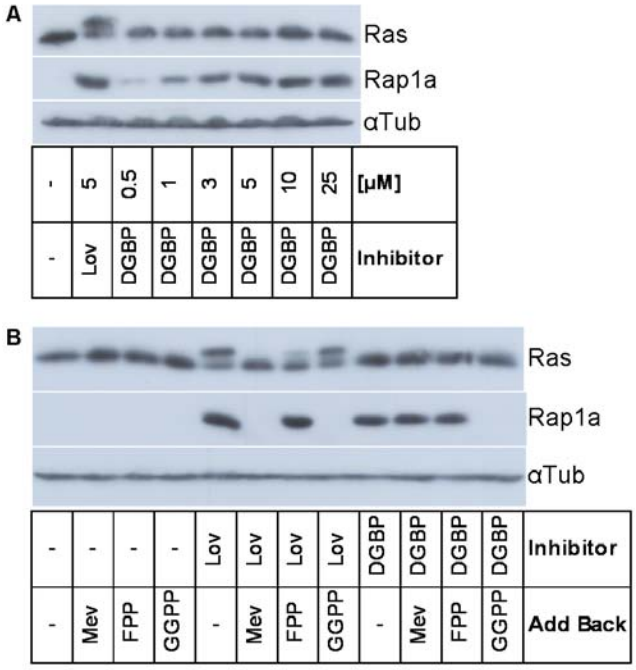


Figure 20. GGPP addition prevents DGBP-induced impairment of protein geranylgeranylation in MDA-MB-231 cells. (A) DGBP inhibits protein geranylgeranylation in a concentration-dependent manner. Cells were treated with several DGBP concentrations for 24 hours. Cells lysis was followed by Western blotting to detect Ras and Rap1a prenylation status. (B) GGPP addition completely prevents DGBP-induced impairment of protein geranylgeranylation. Cells were treated with 5 μM lovastatin or 25 μM DGBP in the presence or absence of 1 mM mevalonate, 20 μM FPP, or 20 μM GGPP for 24 hours. Cell lysis was followed by Western blotting to detect Ras and Rap1a prenylation status.

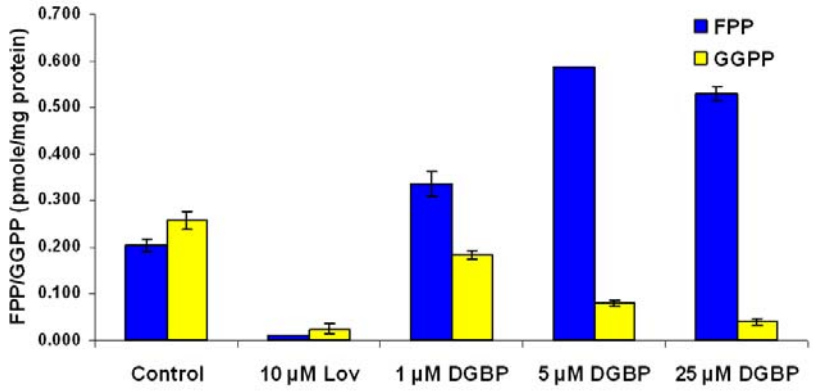


Figure 21. DGBP depletes GGPP and increases FPP levels in MDA-MB-231 cells. Cells were treated with 10 µM lovastatin or various concentrations of DGBP for 24 hours. Extraction and quantification of FPP and GGPP was performed as described in methods. FPP and GGPP levels were normalized to protein concentration measured by BCA assay (mean +/- SEM, n = 2).

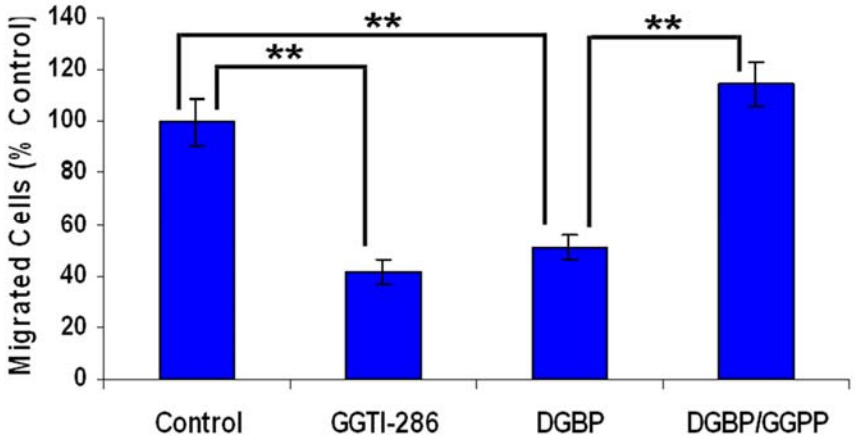


Figure 22. DGBP inhibits migration of MDA-MB-231 cells (transwell migration assay). Cells were treated with 10 μM GGTI-286, 25 μM DGBP, and 30 μM GGPP (mean +/- SEM, n = 3, $p<0.01$**).

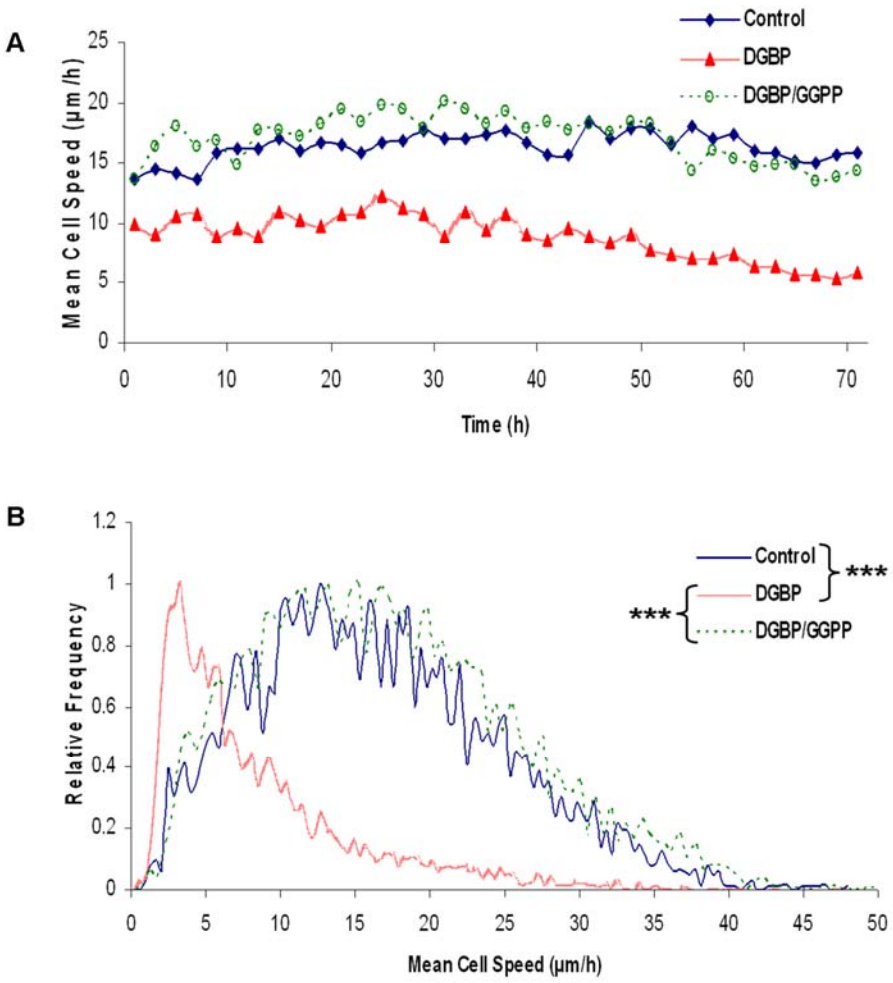


Figure 23. DGBP inhibits migration of MDA-MB-231 cells (LSDCAS microscopy). (A) LSDCAS microscopy. Cells were treated with 25 µM DGBP alone or in combination with 20 µM GGPP. (B) Normalized histogram analysis of data in part A ($p<0.0001$***).

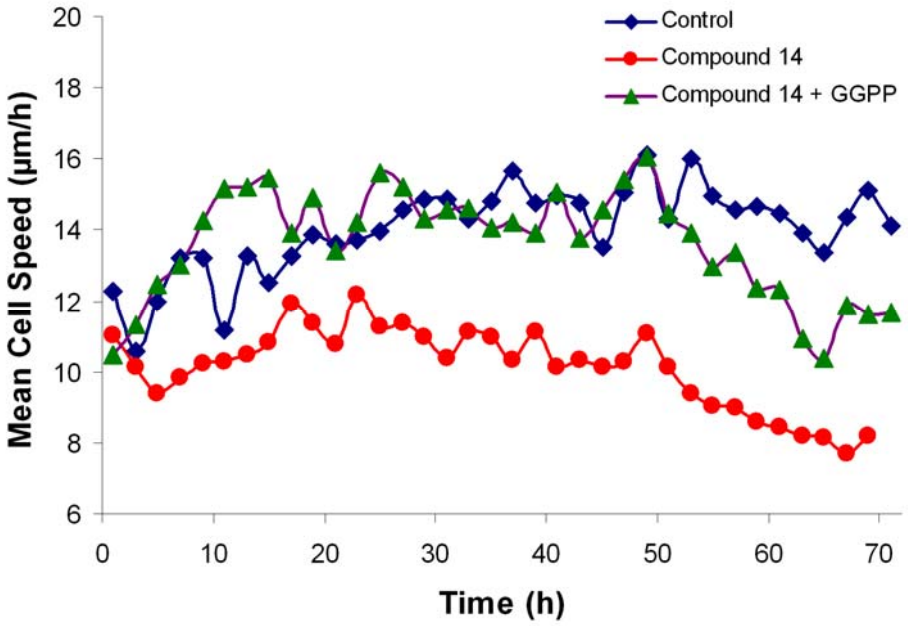


Figure 24. Compound 14 inhibits migration of MDA-MB-231 cells (LSDCAS microscopy). Cells were treated with 25 µM DGBP alone or in combination with 20 µM GGPP.

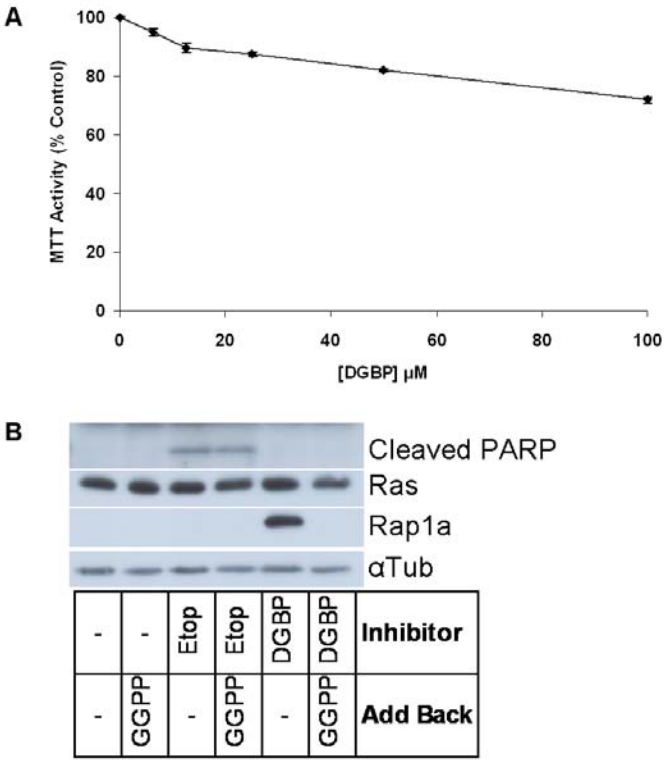


Figure 25. DGBP is not toxic at relevant concentration to MDA-MB-231 cells. (A) MTT assay of cells treated with various concentrations of DGBP for 48 hours (mean +/- SEM, n = 3). (B) Western blot analysis of cells treated with 20 µM etoposide, 25 µM DGBP, and 30 µM GGPP for 48 hours. Cell lysis was followed by Western blotting to detect PARP cleavage and to monitor Ras and Rap1a prenylation.

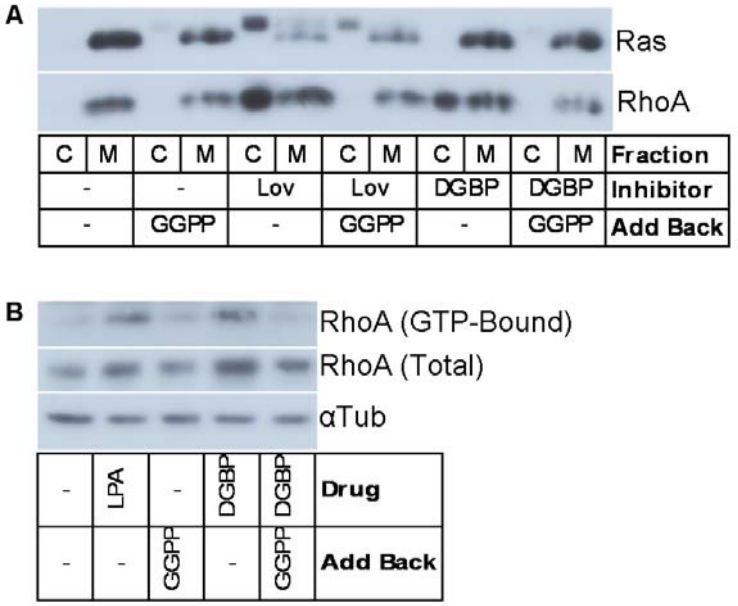


Figure 26. GGPP depletion interferes with localization and GTP binding of RhoA in MDA-MB-231 cells. (A) GGPP depletion leads to mis-localization of RhoA from the membrane to the cytosol. Cells were treated with 5 μM lovastatin, 25 μM DGBP, and 20 μM GGPP for 24 hours. Membrane and cytosolic fractions were isolated as described in methods followed by Western blotting (C = cytosolic fraction, M = membrane fraction). (B) GGPP depletion increases GTP binding to RhoA. Cells were treated with 25 μM DGBP and 20 μM GGPP for 24 hours in serum-free media. 20 μM LPA was added 20 minutes before the end of the experiment. GTP-binding assay was performed as described in methods followed by Western blotting.

CHAPTER V: FDPS AND GGDPS INHIBITION INDUCES AUTOPHAGY IN CANCER CELLS

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 clarified in detail. We hypothesized that inhibition of farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase by bisphosphonates will induce autophagy by depleting cellular geranylgeranyl diphosphate stores and impairing protein geranylgeranylation. We here show that a FDPS inhibitor (zoledronate) and GGDPS inhibitors (digeranyl bisphosphonate and compound 14) 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, digeranyl bisphosphonate, or compound 14 further enhances the formation of LC3-II, indicating these compounds induce autophagic flux. In addition, specific inhibitors of farnesyl transferase and geranylgeranyl transferase I are unable to induce autophagy in our system. Previous studies have demonstrated that inhibition of autophagy enhances the potency of some chemotherapeutics. We show that the addition of bafilomycin A1 (an inhibitor of autophagy processing) enhanced the cytotoxic effects of digeranyl bisphosphonate. These results are the first to demonstrate that bisphosphonates induce autophagy. Our studies suggest that induction of autophagy with these agents may be either dependent upon inhibition of protein geranylgeranylation

by geranylgeranyl transferase II or a consequence of alterations of isoprenoid levels themselves.

Introduction

The isoprenoid biosynthetic pathway (Figure 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) (187), which is the rate-limiting step of cholesterol biosynthesis (188). The nitrogenous bisphosphonates (e.g. zoledronate) target farnesyl diphosphate synthase (FDPS) and are used for bone-related disorders such as osteoporosis (95).

While statins and nitrogenous bisphosphonates are used for discrete 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 (20). Farnesylation is catalyzed by farnesyl transferase (FTase), while geranylgeranylation can be catalyzed by either geranylgeranyl transferase I (GGTase I) or geranylgeranyl transferase II (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 (21).

Macro-autophagy (hereafter referred to as autophagy) is a cellular process which degrades damaged cytoplasmic organelles as well as long-lived, misfolded, or aggregated proteins (Figure 27) (189). 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 is 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.

Cancer cells have been shown to have a lower autophagic rate when compared to normal counterparts (190). However, autophagy is not down-regulated in transformed cells when compared to normal cells under nutrient deprivation (191). Numerous studies have looked at autophagy in response to various anti-cancer treatments. Studies have demonstrated that agents such as tamoxifen (targets the estrogen receptor), suberoylanilide hydroxamic acid (histone deacetylase inhibitor), γ-irradiation, temozolomide (DNA alkylating agent), and imatinib (targets the BCR-ABL oncogene) induce autophagy in cancer cells (192, 193). Therefore, modulation of autophagy has been examined in combination with anti-cancer therapeutics in numerous studies. The majority of these studies demonstrate that autophagy inhibitors enhance toxicity that is induced by anti-cancer therapeutics in wide variety of cancer models (194). Studies with imatinib demonstrated that chloroquine, which inhibits autophagy by increasing pH in the lysosome, potentiated cell death of chronic myeloid leukemia cell lines and primary cancer cells (195). Another study showed that 3-methyladenine, which inhibits

autophagy by interfering with the function of class III Phosphatidylinositol 3-kinases, increases cell death associated with 5-fluorouracil in colon cancer cells (196). Lastly, bafilomycin A1, which interferes with autophagy by inhibiting vacuolar H+-ATPase, has been demonstrated to sensitize glioma cells to temozolomide-induced apoptosis (197).

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 (198). 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 (199). 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, suggesting that the impairment of prenylation can induce autophagy (200). 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 (201). In addition, an inhibitor of isoprenylcysteine carboxyl methyltransferase, an enzyme required in later steps of prenylation processing, induced autophagy in PC3 and HepG2 cells (202, 203). To identify alternate potential drug targets, a synthetic lethal screen was performed with yeast deletion mutants treated with nitrogenous bisphosphonates. This study identified *ATG4*, *ATG11*, *ATG14* and

ATG16 (all autophagy-related genes) hemizygous strains as having increased sensitivity to nitrogenous bisphosphonates (204).

Our collaborators have synthesized digeranyl bisphosphonate (DGBP), which we have shown to be a specific inhibitor of geranylgeranyl diphosphate synthase (GGDPS) (139). We have shown this compound specifically impairs protein geranylgeranylation via depletion of GGPP in various cell types. We hypothesized and provide evidence herein that depletion of GGPP and subsequent impairment of protein geranylgeranylation induced by bisphosphonate inhibitors of FDPS (i.e. zoledronate) or GGDPS (i.e. DGBP and compound 14) results in the induction of autophagy. Furthermore, we show that inhibition of autophagy by bafilomycin A1 enhances DGBP-induced toxicity in PC3 prostate cancer cells.

Materials and 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 % FBS at 5% CO_2 at 37^{o}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 α-Tubulin 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 IgGs were from GE Healthcare, while conjugated anti-goat IgG was from Santa Cruz Biotechnology, Inc. The 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 h), media was removed and cells were washed twice in phosphate-buffered saline. Cells were collected by the trypsin method and lysed in radioimmunoprecipitation buffer supplemented with protease inhibitor cocktail, sodium vanadate, sodium fluoride, and phenylmethylsulphonyl fluoride. Lysates were transferred to a 1.5 ml tube, vortexed several times over 30 minutes, and passed through a 27-gage needle. Lysates were then centrifuged and supernatant transferred to a fresh 1.5 ml tube. All steps were performed at 4^{o}C.

Western Blot Analysis. Protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Proteins were resolved on 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 h at 37^{o}C and proteins visualized using an ECL detection kit.

MTT Assay. 8×10^4 cells/well were allowed to adhere in 24-well plates overnight. Cells were treated with relevant compounds and incubated 45 h. 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 the reference wavelength at 650 nm.

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 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 treated with varying lovastatin, zoledronate, and DGBP concentrations for 24 hours (Fig. 28A). The Ras antibody utilized in these experiments recognizes the modified (farnesylated) and the unmodified form of the protein. The unmodified form of Ras shows a slower migrating, upper-band, on the Ras Western blot. In contrast, the antibody used to detect Rap1a only detects the unmodified form of this protein that is normally geranylgeranylated by GGTase I. Therefore, the appearance of a band on the Rap1a Western blot indicates impairment of protein 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 (Fig. 28A). DGBP interferes with protein geranylgeranylation without disturbing protein farnesylation (Fig. 28A). Here, maximal impairment of Rap1a geranylgeranylation occured 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. These concentrations were selected for use in subsequent experiments.

Isoprenoid biosynthetic pathway inhibitors induce cytotoxicity in a concentration-dependent manner in PC3 cells. In order to assess viability of cells in the presence of isoprenoid biosynthetic pathway inhibitors, MTT assays were performed at 48 hours (Figure 28B). Concentration-dependent cytotoxicity is observed with all three inhibitors. Lovastatin is most potent while zoledronate is least potent in inducing cytotoxicity. The concentration that maximally impairs protein geranylgeranylation for each inhibitor (1μM lovastatin, 100 μM zoledronate, and 25 μM DGBP) results in similar reduction in MTT activity with each of the three drugs.

Bisphosphonates induce autophagy in PC3 cells. To establish if bisphosphonates induce autophagy, PC3 cells were treated with lovastatin, zoledronate, and DGBP for 24 and 48 hours and autophagy assessed (Figure 29A). As previously mentioned, statins have been shown to induce autophagy in PC3 cells (199). Thus, lovastatin was used as a positive control for induction of autophagy in all of our experiments. To assess induction of autophagy, an antibody that detects the LC3-II form of LC3 was also used. The appearance of the LC3-II band on a Western blot is an established method for the detection of autophagy (189, 205). Induction of autophagy (appearance of LC3-II band) is not apparent at 24 hours with the use of isoprenoid pathway inhibitors (Fig. 29A). In contrast, induction of autophagy 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 μM DGBP and 50 μM zoledronate, and this effect is dose-responsive with respect to both drugs.

Bisphosphonate-induced autophagy is dependent on GGPP depletion in PC3 cells. In order to determine if the effects of bisphosphonates on autophagy are dependent on the depletion of specific molecules within the isoprenoid pathway, inhibitors were co-administered with exogenous isoprenoid pathway intermediates for 48 hours (Figure 29B). 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. Similarly, GGPP completely prevents, while FPP only partially prevents autophagic induction by zoledronate. GGPP also entirely prevents induction of autophagy by DGBP.

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 (205). In order to confirm that GGPP depletion by bisphosphonates truly induces autophagy, experiments were performed to evaluate autophagic flux (Figure 30). 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 expression of LC3-II. Dual administration of protease inhibitors with each of the isoprenoid pathway inhibitors further enhances the expression of LC3-II suggesting that bisphosphonates (zoledronate and DGBP) induce autophagy as opposed to interfering with autophagosomal processing. Of note, the lysosomal inhibitors also increase LC3-II formation when compared to control, likely by blocking basal autophagosomal degradation.

Compound 14 induces autophagy and autophagy flux by depleting GGPP in PC3 cells. As in previous chapters of this thesis, we wanted to determine whether the effects seen with GGDPS inhibition are drug-specific. Therefore, we repeated the autophagy

experiments with compound 14, a novel aromatic bisphosphonate inhibitor of GGDPS (Figure 31). As it was seen with DGBP, compound 14 (25 and 100 μM) induces autophagy in PC3 cells as measured by LC3-II formation. The addition of GGPP completely prevents the effects of compound 14 on LC3-II formation in these cells. Similar to DGBP treatment, the addition of protease inhibitors further enhances LC3-II formation, which suggests that compound 14 is inducing autophagy and is not causing an accumulation of LC3-II by interfering with autophagy flux.

Bisphosphonates induce autophagy 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 (Figure 32). Similar to PC3 cells, autophagy is induced by each of the isoprenoid biosynthetic pathway inhibitors (lovastatin, zoledronate, and DGBP) 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, despite pronounced impairment of protein geranylgeranylation. Of note, higher concentrations of zoledronate (250 μM) and DGBP (50 μM) were also unable to induce autophagy in these two cancer cell lines (Figure 33).

Inhibition of either FTase or GGTase I does not induce autophagy in PC3 cells. To determine whether direct impairment of protein farnesylation by FTase or protein geranylgeranylation by GGTase I induce autophagy, inhibitors of these two enzymes were utilized (Figure 34). 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 does not result in the induction of autophagy as measured by LC3-II formation, despite the effective inhibition of their respective target enzymes.

Inhibition of autophagy enhances DGBP-induced cytotoxicity in PC3 cells. In order to assess the role of autophagy on cytotoxicity elicited by zoledronate and DGBP, combinational studies were performed with bafilomycin A1 using MTT assay at 48 hours (Figure 35). Bafilomycin A1 is an inhibitor of fusion between autophagosomes and lysosomes. The combination of zoledronate and bafilomycin A1 did not significantly enhance toxicity when compared to either single agent. In contrast, the combination of DGBP and bafilomycin A1 enhances toxicity when compared to each agent alone.

Discussion

It is well established that statins and nitrogenous bisphosphonates deplete isoprenoid pathway intermediates (17). Some of the cellular effects of these agents have been attributed to the depletion of GGPP (121, 178). Our prior work has explored cellular consequences of GGPP depletion through the utilization of a novel bisphosphonate that directly inhibits GGDPS (179, 206). Other previous work has suggested that statins induce autophagy (199). In this study, we explore the possibility that more specific depletion of GGPP, and thus impairment of protein geranylgeranylation, by bisphosphonates would induce autophagy in PC3 prostate cancer cells.

In order to establish proper experimental parameters for subsequent experiments, Western blotting and MTT assays were performed with isoprenoid pathway inhibitors. Optimal concentrations of the inhibitors were selected based on their maximal impairment of protein geranylgeranylation and toxicity profiles. We have shown for the first time that bisphosphonate inhibitors of FDPS (zoledronate) and GGDPS (DGBP and compound 14) 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 (impairment of protein geranylgeranylation) is the primary mechanism by which zoledronate, DGBP, and compound 14 induce autophagy.

The accumulation of LC3-II can result from induction of autophagy or impaired basal autophagic processing (205). The addition of lysosomal inhibitors was used to determine 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 (199), 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, induction of autophagy is not observed in the presence of lovastatin or bisphosphonates (zoledronate

and DGBP). It has been shown that statins do not induce autophagy in HepG2 cells (198). These results are not due to a lack of functional autophagy, as both HepG2 and MDA-MB-468 have been shown to be capable of autophagic induction (203, 207).

Due to the prevention of bisphosphonate-induced LC3-II accumulation by exogenous addition of GGPP, 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) does not induce LC3-II accumulation despite effective impairment of protein geranylgeranylation. This leads to one possible conclusion that autophagy induced by bisphosphonates is due specifically to the impairment of protein geranylgeranylation by GGTase II. At this point it is not possible to exclude that this is a primary consequence of alteration of FPP and GGPP levels alone. Other studies have shown a novel GGTI when combined with a statin induced autophagy in STS-26T MPNST cells (200). While again cell line differences can be asserted, it is also possible that this novel GGTI specificity is such that it results in the impairment of geranylgeranylation of GGTase II substrates. The lack of commercially available reagents does not allow a detailed examination of this hypothesis at this time. Previous studies suggest farnesyl transferase inhibitors can induce autophagy (201). Our results did not show accumulation of LC3-II due to FTI-277 in PC3 cells. Pan *et al.* speculate that the impairment of Rheb farnesylation by FTIs is responsible for autophagy induction (208). 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 see

induction of autophagy upon treatment with rapamycin, an mTOR inhibitor that can induce autophagy (data not shown).

Nitrogenous bisphosphonates are currently used for treatment of bone-related metastatic cancers (144). In contrast, the inhibition of autophagy is under intense evaluation with respect to anti-cancer applications (192). Therefore, zoledronate and DGBP were combined with bafilomycin A1, an inhibitor of autophagic function, to assess whether inhibition of autophagy would enhance the cytotoxic effect of bisphosphonates. The addition of bafilomycin A1 significantly enhances cytotoxicity induced by DGBP, but not zoledronate. This suggests that the combination of inhibitors of autophagy with GGDPS inhibitors should be further explored as a possible therapeutic strategy.

The data presented herein suggests that bisphosphonates induce autophagy through the depletion of cellular GGPP and thus impairment of protein geranylgeranylation. Future studies could determine if disruption of a GGTase II substrate or substrates or if alterations of intracellular isoprenoid levels alone are responsible for the induction of autophagy.

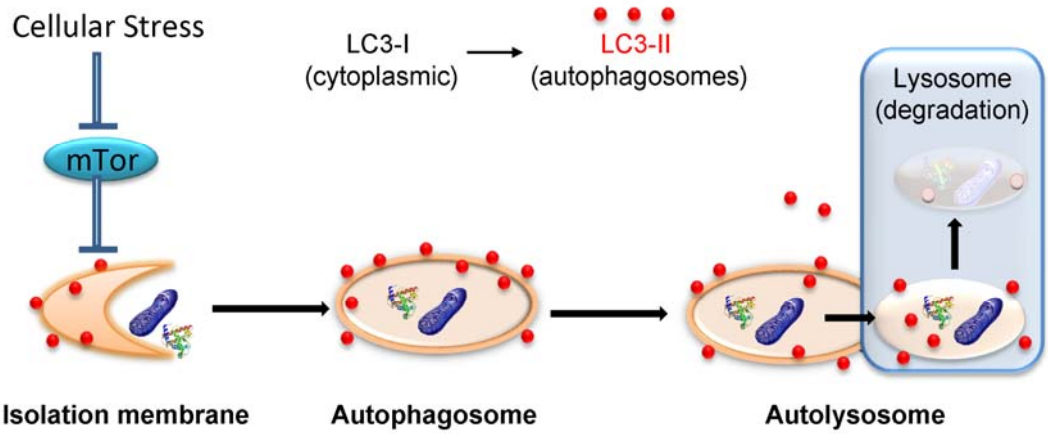


Figure 27. Autophagic processing. This diagram illustrates the sequential steps of autophagy; isolation membrane formation, autophagosome formation, and autolysosome formation.

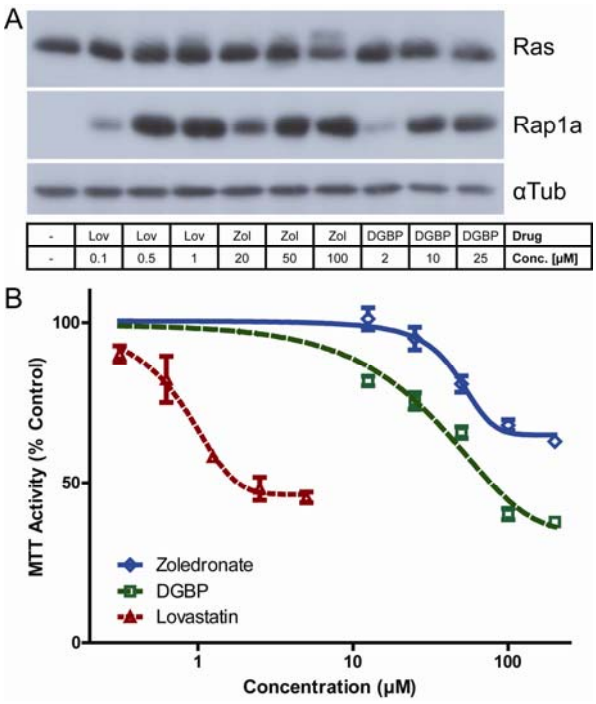


Figure 28. Isoprenoid biosynthetic pathway inhibitors interfere with prenylation and induce cytotoxicity in PC3 cells. (A) Isoprenoid pathway inhibitors interfere with protein prenylation. Cells were treated with several concentrations of isoprenoid pathway inhibitors (lovastatin, zoledronate, and DGBP) for 24 h. Cell lysis was followed by Western blotting to detect prenylation status of Ras and Rap1a. (B) Isoprenoid pathway inhibitors decrease cell viability. MTT assay of cells treated with various concentrations of lovastatin, zoledronate, and DGBP for 48 h (mean +/ SD, n = 3).

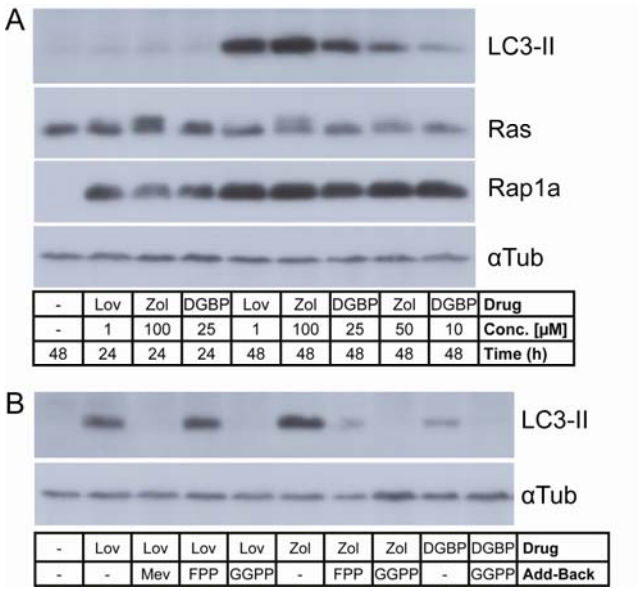


Figure 29. Bisphosphonates induce accumulation of the autophagic marker LC3-II in PC3 cells. (A) Bisphosphonates (zoledronate and DGBP) induce accumulation of LC3-II. Cells were treated with isoprenoid pathway inhibitors (lovastatin, zoledronate, and DGBP) at indicated concentrations for 24 and 48 h. Cell lysis was followed by Western blotting to detect LC3-II formation and monitor Ras and Rap1a prenylation. (B) GGPP addition prevents the accumulation of LC3-II by bisphosphonates (zoledronate and DGBP). 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 h. Cell lysis was followed by Western blotting to detect LC3-II formation.

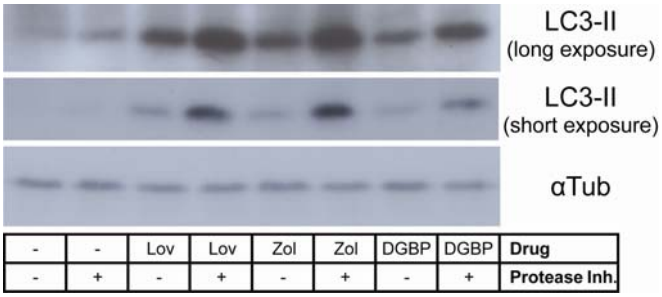


Figure 30. Bisphosphonates induce autophagy in PC3 cells. Cells were treated with isoprenoid pathway inhibitors (1 μM lovastatin, 100 μM zoledronate, and 25 μM DGBP) in the presence or absence of lysosomal protease inhibitors (10 μg/ml Pepstatin A and 10 μg/ml E64-d) for 48 h. Cell lysis was followed by Western blotting to detect LC3-II formation.

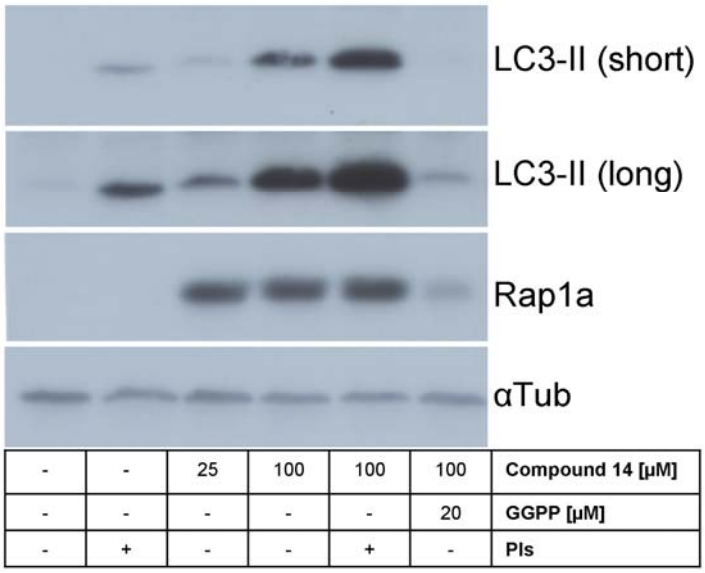


Figure 31. Compound 14 induces autophagy and autophagy flux by depleting GGPP in PC3 cells. Cells were treated with compound 14 in the presence or absence of lysosomal protease inhibitors (10 μg/ml Pepstatin A and 10 μg/ml E64-d) or GGPP for 48 h. Cell lysis was followed by Western blotting to detect LC3-II formation and monitor Rap1a geranylgeranylation.

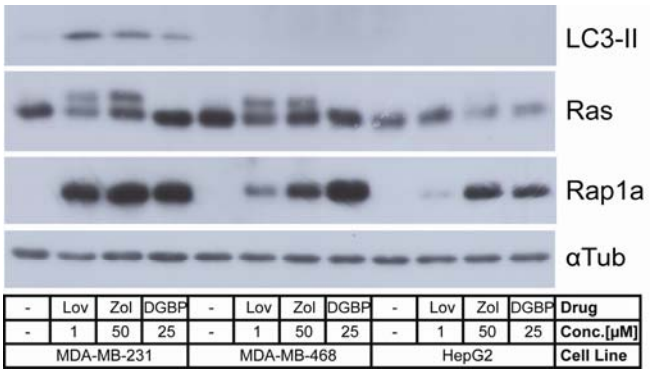


Figure 32. Bisphosphonates induce autophagy in MDA-MB-231 but not in MDA-MB-468 or HepG2 cells. Cells were treated with isoprenoid pathway inhibitors (lovastatin, zoledronate, and DGBP) as indicated for 48 h. Cell lysis was followed by Western blotting to detect LC3-II formation and monitor Ras and Rap1a prenylation.

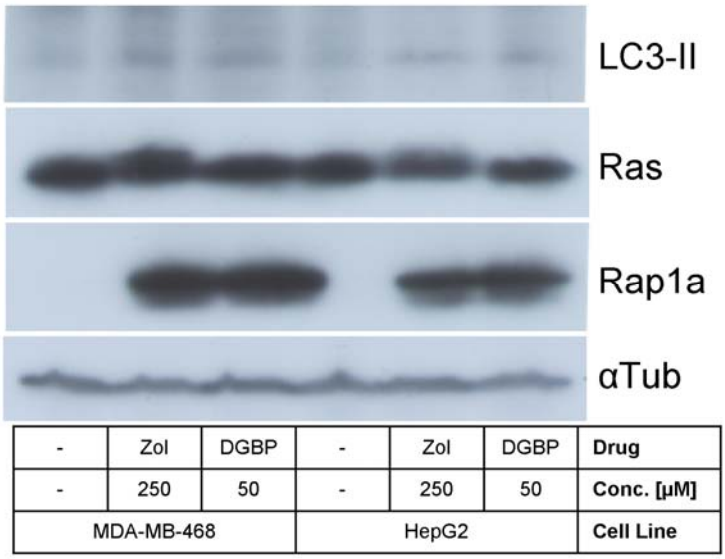
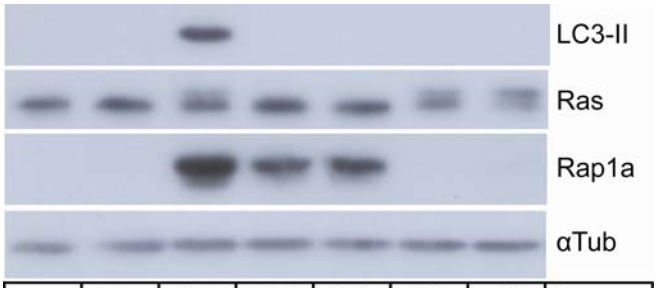


Figure 33. Higher concentrations of bisphosphonates do not induce autophagy in MDA-MB-468 and HepG2 cells. Cells were treated with isoprenoid pathway inhibitors (zoledronate and DGBP) as indicated for 48 h. Cell lysis was followed by Western blotting to detect LC3-II formation and monitor Ras and Rap1a prenylation.



-	-	Lov	GGTI	GGTI	FTI	FTI	Drug
-	-	5	10	25	1	10	Conc.[μM]
-	+	+	-	-	-	-	DMSO

Figure 34. FTase or GGTase I inhibition does not induce autophagy in PC3 cells. Cells were treated with isoprenoid pathway inhibitors (lovastatin, GGTI-2133, and FTI-277) as indicated for 48 h. Cell lysis was followed by Western blotting to detect LC3-II formation and monitor Ras and Rap1a prenylation.

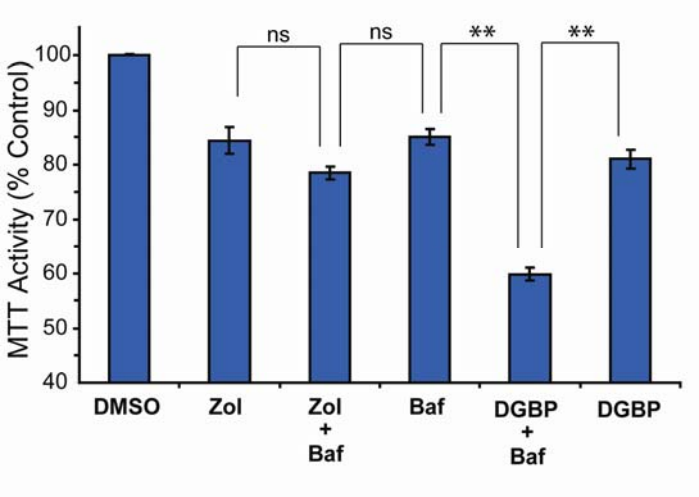


Figure 35. Inhibition of autophagy enhances DGBP-induced cytotoxicity in PC3 cells. MTT assay of cells treated with 50 μM zoledronate or 25 μM DGBP in the presence or absence of 5 nM bafilomycin A1 for 48 h (mean +/ SE, n = 3, ns = not significant, $p<0.01^{**}$).

CHAPTER VI: GGDPS INHIBITION AND THE CELL CYCLE

Abstract

Previous work has demonstrated that isoprenoid pathway inhibitors induce cell cycle arrest in numerous cancer cell lines. These effects have been attributed to increased expression of cyclin-dependent kinase inhibitors such as $p21^{Waf1/Kip1}$ and $p27^{Kip1}$. In this study, we determine whether specific inhibition of geranylgeranyl diphosphate synthase by our lead compound, digeranyl bisphosphonate, induces cell cycle arrest in MDA-MB-231 and MDA-MB-468 breast cancer cells. We demonstrate that DGBP increases the expression of $p27^{Kip1}$ in both breast cancer cell lines. The increase in expression of this cyclin-dependent kinase inhibitor is prevented by exogenous addition of geranylgeranyl diphosphate. Increased expression of $p27^{Kip1}$ is associated with transient increase in nuclear localization of this protein in MDA-MB-468 but not MDA-MB-231 cells. During these experiments, we noticed that GGPP and GGOH did not completely prevent DGBP-induced impairment of Rap1a geranylgeranylation in MDA-MB-468 breast cancer cells. This was also observed in PC3 prostate cancer cells. Additional experiments in PC3 cells demonstrate that Rab6 (GGTase II substrate) is preferentially geranylgeranylated over Rap1a (GGTase I substrate) during GGPP depletion, which suggests hierarchial geranylgeranylation of proteins.

Introduction

As describe in previous chapters, statins and bisphosphonates are used for the treatment of hypercholesteremia and bone related disorders, respectively. The inhibition of the isoprenoid biosynthesis pathway by these agents has been shown to disrupt cell cycle progression in various cancer cells (21). Statins have been shown to prevent the

degradation of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, and thus arrest the cells in G0/G1 phase of the cell cycle (209). The addition of GGPP but not FPP restored the degradation of $p27^{Kip1}$, which led to the activation of cyclin-dependent kinase 2 (Cdk2) and allowed the cells to resume the cell cycle. In a different study, cell cycle arrest by statins was attributed to increased expression $p21^{Waf1/Kip1}$, a different cyclin-dependent kinase inhibitor (210). This study demonstrated that lovastatin increased the binding of $p21^{Waf1/Kip1}$ to Cdk2 and thus interfered with the progression of cancer cell through the cell cycle. Similar to statins, studies with NBPs have demonstrated that these agents have the ability to arrest cells in a certain phase of the cell cycle. One study demonstrated that zoledronate arrested myeloma cells in the S phase of the cell cycle (211). In the same study, the addition of exogenous GGOH prevented the effects of zoledronate on the cell cycle. In addition, zoledronate was shown to induce S-phase cell cycle arrest in other cell models of cancer (212, 213).

Similar to statins and NBPs, studies have been performed with FTIs and GGTIs to assess the effects of these agents on the cell cycle of cancer cells (21). Studies have demonstrated that FTIs can induce G0/G1 and G2/M cell cycle arrest (214). The effects of FTIs on the cell cycle are believed to be cell line specific (215). The G0/G1 cell cycle arrest induced by FTIs has been associated with increased expression of $p21^{Waf1/Kip1}$ (216). Regression of tumors in transgenic mice by an FTI has been attributed in part to it's ability to induce cell cycle arrest (217). Extensive work has also been performed with GGTIs in regards to cell cycle inhibition. It has been demonstrated that GGTIs arrest cell in the G0/G1 phase of the cell cycle through the up-regulation of $p21^{Waf1/Kip1}$ at the transcriptional level (218). More recently, it has been demonstrated that GGTIs also

induce the expression of $p27^{Kip1}$ in breast cancer cells (109). The increase in $p27^{Kip1}$ was due to phosphorylation at Thr187, leading to decreased degradation. This resulted in accumulation of this cyclin-dependent kinase inhibitor in the nucleus.

Literature suggests that isoprenoid biosynthesis inhibitors (statins and NBPs) can induce cell cycle arrest. Restoration of the cell cycle by the addition of GGPP or GGOH (see above) suggests that GGPP depletion may contribute to cell cycle arrest by statins and NBPs. With the development of GGDPS inhibitors (DGBP) in our laboratories, we set out to determine whether the inhibition of this enzyme interferes with cell cycle progression. More specifically, we wanted to determine whether GGPP depletion and the resulting impairment of protein geranylgeranylation leads to cell cycle arrest in MDA-MB-231 and MDA-MB-468 cells. We chose these breast cancer cell lines as a result of recent studies with GGTIs in these two cell lines (109).

Materials and Methods

Cell culture. PC3 prostate cancer cells, MDA-MB-231 breast cancer cells, MDA-MB-468 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in Ham's F-12 (PC3), MEM (MDA-MB-231), and Leibovitz's L-15 (MDA-MB-468) medium supplemented with 10 % FBS at 5% CO_2 and 37^{o}C.

Materials. Doxorubicin, GGPP, and GGOH were purchased from Sigma (St. Louis, MO). Rap1a, Sp1, Rab6, αTub and βTub antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The p27 antibody was from Cell Signaling Technology Inc. (Danvers, MA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit were from GE Healthcare while anti-goat was from Santa Cruz

Biotechnology, Inc. ECL detection kit was obtained from GE Healthcare (Buckinghamshire, UK). The nuclear and cytosol fractionation kit was obtained from BioVision Research Products (Mountain View, CA).

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 and lysed in radioimmunoprecipitation buffer supplemented with protease inhibitor cocktail, sodium vanadate, sodium fluoride, and phenylmethylsulphonyl fluoride. Lysates were transferred to a 1.5 ml tube, vortexed several times over 30 minutes, and passed through a 27-gage needle. Lysates were then centrifuged and supernatant transferred to a fresh 1.5 ml tube. All steps were performed at 4^{o}C.

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

Cell cycle analysis. The procedure was adapted from the Flow Facility at the University of Iowa (Iowa City, IA). Cells were plated in 12-well plates and treated with drugs as described in figures. After the incubation period, the cells were pelleted by trypsin re-suspended in 100 µl of PBS. 3 ml of -20^{o}C 70% ethanol was added to the

cells. After mixing, cells were incubated at 4°C for 1 hour. Cells were then washed two times with 2 ml of PBS and re-suspended in 100 µl of PBS. 100 µl of 1mM RNaseA solution and 200 µl of 100 µg/ml propidium iodine were added to the cells and mixed. Cells were incubated at 4°C for 30 minutes in the dark. Samples were then run on the flow cytometer.

Separation of nuclear and cytosolic proteins. A nuclear/cytosol fractionation kit (Catalog #K266) was purchased from BioVision Research Products (Mountain View, CA). Briefly, cells were plated in T75 flask and allowed to adhere overnight. Cells were treated with indicated compounds and separation of nuclear and cytosolic proteins was performed according to the kit protocol. Proteins were then separated on SDS-PAGE gels and analyzed by Western blotting.

FPP and GGPP quantification. FPP and GGPP levels were determined as described previously (161). Briefly, FPP and GGPP were extracted and used as substrates for incorporation into fluorescent CAAX peptides by farnesyl transferase and geranylgeranyl transferase. Prenylated fluorescent peptides were quantified by fluorescent detection. FPP and GGPP levels were normalized to protein levels measured by BCA assay.

Results

DGBP increases $p27^{Kip1}$ protein expression in MDA-MB-468 and MDA-MB-231 cells. In order to determine if GGDPS inhibition by DGBP alters $p27^{Kip1}$ protein levels, MDA-MB-468 and MDA-MB-231 cells were treated with 25 µM of the drug for 24 and 48 hours (Figure 36). 25 µM DGBP maximally impairs protein geranylgeranylation as measured by accumulation of unmodified Rap1a in MDA-MB-468 and MDA-MB-231

breast cancer cells (data not shown) and this concentration of DGBP will be utilized for all experiments in this chapter. The appearance of a band on the Rap1a blot indicates impaired geranylgeranylation of Rap1a while the appearance of an upper band on the Rab6 blot indicates impaired geranylgeranylation of Rab6. 25 μM DGBP impairs geranylgeranylation of Rap1a (GGTase I substrate) and Rab6 (GGTase II substrate) in a time-dependent manner in MDA-MB-468 and MDA-MB-231 cells. The addition of 20 μM GGPP completely restores Rap1a and Rab6 geranylgeranylation which is impaired by DGBP in MDA-MB-231. Interestingly, 20 μM GGPP restores Rab6 geranylgeranylation completely while Rap1a geranylgeranylation is only partially restored during DGBP treatment in MDA-MB-468 cells. A time-dependent increase in the expression of $p27^{Kip1}$ is observed in both breast cancer cell lines but the change is more pronounced in the MDA-MB-231 cells. The addition of GGPP to DGBP restores the expression of $p27^{Kip1}$ to control levels in both cell lines.

GGPP does not completely restore Rap1a geranylgeranylation in MDA-MB-468 cells. An interesting finding was that 20 μM GGPP is not able to restore Rap1a geranylgeranylation in the presence of 25 μM DGBP in MDA-MB-468 cells (Figure 36). We next determined whether a change in GGPP concentration may completely restore Rap1a geranylgeranylation in MDA-MB-468 cells. MDA-MB-231 cells, in which Rap1a geranylgeranylation was previously restored with 20 μM GGPP in the presence of 25 μM DGBP (206), were included in the study. The addition of 25 μM DGBP impairs Rap1a geranylgeranylation in both MDA-MB-468 and MDA-MB-231 cells (Figure 37). All tested concentrations of GGPP and 10 μM GGOH restore Rap1a geranylgeranylation in MDA-MB-231 cells. Conversely, none of the GGPP concentrations tested are able to

completely restore Rap1a geranylgeranylation in MDA-MB-468 cells. However, GGPP concentration-dependent restoration of Rap1a prenylation is observed in this experiment. Similarly, 10 μM GGOH fails to restore prenylation of Rap1a in the presence of 25 μM DGBP in MDA-MB-468 cells.

DGBP does not significantly increase nuclear $p27^{Kip1}$ in MDA-MB-468 and MDA-MB-231 cells. In order to determine whether the increase in $p27^{Kip1}$ expression results in increased nuclear localization of this protein, nuclear and cytosolic proteins were fractionated. The transcriptional factor, Sp1, was utilized as a nuclear marker while β-tubulin (βtub) was utilized as a cytosolic marker in these studies. DGBP increases the nuclear localization of $p27^{Kip1}$ in MDA-MB-468 cells at 24 hours; this increase is abolished at 48 hours (Figure 38). In these cells, DGBP increases expression of $p27^{Kip1}$ in the cytosol at 48 but not at 24 hours. Addition of exogenous GGPP prevents DGBP-induced expression of $p27^{Kip1}$ in the nucleus and cytosol.

The same study was performed with MDA-MB-231 cells (Figure 39). The effects of DGBP are less pronounced in MDA-MB-231 cells (Figure 39) as compared to MDA-MB-468 (Figure 38) cells. The expression of $p27^{Kip1}$ in the nucleus remains unchanged in the presence of DGBP for 24 and 48 hours. DGBP increases the cytosolic expression of $p27^{Kip1}$ at 24 and 48 hours; this is prevented by exogenous GGPP addition. The separation of nuclear and cytosolic proteins is confirmed by the cytosolic (βtub) and nuclear (Sp1) markers being detected only in their respective fractions (Figures 38 and 39).

DGBP does not significantly alter cell cycle progression in MDA-MB-468 and MDA-MB-231 cells. In order to assess the effects of GGDPS inhibition on cell cycle

progression, MB-468 and MDA-MB-231 breast cancer cells were treated with DGBP in the presence or absence of GGPP. Doxorubicin, a DNA intercalating agent, was used as a positive control for cell cycle arrest since it was previously shown to interfere with cell cycle progression in breast cancer cells (219). Propidium iodine staining demonstrates that DGBP has no significant effect on the cycle progression in MDA-MB-468 (Figure 40) and MDA-MB-231 (Figure 41) breast cancer cells. A slight increase in the G0/G1 phase is observed in MDA-MB-468 (~8% increase) and MDA-MB-231 (~11% increase) cells in the presence of DGBP. The addition of GGPP prevents the effects of DGBP on the cell cycle. As anticipated, doxorubicin arrested cells in the G2/M phase of the cell cycle in both cell lines.

Rab6 is preferentially geranylgeranylated over Rap1a in the presence of DGBP in PC3 cells. It was observed that Rap1a geranylgeranylation cannot be restored with GGPP or GGOH in MDA-MB-468 cells (Figures 36 and 37). An initial finding with PC3 prostate cancer cells demonstrated a similar phenomenon: GGPP and GGOH cannot completely restore Rap1a geranylgeranylation (data not shown). Therefore, a closer analysis of these results was necessary. Due to convenience, more detailed look at this phenomenon was performed in PC3 over MDA-MB-468 cells. In order to determine if restoration of protein geranylgeranylation is time dependent, PC3 cells were treated with DGBP in the presence or absence of GGPP for variable lengths of time (Figure 42). For each time point, cells were pre-treated (3 hours) with GGPP and GGPP was also administered with DGBP at the same time. DGBP interferes with geranylgeranylation of Rap1a (GGTase I substrate) and Rab6 (GGTase II substrate) at all time points as indicated by the appearance of a Rap1a band and an upper Rab6 band, respectively. Dual

administration or pre-treatment of GGPP completely restores Rap1a geranylgeranylation at 9 hours in the presence of DGBP. However, GGPP is not able to completely restore Rap1a geranylgeranylation as indicated by the presence of a Rap1a band at 24 and 48 hours. On the other hand, both GGPP applications restore Rab6 geranylgeranylation as indicated by the absence of the upper Rab6 band in the presence of DGBP at all times tested (9, 24, 48 hours).

Limited repletion of cellular GGPP (depleted by DGBP) by exogenous GGOH in PC3 cells. To determine if DGBP-depleted GGPP can be restored in PC3 cells, 10 μM of exogenous GGOH was co-administered with 25 μM DGBP for 9 and 48 hours (Figure 43). As previously reported in other cell lines, DGBP depletes GGPP while slightly increasing FPP stores when compared to untreated cells (columns 1, 2 and 4) at 9 and 48 hours in PC3 cells. Dual administration of exogenous GGOH partially restores cellular GGPP levels when compared to cells treated with DGBP (columns 2, 3, 4, and 5) at 9 and 48 hour. However, only ~20% of GGPP is restored when compared to untreated cells. Interestingly, cellular FPP levels drop at 9 hours with the addition of GGOH to DGBP-treated cells (columns 1, 2 and 3). Similarly, sole addition of exogenous GGOH decreases cellular FPP levels without affecting cellular GGPP levels when compared to untreated cells (columns 1 and 6).

Discussion

In this chapter of the thesis, we set out to determine whether GGDPS inhibition, which leads to depletion of GGPP resulting in impaired protein geranylgeranylation, interferes with the cell cycles of MDA-MB-231 and MDA-MB-468 breast cancer cells. As described in the introduction, isoprenoid biosynthetic pathway inhibitors have been

shown to alter expression levels of cell cycle proteins, including $p27^{Kip1}$. Similar to other inhibitors of isoprenoid biosynthetic pathway such as statins and NBPs, DGBP increases the expression of $p27^{Kip1}$ in the two breast cancer cell lines. The increase in $p27^{Kip1}$ expression by DGBP is prevented by exogenous GGPP, suggesting high specificity of this effect to GGDPS inhibition. As a control, Rap1a geranylgeranylation was monitored. Interestingly, GGPP is not able to completely restore Rap1a geranylgeranylation in the presence of DGBP. This is a very novel finding. The addition of GGPP completely restores Rap1a geranylgeranylation in the presence of statins, NBPs, and DGBP in all other cell lines tested in our laboratory.

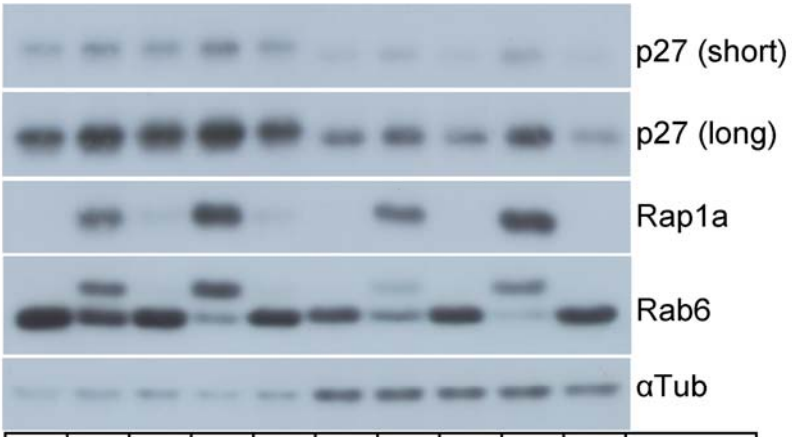
Earlier work has demonstrated that increase in $p27^{Kip1}$ by a GGTI results in increased localization of this cell cycle inhibitor in the nucleus (109). Increased expression of $p27^{Kip1}$ in the nucleus is associated with a better prognosis in breast cancer patients (220). Therefore, we wanted to determine whether the increase in $p27^{Kip1}$ expression leads to increased nuclear localization of this protein. An increase in nuclear localization of $p27^{Kip1}$ is observed in the presence of DGBP in MDA-MB-468 cells at 24 hours. This is completely prevented by exogenous GGPP. However, nuclear localization of $p27^{Kip1}$ returns to control levels at 48 hours. These results suggest that $p27^{Kip1}$ is exported to the cytoplasm with prolonged DGBP treatment. This is evidenced by our results that show increased cytoplasmic localization of this protein at 48 hours. In MDA-MB-231 cells, $p27^{Kip1}$ expression is increased in the cytoplasmic fraction at all time points (no increase in nuclear localization at 24 hours as observed with MDA-MB-468 cells).

In order to assess whether increased expression of $p27^{Kip1}$ is associated with cell cycle arrest, flow cytometry experiments were performed with cells that were treated with DGBP in the presence or absence of GGPP. Doxorubicin, the positive control, arrests both cell types, MDA-MB-231 and MDA-MB-468, in the G2/M phase of the cell cycle. DGBP minimally increases the number of cells in the G0/G1 phase of the cell cycle. These effects are completely prevented by exogenous addition of GGPP. With these results, it is safe to say that cell cycle arrest is not a means by which GGDPS inhibitors, such as DGBP, may be utilized in the treatment of cancers, including breast cancer. There is not a clear explanation that addresses the differences observed with our studies of GGDPS inhibition and previous studies with GGTase I inhibition on cell cycle arrest (109, 218). One possible explanation is the fact that compounds such as DGBP interfere with geranylgeranylation of GGTase I substrates (RhoA, Rac1, Cdc42) and GGTase II substrates (various Rab proteins) by depleting GGPP while GGTIs only interfere with geranylgeranylation of GGTase I substrates. It is also possible that cells bypass cell cycle arrest during GGDPS inhibition and enter other cell processes such as apoptosis and autophagy.

Interestingly, it was found that neither GGPP nor GGOH were able to completely restore Rap1a geranylgeranylation impaired by DGBP in MDA-MB-468 and PC3 cells (PC3 data not shown). As a result, additional experiments were performed in PC3 cells. Time-course experiments demonstrate that GGPP restores Rap1a (GGTase I substrate) and Rab6 (GGTase II substrate) geranylgeranylation at shorter (9 hour) while only Rab6 geranylgeranylation is restored at longer (24 and 48 hour) DGBP exposures. Measurements of cellular FPP and GGPP levels indicate that GGOH restores a small

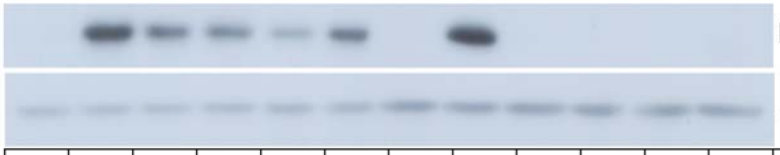
fraction (~20%) of cellular GGPP during DGBP treatment (9 and 48 hours). These results may explain the inability of GGOH to restore Rap1a geranylgeranylation in PC3 cells. We have previously shown that a greater fraction (~80%) of GGPP is restored by 10 μM of exogenous GGOH in K562 cells (179). Rap1a geranylgeranylation was completely restored by GGOH in these cells. It appears that a greater degree of GGPP replenishment is required to restore Rap1a geranylgeranylation when comparing PC3 cells to K562 cells. The complete restoration of Rab6 but not Rap1a geranylgeranylation by GGOH in the presence of DGBP at all times tested suggests hierarchial geranylgeranylation of proteins. In other words, some proteins (Rab6) are preferentially geranylgeranylated over other proteins (Rap1a) during shortage of GGPP and perhaps under normal (no depletion of GGPP) cellular conditions. Interestingly, we also show that GGOH addition in the absence or presence of DGBP decreases cellular FPP levels at 9 hours. It is possible that an increase in cellular GGOH inhibits or modulates upstream isoprenoid biosynthesis pathway enzymes to decrease FPP production. Indeed, it has been shown that GGOH interferes with HMGCR reductase activity in A549 lung adenocarcinoma cells (221). In addition, GGOH has been shown to induce ubiquitin-dependent HMGCR degradation (222).

In this chapter, we demonstrate that GGDPS inhibition does not have a significant impact on the cell cycle. In addition, GGOH add-back experiments suggest hierarchial geranylgeranylation of proteins during GGPP depletion in cells.



-	+	+	+	+	-	+	+	+	+	25 μM DGBP
-	-	+	-	+	-	-	+	-	+	20 μM GGPP
48	24	24	48	48	48	24	24	48	48	Time (h)
MDA-MB-468					MDA-MB-231					Cell Line

Figure 36. DGBP increases $p27^{Kip1}$ protein expression in MDA-MB-468 and MDA-MB-231 cells. Cells were treated with DGBP in the presence or absence of GGPP for 24 and 48 hours. Cell lysis was followed by Western blotting to detect changes in p27 expression and prenylation status of Ras and Rap1a.



-	25	25	25	25	25	-	25	25	25	25	25	DGBP [μM]
-	-	10	20	40	-	-	-	10	20	40	-	GGPP [μM]
-	-	-	-	-	10	-	-	-	-	-	10	GGOH [μM]
MDA-MB-468						MDA-MB-231						Cells

Figure 37. Restoration of Rap1a geranylgeranylation by GGPP and GGOH in the presence of DGBP in MDA-MB-468 and MDA-MB-231 cells. Cells were treated with DGBP in the presence or absence of GGPP and GGOH for 48 hours. Cell lysis was followed by Western blotting to detect prenylation status of Rap1a.

-	25	25	25	25	-	25	25	25	25	DGBP [μM]
-	-	20	-	20	-	-	20	-	20	GGPP [μM]
48	24	24	48	48	48	24	24	48	48	Time (h)
Cytosolic					Nuclear					Fraction

Figure 38. Expression of $p27^{Kip1}$ in the cytosol and nucleus in MDA-MB-468 cells. Cells were treated with DGBP in the presence or absence of GGPP for 24 and 48 hours. Nuclear and cytosolic proteins were separated as described followed by Western blotting to detect expression and localization of p27.Sp1 is a nuclear (N) while βtub (C) is a cytosolic marker.

-	25	25	25	25	-	25	25	25	25	DGBP [μM]
-	-	20	-	20	-	-	20	-	20	GGPP [μM]
48	24	24	48	48	48	24	24	48	48	Time (h)
Cytosolic					Nuclear					Fraction

Figure 39. Expression of $p27^{Kip1}$ in the cytosol and nucleus in MDA-MB-231 cells. Cells were treated with DGBP in the presence or absence of GGPP for 24 and 48 hours. Nuclear and cytosolic proteins were separated as described followed by Western blotting to detect expression and localization of p27. Sp1 is a nuclear (N) while βtub (C) is a cytosolic marker.

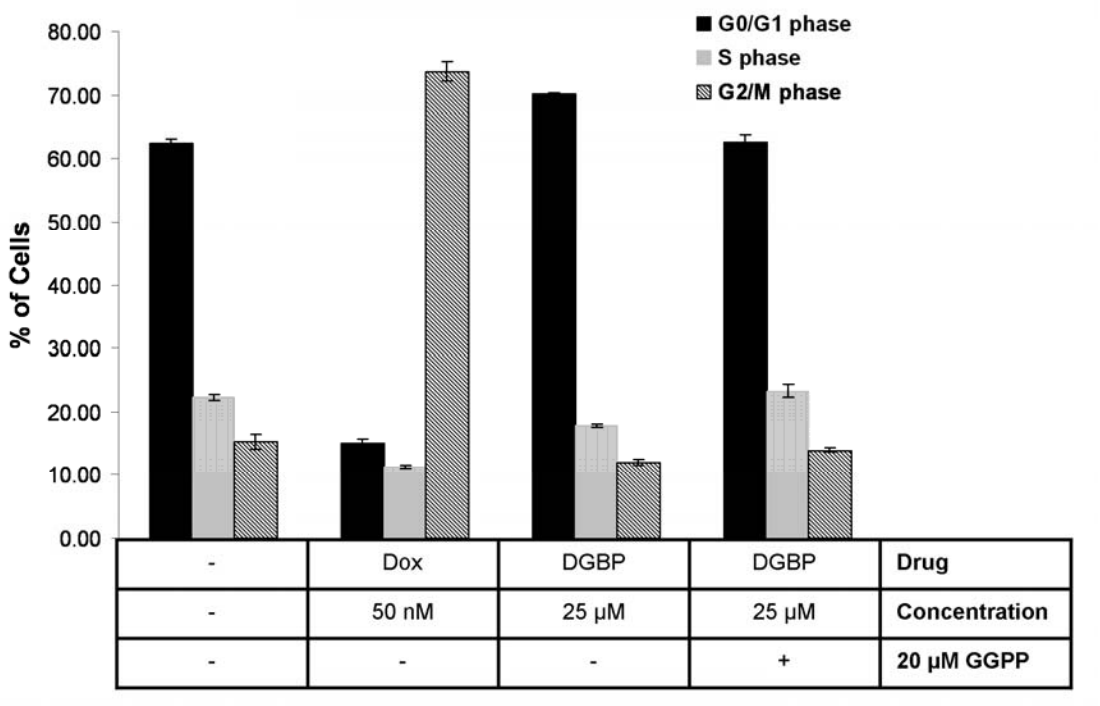


Figure 40. Cell cycle analysis of DGBP-treated MDA-MB-468 cells. Cells were treated with DGBP, GGPP, and doxorubicin as demonstrated for 48 hours. Cell cycle analysis was performed as described in materials and methods.

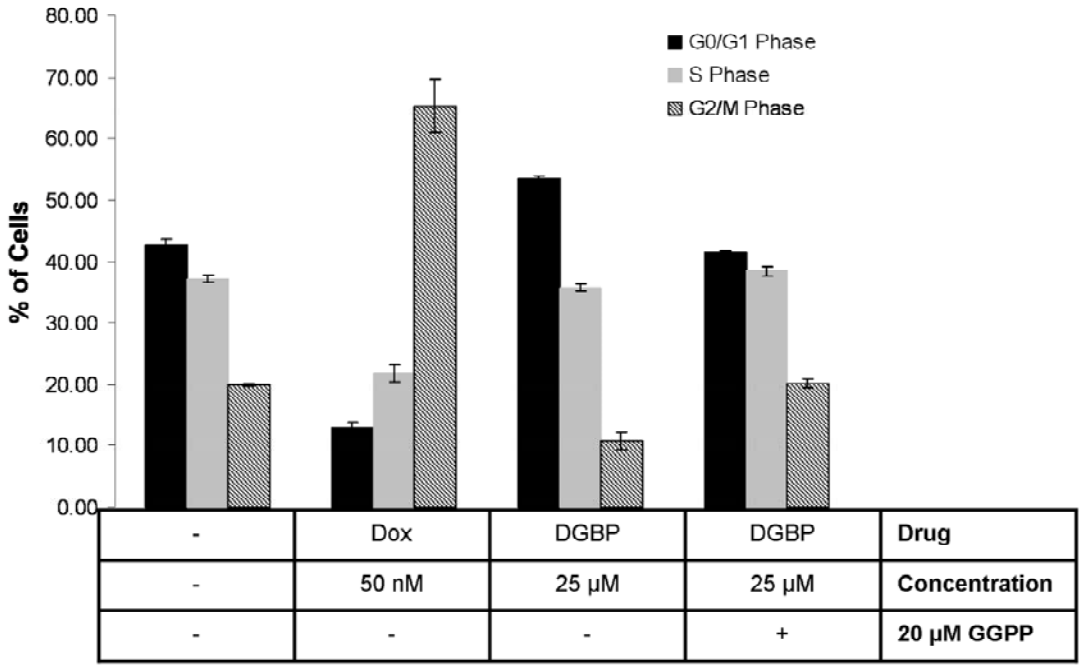
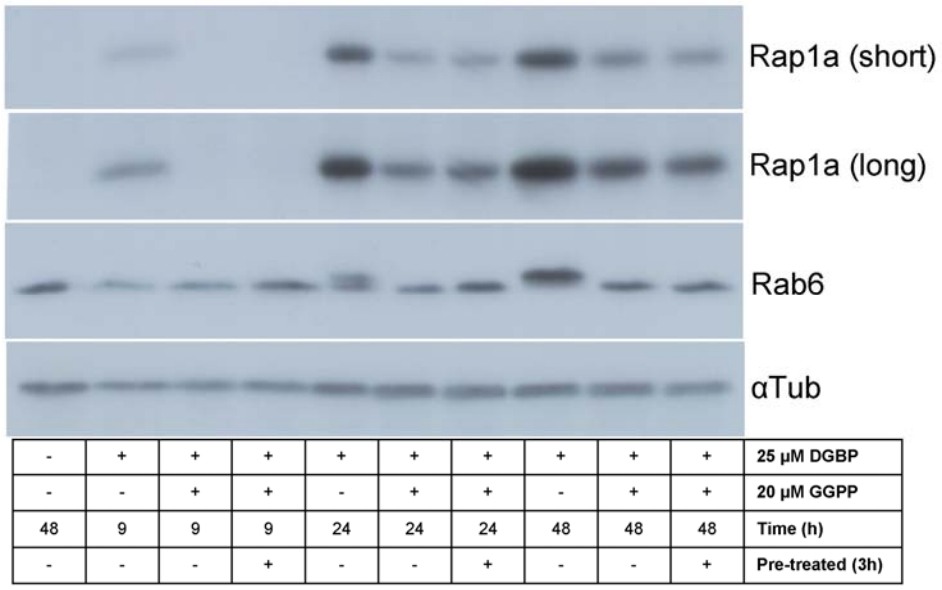


Figure 41. Cell cycle analysis of DGBP-treated MDA-MB-231 cells. Cells were treated with DGBP, GGPP, and doxorubicin as demonstrated for 48 hours. Cell cycle analysis was performed as described in materials and methods.



-	+	+	+	+	+	+	+	+	+	25 μM DGBP
-	-	+	+	-	+	+	-	+	+	20 μM GGPP
48	9	9	9	24	24	24	48	48	48	Time (h)
-	-	-	+	-	-	+	-	-	+	Pre-treated (3h)

Figure 42. Rab6 is preferentially geranylgeranylation over Rap1a in the presence of DGBP in PC3 cells. Cells were treated with DGBP in the presence or absence of GGPP at indicated time. GGPP was added two different ways: Together with or 3 hours prior to the DGBP treatment. Cell lysis was followed by Western blotting to detect the prenylation status of Rap1a and Rab6.

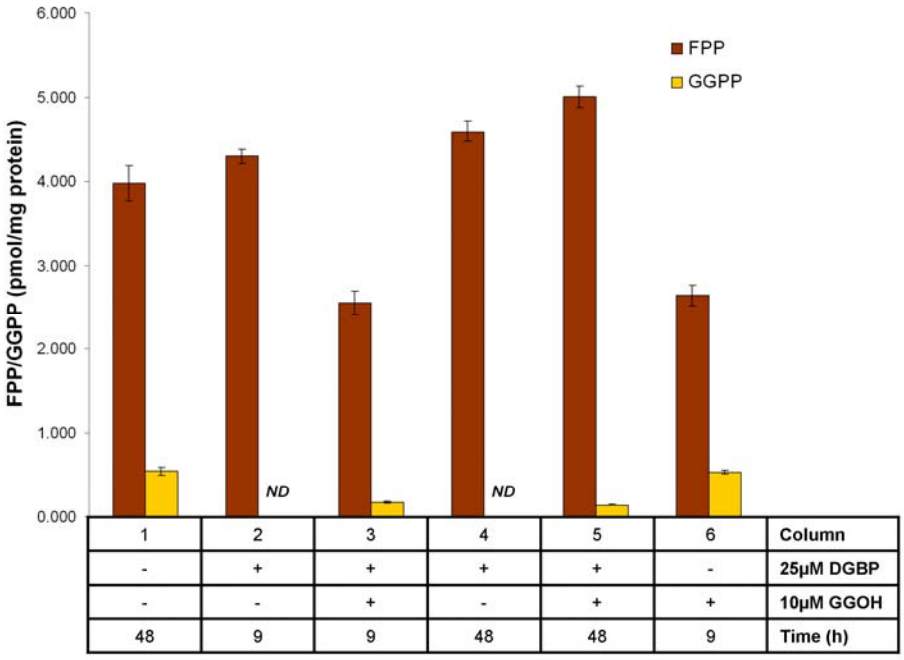


Figure 43. Limited repletion of cellular GGPP (depleted by DGBP) by exogenous GGOH in PC3 cells. Cells were treated with 25 μM DGBP in the presence or absence of 10 μM GGOH as outlined in the chart. Extraction and quantification of FPP and GGPP was performed as described in methods. FPP and GGPP levels were normalized to protein concentration measured by BCA assay (mean +/- SEM, n = 2). *ND* = not detected (below the limit of detection).

CHAPTER VII: SUMMARY

Summary of Results

As discussed in the introductory chapter of this thesis, recent literature suggests that GGPP depletion is the mechanism by which isoprenoid biosynthetic inhibitors (statins and NBPs) interfere with cancer cell proliferation. Add-back experiments with isoprenoid biosynthetic intermediates (FPP and GGPP) illustrate the importance of GGPP to cancer cell survival and function. However, the development of agents that specifically deplete GGPP (do not deplete upstream isoprenoid biosynthetic intermediates such as FPP) would allow for more specific analysis of cancer cell dependence on GGPP.

Our laboratories have therefore developed specific bisphosphonate inhibitors of GGDPS (138-142). Our lead compound, digeranyl bisphosphonate (DGBP) (structure shown in Figure 1), has been shown to inhibit protein geranylgeranylation but not protein farnesylation (138, 139). DGBP has been shown to deplete GGPP but not FPP in tissue culture cells as well some mammalian tissues (139, 143).

The overall hypothesis of this work was to determine if GGDPS inhibition is a potential cancer therapeutic approach. The goal of this research was to assess the effects of GGDPS inhibition, which leads to the depletion of GGPP and inhibition of protein geranylgeranylation, on various aspects of cancer cell survival and function. We also set out to identify novel and perhaps more potent GGDPS inhibitors with the help of Professor Wiemer's chemistry group.

In the second chapter of this thesis, several compounds were assessed as potential GGDPS inhibitors. Rocky Barney (Professor Wiemer's chemistry group) synthesized several aromatic bisphosphonates by modifying the structure of our lead compound,

DGBP. The research herein shows that several of these new compounds are potent *in vitro* inhibitors of GGDPS. The *in vitro* IC_{50} values (nM range) of these compounds are comparable to our lead compound (DGBP). Cell culture work demonstrated that two of these compounds impair protein geranylgeranylation in K562 cells. However, these compounds are not as potent at impairing protein geranylgeranylation as is DGBP. Proliferation assays performed with these novel compounds suggest that more potent inhibition of GGDPS by bisphosphonates leads to increased cellular effects (greater inhibition of DNA synthesis).

In the third chapter of this thesis, we demonstrate that inhibition of geranylgeranyl diphosphate synthase by DGBP induces apoptosis in K562 leukemia cells. This induction of apoptosis is in part dependent upon both GGPP depletion and accumulation of FPP. A combination of either lovastatin or zoledronate with DGBP synergistically inhibits growth and induces apoptosis. These combinations also potently inhibited cellular geranylgeranylation. In addition to DGBP, we show that compound 14, a novel GGDPS inhibitor, induces apoptosis and synergizes with lovastatin. These results support the potential use of combinations of multiple inhibitors of isoprene biosynthesis to inhibit cancer cell growth.

In the fourth chapter of this thesis, we show that DGBP, similar to GGTI-286, significantly inhibits migration of MDA-MB-231 cells as measured by a transwell assay. Similarly, DGBP and compound 14 reduce motility of MDA-MB-231 cells in a time-dependent manner as measured by large scale digital cell analysis system microscopy. DGBP is mildly toxic and does not induce apoptosis in these cells at relevant concentrations. Treatment of MDA-MB-231 cells with DGBP decreases membrane while

increasing cytosolic RhoA localization. In addition, DGBP increases RhoA GTP binding in MDA-MB-231 cells. The specificity of GGDPS inhibition by DGBP is confirmed by exogenous addition of GGPP. GGPP addition prevents the effects of DGBP on migration, RhoA localization, and GTP binding to RhoA in MDA-MB-231 cells. These studies suggest that GGDPS inhibitors are a novel approach to interfere with cancer cell migration.

In the fifth chapter of this thesis, we show that a FDPS inhibitor (zoledronate) and two GGDPS inhibitors (DGBP and compound 14) 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, DGBP, or compound 14 further enhances the formation of LC3-II, indicating these compounds induce autophagic flux. In addition, specific inhibitors of FTase and GGTase I are unable to induce autophagy in our system. Furthermore, the addition of bafilomycin A1 (an inhibitor of autophagy processing) enhances the cytotoxic effects of DGBP. These results are the first to demonstrate that bisphosphonates induce autophagy. Our studies suggest that induction of autophagy with these agents is dependent upon inhibition of protein geranylgeranylation by geranylgeranyl transferase II.

In the sixth chapter of this thesis, we demonstrate that DGBP increases the expression of $p27^{Kip1}$ in MDA-MB-231 and MDA-MB-468 breast cancer cell lines. The increase in expression of this cyclin-dependent kinase inhibitor is prevented by exogenous addition of GGPP. Increased expression of $p27^{Kip1}$ is associated with transient increase in nuclear localization of this protein in MDA-MB-468 but not MDA-MB-231

cells. During these experiments, we noticed that GGPP and GGOH do not completely prevent DGBP-induced impairment of Rap1a geranylgeranylation in MDA-MB-468 breast cancer and PC3 prostate cancer cells. Additional experiments in PC3 cells demonstrate that Rab6 (GGTase II substrate) is preferentially geranylgeranylated over Rap1a (GGTase I substrate) during GGPP depletion, which suggests hierarchial geranylgeranylation of proteins.

In summary, we have identified two novel aromatic bisphosphonate inhibitors of GGDPS. We show that some of the previously reported anti-cancer effects of upstream isoprenoid biosynthetic inhibitors (statins and NBPs) can be achieved through downstream inhibition of GGDPS. Induction of apoptosis, inhibition of migration, and activation of autophagy can be achieved through direct inhibition of GGDPS. On the other hand, cell cycle arrest, which has been demonstrated with statins and NBPs, cannot be achieved with the inhibition of GGDPS.

Future Directions

According to the data presented in this thesis, inhibition of GGPDS appears to be a potential anti-cancer strategy. For the most part, anti-cancer effects that are observed with statins and NBPs can be achieved by inhibiting GGDPS. Due to the downstream location of GGDPS in the isoprenoid biosynthesis pathway, it may be more beneficial to target this enzyme over HMGCR and FDPS, which are located upstream in the isoprenoid biosynthesis pathway, as part of cancer therapy. The inhibition of GGDPS could achieve clinically desired effects: depletion of GGPP and thus inhibition of protein geranylgeranylation, which appears to be a critical component for cancer cell progression

and survival. At the same time, the inhibition of GGDPS does not significantly alter upstream isoprenoids as seen with statins and NBPs.

A reasonable next set of experiments should investigate GGDPS inhibitors in animal models. The first set of experiments in animal models should determine the toxicity profiles of some the most potent *in vitro* and cellular GGDPS inhibitors. It may be possible that some of these inhibitors are toxic and the required drug concentrations for inhibition of protein geranylgeranylation may not be achievable in animals. Therefore, multiple GGDPS inhibitors should be assesses at the same time to increase the chance of identifying a clinically relevant compound. Once a candidate compound has been identified and all the necessary pharmacokinetic studies performed, this lead GGDPS inhibitor should be tested in animal models of cancer. This could include transgenic or xenograft models of cancer. Investigation of cancer models that have altered expression or function of geranylgeranylated proteins should be investigated. A great example would be models of breast cancer in which increased expression of Rho proteins (RhoA, Cdc42, and Rac1) are associated with poor clinical outcomes. In case GGDPS inhibitors are not effective as single agents, they should be considered as part of multi-drug regimen in the treatment of cancer. It may be a good idea to perform cellular assays (synergy experiments) with GGDPS inhibitors in combination with some of the clinically used anti-cancer agents to justify combinational studies in animal.

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