Inhibition of Geranylgeranyl Diphosphate Synthase Induces Apoptosis through Multiple Mechanisms and Displays Synergy with Inhibition of Other Isoprenoid Biosynthetic Enzymes

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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. Because all three enzymes fall in the same biosynthetic pathway and many of the biological effects are due to depletion of downstream products, we hypothesized that simultaneous inhibition of these enzymes would result in synergistic growth inhibition. In this study, we show that inhibition of geranylgeranyl diphosphate synthase induces apoptosis in K562 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 inhibited growth and induced apoptosis. These combinations also potently inhibited cellular 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.

The mevalonate pathway (Fig. 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 several years to treat hypercholesterolemia (Grundy, 1988). The statins inhibit HMG-CoA reductase (HMGCR), depleting cells of downstream isoprenoids, including farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) (Brown and Goldstein, 1980). FPP is a key intermediate in synthesis of cholesterol, and its depletion results in decreased de novo cholesterol synthesis (Andersen and Dietsch, 1978) leading to increased low-density lipoprotein receptor expression and decreased serum low-density lipoprotein cholesterol in vivo (Goldstein and Brown, 1990). 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 (Takai et al., 2001), and the Rho family of GTPases, which are important mediators of cell migration (Burridge and Doughman, 2006). Because prenylation is necessary for the activation of small GTPases, statins have been investigated as potential cytotoxic agents for use in cancer chemotherapy (Swanson and Hohl, 2006).

The nitrogenous bisphosphonates (NBPs), including zoledronate are diphosphate analogs used clinically to treat bone disorders, including osteoporosis and metastatic bone disease (Licata, 2005). These drugs have also been shown in the laboratory to induce apoptosis, and they have direct growth inhibitory effects on malignant cells (Shipman et al., 2005). These drugs have also been shown in the laboratory to induce apoptosis, and they have direct growth inhibitory effects on malignant cells (Shipman et al., 2005).

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ABBREVIATIONS: HMGCR, HMG-CoA reductase; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; NBP, nitrogenous bisphosphonate; FDPS, farnesyl diphosphate synthase; GGDP, geranylgeranyl diphosphate synthase; DGBP, digeranyl bisphosphonate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PI, propidium iodide; PCR, polymerase chain reaction; PARP, poly(ADP-ribose) polymerase; RT, room temperature; PBS, phosphate-buffered saline; CI, combination index; GGOH, geranylgeraniol; GGTase, geranylgeranyl transferase; FOH, farnesol; PC, phosphatidylcholine; SQS, squalene synthase; Lov, lovastatin; GGTL-298, N-4-[2(R)-amino-3-mercaptopropyl]amino-2-naphthylbenzoyl-(L)-leucine methyl ester trifluoroacetate.
Like statins, the clinically used NBPs deplete cells of isoprenoid diphosphates, resulting in the inhibition of post-translational protein prenylation (Reszka and Rodan, 2004). In particular, the NBPs inhibit FPP synthase (FDPS) (van Beek et al., 1999b; Dunford et al., 2001). However, their cellular effects, including induction of apoptosis, may be largely a result of downstream GGPP depletion (Fisher et al., 1999; van Beek et al., 1999a). Based on this rationale, we developed a series of isoprenoid-containing bisphosphonates that specifically inhibit geranylgeranyl diphosphate synthase (GGDPS) (Shull et al., 2006; Maalouf et al., 2007; Wiemer et al., 2007). We have shown that several of these compounds, including digeranyl bisphosphonate (DGBP), can potently inhibit GGDPS in vitro (Wiemer et al., 2007). We have also shown that inhibition of cellular GGDPS leads to depletion of intracellular GGPP levels and not depletion of FPP (Wiemer et al., 2007).

Both statins and NBPs have limited potential as single-agent treatments of malignancies. Inhibition of protein prenylation by the statins does not occur at standard concentrations for treatment of hypercholesterolemia (Lewis et al., 2005). NBPs are limited as anticancer agents because of high bone affinity and low plasma concentrations (Green and Clezardin, 2002). Therefore, if these agents are to be useful for treatment of malignant disease, it is possible that they will need to be used in combination with other agents. This strategy has led to the identification of an increasing number of synergistic interactions between inhibitors of isoprenoid biosynthesis and chemotherapeutic agents (Santini et al., 2006). For example, statins are synergistic with cytosine arabinoside (Holstein and Hohl, 2001a) and paclitaxel (Holstein and Hohl, 2001b). NBPs are synergistic with farnesyl transferase inhibitors (Caraglia et al., 2004), paclitaxel (Jagdev et al., 2001), and imatinib (Kuroda et al., 2003). It is noteworthy that the combination of statins and NBPs is synergistic (Schmidmaier et al., 2006).

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 inhibi-
tion to be studied. 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 noteworthy that inhibition of GGDPs by digeranyl bisphosphonate is synergistic with either HMG-CoA reductase inhibition by lovastatin or FDPS inhibition by zoledronate.

Materials and Methods

Cell Culture and Incubations. K562 leukemia cells were obtained from American Type Culture Collection (Manassas, VA), and they were cultured accordingly. For all experiments, cells were incubated in fresh RPMI 1640 medium containing 10% fetal bovine serum at a concentration of 0.5 x 10^6 cells/ml. For Western blots and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays, 2.5 x 10^6 total cells were incubated for times and concentrations indicated. Annexin V and propidium iodide (PI) analysis required 0.5 x 10^6 total cells. Real-time PCR experiments required 5 x 10^6 total cells.

DNA Synthesis Assays. K562 cells were incubated in 96-well plates and treated with compounds as described previously (Shull et al., 2006). After 22 h, 20 μl of [3H]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. [3H]Thymidine incorporated into cellular DNA was quantified by scintillation counting.

Annexin V and PI Analysis. Annexin V and PI analysis was 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 the supernatant was aspirated. Cells were resuspended in 500 μl of buffer (10 mM HEPES, 150 mM NaCl, 1 mM MgCl2, 5 mM KCl, and 1.8 mM CaCl2, 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 suspension was vortexed. Samples were analyzed using FACS can (BD Biosciences, Franklin Lakes, NJ).

Western Blot Analysis. Protein concentrations were determined using the bichinchoninic acid 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 polyvinylidene difluoride membrane. PARP was resolved using 7.5% gels. Primary and secondary antibodies were added sequentially for 45 min, and proteins were visualized using an enhanced chemiluminescence detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Anti pan-Ras was obtained from InterBio-technology (Tokyo, Japan). Ralp1a (sc-1482), Ralb6 (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-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. Reaction was 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, and then they were 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 (Tong et al., 2005). In brief, isopenoid 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 was 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:

- HMGR (5’-ACAGGCTTTGAATGCTTGGCCC-3’, 5’-GACAT-GCAGCCAAAGACGACATA3’), FDPS (5’-CTTCTGCAGAGTTCCTATCAGAC3’, 5’-TCCAGCCGAGGTCTCCTTGCGAT3’), and SQR (5’-ACTTCCCCAGATCTCCTCCAGT-3’, 5’-TCCAAACCT-TTGGCCGCAAACT-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 (1984) as described in the software manual. For each drug, 48-h IC50 values were determined by thymidine incorporation. Concentration-response curves were generated for each drug and combination using IC50 values as the highest concentration and four 80% dilutions.

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 set as the level of significance.

Results

Inhibition of Geranylgeranyl Diphosphate Synthase Inhibits Growth and Induces Apoptosis in Human Chronic Myelogenous Leukemia 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 [3H]thymidine incorporation (Fig. 2A). DGBP inhibited growth of these cells, with an IC50 value of 55 μM. To determine whether this effect was due to cell death, cells were treated with DGBP, and total cell death was measured using annexin V and PI staining. DGBP induced a concentration- and time-dependent increase in the number of apoptotic and necrotic cells (Fig. 2B). This increase was 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 Geranylgeranyl Diphosphate Synthase 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 (Schreiber et al., 2006). Caspases are cysteine aspartate-specific proteases that are activated by cleavage during early apoptosis (Thornberry and Lazebnik, 1998). Etoposide (50 μM), which is a well-characterized activator of apoptosis, was used as a positive control. DGBP, like etoposide, induced time-dependent cleavage of PARP as demonstrated by the appearance of an 85-kDa band (Fig. 2C). PARP cleavage was first observed at 24 h, and the effect was more pronounced after 72 h, analogous to what was 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 resulted in caspase-3 cleavage. These results indicated that DGBP activates the apoptosis machinery, leading to caspase-3 activation, PARP cleavage, and eventual cell death.

Geranylgeraniol Supplementation Prevents Digerozyll Bisphosphonate-Induced Inhibition of Protein Geranylgeranylation. To determine whether product supplementation could prevent DGBP-mediated inhibition of protein geranylgeranylation, cells were treated with GGOH, which is phosphorylated to form GGPP (Crick et al., 1997). Cells were treated for 48 h with DGBP in the presence or absence of GGOH, and then they were analyzed for inhibition of Rap1a and Rab6 geranylgeranylation by Western blot analysis (Fig. 3). 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 (Fig. 3, lane 2) and GGOH (Fig. 3, lane 3), neither of which limits prenylation. Like GGOH, FOH is phosphorylated to form FPP. As expected, 20 μM lovastatin limited Ras farnesylation and Rap1a and Rab6 geranylgeranylation (lane 5). Lovastatin-induced limitation of Ras farnesylation was prevented by addition of 1 mM mevalonate (Fig. 3, lane 6) or 10 μM FOH (Fig. 3, lane 7), but not 10 μM GGOH (Fig. 3, lane 8). Lovastatin-induced limitation of geranylgeranylation was prevented by addition of mevalonate (Fig. 3, lane 6) or GGOH (Fig. 3, lane 8), but not FOH (Fig. 3, lane 7). Mevalonate alone did not affect prenylation (data not shown). Concentrations of 120 μM zoledronate limited farnesylation and geranylgeranylation (Fig. 3, lane 10), and the effect on each was prevented by addition of
10 μM FOH and 10 μM GGOH, respectively (Fig. 3, lanes 11 and 12). Treatment with 40 μM DGBP did not affect farnesylation, but it inhibited geranylgeranylation of both the GGTase I substrate Rap1a and GGTase II substrate Rab6 (Fig. 3, lane 14), an effect that was completely reversed by addition of 10 μM GGOH (Fig. 3, lane 15).

Unlike HMG-CoA Reductase Inhibition, Apoptosis Induced by Geranylgeranyl Diphosphate Synthase Inhibition Is Not Fully Reversed by Product Supplementation. To determine whether product supplementation could prevent the apoptosis induced by inhibition of GGDS as indicated by PARP and caspase-3 cleavage, cells were treated with 100 μM DGBP for 72 h (Fig. 4A). As controls, cells were treated with either lovastatin, zoledronate, or GGTI-298, a peptidomimetic inhibitor of geranylgeranyl transferase I (Lerner et al., 1995). We were surprised to find that the addition of 10 μM exogenous GGOH did not prevent the ability of 100 μM DGBP to induce apoptotic effects (Fig. 4A, lanes 5 and 9). In fact, addition of 10 μM GGOH to 100 μM DGBP enhanced the cleavage of both PARP and caspase-3, whereas GGOH had no effects on PARP and caspase-3 on its own (Fig. 4A, lane 7). This contrasts to 50 μM lovastatin-induced PARP and caspase-3 cleavage, which was completely prevented by the addition of 1 mM mevalonate (Fig. 4A, lanes 3 and 8). Treatment with 250 μM zoledronate (Fig. 4A, lane 4) displayed minor amounts of cleavage, whereas 20 μM GGTI-298 did not induce any PARP and caspase-3 cleavage (Fig. 4A, lane 6). We performed a TUNEL assay to detect DNA fragmentation that results from the apoptotic signaling cascade, using the same conditions (Fig. 4B). As with PARP and caspase-3 cleavage, 50 μM lovastatin induced DNA fragmentation (Fig. 4B, column 3) that was prevented by the addition of 1 mM mevalonate (Fig. 4B, column 8), whereas 10 μM GGOH (Fig. 4B, column 9) was not able to prevent DNA fragmentation resulting from 100 μM DGBP (Fig. 4B, column 5). GGOH (10 μM) (Fig. 4B, column 7) and 20 μM GGTI (Fig. 4B, column 6) did not lead to DNA fragmentation. Zoledronate (250 μM) (Fig. 4B, column 4) led to DNA fragmentation but not as well as DGBP or lovastatin. Mevalonate alone had no effect on cleavage of PARP or caspase-3 and DNA fragmentation (data not shown).

Inhibition of Geranylgeranyl Diphosphate Synthase Leads to Apoptosis through Two Different Mechanisms. 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 (Fig. 5). Treatment with DGBP concentration-dependently decreased GGPP, whereas it increased FPP. GGOH partially reversed both the increase in FPP and decrease in GGPP.

Previous studies have shown that farnesol and geranylgerrnoi induce apoptosis (Edwards and Ericsson, 1999). Iso- prenoid alcohols, but not diphosphates, inhibit choline phosphotransferase leading to depletion of phosphatidylcholine (PC) (Voziyan et al., 1993; Miquel et al., 1998). 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) (Fig. 6). GGOH (10 μM) prevented apoptotic effects caused by low (25 μM) DGBP (Fig. 6, A and B). Although 10 μM GGOH completely prevented staining, 100 μM PC was not able to significantly impair, annexin V and PI staining (Fig. 6A, columns 4–6) and PARP cleavage (Fig. 6B, lanes 4–6) caused by 25 μM DGBP. However, PC was able to partially impair apoptotic effects caused by high (100 μM) DGBP (Fig. 6, A and C). Addition of 100 μM PC prevented some annexin V and PI staining (Fig. 6A, columns 7 and 9) and most PARP cleavage (Fig. 6C, lanes 4 and 6) caused by 100 μM DGBP. In contrast, the addition of 10 μM GGOH to 100 μM DGBP lead to enhanced annexin V and PI staining (Fig. 6A, columns 7 and 8) and PARP cleavage (Fig. 6C, lanes 4 and 5). Etoposide-induced apoptotic effects were not significantly altered by the addition of PC (Fig. 6, A–C, lanes/columns 2 and 3). Annexin V and PI staining and PARP cleavage were not affected by 10 μM GGOH or 100 μM PC (data not shown).

Inhibition of Geranylgeranyl Diphosphate Synthase Alters Steady-State mRNA Levels of Isoprenoid Biosynthetic Enzymes. 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 (Brown and Goldstein, 1997), 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 tested for altered expression of enzymes involved in isoprenoid biosynthesis via quantitative real-time PCR (Table 1). As characterized previously, treatment with lovastatin or zaragozic acid resulted in increased mRNA expression of HMGCR, FDPS, and SQS. Supporting our hypothesis, treatment with DGBP resulted in decreased expression of HMGCR, FDPS, and SQS mRNA.

Digeranyl Bisphosphonate-Induced Inhibition of Geranylgeranyl Diphosphate Synthase Is Synergistic with Lovastatin and Zoledronate. To further examine the consequences of GGDPS inhibition in the context of other protein geranylgeranylation inhibitors, cells were tested for synergistic interactions between DGBP and lovastatin, zoledronate, or GGTI-298. For these studies, concentration-response curves for each compound were constructed using [3H]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 (1984). The data were analyzed in two ways. First, isobologram analyses were conducted (Fig. 7). A general isobologram with a line of additivity and regions of synergy and antagonism is shown (Fig. 7A). Lovastatin and DGBP were strongly synergistic (CI50 = 0.03) for inhibition of K562 cell growth (Fig. 7B). Zoledronate was also synergistic with DGBP (CI50 = 0.84) (Fig. 7C), but not to the extent of the synergy with lovastatin. GGTI-298 was antagonistic to DGBP (CI50 = 1.64) (Fig. 7D). Although isobologram analysis can determine whether a combination of compounds is synergistic and approximate the magnitude of the synergistic interaction, it does not quantify this at the experimental concentrations per se. 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 displayed stronger synergy than the combination of zoledronate and lovastatin (CI50 = 0.64). This result has been observed in RPMI-8226 and U937 cells as well (data not shown).

To further support the synergy observed in [3H]thymidine incorporation assays, the effects of DGBP and lovastatin, DGBP and zoledronate, or the combination of both were determined by real-time PCR (Table 1). Data are mean ± S.E. (n = 4).

**TABLE 1**

| Differential effects of lovastatin, zaragozic acid, and DGBP on expression of HMGCR, FDPS, and SQS mRNA in K562 cells after 48 h determined by real-time PCR |
|-----------------|-----------------|-----------------|-----------------|
|                  | 20 μM Lovastatin | 5 μM Zaragozic Acid | 10 μM DGBP     |
| HMGCR           | 1.98 ± 0.21     | 1.92 ± 0.15     | 1.05 ± 0.04    |
| FDPS            | 1.87 ± 0.21     | 1.66 ± 0.14     | 0.98 ± 0.06    |
| SQS             | 1.52 ± 0.06     | 1.87 ± 0.34*    | 0.12 ± 0.06    |

*p < 0.05 relative to control for all samples except * p = 0.16.

Fig. 6. The mechanism of DGBP-induced apoptosis is concentration-dependent. K562 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 (n = 3). C, Western blot analysis in cells treated with high (100 μM) DGBP concentration (n = 3).

Inhibition of GGDPS is synergistic with inhibition of HMGCR and FDPS, but it is antagonistic to inhibition of GGTase. K562 cells were treated with varying concentrations of inhibitors alone or in indicated combinations at constant ratios for 48 h. Cell growth was assessed by [3H]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.

Experimental combination index values for K562 cells treated with DGBP in combination with various inhibitors of isoprenoid biosynthesis for 48 h as determined by [3H]thymidine incorporation (n = 4)

| Lov | DGBP | CI  | DGBP | GGTI | CI  | DGBP | Zol | CI  | Lov | Zol | CI  |
|-----|------|-----|------|------|-----|------|-----|-----|-----|-----|-----|-----|
| 20  | 41   | 0.25 | 20   | 12   | 1.57 | 41   | 123 | 0.79 | 20  | 123 | 0.65 |
| 26  | 51   | 0.22 | 26   | 15   | 2.09 | 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 |

Zol, zoledronate.

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 (Pérez-Sala and Mollinedo, 1994), FDPS (Shipman et al., 1997), and protein prenyltransferases (Lebowitz et al., 1997), 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 inhibition of protein geranylgeranylation can be prevented by addition of exogenous GGOH. At low amounts of GGDPS inhibition, apoptotic consequences are able to be prevented by exogenous GGOH. This agrees with previous studies that showed GGOH reverses apoptosis induced by the clinical NBPs (van Beek et al., 1999a). 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 (Miquel et al., 1998). 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 inhibition of protein geranylgeranylation caused by GGDPS inhibition.

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 (Brown and Goldstein, 1997). 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

![Fig. 7. Inhibition of GGDPS is synergetic with inhibition of HMGCR and FDPS, but it is antagonistic to inhibition of GGTase. K562 cells were treated with varying concentrations of inhibitors alone or in indicated combinations at constant ratios for 48 h. Cell growth was assessed by [3H]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.]
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 HMGCRR degradation in pulse-chase experiments (Meigs and Simoni, 1997). Third, both FPP and FOH have been shown to inhibit the mevalonate kinase reaction (Dorsey and Porter, 1968).

We have also shown for that 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 zolendronic acid similarly deplete FPP and GGPP and lack the apoptotic effects caused by FPP accumulation. This line of reasoning also explains why the combination of zolendronic and DGBP is more synergistic than the combination of zolendronic and lovastatin.

The data presented herein suggest that inhibition of GGDPS may be a good therapeutic strategy either alone or in combination with other isoprenoid pathway inhibitors. DGBP may be useful alone or in combination with statins for inhibition of cancerous 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.

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