Isoprenoid Pyrophosphate Analogues Regulate Expression of Ras-Related Proteins†

Sarah A. Holstein,‡ Christine L. Wohlford-Lenane,§ David F. Wiemer,|| and Raymond J. Hohl*,‡,§

Departments of Pharmacology, Internal Medicine, and Chemistry, University of Iowa, Iowa City, Iowa 52242

Received November 25, 2002; Revised Manuscript Received February 20, 2003

ABSTRACT: The isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are synthetic precursors for numerous molecules essential for cellular function as well as substrates in isoprenylation reactions. We have previously demonstrated that depletion of mevalonate results in the upregulation of Ras-related proteins which can be prevented by FPP or GGPP, independent of restoration of protein isoprenylation. To better define the regulatory properties of isoprenoid pyrophosphates, we have investigated the abilities of isoprenoid analogues to regulate the expression of the Ras-related proteins. Farnesyl phosphonic acids potentiate the upregulation of these proteins induced by mevalonate depletion independent of inhibitory activity against farnesyl protein transferase, geranylgeranyl protein transferase I, FPP synthase, or GGPP synthase. The potentiation of RhoB upregulation is at both the mRNA and protein level. The ability of these analogues to serve as functional antagonists of the isoprenoid pyrophosphates is dependent on the nature of the functional group at the head of the molecule, the charge of the molecule, and the length of the isoprenoid chain. Metabolites and additional analogues of isoprenoid pyrophosphates were found to possess agonist properties relative to FPP and GGPP. Interestingly, the structurally related retinoids *all-trans*-retinoic acid and 9-*cis*-retinoic acid also display slight agonist properties. These studies provide evidence for direct roles of FPP and GGPP in regulating transcriptional and post-transcriptional events.

Ras, Rap1a, RhoA, and RhoB belong to the Ras superfamily of small GTPases. These proteins play important roles in regulating diverse cellular functions, including cell survival, proliferation, differentiation, and cytoskeletal organization (*1*, *2*). Out of necessity, these roles require protein expression, proper intracellular localization, and interaction with downstream signal transducing elements. For example, central to the function of these proteins is their association with cellular membranes which is achieved, in part, by isoprenylation (*3*, *4*). Isoprenylation involves the addition of either a 15-carbon farnesyl chain or a 20-carbon geranylgeranyl chain to a cysteine sulfhydryl group near the carboxy terminus. The lipid donors in these reactions, FPP[1] and GGPP, are derived from mevalonate and are intermediates in the isoprenoid biosynthetic pathway.

Critically important, but less well characterized, are the factors that regulate the expression of these small GTPases. We have previously reported that mevalonate depletion, due to HMG-CoA reductase inhibition, results in the upregulation of Ras, Rap1a, RhoA, and RhoB (*5*). This upregulation is mediated transcriptionally for RhoB and translationally/post-translationally for Ras, RhoA, and Rap1a (*5*). Analysis of this upregulation has revealed that it is a consequence of depletion of specific isoprenoid species rather than a diminution of protein isoprenylation (*6*). Specifically, FPP prevents the upregulation of Ras and RhoB induced by mevalonate depletion, while GGPP prevents the upregulation of RhoA, RhoB, and Rap1a (*6*). Thus far, other isoprenoids have not been evaluated as regulators of the transcription and translation of these small GTPases.

There is precedence in the literature for the regulatory roles of farnesyl derivatives. The degradation of HMG-CoA reductase is regulated by farnesol (*7*−*9*). The farnesoid X receptor (FXR), a member of the nuclear receptor superfamily, has been shown to be activated by farnesol and juvenile hormone III (*10*). The mechanisms underlying the isoprenoid pyrophosphate-mediated regulation of the small GTPases are unknown. On the basis of our prior results (*5*, *6*), we reasoned that since FPP itself demonstrates biochemical activities, the use of FPP analogues would allow definition of the structural basis for these activities. We found that the structure−function relationship depends on charge, isoprene chain length, and differences in functional groups. Furthermore, comparisons to members of the retinoid family revealed interesting similarities to select isoprenoid analogues.

EXPERIMENTAL PROCEDURES

Cell Cultures and Reagents. The K562 cell line was purchased from the American Type Culture Collection

† This project was supported by the Leukemia and Lymphoma Society in the form of a Translational Research Grant, the Roy J. Carver Charitable Trust, and the Roland W. Holden Family Program for Experimental Cancer Therapeutics.

* To whom correspondence should be addressed: Department of Internal Medicine, C32 GH, University of Iowa, Iowa City, IA 52242. Telephone: (319) 356-8110. Fax: (319) 353-8383. E-mail: raymond-hohl@uiowa.edu.

‡ Department of Pharmacology.

§ Department of Internal Medicine.

|| Department of Chemistry.

[1] Abbreviations: HMG-CoA, hydroxymethylglutaryl coenzyme A; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; FPTase, farnesyl protein transferase; GGPTase, geranylgeranyltransferase; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; cmpd, compound.

10.1021/bi027227m CCC: $25.00
Published on Web 03/29/2003

(Manassas, VA). The K562 cell line is a human erythroleukemia line that was established from a patient with chronic myelogenous leukemia (*11*). K562 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin, amphotericin (2.5 μg/mL), and glutamine (2 mM). Cells were grown at 37 °C in a 5% CO_2 atmosphere in T-75 culture flasks. Anti-RhoA, anti-RhoB, anti-Rap1a [specific for unmodified Rap1a (*12*), catalog no. sc-1482], anti-β-tubulin, and anti-goat IgG HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The NCC-004 anti-pan RAS antibody (*13*) was kindly provided by S. Hirohashi (National Cancer Center, Tokyo, Japan). Anti-mouse and anti-rabbit HRP-linked antibodies were obtained from Amersham (Piscataway, NJ). Lovastatin, IPP, GPP, FPP, GGPP, *trans,trans*-farnesol (compound **19**, Figure 1B), geranylgeraniol (compound **21**), *trans,trans*-farnesyl acetate (compound **15**), *all-trans*-retinoic acid (compound **23**), and 9-*cis*-retinoic acid (compound **24**) were purchased from Sigma. (*E,E*)-Methyl farnesoate (compound **22**) and (*E,E*)-farnesoic acid (**18**) were purchased from Echelon Biosciences Inc. (Salt Lake City, UT). [1-^{14}C]IPP and [1-^{3}H]GGPP were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Rat recombinant GGPTase I was purchased from Calbiochem (La Jolla, CA). H-ras (CVLL) was obtained from PanVera (Madison, WI).

Chemical Synthesis. The α-hydroxyphosphonic acids **1**, **2**, and **14** (Figure 1A) were prepared as described by Hohl et al. (*14*). Phosphonic acids **3**−**6** and **10**−**13** were synthesized as described by Holstein et al. (*15*). Compound **7** was synthesized using methods previously described (*16*). Compounds **8** and **9** were prepared as described by Cermak et al. (*17*). Dihydrofarnesol (compound **20**) was synthesized using methods previously described (*18*). Dihydrofarnesyl acetate (compound **16**) and geranylgeranyl acetate (compound **17**) were prepared from their corresponding alcohols using standard esterification sequences.

Western Blot Analysis. Cells (1×10^6 cells/mL) were incubated with lovastatin and various isoprenoid species for 24 h. Cells were lysed as previously described (*5*). Protein content was determined using the Lowry method (*19*). Equivalent amounts of cell lysate were resolved by SDS−PAGE, transferred to a polyvinylidene difluoride membrane, and probed with the appropriate antibodies. HRP-linked secondary antibodies and ECL Western blotting reagents (Amersham Biosciences, Inc.) were employed according to manufacturer's protocols.

Northern Blot Analysis. All RNA probes (with the exception of L32) used in this study were generated in our laboratory using the reverse transcriptase polymerase chain reaction with the addition of a RNA polymerase site (Lig'nScribe, Ambion, Austin, TX) to the RT-PCR product. The sequences for RhoA, RhoB, and N-Ras probes were previously described (*5*, *6*). The human L32 probe template was purchased from BD Biosciences PharMingen (San Diego, CA). Isolation of total RNA and Northern blot analysis were performed as previously described (*5*).

GGPTase I Assay. GGPTase I activity was determined using the method of Harwood (*20*) with some modifications. Briefly, reactions were initiated with the addition of 9.7 μL of assay buffer containing 0.12 μg of recombinant GGPTase I (specific activity of 400 units/mg) to 15.3 μL of reaction

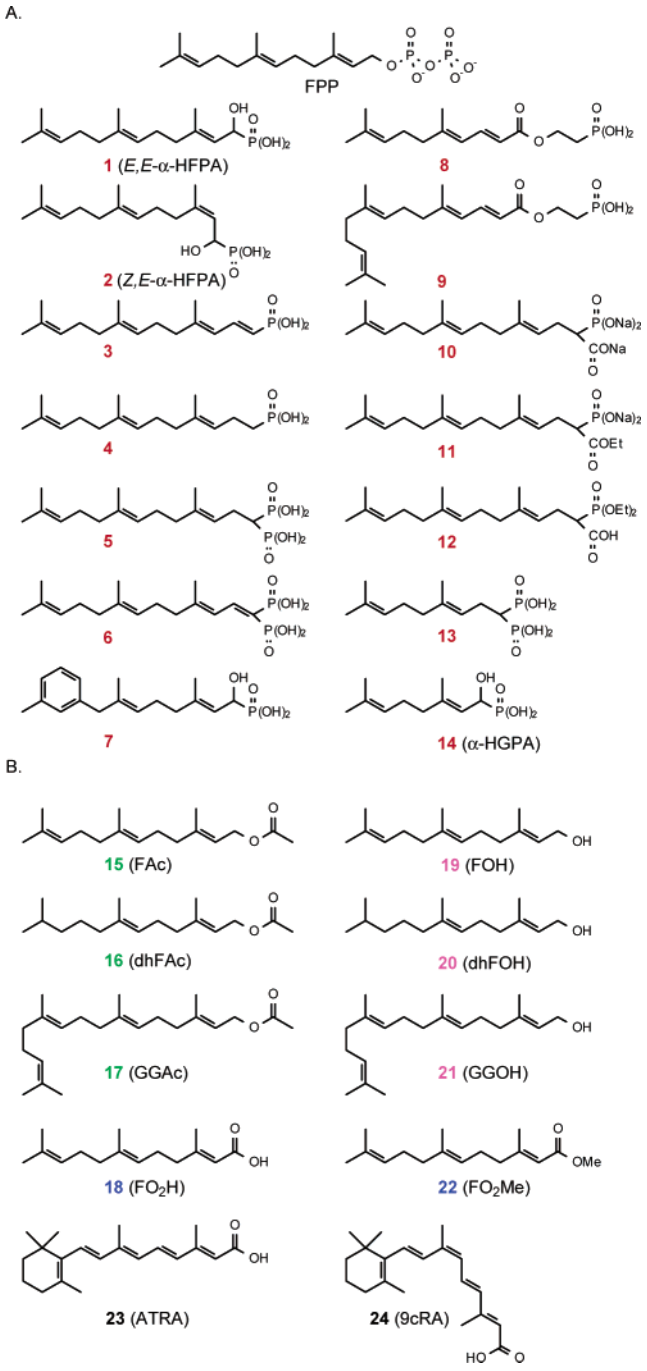

FIGURE 1: Structures of isoprenoid analogues. The abbreviations and compound numbers are shown below the structures. Red is for phosphonates, green for acetates, blue for carboxylic acids/esters, magenta for alcohols, and black for retinoids.

mixture [8 μM H-Ras-CVLL, 0.5 μM [1-^{3}H]GGPP, 5 mM $MgCl_2$, 8 μM $ZnCl_2$, 20 mM Tris-HCl (pH 7.5), and 2 mM dithiothreitol] with or without farnesyl phosphonates. Following incubation at 37 °C for 2 h, reactions were terminated by the addition of 200 μL of a 10% HCl/EtOH mixture. After incubation for an additional 15 min at 37 °C, samples were filtered through P-81 filters by a Brandel harvester. Filters were then washed in EtOH, dried, and counted using liquid scintillation counting.

FPP Synthase Assay. FPP synthase activity was determined using the method of Dunford et al. (*21*). Briefly, isoprenoid phosphonates were preincubated with bovine brain enzyme preparation (*22*, *23*), and the reaction was initiated

following addition to 40 μM [1-^{14}C]IPP and 40 μM GPP in assay buffer [50 mM Tris (pH 7.7), 10 mM NaF, 2 mM $MgCl_2$, 1 mg/mL bovine serum albumin, and 0.5 mM dithiothreitol]. The reaction was allowed to proceed for 30 min at 37 °C and then terminated by the addition of saturated NaCl followed by extraction with water-saturated 1-butanol. Radioactivity of the organic phase was determined by liquid scintillation counting.

GGPP Synthase Assay. GGPP synthase activity was determined using the method of Ericsson et al. (*24*) with modification. In short, the reaction was started by the addition of bovine brain enzyme preparation (preincubated with test compounds) to assay buffer [25 mM imidazole chloride (pH 6.0), 1.0 mM $ZnCl_2$, 0.1 mM dithiothreitol, 100 mM KF, 40 μM [1-^{14}C]IPP, and 40 μM FPP]. After incubation for 1 h at 37 °C, the reaction was terminated by the addition of saturated NaCl. The reaction mixture was then extracted with water-saturated 1-butanol, and the radioactivity of the organic phase was determined via liquid scintillation counting.

RESULTS

Farnesyl Phosphonic Acids Influence the Upregulation of Ras-Related Proteins Induced by Mevalonate Depletion. The isoprenoid species FPP and GGPP have been shown to regulate the expression of Ras and Ras-related proteins (*6*). To further investigate these regulatory roles, the effects of phosphonate analogues of isoprenoid pyrophosphates (Figure 1A, compounds **1**−**14**) on mevalonate depletion-induced upregulation of Ras and Ras-related proteins were examined. K562 cells were incubated with the *E*,*E* and *Z*,*E* isomers of α-hydroxyfarnesyl phosphonic acid (αHFPA) in the presence or absence of 1 or 10 μM lovastatin. Consistent with their effects on FPTase (*14*, *25*), treatment with 10 μM *E*,*E* isomer **1**, but not the *Z*,*E* isomer **2**, resulted in inhibition of Ras processing as evidenced by the appearance of a more slowly migrating protein (Figure 2A). Cotreatment with the *E*,*E* isomer increased the level of accumulation of unmodified Ras induced by low concentrations of lovastatin (1 μM) by 100%. Interestingly, while neither isomer on its own induced an increase in the amount of unmodified Rap1a, combination of either compound with lovastatin potentiated the lovastatin-induced accumulation of unmodified Rap1a by 2−4-fold. In contrast to the ability of FPP to mitigate the mevalonate depletion-induced upregulation of RhoA and RhoB, the structurally similar αHFPA compounds **1** and **2** increased the levels of RhoA and RhoB compared to the levels with lovastatin alone, with the effects most evident in combination with 1 μM lovastatin for RhoA and 10 μM lovastatin for RhoB. To determine whether these effects could be altered by isoprenoid pyrophosphates, cells were cotreated with FPP, FPP and IPP, or GGPP. As shown in Figure 2B, FPP only partially restored the processing of Ras in cells treated with lovastatin and *E*,*E* isomer **1**. While GGPP fully restored Rap1a processing in cells incubated with lovastatin and either isomer, the combination of FPP and IPP was less effective in diminishing levels of unmodified Rap1a in cells treated with the isomers as compared with lovastatin alone. Similar effects were observed for RhoA. While either FPP or GGPP fully prevents the lovastatin-induced upregulation of RhoB (*6*), only GGPP could completely prevent the upregulation in the presence of either isomer.

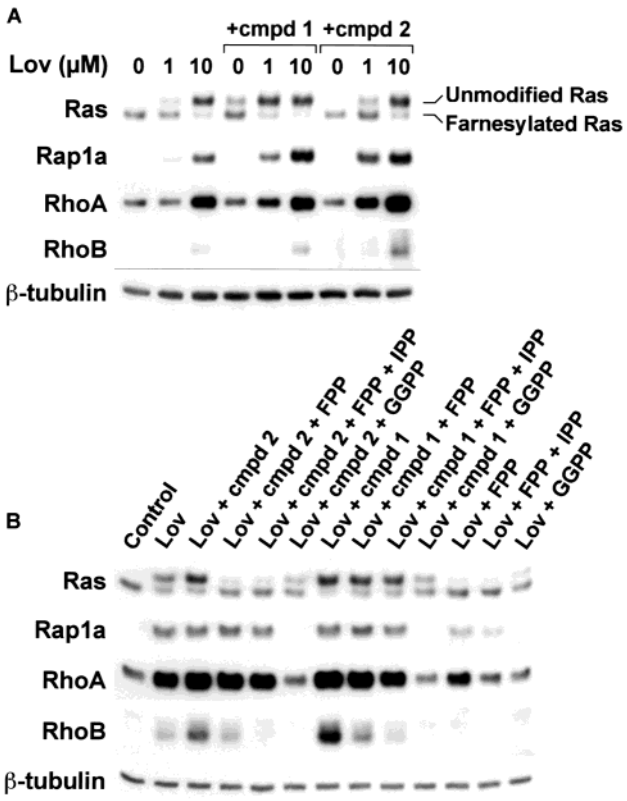

FIGURE 2: α-Hydroxyfarnesyl phosphonic acids alter lovastatin-induced upregulation of Ras-related proteins. (A) K562 cells were incubated with lovastatin and compounds **1** [(*E*,*E*)-α-HFPA] or **2** [(*Z*,*E*)-α-HFPA] for 24 h. (B) Cells were incubated with lovastatin, compound **1** or **2**, 10 μM FPP, 10 μM FPP and 10 μM IPP, or 10 μM GGPP for 24 h. These immunoblots were developed as described in Experimental Procedures. Each lane contains an equivalent amount of protein from cell lysate, and β-tubulin levels are shown as a control. The blots reflect one study that is representative of three independent experiments.

To further investigate the structure−function relationship of the isoprenoids, cells were incubated with additional isoprenoid phosphonates. The vinyl phosphonic acid **3** yielded results similar to those for compound **1** in that there was potentiation of lovastatin's effects on Ras, Rap1a, RhoA, and RhoB (Figure 3A). The unconjugated phosphonic acid **4** displayed properties similar to those of the *Z*,*E* isomer **2** by potentiating the lovastatin-induced upregulation of Rap1a, RhoA, and RhoB (data not shown). Interestingly, the bisphosphonic acids (compounds **5** and **6**) had additional effects. While neither compound altered Ras processing, both induced an accumulation of Rap1a (Figure 3A) and potentiated lovastatin's effects on Rap1a. In addition, both **5** and **6** doubled the amount of RhoA. Similar to the other phosphonic acids, both **5** and **6** markedly potentiated the lovastatin-induced upregulation of RhoA and RhoB. Compounds **7**−**9** had no effects either alone or in combination with lovastatin (data not shown).

Role of Isoprenoid Charge and Chain Length on Expression of Ras-Related Proteins. To more closely examine the effects of the charge of the isoprenoid species on regulation of expression, cells were incubated with different carboxyphosphonate species. As shown in Figure 3B, compound **10**, with the potential of a −3 charge at physiological pH, induced an accumulation of Rap1a and RhoA and potentiated the effects of lovastatin on Rap1a, RhoA, and RhoB levels.

Table 1: Effects of Isoprenoid Phosphonates on FPTase, GGPTase I, FPP Synthase, and GGPP Synthase Activity

	FPTase (fraction of control at 1 μM)[a]	GGPTase I (fraction of control at 1 μM)	FPP synthase (fraction of control at 10 μM)	GGPP synthase (fraction of control at 10 μM)
compound **1**	0.10 (IC_{50} = 65 nM)	1.03	1.18	1.06
compound **2**	0.65	0.96	0.96	1.00
compound **3**	0.56	0.81	0.85	1.13
compound **5**	0.46	0.83	1.03	1.11
compound **6**	0.53	0.92	0.92	1.05
compound **10**	0.65	0.82	0.76	1.10
compound **11**	0.75	0.84	0.85	1.17

[a] FPTase data for compounds **1** and **2** were previously published by Hohl et al. (*14*), and data for compounds **3**, **5**, **6**, **10**, and **11** were previously published by Holstein et al. (*15*). GGPTase I, FFP synthase, and GGPP synthase assays were performed as described in Experimental Procedures.

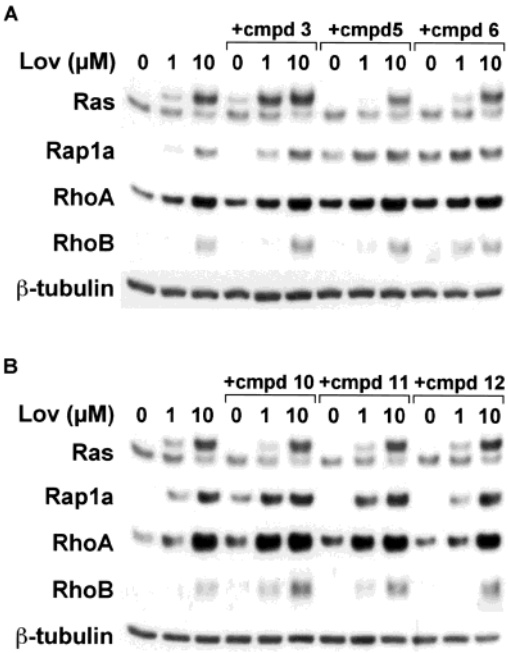

FIGURE 3: Effects of farnesyl phosphonic acids on regulation of Ras-related proteins. K562 cells were incubated for 24 h with lovastatin and compounds **3**, **5**, and **6** (A) or **10**−**12** (B). These immunoblots were developed as described in Experimental Procedures. Each lane contains an equivalent amount of protein from cell lysate. The blots are representative of two independent experiments.

Compound **11**, with the potential of a −2 charge, increased the levels of Rap1a, RhoA, and RhoB, but only in combination with lovastatin. The phosphonate−carboxylic acid derivative **12**, with the potential of a −1 charge, only minimally increased the amount of RhoB induced by lovastatin. Further evidence of the importance of the charge of the isoprenoid species in relation to the effects on protein levels was provided by the failure of the methyl or ethyl esters of compounds **1**−**3** and **5** to alter lovastatin's effects (data not shown).

The effects of the isoprenoid chain length were also examined (data not shown). The geranyl length bisphosphonic acid **13** was found to be less potent than the farnesyl length version **5**. The α-hydroxygeranyl phosphonic acid **14** had no effect either alone or in combination with lovastatin. Finally, incubation with either methylene diphosphonic acid or phosphonoacetic acid did not mimic the effects of compound **5**, **6**, or **10**.

Effects of Farnesyl Phosphonic Acids on Ras-Related mRNA Levels. To determine whether the enhanced upregulation induced by the farnesyl analogues occurs at the level

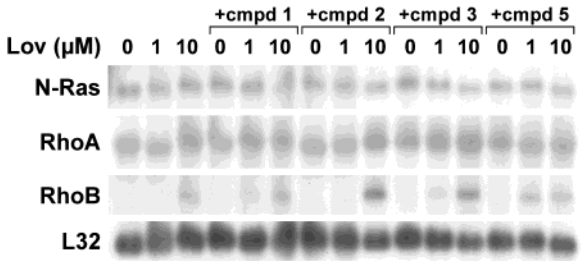

FIGURE 4: Effects of farnesyl phosphonates and lovastatin on N-Ras, RhoA, and RhoB mRNA levels. K562 cells were incubated for 24 h with lovastatin and/or compounds **1**−**3** and **5.** Total RNA was isolated, and Northern blot analysis was performed with N-Ras-, RhoA-, or RhoB-specific riboprobes as described in Experimental Procedures. L32 mRNA levels are shown as a control. The blots are representative of two independent experiments.

of transcription, Northern blot analyses were performed. Cells were incubated with compounds **1**−**3** and **5** both alone and in combination with 1 or 10 μM lovastatin. Consistent with our previous work (*5*, *6*), incubation with lovastatin does not alter steady state levels of N-Ras or RhoA mRNA (Figure 4). Furthermore, while RhoB mRNA is not detectable under control conditions or in cells treated with 1 μM lovastatin, it is detectable in cells incubated with 10 μM lovastatin. None of the farnesyl analogues by themselves altered steady state mRNA levels of N-Ras or RhoA, or induced RhoB. In addition, the combination of the analogues and lovastatin did not alter N-Ras or RhoA mRNA levels compared to control levels. However, addition of compound **1**, **3**, or **5** to 1 μM lovastatin resulted in detectable levels of RhoB mRNA, and the amount of RhoB induced by 10 μM lovastatin was further increased with compound **2**.

Relationship of Isoprenoid Activities to Enzyme Inhibition. The effects of the isoprenoid phosphonates might be direct through antagonism of the isoprenoid pyrophosphates or indirect through inhibition of isoprenylation or synthesis of isoprenoid pyrophosphates. As shown in Table 1, the inhibitory activities of compounds **1**−**3**, **5**, **6**, **10**, and **11** with respect to FPTase vary significantly, with compound **1** being the most effective inhibitor. Consistent with the importance of the 20-carbon chain length for inhibitory activity (*26*, *27*), the farnesyl phosphonates were found to have little or no inhibitory activity against GGPTase I (Table 1), with none of the compounds reaching an IC_{50} even at 10 μM (data not shown). The effects of the compounds on FPP synthase and GGPP synthase activities were also examined. Compounds **3**, **10**, and **11** only minimally inhibited FPP synthase activity at 10 μM, and none of the compounds inhibited GGPP synthase (Table 1).

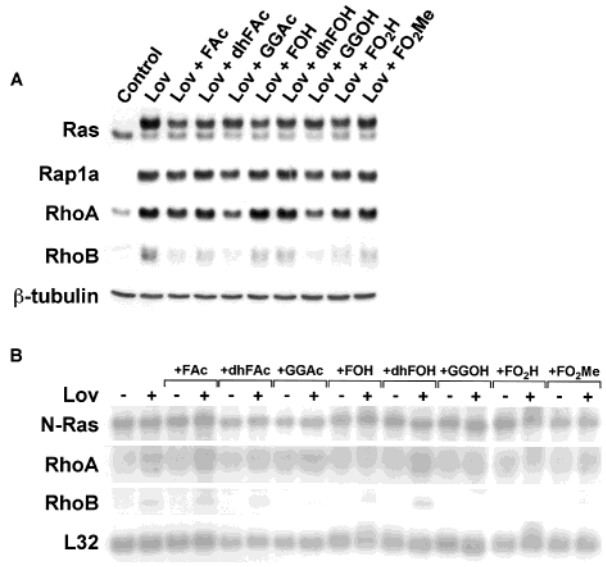

FIGURE 5: Isoprenoid pyrophosphate derivatives mitigate the upregulation of Ras-related proteins induced by mevalonate depletion. K562 cells were incubated for 24 h in the presence of 10 μM lovastatin (Lov), 100 μM farnesyl acetate (FAc, **15**), 100 μM dihydrofarnesyl acetate (dhFAc, **16**), 100 μM geranylgeranyl acetate (GGAc, **17**), 10 μM farnesol (FOH, **19**), 10 μM dihydrofarnesol (dhFOH, **20**), 10 μM geranylgeraniol (GGOH, **21**), 100 μM farnesoic acid (FO_2H, **18**), or 100 μM methyl farnesoate (FO_2Me, **22**). Immunoblots (A) and Northern blots (B) were developed as described in Experimental Procedures. β-Tubulin protein and L32 mRNA levels are shown as controls. The blots represent two (Northern) or three (immunoblot) independent experiments.

Effects of Isoprenoid Pyrophosphate Derivatives on Ras-Related Protein Expression. Because the isoprenoid phosphonates were found to antagonize the effects of isoprenoid pyrophosphates, we next determined whether derivatives of the isoprenoid pyrophosphates could mimic the effects of the pyrophosphates. The alcohol derivatives of FPP and GGPP (compounds **19** and **21**, respectively) and their acetates (compounds **15** and **17**, respectively), as well as farnesoic acid **18** and its methyl ester **22** (Figure 1B), were tested for their ability to affect mevalonate depletion-induced upregulation of Ras-related proteins. The 10,11-dihydro derivatives of farnesyl acetate (compound **16**) and farnesol (**20**) were also used. As shown in Figure 5A, none of the compounds restored the farnesylation of Ras or the geranylgeranylation of Rap1a. However, both geranylgeranyl acetate **17** and geranylgeraniol **21** reduced the lovastatin-induced upregulation of RhoA by 40%. All of the compounds lessened the mevalonate depletion-induced upregulation of RhoB by at least 35%, with compounds **15**, **17**, **18**, and **21** most effectively preventing the upregulation. Geraniol and geranyl acetate, the 10-carbon versions of compounds **19** and **15**, respectively, had no effect on the induced upregulation of these proteins (data not shown). In control experiments without lovastatin, none of the isoprenoids altered Ras or Ras-related protein levels (data not shown).

The effects of compounds **15**−**22**, both alone and in combination with lovastatin, on mRNA levels of the small GTPases were also examined. As shown in Figure 5B, none of the compounds by themselves altered N-Ras or RhoA mRNA levels or induced expression of RhoB mRNA. However, consistent with the protein data, the compounds which most effectively prevented the upregulation of RhoB protein induced by mevalonate depletion also most effectively prevented the upregulation of RhoB mRNA (compounds **17**, **18**, and **21**).

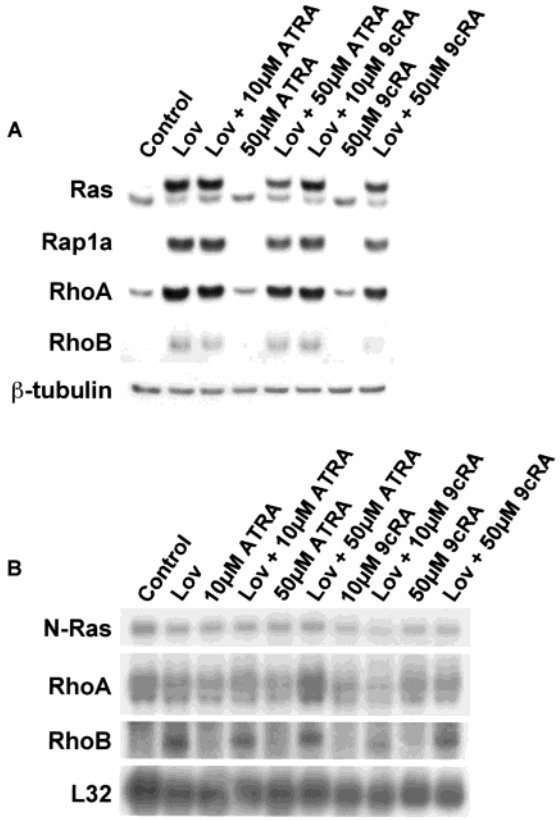

FIGURE 6: Effects of retinoic acids on lovastatin-induced upregulation of Ras-related proteins. K562 cells were incubated for 24 h with 10 μM lovastatin (Lov), *all-trans*-retinoic acid (ATRA, **23**), or 9-*cis*-retinoic acid (9CRA, **24**). Immunoblots (A) and Northern blots (B) were developed as described in Experimental Procedures. β-Tubulin protein and L32 mRNA levels are shown as controls. The blots are representative of duplicated experiments.

The ability of farnesoic acid **18** and the other isoprenoid metabolites to modify the lovastatin-induced upregulation of the Ras-related proteins raised the question of whether other related isoprenoids had similar regulatory properties. *all-trans*-Retinoic acid (ATRA, **23**) and 9-*cis*-retinoic acid (9cRA, **24**) are members of the retinoid family (ultimately derived from GGPP) and modulate gene expression by interacting with their receptors which are ligand-dependent transcription factors (*28*). The ability of ATRA and 9cRA to alter the expression of the Ras-related proteins both alone and in combination with lovastatin was examined. As shown in Figure 6A, neither 50 μM ATRA nor 50 μM 9cRA alone (or 10 μM, data not shown) altered the levels of Ras, Rap1a, RhoA, or RhoB protein. However, higher concentrations of either retinoid partially reduced the upregulation of Ras (30%), Rap1a (20−40%), RhoA (20−30%), and RhoB (20−60%) induced by lovastatin. The retinoic acids, either alone or in combination with lovastatin, did not alter the levels of N-Ras or RhoA mRNA (Figure 6B). The upregulation of RhoB mRNA induced by lovastatin was not significantly altered by either retinoid (Figure 6B). The effects of compounds **1**−**24** on Ras-related expression as described above are summarized in Table 2.

Table 2: Summary of the Effects of Isoprenoid Analogues on Ras-Related Protein Levels in the Absence (−lov) or Presence (+lov) of Mevalonate Depletion[f]

	Ras		Rap1a		RhoA		RhoB	
Compound	- lov[a]	+ lov	- lov	+ lov	- lov	+ lov	- lov	+ lov
1, 3	↑[b]	↑[c]	--[d]	↑	--	↑	--	↑
2, 4, 11	--	--	--	↑	--	↑	--	↑
5, 6, 10	--	--	↑	↑	↑	↑	--	↑
7-9, 12-14	--	--	--	--	--	--	--	--
15, 16, 18, 19, 20, 22	--	--	--	--	--	--	--	↓[e]
17, 21	--	--	--	--	--	↓	--	↓
23, 24	--	↓	--	↓	--	↓	--	↓

[a] Lovastatin (lov). [b] Increased level of protein expression compared to that of the untreated control (↑). [c] Increased level of protein expression compared to that of the lovastatin-treated control (↑). [d] No effect observed (- -). [e] Decreased level of protein expression compared to that of a lovastatin-treated control (↓). [f] Red is for phosphonates, green for acetates, blue for carboxylic acids/esters, magenta for alcohols, and black for retinoids.

DISCUSSION

FPP is a central metabolite in the isoprenoid pathway and is positioned at the branch point that precedes sterol and nonsterol products. As such, FPP serves as a substrate for FPTase, squalene synthase, and GGPP synthase. FPP analogues display differential inhibitor properties for these enzymes. For example, (*E*,*E*)-α-HFPA (compound **1**) competitively inhibits FPTase (*14*, *25*), but not squalene synthase (*14*) or GGPP synthase (Table 1). The bisphosphonic acid compound **5** inhibits squalene synthase (*29*) and to a lesser extent FPTase (*15*) but not GGPP synthase (Table 1). That these two compounds (**1** and **5**) differentially inhibit the same enzymes allows for the use of FPP analogues as probes for the distinct FPP binding pockets of these known enzymes (*30*−*32*) as well as the binding sites of as yet uncharacterized targets. Our recent findings suggest that FPP's biochemical activities are greater than simply serving as substrates for these enzymes, and instead include regulation of transcriptional and post-transcriptional events (*5*, *6*). We therefore hypothesized that FPP analogues (Figure 1) are useful tools for analyzing the roles of isoprenoids in regulating these events.

(*E*,*E*)-α-HFPA (compound **1**) potentiated the upregulation of Ras, Rap1a, RhoA, and RhoB induced by mevalonate depletion, while its diastereomer (*Z*,*E*)-α-HFPA (compound **2**) potentiated the upregulation of Rap1a, RhoA, and RhoB (Figure 2A). The potentiated upregulation of Ras by (*E*,*E*)-α-HFPA (compound **1**) might be a consequence of the ability of compound **1** to serve as an FPTase inhibitor (Table 1). However, compound **2** is not an effective FPTase inhibitor in the enzyme assay (Table 1) and does not inhibit Ras processing in intact cells (Figure 2A). Therefore, the potentiated upregulation of Rap1a, RhoA, and RhoB by both compounds **1** and **2** appears to be independent of any effects on FPTase. Further evidence to support the direct regulatory roles of α-HFPAs is their lack of inhibitory activities against either GGPTase or enzymes in the isoprenoid biosynthetic pathway (Table 1). The addition of FPP was not sufficient to completely prevent the upregulation of RhoB induced by the combination of the analogues and lovastatin, but does completely prevent the upregulation by lovastatin alone. This suggests that compounds **1** and **2** are capable of directly antagonizing the effects of FPP. We have previously shown in lovastatin-treated cells that the combination of FPP and IPP closely mimics the effects observed with GGPP, presumably due to restoration of GGPP synthesis (*6*). That FPP and IPP are less effective in cells treated with both lovastatin and either compound **1** or **2**, despite the lack of GGPP synthase inhibitory activity (Table 1), indicates additional as yet unknown activities for compounds **1** and **2**. GGPP completely prevents the upregulation of Rap1a, RhoA, and RhoB induced by lovastatin, both alone and in combination with compounds **1** and **2**. These findings indicate differences between RhoB and either Rap1a or RhoA regulation and emphasize for RhoB greater influence from GGPP than FPP.

To better delineate these phenomena, other isoprenoids with known biochemical activities were studied. We have previously reported that the vinyl phosphonic acid **3** is an FPTase inhibitor (*15*). As shown in Figure 3A, compound **3** yielded results similar to those for compound **1**, while compound **4** displayed properties similar to those of compound **2**. Compound **4**, a comparatively weak inhibitor of FPTase (Table 1) (*15*), potentiates the upregulation of Rap1a, RhoA, and RhoB induced by lovastatin. Thus, the potentiation by both compounds **3** and **4**, as for compounds **1** and **2**, is independent of FPTase inhibition.

Notably, compound **5**, the bisphosphonic analogue of compound **4**, not only potentiates the upregulation of Rap1a, RhoA, and RhoB induced by mevalonate depletion but also upregulates Rap1a and RhoA in the absence of lovastatin

(Figure 3A). It is possible that the regulatory effects of the isoprenoid analogues might result from interference with other key enzymes in the isoprenoid pathway. However, none of the tested analogues were found to inhibit GGPTase I, FPP synthase, or GGPP synthase (Table 1). While compound **5** has also been reported to be a squalene synthase inhibitor (*33*), the observed potentiated upregulation is unlikely to be a consequence of such inhibition as the combination of another squalene synthase inhibitor (zaragozic acid A) and lovastatin does not produce the same effects (*6*). The ability of another bisphosphonic acid (compound **6**) (Figure 3A) to influence levels of Rap1a, RhoA, and RhoB suggests a relationship between the charge of the analogue headgroup and its regulatory effects. Use of the carboxyphosphonate compounds (**10**−**12**) allowed for careful dissection of this effect (Figure 3B). While compound **10**, with a potential charge of −3, mimicked the effects of the bisphosphonic acids **5** and **6** (potential charge of −4), compound **11**, with a potential charge of −2, mimicked the effects of the monophosphonic acids (**1**−**4**) with respect to Rap1a, RhoA, and RhoB. That compound **12**, with a potential charge of −1, as well as the esters of compounds **1**−**3** and **5** (zero charge), failed to demonstrate an effect suggests a threshold phenomenon. That is, compounds must possess a minimum charge of −2 to produce the potentiated upregulation and a charge of −3 or greater to yield upregulation of these proteins in the absence of mevalonate depletion. This finding is reassuring because it implies cellular internalization of these compounds regardless of charge. This is consistent with the hypothesis that these compounds regulate protein expression competitively with respect to FPP and that the site of mutual binding is positively charged and/or accommodates positive counterions. Of relevance is the fact that known FPP binding pockets contain magnesium ions (*32*) as well as positively charged side chains (*31*).

While the identity and charge of the functional group were found to be important, the structure and length of the isoprene chain were also shown to contribute to the regulatory effects. Modification of the tail of compound **1**, to yield compound **7**, resulted in the loss of potentiating activity (data not shown). The 10-carbon versions of compounds **1** and **5** (**14** and **13**, respectively) were found to be largely inactive (data not shown). Taken to the extreme, the use of methylene diphosphonic acid and phosphonoacetic acid (the headgroups of compounds **5**, **6**, and **10**) demonstrated the necessity of an isoprene tail as neither compound had an effect on regulation (data not shown). These studies demonstrated a minimum chain length requirement of the equivalent of three isoprenoid units.

The abilities of the isoprenoid molecules to alter small GTPase protein levels might be a result of parallel effects on transcriptional events. We have shown that isoprenoid pyrophosphates prevent upregulation of RhoB mRNA induced by mevalonate depletion independent of protein isoprenylation restoration (*6*). We now demonstrate a more direct role in which FPP analogues can influence FPP-mediated transcriptional regulation. The FPP analogues **1**−**3** and **5** potentiate the upregulation of RhoB mRNA induced by mevalonate depletion (Figure 4). This is in contrast to the effects of FPP (*6*). These findings support the hypothesis that there is an isoprenoid-binding transcription factor, at least for RhoB. While such a binding protein may have been described as in the case of farnesol's interaction with FXR (*10*, *34*), its binding element is not readily identified in the known RhoB promoter (*35*, *36*). For RhoA and Ras, the regulatory influence of these isoprenoids is even more complex and must involve translational/post-translational events like those we have previously attributed to the endogenous isoprenoid pyrophosphates (*5*, *6*). It seems likely that this regulation is mediated by an isoprenoid-binding factor. In contrast to the isoprenoid pyrophosphates, which prevent the upregulation of these small GTPase proteins induced by mevalonate depletion, the isoprenoid analogues (compounds **1**−**6**, **10**, and **11**) potentiate this upregulation. In this context, these analogues are functional antagonists for FPP and GGPP, and conformational analysis of the chemical requirements for these effects will aid definition of the isoprenoid binding factor's structure.

Some isoprenoid analogues display agonist activities in comparison to FPP and GGPP. Like FPP and GGPP, compounds **15**−**22** all suppress the mevalonate depletion-induced upregulation of RhoB protein (Figure 5A) and mRNA (Figure 5B), although to varying degrees. These effects might be explained by the metabolic conversion of these compounds to the corresponding pyrophosphates. However, this is unlikely because none of these compounds restore protein isoprenylation (Figure 5A). Our data are also useful in providing more evidence for a direct role of isoprenoid pyrophosphates in regulating expression. It is possible that FPP and GGPP might be hydrolyzed to their corresponding alcohols (compounds **19** and **21**, respectively) and that these alcohols, or their oxidation products, are responsible for regulating expression. This is unlikely because farnesol (**19**) and farnesoic acid (**18**), even at high concentrations, are unable to completely suppress the upregulation of RhoB. Thus, while isoprenoid phosphonic acids have properties that are antagonistic to those of the endogenous pyrophosphates, isoprenoid alcohols, acetates, and carboxylic acids serve as functional agonists (Table 2). The complexity of the isoprenoid pyrophosphate-mediated regulation of Ras/RhoA (translational/post-translational) and RhoB (transcriptional) (*6*) makes it difficult to correlate the regulatory activities of the isoprenoid analogues with their chemical structures.

Although in aggregate these results strongly implicate FPP and GGPP as being the dominant regulatory species, there is nonetheless detectable activity for the related compounds **15**−**22**. This can be explained by the latter compounds having less affinity for the putative FPP and GGPP binding sites. In this context, it is interesting to note that the structurally related retinoids (compounds **23** and **24**) displayed activities (Figure 6) at concentrations comparable to those of the isoprenoids, albeit at higher concentrations than are known to alter the expression of genes with known retinoid responsive elements. This observation may be explained by the direct interaction of these retinoids with the putative isoprenoid pyrophosphate binding sites. The extent to which there is overlap between isoprenoid pyrophosphates and retinoids with regard to their regulatory activities remains to be determined. Our current studies, in addition to demonstrating the utility of FPP analogues as useful biochemical tools, further delineate roles for isoprenoids in regulating the expression of Ras-related proteins. We have identified agonists and antagonists with regard to

FPP and GGPP for the putative isoprenoid binding factors. The mechanisms underlying these effects are both transcriptional and post-transcriptional. These results will aid in the eventual identification of the responsible isoprenoid-binding regulatory elements that regulate small GTPase expression.

ACKNOWLEDGMENT

We thank Dr. Diana Cermak for her work in originally preparing many of the isoprenoid analogues.

REFERENCES

1. Vojtek, A. B., and Der, C. J. (1998) Increasing complexity of the Ras signaling pathway, *J. Biol. Chem. 273*, 19925−19928.
2. Van Aelst, L., and D'Souza-Schorey, C. (1997) Rho GTPases and signaling networks, *Genes Dev. 11*, 2295−2322.
3. Schafer, W. R., Kim, R., Sterne, R., Thorner, J., Kim, S. H., and Rine, J. (1989) Genetic and pharmacological suppression of oncogenic mutations in ras genes of yeast and humans, *Science 245*, 379−385.
4. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) All ras proteins are polyisoprenylated but only some are palmitoylated, *Cell 57*, 1167−1177.
5. Holstein, S. A., Wohlford-Lenane, C. L., and Hohl, R. J. (2002) Consequences of mevalonate depletion. Differential transcriptional, translational, and post-translational up-regulation of Ras, Rap1a, RhoA, AND RhoB, *J. Biol. Chem. 277*, 10678−10682.
6. Holstein, S. A., Wohlford-Lenane, C. L., and Hohl, R. J. (2002) Isoprenoids influence the expression of Ras and Ras-related proteins, *Biochemistry 41*, 13698−13704.
7. Meigs, T. E., Roseman, D. S., and Simoni, R. D. (1996) Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase degradation by the nonsterol mevalonate metabolite farnesol in vivo, *J. Biol. Chem. 271*, 7916−7922.
8. Correll, C. C., Ng, L., and Edwards, P. A. (1994) Identification of farnesol as the non-sterol derivative of mevalonic acid required for the accelerated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, *J. Biol. Chem. 269*, 17390−17393.
9. Meigs, T. E., and Simoni, R. D. (1997) Farnesol as a regulator of HMG-CoA reductase degradation: characterization and role of farnesyl pyrophosphatase, *Arch. Biochem. Biophys. 345*, 1−9.
10. Forman, B. M., Goode, E., Chen, J., Oro, A. E., Bradley, D. J., Perlmann, T., Noonan, D. J., Burka, L. T., McMorris, T., Lamph, W. W., Evans, R. M., and Weinberger, C. (1995) Identification of a nuclear receptor that is activated by farnesol metabolites, *Cell 81*, 687−693.
11. Lozzio, C. B., and Lozzio, B. B. (1975) Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome, *Blood 45*, 321−334.
12. Reszka, A. A., Halasy-Nagy, J., and Rodan, G. A. (2001) Nitrogen-bisphosphonates block retinoblastoma phosphorylation and cell growth by inhibiting the cholesterol biosynthetic pathway in a keratinocyte model for esophageal irritation, *Mol. Pharmacol. 59*, 193−202.
13. Kanai, T., Hirohashi, S., Noguchi, M., Shimoyama, Y., Shimosato, Y., Noguchi, S., Nishimura, S., and Abe, O. (1987) Monoclonal antibody highly sensitive for the detection of ras p21 in immunoblotting analysis, *Jpn. J. Cancer Res. 78*, 1314−1318.
14. Hohl, R. J., Lewis, K. A., Cermak, D. M., and Wiemer, D. F. (1998) Stereochemistry-dependent inhibition of RAS farnesylation by farnesyl phosphonic acids, *Lipids 33*, 39−46.
15. Holstein, S. A., Cermak, D. M., Wiemer, D. F., Lewis, K., and Hohl, R. J. (1998) Phosphonate and bisphosphonate analogues of farnesyl pyrophosphate as potential inhibitors of farnesyl protein transferase, *Bioorg. Med. Chem. 6*, 687−694.
16. Mechelke, M. F. (1998) "Heads and Tails": The Design and Synthesis of Novel RAS Farnesyl Protein Transferase Inhibitors, Ph.D. Thesis, University of Iowa, Iowa City, IA.
17. Cermak, D. M., Wiemer, D. F., Lewis, K., and Hohl, R. J. (2000) 2-(Acyloxy)ethylphosphonate analogues of prenyl pyrophosphates: synthesis and biological characterization, *Bioorg. Med. Chem. 8*, 2729−2737.
18. Mechelke, M. F., and Wiemer, D. F. (1998) Preparation of (2*E*,6*E*)-10,11-Dihydrofarnesol via a (Bisphenyl)dithioacetal Reduction, *Tetrahedron Lett. 39*, 9609−9612.
19. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem. 193*, 265−275.
20. Harwood, H. J., Jr. (1995) Protein farnesyltransferase: measurement of enzymatic activity in 96-well format using TopCount microplate scintillation counting technology, *Anal. Biochem. 226*, 268−278.
21. Dunford, J. E., Thompson, K., Coxon, F. P., Luckman, S. P., Hahn, F. M., Poulter, C. D., Ebetino, F. H., and Rogers, M. J. (2001) Structure−activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates, *J. Pharmacol. Exp. Ther. 296*, 235−242.
22. Moores, S. L., Schaber, M. D., Mosser, S. D., Rands, E., O'Hara, M. B., Garsky, V. M., Marshall, M. S., Pompliano, D. L., and Gibbs, J. B. (1991) Sequence dependence of protein isoprenylation, *J. Biol. Chem. 266*, 14603−14610.
23. Khan, S. G., Mukhtar, H., and Agarwal, R. (1995) A rapid and convenient filter-binding assay for ras p21 processing enzyme farnesyltransferase, *J. Biochem. Biophys. Methods 30*, 133−144.
24. Ericsson, J., Runquist, M., Thelin, A., Andersson, M., Chojnacki, T., and Dallner, G. (1993) Distribution of prenyltransferases in rat tissues. Evidence for a cytosolic all-trans-geranylgeranyl diphosphate synthase, *J. Biol. Chem. 268*, 832−838.
25. Pompliano, D. L., Rands, E., Schaber, M. D., Mosser, S. D., Anthony, N. J., and Gibbs, J. B. (1992) Steady-state kinetic mechanism of Ras farnesyl:protein transferase, *Biochemistry 31*, 3800−3807.
26. Macchia, M., Jannitti, N., Gervasi, G., and Danesi, R. (1996) Geranylgeranyl diphosphate-based inhibitors of post-translational geranylgeranylation of cellular proteins, *J. Med. Chem. 39*, 1352−1356.
27. Gibbs, B. S., Zahn, T. J., Mu, Y., Sebolt-Leopold, J. S., and Gibbs, R. A. (1999) Novel farnesol and geranylgeraniol analogues: A potential new class of anticancer agents directed against protein prenylation, *J. Med. Chem. 42*, 3800−3808.
28. Mangelsdorf, D. J., Kliewer, S. A., Kakizuka, A., Umesono, K., and Evans, R. M. (1993) Retinoid receptors, *Recent Prog. Horm. Res. 48*, 99−121.
29. Ciosek, C. P., Jr., Magnin, D. R., Harrity, T. W., Logan, J. V., Dickson, J. K., Jr., Gordon, E. M., Hamilton, K. A., Jolibois, K. G., Kunselman, L. K., and Lawrence, R. M. (1993) Lipophilic 1,1-bisphosphonates are potent squalene synthase inhibitors and orally active cholesterol lowering agents in vivo, *J. Biol. Chem. 268*, 24832−24837.
30. Park, H. W., Boduluri, S. R., Moomaw, J. F., Casey, P. J., and Beese, L. S. (1997) Crystal structure of protein farnesyltransferase at 2.25 angstrom resolution, *Science 275*, 1800−1804.
31. Strickland, C. L., Windsor, W. T., Syto, R., Wang, L., Bond, R., Wu, Z., Schwartz, J., Le, H. V., Beese, L. S., and Weber, P. C. (1998) Crystal structure of farnesyl protein transferase complexed with a CaaX peptide and farnesyl diphosphate analogue, *Biochemistry 37*, 16601−16611.
32. Pandit, J., Danley, D. E., Schulte, G. K., Mazzalupo, S., Pauly, T. A., Hayward, C. M., Hamanaka, E. S., Thompson, J. F., and Harwood, H. J., Jr. (2000) Crystal structure of human squalene synthase. A key enzyme in cholesterol biosynthesis, *J. Biol. Chem. 275*, 30610−30617.
33. Patel, D. V., Schmidt, R. J., Biller, S. A., Gordon, E. M., Robinson, S. S., and Manne, V. (1995) Farnesyl diphosphate-based inhibitors of Ras farnesyl protein transferase, *J. Med. Chem. 38*, 2906−2921.
34. Edwards, P. A., and Ericsson, J. (1999) Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway, *Annu. Rev. Biochem. 68*, 157−185.
35. Fritz, G., and Kaina, B. (1997) rhoB encoding a UV-inducible Ras-related small GTP-binding protein is regulated by GTPases of the Rho family and independent of JNK, ERK, and p38 MAP kinase, *J. Biol. Chem. 272*, 30637−30644.
36. Fritz, G., and Kaina, B. (2001) Transcriptional activation of the small GTPase gene rhoB by genotoxic stress is regulated via a CCAAT element, *Nucleic Acids Res. 29*, 792−798.

BI027227M