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In vitro studies in a myelogenous leukemia cell line suggest an organized binding of geranylgeranyl diphosphate synthase inhibitors

Jacqueline E. Reilly^a, Xiang Zhou^b, Huaxiang Tong^c, Craig H. Kuder^c, David F. Wiemer^b, and Raymond J. Hohl^{a,c,d,*}

Jacqueline E. Reilly: jacqueline-reilly@uiowa.edu; Xiang Zhou: xiang-zhou@uiowa.edu; Huaxiang Tong: huaxiang-tong@uiowa.edu; Craig H. Kuder: craig.kuder@gmail.com; David F. Wiemer: david-wiemer@uiowa.edu; Raymond J. Hohl: rhohl@hmc.psu.edu

^aDepartment of Pharmacology, University of Iowa, 375 Newton Rd, 5219 MERF, Iowa City IA, 52242, USA; Tel.: 1-319-335-8272

^bDepartment of Chemistry, E531 Chemistry Building, University of Iowa, Iowa City IA, 52242, USA; Tel.: 1-319-335-1365

^cDepartment of Internal Medicine, University of Iowa, 375 Newton Rd, 5219 MERF, Iowa City IA, 52242, USA; Tel.: 1-319-335-8272

^dDepartments of Medicine and Pharmacology, Pennsylvania State University, Penn State Hershey Cancer Institute, 500 University Dr, Hershey PA, 17033-0850, USA; Tel.: 1-717-531-8401; Fax: 1-717-531-0647

Abstract

A small set of isoprenoid bisphosphonates ethers have been tested in the K562 chronic myelogenous leukemia cell line to determine their impact on isoprenoid biosynthesis. Five of these compounds inhibit geranylgeranyl diphosphate synthase (GGDPS) with IC₅₀ values below 1 μ M in enzyme assays, but in cells their apparent activity is more varied. In particular, the isomeric *C*-geranyl-*O*-prenyl and *C*-prenyl-*O*-geranyl bisphosphonates are quite different in their activity with the former consistently demonstrating greater impairment of geranylgeranylation in cells but the latter showing greater impact in the enzyme assays with GGDPS. Together, these findings suggest an organized binding of these inhibitors in the two hydrophobic channels of the geranylgeranyl diphosphate synthase enzyme.

Graphical abstract

*Corresponding Author: Raymond J. Hohl, Director of Penn State Hershey Cancer Institute, Pennsylvania State University, Penn State Hershey Cancer Institute, 500 University Dr Hershey, PA, 17033-0850, Phone: 1-717-531-8401, Fax: 1-717-531-0647, rhohl@hmc.psu.edu.

Keywords

isoprenoid; protein isoprenylation; geranylgeranyl diphosphate synthase; bisphosphonate; statin; chronic myelogenous leukemia (CML)

1. INTRODUCTION

The isoprenoid biosynthetic pathway (IBP, Figure 1) provides critical substrates for posttranslational modification of proteins that modulate cell signaling and regulatory processes [1–9]. The enzymes of this pathway have drawn significant attention aimed at development of both biological probes and potential drugs. Clinically relevant inhibitors of the IBP include statins such as lovastatin (**1**), which inhibit hydroxymethylglutaryl co-enzyme A (HMG-CoA) reductase and are used to treat hypercholesterolemia, and the nitrogenous bisphosphonates (NBPs) such as zoledronate (**2**), which inhibit the enzyme farnesyl diphosphate synthase (FDPS) and are used to treat osteoporosis (Figure 1) [10, 11]. Studies in a variety of cancer cell lines have found that both statins and NBPs reduce cell proliferation, invasion, and migration [3, 7, 12–15]. However, because both the statins and the NBPs inhibit enzymes at relatively early steps of the IBP, the specific isoprenoid(s) responsible for altering malignant cell properties is(are) less clear.

Some time ago our lab reported the synthesis of digeranyl bisphosphonate (DGBP, **3**) and its activity as a novel inhibitor of geranylgeranyl diphosphate synthase (GGDPS), the enzyme responsible for production of geranylgeranyl diphosphate (GGPP) (Figure 1) [16–18]. The diphosphate GGPP is critical for protein geranylgeranylation[19], a posttranslational modification important in membrane localization of the Ras family of small GTPases [20]. Subsequent work with the human breast cancer cell line MDA-MB-231 found that DGBP significantly inhibits cell motility and migration, suggesting that geranylgeranylated proteins play a critical role in cell malignancy [21]. The significance of geranylgeranylated proteins also is supported by a very recent study that showed knockdown of GGDPS induced significant cell death *in vitro* and reduced U87 glioblastoma tumor growth *in vivo* [22].

Recently, our lab reported the synthesis of a library of six new bisphosphonates, basing their structural design on the parental compound DGBP (Figure 2, **3**) with the alterations of an additional oxygen on the geminal carbon and varied isoprenoid chain lengths (Figure 2 compounds **4–9**) [23]. The additional oxygen substituent was intended to lower each compound's pK_{a3} [24] and consequently increase biological activity, while the varying isoprenoid lengths were prepared to identify the better structure(s) for potentially targeting GGDPS. Initial work with the isolated enzyme GGDPS found that compounds **4**, **5**, **6**, **8**, and **9** all have IC_{50} values below 1 μ M [23]. However, these bisphosphonates could act at more

than one target in the IBP [25], or even bind at other sites that recognize diphosphates. Furthermore, one intriguing aspect of our prior studies was the difference in activity observed for the two isomeric compounds **6** and **8**. In assays with the isolated enzyme, compound **8** was both more potent as an inhibitor of GGDPS and much less effective as an inhibitor of the related enzyme FDPS (Figure 2), while compound **6** showed comparable, sub-micromolar activity against both enzymes. This difference suggests that there may be an organized binding of these compounds in the active site of GGDPS rather than a random placement of the hydrophobic chains into the enzyme channels that accept farnesyl diphosphate (FPP) and release GGPP [26]. To explore the structure activity relationships of these new compounds in more detail, we have extended our studies of these bisphosphonates to cell assays with the K562 chronic myelogenous leukemia cell line, a cell line that we have used consistently throughout our studies of bisphosphonates [16–18]. The results of those studies are reported herein.

2. MATERIALS AND METHODS

2.1 Compound synthesis

All compounds were synthesized at the University of Iowa, Department of Chemistry (Iowa City, IA) as previously described.[23]

2.2 Cell culture

The K562 chronic myelogenous leukemia cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and grown at 37 °C with 5% CO₂ in RPMI 1640 media containing 10% heat-inactivated fetal bovine serum, 1% pen-strep, 1% amphotericin B, and 1% L-glutamine (Life Technologies, Grand Island, NY).

2.3 MTT activity

K562 cells were plated at 50,000 cells/well in RPMI 1640 media lacking phenol red and treated immediately for 20, 44, or 68 hrs. After 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO) was added at 0.6 mg/mL, the experiment was continued for 4 hrs at 37 °C followed by addition of the MTT stop solution (10% 1N HCl, 10% Triton X-100, 80% 2-propanolol (Research Products International Corp, Mount Prospect, IL)) with gentle agitation overnight. Absorbance was measured at 540 nm with a reference wavelength at 650 nm.

2.4 FPP/GGPP quantification

Both FPP and GGPP levels were quantified as previously reported [27] using HPLC analysis. Briefly, cells were incubated with the compounds for 48 hrs. Isoprenoid pyrophosphates were extracted by incubation with extract solvent twice and dried under a stream of nitrogen. The resultant residue was incorporated into fluorescent dansyl peptide D*-GCVLS or D*-GCVLL by farnesyltransferase or geranylgeranyl transferase I or II. The enzymatic reaction was terminated by addition of acetonitrile and hydrogen chloride, and analyzed by HPLC equipped with fluorescent detection at an excitation wavelength of 335 nm and emission wavelength of 528 nm.

2.5 TX-114 separation

After 48 hrs of treatment, K562 cells were lysed in TX-114 (1% TX-114, 20 mM Tris, 150 mM NaCl (Research Products International Corp, Mount Prospect, IL)) followed by incubation at 37 °C until the lysates became cloudy. Lysates were centrifuged at 14000g for 2 min immediately followed by separation of the aqueous (supernatant) and detergent (pellet) phases. TX-114 was added to the aqueous phase at 1% of its volume. Buffer B (20 mM Tris and 150 mM NaCl) was added to the detergent phase to bring the detergent phase to ¼ the volume of the aqueous phase. Both phases were incubated at 37 °C until lysates became cloudy followed by centrifugation at 14000g for 2 min, immediate separation of each phase, and the addition of TX-114 or Buffer B, repeating an additional three more times. Following the final separation of the phases, Buffer B was added to the detergent phases to bring them to the same volume as the aqueous phases. Protein was quantified by BCA assay (Life Technologies, Grand Island, NY) [28].

2.6 Western blot analysis

K562 cells were plated at 3 million cells/plate and immediately treated for 48 hrs. Cells were lysed in RIPA buffer (0.15 M NaCl, 0.05 M Tris HCl, 1% w/v sodium deoxycholate, 1% w/v SDS, 1% w/v Triton X-100, 1 mM EDTA (Research Products International Corp, Mount Prospect, IL)) or TX-114 as indicated, both containing protease inhibitor cocktail and phenylmethanesulfonyl fluoride (Sigma Aldrich, St. Louis, MO). Protein concentrations were quantified by BCA assay [28]. Equal protein quantities were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes overnight. Membranes were blocked at 37 °C for 45 min in 5% non-fat milk followed by incubation with primary antibodies at 4 °C overnight and secondary antibodies at 37 °C for 1 hr. Proteins were visualized using ECL detection (GE Healthcare, Buckinghamshire, UK) and quantified by densitometry using Image J. [29] Anti-pan-Ras was obtained from Inter-Biotechnology (Tokyo, Japan). Rap1A (sc-1482), Rab6 (sc-310), β -tubulin (sc-9140), calnexin (sc-23954), and secondary antibody donkey anti-goat IgG-HRP (sc-2033) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary HRP-conjugated anti-rabbit (NA934) and anti-mouse (NXA931) antibodies were obtained from GE Healthcare (Buckinghamshire, UK).

2.7 FOH and GGOH add-back assays

FOH and GGOH were obtained from Sigma-Aldrich (St. Louis, MO) and diluted in methanol. K562 cells were plated at 3 million cells/plate and immediately treated with the indicated concentrations of each compound with or without the simultaneous addition of 40 μ M FOH or GGOH [30]. Cell lysis and Western blot analysis of Rap1A was completed as detailed above.

2.8 Statistical analysis

Statistical analysis was performed using the Welch Two-Sample t-test with the R Project for Statistical Computing software.

3. RESULTS

3.1 The novel bisphosphonates are not toxic to the K562 myelogenous leukemia cell line at micromolar concentrations

To determine the toxicity of the six novel bisphosphonates in the human chronic myelogenous leukemia cell line K562, MTT activity assays were performed after 24 hrs (Figure 3A), 48 hrs (Figure 3B), or 72 hrs (Figure 3C) of treatment. Compounds **3**, **4**, **6**, **8**, and **9** show a dose-dependent reduction in MTT activity that is amplified from 24 hrs to 72 hrs. After 72 hrs of treatment, MTT activity was reduced to levels below 80% at concentrations of 2.5 μ M for compounds **4** and **6**, 10 μ M for compounds **3** and **8**, and 100 μ M for compound **9**. At concentrations as high as 100 μ M for 72 hrs, only compounds **3** and **9** reduced MTT activity below 50%. Conversely, compounds **5** and **7** caused no alteration in MTT activity even after 72 hrs of 100 μ M treatments.

3.2 Compounds do not alter Ras farnesylation

To compare the ability of the novel bisphosphonates to alter protein farnesylation, K562 cells were treated with increasing concentrations of each compound for 48 hrs. The Ras antibody used detects both the prenylated and unprenylated forms of the protein. The prenylated form consists of the lower band seen in all lanes while the unprenylated form is the upper band seen only in the positive control lovastatin (Figure 4). Analyses of cell lysates found none of the compounds alter Ras farnesylation at concentrations as high as 10 and 100 μ M as shown by the lack of an upper unprenylated band (Figure 4).

3.3 Compound 6 most potently reduces Rap1A geranylgeranylation

In order to compare the compounds' ability to alter geranylgeranylation we first performed Western blot analysis on Rap1A, a protein normally isoprenylated by geranylgeranyl protein transferase-1 (GGPTase I) (Figure 1). The Rap1A antibody used detects only the unprenylated form of Rap1A, and therefore the presence of a band indicates a reduction in Rap1A geranylgeranylation. Comparing the novel bisphosphonates, compound **6** was found to be the most potent, reducing Rap1A geranylgeranylation at easily visualized levels beginning at 0.4 μ M (Figure 5C). Comparatively, compounds **4** (Figure 5A) and **9** (Figure 5F) began to reduce Rap1A geranylgeranylation detectably at 2.5 μ M, compound **8** (Figure 5E) at 5 μ M, and compound **7** (Figure 5D) at 50 μ M, while compound **5** had no effect at concentrations as high as 100 μ M (Figure 5B).

3.4 Compounds 6 and 9 most potently reduce Rab6 geranylgeranylation

Next we performed Western blot analysis on Rab6, a protein normally isoprenylated by geranylgeranyl protein transferase-2 (GGPTase II) (Figure 1). The Rab6 antibody used detects both the prenylated and unprenylated forms. However, the two forms are unable to be separated on one gel requiring the additional step of a Triton-X 114 (TX-114) separation of the cell lysates. Due to the additional time-restrictive step of partitioning the aqueous and detergent phases, only one concentration was utilized for each compound. Comparison of compounds **4** (Figure 6A), **6** (Figure 6C), **7** (Figure 6D), **8** (Figure 6E), and **9** (Figure 6F), indicate that all reduce Rab6 geranylgeranylation at 10 μ M concentrations as shown by the

presence of an aqueous band and a corresponding reduction in the detergent band. Comparing each compound to the parental compound **3** at 10 μ M, compounds **6** and **9** more potently reduce Rab6 prenylation with aqueous bands on average 11% and 18% greater than those generated by treatment with compound **3**, respectively (Figure 6). Conversely, compound **5** does not alter Rab6 geranylgeranylation at concentrations as high as 100 μ M (Figure 6B).

3.5 The addition of GGOH but not FOH abolishes the bisphosphonate effect on Rap1A geranylgeranylation

In order to clarify further the mechanism(s) by which the alkyl bisphosphonate ethers are altering geranylgeranylation *in vitro*, we performed add back studies through the simultaneous addition[30] of farnesol (FOH) or geraniol (GGOH) with each compound for 48 hrs followed by Western blot analysis of Rap1A. Using densitometry to quantify the alterations in Rap1A geranylgeranylation we found the addition of FOH to cause no alterations in the ability of compounds **3**, **4**, **6**, **7**, and **9** to alter Rap1A prenylation (Figure 7A,C,D,F). Interestingly, the addition of FOH to compound **8** consistently caused a significant *increase* ($p < 0.05$) in the compound's ability to reduce Rap1A prenylation as shown by a 1.2 ± 0.05 fold increase in the density of the Rap1A band (Figure 7E). Conversely, the addition of GGOH abolished the ability of compounds **3**, **4**, **6**, **7**, **8**, and **9** to alter Rap1A prenylation as shown by the lack of a detectable Rap1A band (Figure 7A,C–F). Consistent with previous findings, compound **5** showed no detectable changes in Rap1A prenylation at concentrations as high as 100 μ M and so the addition of FOH and GGOH caused no changes in compound **5** activity (Figure 7B).

3.6 Compounds cause a dose-dependent reduction in GGPP levels

In order to determine the impact of the novel bisphosphonates on the protein isoprenylation precursors FPP and GGPP, K562 cells were treated for 48 hrs with increasing concentrations of each compound. Analyses of FPP levels found compounds **3**, **4**, **7**, and **9** to cause minimal to no alteration at concentrations as high as 10 μ M (Figure 8). Conversely, at 10 μ M concentrations compounds **6** and **8** caused a 444% and 296% increase in FPP levels respectively (Figure 8). Analyses of GGPP levels found compounds **3**, **4**, **6**, **8**, and **9** to reduce levels by 90% at 10 μ M concentrations whereas compound **7** caused no alteration at 10 μ M concentrations (Figure 8). Compound **6** was found to be the most potent, reducing GGPP levels by 93% even at 1 μ M compared to 60% reduction by all other tested compounds at the same concentration (Figure 8). Compound **5** was not analyzed due to the observed lack in activity against Rap1A and Rab6 prenylation at concentrations as high as 100 μ M.

4. DISCUSSION

Previous work in our laboratory has generated a novel library of six bisphosphonate compounds capable of inhibiting GGDPS at concentrations below 1 μ M while having little to no activity against FDPS [23]. Based on the data from studies with the isolated GGDPS enzyme (Figure 2),[23] we expected the greatest biological activity *in vitro* to be found with compound **9**. In reality, compound **6** consistently was found to be the most potent in its

ability to reduce GGPP and protein geranylgeranylation despite its GGDPS IC₅₀ being ~3-fold less potent than the parental compound **3** and ~8-fold less potent than compound **9** (Table 1). Compound **6** was found to have activity against FDPS in isolated enzyme assays (~1.2 fold less potent than its activity against GGDPS), suggesting its ability to impact two sites of the IBP may account for its high biological activity. However, we did not observe alteration of Ras farnesylation at concentrations up to 10 μ M (data not shown) suggesting that the high biological activity of compound **6** against GGPP and geranylgeranylation is not due to inhibition of FDPS. Compound **6** also caused significant increases in FPP, a finding that would not be expected if it were inhibiting FDPS at relevant concentrations *in vitro*, and the addition of GGOH but not FOH abolished the effect of compound **6** on Rap1A geranylgeranylation. Finally compound **6** also has shown activity at similar concentrations in three human-derived prostate cancer cell lines (data not shown) [31].

A second interesting finding is the observed difference in the biological activity of the two prenyl-geranyl isomers **6** and **8**. Both compounds caused increases in FPP levels but compound **6** reduced GGPP levels by 93% at 1 μ M as compared to 60% for compound **8**. Analysis of Rap1A geranylgeranylation found compound **6** to reduce isoprenylation at 0.4 μ M as compared to 5 μ M for compound **8**. Unexpectedly, the simultaneous addition of FOH to assays with compound **8** caused a significant increase in its ability to reduce Rap1A geranylgeranylation which was not seen with compound **6**. Previous work has found FOH to increase the degradation of HMG-CoA (hydroxymethylglutaryl co-enzyme A) reductase upstream of compound **8** inhibition (Figure 1) [32, 33]. One possible explanation for the observed enhancement of compound **8** activity against Rap1A prenylation may be a reduction in available GGPP substrates by exogenous FOH. However, the same phenomenon would be expected to occur with all compound treatments, suggesting a mechanism specific to compound **8**.

Crystallographic analyses of the GGDPS enzyme by the Oldfield group found parental compound **3** to occupy both the channel for FPP entrance and that where GGPP is released [26]. Prior to the present work, it was unclear if the placement of two different isoprenoid chains would affect the ability of the compounds to inhibit GGDPS. For the isomeric pair of compounds **6** and **8**, the isomer **8** is both more potent and more selective for inhibition of GGDPS over FDPS in assays with the isolated enzymes. However, in these cell assays the impact of compound **6** is consistently greater on geranylgeranylation of both Rap1A and Rab6, and it also more effectively lowers GGPP and raises FPP concentrations. Furthermore, addition of FOH increases the potency of compound **8**, while compound **6** is unaffected in this assay. These clear differences in activity suggest an organized binding with the C-geranyl-*O*-prenyl structure of compound **6** more effective than the corresponding C-prenyl-*O*-geranyl compound **8** in all of the cellular assays. The electronic effects on the acidity of these two isomers should be virtually identical, and while metabolic cleavage of C-P bonds is known,[34, 35] it would be surprising to find a significant difference in the metabolic stability of such closely related compounds.[36] One possibility that cannot be excluded is that the longer overall chain length of the *O*-geranyl compound has an impact on its binding. Future work defining the interaction of the prenyl-geranyl isomers with the FDPS and GGDPS enzymes will help clarify the nature of this binding in our compound library as well

as provide information that should facilitate development of even more potent IBP inhibitors.

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NONSTANDARD ABBREVIATIONS

DGBP	digeranyl bisphosphonate
FDPS	farnesyl diphosphate synthase
FOH	farnesol
FPP	farnesyl diphosphate
GGDPS	geranylgeranyl diphosphate synthase
GGOH	geraniol
GGPP	geranylgeranyl diphosphate
GGPTase	geranylgeranyl protein transferase
HMG-CoA	hydroxymethylglutaryl co-enzyme A
IBP	isoprenoid biosynthetic pathway
NBP	nitrogenous bisphosphonates

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

TABLE 1

Effect of bisphosphonate ethers on Rap1A and Rab6 geranylgeranylation, and FPP and GGPP levels. Concentrations at which compounds alter Rap1A geranylgeranylation are given. Rab6 unprenylated (aqueous) bands were quantified by densitometry and calculated as a percentage of the untreated controls. The percent difference between the indicated compound and DGBP at 10 μ M DGBP are shown below. Quantification of FPP and GGPP levels was established in the presence of 10 μ M compound for 48 hrs.

Compound	Rap1A (GGTase-I) (μ M)	Rab6 (GGTase-II) (% diff)	FPP (% control)	GGPP (% control)
3	5	NA	182%	10%
4	2.5	5%	186%	5%
6	0.4	11%	444%	4%
7	50	-27%	189%	96%
8	5	-1%	296%	13%
9	2.5	18%	109%	9%