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Digeranyl bisphosphonate inhibits geranylgeranyl pyrophosphate synthase

Andrew J. Wiemer [a,*], Huaxiang Tong [b], Kelly M. Swanson [b], Raymond J. Hohl [a,b,c]

[a] *Molecular and Cellular Biology Interdisciplinary Program, University of Iowa, Iowa City, IA 52242, USA*
[b] *Department of Internal Medicine, University of Iowa, Iowa City, IA 52242, USA*
[c] *Department of Pharmacology, University of Iowa, Iowa City, IA 52242, USA*

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Abstract

A primary cellular target of the clinical nitrogenous bisphosphonates is the isoprenoid biosynthetic pathway. Specifically these drugs inhibit the enzyme farnesyl pyrophosphate synthase and deplete cells of larger isoprenoids. Inhibition of this enzyme results in impaired processing of both farnesylated and geranylgeranylated proteins. We recently showed that isoprenoid-containing bisphosphonates such as digeranyl bisphosphonate inhibit protein geranylgeranylation and not farnesylation. Here, we show that this impairment results from potent and specific inhibition of geranylgeranyl pyrophosphate synthase, which leads to enhanced depletion of intracellular geranylgeranyl pyrophosphate relative to the nitrogenous bisphosphonate zoledronate.

Keywords: Bisphosphonate; Geranylgeranyl pyrophosphate synthase; Farnesyl pyrophosphate synthase; Isoprenoid; Mevalonate; Prenylation; Ras; Zoledronate; Digeranyl bisphosphonate

Bisphosphonates are commonly used for treatment of diseases associated with bone resorption such as osteoporosis and metastatic bone disease. The primary cellular mechanism of the clinical bisphosphonates is inhibition of osteoclast-mediated bone resorption [1–3], although they have also been shown to directly inhibit cellular growth some cancers including multiple myeloma [4,5]. The nitrogenous bisphosphonates, including zoledronate, inhibit isoprenoid biosynthesis by targeting the mevalonate pathway, which subsequently impairs protein prenylation of the Ras superfamily of small GTPases [6]. Inhibition of GTPase prenylation results in altered cell signaling pathways. These pathways include the Ras family which controls gene expression [7], the Rho family which controls cytoskeleton rearrangement [8] and the Rab family which controls vesicular trafficking [9].

It has been shown that the nitrogenous bisphosphonates directly inhibit the enzyme farnesyl pyrophosphate (FPP) synthase [10–14], however, many of the phenotypic effects of bisphosphonates on both osteoclasts and cancer cells result from depletion of intracellular geranylgeranyl pyrophosphate (GGPP) [15,16]. We and others have hypothesized that alterations of the bisphosphonate structure may serve to increase their affinity for specific enzymes in the isoprenoid biosynthetic pathway. Indeed, it has recently been shown that some bisphosphonates inhibit GGPP synthase *in vitro* with varying potencies [17]. Additionally, our laboratory has previously synthesized novel bisphosphonates which contain an isoprenoid moiety that can specifically impair protein geranylgeranylation and not farnesylation [18].

In this study, we identified the mechanism by which the most potent novel isoprenoid bisphosphonate, digeranyl bisphosphonate, inhibits protein geranylgeranylation. Here, we demonstrate that GGPP synthase is the intracellular target of digeranyl bisphosphonate. Digeranyl bisphosphonate-induced inhibition of geranylgeranylation

* Corresponding author. Fax: +1 319 335 8891.
E-mail address: Andrew-Wiemer@uiowa.edu (A.J. Wiemer).

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results from depletion of intracellular GGPP levels in RPMI-8226 cells.

Materials and methods

Chemicals. Zoledronate was purchased from Novartis (Basel, Switzerland). Digeranyl bisphosphonate (Fig. 1c) was synthesized as described previously [18]. Lovastatin and isoprenoid pyrophosphates were obtained from Sigma–Aldrich (St. Louis, MO).

Cell culture. Human-derived RPMI-8226 myeloma cells were obtained from ATCC (Manasas, VA) and cultured according to manufacturer's protocol. Suspension cultures (1×10^6 cells/ml) were incubated for 24 h with the indicated compound concentrations. Western blot analyses required 4 million cells per treatment and FPP/GGPP detection required 6 million cells per condition.

FPP and GGPP synthase assays. Plasmids containing GST-tagged recombinant human FPP synthase or GGPP synthase were expressed in BL21 gold bacteria by induction with IPTG. Proteins were purified by column chromatography. FPP synthase assays were performed in a method modified from Dunford [13]. Each reaction mixture contained 20 μM GPP, 40 μM ^{14}C-IPP, and FPP synthase (activity = 80 nmol/min) in 50 μL of reaction buffer (50 mM Tris, pH 7.7, 10 mM NaF, 2 mM $MgCl_2$, and 1 mg/mL BSA). For GGPP synthase assays each reaction mixture contained 20 μM FPP, 40 μM ^{14}C-IPP, and GGPP synthase (activity = 133 nmol/min) in 35 μL of reaction buffer (50 mM Imidazole, pH 7.5, 0.5 mM $MgCl_2$, and 0.5 mM $ZnCl_2$). Following a 10 min pre-incubation with inhibitors, reactions were initiated by simultaneous addition of ^{14}C-IPP and GPP or FPP. Reactions were allowed to proceed for 1 h at 37 °C, at which time no more than 20% of available substrate was used. Following incubations longer isoprenoids were extracted with 1 mL butanol and washed three times with 300 μL water. The amount of ^{14}C incorporation into longer isoprenoids was detected by liquid scintillation counting.

FPP/GGPP quantification. FPP and GGPP levels were determined as we previously reported by reverse phase HPLC [19]. Briefly, isoprenoid pyrophosphates were extracted from cells and used as substrates for incorporation into fluorescent GCVLS or GCVLL peptides by farnesyltransferase or geranylgeranyl transferase I. The prenylated fluorescent peptides were separated by reverse phase HPLC and quantified by fluorescence detection.

Western blot analysis. Protein concentrations were determined using BCA method (Thermo Fisher, Waltham, MA). Proteins were separated on a 12% gel and transferred to a PDVF membrane. Primary and secondary antibodies were added and proteins were visualized using an ECL chemiluminescence detection kit. Anti pan-Ras was obtained from Inter-Biotechnology (Tokyo, Japan). All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, California).

Detection of cell cycle distribution and apoptosis. Two million cells were pelleted, washed, and resuspended in 200 μL PBS. Three milliliters of −20 °C 70% EtOH was added and then incubated for 1 h at 4 °C. Cells were washed twice with 2 ml PBS and resuspended in 100 μL PBS. RNase A solution (10 mg/ml) was added at 1:100. Two hundred microliters of PI solution (50 μg/ml) was added to the cell suspension which was incubated for 30 min before flow cytometric analysis (FACScan, Beckton–Dickinson).

Results and discussion

In recent years, many studies have identified FPP synthase as the molecular target of bisphosphonates while implicating GGPP as the most important depleted isoprenoid. However, few attempts have been made at development of GGPP synthase inhibitors, and the most potent bisphosphonate inhibitor of GGPP synthase described to date is the saturated 1-hydroxydecane-1,1-bisphosphonate, which has a reported *in vitro* IC_{50} of approximately 700 nM [17]. We have recently described the synthesis of a novel class of bisphosphonates that inhibit protein geranylgeranylation, and the current studies sought to demonstrate the potency and specificity of one of these compounds, digeranyl bisphosphonate, towards inhibition of GGPP synthase [18].

Inhibition of FPP synthase and GGPP synthase are displayed in Fig. 2. As has been shown previously, zoledronate is a potent inhibitor of FPP synthase (Fig. 2a). Zoledronate also demonstrates weak inhibition of GGPP synthase *in vitro* ($IC_{50} > 100$ μM). Digeranyl bisphosphonate demonstrates potent inhibition for GGPP synthase, with an experimental IC_{50} of approximately 200 nM. This IC_{50} value is lower than the reported IC_{50} value for 1-hydroxydecane-1,1-bisphosphonate (720 nM) [17]. Additionally, digeranyl bisphosphonate did not inhibit FPP synthase at the concentrations tested. This data, as well as our previous finding that at least one side chain must be of geranyl or longer length for inhibition of geranylgeranylation [18] is consistent with the structure–activity relationship

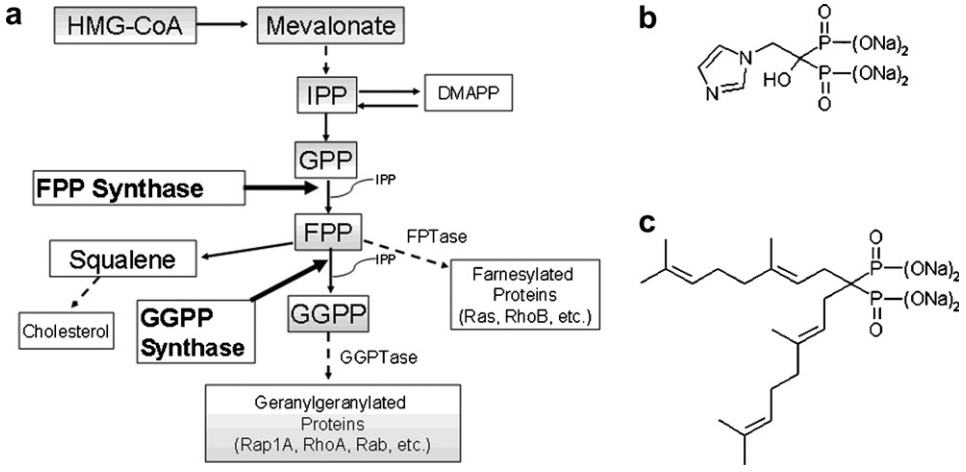

Fig. 1. (a) The mevalonate pathway. (b) Structure of zoledronate. (c) Structure of digeranyl bisphosphonate.