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Multi-target-directed design, syntheses, and characterization of fluorescent bisphosphonate derivatives as multifunctional enzyme inhibitors in mevalonate pathway

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ABSTRACT

Background: Mevalonate pathway is an important cellular metabolic pathway present in all higher eukaryotes and many bacteria. Four enzymes in mevalonate pathway, including MVK, PMK, MDD, and FPPS, play important regulatory roles in cholesterol biosynthesis and cell proliferation.
Methods: The following methods were used: cloning, expression and purification of enzymes in mevalonate pathway, organic syntheses of multifunctional enzyme inhibitors, measurement of their IC_{50} values for above four enzymes, kinetic studies of enzyme inhibitions, molecular modeling studies, cell viability tests, and fluorescence microscopy.
Results and conclusions: We report our multi-target-directed design, syntheses, and characterization of two blue fluorescent bisphosphonate derivatives compounds **15** and **16** as multifunctional enzyme inhibitors in mevalonate pathway. These two compounds had good inhibition to all these four enzymes with their IC_{50} values at nanomolar to micromolar range. Kinetic and molecular modeling studies showed that these two compounds could bind to the active sites of all these four enzymes. The fluorescence microscopy indicated that these two compounds could easily get into cancer cells.
General significance: Multifunctional enzyme inhibitors are generally more effective than single enzyme inhibitors, with fewer side effects. Our results showed that these multifunctional inhibitors could become lead compounds for further development for the treatment of soft-tissue tumors and hypercholesteremia.

1. Introduction

The mevalonate pathway is an important cellular metabolic pathway present in all higher eukaryotes and many bacteria, and has so far been exploited in the design of drugs treating and preventing human diseases, including cardiovascular diseases and osteoporosis [1]. This pathway is also a potentially important target for the treatment of tumors especially bearing mutations in protein p53 [2,3]. Farnesyl pyrophosphate synthase (FPPS) is a key regulatory enzyme in the mevalonate pathway [4]. Besides, mevalonate pathway also contains a unique series of three sequential ATP-dependent enzymes that convert mevalonate to isopentenyl diphosphate (IPP): mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and mevalonate 5-diphosphate decarboxylase (MDD). These three enzymes catalyze consecutive steps downstream from HMG-CoA reductase in the mevalonate pathway, and are responsive to cholesterol in-take in animals. The blockade of the mevalonate pathway is a concept that has found widespread clinical use, with statins as drugs that inhibit HMG-CoA reductase and reduce cholesterol biosynthesis [5], and nitrogen-containing bisphosphonates (*N*-BPs) as drugs for osteoporosis therapy that target FPPS and inhibit protein prenylation [6]. Some multiple inhibitory compounds, including statins [7], bisphosphonates [8], farnesyl transferase inhibitors (FTIs), and geranyl geranyltransferase inhibitors (GGTIs) [9], have been developed previously targeting mevalonate pathway. Statin's side effects have been the subject of much controversy over the past few decades, since more and more research is revealing serious potential adverse reactions from statin medications [10–12].

Bisphosphonate therapy has been considered as standard therapy in the management and care of cancer patients with metastatic bone disease and patients with osteoporosis with fewer side effects [13–15]. In addition, bisphosphonates can be easily modified in various positions of their P-C-P structure. Its central carbon has great metabolic stability, and also provides a scaffold that can be modified

Abbreviations: FPP, farnesyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; GPP, geranyl diphosphate; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl diphosphate; MDD, mevalonate 5-diphosphate decarboxylase, also known as mevalonate 5-pyrophosphate decarboxylase or MPD; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; MVAPP, mevalonate 5-diphosphate; MVK, mevalonate kinase; *N*-BPs, nitrogen-containing bisphosphonates; PMK, phosphomevalonate kinase; PP, pyrophosphate or diphosphate; TsCl, toluene sulfonyl chloride

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with additional substituents, such as the hydroxyl group and heteroaromatic rings found in risedronate and zoledronate [16,17]. However, these bisphosphonates all bear substantial negative charge at physiological pH, which limit the compounds' ability to penetrate the cell membrane. In an effort to circumvent this perceived limitation, some biodegradable protecting groups have been used to mask a negative charge until after penetration of the cell membrane [18,19]. Further investigation and improvement of bisphosphonates are still required.

Our strategy in the design of novel multifunctional inhibitors based on bisphosphonates is to link some known enzyme inhibitors with covalent chemical bond in the P-C-P backbone, which targets on four different enzymes in the mevalonate pathway, including MVK, PMK, MDD, and FPPS. In the present paper, we report our multi-target-directed design, syntheses, and characterization of two bisphosphonate derivatives as multifunctional enzyme inhibitors in the mevalonate pathway, which mainly contain the following functional groups: the first one is bisphosphonate targeting on FPPS; the second one is geranyl group with aromatic substitution targeting on ATP binding site of MVK, PMK, and MDD; and the third one is mevalonate group with fluorine substitution targeting on mevalonate binding site of MVK, PMK, and MDD.

2. Materials and Methods

2.1. Materials

Lactate dehydrogenase, pyruvate kinase, phosphoenol pyruvate, NADH, (*RS*)-mevalonic acid lactone, and ATP were purchased from Sigma. *Taq* DNA polymerase, HB101 competent cells, and *E. coli* strain BL21(DE3) competent cells came from Invitrogen Life Technologies. Synthesized oligonucleotides were obtained from Tech Dragon Company of Hong Kong. T4 DNA ligase and restriction enzymes came from MBI Fermentas of Germany. All other reagents were of research grade or better and were obtained from commercial sources. The rat liver MVK, MDD, and FPPS were obtained and assayed as previously described [20–22]. Tris buffer was used instead of phosphate buffer in enzyme storage and enzyme assay.

2.2. Cloning, expression, and purification of rat PMK

A rat liver Quick-Clone cDNA library was purchased from Clontech (Palo Alto, CA). Standard cloning technology was used to clone the gene of rat PMK. DNA sequencing of the cloned rat PMK gene was performed, and the inserted gene sequence was identified to be the same as previously deposited in NCBI without any mutation. Established methods were used to prepare rat PMK [20], and the protein was purified to apparent homogeneity as shown in SDS-PAGE in supporting information, which was stored at −80 °C in 50 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, 5% glycerol, and 5 mM β-mercaptoethanol. The activity of the enzyme was assayed spectrophotometrically following the decrease in absorbance at 340 nm as reported previously [23].

2.3. Organic syntheses of blue fluorescent bisphosphonate derivatives

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Varian Mercury 300 MHz NMR spectrometer at room temperature. Chemical shifts are reported in ppm on the δ scale relative to the internal standard TMS. Flash chromatography was performed in columns of various diameters with silica gel by elution with appropriate solvents. Analytical thin layer chromatography (TLC) was carried out on Merck silica gel 60G254 plates (25 mm), and developed with appropriate solvents, and was visualized either with UV light or by dipping into a staining solution of potassium permanganate and then heating. AG MP-1M 100–200 resin, chloride form was from Bio-Rad. Dowex 50 WX2-100 cation-exchange resin was purchased from Aldrich. All other reagents were of research grade or better, and were obtained from commercial sources. Organic syntheses of bisphosphonate derivatives were shown in Scheme 1.

Synthesis of tetraethyl ethenylidenebisphosphonate (**3**). The compound was synthesized following a reported procedure with minor modification [24]. Paraformaldehyde (10.4 g, 0.35 mol) and diethylamine (5.08 g, 0.069 mol) were combined in 0.2 L of methanol, and the mixture was warmed until clear. Compound **1** (20.0 g, 0.069 mol) was added, and the resulting mixture was heated under reflux for 24 h. Then additional 0.2 L of methanol was added, and the solution was concentrated under vacuum at 35 °C. Toluene (0.1 L) was added, and the solution was again concentrated. This last step was repeated to ensure complete removal of methanol to give the product **2** as a clear liquid. ^{1}H NMR ($CDCl_3$) δ 4.02 (m, 8 H, OCH_2CH_3, $J=7.3$ Hz), 3.63 (overlapping m, 2H, CH_3OCH_2, $J=5.4$ Hz and 15.6 Hz), 3.20 (s, 3H, CH_3O), 2.52 (tt, 1H, PCHP, $J=6.0$ Hz and 24.01 Hz), 1.18 (t, 12H, CH_2CH_3, $J=7.1$ Hz).

p-Toluenesulfonic acid monohydrate (0.50 g) was added, and the mixture was heated under reflux. Methanol was removed from the reaction mixture either by collection in a Dean-Stark trap or by adsorption into 4 Å molecular sieves contained in a Soxhlet extractor. After 14 h, the solution was concentrated. The crude product was diluted with 1 L of chloroform, and washed with water (2×150 mL). The solution was dried over $MgSO_4$ and concentrated to give the product **3**. ^{1}H NMR ($CDCl_3$) δ 6.98 (distorted dd, 2H, $H_2C=$, $J=33.8$ Hz and 37.71 Hz), 4.32–4.00 (m, 8H, OCH_2CH_3), 1.32 (t, 12H, OCH_2CH_3, $J=$ 7.1 Hz). This NMR data is consistent with that reported previously [24].

Synthesis of 2-(3-chloropropyl)-2-methyl-1,3-dioxolane (**4**). A mixture of 5-chloro-2-pentanone (19.6 g, 158 mmol), ethylene glycol (49.7 g, 800 mmol), and *p*-toluenesulfonic acid monohydrate (300 mg, 1.6 mmol) was heated in 500 mL of toluene under reflux with a Dean–Stark trap for 24 h. The mixture was then washed with 10% aq. $NaHCO_3$ solution (3×30 mL), followed by brine (3×30 mL). The organic phase was dried over anhydrous Na_2SO_4. After filtration, the solvent was removed under reduced pressure, and the residual liquid was distilled to give 24 g (92%) of compound **4** as a colorless liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.95 (4 H, m, OCH_2CH_2O), 3.60 (2 H, t, $J=7.0$ Hz, CH_2Cl), 1.85 (4 H, m, CH_2-CH_2), 1.35 (3 H, s, CH_3).

Synthesis of tetraethyl 5-(2-methyl-1,3-dioxolan-2-yl) pentane-1, 1-diyldiphosphonate (**6**). A titrated solution containing 1.66 mmol of the Grignard reactant of the compound **4** (0.062–2.44 M) in THF was slowly added to a magnetically stirred solution of tetraethyl ethenylidenebisphosphonate **3** (500 mg, 1.66 mmol) in dry THF (10 mL) at −15 °C under Ar. The reaction progress was followed by using TLC, and the reaction was usually complete at the end of the addition. The mixture was warmed to room temperature, and then slowly poured into a saturated solution of NH_4Cl (20 mL). The mixture was extracted with ether (2×20 mL), and the combined organic layers were dried and concentrated under reduced pressure. The crude residue was purified by using flash chromatography with appropriate eluent to give the product **6** as an oil. MS (ESI): m/z 431 $(M+H)^+$.

Synthesis of (*E*)-3,7-dimethyl-octa-2,6-dienyl acetate (**7**). To a mixture of alcohol/phenol/amine (1 mmol) and acetic anhydride (1.2 mmol), $La(NO_3)_3 \cdot 6H_2O$ (10 mol%) was added. After completion of the reaction as monitored by using TLC, water was added to the reaction mixture, and the product was extracted into ethyl acetate (3×20 mL). The combined organic layers were washed with brine and concentrated in vacuum, which was purified by using silica gel column chromatography to afford the acetylated product **7**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.32 (br t, 1H), 5.06 (m, 1H), 4.57 (d, 2H, $J=$ 7.5Hz), 2.03 (s, 3H), 2.03–2.09 (m, 4H), 1.68 (s, 3H), 1.66 (s, 3H), 1.58 (s, 3H).

Synthesis of (*2E*, *6E*)-3,7-dimethyl-8-oxoocta-2,6-dienyl acetate (**8**). Geranyl acetate (**7**) (6.84 g, 34.9 mmol) pre-dissolved in 100 mL of 95% ethanol was added dropwise over 40 min to a refluxing solution of SeO_2 (5.8 g, 52 mmol) in 300 mL of 95% ethanol. The mixture was heated

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