Research Article

Structure-Based Inhibitors Exhibit Differential Activities against *Helicobacter pylori* and *Escherichia coli* Undecaprenyl Pyrophosphate Synthases

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*Helicobacter Pylori* colonizes the human gastric epithelium and causes diseases such as gastritis, peptic ulcers, and stomach cancer. Undecaprenyl pyrophosphate synthase (UPPS), which catalyzes consecutive condensation reactions of farnesyl pyrophosphate with eight isopentenyl pyrophosphate to form lipid carrier for bacterial peptidoglycan biosynthesis, represents a potential target for developing new antibiotics. In this study, we solved the crystal structure of *H. pylori* UPPS and performed virtual screening of inhibitors from a library of 58,635 compounds. Two hits were found to exhibit differential activities against *Helicobacter Pylori* and *Escherichia coli* UPPS, giving the possibility of developing antibiotics specially targeting pathogenic *H. pylori* without killing the intestinal *E. coli*.

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1. INTRODUCTION

Undecaprenyl pyrophosphate synthase (UPPS) catalyzes consecutive condensation reactions of farnesyl pyrophosphate (FPP) with eight molecules of isopentenyl pyrophosphate (IPP) to form C55 undecaprenyl pyrophosphate (UPP), which acts as a lipid carrier to mediate bacterial peptidoglycan biosynthesis [1, 2]. This enzyme belongs to a group of cis-prenyltransferases which catalyze cis-double bonds during IPP condensation reactions [3, 4]. UPPS was first cloned from *Micrococcus luteus* and *Escherichia coli*, and their amino acid sequences were found conserved among the cis-prenyltransferases, but totally different from those of the trans-prenyltransferases [5–7], implying different catalytic mechanism [8, 9].

*Helicobacter Pylori* is a pathogen which causes chronic inflammation in the stomach [10]. The infection may evolve to peptic ulcerations and gastric neoplasias. Due to its unusual ability to survive in stomach under the low pH condition via proton pumps, *H. Pylori* infection becomes wide spreading and accounts for the increased cases of stomach carcinogenesis [11]. Antibiotics, such as proton pump inhibitors (PPI), amoxicillin, and clarithromycin, are used to treat the infected patients. When failed, empirical quadruple therapy (PPI-bismuth-tetracyclin-metronidazole) is then used as the second-line therapy [12]. Since UPPS is essential for bacterial survival, it could possibly serve as a target for new antibiotics. Even though the complex structures of *E. coli* UPPS with the FPP substrate or with its analogue (farnesyl thiopyrophosphate, FsPP) and IPP have been obtained [9, 13], no UPPS structure-derived inhibitors have been reported so far. As shown in this study, we solved the crystal structures of *H. pylori* UPPS and performed structure-based inhibitor discovery. Two hits were discovered through computer virtual screening from 58,635 compounds, which exhibited different level of inhibition against *E. coli* and *H. pylori* UPPS.

2. MATERIALS AND METHODS

2.1. Overexpression of *H. pylori* UPPS

The gene encoding UPPS from the *H. pylori* (ATCC43504) genomic DNA was amplified by using polymerase chain
reaction (PCR). The forward primer 5′-GATTTAGA-
GGGTGCTTGGATAGCCTCTCAA-3′ and reverse primer
5′-AGAGGAGCTTAGAGCCCTAGCTTTTAA-
TTCCCC-3′ were utilized in the PCR. The PCR product was
purified from 0.8% agarose gel electrophoresis. The DNA
product was ligated with pET-32×a/LiC vector and trans-
formed into E. coli BL21 (DE3) for protein expression as pre-
viously described for expressing E. coli UPPS [14].

The C234A mutant was prepared by using QuikChange
Site-Directed Mutagenesis Kit in conjunction with the wild-
type gene template in the pET32×a/LiC vector. The muta-
genic forward primer was 5′-CGCAATTCGGGAAATT-
AAA GCC TAGTGAGGCTTCTCAA-3′. The procedure of
mutagenesis utilized a supercoiled double-stranded DNA
(dsDNA) vector with an insert of interest and two synthetic
forward and backward primers containing the desired muta-
tion. The mutation was confirmed by sequencing the entire
UPPS mutant gene of the plasmid obtained from overnight
culture. The correct construct was subsequently transformed
to E. coli BL21(DE3) for protein expression. The procedure
for protein purification followed our reported protocol [15].
Each purified mutant UPPS was verified by mass spectro-
scopic analysis and its purity (>95%) was checked by SDS-
PAGE.

2.2. Crystallization and data collection

H. pylori C234A UPPS mutant was crystallized using the
hanging drop method from Hampton Research (Laguna
Niguel, Calif, USA) by mixing 2 μL of the UPPS solution
(10 mg/mL in 25 mM Tris, 150 mM NaCl, pH 8.0) with 2 μL
of the mother liquor (0.15 M KSCN, 15% PEG600, and 2%
PEG5KMME), and equilibrating with 500 μL of the mother
liquor. Within 4 days, crystals grew to dimensions of about
0.5×0.5×0.2 mm, and then the crystals were soaked with a
cryo.protectant solution of 0.2 M KSCN, 30% PEG600, and
5% PEG5KMME for 1 day. The structure of the C234A H.
pylori UPPS in complex with FsPP was obtained by soaking
the crystals with cryoprotectant solution of 2.5 mM MgCl2,
2.5 mM IPP, 2.5 mM FsPP, 0.15 M KSCN, 15% PEG600, and
2% PEG5KMME. However, only the pyrophosphate of FsPP
was found in the complex structure. The X-ray diffraction
datasets for the structures of the C234A UPPS mutant and
the complex with FsPP were collected to 1.88 A and 2.5 A
resolution, respectively. Data for the C234A UPPS crystals
were collected at beam line BL17B2 of the National Syn-
chrotron Radiation Research Center (NSRRC, Hsinchu, Tai-
wan). Data for the C234A UPPS complexed with FsPP were
collected in house using a Rigaku MicroMax002 X-ray gen-
erator equipped with an R-Axis IV++ image plate detector.
The diffraction data were processed using the programs of
HKL and HKL2000 [16]. Statistics for the dataset are listed
in Table 1. Prior to use in structural refinements, 5% ran-
domly selected reflections were set aside for calculating Rfree
as a monitor [17].

2.3. Structure determination and refinement

The crystal structure of C234A UPPS was determined by
molecular replacement method using the Crystallography &

| Table 1: Data collection and refinement statistics for the or-
| thorhombic H. pylori UPPS crystals of the apoenzyme and the com-
| plex with thiopyrophosphate. C234A mutation was included to pre-
| vent intramolecular disulfide bond formation. |
| --- |
| **H. pylori UPPS** | **H. pylori UPPS +** |
| **PPi** | **Data collection** |
| **Space group** | P2₁2₁2₁ |
| **Resolution (Å)** | 25 to 1.88 (1.95 to 1.88) |
| **Unit cell dimensions** | 50 to 2.5 (2.59 to 2.5) |
| a, b, c (Å) | 49.63, 58.91, 153.43 |
| **No. of reflections** | 201711 (18692) |
| **Unique** | 35917 (3338) |
| **Completeness (%)** | 95.4 (90.3) |
| **Rmerge (%)** | 5.5 (43.3) |
| **I/σ(I)** | 30.7 (4.1) |
| **Refinement** | 137910 (12888) |
| **No. of reflections** | 34629 (3038) |
| **Rwork (%)** | 19.34 (22.91) |
| **Rfree (%)** | 24.00 (30.02) |
| **Mean B-values (Å²)** | 0.0193 |
| **Bond angles (°)** | 1.817 |
| **No. of all non-H atoms** | 3463 |
| **No. of water molecules** | 581 |
| **Mean B-values (Å²)** | 39.54 |
| **Ramachandran plot (%)** | 92.1 |
| **Most favored** | 92.3 |
| **Additionally allowed** | 7.9 |

NMR System (CNS) program [18]. The orthorhombic crys-
tal contained one UPPS dimer in an asymmetric unit. The
models of PDB 1V7U (E. coli UPPS structure bound with
FPP, chain A) [13] were used as search model to yield a good
resolution for the H. pylori UPPS. The space group was
determined as P2₁2₁2₁. With all solvent and cofactor molecules
removed, the model yielded an initial R-factor of 0.50 using
all positive reflections at 1.88 Å resolution upon rigid-body
refinement.

The 2Fo-Fc difference Fourier map showed clear electron
densities for most amino acid residues. The residues of
 catastrophic loop of 58–67 in chain A, 56–71 and 150–158
in chain B were disordered. Subsequent refinement with in-
corporation of 581 water molecules according to 1.0 σ map
level yielded R and Rfree values of 0.193 and 0.240, respec-
tively, at 1.88 Å resolution. By employing similar procedures,
the C234A H. pylori UPPS and the FsPP-complexed struc-
tures were refined with the addition of cofactor and solvent
molecules. All manual modifications of the models were performed on an SGI Fuel computer using the program O [19]. Computational refinements, which included maximal likelihood and simulated-annealing protocols, were carried out using CNS. The programs MolScript [20], and Raster3D [21] were used in producing figures.

2.4. Computer screening to identify the inhibitors

The X-ray structure of H. pylori UPPS reported here and the complex structure of E. coli UPPS (PDB code 1V7U) were chosen as the templates in the virtual screening. The program GOLD V2.1 was used to screen Maybridge database, a commercially available compound database obtained from Maybridge Chemical Company (Tintagel, Cornwall, England). The binding pocket for the docking study was defined as a 15 Å radius sphere centered on the active site Asp13 of H. pylori UPPS or Asp26 of E. coli UPPS. The scoring function, GoldScore, implemented in GOLD was used to rank the docking positions of the compounds. 26 compounds with the highest score ranked by GoldScore were selected for inhibition assays.

2.5. IC\textsubscript{50} determination

The IC\textsubscript{50} values of the two hits were measured in a buffer of 100 mM Hepes (pH 7.5), 50 mM KCl, 0.5 mM MgCl\textsubscript{2}, and 0.1% Triton X-100, containing 0.05 μM of E. coli or H. pylori UPPS. The concentrations of inhibitors used were ranged from 0 to 500 μM. To obtain the IC\textsubscript{50}, the dose-response curves were fitted with the equation, A(I) = A(0) × (1 − [I/(1+ IC\textsubscript{50}))], where A(I) is the enzyme activity with inhibitor concentration I, A(0) is enzyme activity without inhibitor, and I is the inhibitor concentration.

3. RESULTS

3.1. 3D structures of H. pylori UPPS

To develop structure-based inhibitors, the crystal structures of H. pylori UPPS were solved in this study. One is the structure of H. pylori UPPS containing C234A mutation to prevent intra-molecular disulfide bond formed during the long period of crystallization process (Figure 1(a)), and the other is the structure of C234A complexed with FsPP, but only the pyrophosphate portion is visible (Figure 1(b)). The C234A mutant has unchanged kinetic property compared with the wild type (k\textsubscript{cat}, FPP K\textsubscript{m} and IPP K\textsubscript{m} of C234A were 0.20 ± 0.08 s\textsuperscript{-1}, 0.15 ± 0.04 μM and 9.6 ± 0.2 μM, almost equal to 0.22 ± 0.05 s\textsuperscript{-1}, 0.11 ± 0.02 μM and 9.2 ± 0.1 μM for the wild type, resp.). The overall structure of H. pylori UPPS was similar to that of E. coli UPPS [22]. The protein is a dimer and each subunit contains a catalytic domain and a pairing domain. Two subunits are tightly associated through the central β-sheet and a pair of long α-helices (a5 and a6). However, H. pylori UPPS has a 1.5-turn shorter a5 helix in the dimer interface. This may weaken the dimer formation for H. pylori UPPS. The catalytic domain is composed of six β-strands and four β-helices and the central tunnel-shaped active site is surrounded by 2 α-helices (α2 and α3) and 4 β-strands (βA-βB-βD-βC) (Figure 1(a)).

At the bottom of the tunnel, a large amino acid F124 occupies a similar position to that of L137 at the bottom of E. coli UPPS tunnel, which is a key residue to shield the final product and determine its chain length [22]. At the top of this tunnel, several amino acids including D13, R17, R26, H30, F57, S58, R180, and E184 are located in the substrate binding site (Figure 1(b)). The position of the pyrophosphate (shown in black sticks in Figure 1(b)) of FsPP in the complex is almost identical to that of the FPP pyrophosphate in the E. coli UPPS active site [13].

The positions of the α3 helix in the two subunits of H. pylori UPPS are slightly different (Figure 1(a)), resembling the open and closed forms of E. coli UPPS [22]. H. pylori UPPS A-chain strongly resembles the Triton-bound open form of E. coli UPPS [23], with root mean square deviation (r.m.s.d.) of 0.78 Å for 200 match pairs of α-carbon atoms. Compare to the closed-form structure of E. coli UPPS with FsPP and IPP bound [9], the H. pylori UPPS B-chain is with the r.m.s.d. of 1.08 Å for 191 match pairs of α-carbon atoms. This suggests a conformational change in the H. pylori UPPS reaction.

3.2. Virtual screening of the H. pylori UPPS inhibitors

Based on the structures, computer virtual screening was carried out to search for selective inhibitors of E. coli and H. pylori UPPS. The screening procedure is summarized in Figure 2. The crystal structure of E. coli UPPS bound with FPP (1V7U) was used as a template first for the virtual screening since the electron density of a small loop responsible for conformational change near the active site is not visible in H. pylori UPPS, which might confound the virtual screening result. A compound database containing 58,635 compounds available from Maybridge Chemical Company were screened using the program GOLD V2.1. Each compound in the database was docked into the active site of E. coli UPPS, defined as 15 Å radius sphere around Asp26, an essential residue responsible to coordinate with the catalytic Mg\textsuperscript{2+}. The docked molecules were then ranked by the GoldScore fitness function, according to the sum of H-bond energy, van der Waals energy, internal ligand van der Waals and internal torsional strain energy. The top 26 compounds ranked by GoldScore were then purchased and experimentally evaluated for their ability to inhibit H. pylori and E. coli UPPS.

3.3. Inhibition against E. coli and H. pylori UPPS

Of these 26 compounds, 2 compounds numbered BTB06061 and HTS04781, were found inhibitory to H. pylori UPPS almost equally with IC\textsubscript{50} values of 350 μM and 362 μM, respectively (Figure 3). The IC\textsubscript{50} values of these two compounds against the C234A and wild-type enzyme were almost equal. As revealed by the predicted models shown in Figures 3(a) and 3(b), two inhibitors are likely bound to H. pylori UPPS with a similar orientation to that of the substrate FPP. The sulfur atom in the thiazole ring of BTB06061 may form H-bonds with Asn15 and His30 while the SO\textsubscript{2} group is hydrogen bound with Met12. In addition,
Figure 1: Crystal structures of H. pylori UPPS. (a) Two subunits of the apoenzyme are superimposed. The most obvious disposition occurs in α3 helix which adopts an open form and a closed form in subunit A and B, respectively. At the top of the tunnel-shaped crevice surrounded by 2α-helices and 4β-strands is the substrate-binding site. Phe124 located at the bottom of the H. pylori UPPS tunnel adopts a similar position to that of Leu137 in E. coli UPPS, essential for determining product chain length. (b) Superimposition of active site structures of H. pylori UPPS with FsPP and E. coli UPPS with FsPP, Mg^{2+}, and IPP [9]. The active site residues in H. pylori UPPS are shown in pink and those in E. coli UPPS in white for carbon-carbon bonds in ball-and-stick model. The thiopyrophosphate (visible in crystal structure) is shown in black, the nitrogen atoms and Mg^{2+} ion are shown in blue, and oxygen atoms are shown in red. Asp13 in H. pylori UPPS occupies a similar position to that of Asp26 in E. coli UPPS to coordinate with an Mg^{2+} for binding with the pyrophosphate leaving group of FPP.

Figure 2: The flow chart for computer screening of H. pylori UPPS inhibitors. The active zone for screening was focused on Asp13, an important amino acid residue for coordinating with catalytic Mg^{2+}. In parentheses are the numbers of compounds. BTB06061 and HTS04781 are the final hits.

In this paper we describe the crystal structures of UPPS from H. pylori, a wide-spreading and life-threatening pathogen, and the first structure-derived inhibitors from computer virtual screening. Although a high-throughput screening has been performed for UPPS by a pharmaceutical company [24], none of the inhibitors have been reported. So far, a series of IPP analogues with a dicarboxylate moiety in place of the diphosphate were synthesized and the E-pentenylbutanedioic acid showed inhibition of UPPS with an IC_{50} of 135 μM[25]. Based on the known structure of UPPS (9), two carboxylate groups may coordinate with the catalytic Mg^{2+} ion which was bound with the pyrophosphate group of the substrates. Recently, we reported some bisphosphonates, which inhibited cis-type FPPs, which could also inhibit cis-type UPPS with sub-μM IC_{50} when containing suitable hydrophobic side-chains [26]. The crystal
structures show that four molecules of inhibitors are bound in the active site and one of them occupies the FPP site with a phosphate group chelating with the Mg²⁺. Here, we report the first two novel inhibitors identified from a randomized compound library through virtual screening. These two inhibitors likely occupy the FPP site of H. pylori UPPS based on computer modeling. Two inhibitors displayed similar inhibition against H. pylori UPPS, but very different inhibition on E. coli UPPS. The one with bulky skeleton did not inhibit E. coli UPPS, likely owing to the partially blocked opening at the top of tunnel by the flexible loop in the E. coli UPPS active site. Our results shed light on the possibility of developing antibiotics specially targeting pathogenic H. pylori without killing the intestinal E. coli.

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