Experimentally Validated Novel Inhibitors of *Helicobacter pylori* Phosphopantetheine Adenylyltransferase Discovered by Virtual High-Throughput Screening

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Abstract

*Helicobacter pylori* is a major etiologic agent associated with the development and maintenance of human gastritis. The goal of this study was to develop novel antibiotics against *H. pylori*, and we thus targeted *H. pylori* phosphopantetheine adenyllyltransferase (*Hp*PPAT). PPAT catalyzes the penultimate step in coenzyme A biosynthesis. Its inactivation effectively prevents bacterial viability, making it an attractive target for antibacterial drug discovery. We employed virtual high-throughput screening and the *Hp*PPAT crystal structure to identify compounds in the PubChem database that might act as inhibitors of *Hp*PPAT. d-amethopterin is a potential inhibitor for blocking *Hp*PPAT activity and suppressing *H. pylori* viability. Following treatment with d-amethopterin, *H. pylori* exhibited morphological characteristics associated with cell death. d-amethopterin is a mixed inhibitor of *Hp*PPAT activity; it simultaneously occupies the *Hp*PPAT 4'-phosphopantetheine- and ATP-binding sites. Its binding affinity is in the micromolar range, implying that it is sufficiently potent to serve as a lead compound in subsequent drug development. Characterization of the d-amethopterin and *Hp*PPAT interaction network in a docked model will allow us to initiate rational drug optimization to improve the inhibitory efficacy of d-amethopterin. We anticipate that novel, potent, and selective *Hp*PPAT inhibitors will emerge for the treatment of *H. pylori* infection.

Introduction

More than 50% of the human population is infected with *Helicobacter pylori* [1,2], a pathogenic bacterium that causes chronic gastritis, peptic ulcers, and gastric carcinoma [3–5]. Various antibiotics, including amoxicillin, clarithromycin, and lansoprazole, have been used to treat *H. pylori* infection [6,7]; however, bacterial strains have often become resistant to these drugs, preventing effective treatment [8]. Thus, novel antibiotics against *H. pylori* are vital.

Coenzyme A (CoA) is an essential metabolic cofactor found in all living organisms, including bacteria, and is involved in many biosynthetic and degradative metabolic pathways, including the citric acid cycle and fatty-acid synthesis [9]. However, the bacterial enzyme phosphopantetheine adenylyltransferase (PPAT), which catalyzes the conversion of 4'-phosphopantetheine (Ppant) to 3'-dephospho-CoA in the penultimate step of CoA biosynthesis [10–13], shares an approximately 6% sequence identity with human PPAT [14,15]. Consequently, bacterial PPAT is an appropriate target for rational drug design [16].

Crystal structures of bacterial PPATs in both their free forms and complexed with various ligands are available [11,12,17–20]. PPAT has a homohexameric quaternary structure; each monomer contains 5 parallel β-strands and 6 α-helices that fold into a canonical dinucleotide-binding domain. Many of the residues involved in substrate binding are conserved, including Pro8–Thr10, His18, Lys42, Leu73, Leu74, Arg88, Arg91, Asp95, Tyr98, Glu99, Asn106, Ser129, and Ser130 [21]. An inhibitor of *Escherichia coli* PPAT (EcPPAT) has been developed [22] that does not inhibit porcine PPAT, suggesting that the active-site residues of bacterial and mammalian PPATs differ substantially. Moreover, genetic footprinting studies have revealed that the inhibition of EcPPAT...
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prevents E. coli growth [16,23,24]; thus, bacterial PPAT has potential as an antibacterial target for drug discovery.

We recently reported the crystal structure of PPAT obtained from H. pylori (HpPPAT, PDB ID: 3OTW) [21]. In our current study, we use virtual high-throughput screening (vHTS) with the HpPPAT crystal structure, and compound structures retrieved from the PubChem compound database (http://pubchem.ncbi.nlm.nih.gov/ Accessed 2012) to identify novel inhibitors of HpPPAT that could serve as lead compounds for the design of antibiotics that target H. pylori infection. The vHTS computational screening technique automatically and individually docks compounds from a specified database into the active site of a target protein, and estimates the binding affinity of the target protein toward the docked compound by using scoring functions [25–27]. Two docking programs, CDOCKER [28] and LigandFit [29], were used to screen a large number of compounds that are available in the PubChem compound database. The top ranked consensus compounds were then subjected to steady-state kinetic inhibition assays of the HpPPAT-catalyzed forward reaction. These compounds were also incubated with H. pylori to characterize their antimicrobial activities. We used a steady-state kinetic inhibition assay and isothermal titration calorimetry (ITC) to characterize the D-amethopterin inhibition mechanism, the most effective overall inhibitor. Transmission electron microscopy (TEM) was performed to characterize the morphology of H. pylori after treatment with D-amethopterin. Finally, by examining the docked model of D-amethopterin and HpPPAT, we were able to propose a D-amethopterin binding mode and identify important interactions between D-amethopterin and HpPPAT, allowing us to rationally optimize the structure of D-amethopterin for the development of an antibiotic against H. pylori.

Materials and Methods

Materials

ATP, imidazole, Luria-Bertani broth, MgCl₂, Tris-HCl, and NaCl were supplied by USB Corporation (Cleveland, OH). The compounds (omeprazole, T556874, zanamivir, sulfamate, amethopterin) used for inhibitory assays were purchased from Sigma-Aldrich (St. Louis, MO). Isopropyl β-d-thiogalactopyranoside and kanamycin were purchased from Promochem (Taipei, Taiwan). Tris(2-carboxyethyl) phosphine hydrochloride was obtained from Acros Organics (Fair Lawn, NJ). P Pant was supplied by Enamine Ltd. (Kiev, Ukraine). All chemicals used were of analytical grade.

Virtual high-throughput screening (vHTS)

The crystal structure of the HpPPAT-CoA complex was retrieved from the Protein Data Bank (PDB code 3OTW) [21]. The complex has a hexameric structure; each HpPPAT monomer binds one CoA molecule with the same orientation. Thus, HpPPAT monomers could be used for vHTS. Prior to the docking study, the CoA molecule, water molecules, and sulphate were removed from the model. Explicit hydrogen atoms were added using Discovery Studio (DS) 2.0 software (Accelrys Inc., San Diego, CA). Protein atoms were typed according to the CHARMM force field [30]. Next, apo-HpPPAT was energy minimized using the steepest descent algorithm available in DS 2.0 [31]. The compounds used were obtained. Using “enzyme inhibitor” as the query string, we retrieved 407 compounds for docking from the PubChem compound library (http://pubchem.ncbi.nlm.nih.gov/ Accessed 2012). All compounds were prepared using the Prepare Ligands protocol in DS 2.0.

The CDOCKER [28] and LigandFit [29] routines in DS 2.0 were used to find potential inhibitors of HpPPAT. The docking parameters for the 2 programs were set to their default values. CDOCKER is a grid-based molecular dynamics docking algorithm that employs the CHARMM force field. The binding pocket was defined as a sphere with a radius of 15 Å, centered on the CoA molecule in the HpPPAT-CoA complex, and encompassing the entire CoA structure. Ten random conformations for each compound were generated from the initial structure by using 1000 molecular dynamics steps at a temperature of 1000 K. Simulated annealing was then used to optimize the conformation of each compound at the HpPPAT binding site, with 2000 heating steps and a target temperature of 700 K. The simulation used 5000 cooling steps to a target cooling temperature of 300 K. Refinement of the docked poses was performed using the full potential.

LigandFit is a shape-based method used to dock ligands into the active site of a protein. The HpPPAT binding pocket was identified using the option “Find sites from Receptor Cavities” in DS 2.0. The active-site points were then manually edited to ensure that the CoA molecular structure would include these points. Monte Carlo simulations (15,000 trials) using a Class II Force Field [32] were employed to generate ligand poses. The grid extension was 3 Å, and the non-bonded cutoff distance was set to 10 Å with a distance-dependent dielectric constant. To avoid identical conformations, the root-mean-square-deviation cutoff was 1.5 Å, with a score threshold of 20 kcal/mol. A 1000-step rigid-body energy minimization was performed, and the top 10 docked conformations for each ligand were saved.

The scoring functions LigScore2 [33], PLP2 [34], Jain [35] and PMF [36] were used to evaluate and rank all docked ligand conformations. The steepest available descent algorithm was applied with the CHARMM force field to refine the best docked poses, using DS 2.0 and PyMOL (DeLano Scientific; http://www.pymol.org Accessed 2002.) [37] to visualize and inspect the docked poses. The HpPPAT-compound interactions were identified using LIGPLOT v4.0 [38].

Protein Expression and Purification

Gene encoding for wild-type (WT) HpPPAT was cloned, expressed, and purified as previously described [21]. The E. coli BL21(DE3) cells (Yeastern Biotech, Taipei, Taiwan) bearing a PET-28a (+) vector (Novagen, Whitehouse Station, NJ) that contained the WT HpPPAT gene tagged at the N terminus with hexahistidine (Hist₅) were inoculated into a 500 mL Luria-Bertani medium containing 30 mg/mL kanamycin, at 37 °C. When OD₆₀₀ of the culture reached 0.7, isopropyl β-d-1-thiogalactopyranoside (final concentration, 1 mM) was added to the culture to induce protein expression. After 20 h of
incubation at 20 °C, the cells were harvested by centrifugation using an Eppendorf 5810-R Centrifuge (Eppendorf, Hauppauge, NY) at 8000 × g for 20 min and 4 °C. The cell pellet was suspended in a solution of ice-cold Tris-HCl (20 mM) at pH 7.9, imidazole (80 mM), and NaCl (500 mM), and lysed on ice with a Misonix Sonicator 3000 (Misonix Inc., Farmingdale, NY).

The lysate was centrifuged at 7245 × g for 20 min at 4 °C, and the supernatant was applied to a 10 mL immobilized-Co<sup>2+</sup>-affinity column (BD Biosciences, Franklin Lakes, NJ), which had been pre-equilibrated with 20 mM Tris-HCl at pH 7.9, 100 mM imidazole, and 500 mM NaCl. After loading the lysate, the column was washed with the pre-equilibration buffer, and then the His<sub>6</sub>-tagged protein was eluted in a solution of 20 mM Tris-HCl at pH 7.9, containing imidazole (300 mM), and NaCl (500 mM). A Centricon Plus-20 centrifugal filter (Millipore, Billerica, MA) was used to remove the imidazole and to concentrate the protein. Purified HpPPAT was characterized by SDS-PAGE (12% w/v acrylamide gel), and its concentration was determined using Bio-Rad Protein Assay kit reagents (Bio-Rad, Hercules, CA) and BSA as the standard.

Quantitative HpPPAT inhibition assay

For the inhibitory assay, we used EnzChek Pyrophosphate Assay kit reagents (E-6645, Invitrogen, Grand Island, NY) [39]. Various concentrations of tested compounds in H<sub>2</sub>O were added to reaction mixtures containing MgCl<sub>2</sub> (6 mM), purine nucleoside phosphorylase (1 U/mL), inorganic pyrophosphatase (0.03 U/mL), 2-amin-6-mercaptop-7-methylpurine ribonucleoside (0.2 mM), tris(2-carboxyethyl)phosphine hydrochloride (1 mM), and saturating concentrations of Ppant (60 µM) and ATP (3000 µM) with respect to the enzyme concentration. Afterward, HpPPAT (final concentration, 25 nM) in a solution of 20 mM Tris-HCl at pH 7.9 and NaCl (125 mM) was added to each solution. The absorbance of each reaction was measured in a 1 cm path-length cuvette, at 360 nm for 120 s at 25 °C, using a Hitachi UV-visible U-3300 spectrophotometer. Residual activity was defined as the ratio of the turnover rate of HpPPAT in the presence or absence of a test compound. Three independent assays were performed for each compound.

Steady-state kinetic inhibition assay using δ-Amethopterin

To determine the inhibition mode of δ-amethopterin, a steady-state kinetic inhibition assay was performed using the procedure described in the preceding section, with the difference that the concentrations of Ppant and ATP were varied. The concentration of Ppant or ATP was fixed at a saturating concentration with respect to the enzyme concentration (25 µM and 1.6 mM, respectively), whereas the concentration of the other substrate was varied. The concentration of δ-amethopterin in each reaction was 0, 200 µM, or 250 µM. Each set of experimental data was fitted by nonlinear regression by using Prism 5 software (GraphPad Software Inc., La Jolla, CA) to determine the kinetic parameters. Assays were repeated at least 3 times, and the data points at each time point are reported as mean averages.

Isothermal titration calorimetry (ITC)

To measure the binding affinity of HpPPAT for δ-amethopterin, we used a Nano ITC Low Volume system (TA Instruments–Waters LLC, New Castle, Delaware). The HpPPAT (80 µM, 190 µl) in 20 mM Tris-HCl at pH 7.9 and 125 mM NaCl was titrated with δ-amethopterin (608 µM, 50 µl), and the heat change for each titration was recorded at 25 °C. The mixtures were stirred at 300 rpm. Data were collected and analyzed using NanoAnalyze software (TA Instruments–Waters LLC). The titration curve was fitted to the independent-site binding model.

Antimicrobial assay

The antimicrobial activities of the tested compounds were determined as previously described [40]. In brief, H. pylori strain 26695 (1 × 10<sup>7</sup> colony-forming units; ATCC#700392, Biosource Collection and Research Center, Hsinchu, Taiwan) was cultured in 3 mL Brucella Broth (Franklin Lakes, NJ) supplemented with 5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub> (microaerophilic conditions) at 37 °C. After 24 h of incubation, each compound was added at 200 µM or 2000 µM to a culture and then incubated for 5 d. After incubation, OD<sub>600</sub> was measured for each culture as an estimation of the antimicrobial activity of the compound. Three independent experiments were performed for each compound. In addition, TEM (JEOL-1400 microscope; Jeol Ltd., Tokyo, Japan) was employed to characterize the H. pylori morphology at the completion of the δ-amethopterin treatment.

Dynamic light scattering

To examine whether the protein or compounds will precipitate, the dynamic light scattering (DLS) analysis was performed with ZetasizerNano S (Malvern Instruments; Spectris, Egham, UK). PPAT protein (4 µg/µl) and D-amethopterin (0.2 mM or 2 mM) in buffer (20 mM Tris, 125 mM NaCl, pH 7.9) were loaded in 1mm path length cuvette (Ratiolab®) and monitored at room temperature (25°C). All sample solutions were filtered through a membrane with 0.22 µm minisart filter.

Results

vHTS

To develop novel antibiotics against H. pylori, we screened 407 PubChem listed compounds against our recently reported crystal structure of HpPPAT (PDB ID: 3OTW) [21] by using CDOCKER and LigandFit. Each retrieved compound was docked into the HpPPAT binding site by using its top 10 energy-minimized conformations; the ranked lists of all docked poses were generated according to the appropriate scoring function; that is, DockScore for LigandFit or the CDOCKER interaction energy. We employed a set of scoring functions such as LigScore2, PLP2, Jain, and PMF to rescore and re-rank the docked poses. The 250 top-ranked docked poses according to each scoring function were retained and compared. If the same compound was found in at least 3 lists, it was retained for further analysis. We retained 89 LigandFit
were also included to do docking and score functions analysis. Methylthio-pyrazoloquinolone) from the previous study (41) structures, with inhibitors (cpd 11 listed in Table S1 in File S1. The scores of these compounds and that of the dinucleotide-binding site in HpPPAT. The 3 compounds, 72440, 24906324, and 25200568, share similar chemical structures, with 72440 having the greatest rank and highest score among the 12 compounds. In addition, the natural substrates of HpPPAT (Ppant and ATP) and two EcPPAT inhibitors (cpd11 (7-iodo-pyrazoloquionolone) and cpd12 (7-methylthio-pyrazoloquinolone) from the previous study [41]) were also included to do docking and score functions analysis. The scores of these compounds and that of 72440 were all listed in Table S1 in File S1. The 72440 compound displayed similar or higher scores than natural substrates and EcPPAT inhibitors, suggesting that 72440 is a potential competitive inhibitor against HpPPAT. Thus, we tested its inhibitory activity against HpPPAT. We also tested 4594, 20112027, 676113, and 11946759, which are commercially available and are readily soluble in water.

**HpPPAT inhibitory assay**

With the exception of compound 20112027, all aforementioned compounds provided approximately 40% or greater inhibition of HpPPAT activity at 2000 µM (Figure 1). At this concentration, 72440 completely inhibited HpPPAT activity, and at 200 µM it provided the greatest degree of inhibition among all of the candidate compounds (Figure 1), indicating that it is a potent inhibitor of HpPPAT activity.

**Antimicrobial assay.** 4594, 72440, 676113, 11946759, and 20112027 were incubated with H. pylori to assess their antimicrobial activities [40]. The density of H. pylori cells (OD600) decreased significantly with an increasing concentration of 72440, 676113, or 20112027 (Figure 2), suggesting that these compounds suppress H. pylori viability. Even at the 2 greatest concentrations tested (8 µM and 10 µM), compounds 4594 and 11946759 did not significantly suppress H. pylori viability (Figure 2). Although 676113 exhibited the greatest antimicrobial activity, it was a weaker inhibitor of HpPPAT activity than was 72440 (Figure 2), suggesting that 676113 may interfere with the functions of proteins other than

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### Table 1. Ranks for the Docked Poses of the 12 Best Compounds.

| CID | Name          | Mw  | CDOCKER    | DockScore | PLP2 | PMF | CIE |
|-----|---------------|-----|------------|-----------|------|-----|-----|
| 4594| Omeprazole    | 345.4| 122        | 249       | 240  | -   | -   |
| 72440| ω-Amethopterin | 454.4| 37         | 167       | 3    | 122 | 3   |
| 100450| Lactoylglutathione lyase | 388.4| 68         | 210       | 72   | 221 | 14  |
| 365754| AC1L7TH4      | 418.6| 124        | -         | 111  | -   | 32  |
| 676113| AC1L7DVWJ     | 226.2| 124        | -         | 229  | -   | 247 |
| 5362033| Enalaprilat   | 348.4| 95         | 246       | 97   | -   | 23  |
| 11946759| T5568746     | 368.5| 173        | -         | 83   | 237 | -   |
| 13216867| SureCN9327107| 416.5| 53         | 172       | 6    | 141 | 4   |
| 20112027| Zanamivir    | 332.3| 187        | 249       | 236  | -   | -   |
| 24906324| 2qk8         | 452.4| 56         | 187       | 14   | 200 | 6   |
| 25200568| CPD-6041     | 452.4| 187        | 248       | 238  | -   | -   |
| 25201794| A835543      | 334.3| 143        | -         | 149  | -   | 35  |

The 250 top-ranked docked poses according to each scoring function are shown. Dash line means the ranks of this compound is out of 250.

a CID, PubChem compound ID; CIE, CDOCKER interaction energy.
HpPPAT, which are required for *H. pylori* viability. Conversely, 72440 showed effective inhibition of both HpPPAT activity and *H. pylori* viability.

**Steady-state inhibition assay**

Compound 72440, also known as d-amethopterin, inhibits *Pneumocystis carinii* dihydrofolate reductase activity [42]. To characterize how 72440 inhibits HpPPAT activity, we performed a steady-state kinetic inhibition assay [41,43]. Various concentrations of d-amethopterin were incubated with HpPPAT while holding the concentration of one of the substrates—ATP or Ppant—constant, and varying the other (Figure 3). Here, the dynamic light scattering test was performed to confirm d-amethopterin has no aggregation and precipitation. The dynamic light scattering data were shown in Figure S1 in File S1. The HpPPAT turnover rate decreased with increasing d-amethopterin concentration when the Ppant concentration was varied and that of ATP (1.6 mM) was held constant (Figure 3A). Nonlinear regression curve fitting indicated that d-amethopterin is a mixed inhibitor of Ppant (K_i = 362.9 µM), suggesting that d-amethopterin binds to both free HpPPAT and to the HpPPAT-substrate complex [44]. Similarly, at a saturating concentration of Ppant (25 µM), d-amethopterin reduces the HpPPAT turnover rate over a wide range of ATP concentrations (Figure 3B). d-Amethopterin also exhibits mixed inhibition against ATP (K_i = 267.4 µM).

**ITC**

The binding affinity of HpPPAT for d-amethopterin was determined by ITC. The binding of d-amethopterin is an exothermic process, and the binding heat gradually decreases with increasing d-amethopterin concentration (Figure 4). Fitting of the titration curve through several repeating, we obtained a K_d value of 31.16 ± 0.34 µM. The n value is 1.071 ± 0.211. The experimentally determined standard-state enthalpy (ΔH°) and the calculated standard-state entropy (ΔS°) for d-amethopterin binding were -12.10 ± 3.74 kJ/mol and 45.69 ± 1.23J/mol/K, respectively.

**Binding model**

The steady-state kinetic inhibition assay revealed that d-amethopterin is a mixed inhibitor against Ppant and ATP because d-amethopterin can simultaneously occupy the ATP- and Ppant-binding sites. Comparing the docked poses obtained using CDocker and LigandFit revealed that only the CDocker model predicts that d-amethopterin binds with both
the ATP- and Ppant-binding sites (Figure 5A). The benzoyl and terminal pteridine rings in D-amethopterin occupy the ATP-binding site. In the LIGPLOT representation (Figure 5B), the side-chain oxygen atom in Asp12, hydrogen bonds with the amino group of the terminal pteridine ring on D-amethopterin. In addition, the C-terminal glutamate in D-amethopterin occupies a

Figure 2. Antimicrobial activity. The H. pylori cells were cultured in the presence of various concentrations of the candidate compounds. Relative activity was defined as the ratio of the OD600 values before and after treatment with the compound.

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Figure 3. HpPPAT inhibition by D-amethopterin. A steady-state kinetic inhibition assay was performed to determine the inhibition mode of D-amethopterin against HpPPAT. The turnover rates of HpPPAT were calculated for different concentrations of D-amethopterin. (A) Initial rates of the HpPPAT-catalyzed reaction were obtained at different Ppant concentrations, while ATP was held at a saturating concentration. (B) Initial rates of the HpPPAT-catalyzed reaction were measured at different ATP concentrations, while Ppant was held at a saturating concentration.

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position that corresponds to the CoA pantetheine arm (Figure 5C), and hydrogen bonds with the side chains of 3 conserved HpPPAT residues (Thr10, Ser39, and Lys42) (Figure 5B). We also found that 6 HpPPAT residues (Pro8, Gly9, His18, Arg88, Tyr98, and Arg133) have nonpolar interactions with D-amethopterin (Figure 5B). With the exception of Arg133, these 6 residues are highly conserved in bacterial PPATs for substrate binding.

Transmission electron microscopy (TEM)

We used TEM to characterize the morphology of H. pylori after D-amethopterin treatment (Figure 6). The TEM image of untreated H. pylori showed that the cells had a normal helical bacillary appearance. However, H. pylori treated with D-amethopterin exhibited a coccoid morphology. It has been postulated that coccoid H. pylori cannot be cultured and that the coccoid shape is a morphologic manifestation of H. pylori death [45].

Discussion

Using vHTS and the DS consensus scores facilitated the effective identification of potential inhibitors. Four of the 5 candidate compounds exhibited inhibitory activity against HpPPAT. D-amethopterin in particular acted as an inhibitor of both HpPPAT and H. pylori viability. Moreover, treatment with this mixed inhibitor converts the H. pylori morphology into a coccoid form, which is associated with H. pylori death. The CDOCKER docked pose for D-amethopterin received the highest score, and therefore, ranked first among the candidate compounds (Table 1), and this is consistent with our experimental results.

The steady-state kinetic inhibition assay revealed that D-amethopterin is a mixed inhibitor against Ppant and ATP, with micromolar $K_i$ values (Figure 3). ITC data suggested that $K_d$ also falls in the micromolar range, indicating that D-amethopterin is an appropriate lead compound for drug development.

Although D-amethopterin binds HpPPAT, its binding enthalpy is small, as revealed by ITC measurements. Thus, the number
of specific inhibitor–HpPPAT interactions must be increased to enhance the inhibitory potency of D-amethopterin. Given our detailed binding model (Figure 5), we proposed that 2 functional groups in D-amethopterin require modification. The D-amethopterin benzoyl ring lies in a position that corresponds to the phosphate group in CoA. In the CoA-HpPPAT complex, the Thr10, Lys42, Arg88, and Tyr98 side chains form hydrogen bonds with the CoA phosphate group [21]. However, in the CDOCKER model, only nonpolar interactions exist, and therefore, this ring structure should be modified to allow hydrogen bond formation. In addition, the C-terminal glutamate of D-amethopterin occupies only a part of the Ppant-binding site, suggesting that an extension of the D-amethopterin C-terminal region might be possible. In the crystal structure of the CoA-HpPPAT complex, the CoA pantetheine arm makes nonpolar contacts with the conserved residues Pro8, Gly9, Ala37, Leu73, Leu74, and Asn106 [21]. Therefore, substituting additional nonpolar groups onto the D-amethopterin C-terminal region might increase the binding affinity of D-amethopterin toward HpPPAT, thereby enhancing its inhibitory potency.

Finally, although a number of EcPPAT inhibitors have been developed [22,41], their chemical structures are relatively different from that of D-amethopterin (Figure 7). The EcPPAT inhibitors were designed using the Ppant structure as a template [22]; another class of inhibitors is the ATP-competitive pyrazolo-quinolone [41]. However, despite these inhibitors having significant inhibitory activities against EcPPAT, they do not possess antibacterial activity. By contrast, D-amethopterin inhibits HpPPAT-catalyzed reactions and suppresses H. pylori viability. D-amethopterin is an inhibitor of P. carinii dihydrofolate reductase (DHFR) [42], and has been used in cancer chemotherapy treatments, as an antibiotic, and as an antiprotozoal agent [46,47]. D-amethopterin acts as a folate antagonist for inhibiting DHFR activity. Structural [48] and mutagenesis [49] studies have revealed that carboxylic groups in D-amethopterin make significant contributions to hydrogen bonding and electrostatic interactions with DHFR. The C7 atom of the pteridine ring in D-amethopterin also participates in essential nonpolar contacts with DHFR. Therefore, these D-amethopterin functional groups must be modified so that DHFR

Figure 5. Model of HpPPAT complexed with D-amethopterin. (A) D-Amethopterin lies in the dinucleotide-binding site of HpPPAT. The HpPPAT electrostatic potential surface is shown, and D-amethopterin is shown as a stick model. Positively and negatively charged surface regions of HpPPAT are in blue and red, respectively. (B) Interactions between HpPPAT and D-amethopterin according to LIGPLOT. Green dashed lines indicate hydrogen bonds. The HpPPAT residues that participate in nonpolar interactions with D-amethopterin are represented as spoked arcs. (C) The structure of CoA in the crystal structure of the CoA-HpPPAT complex (PDB code 3OTW) is superimposed onto that of D-amethopterin in the CDOCKER model. D-Amethopterin is shown as a yellow stick model, and CoA is shown as a ball-and-stick model.

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and cancer-related effects are eliminated from the rational design of d-amethopterin. Accordingly, the results of our study suggest that d-amethopterin or rationally designed derivatives could be applied to other treatments, such as for *H. pylori* infection.

**Conclusions**

In this study, vHTS was used to identify novel inhibitors of *Hp*PPAT. We screened 407 compounds by using CDOCKER and LigandFit, and identified d-amethopterin, which inhibits *Hp*PPAT activity and *H. pylori* viability, as a promising inhibitor. The *H. pylori* treated with d-amethopterin exhibits morphologic characteristics associated with cell death. This compound has sufficient inhibitory potency that it could be used as a lead compound for drug development. Our studies also revealed that the d-amethopterin binding affinity (*K_d*) and inhibitory potency (*K_i*) toward *Hp*PPAT are in the micromolar range, and that d-amethopterin acts as a mixed inhibitor in the suppression of *Hp*PPAT activity. d-Amethopterin can bind stably to the *Hp*PPAT active site by hydrogen bonding and nonpolar interactions. The conserved *Hp*PPAT residues Pro8–Thr10, Asp12, His18, Ser39, Lys42, Arg88, Tyr98, and Arg133 participate in these interactions. Moreover, on the basis of our binding model, we propose a rational drug optimization involving modification of the benzoyl ring and of the C-terminal region in d-amethopterin to increase d-amethopterin–*Hp*PPAT interactions and improve the inhibitory efficacy of d-amethopterin. We anticipate that novel, potent, and selective *Hp*PPAT inhibitors will emerge for the treatment of *H. pylori* infection because of this study.

**Supporting Information**

File S1. This file contains Table S1 and Figure S1. Table S1, Scores for the Docked Poses of the d-Amethopterin, ATP, Ppant and two EcPPAT inhibitors (7-iodo-pyrazoloquinolone (cpd11) and 7-methylthio-pyrazoloquinolone (cpd12) of [41]). Figure S1, Dynamic light scattering analysis of different concentrations of d-amethopterin and *Hp*PPAT.
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