RESEARCH LETTER

Discovery of a compound which acts as a bacterial UMP kinase PyrH inhibitor

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Abstract

PyrH is a member of the UMP kinase family that catalyses the conversion of UMP to UDP, an essential step in the pyrimidine metabolic pathway in a variety of bacteria including those causing community-acquired respiratory tract infections (RTIs). In this study, we have developed a luminescence-based kinase assay of PyrH and evaluated the inhibitory activity of PYRH-1 (sodium 3-[4-tert-butyl-3-(9H-xanthen-9-ylacetamino)phenyl]-1-cyclohexylmethylpropoxycarbonyloxy acetate). PYRH-1 inhibits PyrH derived from both Streptococcus pneumoniae and Haemophilus influenzae with IC₅₀ (concentration of inhibitor giving a 50% decrease in enzyme activity) values of 48 and 75 μM, respectively, whose inhibitory activity against S. pneumoniae PyrH was far higher compared with that of UTP (IC₅₀ = 710 μM), an allosteric PyrH inhibitor. The molecular interaction analysis by surface plasmon resonance suggested that PYRH-1 directly interacts with S. pneumoniae PyrH at one-to-one molar ratio. Finally, PYRH-1 was shown to have antimicrobial activity against several different bacteria causing RTIs, such as S. pneumoniae, Staphylococcus aureus, H. influenzae (acrA knockout strain), suggesting that PYRH-1 is a prototype chemical compound that can be harnessed as an antimicrobial drug with a novel mode of action by targeting bacterial PyrH.

Introduction

Although numerous antibiotics for community-acquired bacterial respiratory tract infection (RTIs) have been discovered, thus far, most of them target the same or functionally similar molecules that are essential for bacterial growth. Because emerging antibiotic-resistant bacteria, such as multidrug-resistant Streptococcus pneumoniae and β-lactamase-negative and ampicillin-resistant Haemophilus influenzae (BLNAR), are posing threats, especially to immunocompromised patients, there is an unmet medical need to provide antibiotics with novel modes of action for reducing infections associated with such bacteria.

Recent progress in the genome projects (Fleischmann et al., 1995; Hoskins et al., 2001; Kuroda et al., 2001) has decoded the genome structure of a variety of organisms such as S. pneumoniae, Staphylococcus aureus and H. influenzae, thereby creating opportunities to design molecular targeting strategies for discovering agents that specifically attack pathogens. In fact, a number of studies in pharmaceutical companies and academia have developed screening platforms based on enzymatic assay and structure-based drug design. As a result, inhibitors that target enzymes such as N-acetylglucosamine-1-phosphate uridylytransferase (GlmU), NAD⁺-dependent DNA ligases (LigA), enoyl-[acyl-carrier-protein] reductase (FabI) and met tRNA synthase, as well as those that inhibit cell wall synthesis, were discovered and validated for their antimicrobial activity (Lynn, 2003; Payne et al., 2007; Meier et al., 2008; Pereira et al., 2009).

In this study, we evaluate the inhibitory activity of PYRH-1 (sodium 3-[4-tert-butyl-3-(9H-xanthen-9-ylacetamino)phenyl]-1-cyclohexylmethylpropoxycarbonyloxy acetate) as a potential antimicrobial agent by targeting the bacterial UMP kinase, PyrH, which serves as a kinase in de novo pyrimidine biosynthesis pathway required for the growth of certain bacteria such as S. pneumoniae (Thanassi et al., 2002; Song et al., 2005) and H. influenzae (Akerley et al., 2002). PYRH-1 was discovered in the course of a 1536-well high throughput screening of an
in-house large chemical library by the selection of chemicals directly inhibiting PyrH of *S. pneumoniae*. To test the inhibitory activity of PYRH-1 against PyrH, we used a luminescence-based ATP quantitative reagent. Moreover, molecular interaction analysis between PYRH-1 and *S. pneumoniae* PyrH by surface plasmon resonance (SPR) and susceptibility tests of PYRH-1 against some bacteria were performed. This is the first report that PYRH-1 inhibits PyrH.

**Materials and methods**

**Bacterial strains and growth conditions**

Bacterial strains used in this study are described in Table 1. *Escherichia coli* DH5α (competent high *E. coli* DH5α, Toyobo Co., Ltd.) was used for the cloning of PyrH. *Escherichia coli* Rosetta-Gami B (DE3) (Novagen) was used as the host for recombinant protein expression. These were grown at 35 °C in Luria–Bertani (LB) broth or LB agar (BD Biosciences) containing 100 µg mL⁻¹ of carbenicillin (Sigma). The culture medium used for each bacterium is as follows: *S. pneumoniae*, cation-adjusted Mueller–Hinton Broth (CAMHB; BD Biosciences) containing 5% of lysed horse blood (Nippon Bio-Test Laboratories Inc.) or Todd Hewitt Broth (Becton, Dickinson and Co.); *S. aureus* and *E. coli*, CAMHB; *H. influenzae*, Haemophilus test medium [CAMHB containing 5 mg mL⁻¹ of Yeast Extract (BD Biosciences), 15 µg mL⁻¹ of Hemin (Sigma) and 15 µg mL⁻¹ of β-NAD (Sigma)].

**DNA preparation**

*Streptococcus pneumoniae* TIGR4 and *H. influenzae* Rd KW20 genomic DNA were extracted with a DNeasy Tissue Kit (Qiagen). Plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (Qiagen). PCR products and plasmids digested by restriction enzyme were purified with a QIAquick PCR Purification Kit (Qiagen). PCR products digested by restriction enzyme were purified with a MinElute Reaction Cleanup Kit (Qiagen).

**Cloning of the pyrH gene**

The open reading frame of the pyrH gene was amplified from *S. pneumoniae* TIGR4 genomic DNA with primers SpPyrH-N-XhoI (5’-CCG CTC GAG TTA TTC CTT TTC TTC GAT ATT ATT TGA AAC TGT TG-3’) and SpPyrH-C-BamHI (5’-CGC GGA TTA TTC CTT TTC TTC TTC TTC GAT ATT TGA AAC TGT TG-3’). The open reading frame of pyrH was amplified from *H. influenzae* Rd KW20 genomic DNA with primers HiPyrH-N-XhoI (5’-CCG CTC GAG ATG AGC CAA CCA ATT TAT AAA CGT ATT TTA TTG A-3’) and HiPyrH-C-BamHI (5’-CGC GGA TGC CTA ACA AAT AGT GGT GCC TTC TTG AG-3’). PCR products were digested with XhoI and BamHI and then introduced into the pET15b (Novagen) expression vector (pET15b-SpPyrH and pET15b-HiPyrH, respectively). The sequences of the cloned DNA fragments were verified as the pyrH gene ORF of *S. pneumoniae* (GenBank accession nos. AE005672) and that of *H. influenzae* (GenBank accession nos. L42023) by DNA sequencing. Then, *E. coli* Rosetta-Gami B (DE3) was transformed with pET15b-SpPyrH or pET15b-HiPyrH according to the manufacturer’s instructions.

**Expression and purification of recombinant PyrH**

After the transformed Rosetta-Gami B (DE3) cells were cultivated at 37 °C for 3 h in 250 mL of LB broth containing 100 µg mL⁻¹ of carbenicillin, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added and then further cultivated at 30 °C for 3–4 h. After centrifugation, the pellets (= cells) were resuspended in 10 mL of B-PER reagent (Thermo Fisher Scientific Inc.), incubated at room temperature for 30 min and then sonicated with

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain no.</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><em>S. pneumoniae</em></td>
<td>TIGR4</td>
<td>This strain was obtained from American Type Culture Collection and used for the cloning of pyrH</td>
</tr>
<tr>
<td></td>
<td>1414021</td>
<td>This strain was clinically isolated in Japan in 2002 and used for the measurement of the MIC</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>Rd KW20</td>
<td>This strain was obtained from American Type Culture Collection and used for the cloning of pyrH</td>
</tr>
<tr>
<td></td>
<td>Rd KW20 ΔacrA</td>
<td>This strain was constructed by deleting the acrA gene and replacing it with a gene that confers resistance to chloramphenicol (cat)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>DH5α</td>
<td>This strain was obtained from TOYOBO Co., Ltd. and used for the cloning of pyrH</td>
</tr>
<tr>
<td></td>
<td>Rosetta-Gami B (DE3)</td>
<td>This strain was obtained from Novagen and used for the expression of PyrH</td>
</tr>
<tr>
<td></td>
<td>K-12 MG1655 ΔtolC</td>
<td>This strain was constructed by deleting the tolC gene and used for the measurement of the MIC</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>ATCC6538P</td>
<td>This strain was obtained from American Type Culture Collection and used for the measurement of the MIC</td>
</tr>
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</table>
DMSO/MeOH (70/30 [v/v]), 50 mM Tris of test inhibitor, which was diluted twofold serially in or HiPyrH (1.5 units per well) was mixed with 2% (v/v) white 96-well half area plate. SpPyrH (2.5 units per well) and the following luciferase reaction were carried out in a reagent, Kinase-Glo (Promega). The UMP kinase reaction measured using the luminescence-based ATP quantitativestrate, ATP, after the reaction. The amount of ATP was determined as the lowest concentration of the compound that completely inhibited the viable growth of the organism in the microdilution wells.

**UMP kinase assay**

PyrH synthesizes UDP according to the following scheme: UMP + ATP to UDP + ADP. To determine UMP kinase activity in vitro, we examined the amount of residual substrate, ATP, after the reaction. The amount of ATP was measured using the luminescence-based ATP quantitative reagent, Kinase-Glo (Promega). The UMP kinase reaction and the following luciferase reaction were carried out in a white 96-well half area plate. SpPyrH (2.5 units per well) or HiPyrH (1.5 units per well) was mixed with 2% (v/v) of test inhibitor, which was diluted twofold serially in DMSO/MeOH (70/30 [v/v]), 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 2 mM MgCl₂, 0.2 mM UMP and 5 mM ATP in a total volume of 50 μL. After 2.5-h incubation at 30 °C, 50 μL of the Kinase-Glo Assay reagent was added to initiate the luciferase reaction and was incubated for 10 min at room temperature. The levels of luminescence were measured using an ARVO luminometer (Perkin Elmer Co., Ltd.) and expressed in relative luminescence units (RLU). The inhibitory activity (%) against UMP kinase was calculated according to the following formula: % of inhibition = 100 − (sample − minus-enzyme control)/(plus-enzyme control − minus-enzyme control) × 100. For the plus-enzyme control, an UMP assay was performed in the absence of inhibitor. In the minus-enzyme control, sterile water instead of enzyme was used. The IC₅₀ were calculated using a linear regression standard curve to predict the concentration of compound needed for 50% inhibition. One unit of activity was defined as the amount of enzyme required to degrade 0.1 nmol of ATP in 120 min at 30 °C under the conditions described above.

**Susceptibility tests**

The minimum inhibitory concentrations (MICs) were determined by a standard microdilution broth method (National Committee for Clinical Laboratory Standards, 2003) with slight modifications. Briefly, the inoculum size was ~5 × 10⁵ CFU mL⁻¹ in the final assay volume of 50 μL. The microdilution plates inoculated with bacteria were incubated at 35 °C for 18–20 h, and the MIC was determined as the lowest concentration of the compound that completely inhibited the viable growth of the organism in the microdilution wells.

**Equilibrium analysis by SPR**

Equilibrium analysis by SPR was performed using a Biacore3000 and the CM5 sensor chip (GE Healthcare Japan). SpPyrH was covalently coupled to CM5 using a standard amine coupling method according to the manufacturer’s protocol. Briefly, CM5 was activated by injecting a mixture of 20 mM N-hydroxysuccinimide (NHS) and 80 mM 1-ethyl-3-(3-diethylaminopropyl) carbodiimide hydrochloride. After being diluted tenfold with acetate buffer (pH 4.8), SpPyrH (0.1 mg mL⁻¹) was injected at 10 μL min⁻¹ for 7 min and then CM5 was inactivated by 1 M ethanolamine hydrochloride (pH 8.5) to block the residual NHS ester groups. Running buffer (10 mM Hepes (pH7.4), 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant (GE Healthcare Japan), 5% DMSO) was used in all binding experiments. All compounds dissolved in DMSO were diluted 1 : 20 with the running buffer without 5% DMSO. The samples were injected at 30 μL min⁻¹ for 2 min. The response was measured in resonance units (RU), and data analysis of the sensorsgrams was performed using BLAevaluation software ver. 3.1 and the response at the equilibrium phase of interaction was obtained using the software program ‘equilibrium analysis model’.

**Results and discussion**

**Purification of recombinant *S. pneumoniae* PyrH (SpPyrH) and *H. influenzae* PyrH (HiPyrH) proteins**

To obtain recombinant PyrH proteins, the SpPyrH or HiPyrH, each tagged with 6xHis at NH2-terminus, was expressed in *E. coli* and then purified using the Ni-affinity resin. When purified SpPyrH or HiPyrH protein was examined by SDS–PAGE followed by Coomassie staining, a prominent band was detected of 29.2 or 28.3 kDa in size, respectively, which was deduced as the molecular weight of SpPyrH or HiPyrH (Fig. 1a and c). These
proteins were also detected by Western blotting analysis with anti-6xHis antibody, suggesting that each of these proteins is an authentic target protein (Fig. 1b and d). The protein of ~60 kDa in size was detected in Coomassie staining but not in Western blotting, suggesting that this protein is an endogenous protein in *E. coli* having an affinity for Ni-NTA agarose resin or is associated with SpPyrH (Fig. 1b).

**Establishment of UMP kinase assay**

To evaluate the level of UMP kinase activity, the amount of residual substrate, ATP, in the reaction was measured. As shown in Fig. 2a and b, the levels of RLU, which reflect the amount of ATP decreased in a dose-dependent fashion with an increasing amount of SpPyrH or HiPyrH in the reaction, suggesting that the level of PyrH kinase activity inversely correlates with the amount of residual ATP. Furthermore, the amount of UMP in the reaction, another substrate of PyrH, correlates with that of residual ATP, while reference reaction (no enzyme control) did not affect the RLU levels (Fig. 2c and d).

To confirm that this assay system is applicable to the evaluation of PyrH kinase inhibitors, we validated the performance using UTP, a known physiological inhibitor of PyrH. As a result, addition of UTP dose dependently increased the level of RLU, suggesting that UTP
inhibition of kinase activity of SpPyrH and HiPyrH was detected as $IC_{50} = 710$ and 71 $\mu$M, respectively (Table 2).

Characterization of PYRH-1

PYRH-1 was tested for molecular interaction with SpPyrH by SPR equilibrium analysis (Fig. 3). The RU of substrate UMP and PYRH-1 converged at a theoretical maximum resonance ($R_{max}$) value (83 and 222 RU, respectively) in the range of 4–1000 $\mu$M or 2.5–40 $\mu$M, suggesting the specific and direct binding of SpPyrH and PYRH-1 at a one-to-one molar ratio (Fig. 4) with the $IC_{50}$ against SpPyrH being less than that of UTP.

We further examined the MIC of PYRH-1 for bacterial strains such as *S. pneumoniae*, *S. aureus* and *H. influenzae ΔacrA* (*acrA*, a member of AcrAB-TolC efflux pump system, deletion strain) and *E. coli ΔtolC*. Because it is reported that the AcrAB-TolC efflux pump system in *H. influenzae* alters the susceptibility of the organism to various classes of antimicrobial compounds (Trepod & Mott, 2004), we used the AcrAB-TolC efflux pump deletion strains. As a result, PYRH-1 had antimicrobial activities against *S. pneumoniae* with MIC = 64 $\mu$g mL$^{-1}$, *S. aureus* with MIC = 2 $\mu$g mL$^{-1}$ and *H. influenzae ΔacrA* with MIC = 1 $\mu$g mL$^{-1}$ but not for *E. coli ΔtolC*.

Taken together, we evaluated PYRH-1 as a kinase inhibitor of PyrH via direct molecular interaction. Although the antimicrobial activity of PYRH-1 was not sufficient for therapeutic use, we are further characterizing SAR (structure–activity relationships) in the PYRH-1 class of compounds to facilitate discovery of new antimicrobial agents.

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References


