The Role of a Novel Auxiliary Pocket in Bacterial Phenylalanyl-tRNA Synthetase Druggability

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**Background:** Phenylalanyl-tRNA synthetase inhibitors have been shown to be efficacious in animal models of infection. Inhibitors occupy a newly identified hydrophobic auxiliary binding pocket. Compound binding in this pocket leads to high screening hit rates, resistance frequencies, and elevated plasma protein binding.

**Significance:** New inhibitors may be identified by avoiding the auxiliary pocket.

The antimicrobial activity of phenyl-thiazolylurea-sulfonamides against *Staphylococcus aureus* PheRS are dependent upon phenylalanine levels in the extracellular fluids. Inhibitor efficacy in animal models of infection is substantially diminished by dietary phenylalanine intake, thereby reducing the perceived clinical utility of this inhibitor class. The search for novel antibacterial compounds against Gram-negative pathogens led to a re-evaluation of this phenomenon, which is shown here to be unique to *S. aureus*. Inhibition of macromolecular syntheses and characterization of novel resistance mutations in *Escherichia coli* demonstrate that antimicrobial activity of phenylthiazolylurea-sulfonamides is mediated by PheRS inhibition, validating this enzyme as a viable drug discovery target for Gram-negative pathogens. A search for novel inhibitors of PheRS yielded three novel chemical starting points. NMR studies were used to confirm direct target engagement for phenylalanine-competitive hits. The crystallographic structure of *Pseudomonas aeruginosa* PheRS defined the binding modes of these hits and revealed an auxiliary hydrophobic pocket that is positioned adjacent to the phenylalanine binding site. Three viable inhibitor-resistant mutants were mapped to this pocket, suggesting that this region is a potential liability for drug discovery.

Protein translation has proven to be a rich source of antibacterial drug discovery targets. An essential step in this process is the aminoacylation of tRNAs. Inhibition of a single aminoacyl tRNA synthetase (aaRS) halts translation or leads to the misincorporation of amino acids. The presence of aaRSs among bacterial species varies widely, which limits the potential clinical spectrum of aaRS inhibitors. Complete genome analysis reveals that a full complement of 20 canonical aaRSs is uncommon (1). More often, tRNA-dependent amidotransferases acting on Asp-tRNA and Gln-tRNA compensate for the absence of AsnRS and GlnRS, which narrows the spectrum of AsnRS or GlnRS inhibitors. Conversely, multiple aaRS isozymes can be present among bacterial strains, which also limits the spectrum of an inhibitor to a subset of a population when it acts against only one of these isozymes. For example, one of two MetRS paralogs was identified in different *Streptococcus pneumoniae* strains, yielding subpopulations with differing sensitivities to a tetrahydroquinoline inhibitor (2). A variation on this theme is the occurrence of mupirocin-resistant IleRS isozymes in *Staphylococcus aureus*, MupA and MupB. Although their prevalence was low when the topical IleRS inhibitor mupirocin was launched, their presence has given rise to increased clinical resistance (3). In contrast to these examples, the genes encoding PheRS subunits, *pheS* and *pht*, have been found in all genomes as single copies, highlighting the potential for an agent with broad spectrum antibacterial activity (1).

aaRSs can be divided into two structural classes, I and II (4), and subdivided into three subclasses (5). This partition is correlated with biochemical differences, including the bound conformation of ATP and tRNA, the location of the aminoacylated hydroxyl group on the terminal ribose of the tRNA, and the formation of either catalytic α-monomers or obligate α₂-homodimers (6).

PheRS is a class II aaRS with an additional β-subunit that forms a (αβ)₂ heterotetramer (7, 8). Unlike other class II aaRSs, PheRS aminoacylates the tRNA at the 2'- and not the 3'-hydroxyl position (9, 10). The smaller catalytic α-subunit PheS (350 residues) catalyzes the initial acyl transfer reaction that converts phenylalanine and ATP into the stable intermediate phenylalanyl-adenylate. Phenylalanine is subsequently transferred to the 3’-end of tRNAPhe (11). The larger β-subunit PheT (800 residues) mediates the physical interaction with the tRNAPhe molecule and exclusively carries out the editing func-

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*This article contains supplemental text, Tables S1 and S2 and Figs. S1 and S2. The atomic coordinates and structure factors (codes 4P71, 4P72, 4P73, 4P74, and 4P75) have been deposited in the Protein Data Bank (http://wwpdb.org/). Present address: Firmenich Inc., 450 E 29th St., Suite 405, New York, NY 10016. To whom correspondence should be addressed: Dept. of Biosciences, Infection Innovative Medicines Unit, AstraZeneca R&D Boston, 35 Gatehouse Dr., Waltham, MA 02451. Tel.: 781-839-4592; Fax: 781-839-4600; E-mail: Ed.Buurman@astrazeneca.com. The abbreviations used are: aaRS, aminoacyl tRNA synthetase; CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration; RMSD, root mean square deviation.
tion of the enzyme by cleaving tRNA molecules that are mischarged with tyrosine (7, 8, 12).

Eukaryotes contain distinct cytosolic and mitochondrial PheRS homologs. Although the structural architecture of cytosolic PheRS is similar to bacterial PheRS, differences in functionally important residues have been described (13). Mitochondrial PheRS is a monomeric protein that appears to have evolved from an αβ chimeric protein (14). These structural differences hold promise for the development of selective inhibitors, as demonstrated by the discovery of a wide array of bacterial PheRS inhibitors with broad biochemical spectrum and selectivity against their human counterparts, such as spirocyclic furans and pyrrolidines (15, 16), ethanolamines (17), and benzyl phenyl ethers (18). Unfortunately, the antimicrobial activity of these compounds was marginal.

A significant advance came with the discovery of phenylthiazolylurea-sulfonamides with both antimicrobial activity and reduced organ burdens in sepsis models of S. aureus and S. pneumoniae in mice (19). Consistent with these compounds acting competitively with substrate, the addition of phenylalanine to the growth medium reduced the in vitro antimicrobial potency against S. aureus. Subsequent studies showed that phenylalanine blood levels in mice were dependent on their diet, which reduced efficacy in regularly fed mice. Assuming these findings could be extrapolated to humans, the clinical utility of phenylalanine-competitive PheRS inhibitors as antimicrobial agents appeared limited.

Although phenyl-thiazolylurea-sulfonamides inhibitors showed antimicrobial activity against the Gram-negative pathogens Haemophilus influenzae and Moraxella catarrhals, and inhibited PheRS isolated from other Gram-negative species, phenylalanine complementation was marginal compared with S. aureus (19). This observation suggested that phenylalanine serum levels would not limit the clinical utility of PheRS inhibitors against Gram-negative pathogens or that the cellular target of phenyl-thiazolylurea-sulfonamides differs between Gram-positive and Gram-negative pathogens (20).

The mechanism of growth inhibition in Gram-negative pathogens was investigated through the isolation of resistant mutants. To rationalize the impact of these mutations, the crystal structure of Pseudomonas aeruginosa PheRS was determined in complex with four distinct chemical scaffolds. Combined with NMR competition data, these structures were used to define the inhibitor binding mode. In addition, an auxiliary hydrophobic pocket was identified adjacent to the phenylalanine binding pocket into which the inhibitors bind but not phenylalanine. Recognition of this drug discovery liability will be critical for future success in exploitation of this enzyme as an antimicrobial target.

**MATERIALS AND METHODS**

**Strains**—S. pneumoniae NCTC 7464, S. aureus RN4220 (21), P. aeruginosa PAO1 (22), H. influenzae ATCC 51907, and Escherichia coli ATCC27325 were used in this study. Efflux-negative strains H. influenzae acrB::cap and E. coli tolC::Tn10 and P. aeruginosa mexABC::Tn10 were derived as previously described (20, 21).

**Determination of Antimicrobial Activity**—Antimicrobial activity was determined using CLSI conditions (23) unless otherwise stated. The defined medium used in this study was similar to that described previously (19) and consisted of M9 medium with 0.4% glucose (24) supplemented with 100 μM of all canonical amino acids except phenylalanine.

**Inhibition of Macromolecule Biosynthesis**—The incorporation of radiolabeled metabolic precursors was performed in H. influenzae as described (25, 26), and Mueller Hinton II broth was used for S. aureus and E. coli tolC instead of haemophilus test medium.

**Isolation and Characterization of Resistant Mutants**—Resistant mutants of E. coli tolC were isolated as described previously (20). Compound 1a or 1b was added at 1.6, 3.2, 6.3, and 12.5 μM to Mueller Hinton II agar plates. An inoculum of 10⁵ cells was used to determine the agar MIC values, which were 1.6 and 6.3 μM, respectively. Larger inocula of 10⁶–10⁷ cells yielded resistant colonies at a frequency of 10⁻⁸–10⁻⁷ at 2–4-fold above the agar MIC value. The pheS genes of 18 mutants were PCR-amplified and sequenced, leading to the identification of three unique single residue mutations in PheS. These isolates have been deposited in the Yale University E. coli Genetic Stock Center.

**Aminocacylation Assay**—Compounds were solubilized in DMSO. Serial 2-fold dilutions covering two concentration ranges, 10 mM to 19.5 μM and 100 μM to 195 mM, were prepared. 0.6 μl/well of the diluted compound solutions (50× the final assay concentration) were added to white 384-well polystyrene assay plates (Thermo Fisher Scientific/Matrix Technology Corp., Hudson, NH). Uninhibited control wells (MAX) received 2 μl of a solution containing 30% (v/v) DMSO and 15 mM phenylalanine (Sigma-Alrich). Assays were performed in a buffer consisting of 50 mM Tris-HCl (pH 8.0), 50 mM NH₄Cl, 10 mM MgCl₂, 2 mM DTT, 0.005% Tween 20 (Surfact-Amps-20, Thermo Fisher Scientific/Pierce Protein Research products, Suwanee, GA), and 0.1 mM EDTA-NaOH (pH 8.0). Compounds were preincubated with 15 μl/well of either 2 mM E. coli PheRS, 2 mM H. influenzae PheRS, or 0.8 mM P. aeruginosa PheRS in buffer for 30 min at 2× final assay concentrations. The reactions were initiated with 15 μl/well of a 2× substrate solution in buffer containing 2 μM E. coli phenylalanine tRNA (Sigma-Alrich), 100 μM ATP, and 2 μM [³H]Phe with a specific radioactivity of 6.3 Ci/mmol (PerkinElmer Life Sciences). The reactions were quenched after 30 min with 15 μl/well of a solution containing 4 mg/ml PVT/PEI/WGA type A SPA beads (PerkinElmer Life Sciences), 262 mM sodium citrate (pH 2.0), and 150 mM NaCl. The plates were sealed with transparent film (PerkinElmer Life Sciences). The beads were allowed to settle for a minimum of 2 h before scintillation counting with a TopCount plate reader (PerkinElmer Life Sciences). [³H] counts (CPM) for aminoacylated tRNA were measured for 1 min/well. The percentage of inhibition was calculated using the equation: % inhibition = 100 × (1 – (CPM – MIN))/(MAX – MIN)), where MAX and MIN are the averages of 32 wells each per plate. Typical values of MIN and MAX were 35 and 720 CPM, respectively.

The IC₅₀ values (concentration of inhibitor producing 50% inhibition) were calculated for each compound dilution series by nonlinear least squares regression using the equation: %
inhibition = 100 - [I]/(IC_{50} + [I])^{n}) where [I] is the inhibitor concentration, and n is the Hill slope.

**PhRS Expression Plasmids—pheS and pheT** genes were PCR-amplified and ligated using the pET-46 EK/LIC Cloning Kit with the LIC Duet Minimal Adaptor as described by the manufacturer (EMD Millipore, Billerica, MA). This system brings pheS and pheT expression under the control of the T7lac promoter encoded within the expression plasmid pETDuet-1.

All oligonucleotides were custom synthesized (Eurofins MWG Operon, Huntsville, AL). Plasmid pSMM84 encodes truncated forms of *P. aeruginosa* PheS and PheT, expressing residues 80–338 of PheS with an N-terminal His_{6} tag cloned into the Sacl and Sall sites, while encoding residues 1–791 of PheT cloned into the Ndel and KpnI sites. Plasmid pSMM107 expresses a full-length N-terminal His_{6}-tagged PheS and a full-length PheT from *P. aeruginosa*. The reverse PCR primer for the C-terminal end of pheS used for construction of pSMM107 encodes two additional stop codons to prevent readthrough. Plasmids pH1504 and pH1514 were cloned as described for pSMM107. Plasmid pH1504 expresses full-length His_{6}-tagged PheS and full-length PheT from *H. influenzae*, whereas plasmid pH1514 expresses full-length His_{6}-tagged PheS and full-length PheT from *E. coli*.

**PhRS Overexpression—E. coli** BL21(DE3) (Invitrogen) was transformed with plasmid pSMM84, plated on LB agar containing 100 μg/ml ampicillin (Sigma-Aldrich), and grown overnight at 37 °C. A single transformant was inoculated into a 100-mL starter culture of LB broth containing 100 μg/ml ampicillin and grown overnight at 37 °C. The overnight culture was diluted to an initial A_{600} of 0.1 in four 1-L cultures and grown at 37 °C with aeration until the A_{600} reached 0.3. The cultures were then transferred to 16 °C and grown until the A_{600} reached 0.6, at which point isopropyl β-D-thiogalactopyranoside (Acros Organics) was added to a final concentration of 0.3 mM and grown overnight. The cells were harvested by centrifugation at 5,000 × g for 15 min at 25 °C and frozen. Overexpression of PhRS using plasmids pSMM107, pH1504, and pH1514 were performed as described with the following modifications. Plasmid pSMM107 was transformed into *E. coli* HMS174(DE3) (EMD Millipore) and grown at 30 °C until the A_{600} reached 0.5, and induction was performed at room temperature overnight. Plasmids pH1504 and pH1514 were transformed into *E. coli* BL21(DE3)pLysS (EMD Millipore) and grown at 30 °C until the A_{600} reached 0.5. Expression from the plh1504 plasmid was induced at room temperature overnight, whereas the pH1514 plasmid was induced at 30 °C for 4.5 h at room temperature.

**PhRS Purification**—Cell pellets were suspended in 50 mL of lysis buffer consisting of Buffer A (25 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 5% glycerol) and one EDTA-free Protease inhibitor mixture tablet (Roche Molecular Biochemical). Use of a French pressure at 18,000 p.s.i. twice at 4 °C disrupted cells, and the crude extract was centrifuged at 150,000 × g for 30 min at 4 °C. The clarified supernatant was applied to a HiTrap Ni^{2+} chelating column (GE Healthcare) pre-equilibrated with Buffer A. The column was washed with Buffer A, and the PhRS complex was eluted using a linear imidazole gradient in Buffer A. Fractions containing the PhRS complex were pooled and characterized by SDS-PAGE and analytical LC-MS. The purified protein was stored at −80 °C.

The N-terminal His_{6} tag was proteolytically removed from the truncated PhRS complex (residues 80–338 of PhRS with an N-terminal His_{6} tag and residues 1–791 of untagged PheT) for crystallization. Specifically, thrombin (EMD Millipore) was added to the pooled fractions and dialyzed against 1 liter of 25 mM Tris–HCl (pH 8.0), 0.1 M NaCl, and 5% glycerol at 4 °C overnight. The dialyzed sample was then reapplied to a HiTrap Ni^{2+} chelating column pre-equilibrated with Buffer A. The fractions containing the PhRS complex were pooled and concentrated using an Amicon Ultracell-10K filtration concentrator (EMD Millipore). The concentrated sample was further purified by size exclusion chromatography using a Sephacryl S300 (HR 26/60) column (GE Healthcare) pre-equilibrated with Buffer B (25 mM Tris–HCl, pH 8.0, 1 mM DTT, 1 mM EDTA 10% glycerol and 150 mM NaCl). Fractions containing the PhRS complex were pooled and characterized by SDS-PAGE and analytical LC-MS.

**Protein Engineering**—The rational protein engineering approach applied by Evdokimov et al. (27) for *Staphylococcus haemolyticus* PhRS was utilized to engineer the *P. aeruginosa* PhRS complex for crystallographic studies. Briefly, a variety of full-length and N-terminal truncation constructs were designed to remove expected regions of disorder within PheS and to introduce a series of surface entropy reduction mutations within PheT to eliminate the formation of inefficient crystal contacts that would lead to low resolution and/or anisotropic diffraction and irreproducible crystallization. A structure-based protein sequence alignment of *P. aeruginosa, S. haemolyticus*, *E. coli*, and *T. thermophilus* PhRS was used to visualize the expected impact of the truncation and mutagenesis strategy. From these efforts, a bicistronic expression plasmid encoding residues 80–338 of PhS with a cleavable N-terminal His_{6} tag and residues 1–791 of untagged PheT was constructed. The N-terminal His_{6} tag was proteolytically cleaved with thrombin prior to crystallization studies to minimize the disorder of the N terminus of PheS. Using this truncated protein, crystallization screening identified initial crystal growth conditions that were extensively optimized, followed by the development of a suitable cryoprotection protocol.

**Crystallography**—All x-ray diffraction data were collected at cryogenic temperature using synchrotron radiation at Beamlines ID-17 and ID-24 at the Advance Photon Source. Diffraction data collected at IMCA-CAT was processed with XDS (28) and scaled using SCALA (29), as implemented in the autoPROC routines from Global Phasing (30). Diffraction data collected at NE-CAT were processed using HKL2000 (31). These crystals belong to the monoclinic space group C2, contain two PhRS heterodimers per asymmetric unit, and have a Matthews’ coefficient of 2.87 Å^{3}/Da (corresponding to an estimated solvent content of 57%) (supplemental Table S1).

The structure of apo PhRS from *P. aeruginosa* was solved at 2.79 Å resolution by molecular replacement using the program MOLREP with the coordinates of PhRS from *S. haemolyticus*.
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TABLE 1
Antimicrobial activity of phenyl-thiazole-sulfonamide PheRS inhibitors

|       | CLSI         | Defined medium (+100 μM Phe) |
|-------|--------------|-----------------------------|
|       | Sau| Spn| Hin| Hin*| Eco*| Pae*| Sau| Eco*| Pae* |
| 1a    | >100| 50| 6.25| 0.78| 3.1| >100| 12.5 (>50)| 0.4 (0.8)| >100 (>100) |
| 1b    | 50| 50| 12.5| 1.56| 6.3| 100| 1.6 (25)| 3.1 (6.2)| 50 (50) |

(Protein Data Bank accession code 2RHQ) as the search model (27, 32). The structures of all liganded PheRS complexes were subsequently determined using the coordinates of the apo structure. Examination of the electron density maps for all liganded complexes showed clear unambiguous difference density with the expected molecular features of the compound (supplemental Fig. S1). Sequential rounds of manual rebuilding using Coot (33) and refinement using autoBUSTER with TLS, NCS, and target restraints (34), produced models for apo and liganded PheRS complexes. The final refinement statistics are shown in supplemental Table S1. All figures were prepared using PyMOL (Schrodinger). The coordinates and structure factors have been deposited with the Protein Data Bank with accession codes 4P71, 4P72, 4P73, 4P74, and 4P75.

NMR Binding Studies—All NMR spectra were acquired at 298 K with a 600 MHz NMR instrument (Bruker, Billerica MA) with an AVANCE III console and a triple-resonance cryogenic probe. In the WaterLOGSY experiments (35), the first water-selective 180° Sinc pulse was 6 ms long, and a weak rectangular pulse field gradient was applied during the mixing time (1.8 s). A gradient recovery time of 2 ms was introduced after the mixing time. Water suppression was achieved by the excitation sculpting scheme (36) and the water-selective 180° Sinc pulse was 3 ms long. The data were collected with a sweep width of 9157 Hz, 0.45-s acquisition time, and 1.8 s for the relaxation delay. For each experiment 128 scans were recorded, requiring 9 min/spectrum. The data were zero-filled to 32,768 complex points and multiplied by an exponential function (line broadening, 3 Hz) prior to Fourier transformation.

Chemistry—Synthesis of compounds was performed in house and is described in the supplemental information. Compound purity was determined by LC/MS shortly before biological testing and was >90%.

Physicochemical Properties—Plasma protein binding and logD7.4 determinations were performed as described (37). Equilibrium solubility was measured according established methods (38).

RESULTS

Phenyl-thiazolylurea-sulfonamides act via PheRS in Gram-negative Species—The antimicrobial activity of two representative phenyl-thiazole-sulfonamides, compounds 1a and 1b (19), were evaluated under CLSI conditions (23). Despite limited compound solubility under these conditions (50–200 μM), MIC values were determined (Table 1). The addition of 100 μM phenylalanine did not increase the observed MIC values for any of the evaluated bacterial strains because these media contain 2 mM phenylalanine. An equivalent experiment was performed using defined minimal salts medium. The results showed increased MIC values following the addition of phenylalanine for S. aureus only, whereas the MIC values for E. coli tolC and P. aeruginosa mexABCDXY remained constant. Taken together, these results are consistent with the previously described phenylalanine complementation data for S. aureus PheRS (19).

To determine whether an alternative mechanism of growth inhibition could be utilized by Gram-negative pathogens, radiolabeled precursor incorporation studies were conducted in the presence of compound 1a. Whereas the translation inhibitor erythromycin showed selective inhibition of protein synthesis (Fig. 1a), mupirocin (Fig. 1c) and compound 1a (Fig. 1d) showed concomitant inhibition of RNA synthesis with weaker inhibition of DNA synthesis. This incorporation pattern is similar to that caused by the RNA polymerase inhibitor rifampin (Fig. 1b) and is consistent with the inhibition of RNA polymerase upon binding of (p)pGpp following binding of uncharged tRNA to the ribosomal A-site as part of the stringent response (39, 40). Similar effects were observed in S. aureus (Fig. 2, a–d) and H. influenzae (data not shown).

To confirm direct target engagement of compound 1a with PheRS, resistant mutants of E. coli tolC were isolated at a fre-
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The architecture of the P. aeruginosa (αβ)₂ heterotetramer is consistent with previously described bacterial PheRS structures (Fig. 3). The smaller component of the heterotetramer, PheS, consists of a central globular domain and a N-terminal extension that forms supplemental interactions with PheT and the tRNA\text{Phe} molecule. PheS from P. aeruginosa is most structurally homologous to PheS from the engineered form of S. haemolyticus (RMSD of 1.07 Å of 229 aligned residues; sequence identity of 55%), PheS from E. coli (RMSD of 1.17 Å over 224 aligned residues; sequence identity of 70%), and PheS from T. thermophilus (RMSD of 1.33 Å over 219 aligned residues; sequence identity of 53%).

The larger component of the (αβ)₂ heterotetramer, PheT, is composed of four globular domains, two of which do not form any contacts with PheS and exclusively mediate interactions with the tRNA\text{Phe} molecule. PheT from P. aeruginosa shows the highest level of structural homology with PheT from E. coli (RMSD of 2.31 Å over 705 aligned residues; sequence identity of 45%), PheT from the engineered form of S. haemolyticus (RMSD of 2.65 Å of 716 aligned residues; sequence identity of 30%), and PheT from T. thermophilus (RMSD of 2.38 Å over 638 aligned residues; sequence identity of 36%).

The phenylalanyl-adenylate binding pocket of P. aeruginosa PheRS is composed exclusively of residues from PheS. The crystal structure of E. coli PheRS in complex with AMP and phenylalanine previously defined the positions of these substrates within the binding pocket (42). In comparing the E. coli structure to the apo structure of P. aeruginosa PheRS, all residues that are expected to interact with the phenylalanine or AMP substrates are invariant between these isoforms, except for residue His-90, which stabilizes the carbonyl oxygen of the phenylalanine molecule at the base of the binding pocket (Fig. 4a). The crystal structure of PheRS from T. thermophilus in complex with a phenylalanyl-adenylate analog (44) further defines the scope of the pocket and its shape similarity to the P. aeruginosa enzyme. A sequence alignment of Gram-positive and Gram-negative PheRS isozymes, highlighting residue sequence conservation in PheS, is presented in supplemental Fig. S1.

Phenyl-thiazolylurea-sulfonamides—The structure of compound 1a in complex with P. aeruginosa PheRS was solved at 3.03 Å resolution (supplemental Table S1 and Fig. S2a). Comparisons to the liganded S. haemolyticus enzyme (27) showed nearly perfect superposition within the binding site. Although the phenyl-sulfonamide core occupies a similar position within the binding site as phenylalanine, the thiazolylurea component extends much deeper into an auxiliary hydrophobic pocket (Fig. 4b). This auxiliary hydrophobic pocket is located below the bound phenylalanine in the E. coli PheRS structure. Two hydrogen bonds are formed between the urea and side chain of

FIGURE 2. Compound 1a preferentially inhibits leucine and uridine incorporation into macromolecules of S. aureus, followed by thymidine incorporation. Protein synthesis inhibition (leucine incorporation; black diamonds), RNA (uridine incorporation; white squares), and DNA (thymidine incorporation; white circles) were measured as described under “Materials and Methods.” The incorporation rates of added precursors into uninhibited cells were 27, 3400, and 67 μmol/h/A₅₀₀ respectively. a, erythromycin; b, rifampin; c, mupirocin; d, compound 1a.
## TABLE 2
Biochemical potency, antimicrobial activity and physicochemical properties of PheRS inhibitors

IC<sub>50</sub> values are expressed in μM and were determined against the isozymes from *E. coli* and *P. aeruginosa*. MIC values determined under CLSI conditions are expressed in μM and were determined against *H. influenzae acrB*::*cap* (Hin*), *E. coli tolC*::*Tn*<sup>10</sup> (Eco*), and *P. aeruginosa* mexABC*<sub>DXY</sub>* (Pae*). The activity against parental strains is indicated in parentheses.

| Compound | IC<sub>50</sub> (μM) | MIC (μM) | Physicochemical properties<sup>a</sup> |
|----------|-----------------|----------|-------------------------------------|
|           | Eco | Pae | Hin* | Eco* | Pae* | LogD<sub>7.4</sub> | Eq sol (μM): | F<sub>u, hu</sub> (%) |
| 1a        | 0.0027 | 0.022 | 0.8 (25) | 1.6 (>200) | 200 (>200) | 3.2 | 2 | <1 |
| 1b        | 0.0022 | 0.013 | 1.6 (50) | 3.1 (>200) | >200 (>200) | >3.5 | 16 | <1 |
| 2a        | 1.4 | 82 | 50 (200) | 200 (>200) | >200 (>200) | 2.5 | 52 | 6.7 |
| 2b        | 0.56 | 41 | 200 (>200) | >200 (>200) | >200 (>200) | 1.9 | 97 | 31 |
| 2c        | 0.1 | 34 | 100 (>200) | 200 (>200) | >200 (>200) | 2.2 | 23 | 10 |
| 3a        | 0.06 | 10 | 100 (200) | 200 (>200) | >200 (>200) | 2.5 | 208 | 2.4 |
| 3b        | 0.08 | 11 | n.d. (>200) | >200 (>200) | >200 (>200) | 3.2 | 82 | 11 |
| 3c        | 1.0 | 59 | 100 (>200) | >200 (>200) | >200 (>200) | 2.3 | 84 | n.d. |
| 3d        | 16 | >200 | 100 (>200) | >200 (>200) | >200 (>200) | 2.8 | 64 | 7.1 |
| 3e        | 0.07 | 4.2 | >200 (>200) | >200 (>200) | >200 (>200) | 3.5 | 11 | n.d. |
| 4a        | 1.4 | 80 | 200 (>200) | >200 (>200) | >200 (>200) | 2.2 | 84 | 15 |
| 4b        | 0.10 | 13 | n.d. (50) | >200 (>200) | >200 (>200) | 2.1 | 55 | 5.9 |
| 4c        | 0.19 | 26 | 100 (>200) | >200 (>200) | >200 (>200) | 2.4 | 39 | n.d. |

<sup>a</sup> Eq sol, equilibrium solubility; F<sub>u, hu</sub>, human serum unbound fraction; n.d., not determined.
residue Glu-131, and another is formed between the side chain of residue Gln-95 and the thiazole ring (Fig. 5a). The sulfonamide oxygen forms a water-mediated interaction with the side chain of residue Gln-129 and the main chain amide of residue Gly-203. However, the piperazine substituent of compound 1a does not form any electrostatic interactions, nor does it overlap with the AMP molecule.

Search for Novel Inhibitory Scaffolds—Although the phenyl-thiazolylurea-sulfonamides showed sufficient cellular activity to determine efficacy in animal models of infection (19), their low aqueous solubility (2 μM) and plasma protein binding of >99% (Table 2) could make formulation of an intravenous clinical product challenging and the dosage high. An attempt was made to identify alternative chemical scaffolds with more attractive physicochemical properties, in particular, those with a lower lipophilicity (logD7.4). High throughput screening of the corporate compound library yielded many hits. Three novel scaffolds passed our selection criterion of calculated lipophilicity (clogD7.4) <3 and were resynthesized and pursued.

Pyridinyl Anilines—A series of pyridinyl anilines were synthesized, and the crystal structure of P. aeruginosa PheRS in complex with compound 2a was determined at 2.62 Å resolution (supplemental Table S1 and Fig. S2b). The structure shows that the aniline-linked chloropyridyl forms a single hydrogen bond with the side chain of residue Glu-131 in contrast to the multiple hydrogen bonds of the phenyl-thiazolylurea-sulfonamide 1a (Fig. 5, a and b). As observed with the thiazolylurea of compound 1a, the aniline-linked chloropyridyl group is positioned in the auxiliary hydrophobic pocket. NMR binding studies were used to confirm that the binding of this compound to P. aeruginosa PheRS was competitive with the phenylalanine substrate (Fig. 6). Substitution of the lipophilic chlorine with a more polar cyano moiety (compound 2b) produced a small enhancement in the inhibition of both E. coli and P. aeruginosa PheRS, a significant reduction in logD7.4, and concomitant improvements in solubility and protein binding (Table 2).

A wider set of amides was synthesized to potentially form additional electrostatic interactions with residues lining the AMP binding pocket. As shown by 2c, although desirable physicochemical properties were maintained, only modest gains in potency were achieved. These results suggest that within this series, few, if any, additional binding interactions within the AMP binding pocket were established.

Trifluoromethyl Pyrazoles—The structure of compound 3a, an exemplar trifluoromethyl pyrazole, was determined at 2.96 Å resolution (supplemental Table S1 and Fig. S2c). The liganded structure demonstrates that the pyrazole ring forms electrostatic interactions with the side chains of residues Gln-95 and Glu-131 (Fig. 5c). Analogous to the thiazolylurea of 1a, the CF₃ substituent is located in the auxiliary hydrophobic pocket of PheRS. The methoxyphenyl group extends toward the bottom of the AMP binding site, and the methoxy substituent is located near the position of the sulfonamide of compound 1a. NMR binding studies confirmed that this compound is competitive with the phenylalanine substrate (Fig. 6).

The structure-activity relationship of pyrazole ring substituents revealed the importance of the CF₃ group. Removing one of three fluorines (3c) decreased the biochemical potency significantly (Table 2). Changing the position of the nitrogen to the isomeric trifluoromethyl imidazole (3d versus 3a) rendered this analog inactive. Taken together, these data indicate that the strength of the electrostatic interaction formed between the pyrazole NH and the side chains residues Gln-95 and Gly-131 is important to maintain enzyme inhibition. Attempts to homologate the methoxy group were also pursued. However, as observed with 3e, only marginal improvements in potency and physicochemical properties were achieved.

Thiazole Amides—The crystal structure of P. aeruginosa PheRS with the thiazole amide compound 4a was also determined at 2.70 Å resolution (supplemental Table S1 and Fig. S2d). Competition with phenylalanine was confirmed by NMR binding experiments (Fig. 6). The ether-linked methylphenyl group overlaps with the position of the phenyl-thiazolylurea of 1a, within the auxiliary hydrophobic pocket, and the ether oxy-
specific electrostatic interactions are observed with the ether hydrogen bonds with the side chain of residue Glu-131, no specific electrostatic interaction is observed with the ether hydrogen bond with the enzyme. The thiazole core of 4a is perfectly superimposed on the phenyl ring of 1a. In addition, the amide-linked alkyl sulfone forms a hydrogen donor acceptor interaction with the side chain of residue Gln-129 and the backbone amide of residue Gly-230. The alkyl substituent is positioned near the location of the ribose of the AMP substrate.

Thiazole amides such as 4a, with a non-phenyl group at their core, fill the hydrophobic pocket with a phenyl ether and point an amide toward the remainder of the binding site. Although substitution of the methyl phenol with the slightly more lipophilic chlorophenol results in increased activity, introduction of polar substituents in this region proved deleterious (data not shown). Modification of the amine portion of these amides resulted in significant improvements in potency as exemplified by the cyanomethyl (4b) and sulfonamidomethyl (4c). The addition of these groups should make the amide significantly more acidic and offers the possibility of forming an additional hydrogen bond with the enzyme.

DISCUSSION

Target-based antibacterial discovery aims to convert biochemical inhibitors into efficacious clinical candidates. The first step in this process is to chemically modify the inhibitor to maximize antimicrobial activity. For this strategy to succeed, it is critical that the desired intracellular mechanism of growth inhibition is validated for the structure-activity relationships to be meaningful. aaRSs are among the first antibacterial target classes to be pursued in this manner. The litmus test to verify the intracellular inhibition of an aaRS has been the addition of the relevant amino acid to the growth medium (45). Only those inhibitors that are competitive with the amino acid substrate should reduce antibacterial activity.

Although this premise is true for phenyl-thiazolylurea-sulfonamide inhibitors of S. aureus PheRS, reduction of antibacterial activity was not observed in E. coli, P. aeruginosa (Table 1), S. pneumoniae, M. catarrhalis, or H. influenzae (19). As part of this work, phenyl-thiazolylurea-sulfonamide-resistant mutants of E. coli were isolated with single residue PheS mutations, indicating that antibacterial activity is driven by the inhibition of its catalytic activity. Differences in phenylalanine complementation against identical PheRS inhibitors likely reflect the presence of distinct phenylalanine uptake mechanisms between S. aureus and Gram-negative pathogens. This is a key finding for drug discovery because Beyer et al. (19) linked variations in serum phenylalanine concentrations to efficacy changes of PheRS inhibitors in animal models of S. aureus infection, leading to the deprioritization of PheRS as an antibacterial target. Our results predict that efficacy against Gram-negative pathogens will be independent from dietary phenylalanine, thereby restoring the clinical promise for PheRS inhibitors.

Although phenyl-thiazole-sulfonamides have been shown to be efficacious, their low aqueous solubility and high serum protein binding make it difficult to calculate tissue exposure levels and predict animal efficacy. Alternative less hydrophobic chemical scaffolds were sought by screening the corporate compound collection. Consistent with previous studies (46), PheRS inhibitors were straightforward to obtain. X-ray crystal-
lography using *P. aeruginosa* PheRS combined with NMR binding studies were used to show that these inhibitors bind in the phenylalanine binding pocket of PheRS and extend into the auxiliary hydrophobic pocket (Figs. 4–6). Structure-guided design was used to improve the physicochemical properties of these inhibitors by extending into the more hydrophilic AMP-binding pocket, producing compounds with improved aqueous solubility and lower protein binding. Although exploration of three distinct chemical scaffolds afforded different vectors to extend into the AMP-binding pocket, the resulting biochemical potencies did not improve significantly.

The isolation of three *E. coli* resistant mutants provided valuable insights. When viewed in light of the structure of *P. aeruginosa* PheRS with compound 1a, residues Val-207 and Val-211 are positioned at the base of the phenylalanyl-adenylate binding pocket and are in close proximity to the thiazole ring (Figs. 5a and 7). The *E. coli* resistance mutants, V275E and V279E (corresponding to residues Val-207 and Val-211 in the...
P. aeruginosa PheRS structure) would introduce negatively charged side chains into the binding pocket that would significantly change its hydrophobicity and shape, clearly abolishing inhibitor binding. The third E. coli resistance mutation, A189E (C110E in P. aeruginosa PheRS), is positioned directly in front of the α-helix that forms an integral component of the phenylalanyl-adenylate binding pocket (Fig. 7). The presence of a glutamate residue at this position would impact the integrity of the phenylalanyl-adenylate binding pocket.

Viable E. coli resistance mutations could be isolated because compound 1a extends into the auxiliary hydrophobic pocket, which is not involved in phenylalanine binding or the aminoclylation of tRNA\textsubscript{Ph}. Interestingly, resistance to toxic halogenated phenylalanine analogs was identified by mutating residue Ala-226 of P. aeruginosa PheS, which is positioned just below the phenylalanine molecule (47).

The phenylalanine binding site of PheRS can accommodate the binding and aminoclylation of other amino acids, including tyrosine. To minimize the misincorporation rate, PheRS evolved an editing domain to catalyze the hydrolysis of tyrosyl-adenylate, instead of evolving a smaller binding pocket with increased selectivity. Although this structural feature holds promise for the design of proteins with novel functionality (48), it is a liability for drug discovery. First, the hydrophobicity of the pocket results in identification of chemical starting points with poor physicochemical properties, including the phenyl-thiazolylurea-sulfonamides and the scaffolds described here. Second, given the abundance of structurally related chemical compounds in our corporate compound collection, those compounds with more attractive physicochemical properties (potentially binding in the more hydrophilic ATP binding site of PheRS) would be assigned a lower relative ranking for resynthesis and characterization given their reduced biochemical potencies. Third, resistance mutations positioned within the auxiliary binding site occurred at a rate of $10^{-8}$–$10^{-7}$. This observation suggests that PheRS inhibitors would be susceptible to the rapid development of clinical resistance, as observed for inhibitor AN3365/GSK2251052, which inactivates the non-essential editing function of LeuRS (49). This liability is ampliﬁed by high bacterial growth rates; similar pockets have been successfully exploited in other therapeutic areas (50).

Silver (51) previously highlighted the clinical resistance risk associated with single gene target inhibitors and suggested that despite this liability, they may have clinical utility. Because there is no functional redundancy for PheRS (unlike, for example, peptide deformylase (52)), alternative lead generation approaches could be pursued. An enzymatic screen could be performed using the resistance mutants described here, followed by hit confirmation using the wild-type enzyme. This is likely to lead to the identiﬁcation of inhibitors that span phenylalanine and ATP binding sites. We also envision the design of substrate or transition state analogs and their potential utility in a screening cascade (53). Irrespective of the approach taken, inhibitor binding in the auxiliary pocket should be avoided, thus ensuring mutations would be rendered unviable by pre-

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**FIGURE 6. New scaffolds prevent binding of phenylalanine to P. aeruginosa PheRS.** One-dimensional WaterLOGSY spectral comparison of 200 μM phenylalanine binding to 10 μM P. aeruginosa PheRS in the absence (top spectrum) and presence of 200 μM compounds 2a, 3a, or 4a. Signals of two protons (7.25–7.32 ppm) from the phenyl ring on phenylalanine are boxed in dashed lines and compared in the WaterLOGSY spectra. Signal decrease indicates displacement of phenylalanine by the compound. The NMR samples were prepared in 50 mM HEPES (pH 7.5), 5 mM MgCl\textsubscript{2}, 5 mM DTT, 0.1 mM EDTA, and 5% deuterated water. The spectra were acquired at 298 K.

**FIGURE 7. Resistance mutations.** E. coli resistance mutants have been mapped onto the crystal structure of P. aeruginosa PheRS in complex with compound 1a. Residues Cys-110, Val-207, and Val-211 are shown as green sticks and labeled. The helix that forms an integral part of the phenylalanyl-adenylate binding pocket is colored purple. Compound 1a is shown with pink carbon atoms, blue nitrogen atoms, red oxygen atoms, and yellow sulfur atoms.

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4 ClinicalTrials.gov (February 13, 2014) GSK2251052 in complicated urinary tract infection.
ventring binding of both inhibitor and substrate to PheRS, thereby minimizing the rate of resistance.

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