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Lysyl-tRNA Synthetase from *Pseudomonas aeruginosa*: Characterization and Identification of Inhibitory Compounds

Samantha Balboa[1,2], Yanmei Hu[1,3], Frank B. Dean[1], and James M. Bullard[1]

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that causes nosocomial infections and has highly developed systems for acquiring resistance against numerous antibiotics. The gene (*lysS*) encoding *P. aeruginosa* lysyl-tRNA synthetase (LysRS) was cloned and overexpressed, and the resulting protein was purified to 98% homogeneity. LysRS was kinetically evaluated, and the K_m values for the interaction with lysine, adenosine triphosphate (ATP), and tRNA^{Lys} were determined to be 45.5, 627, and 3.3 µM, respectively. The k_{cat}^{obs} values were calculated to be 13, 22.8, and 0.35 s^{-1}, resulting in k_{cat}^{obs}/K_M values of 0.29, 0.036, and 0.11 s^{-1}µM^{-1}, respectively. Using scintillation proximity assay technology, natural product and synthetic compound libraries were screened to identify inhibitors of function of the enzyme. Three compounds (BM01D09, BT06F11, and BT08F04) were identified with inhibitory activity against LysRS. The IC$_{50}$ values were 17, 30, and 27 µM for each compound, respectively. The minimum inhibitory concentrations were determined against a panel of clinically important pathogens. All three compounds were observed to inhibit the growth of gram-positive organisms with a bacteriostatic mode of action. However, two compounds (BT06F11 and BT08F04) were bactericidal against cultures of gram-negative bacteria. When tested against human cell cultures, BT06F11 was not toxic at any concentration tested, and BM01D09 was toxic only at elevated levels. However, BT08F04 displayed a CC$_{50}$ of 61 µg/mL. In studies of the mechanism of inhibition, BM01D09 inhibited LysRS activity by competing with ATP for binding, and BT08F04 was competitive with ATP and uncompetitive with the amino acid. BT06F11 inhibited LysRS activity by a mechanism other than substrate competition.

Keywords

lysyl-tRNA synthetase (LysRS), protein synthesis, *Pseudomonas aeruginosa*, aminoacyl-tRNA synthetase, drug discovery, antibiotics

Introduction

Antibiotic resistance in bacteria is a growing global concern that has the potential to disrupt the fabric of society. This concern is exacerbated by the fact that discovery and development of novel antibiotics have dwindled to zero over the past decade. With the rise of bacteria that are resistant to multiple antibiotics, antimicrobial "superbugs," it is imperative that new antibiotics are discovered. *Pseudomonas aeruginosa* is a gram-negative, opportunistic pathogen responsible for 10 to 15% of nosocomial infections worldwide.[1] It is also the principal cause of mortality in patients with cystic fibrosis.[1] This pathogen contains many intrinsic properties that enable it to resist multiple drugs. These properties include the existence of at least five families of efflux pumps known for removal of antibiotics that gain entry into the cell and a cell membrane that already has a low permeability to antibiotics due to modification of certain porin proteins.[2] *P. aeruginosa* also has the ability to take up genes

through horizontal transfer that allows production of antibiotic-inactivating enzymes and proteins that prevent the dissemination of antibiotics across cell membranes. This pathogen also has the propensity to develop antibiotic resistance through genomic mutations. Examples of this include

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the mutation of genes encoding proteins targeted by antibiotics, resulting in a reduced susceptibility to the activity of the antibiotics.

Protein synthesis has long been a validated target for development of antibiotics, and aminoacyl-tRNA synthetases (aaRSs) play an indispensable role in protein synthesis. Aminoacyl tRNA synthetases are essential enzymes that attach specific amino acids to the cognate tRNA. Once the tRNA is charged with the correct amino acid, the ribosome transfers the amino acid from the aminoacylated tRNA to a growing peptide chain during protein biosynthesis, putting the aaRSs at the basis of gene expression by acting to interpret the genetic code between the interface of nucleic acids in mRNA and the amino acid sequence in proteins. Lysyl-tRNA synthetase (LysRS) binds its three substrates (tRNA^{Lys}, adenosine triphosphate [ATP], and lysine) and catalyzes the aminoacylation reaction to form Lys-tRNA^{Lys}. The loss of LysRS activity in *Escherichia coli* has been shown to be deleterious to bacterial cell viability,[3] indicating that the essentiality of the enzyme function of bacterial LysRS in protein synthesis is a validated target for discovery of antibacterial agents.

LysRS is a class II aminoacyl tRNA synthetase, characterized by an active site structure formed by three structural elements: motifs 1, 2, and 3. LysRS is further grouped, along with asparginyl- and aspartyl-tRNA synthetases (AsnRS and AspRS), into subclass IIb based on the presence of an oligonucleotide binding domain within its structure. LysRS appears to be functional in a α_2 dimer form, as are all subclass IIa and IIb enzymes.[4]

Scintillation proximity assay (SPA) technology has been previously used to measure aaRS activity in the aminoacylation reaction.[5] In this study, a screening platform based on SPA technology was developed to measure the aminoacylation activity of LysRS as well as the chemical compound inhibition of that activity in a high-throughput screening format. Two chemical compound libraries (synthetic and natural compounds) containing 1690 chemical compounds were screened against the activity of LysRS from *P. aeruginosa*. Three individual compounds were confirmed to have inhibitory effects against the enzymatic activity of the LysRS. These compounds were further characterized for inhibition of enzymatic activity, inhibition of bacterial growth, mode of action, mechanism of inhibition, and toxicity issues.

Methods and Materials

Materials

All chemical compounds used in this study were obtained from Fisher Scientific (Pittsburg, PA). Radioactive isotopes, SPA beads, and 96-well plates were obtained from PerkinElmer (Waltham, MA). The synthetic compound library was from TimTec LLC (Newark, DE), and the natural product library was from MicroSourceDiscovery Systems, Inc. (Gaylordsville, CT). The chemical compounds were obtained as 10 mM stock solutions dissolved in DMSO and stored at −20 °C. These compounds were thawed immediately before screening. The compounds have an average purity of 95%, with the minimum purity at least 90%.

Cloning and Purification of P. aeruginosa LysRS

The gene encoding *P. aeruginosa* LysRS was obtained through PCR amplification (MJ Mini Thermo Cycler, Bio-Rad, Hercules, CA) using *P. aeruginosa* PAO1 (ATCC 47085) genomic DNA as a substrate. A forward primer consisting of the sequence 5′-ATAAGCTAGCAGCGACCA ACAACTCGACCA-3′ was designed to add an *Nhe*I restriction site to the 5′ end of the gene, and the reverse primer consisting of the sequence 5′-ATATGGATCCTCAGGC CTGCGGGC-3′ was designed to add a *Bam*HI restriction site to the 3′ end of the gene during PCR. The PCR product was inserted into a pET-28b(+) plasmid (Novagen, Darmstadt, Germany) digested with *Nhe*I/*Bam*HI. The recombinant plasmid was transformed into *E. coli* Rosetta 2(DE3) Singles Competent Cells (EMD Millipore, Danvers, MA).

The *E. coli* bacterial cultures containing the plasmid were grown in Terrific Broth containing 25 μg/mL kanamycin and 50 μg/mL chloramphenicol at a temperature of 37 °C until an optical density (A_{600}) of 0.6 to 0.8 was reached. Overexpression of *P. aeruginosa* LysRS in the cultures was induced by addition of isopropyl β-D-1-thiogalactopyranoside to a concentration of 0.25 mM. The culture was grown for 2 h postinduction. Then, the cells were harvested by centrifugation (10,000 *g*, 30 min, 4 °C). Fraction I lysates were prepared as described,[6] and *P. aeruginosa* LysRS was purified, fast frozen in liquid nitrogen, and stored at −80 °C as previously described.[6] The published procedure was modified as follows: the initial purification step used precipitation of *P. aeruginosa* LysRS between 40% and 70% saturated ammonium sulfate, and affinity chromatography was performed using Perfect Pro Ni-NTA resin (5-Prime).

Gel Electrophoresis and Protein Analysis

The proteins in each fraction were visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 4% to 12% polyacrylamide premade gradient gels (Novex NuPAGE; Invitrogen, Grand Island, NY) with 3-(N-morpholino)propanesulfonic acid as the running buffer (Invitrogen) followed by staining with Coomassie.

EZ-Run Rec Protein Ladder (Fisher Scientific) was used as a protein standard. Coomassie Protein Assay Reagent (Thermo Scientific, Waltham, MA) was used to determine protein concentrations with bovine serum albumin as a standard.

ATP:PP_i Exchange Reactions

The rate of the formation of an aminoacyl-adenylate in varying concentrations of ATP and lysine (Lys) was determined using ATP:PP$_i$ exchange reactions. These reactions were carried out in 100 μL reactions containing 50 mM Tris-HCl (pH 7.5), 10 mM KF, 2 mM [^{32}P]PP$_i$, 10 mM MgOAc, and 0.1 μM *P. aeruginosa* LysRS at 37 °C as described.[7] In reactions to determine initial velocity, the reactions were stopped at 1, 2, 3, 4, and 5 min intervals. The concentration of lysine was held constant at 2 mM in the reactions in which ATP concentrations were varied (50, 100, 200, 300, 400, and 500 μM), and the concentration of ATP was held constant at 2 mM in the reactions in which lysine concentrations were varied (12.5, 25, 50, 100, 150, 200, and 250 μM). The initial velocities were determined for exchange of PP$_i$ and fit to the Michaelis-Menten steady-state model using XLfit (IDBS, Boston, MA). From these data, the kinetic parameters K_M and V_{max} were obtained and used to determine the observed turnover number (k_{cat}^{obs}) for the interactions of *P. aeruginosa* LysRS with ATP and lysine.

Aminoacylation Assays

The rate of catalysis of LysRS in the aminoacylation of tRNA was measured using SPAs as described.[6] Briefly, the concentrations of components in the aminoacylation reactions (50 μL) were 50 mM Tris-HCl (pH 7.5), 0.5 mM spermine, 10 mM MgOAc, 2.5 mM ATP, 75 μM [^{3}H]Lys, and 0.03 μM *P. aeruginosa* LysRS. Reactions were initiated by the addition of tRNA. The concentrations of tRNA^{Lys} in the assays were 1, 2, 3, 4, and 5 μM (the amount/percentage of tRNA^{Lys} was determined from the total tRNA as described[8]). The tRNA was from *E. coli* MRE 600 (Roche, Mannheim, Germany). Reactions were stopped by the addition of ethylenediaminetetra-acetic acid (EDTA) to 50 mM at 1 min time intervals between 1 and 5 min. Initial velocities for tRNA aminoacylation were calculated for all concentrations of tRNA, and these data were fit to the Michaelis-Menten steady-state model using XLfit (IDBS) to determine the K_M, V_{max}, and k_{cat}^{obs} values.

Chemical Compound Screening

To screen for chemical compounds with the potential to inhibit *P. aeruginosa* LysRS, tRNA aminoacylation was monitored using SPAs as previously described.[6] The reactions were carried out in 96-well microtiter plates (Costar),

and the compounds were dissolved in DMSO at a concentration of 3.3 mM. The final compound concentration in the assay was 132 μM. The concentrations of *P. aeruginosa* LysRS and tRNA^{Lys} were 0.03 and 4.0 μM, respectively. To determine IC$_{50}$ values, the test compounds were serially diluted from 200 μM to 0.4 μM into the SPA-based aminoacylation assays, and the enzyme concentration was 0.01 μM. The curve fits and IC$_{50}$ values were determined using the sigmoidal dose-response model in XL*fit* 5.3 (IDBS).

Microbiological Assays

Minimum inhibitory concentration (MIC) assays for the chemical compounds were performed using broth microdilutions according to the Clinical and Laboratory Safety Standards Institute (CLSI) guideline M7-A7.[9] MIC values were determined for *E. coli* (ATCC 25922), *E. coli tolC* mutant (W4573:*tolC*::Tn*10*), *Enterococcus faecalis* (ATCC 29212), *Haemophilus influenzae* (ATCC 49766), *Moraxella catarrhalis, P. aeruginosa* (ATCC 47085), *P. aeruginosa* PAO200 (efflux pump mutant), *P. aeruginosa* hypersensitive strain (ATCC 35151), *Staphylococcus aureus* (ATCC 29213), and *Streptococcus pneumonia* (ATCC 49619). Quality control of obtained MIC data was maintained by MIC determination for inhibitory antibiotics specific for each bacterial strain as described[7] in "Table 1: Summary of Etest® Performance, Interpretive Criteria and Quality Control Ranges."[10]

Time-kill studies were performed to determine the mode of inhibition of the chemical compounds using *M. catarrhalis* and *S. aureus* according to the CLSI document M26-A.[11] The growth media was brain heart infusion and trypticase soy broth (Remel, Lenexa, KS), respectively. The same growth media were used in MIC and time-kill studies.

Binding Mode Assay

To determine if the hit compounds competed with either ATP or the amino acid for binding, IC$_{50}$ values were determined using aminoacylation assays as described.[7] The *P. aeruginosa* LysRS concentration was set at 0.01 μM. For competition with ATP, IC$_{50}$ values were determined in reactions containing varying ATP concentrations (25, 50, 100, 250, 500, 1000 μM). To determine the competition with lysine, the same assays were performed with varying concentrations of the amino acid (25, 50, 100, 200, 300 μM). The final compound concentrations in the IC$_{50}$ assays ranged from 200 to 0.4 μM.

Cytotoxicity Testing

The toxic effect of each compound on the growth of human cell cultures was determined as described[6] using human embryonic kidney 293 cells (HEK-293). The Trevigen

TACS MTT Cell Proliferation Assay Kit (Gaithersburg, MD) was used to assess the effects on human cell viability. The MTT assays were carried out in triplicate at each compound concentration. The concentration of test compounds ranged from 400 µg/mL to 25 µg/mL, and the concentration of the control, staurosporine, ranged from 1 to 0.001 µg/mL. Student's two-tiered *t* test was used to assess statistical significance.

Results

Sequence Analysis

The structure of LysRS has been primarily analyzed from studies of LysRS from *E. coli*. In *E. coli*, there have been two genes identified encoding two distinct forms of LysRS.[12] First, there is the constitutive *lys*S gene expressing one form of LysRS (LysS), and the second gene, *lys*U, which is an inducible gene resulting in the synthesis of the second form of LysRS (LysU). These two proteins share 93.3%/88.5% similarity/identity in their amino acid sequences. The crystal structures of both LysS (PDB ID: 1BBW and 1BBU) and LysU (PDB ID:1LYL and 1EIT/1E1O) have been solved in both an apo-structure and bound to lysine/ATP, respectively.[13,14] The amino acid sequence of LysRS from *P. aeruginosa* is equally similar to both forms of LysRS from *E. coli* and shares 72%/60% similarity/identity in amino acid conservation with each of them (**Fig. 1**). The crystal structure of LysRS has been solved from other bacterial sources, including that from *Burkholderia thailandensis*,[15] a member of the β-subdivision of the proteobacteria, and *Thermus thermophilus* (*Tth*),[16] from the Deinococcus-Thermus subphyla, and each shares amino acid conservation (69%/57% and 56%/44% similarity/identity, respectively) with LysRS from *P. aeruginosa.* In lieu of a crystal structure of *P. aeruginosa* LysRS, a comparison of the amino acid sequence of LysRS from *P. aeruginosa* with that of these homologs, from whom the crystal structures has been solved, allows an estimation of the structure of the active site regions in the *P. aeruginosa* enzyme.

The active site composed of the three structural motifs (motif 1, 2, and 3) is contained within the large C-terminal catalytic domain and is highly conserved (**Fig. 1**). Motif 1 appears to be involved in the dimerization of the two catalytic domains in the α2 dimer formation, whereas motifs 2 and 3 contain residues involved in substrate binding. The residues in the active site that have been shown to interact with the substrates lysine and ATP are mostly identical. The lysine *ε*-amino group interacts via hydrogen bonding with Tyr280, Glu428, and Glu240, whereas Asn424, Arg262, and Gly216 interact with the α-carboxylic acid group and Glu278 and Glu240 stabilize the α-amino group (*E. coli* numbering).[13,14] These residues are strictly conserved in all LysRS analyzed, with the only variation being that Asn424 is replaced with a proline in *Tth* LysRS. ATP binding is stabilized by hydrogen bonding between Glu414 and Glu421 and the α- and β-phosphates. The guanidinium group of Arg262 also interacts with α-phosphate, holding it in position for a nucleophilic attack by the carboxylic acid group of lysine. Arg480 and His270, along with magnesium ions, form salt bridges with the γ-phosphate, whereas Glu264, His270, and Asn271 make direct contacts with the γ-phosphate or the adenine ring. The specificity for ATP binding occurs as a result of hydrogen bonds formed between Asn271 and the N1 and 6-amino group of the adenine base. The adenine base of ATP is further stabilized by π-bonds, formed as it is stacked between the ring of Phe274 and the side chain of Arg480. The 2′ and 3′ hydroxyl groups of the ribose sugar form hydrogen bonds with Glu421. The amino acid residues interacting with and stabilizing ATP binding are strongly conserved in the five proteins aligned in **Figure 1**, with the Glu414 being replaced with an aspartic acid in the *Tth* LysRS.

The nucleotides forming the anticodon of tRNA^{Lys} are important identity elements for cognate aminoacylation by LysRS[17] and are recognized by amino acid residues in the small N-terminal domain of the enzyme. The crystal structure of LysRS bound to tRNA^{Lys} has been solved for both the *Tth* and *E. coli* enzymes.[16] The two tRNAs vary at the first nucleotide of the anticodon, which results in C-34 and a modified U-34 (mnm^{5}s^{2}U-34) in *Tth* and *E. coli* tRNA^{Lys}, respectively. The nucleotide in *P. aeruginosa* tRNA^{Lys} at this position corresponds to U-34, but any modification is unknown.[18] In *Tth* LysRS, this nucleotide is stabilized on one side by the hydrophobic residues Phe71, Tyr84, and Phe112 (*Tth* numbering) and on the other side by Lys115. The hydrophobic residues are conserved with only slight variations. However, Lys115 is not well conserved (**Fig. 1**), which may be understood considering the variation at C-34 in the anticodon of the different tRNAs. The central anticodon nucleotide, U-35, is stacked between Phe71 and U-36 and further stabilized by hydrogen bonds formed with Arg64, Gln82, and Thr114. These residues are strictly conserved in the sequences analyzed, with the similar replacement of threonine by serine in *P. aeruginosa* LysRS. The nucleotide U-36 is stacked against U-35 on one side and stabilized by hydrogen bonding with His73, Thr116, and Glu118 on the other side. The residues at positions 73 and 116 are only partially conserved, but Glu118, which is a key residue that distinguishes a U from a C at this nucleotide position, is highly conserved. This level of sequence conservation indicates that recognition of the anticodon follows a similar mechanism in the different enzymes.

Protein Expression and Enzymatic Characterization

P. aeruginosa LysRS was purified to greater than 98% homogeneity as described in the "Methods and Materials"
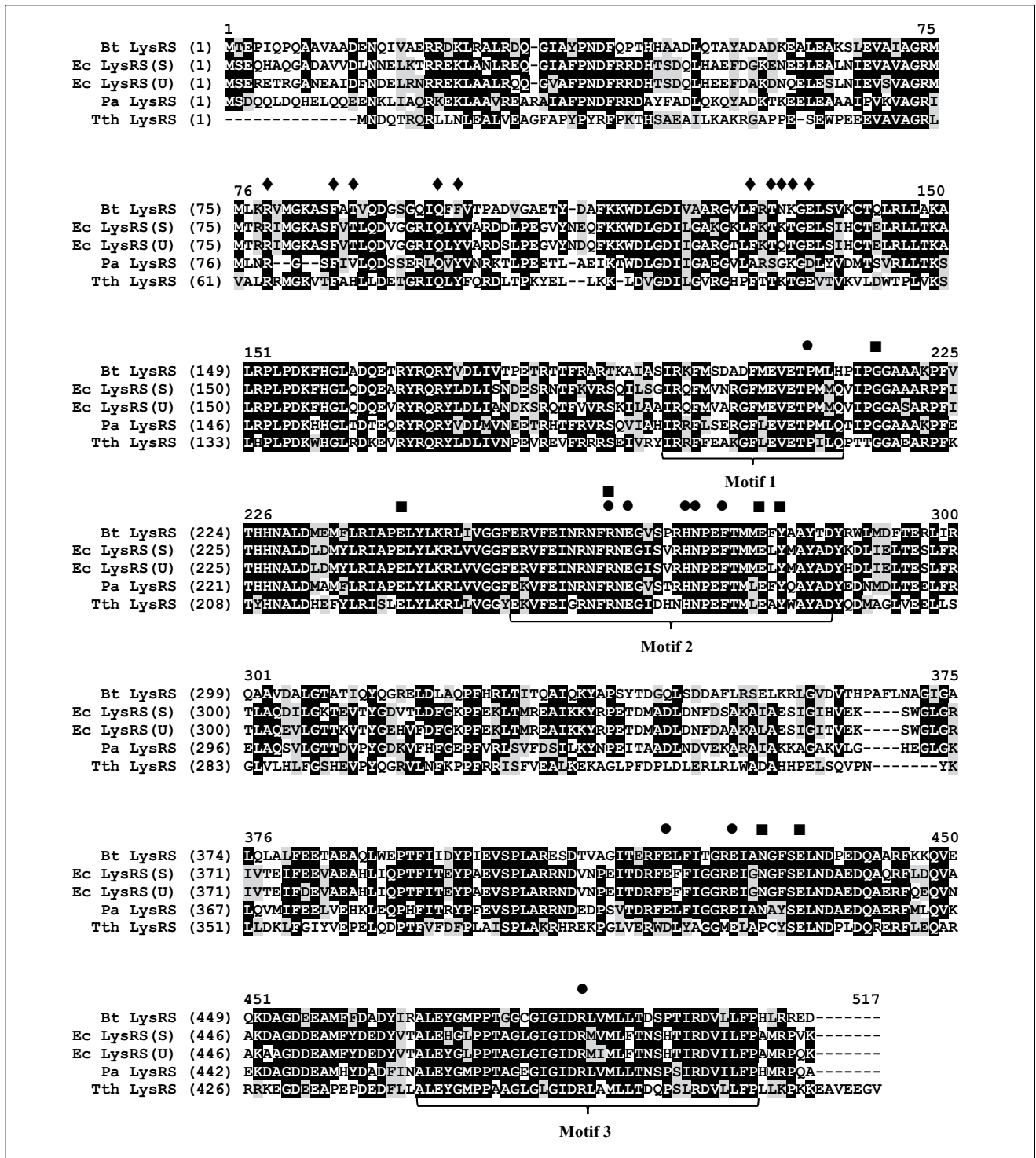
Figure 1. Alignment of the amino acid sequence of *P. aeruginosa* lysyl-tRNA synthetase (LysRS) with homologs. The protein sequences of LysRS from Bt, *Burkholderia thailandensis*; Ec, *E. coli*; Pa, *P. aeruginosa*; and Tth, *T. thermophilus* were downloaded from the National Center for Biotechnology Information. Accession numbers for LysRS protein sequences of *B. thailandensis, E. coli* (S), *E. coli* (U), *P. aeruginosa*, and *T. thermophilus* are AOJ57238, AAA83071, Q8FAT5, NP_252390, and P41255, respectively. Sequence alignments were performed using Vector NTI Advance 11.5.4 (Invitrogen). Identical residues are indicated by white letters on a black background, whereas similar sequences are black letters on a gray background. The three structural motifs (1, 2, and 3) are indicated. Amino acids that interact with adenosine triphosphate (●), lysine (■), and tRNA^Lys (♦) are indicated above the aligned residues.
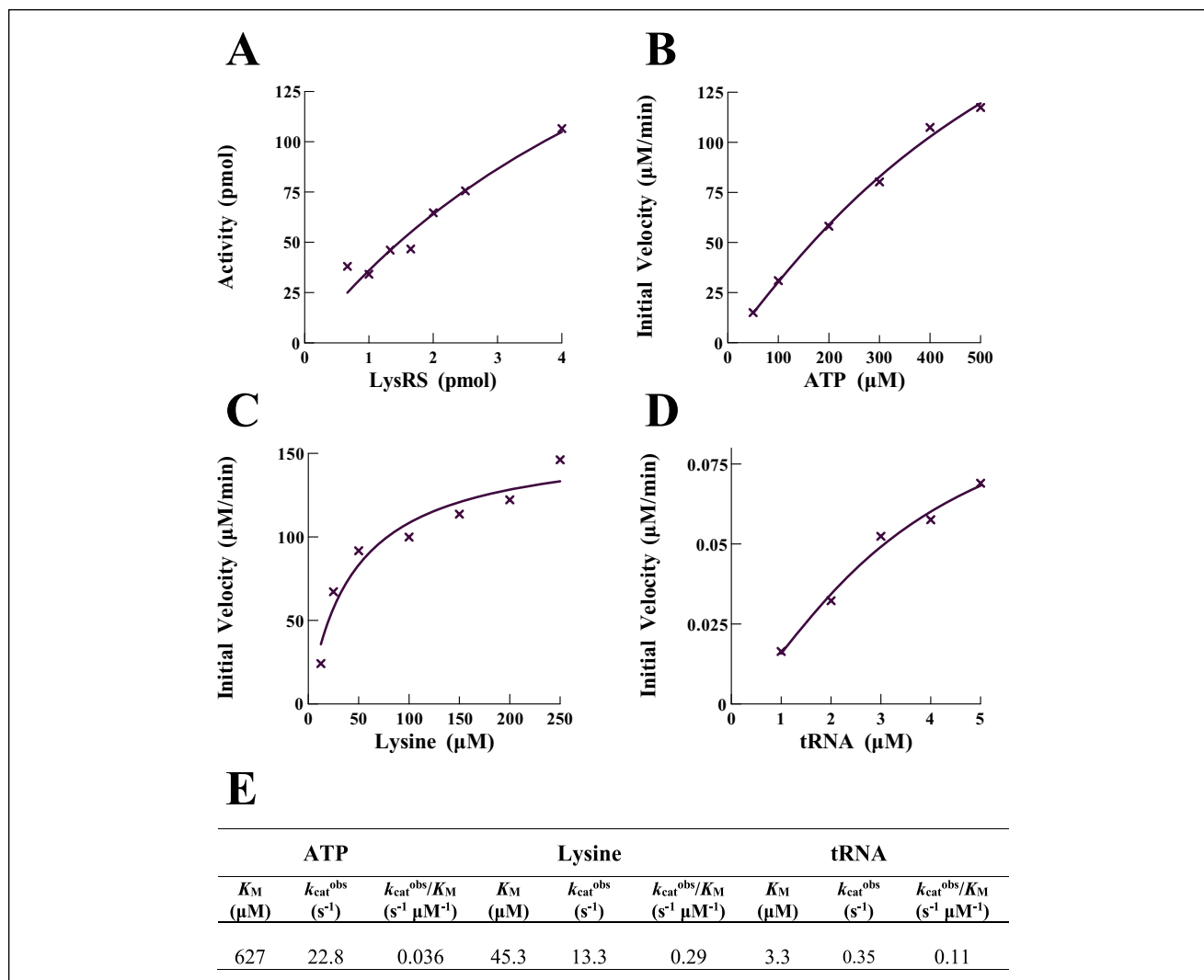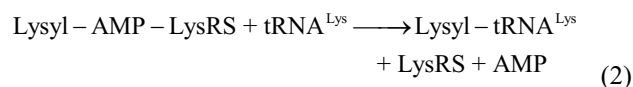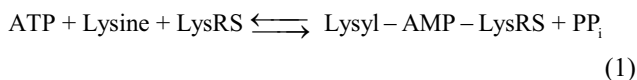
Figure 2. Determination of the activity of *P. aeruginosa* lysyl-tRNA synthetase (LysRS) and the kinetic parameters governing interactions with its three substrates: adenosine triphosphate (ATP), lysine and tRNA^{Lys}. *P. aeruginosa* LysRS was titrated into the aminoacylation assay (**A**) as described in the "Methods and Materials" section in amounts varying from 0.5 to 4 pmol enzyme. Background activity was minimal and was subtracted from values at all concentrations of LysRS. Initial velocities for the interaction of LysRS with both ATP (**B**) and lysine (**C**) were determined using the ATP:PP$_i$ exchange reaction. The initial velocity for the interaction of LysRS with tRNA was determined using the aminoacylation reaction (**D**). The concentration of LysRS in the aminoacylation reactions and the exchange reactions was 0.03 μM and 0.1 μM, respectively. Initial velocities were determined, and the data were fit to a Michaelis-Menten steady-state model using XL*fit* 5.3 (IDBS) to determine K_M and V_{max}. The kinetic parameters were calculated from these data for the interaction of LysRS with the three substrates (**E**).

section (**Suppl. Fig. S1**). The primary function of LysRS is to attach lysine through the aminoacylation reaction to the cognate tRNA, and LysRS from *P. aeruginosa* was initially assayed for this activity (**Fig. 2A**). The aminoacylation reaction follows the typical two-step mechanism in which the enzyme condenses lysine and ATP, forming an aminoacyl-adenylate intermediate with a pyrophosphate as the leaving group (1). This is followed by the transfer of the activated amino acid intermediate to the 5′-end of the tRNA (2).

$$ATP + Lysine + LysRS \rightleftharpoons Lysyl - AMP - LysRS + PP_i \tag{1}$$

$$Lysyl - AMP - LysRS + tRNA^{Lys} \longrightarrow Lysyl - tRNA^{Lys} + LysRS + AMP \tag{2}$$

The first step of the reaction is reversible in the absence of the tRNA and can be monitored using the ATP:PP$_i$

exchange reaction to determine the interaction of the enzyme with its substrates, ATP and lysine. The ATP:PP$_i$ exchange assay monitors the ability of the enzyme to form an aminoacyl-adenylate, followed by reversal of the reaction and subsequent radioactive labeling of ATP in the presence of saturating amounts of [^{32}P]PP$_i$ (**Suppl. Fig. S2**). Initial velocities were determined in reactions when the concentration of lysine was held constant (2 mM) and ATP concentrations were varied (50–500 μM), and the resulting data were fit to the Michaelis-Menten steady-state model (**Fig. 2B**). From these data, the kinetic parameters K_M and V_{max} were obtained and used to determine the observed turnover number (k_{cat}^{obs}). The kinetic parameters, K_M, k_{cat}^{obs}, and k_{cat}^{obs}/K_M, for interaction of *P. aeruginosa* LysRS with ATP were determined to be 627 μM, 22.8 s^{-1}, and 0.036 s^{-1}μM^{-1}, respectively. To characterize the interaction of *P. aeruginosa* LysRS with the amino acid, the concentration of ATP was held constant (2 mM), the concentration of lysine was varied (12.5–250 μM), and the data were also fit to the Michaelis-Menten steady-state model (**Fig. 2C**). The K_M, k_{cat}^{obs}, and k_{cat}^{obs}/K_M for the interaction of LysRS with lysine were determined to be 45.3 μM, 13.3 s^{-1}, and 0.29 s^{-1}μM^{-1}, respectively.

The second step of the aminoacylation reaction involves the transfer of the activated amino acid to the cognate tRNA and tends to be the rate-limiting step in the total reaction. In the assays used to determine the kinetic interaction of LysRS with tRNA^{Lys}, the concentration of ATP was 2.5 mM, of lysine was 75 μM, and of tRNA^{Lys} varied from 1 to 5 μM (**Fig. 2D**). In these reactions, the kinetic parameters K_M, k_{cat}^{obs}, and k_{cat}^{obs}/K_M were determined to be 3.3 μM, 0.35 s^{-1}, and 0.11 s^{-1}μM^{-1}, respectively (**Fig. 2E**).

Chemical Compound Screening

Using SPA technology, two chemical compound libraries were screened against the function of *P. aeruginosa* LysRS for identification of compounds with inhibitory effects. One compound library, the Anti-infective Library from TimTec LLC, contained 890 distinct compounds of low-molecular-weight, druglike molecules with scaffolds found in antiseptic agents with antibacterial, antifungal, and antimicrobial activities. The second compound library was from the NatProd Collection from Microsource Discovery Systems and was composed of 800 natural products, including simple and complex oxygen heterocycles, alkaloids, sesquiterpenes, diterpenes, pentacyclic triterpenes, and sterols. Chemical compounds were dissolved in DMSO for a final concentration of 3.3 mM.

The concentration of LysRS for use in SPAs was 0.03 μM (~1.6 pmol), which was in the linear range of the titration curve and allowed maximum sensitivity to enzymatic inhibition (**Fig. 2A**). Next, tRNA was titrated into the assay

to determine the concentration for use in the screening assay with the goal that the amount of tRNA^{Lys} used would be within the linear region of the reaction-detection time (**Suppl. Fig. S3**). From the titration reactions, 4 μM tRNA^{Lys} was selected for use in the screening assays. The screening reactions contained 2 μL of compound dissolved in 100% DMSO, resulting in final DMSO concentrations of 4%. Therefore, the function of *P. aeruginosa* LysRS was analyzed in the presence of increasing amounts of DMSO. There was no significant decrease of activity observed in aminoacylation reactions containing up to 10% DMSO (**Suppl. Fig. S4**).

Initial screening assays contained chemical compounds at a concentration of 132 μM and were carried out as single-point assays. Compounds that inhibited the enzymatic activity by 50% or more were considered hit compounds. Thirty compounds that were identified as hits in the initial screen were reassayed in triplicate. From these 30 initial hits, after confirmation and structure analysis, three compounds (BM01D09, BT06F11, and BT08F04) were selected for additional analysis (**Fig. 3**). Initially, the IC$_{50}$ values were determined using aminoacylation assays, in which the hit compounds were serially diluted from 200 μM to 0.4 μM (**Fig. 4A–C**). The IC$_{50}$ values for inhibition of LysRS for BM01D09, BT06F11, and BT08F04 were determined to be 17, 30, and 27 μM, respectively.

Microbiological Assays

The three confirmed hit compounds were tested in broth microdilution assays to determine MIC values. The tests were performed against a panel of 10 pathogenic bacteria, including efflux pump mutants of *E. coli* and *P. aeruginosa* and a hypersensitive strain of *P. aeruginosa* (**Suppl. Table S1**). The three compounds exhibited modest MICs against the mutant forms of *E. coli* and *P. aeruginosa*. However, none of the compounds inhibited the growth of the wild-type strains of these bacteria. The compounds also had moderate activity against the growth of gram-positive *E. faecalis* and *S. aureus* but lacked activity against *S. pneumoniae*. All three compounds were observed to have good activity against *M. catarrhalis*.

To determine the global mode of inhibition of the growth of bacterial cultures, the compounds were tested in time-kill kinetic assays. The compounds were tested at concentrations of four times the MIC and at time points between 0 and 24 h against cultures of *S. aureus* and *M. catarrhalis* (**Fig. 4D,E**). All three compounds were shown to be bacteriostatic against the gram-positive pathogen, *S. aureus*. The bacteria displayed constant growth but a decrease in colony-forming units of 2 to 6 log$_{10}$ compared with the control during the initial 6 h. BM01D09 was also observed to inhibit growth of *M. catarrhalis* in a bacteriostatic mode of

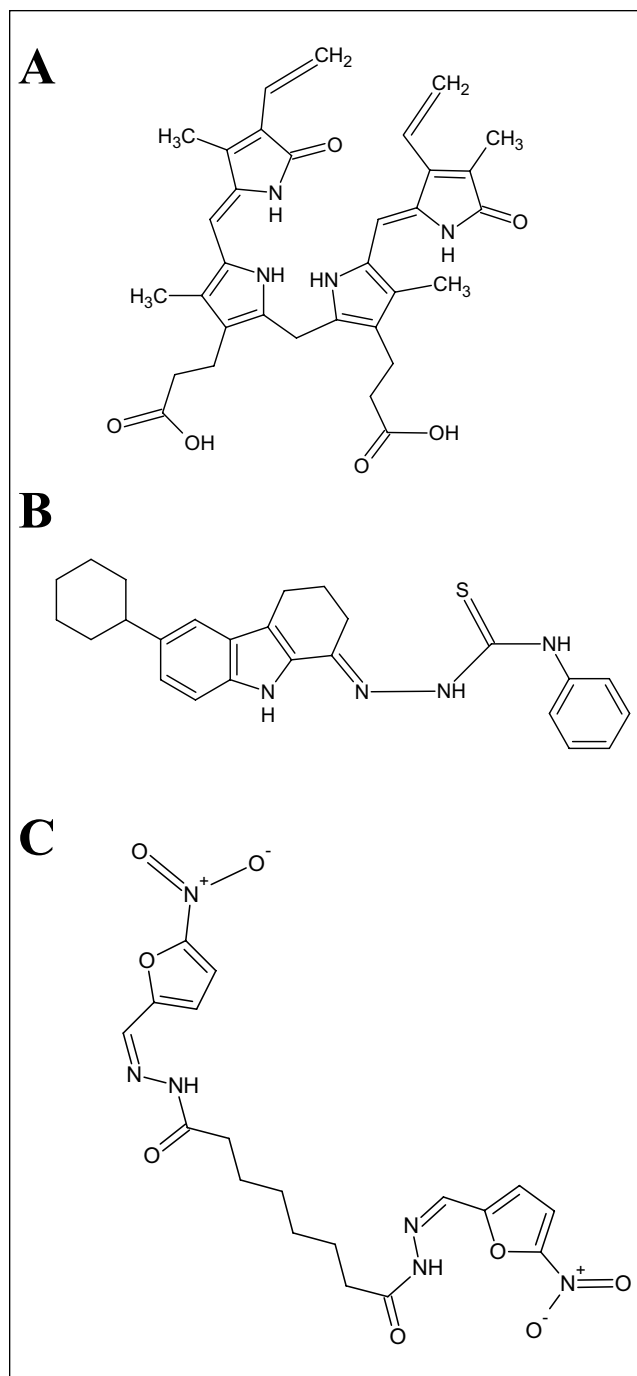**Figure 3.** The chemical structure of the hit compounds. The structure of BM01D09 (**A**), BT06F11 (**B**), and BT08F04 (**C**).

inhibition. However, BT06F11 and BT08F04 exhibited a bactericidal mode of action and inhibited bacterial growth by killing the bacteria in cultures of *M. catarrhalis*.

Mechanism of Action

The binding sites for the substrates ATP and lysine are located in the active region of the catalytic domain of

LysRS, and the small molecules identified are similar in molecular mass to that of both lysine and ATP. Therefore, it was important to test whether the hit compounds interfered with substrate binding as a possible mechanism of inhibition. The three inhibitor compounds were tested in competition assays with varying amounts of ATP or lysine. The mechanism of inhibition with respect to ATP was determined at various ATP concentrations, ranging above and below the K_M (25 to 1000 μM) while holding the amino acid concentration constant. To determine the mechanism of inhibition with respect to the amino acid, the same assay was used, except ATP was held constant at saturating concentrations (2.5 mM) and the IC_{50} was determined at different concentrations of lysine (25 to 300 μM). The lysine concentrations ranged from approximately twofold below to sixfold above the K_M. In these assays, the IC_{50} values for BT06F11 remained constant at all concentrations of both ATP and lysine, which is characteristic of a noncompetitive inhibitor.[19]

In assays containing BM01D09, there was no change in the IC_{50} at varying concentrations of lysine. However, as the concentration of ATP was increased, the IC_{50} also increased (**Fig. 5A**). For inhibitors that are competitive with ATP and noncompetitive with the amino acid (lysine), the presence of the noncompetitive inhibitor does not interfere with the competitive inhibition of ATP, and the relationship is as follows:

$$IC_{50} = \left(1 + [ATP]/K_M^{ATP}\right)K_i.^{18}$$

In the competition assays containing BT08F04, when the inhibition was analyzed in the presence of increasing concentrations of ATP, the IC_{50} was observed to increase, indicating competitive inhibition (**Fig. 5B**). As the lysine concentration was increased, the IC_{50} values decreased, which is characteristic of an uncompetitive inhibitor (**Fig. 5C**). For the LysRS aminoacylation assay with inhibitors that are lysine uncompetitive and ATP competitive, the relationship is as follows:

$$IC_{50} = \left(1 + [ATP]/K_M^{ATP}\right)\left(1 + K_M^{Lys}/[Lysine]\right)K_i.^{18}$$

These equations were used to calculate K_i for the hit compounds using IC_{50} values determined at 75 μM lysine and 2.5 mM ATP. In multiple determinations, BM01D09 and BT08F04 had mean K_i values of 3.5 and 3.4 μM, respectively, when tested against *P. aeruginosa* LysRS.

Cytotoxicity Assays

An initial step in drug discovery is the prediction of chemical toxicity using human cell systems. The potential for inhibition of human cytoplasmic LysRS (hcLysRS) or
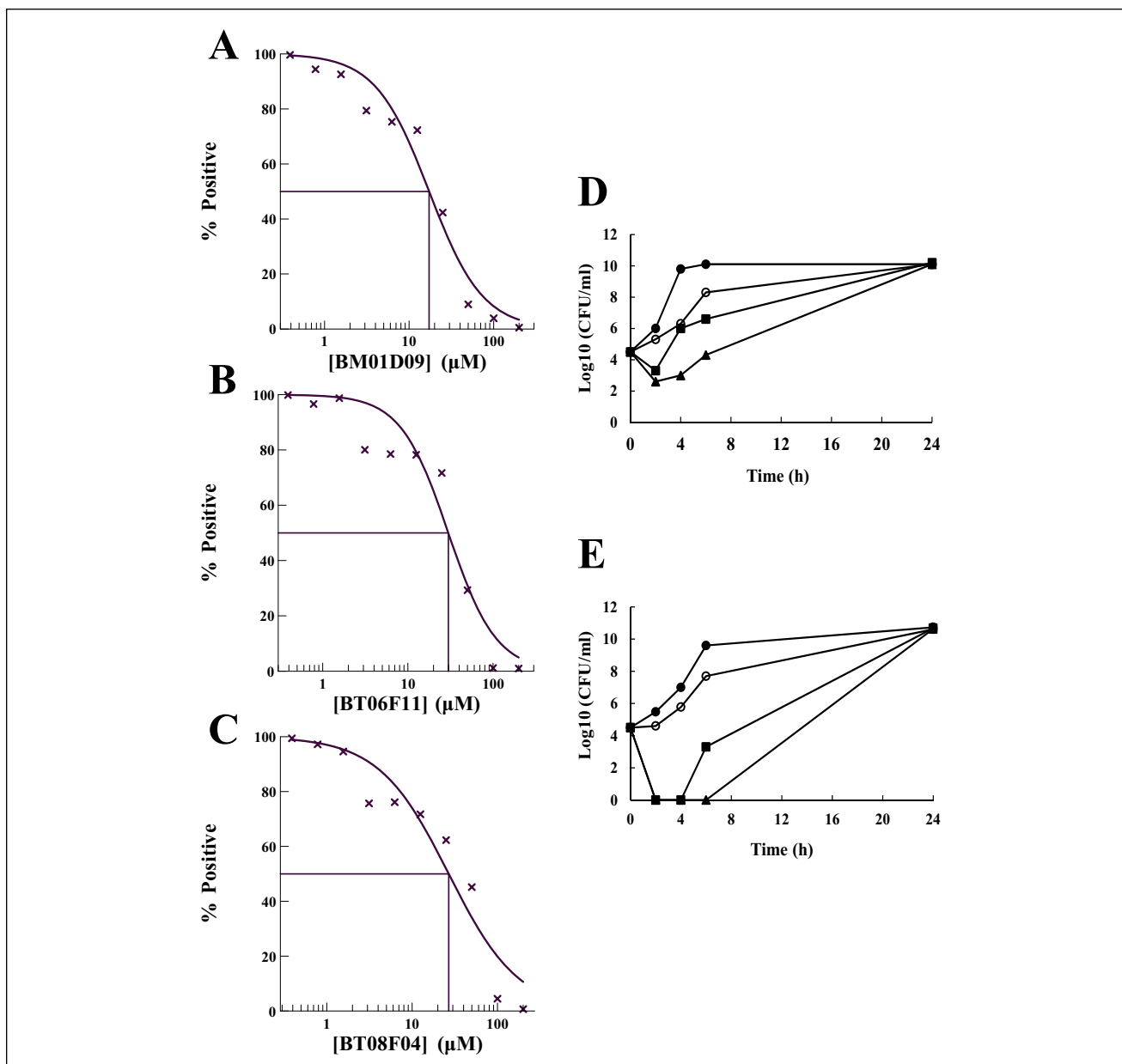
Figure 4. Characterization of enzymatic and bacterial inhibition by BM01D09, BT06F11 and BT08F04. IC_{50} values for the inhibitory potency of BM01D09 (**A**), BT06F11 (**B**), and BT08F04 (**C**) against the aminoacylation activity of *P. aeruginosa* LysRS were 17 μM, 30 μM, and 27 μM, respectively. The compounds were serially diluted from 200 μM to 0.4 μM into aminoacylation assays containing *P. aeruginosa* LysRS at 0.01 μM. "% Positive" indicates the percentage of activity observed relative to activity in assays in which only DMSO was added to the assay in the absence of compound. The curve fits and IC_{50} values were determined using the sigmoidal dose-response model in XL*fit* 5.3 (IDBS). The activity of the hit compounds against the growth of cultures containing (**D**) *S. aureus* and (**E**) *M. catarrhalis* bacteria were determined using broth microdilution susceptibility testing. Compounds were added to bacterial cultures at 4×minimum inhibitory concentration. Samples were analyzed by plating and determination of colony-forming units at 0, 2, 4, 6, and 24 h. Open circles (○) represent cultures containing BM01D09, filled squares (■) represent cultures containing BT06F11, and filled triangles (▲) represent cultures containing BT08F04. Filled circles (●) represent the growth of control cultures containing only DMSO in the absence of compound.
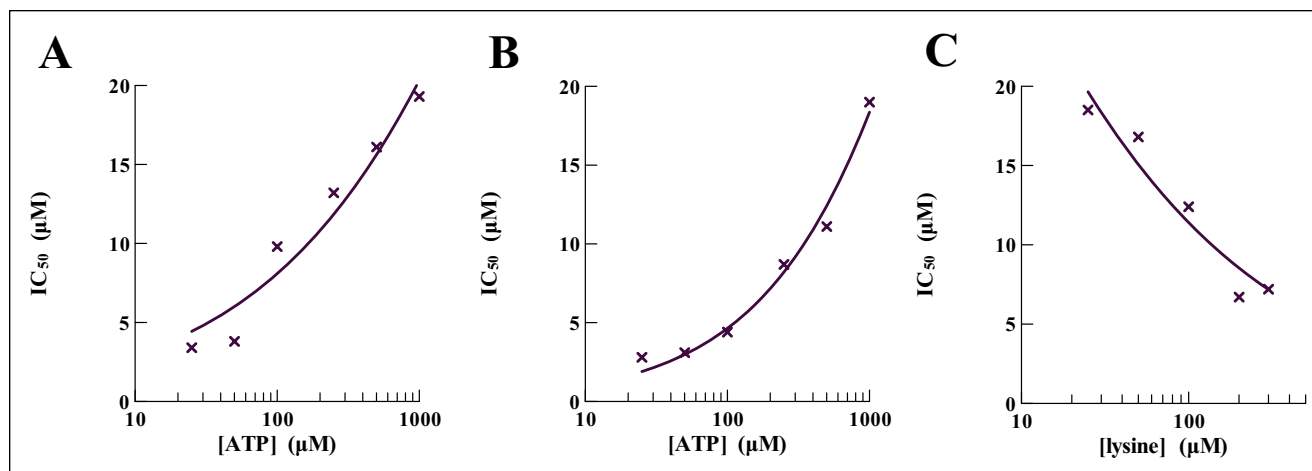
Figure 5. Hit compound competition with adenosine triphosphate (ATP) and lysine for active site binding. In aminoacylation assays containing BM01D09, the IC_{50} values increased as the concentration of ATP was increased, indicating competitive inhibition (**A**). In competition assays containing BT08F04, the IC_{50} values increased with increasing concentrations of ATP, indicating competitive inhibition (**B**), and decreased when the concentration of lysine increased, which is characteristic of an uncompetitive inhibitor (**C**). *P. aeruginosa* lysyl-tRNA synthetase (LysRS) concentration was set at 0.01 µM in these assays. Background amounts of free [^{3}H]Lys in the absence of LysRS were insignificant.

another potential target in human cells is a concern, so the cytotoxicity of the test compounds was determined in HEK-293 cell cultures using the Trevigen TACS MTT Cell Proliferation Assay Kit. MTT assays were performed at compound concentrations ranging between 25 and 400 µg/mL for 24 h. BT06F11 was not observed to be toxic to cell growth at any concentrations tested (**Fig. 6A**). BM01D09 exhibited a cytotoxicity CC_{50} value of 370 µg/mL (**Fig. 6B**). However, BT08F04 inhibited HEK-293 cell cultures with a CC_{50} of 61 µg/mL (**Fig. 6C**). Staurosporine, a potent inhibitor of human cell culture growth, was used as a comparator in the studies. Staurosporine was observed to inhibit cell growth with a CC_{50} of 0.003 µg/mL (**Fig. 6D**).

Discussion

Bacterial resistance has become widespread and constitutes a threat to modern health care. A noteworthy development is that strains of certain bacteria, including *P. aeruginosa*, have arisen that are resistant to all approved antibacterials. These findings increase the impetus for identifying new types of antibiotics that are effective against different targets than those of current therapies. The aaRSs are vital for cell growth in all organisms, yet bacterial forms are divergent in amino acid primary structure from those of their eukaryotic counterparts, making them ideal targets for development of new antibacterial agents. An example of this is that the human cytoplasmic and mitochondrial LysRS (encoded by the same gene and expressed by means of alternative splicing[20]) contain only 34.8% and 33.2% amino

acid conservation when compared with that of *P. aeruginosa* LysRS. In contrast, the two forms of LysRS from *E. coli* (LysRS-S and LysRS-U) contain more than 60% conserved amino acids compared with that of *P. aeruginosa* LysRS. Thus, the eukaryotic and prokaryotic forms of LysRS are much more diverse than forms of LysRS within the prokaryotic grouping, which indicates that an inhibitor of bacterial LysRS may have broad-spectrum activity against other bacteria yet no effect on the eukaryotic counterpart. The structures of *E. coli* and *Tth* LysRS were previously solved, and when the amino acid sequence from these enzymes was compared with that of *P. aeruginosa* LeuRS, the critical amino acid residues were found to be strictly conserved, indicating likely conservation of structure as well as function.

P. aeruginosa LysRS was expressed and purified, and the kinetic parameters for activity were determined relative to its three substrates, lysine, ATP, and tRNA^{Lys}. Using SPA technology, natural product (800) and synthetic (890) compounds were screened to identify compounds with the ability to inhibit enzymatic activity. The screening assays were robust and resulted in Z′ and Z factors of approximately 0.635 and 0.434, respectively, across all plates. The signal-to-background ratio of the compounds to the EDTA controls was approximately 4.2:1. After confirmation assays and structure analysis, three compounds (BM01D09, BT06F11, and BT08F04) were selected for further analysis. All three of the hit compounds inhibited the function of *P. aeruginosa* LysRS with IC_{50} values in the low micromolar range. The three compounds were tested for the ability to inhibit the
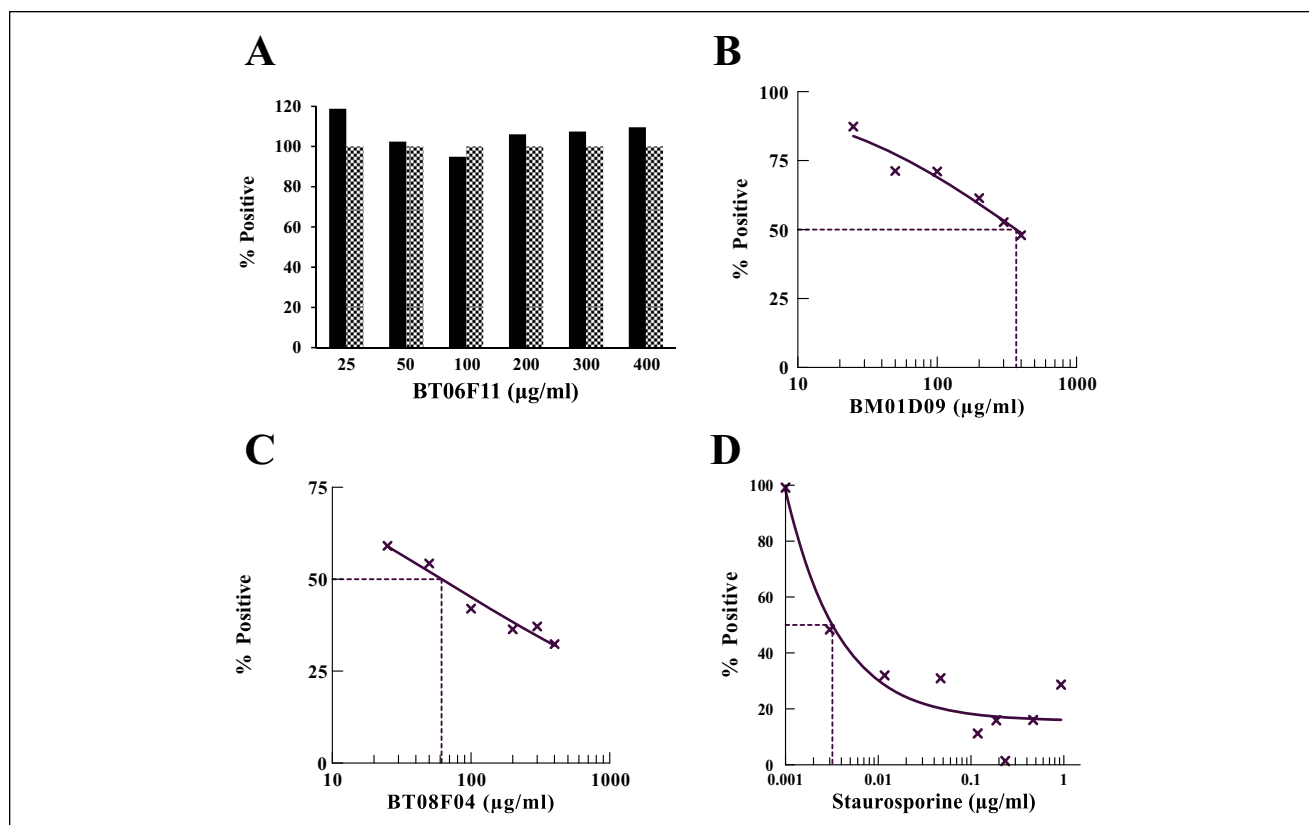
Figure 6. Toxicity of the hit compounds in cultures of human cell lines. Toxicity of the hit compounds was measured using human embryonic kidney 293 (HEK-293) cell cultures. The compound concentration ranged from 25 to 400 µg/mL. The data points represent the average values for assays carried out in triplicate. "% Positive" indicates the percentage of activity observed relative to activity in assays in which only DMSO was added to the assay in the absence of compound. The curve fits and CC_{50} values were determined using the sigmoidal dose-response model in XL*fit* 5.3 (IDBS). (**A**) BT06F11 was not toxic at any concentration tested. The hatched column represent the growth of cultures in the positive control assays, and the solid column represents the cultures containing the indicated concentration of BT06F11. The CC_{50} values for (**B**) BM01D09 and (**C**) BT08F04 were 370 µg/mL and 61 µg/mL, respectively. The control staurosporine (**D**) was serially diluted in assays from 1 to 0.001 µg/mL and exhibited a CC_{50} of 0.003 µg/mL.

growth of 10 clinically important bacteria. All three compounds had modest MICs against two gram-positive bacteria (*E. faecalis* and *S. aureus*) and also modest MICs against the efflux pump mutants of *E. coli* and *P. aeruginosa* but almost no activity against the wild-type forms of these gram-negative bacteria, indicating that this lack of inhibition is likely efflux mediated. There was, however, good activity against the gram-negative bacteria, *M. catarrhalis*. The three hit compounds displayed a bacteriostatic global mode of action against the gram-positive *S. aureus*, but when tested against *M. catarrhalis*, BM01D09 was observed to be bacteriostatic, whereas both BT06F11 and BT08F04 inhibited growth by killing the bacteria. Static inhibition (bacteriostatic) of the bacteria would be the expected result of inhibition of the aminoacylation activity of a bacterial aaRS. This occurs because inhibition of an aaRS mimics amino acid starvation in protein biosynthesis, which would elicit the stringent response resulting in static growth of the

cultures but not necessarily killing the bacteria. The bactericidal activity of BT06F11 and BT8F04 against gram-negative bacteria may potentially be due to an unknown secondary role of LysRS, such as has been observed for many eukaryotic forms of the enzyme. Also, paralogs have been observed for many members of the aaRS group of enzymes,[21] and inhibition of the activity of an essential paralog could potentially lead to the bactericidal activity observed. There is also the possibility that the bactericidal activity of the compounds on the bacteria may be due to an off-target effect in addition to inhibition of LysRS.

The compound BM01D09 from the natural product library was identified as bilirubin, a catabolite of heme metabolism. Recently, a number of studies have identified bilirubin as a major cytoprotectant against a number of physiological ailments in adults.[22] Human newborns are highly susceptible to infection by an array of pathogenic organisms, yet through an innate immune response, they

have the ability to circumvent many of these infections. However, several innate immune mechanisms may not be fully developed in neonates, and an adaptive immunity is not present, leaving infants susceptible to some infections.[23] Almost all newborn infants display some level of jaundice caused by elevated levels of bilirubin. At present, there has been no direct link between the elevated levels of bilirubin in infants and the ability to fight off bacterial infections. However, recent evidence indicates that bilirubin is inhibitory to the growth of gram-positive bacterial cultures,[24] suggesting that the higher level of bilirubin in infants may play an early role in innate immunity. In competition assays, bilirubin was shown to be noncompetitive with lysine but was competitive with ATP for LysRS binding. ATP utilization as a substrate is prevalent in both bacterial and eukaryotic cells, and nonspecific inhibition resulting from ATP competition might decrease the utility of the compound as a potential drug candidate. However, if the competition with ATP for binding is specific for LysRS, this would increase the potential of bilirubin as a therapeutic agent. An example is the specificity of cladosporin as a potent ATP competitive inhibitor of the eukaryotic LysRS.[25] The cladosporin study, along with the fact that this natural product library was screened in our laboratory against several aaRS enzymes (PheRS, GluRS, MetRS, GlnRS, LeuRS, ProRS, ArgRS, AspRS, and HisRS) from *P. aeruginosa*, all of which have ATP as a substrate, and the fact that there was no inhibition observed, is indicative of specificity. When tested for toxicity in human cell cultures, bilirubin was observed to have a CC_{50} of 370 μg/mL, which is 6- to 20-fold higher than the MICs against cultures of the susceptible bacteria, which provides an acceptable starting place for development of a lead series of antibacterial compounds.

The hit compound BT06F11 (PubChem Compound Database; CID 135479627) (1-[(~›E‹)-(6-cyclohexyl-2,3,4,9-tetrahydrocarbazol-1-ylidene)amino]-3-phenylthiourea) was searched against the PubChem BioAssay Database for biological activity (accessed February 22, 2019), but no biological information was found associated with this compound (https://pubchem.ncbi.nlm.nih.gov/compound/135479627). BT06F11 was not observed to be competitive with either ATP or the amino acid when tested against these substrates. It is possible that this compound could interfere with binding of the tRNA or bind elsewhere on the enzyme, possibly inhibiting function by an allosteric mechanism. However, future structural studies will be required to better understand the mechanism of action. Also, this compound was not toxic to human cell cultures at any concentration tested, which is very advantageous for the development of a bacterial inhibitor.

The hit compound BT08F04 (PubChem Compound Database; CID 9640850) (~›N‹,~›N‹'-bis[(~›E‹)-(5-nitrofuran-2-yl)methylideneamino]decanediamide) was also searched against the PubChem BioAssay Database for

biological activity (accessed February 22, 2019), and no biological information was found associated with this compound. Interestingly, BM08F04 is composed of two nitrofurans attached to each end of a molecule composed of two methylformic hydrazide moieties separated by a six-carbon chain. Nitrofurans are a class of synthetic broad-spectrum antibiotics including furazolidone, furaltadone, nitrofurantoin, and nitrofurazone.[26] The nitrofurans are particularly active against gram-positive bacteria, but little activity has been observed against *P. aeruginosa*. The issue of toxicity (CC_{50} = 60 μg/mL) to human cell cultures could be an obstruction for the development of this compound as an antibacterial agent.

With concern about the global rate at which pathogens are becoming resistant to existing antibiotics and causing significant health-related problems, the interest in finding new compounds has increased. Targeting the bacterial aaRSs has been effective, and numerous new compounds have been identified that inhibit the bacterial forms of these enzymes, even though only one antibiotic, mupirocin, is currently being marketed. There are numerous aaRS inhibitors that have been discovered and developed by a variety of research groups that have gone into preclinical and clinical studies yet have not been marketed for various reasons.[27] The primary reason appears to be the development of resistance against compounds targeting single aaRS enzymes; however, there has been some success in overcoming this issue by targeting more than one aaRS at a time. At present, the bacterial form of LysRS has been identified as a target for only one of these newly identified compounds. During a high-throughput phenotypic screen, a pyrazolopyrimidinedione compound series was identified as a LysRS inhibitor by a group at Pfizer Worldwide Research and Development.[28] This compound was also shown to be an ATP competitor, but additional work has not been published. Three compounds were identified as inhibitors of LysRS in this study, and additional structure-activity relationship studies to enhance potency will be required to determine if these compounds have the potential for development as antibacterial agents.

Declaration of Conflicting Interests

Funding

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