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Corona, A., Palmer, S. O., Zamacona, R., Mendez, B., Dean, F. B., & Bullard, J. M. (2018). Discovery and characterization of chemical compounds that inhibit the function of aspartyl-tRNA synthetase from Pseudomonas aeruginosa. SLAS DISCOVERY: Advancing Life Sciences R&D, 23(3), 294-301. https://doi.org/10.1177/2472555217744559

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SLAS Discovery
2018, Vol. 23(3) 294–301
© 2017 Society for Laboratory
Automation and Screening
DOI: 10.1177/2472555217744559
journals.sagepub.com/home/jbx
SAGE

Discovery and Characterization of Chemical Compounds That Inhibit the Function of Aspartyl-tRNA Synthetase from *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa, an opportunistic pathogen, is highly susceptible to developing resistance to multiple antibiotics. The gene encoding aspartyl-tRNA synthetase (AspRS) from *P. aeruginosa* was cloned and the resulting protein characterized. AspRS was kinetically evaluated, and the K_M values for aspartic acid, ATP, and tRNA were 170, 495, and 0.5 µM, respectively. AspRS was developed into a screening platform using scintillation proximity assay (SPA) technology and used to screen 1690 chemical compounds, resulting in the identification of two inhibitory compounds, BT02A02 and BT02C05. The minimum inhibitory concentrations (MICs) were determined against nine clinically relevant bacterial strains, including efflux pump mutant and hypersensitive strains of *P. aeruginosa*. The compounds displayed broad-spectrum antibacterial activity and inhibited growth of the efflux and hypersensitive strains with MICs of 16 µg/mL. Growth of wild-type strains were unaffected, indicating that efflux was likely responsible for this lack of activity. BT02A02 did not inhibit growth of human cell cultures at any concentration. However, BT02C05 did inhibit human cell cultures with a cytotoxicity concentration (CC_{50}) of 61.6 µg/mL. The compounds did not compete with either aspartic acid or ATP for binding AspRS, indicating that the mechanism of action of the compound occurs outside the active site of aminoacylation.

Keywords

aspartyl-tRNA synthetase, protein synthesis, *Pseudomonas aeruginosa*, drug discovery, antibiotics

Introduction

The report of the World Health Organization in 2014 on the global surveillance of antimicrobial resistance stated that antibiotic resistance is no longer a prediction for the future; it is a current worldwide problem, and is putting at risk the treatment of an increasing range of infections.[1] Bacteria have genetic mechanisms for the development of resistance against antibiotics. They may demonstrate natural resistance to certain types of antibiotics, yet the emerging problem is their ability to become resistant by genetic mutation or by acquiring genetic information from other pathogens to multiple compounds in the current armamentarium of antibiotics. These multi-drug-resistant (MDR) bacteria have become a major threat during the treatment of nosocomial and community-acquired infections, and because of their involvement in chronic infections. *Pseudomonas aeruginosa* is opportunistic and has developed resistance mechanisms, both intrinsic and acquired, against multiple antibiotics. Because of this capacity for developing resistance, it is now part of an elevated group of pathogenic MDR bacteria referred to as "superbugs."[2]

Aminoacyl-tRNA synthetases are a family of enzymes with the primary function of attaching the correct amino acid to the cognate tRNA. As such, this entire family is indispensable in its unique role in protein biosynthesis. Aspartyl-tRNA synthetase (AspRS), encoded by the *asp*S gene, catalyzes the reaction resulting in the aminoacylation of $tRNA^{Asp}$. AspRS is a class II aminoacyl-tRNA synthetase characterized by an active site structure formed by three structural motifs. AspRS is further grouped, along with asparginyl- and lysinyl-tRNA synthetases (AsnRS and

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Received Sept 12, 2017, and in revised form Oct 27, 2017. Accepted for publication Nov 6, 2017.

Supplementary material is available online with this article.

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LysRS), into subclass IIb, based on the presence of an oligonucleotide binding (OB) domain within its structure. There are two forms of AspRS. One form only aminoacylates $tRNA^{Asp}$, producing Asp-$tRNA^{Asp}$; this form is called the discriminating form (D-AspRS). The other form of AspRS, called nondiscriminating AspRS (ND-AspRS), will attach aspartic acid to either $tRNA^{Asp}$, forming Asp-$tRNA^{Asp}$, or $tRNA^{Asn}$, producing Asp-$tRNA^{Asn}$. The aspartic acid attached to $tRNA^{Asn}$ is then converted to asparagine in a transamidase reaction catalyzed by the heterotrimeric amidotransferase GatCAB.[3] Unlike many other bacteria in the proteobacteria phylum, *P. aeruginosa* contains the nondiscriminating form of AspRS.

AspRS from *P. aeruginosa* was cloned and purified, and the kinetic parameters (K_M, V_{max}, and the observed reaction rate, k_{cat}^{obs}) for interaction with its substrates were experimentally determined. Using scintillation proximity assay (SPA) technology, AspRS was developed into a screening platform[4] and used to screen 1690 natural and synthetic compounds for inhibitory activity. Two compounds were identified that inhibited the activity of the enzyme. These compounds were further characterized for inhibition of enzymatic activity, inhibition of bacterial growth, mode of action, mechanism of inhibition, and toxicity issues.

Materials and Methods

Materials

Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). All chemicals were from Fisher Scientific (Pittsburgh, PA). DNA was sequenced by Functional Biosciences (Madison, WI). Radioactive isotopes were from PerkinElmer (Waltham, MA). *Escherichia coli tol*C mutant, *P. aeruginosa* PAO200 (efflux pump mutant), and *P. aeruginosa* hypersensitive strain (ATCC 35151) were a kind gift from Urs Ochsner (Crestone Pharma, Boulder, CO). All other bacteria were from the American Type Culture Collection (ATCC, Manassas, VA). The natural product library was from MicroSource Discovery Systems, Inc. (Gaylordsville, CT), and the synthetic compound library was from TimTec LLC (Newark, DE). Compounds stocks were dissolved in DMSO to a concentration of 10 mM, stored at –20 °C, and thawed immediately before analysis. The compounds have an average purity of 95%, and the minimum purity is at least 90%.

Gel Electrophoresis and Protein Analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4%–12% polyacrylamide precast gels (Novex NuPAGE, Invitrogen, Waltham, MA) with MOPS running buffer (Invitrogen). EZ-Run *Rec* Protein Ladder was from Fisher Scientific. Gels were stained with Simply Blue Safe Stain (Invitrogen). Protein concentrations were determined using Coomassie Protein Assay Reagent (Thermo Scientific, Waltham, MA) with bovine serum albumin as the standard.

Cloning and Purification of P. aeruginosa *AspRS*

The gene (*asp*S) encoding *P. aeruginosa* AspRS was amplified by PCR (Bio-Rad MJ Mini Thermo Cycler) from *P. aeruginosa* PAO1 (ATCC 47085) genomic DNA using the forward primer 5′-CACCATGATGCGCAGCCACTATTGC-3′, which contained a 5′-CACC sequence for insertion into the pET101/D-TOPO directional plasmid, and the reverse primer 5′-TCA ATGGTGATGGTGATGGTGAGAACCCCTCGG CCTTCGGCTGCTC-3′, which was designed to add six histidine amino acid residues to the C-terminus of AspRS. The PCR product was inserted into pET101/D-TOPO and transformed into *E. coli* Rosetta 2(DE3) Singles Competent Cells (Novagen, Darmstadt, Germany).

Bacterial cultures were grown in Terrific Broth containing 50 µg/mL of ampicillin and 50 µg/mL of chloramphenicol. The cultures were grown at 37 °C, and expression of the target protein was induced at an optical density (A_{600}) of 0.6–0.8 by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to 0.25 mM. Growth of bacterial culture was continued for 3 h postinduction, and the bacteria were harvested by centrifugation (10,000*g*, 30 min, 4 °C). Bacterial cells were lysed and fraction I lysate was prepared as previously described.[5] *P. aeruginosa* AspRS was purified to greater than 95% homogeneity using nickel–nitrilotriacetic acid (NTA) affinity chromatography (Perfect Pro, 5 Prime), followed by dialysis (two times) against a buffer containing 20 mM Hepes-KOH (pH 7.0), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 10 % glycerol. Purified proteins were fast frozen in liquid nitrogen and stored at –80 °C.

ATP:PP$_i$ Exchange Reactions

ATP:PP_i exchange reactions (100 µL) were carried out at 37 ° for 20 min in 50 mM Tris-HCl (pH 7.5), 10 mM KF, 8 mM MgOAc, 1 mM dithiothreitol (DTT), 2 mM [^{32}P]PP_i (50 cpm/pmol), and 0.1 µM *P. aeruginosa* AspRS as previously described.[4] When the concentration of aspartic acid was varied (50, 100, 150, 200, 300, 400, 500, and 600 µM), the concentration of ATP was held at 2 mM, and when the concentration of ATP was varied (50, 100, 150, 200, 300, 400, 500, and 600 µM), the concentration of aspartic acid was held constant at 2 mM. The reactions were stopped at 1, 2, 3, 4, and 5 min time points. The K_M, V_{max}, and the k_{cat}^{obs} for the interactions of *P. aeruginosa* AspRS with ATP and aspartic acid were determined by plotting the initial velocities for exchange of PP_i at each concentration of the varied substrate, and fitting these data to the Michaelis–Menten steady-state model using XLfit (IDBS, Alameda, CA).

Timed Aminoacylation Assays

Aminoacylation was measured using filter binding assays (50 μL) containing 50 mM Tris-HCl (pH 7.5), 7.5 mM $MgCl_2$, 2.5 mM ATP, 1 mM DTT, 75 μM [^{3}H] aspartic acid, and 0.05 μM *P. aeruginosa* AspRS, as previously described.[4] The $tRNA^{Asp/Asn}$ concentrations were 0.075, 0.15, 0.36, 0.48, 0.6, 1.2, 2.4, and 3.0 μM. Assays were stopped at time intervals between 1 and 5 min. Initial velocities for aminoacylation were calculated for all tRNA concentrations, and the data were fit to the Michaelis–Menten steady-state model using XLfit (IDBS) to determine the kinetic parameters (K_M, V_{max}, and k_{cat}^{obs}).

Chemical Compound Screening

tRNA aminoacylation was monitored using a SPA as previously described.[4] The screening reactions were in 96-well microtiter plates (Costar). Compounds were dissolved in 100% DMSO, and the final concentration of compounds in the screening assay was 132 μM. Briefly, 2 μL of compound (3.3 mM) was mixed with 33 μL of the protein/substrate mix described above for the aminoacylation assay (minus tRNA). The concentration of *P. aeruginosa* AspRS in the screening assays was set at 0.05 μM. Positive control reactions contained only DMSO (2 μL) with no compound, and negative control reactions contained 2 μL of EDTA (0.5 M). This mixture was incubated at ambient temperature for 15 min, and then reactions were started by the addition of 15 μL of *E. coli* tRNA (40 μM total tRNA or 2.5 μM $tRNA^{Asp/Asn}$), followed by incubation for 60 min at 37 °C. Reactions were stopped by the addition of 5 μL of 0.5 M EDTA. Four hundred micrograms of yttrium silicate (Ysi) poly-L-lysine-coated SPA beads (PerkinElmer) in 150 μL of 300 mM citric acid were added and allowed to incubate at room temperature for 1 h. The plates were analyzed using a 1450 MicroBeta (Jet) liquid scintillation/luminescent counter (Wallac, Waltham, MA). For IC_{50} determination, assays were as described above, with the test compounds serially diluted from 200 to 0.4 μM.

Microbiological Assays

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method performed in 96-well microtiter plates according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guideline M7-A7.[6] MIC values were determined for *E. coli* (ATCC 25922), *E. coli tol*C mutant (W4573:*tolC*::Tn*10*), *Enterococcus faecalis* (ATCC 29212), *Haemophilus influenzae* (ATCC 49766), *P. aeruginosa* (ATCC 47085), *P. aeruginosa* PAO200 (efflux pump mutant), *P. aeruginosa* hypersensitive strain (ATCC 35151), *Staphylococcus aureus* (ATCC 29213), and *Streptococcus pneumoniae* (ATCC 49619).

Time-kill studies were performed using *H. influenzae* and *S. aureus* as previously described,[7] according to CLSI document M26-A. Growth media was *Haemophilus* test medium for *H. influenzae* and brain heart infusion for *S. aureus* (Becton, Dickinson and Company, Franklin Lakes, NJ). These same growth media were used in MIC and time-kill studies.

In Vitro Cytotoxicity Test

To determine the effect of the hit compound on the growth of human cell cultures, in vitro cytotoxicity testing was carried out as described using human embryonic kidney 293 (HEK293) cells.[4] The Trevigen TACS MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) Cell Proliferation Assay Kit (Gaithersburg, MD) was used to analyze the impact of the hit compound on human cell proliferation and/or viability. The assays were carried out in triplicate. The control staurosporine was serially diluted in assays from 1 to 0.001 μg/mL, and the hit compound was tested in assays ranging from 25 to 400 μg/mL.

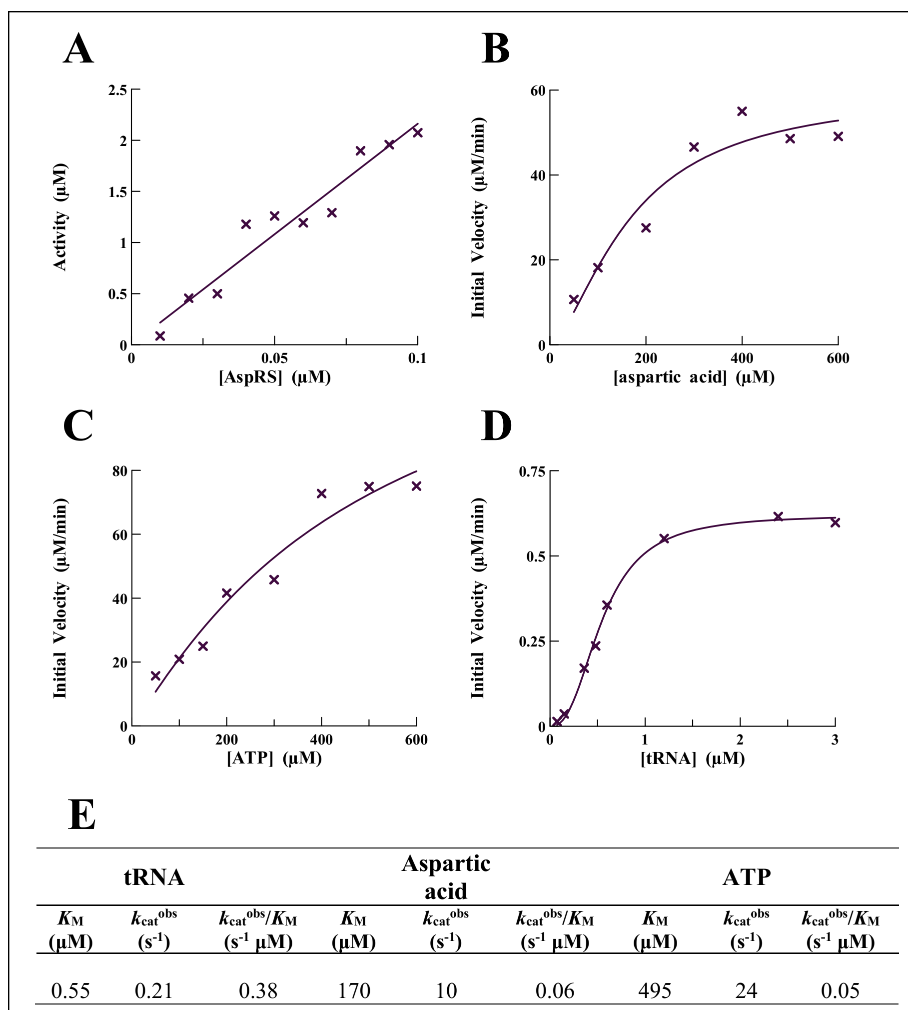
Binding Mode Assay

To determine the mechanism of action of the hit compounds with respect to the substrates, aspartic acid, and ATP, IC_{50} values were determined using SPAs as described above. Final compound concentrations in the IC_{50} reactions were serially diluted from 200 to 0.4 μM. Competition with ATP was determined at 25, 50, 100, 250, 500, and 1000 μM ATP, while the aspartic acid concentration was held constant at 75 μM. Competition with the amino acid was determined at 25, 50, 100, 200, and 300 μM aspartic acid, while the ATP concentration was held constant at 2.5 mM. Positive controls contained only DMSO (2 μL) without compound. Background amounts of free [^{3}H]Asp in the absence of AspRS were insignificant.

Results and Discussion

Protein Expression and Characterization

The AspRS gene (*asp*S) from *P. aeruginosa* was cloned and expressed, and the resulting protein was fused to a C-terminal histidine tag and purified in an active form to greater than 98% homogeneity (**Suppl. Fig. S1**). In aminoacylation assays, *P. aeruginosa* AspRS was shown to be active in attaching radiolabeled aspartic acid to tRNA (**Fig. 1A**). The aminoacylation of a particular tRNA with the cognate amino acid by the aminoacyl-tRNA synthetases occurs as a two-step reaction. In the first step, the enzyme condenses the amino acid and ATP, forming an aminoacyl-adenylate intermediate with a pyrophosphate as the leaving group. This reaction is reversible, for the majority of the aaRS proteins, in the absence of tRNA. Interaction of the enzyme with both the amino acid and ATP can be monitored using the ATP:PP_i exchange reaction. To determine the kinetic parameters with respect to ATP, the amino acid concentration was held



tRNA			Aspartic acid			ATP		
K_M (μM)	k_{cat}^{obs} (s^{-1})	k_{cat}^{obs}/K_M (s^{-1} μM)	K_M (μM)	k_{cat}^{obs} (s^{-1})	k_{cat}^{obs}/K_M (s^{-1} μM)	K_M (μM)	k_{cat}^{obs} (s^{-1})	k_{cat}^{obs}/K_M (s^{-1} μM)
0.55	0.21	0.38	170	10	0.06	495	24	0.05

Figure 1. Determination of the activity of *P. aeruginosa* AspRS and the kinetic parameters governing interactions with its three substrates: aspartic acid, ATP, and tRNA. (**A**) *P. aeruginosa* AspRS was titrated into the aminoacylation assay as described in Materials and Methods at concentrations between 0.01 and 0.1 μM. Background activity was minimal and was subtracted from values at all concentrations of AspRS. Initial velocities for the interaction of AspRS with (**B**) aspartic acid and (**C**) ATP were both determined using the ATP:PP_i exchange reaction. (**D**) The initial velocity for the interaction of AspRS with tRNA was determined using the aminoacylation reaction. The concentration of AspRS in the aminoacylation reactions and exchange reactions was 0.05 μM and 0.1 μM, respectively. Initial velocities were determined and the data were fit to a Michaelis–Menten steady-state model using XLfit 5.3 (IDBS) to determine K_M and V_{max}. (**E**) The kinetic parameters were determined for interaction of AspRS with the three substrates.

constant at 2 mM, while ATP concentrations were varied between 50 and 600 μM. Alternatively, to determine the kinetic parameters with respect to the amino acid, the ATP concentration was held constant at 2 mM, while the aspartic acid concentrations were varied between 50 and 600 μM. To determine maximum velocity (V_{max}), the initial velocities were determined at each substrate concentration and fit to the Michaelis–Menten steady-state model using XLfit (IDBS) (**Fig. 1B,C**). From these data, the kinetic parameters, K_M, k_{cat}^{obs}, and k_{cat}^{obs}/K_M, for interaction of *P. aeruginosa* AspRS with the amino acid were determined to be 170 μM, 10 s^{-1}, and 0.06 s^{-1} $μM^{-1}$, respectively (**Fig. 1E**). The same kinetic parameters for the interaction with ATP were 495 μM, 24 s^{-1}, and 0.05 s^{-1} $μM^{-1}$, respectively.

Next, the kinetic interactions of *P. aeruginosa* AspRS with tRNA were determined using the aminoacylation reaction. The initial rate for aminoacylation of $tRNA^{Asp/Asn}$ was determined at several different concentrations of $tRNA^{Asp/Asn}$ (0.075, 0.15, 0.36, 0.48, 0.6, 1.2, 2.4, and 3.0 μM) while holding the concentrations of ATP and aspartic acid constant at 2.5 mM and 100 μM, respectively (**Fig. 1D**). The initial velocities were modeled by fitting them to the Michaelis–Menten steady-state model. The K_M and k_{cat}^{obs} values for the interaction of *P. aeruginosa* AspRS with the tRNA were determined to be 0.55 μM and 0.21 s^{-1}, which gave a k_{cat}^{obs}/K_M value of 0.38 s^{-1} $μM^{-1}$. The K_M for *E. coli* AspRS with $tRNA^{Asp}$ was previously determined to be 0.6 μM, which is almost identical to that observed with *P. aeruginosa* AspRS.[8]

Screening Chemical Compounds against the Activity of P. aeruginosa *AspRS*

Using SPA technology, the activity of AspRS in the aminoacylation assay was screened against two chemical compound libraries with diverse chemical structures.[9,10] Initially, all buffer components of the aminoacylation reaction were individually titrated into the assay to determine the optimal concentration of each (data not shown). The concentration of AspRS for use in SPAs was determined to be 0.05 μM,

which yielded the maximum sensitivity to enzymatic inhibition (**Fig. 1A)**. Finally, tRNA was titrated into the assay to determine the concentration of $tRNA^{Asp/Asn}$ (2.5 µM) to be used in the screening assays (**Suppl. Fig. S2**). One compound library contained 890 synthetic compounds, and the other contained 800 natural products and their derivatives. The assay detects the ability of AspRS to aminoacylate $tRNA^{Asp/Asn}$ and, in the presence of a chemical compound, to measure the effect of the compound on the activity of AspRS. Since chemical compounds were dissolved in 100% DMSO, resulting in final DMSO concentrations in screening assays of 4%, the ability of *P. aeruginosa* AspRS to function in the presence of increasing amounts of DMSO was determined. There was no decrease of activity observed in aminoacylation assays containing up to 10% DMSO (data not shown). Initial screening assays contained chemical compounds at a concentration of 132 µM and were carried out as single-point assays. Compounds observed to inhibit enzymatic activity by at least 50% were reassayed in duplicate using filter binding assays, as described. These assays resulted in 23 confirmed hit compounds from both the synthetic compound and the natural product compound libraries. The IC_{50} values for inhibition of aminoacylation activity for these compounds were determined, and the values ranged between 2.5 and 50 µM for all compounds (**Suppl. Table S1**).

Microbiological Assays

All 23 confirmed hit compounds were tested in broth microdilution assays to determine MICs. The tests were performed against a panel of nine pathogenic bacteria, including efflux pump mutants of *E. coli* and *P. aeruginosa* and a hypersensitive strain of *P. aeruginosa* (**Suppl. Table S1**). Although there was similarity in the biochemical activity of the compounds, only two compounds (BT02A02 and BT02C05) (**Fig. 2A**,**B**) were observed to significantly inhibit the growth of bacteria in cultures (**Table 1**). In enzymatic inhibition, BT02A02 and BT02C05 inhibited the activity of *P. aeruginosa* AspRS with IC_{50} values of 11.5 and 39 µM, respectively (**Fig. 2C**,**D**). Both compounds exhibited moderate MICs against the Gram-negative *H. influenzae*, against the efflux mutant forms of *E. coli* and *P. aeruginosa*, and against the hypersensitive form of *P. aeruginosa*. However, no inhibition was observed against the wild-type strains of *E. coli* and *P. aeruginosa*. This indicates that efflux may be responsible for the lack of activity against the wild-type strains of both *E. coli* and *P. aeruginosa*. The two compounds inhibited the growth of cultures of the three Gram-positive bacteria (*E. faecalis, S. aureus*, and *S. pneumoniae*) with moderate to good MICs.

To determine the method of inhibition of the growth of bacterial cultures, BT02A02 and BT02C05 were tested in time-kill kinetic assays. The compounds were tested at concentrations four times the MIC and at time points between 0 and 24 h against cultures of *H. influenzae* and *S. aureus*. Both of these compounds were shown to be bacteriostatic against the Gram-negative and Gram-positive pathogens (**Fig. 2E**,**F**).

Table 1. Minimum Inhibitory Concentration of the Hit Compounds against Selected Bacteria.

Bacteria	MIC (µg/mL)	
	BT02A02	BT02C05
E. coli (ATCC 25922)	128[a]	128
*E. coli tol*C (efflux mutant)	32	32
E. faecalis (ATCC 29212)	8	8
H. influenzae (ATCC 49766)	16	32
P. aeruginosa (ATCC 47085)	128	128
P. aeruginosa PAO200 (efflux mutant)	16	64
P. aeruginosa (hypersensitive)	16	32
S. aureus (ATCC 29213)	16	16
S. pneumoniae (ATCC 49619)	64	64

[a]The MIC for each compound against each of the bacteria was determined in three separate MIC assays.

Compounds BT02A02 and BT02C05 Were Not Toxic to Mammalian Cell Cultures

To test for potential cytotoxicity, the compounds were tested against human cell lines using the Trevigen MTT cell proliferation assay to determine the impact on human cells in culture. HEK293 cells were dosed with either a control compound, staurosporine, or the hit compounds BT02A02 and BT02C05 (**Fig. 3**). The cells were treated with 25–400 µg/mL of each compound separately for 24 h under standard tissue culture conditions in triplicate. BT02A02 was not toxic to the cells at any concentration tested (**Fig. 3A**). MICs for this compound ranged from 8 to 64 µg/mL against susceptible bacteria and were not toxic in the human cell cultures up to 400 µg/mL. This level of difference between MIC and cytotoxicity is advantageous in the development of potential therapeutic agents. Some toxicity was observed at higher concentrations of BT02C05, which is a matter of concern. When comparing the CC_{50}, which was 61.6 µg/mL (**Fig. 3B**), with the MIC, which ranged from 8 to 64 µg/mL against susceptible bacteria, there is less difference than observed with BT02A02. However, even at these concentrations, when compared with the control staurosporine (0.003 µg/mL), the cytotoxic potential was 20,000-fold lower (**Fig. 3C**).

Mechanism of Action

The binding sites of both ATP and aspartic acid are located in the active region of the catalytic domain formed by the

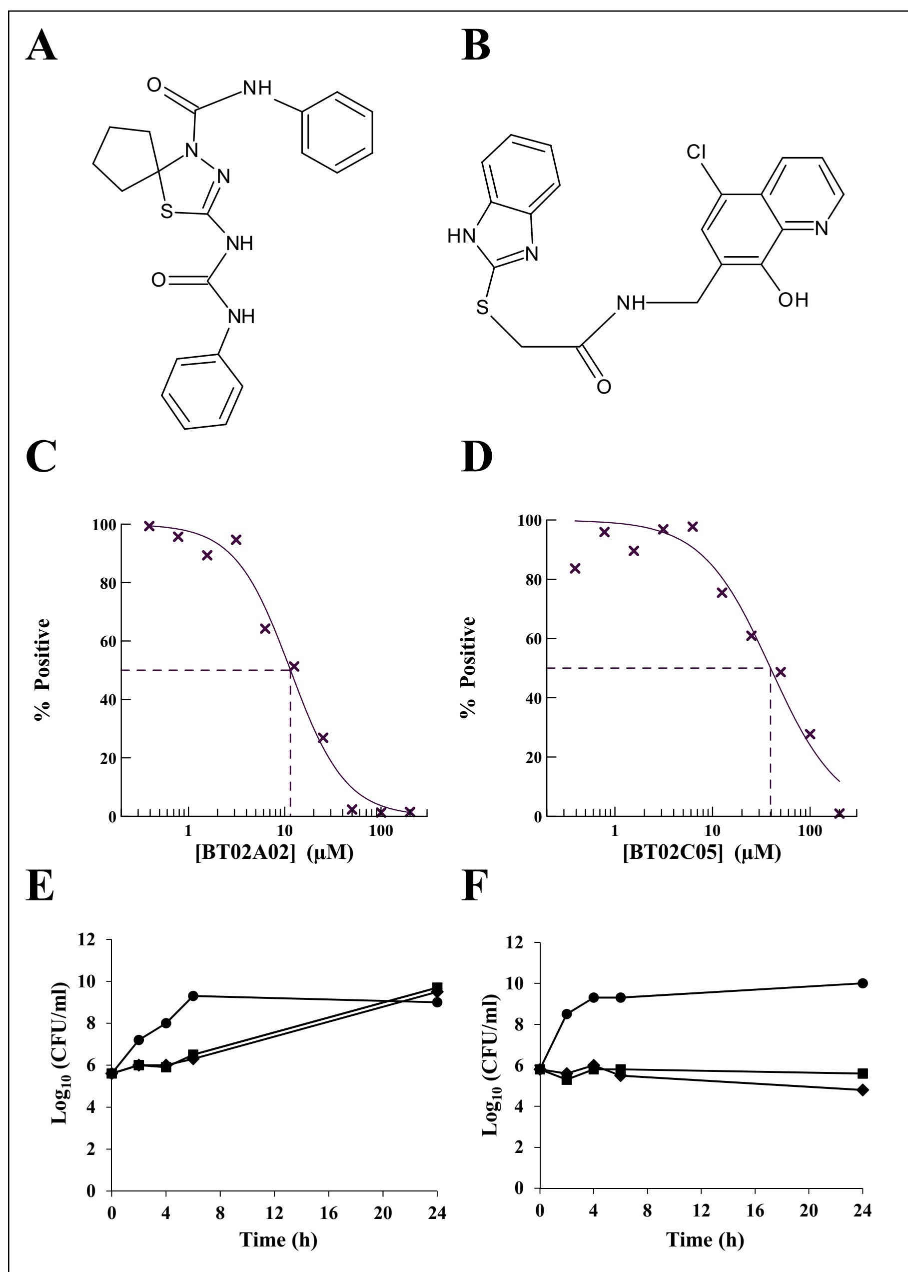


Figure 2. Chemical structure of the hit compounds and characterization of inhibition. The structures of (**A**) BT02A02 and (**B**) BT02C05 are shown. IC_{50} values for the inhibitory activity of (**C**) BT02A02 and (**D**) BT02C05 against *P. aeruginosa* AspRS were 11.5 and 46.2 μM, respectively. IC_{50} values were determined with the test compounds serially diluted from 200 to 0.4 μM into aminoacylation assays containing *P. aeruginosa* AspRS at 0.05 μM. "% Positive" indicates the percent of activity observed relative to activity in assays where 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 XLfit 5.3 (IDBS). The activities of the hit compounds against the growth of cultures containing (**E**) *H. influenzae* and (**F**) *S. aureus* were determined using broth microdilution susceptibility testing. Compounds were added to bacterial cultures at four times the MIC. Samples were analyzed by plating and determination of colony forming units (CFU) at 0, 2, 4, 6, and 24 h. Filled squares (■) represent cultures containing BT02A02, and filled diamonds (◆) represent cultures containing BT02C05. Filled circles (●) represent the growth of control cultures in the absence of compound.

three structural motifs of AspRS. Since the small molecules primarily used in drug screening are similar in molecular mass to that of both ATP and the amino acid, it was important to test whether the compounds interfered with the binding of either of these substrates as a possible mechanism of action for the observed inhibition. An inhibitory compound that competes with ATP for binding may be a poor candidate for drug development, since enzymes that utilize ATP as a substrate are prevalent in both bacterial and eukaryotic cells. However, since the amino acid uniquely binds AspRS, likely in an induced fit mechanism, competitive inhibition could be very specific.[11] Both compounds were tested in competition assays with varying amounts of ATP or aspartic acid. The mechanisms of inhibition of both inhibitors were determined with respect to ATP. IC_{50} values were determined using the tRNA aminoacylation assay at various ATP concentrations (25, 50, 100, 250, 500, and 1000 μM), while holding the concentration of the amino acid at 75 μM. To determine the mechanism of inhibition with respect to aspartic acid, the same assay was used, except that ATP was held constant at a saturating concentration (2.5 mM) and the IC_{50} was determined at different concentrations of aspartic acid (25, 50, 100, 200, and 300 μM). The IC_{50} values for BT02A02 and BT02C05 remained constant at all

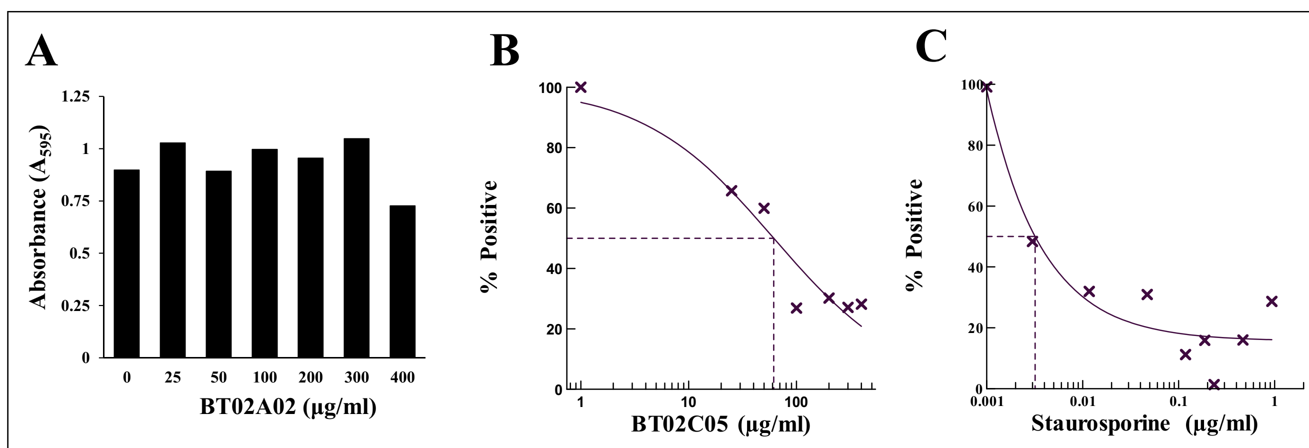


Figure 3. Determination of toxicity relative to the control staurosporine in human cell cultures. MTT assays were performed after 24 h of exposure to the indicated dose of drug under standard tissue culture conditions as described in Materials and Methods. (**A**) Effect observed after treatment with BT02A02. (**B**) To determine the CC_{50} of BT02C05, the compound concentration ranged from 0 to 400 μg/mL. (**C**) The control staurosporine was serially diluted in assays from 1 to 0.001 μg/mL to determine CC_{50}. "% Positive" indicates the percent of growth of cell cultures containing compound observed relative to the growth of cell cultures where only DMSO was added to the cells in the absence of compound. The data points represent an average value for assays carried out in triplicate. The curve fits and CC_{50} values were determined using the Sigmoidal Dose-Response Model in XLfit 5.3 (IDBS).

concentrations of both ATP and aspartic acid (**Suppl. Fig. S3**), which is characteristic of a noncompetitive inhibitor.[12] We conclude that BT02A02 and BT02C05 inhibit the function of AspRS by a mechanism different than direct competition with either substrate in the active site.

The AspRS has been well studied, and the crystal structure of AspRS in apo forms and bound with ligands has been solved from various organisms, including both the mitochondrial and cytoplasmic forms from humans. The structures of the ND-AspRS from *P. aeruginosa* with $tRNA^{Asn}$ alone, or in a complex with Asp-$tRNA^{Asn}$ and the amidotransferase GatCAB, have recently been solved[13] and confirm that the synthesis of Asn-$tRNA^{Asn}$ is tRNA dependent. Both $tRNA^{Asp}$ and $tRNA^{Asn}$ are aminoacylated initially by the ND-AspRS, and GatCAB then recognizes and binds the AspRS/Asp-$tRNA^{Asn}$ complex before it dissociates, forming an ND-AspRS/Asp-$tRNA^{Asn}$/GatCAB complex, in which the aspartic acid attached to $tRNA^{Asn}$ is converted to asparagine. Inhibition of the function of AspRS from *P. aeruginosa* would then have the potential to block the formation of Asp-$tRNA^{Asp}$ or Asn-$tRNA^{Asn}$ during protein biosynthesis, potentially having double the antibacterial effect of a compound that would specifically inhibit one of the other aminoacyl-tRNA synthetases.

In the present study, we have kinetically characterized AspRS from *P. aeruginosa* and used this information to develop a screening platform using SPA technology to identify inhibitors of activity. The screening assays were robust and resulted in Z′ and Z factors of approximately 0.44 and 0.31, respectively, across all plates. Two chemical compound libraries (1690 compounds) were screened and the confirmed hit compounds assayed for inhibition of enzymatic activity, as well as for the ability to negatively affect the growth of a panel of pathogenic bacteria in cultures. Two compounds, BT02A02 and BT02C05, were identified that had broad-spectrum antibacterial activity. Both compounds inhibited the Gram-negative bacteria *H. influenzae*, as well as efflux mutant strains of *E. coli* and *P. aeruginosa*. However, the wild-type strains of *E. coli* and *P. aeruginosa* were not affected by the compounds, suggesting that the compounds are taken up by the wild-type strains but are likely pumped out of the cells before reaching an effective concentration.

In time-kill assays, both BT02A02 and BT02C05 were bacteriostatic against both Gram-positive and Gram-negative pathogens. The compounds were tested against *H. influenzae* and *S. aureus*, and there was constant growth in the presence of the compound, but there was a decrease in the growth rate of 3–4 log_{10} units, compared with the control, during the times tested. Inhibition of aminoacylation activity results in depletion of charged tRNA, which mimics amino acid starvation and elicits the stringent response resulting in static bacterial growth, but not necessarily killing the bacteria.

When tested for toxicity against the HEK293 human cell line, BT02A02 did not exhibit toxicity at any concentration tested, up to 400 μg/mL. Compound BT02C05 had a CC_{50} of 61.6 μg/mL in these assays. The control, staurosporine, a potent protein kinase inhibitor that is used as a positive control for cytotoxicity, inhibited growth of the human cell cultures with a CC_{50} of 0.003 μg/mL. These results indicate that BT02A02 may be amenable for development as a therapeutic against bacterial infections. However, BT02C05 may require further modification to increase potency, while reducing cytotoxic effects.

Even though the structure and function of AspRS have been widely studied, little data exist on the discovery of compounds that have the potential to target the enzyme as an antibacterial. Two types of AspRS inhibitors have been described previously, and both appear to inhibit by competition with the aminoacyl-adenylate. One type of inhibition is via direct competition by nonhydrolyzable analogs of the adenylate,[14] and the other is by inhibition by microcin C (McC) and analogs.[15] McC and its analogs are peptides linked to a modified AMP and have been shown to inhibit the activity of AspRS, also by competition with the adenylate, but they also appear to be prone to the development of resistance and short-lived in the cells. Since we have shown that the compounds identified in this study are not competitive with ATP or the aspartate, the mechanism of action likely occurs outside the active site. Both BT02A02 and BT02C05 contain internal carboxamide functional groups, which are characteristic of certain antibiotics,[4] but are also contained within numerous other drugs. Information for both BT02A02 and BT02C05 is available in the PubChem Substance and Compound database through the unique chemical structure identifiers CID: 17138966 and CID: 24208376, respectively. There was no bioassay information available on either compound.

The discovery of two compounds, BT02A02 and BT02C05, which inhibit AspRS by a mechanism that previously has not been described, represents a promising approach for the development of a novel type of antibiotic.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The authors are grateful for the financial support provided by the National Institutes of Health (grant number: 1SC3GM098173). The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health. A portion of graduate student support was from a departmental grant from the Robert A. Welch Foundation (grant no. BG-0017). Partial undergraduate support was from an National Institutes of Health UTRGV RISE program, grant no. 1R25GM100866, and the Office of the Secretary of Defense for Research and Engineering through the U.S. Army Research Office, CSTEM grant no. W911NF-11-1-0150.

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