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Identification and Characterization of a Chemical Compound that Inhibits Methionyl-tRNA Synthetase from *Pseudomonas aeruginosa*

Sara Robles¹, Yanmei Hu^{1,2}, Tahyra Resto¹, Frank Dean¹, and James M. Bullard^{1,*}

¹Chemistry Department, The University of Texas-RGV, 1201 W. University Drive, Edinburg, TX 78541, USA

Abstract

Background—*Pseudomonas aeruginosa* is an opportunistic pathogen problematic in causing nosocomial infections and is highly susceptible to development of resistance to multiple antibiotics. The gene encoding methionyl-tRNA synthetase (MetRS) from *P. aeruginosa* was cloned and the resulting protein characterized.

Methods—MetRS was kinetically evaluated and the K_M for its three substrates, methionine, ATP and tRNA^{Met} were determined to be 35, 515, and 29 μ M, respectively. *P. aeruginosa* MetRS was used to screen two chemical compound libraries containing 1690 individual compounds.

Results—A natural product compound (BM01C11) was identified that inhibited the aminoacylation function. The compound inhibited *P. aeruginosa* MetRS with an IC₅₀ of 70 μ M. The minimum inhibitory concentration (MIC) of BM01C11 was determined against nine clinically relevant bacterial strains, including efflux pump mutants and hypersensitive strains of *P. aeruginosa* and *E. coli*. The MIC against the hypersensitive strain of *P. aeruginosa* was 16 μ g/ml. However, the compound was not effective against the wild-type and efflux pump mutant strains, indicating that efflux may not be responsible for the lack of activity against the wild-type strains. When tested in human cell cultures, the cytotoxicity concentration (CC₅₀) was observed to be 30 μ g/ml. The compound did not compete with methionine or ATP for binding MetRS, indicating that the mechanism of action of the compound likely occurs outside the active site of aminoacylation.

Conclusion—An inhibitor of *P. aeruginosa* MetRS, BM01C11, was identified as a flavonoid compound named isopomiferin. Isopomiferin inhibited the enzymatic activity of MetRS and displayed broad spectrum antibacterial activity. These studies indicate that isopomiferin may be amenable to development as a therapeutic for bacterial infections.

*Address correspondence to this author at the Chemistry Department, SCNE. 3.320, The University of Texas-RGV, 1201 W. University Drive, Edinburg, TX 78541, USA; Tel: 956-665-2950; Mobile: 303-775-5100; Fax: 956-665-5006; james.bullard@utrgv.edu.

²Current address: Department of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona, Tucson, Arizona 85721, USA

CONFLICT OF INTEREST

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Keywords

Protein synthesis; *in vitro* screening; tRNA aminoacylation; drug discovery; antibiotics; *Pseudomonas*.

INTRODUCTION

Antibiotic resistance is an issue of growing public concern. The rise of so-called ‘superbugs’ or bacteria that are resistant to multiple classes of antibiotics has marked the beginning of an era where current antibiotic treatments are no longer sufficient [1]. *Pseudomonas aeruginosa*, a Gram-negative pathogen, is a member of this group of bacteria and has a high intrinsic resistance to antibiotics due to numerous efflux systems and low permeability of its membrane. This bacterial pathogen is the leading cause of pulmonary infections in cystic fibrosis patients and a significant contributor to hospital acquired infections [2].

Methionyl-tRNA synthetase (MetRS), encoded by the *metS* gene, binds three natural substrates (ATP, methionine, and tRNA) and catalyzes the reaction resulting in the aminoacylation of tRNA^{Met}. MetRS is a class I aminoacyl tRNA synthetase (aaRS) characterized by an active site structure formed by a Rossmann fold containing two structural motifs (HIGH and KMSKS) [3]. MetRS is further grouped along with isoleucyl-, valyl-, leucyl-, cysteinyl- and arginyl-tRNA synthetases (IleRS, ValRS, LeuRS, CysRS and ArgRS) into a subclass Ia based on sequence conservation. A unique property of MetRS is its ability to recognize and charge two tRNA^{Met} substrates: the elongator tRNA^{Met} and the initiator tRNA^{Met}. MetRS thus plays a crucial role during the initiation phase and the elongation phase of protein biosynthesis.

Most bacterial isolates contain one of the two major forms of MetRS (MetRS1 and MetRS2) that are distinct at the amino acid sequence level and are encoded by two different genes, *metS1* and *metS2*, respectively. MetRS1 is the form typically found in Gram-positive bacteria while MetRS2 appears to be the product of archaea and eukaryotic organisms as well as the form found in Gram-negative bacteria [4]. There are exceptions to this in that certain Gram-positive bacteria, in particular *Streptococcus pneumoniae*, contain genes encoding both forms of MetRS, which is likely the result of acquisition through horizontal gene transfer [5]. In addition, structural studies have divided MetRS into four additional families based on the number of Zn-binding knuckle motifs and the number of Zn²⁺ atoms bound to the motifs. MetRS2 contains two Zn-binding knuckles and either two Zn²⁺ (A family) or one Zn²⁺ atom bound (B family). MetRS1 orthologs contain only one Zn-binding knuckle motif and either one (C family) or no Zn²⁺ atoms bound (D family). Sequence analysis indicates that MetRS from *P. aeruginosa* is of the MetRS2 form and belongs to the B structural family based on Zn-binding knuckle motifs.

MetRS from *P. aeruginosa* was purified and the kinetic parameters (K_M , V_{max} and k^{cat}) for interaction with its natural substrates were experimentally determined. It was then developed into a screening platform using scintillation proximity assay (SPA) technology [6] and used to screen 1690 natural and synthetic compounds for inhibitory activity. One compound was identified that inhibited the activity of the enzyme. This compound was further characterized

for inhibition of enzymatic activity and bacterial growth, mode of action, mechanism of inhibition and toxicity issues.

METHODS AND MATERIALS

Materials

All chemicals were obtained from Fisher Scientific (Pittsburg, PA). Radioactive isotopes, SPA beads and 96-well screening plates were from PerkinElmer (Waltham, MA). *E. coli* tolC mutant, *P. aeruginosa* PAO200 (efflux pump mutant) and *P. aeruginosa* hypersensitive (ATCC® 35151™) strains were a kind gift from Urs Ochsner (Crestone Pharma, Boulder, CO). All other bacteria were from American Type Culture Collection (ATCC) (Manassas, VA). The synthetic compound library was from TimTec LLC (Newark, DE) and the natural product library was from MicroSourceDiscovery Systems, Inc. (Gaylordville, CT). Compounds were supplied as 10 mM stocks dissolved in dimethyl sulfoxide (DMSO), 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%.

Cloning and Purification of *P. aeruginosa* MetRS

The gene encoding *P. aeruginosa* MetRS 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 (5'-ATATGCTAGCTCCGAACCGCAAGATC-3'), designed to add an *NheI* restriction site to the 5' end of the gene and the reverse primer (5'-CTCTAAGCTTTTACTTGACGCGCTGGC-3') which was designed to add a *HindIII* restriction site to the 3' end of the gene, were used in the PCR. The PCR product was inserted into a pET-28b(+) plasmid (Novagen) digested with *NheI/HindIII*. The recombinant plasmid was transformed into *E. coli* Rosetta 2(DE3) Singles Competent Cells (EMD Millipore, Danvers, MA).

The *E. coli* bacterial cultures were grown in Terrific Broth containing 25 µg/mL of kanamycin and 50 µg/mL of chloramphenicol at a temperature of 37 °C to an optical density (A_{600}) of 0.6–0.8. The over-expression of *P. aeruginosa* MetRS in the cultures was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a concentration of 0.25 mM. Growth of the culture was continued for 2 hours post-induction, and cells were harvested using centrifugation (10,000 *g*, 30 min, 4 °C). Fraction I lysates were prepared as previously described [7]. *P. aeruginosa* MetRS was purified to more than 98% homogeneity as previously described [6].

Gel Electrophoresis and Protein Analysis

Proteins were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–12% polyacrylamide precast gradient gels (Novex NuPAGE; Invitrogen, Grand Island, NY) with 3-(N-morpholino) propanesulfonic acid (MOPS) as the running buffer (Invitrogen). 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 [8].

Timed tRNA Aminoacylation Assays

Aminoacylation was measured using filter binding assays (50 μ L) containing 50 mM Tris-HCl (pH 7.5), 7.5 mM $MgCl_2$, 2.5 mM ATP, 1 mM dithiothreitol (DTT), 75 μ M [3H] methionine, and 0.1 μ M *P. aeruginosa* MetRS as previously described [9]. Assays were stopped at time intervals between 1 and 5 min. The tRNA concentrations were varied in six different sets of assays and contained 10, 30, 50, 70, 90 or 110 μ M total tRNA (0.5, 1.5, 2.5, 3, 4, 5 μ M tRNA^{Met}). From the timed assays, initial velocities were measured and the kinetic parameters (K_M and k^{cat}) were determined by fitting the data to the Michaelis-Menten steady-state model using XLfit (IDBS).

Phosphate Exchange Reactions

ATP:PP_i exchange reactions (100 μ L) were carried out at 37 °C in 50.0 mM Tris-HCl (pH 7.5), 10 mM KF, 2 mM [^{32}P]PP_i, 7.5 mM $MgCl_2$, 0.1 μ M *P. aeruginosa* MetRS as previously described [10]. The reactions were stopped at 1, 2, 3, 4, and 5 min intervals. In the reactions in which the concentration of ATP was varied (25, 50, 100, 200, 300, and 400 μ M), the methionine concentration was held constant at 2 mM; alternatively, when the concentration of the amino acid was varied (2.5, 5, 10, 20, 30 μ M), the ATP concentration was at 2 mM. Initial velocities were determined for exchange of PP_i and the kinetic parameters (K_M , V_{max} and K^{cat}) for the interactions of *P. aeruginosa* MetRS with ATP and methionine were determined and fit to the Michaelis-Menten steady-state model using XLfit (IDBS).

Chemical Compound Screening

In compound screening assays, the aminoacylation of tRNA was monitored using SPA technology as previously described [10]. The reactions were carried out in 96-well microtiter plates (Costar). Compounds were dissolved in 100% DMSO to a concentration of 3.3 mM, and the final concentration of compounds in the screening assay was 132 μ M. The concentration of *P. aeruginosa* MetRS in the screening assays was set at 0.1 μ M. To determine IC₅₀ values, the compounds were serially diluted into the assay at concentration ranging from 200 μ M down to 0.4 μ M.

Microbiological Assays

Minimum inhibitory concentration (MIC) for the hit compound was obtained using broth microdilutions following Clinical and Laboratory Safety Standards Institute (CLSI) guideline M7-A7 [11]. MIC values were determined for *E. coli* (ATCC 25922), *E. coli* toK mutant, *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). Quality control (QC) of MIC data and culture purity was maintained by MIC determination for antibiotics specific for each bacterial strain [10] as described in Table 1: Summary of Etest® Performance, Interpretive Criteria and Quality Control Ranges (Biomerieux).

Time-kill studies were performed to determine mode of inhibition of the hit compound using *H. influenzae* and *S. aureus* based on the MIC assay results, according to the CLSI

document M26-A [12]. Growth media was Haemophilus Test Broth and Trypticase Soy Broth from Remel (Lenexa, KS).

Cytotoxicity Testing

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 cells (HEK-293) [6]. 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 control staurosporine was serially diluted in assays from 1 to 0.001 µg/ml and the hit compound was tested in assays ranging from 100 µg/ml to 1 µg/ml.

Binding Mode Assay

To determine the mechanism of action of the hit compound with respect to ATP and methionine, IC₅₀ values were determined using SPA assays as described above. Final compound concentrations in each IC₅₀ reaction ranged from 200 to 0.4 µM. Competition with ATP was determined at 25, 50, 100, 250, 500, 1000 µM ATP while the methionine concentration was 75 µM. Competition with the amino acid was determined at 25, 50, 100, 200, 300 µM methionine while the ATP concentration was 2.5 mM. Positive controls contained only DMSO (2 µl) without compound.

RESULTS AND DISCUSSION

Sequence Analysis

The crystal structures of bacterial MetRS from *E. coli* [13, 14] and *Thermus thermophilus* [15] and *Brucella melitensis* [16] in an apo-form and bound to its substrates have been solved. Also the MetRS structure from the archaea *Pyrococcus abyssi* is known [17]. The amino acid sequence of MetRS from *P. aeruginosa* exhibits a high degree of conservation when compared with corresponding enzymes of the γ -subdivision of the proteobacteria resulting in 73% sequence similarity and 61% sequence identity (Table 1). The level of conservation is reduced considerably when compared with MetRS from other phyla and when compared with either human mitochondrial MetRS (hmMetRS), or human cytosolic MetRS (hcMetRS), there is only 18% conservation of the amino acid residues.

MetRS binds methionine (Met) through an induced-fit mechanism [14] and the active site residues, Y15, W253 and H301 (*E. coli* numbering), that have been shown to directly interact with the amino acid substrate are contained within the Rossmann fold in the catalytic domain and are strictly conserved between *E. coli*, *T. thermophilus* and *P. aeruginosa* MetRS (Fig. 1). These residues, along with others, make up the methionine binding pocket of MetRS, in which the amino acid residues are conserved between *E. coli* and *P. aeruginosa* MetRS, but greater variation occurs in the *T. thermophilus* enzyme. MetRS must discriminate between the binding of methionine and homocysteine (a precursor of cysteine). Two amino acids, L13 and Y260, that are located in the methionine binding pocket form H-bonds with the δ -sulfur atom of methionine and, along with W253 and Y15,

create the binding pocket surrounding the side-chain, resulting in specificity for methionine [18][14]. L13 and Y260 are conserved in MetRS from *E. coli* and *P. aeruginosa*.

Binding of the ATP is stabilized by several residues. In *E. coli* MetRS, V326 and H23 appear to form H-bonds with the adenine base [14]. In both *P. aeruginosa* and *T. thermophilus*, the valine at position 326 is replaced by the similar leucine. The ribose sugar of ATP is stabilized by interactions with E27, G294, and D296. The amino acids at positions 294 and 296 are conserved in all three MetRS enzymes. However, the glutamic acid at position 27 is replaced with a threonine in *T. thermophilus* MetRS. The two histidine residues in the HLGH (HIGH) motif interact with the adenosine moiety of the ATP and H24 likely stabilizes the phosphate in the aminoacyladenylate [14]. These amino acids are also strictly conserved in the three MetRS proteins.

A number of amino acids have been shown to interact with and proofread the cognate tRNA [13]. In sequence alignments (Fig. 1) these amino acids are fairly well conserved between MetRS from *E. coli*, *P. aeruginosa* and *T. thermophilus*. At only one position, D456 (*E. coli* numbering), is there a lack of conservation. The aspartic acid in *E. coli* is replaced with an alanine and an asparagine in *P. aeruginosa* and *T. thermophilus*, respectively. This is interesting since D456 in *E. coli* is one of two amino acid residues behaving as “anti-determinants” contributing to rejection of non-methionine anticodon containing tRNAs [19].

MetRS is a known zinc metalloprotein, in which binding of Zn^{2+} is required for enzymatic activity [20][21]. Sequence alignment of the amino acids from various MetRS proteins identified four families in context of the number of zinc-binding ligands and the number of Zn^{2+} bound [13]. *E. coli* contains four zinc-binding motifs (1–4), but only motif 2 and motif 3 actively bind Zn^{2+} (Fig. 1). Alternatively, *T. thermophilus* MetRS contains only two zinc-binding motifs and binds one Zn^{2+} [15]. In the alignment in Fig. 1, MetRS from *P. aeruginosa* aligns more closely with that of *E. coli*, containing all four of the zinc-binding motifs, and with the lack of cysteine residues in motifs 1 and 3, like *E. coli* MetRS, likely only binds one Zn^{2+} in what is considered the distal knuckle.

Expression and Characterization of MetRS from *P. aeruginosa*

Methionyl-tRNA synthetase from *P. aeruginosa* was cloned, overexpressed in *E. coli* and purified to greater than 98% homogeneity as visualized by SDS-PAGE and shown to be active in the aminoacylation assay (Fig. 2A). The primary physiological function of an aminoacyl tRNA synthetase (aaRS) is the catalytic attachment of an amino acid to its cognate tRNA. This reaction occurs via two distinct enzymatic steps catalyzed without the amino acid substrate being released from the enzyme. In the first step the enzyme catalyzes the condensation of the amino acid and another substrate, ATP, forming an aminoacyl adenylate intermediate with the release of an inorganic pyrophosphate (PP_i). This reaction is reversible in the absence of cognate tRNA and can be used to monitor the interaction of the enzyme with the amino acid and ATP. The kinetic parameters governing the interaction of *P. aeruginosa* MetRS with methionine and ATP were determined using the ATP: PP_i exchange reaction as described under the “Methods and Material” section. To determine the kinetic parameters with respect to ATP, the amino acid concentration was held constant while ATP concentrations were varied between 25 and 400 μM . Alternatively, to determine the kinetic

parameters with respect to the amino acid, the ATP concentration was held constant while the methionine concentrations were varied between 2.5 and 30 μM . The initial velocities were determined and fit to the Michaelis-Menten steady-state model using XLfit (IDBS) (Fig. 2B–C). The kinetic parameters, K_M , k^{cat} , and k^{cat}/K_M , for interaction of *P. aeruginosa* MetRS with ATP were determined to be 515 μM , 24.2 sec^{-1} , and 0.05 $\text{s}^{-1}\mu\text{M}^{-1}$, respectively (Table 2). The same kinetic parameters for the interaction with methionine were 35 μM , 37.2 sec^{-1} , and 1.1 $\text{s}^{-1}\mu\text{M}^{-1}$, also respectively. These values are similar to the kinetic interactions previously observed for *E. coli* MetRS with ATP ($K_M = 528 \mu\text{M}$, $k^{cat} = 74 \mu\text{M}$) and methionine ($K_M = 21 \mu\text{M}$, $k^{cat} = 74 \mu\text{M}$) [22].

In the second step of the reaction the methionine is released from the aminoacyl adenylate and transferred to the acceptor end of the cognate tRNA^{Met} . This step tends to be the rate limiting step and the turnover number for the entire aminoacylation reaction is well below that for formation of the aminoacyl adenylate. In these reactions the concentration of Met and ATP were saturating and the concentration of tRNA^{Met} was varied between 0.5 and 5 μM . The initial velocities were determined and fit to the Michaelis-Menten steady-state model (Fig. 2D) and the K_M , k^{cat} , and k^{cat}/K_M values for tRNA were determined to be 29.0 μM , 1.90 sec^{-1} , and 0.07 $\text{s}^{-1}\mu\text{M}^{-1}$ respectively.

Chemical Compound Screening

The chemical compound screening assays were based on the aminoacylation assay and modified for monitoring using SPA technology. Initially, the non-enzymatic components of the aminoacylation reaction were individually titrated into the assay to ensure optimal concentrations for maximum signal. Next, MetRS was titrated into the assay to determine the concentration yielding maximum sensitivity to enzymatic inhibition; this resulted in a screening concentration of 0.1 μM (Fig. 2A). Using this optimized assay, two chemical compound libraries were screened against the activity of *P. aeruginosa* MetRS. One library contained 800 compounds composed of natural products including simple and complex oxygen heterocycles, alkaloids, sesquiterpenes, diterpenes, pentacyclic triterpenes, and sterols from Microsource Discovery Systems (Gaylordville, CT) [9]. The other library contained 890 low molecular weight synthetic compounds with scaffolds based on known anti-bacterial, anti-fungoid, and anti-microbial agents from TimTec LLC (Newark, DE) [23]. The chemical compounds (3.3 mM) were dissolved in DMSO and screening reactions (50 μl) contained 2 μl of compound resulting in a final compound concentration of 132 μM . This also resulted in 4% DMSO in each reaction. To determine the effect of DMSO on the activity of MetRS, it was titrated into the assay up to 10% final concentration. There was no decrease in enzymatic activity observed at 4% DMSO (data not shown). The screening reactions were very robust with an average signal to background ratio of 10:1. The Z' and Z factors across all plates averaged 0.7 and 0.5, respectively. Screening assays were performed as single point assays and compounds observed to inhibit at least 50% of enzymatic activity were confirmed in additional assays performed in triplicate. The initial screen resulted in eleven confirmed hit compounds. These compounds were analyzed to determine IC_{50} values. Only two compounds, BM01C11 (70 μM) and BM01F04 (8.3 μM), both from the natural compound library, were observed to have an IC_{50} value below 200 μM . BM01F04 was identified as a compound named hieracin, also called Tricetin. The PubChem BioAssay

database was searched for biological activity of hieracin and it was found to have inhibitory activity against a number of eukaryotic and viral targets as well as antibacterial activity [24]. This compound will not be analyzed further. BM01C11 was identified as a compound named isopomiferin (Fig. 3). The Pub-Chem BioAssay database was also searched for biological activity of isopomiferin and a close conformer, isopomiferin dimethyl ether. There was no inhibitory bioactivity found for this compound and it will be investigated further.

Microbiological Assays

Isopomiferin was tested in broth microdilution assays to determine minimum inhibitory concentrations (MIC) against nine pathogenic bacteria (Table 3). The organisms used to determine the MICs were chosen using Clinical Laboratory Standards Institute guidelines [11] along with efflux and hypersensitive mutant strains of *E. coli* and *P. aeruginosa*. There was no inhibition of wild-type *E. coli* or *P. aeruginosa* observed at concentrations below 128 µg/ml. There was also no inhibitory activity observed with the efflux pump mutant of *P. aeruginosa* and only a slight decrease in the MIC with the efflux pump mutant of *E. coli*. However, isopomiferin inhibited the growth of cultures of the hypersensitive strain of *P. aeruginosa* with a moderately low MIC of 16 µg/ml, indicating that efflux may not be responsible for the lack of activity against the wild-type strains. The hypersensitive strain of *P. aeruginosa* is susceptible to numerous antibiotics because of an increase in outer membrane permeability partially due to a malfunction in porin protein F [25]. Therefore, the inability of isopomiferin to effect the growth of wild-type and efflux pump mutant strains of *P. aeruginosa* may be a permeability issue. The other Gram-negative bacteria, *Haemophilus influenzae*, does not contain a porin protein F and was susceptible to the compound. Isopomiferin inhibited growth of cultures of the Gram-positive bacteria, *Enterococcus faecalis* and *Staphylococcus aureus*, but the compound did not inhibit the growth of *Streptococcus pneumoniae* at a concentration lower than 128 µg/ml. This may be due to the fact that *S. pneumoniae* contains two genes (*metS1* and *metS2*) encoding two distinctly different forms of MetRS [4] and both forms of the protein may not be compromised by the compound.

Next, to determine the mode of inhibition in bacterial cultures, time kill studies were performed with isopomiferin. Based on MIC results, isopomiferin was tested against cultures of *H. influenzae* and *S. aureus*. The cultures were analyzed between 0 and 24 hours and contained compounds at four times the MIC. *H. influenzae* grown in the presence of isopomiferin was observed to have constant growth in the presence of compounds, but a decrease of 2 to 5 log₁₀ colony forming units (CFU) compared to the control during the twenty-four hour period (Fig. 4). This is indicative of bacteriostatic inhibition and occurs, in this case, because inhibition of aminoacylation by an amino-acyl tRNA synthetase produces the same results as amino acid starvation, and likely activates the stringent response, which results in static bacterial growth [26]. The effect of isopomiferin on cultures containing *S. aureus* were observed to be bactericidal and no CFUs were observed in the first six hours of growth. However, increased growth was observed at 24 hours, possibly because the compounds lost potency over extended time periods. *S. aureus* contains MetRS1 and *H. influenzae* contains MetRS2, and the different forms of the synthetase may contribute to the difference observed in the mode of inhibition. The reason for the bactericidal activity is not

clear, but many aaRS proteins have secondary roles that could be affected by the compound leading to cell death. MetRS contains an editing function which prevents the misaminoacylation of tRNA^{Met} with homocysteine (Hcy). Blocking this editing function could result in the incorporation of Hcy into nascent proteins, which in turn would inactivate the biological activity of these proteins, possibly leading to cell death [27]. Alternatively, inhibition of the editing function could result in the misaminoacylation of the initiator tRNA^{Met}, thus blocking initiation of protein synthesis.

Cytotoxicity Assays

Mammalian cells contain a cytoplasmic as well as a mitochondrial form of MetRS, which have many structural similarities with bacterial MetRS. This presents the potential that a compound which is active in inhibition of bacterial MetRS may have cytotoxic effects on eukaryotic cells. It was therefore important to analyze the potential for toxicity by isopomiferin in eukaryotic cells. Predicting toxicity of chemicals using human cell systems instead of whole animal systems is a viable first step in drug discovery, therefore isopomiferin was tested for cytotoxicity in human embryonic kidney 293 (HEK-293) cell cultures using the Trevigen TACS[®] MTT Cell Proliferation Assay Kit (Fig. 5). Staurosporine, a potent inhibitor of human cell cultures [28], was used as a comparator in the studies. Staurosporine was observed to inhibit cell growth with a CC₅₀ of 0.003 µg/ml. MTT assays were performed at isopomiferin concentrations between 1 and 100 µg/ml for 24 hours. Isopomiferin exhibited a CC₅₀ of near 30 µg/ml, which when compared to the toxicity of many antibiotics appears problematic for therapeutic use. However, the CC₅₀ of isopomiferin is 10,000-fold higher than that of staurosporine, and when compared with other antibiotics, erythromycin and tetracycline, the toxicity profile was similar [29].

Mechanism of Action

The binding sites of both ATP and methionine are located in the Rossman fold active site region of MetRS. Small molecules primarily used in drug screening have a molecular mass that is similar to both ATP and the amino acid. It is therefore prudent as an initial determination of the mechanism of action to ascertain if a hit compound interferes with binding of one or both of these substrates. An inhibitory compound that competes with ATP for binding may be a poor candidate for drug development, since ATPase enzymes are prevalent in both bacterial and eukaryotic cells. However, since methionine uniquely binds MetRS in an induced fit mechanism, competitive inhibition of this mechanism could be very specific. To understand the mechanism of action relative to substrate binding, isopomiferin was tested in competition assays with varying amounts of ATP or methionine (Fig. 6). The compound was not observed to compete with either ATP or methionine for binding, indicating that the binding region of isopomiferin is likely outside the active site for aminoacylation. In the assays, as the concentration of either ATP or methionine was increased the IC₅₀ remained constant, indicating a non-competitive form of inhibition [30].

CONCLUSION

The amino acid sequence of *P. aeruginosa* MetRS, when compared to homologs obtained from a variety of bacterial species was, as expected, more similar (greater than 60%

sequence conservation) to that of other MetRS proteins from the gamma subdivision of the proteobacteria (Table 1). In particular, the critical amino acid residues in *E. coli* MetRS are strictly conserved in *P. aeruginosa* MetRS. However, when compared with human cytosolic or mitochondrial MetRS, there is little conservation of the primary sequence, indicating that a compound that inhibits the function of the bacterial MetRS may be ineffective against the human forms of MetRS. In the present work, *P. aeruginosa* MetRS was developed into a screening platform based on scintillation proximity assay technology and used to screen two chemical compound libraries (1690 compounds) for inhibitors of aminoacylation activity. The screening assays were robust and eleven compounds from the compound libraries were identified and confirmed as hit compounds. Two of the compounds, isopomiferin (BM01C11) and hieracin (BM01F04), both natural products, inhibited MetRS activity with IC₅₀s of 70 μ M and 8.3 μ M, respectively. Hieracin was discarded due to promiscuous inhibitory activity observed in bioassay data (National Center for Biotechnology Information. PubChem Compound Database; CID=5281701, <https://pubchem.ncbi.nlm.nih.gov/compound/5281701> (accessed Feb. 9, 2017). Isopomiferin displayed inhibition against bacterial growth from a panel of both Gram-negative and Gram-positive bacteria. Moderate activity was observed against *S. aureus*, *E. faecalis*, and *H. influenzae*. However, the wild-type and efflux pump mutant strains of *E. coli* and *P. aeruginosa* were not affected by the compounds, indicating that the mechanism of resistance may not be due to efflux. Cultures of the antibiotic hypersensitive strain of *P. aeruginosa* were inhibited by isopomiferin. This suggests that the lack of inhibitory activity against the wild-type and efflux pump mutant strains may be due to an inability to gain entry into the cells.

When isopomiferin was tested in human cell cultures, the CC₅₀ was observed to be near 30 μ g/ml. When compared with a small subset of antibiotics, this is not unusual. However, when compared with the majority of antibiotics in use, this is a relatively high toxicity level. The toxicity issue with isopomiferin will need to be addressed in future structure activity relationship (SAR) studies. Isopomiferin did not interfere with MetRS binding of methionine or with ATP, and would appear to inhibit the activity of MetRS by another mechanism. It is possible that the compound may interact with the Zn⁺⁺ binding motif interfering with coordination of the metal due to the catechol moiety or it may bind at an unknown site thereby inducing a conformational change in the protein leading to inactivation of enzymatic function. In this era of multi-drug resistant bacteria, compounds that interact with molecular targets differently, possibly with a different mechanism of action, or with different binding sites, would be advantageous. This is evidenced by the low level of clinical resistance observed, even after many years of use, against mupirocin that occurs by mutations in the IleRS protein itself. Mupirocin blocks the binding site of the unique aminoacyl adenylate intermediary molecule [31]. Higher levels of resistance come from the acquisition of the *mupA* gene, normally from outside sources [32]. Also, the structure of isopomiferin offers several avenues in which chemical modifications can be made in attempts to increase potency against the growth of bacteria and to decrease toxicity observed in human cell lines.

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LIST OF ABBREVIATIONS

aaRS	Aminoacyl-tRNA synthetase
AS	Ammonium sulfate
ATP	Adenosine triphosphate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
IPTG	Isopropyl b-D-1-thiogalactopyranoside
KF	Potassium fluoride
KPi	Potassium phosphate
Met	Methionine
MgOAc	Magnesium acetate
MOPS	3-(N-morpholino)propanesulfonic acid
SAR	Structure-activity relationship
SPA	Scintillation proximity assay
TCA	Trichloroacetic acid

References

1. Breidenstein EB, Fuente-Nunez C, Hancock RE. *Pseudomonas aeruginosa*: all roads lead to resistance. Trends Microbiol. 2011; 19:419–26. [PubMed: 21664819]
2. Giamarellou H, Antoniadou A. Antipseudomonal antibiotics. Med Clin North Am. 2001; 85:19–42. [PubMed: 11190351]
3. Eriani G, Delarue M, Poch O, Gangloff J, Moras D. Partition of tRNA synthetases into two classes based on mutually exclusive sets of sequence motifs. Nature. 1990; 347:203–6. [PubMed: 2203971]
4. Gentry DR, Ingraham KA, Stanhope MJ, et al. Variable sensitivity to bacterial methionyl-tRNA synthetase inhibitors reveals subpopulations of *Streptococcus pneumoniae* with two distinct methionyl-tRNA synthetase genes. Antimicrob Agents Chemother. 2003; 47:1784–9. [PubMed: 12760849]
5. Brown JR, Gentry D, Becker JA, Ingraham K, Holmes DJ, Stanhope MJ. Horizontal transfer of drug-resistant aminoacyl-transfer-RNA synthetases of anthrax and Gram-positive pathogens. EMBO Rep. 2003; 4:692–8. [PubMed: 12792655]

6. Hu Y, Guerrero E, Keniry M, Manrique J, Bullard JM. Identification of chemical compounds that inhibit the function of glutamyl-tRNA synthetase from *Pseudomonas aeruginosa*. J Biomol Screen. 2015; 20:1160–70. [PubMed: 26116192]
7. Cull MG, McHenry CS. Purification of *Escherichia coli* DNA polymerase III holoenzyme. Methods Enzymol. 1995; 262:22–35. [PubMed: 8594350]
8. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–54. [PubMed: 942051]
9. Hu Y, Keniry M, Palmer SO, Bullard JM. Discovery and analysis of natural product compounds inhibiting protein synthesis in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 2016; 60:4820–9. [PubMed: 27246774]
10. Hu Y, Palmer SO, Munoz H, Bullard JM. High throughput screen identifies natural product inhibitor of phenylalanyl-tRNA synthetase from *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*. Curr Drug Discov Technol. 2015; 11:279–92.
11. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically: approved guidelines M7-A7. CLSI, Wayne PA. M7-A7. 2006. Clinical and Laboratory Standards Institute.
12. Clinical and Laboratory Standards Institute. Methods for determining bactericidal activity of antimicrobial agents: approved guideline M26-A. CLSI; Wayne, PA: 2002. p. M26-A
13. Mechulam Y, Schmitt E, Maveyraud L, et al. Crystal structure of *Escherichia coli* methionyl-tRNA synthetase highlights species-specific features. J Mol Biol. 1999; 294:1287–97. [PubMed: 10600385]
14. Crepin T, Schmitt E, Mechulam Y, et al. Use of analogues of methionine and methionyl adenylate to sample conformational changes during catalysis in *Escherichia coli* methionyl-tRNA synthetase. J Mol Biol. 2003; 332:59–72. [PubMed: 12946347]
15. Sugiura I, Nureki O, Ugaji-Yoshikawa Y, et al. The 2.0 Å crystal structure of *Thermus thermophilus* methionyl-tRNA synthetase reveals two RNA-binding modules. Structure. 2000; 8:197–208. [PubMed: 10673435]
16. Ojo KK, Ranade RM, Zhang Z, et al. *Brucella melitensis* methionyl-tRNA-synthetase (MetRS), a potential drug target for Brucellosis. PLoS One. 2016; 11:e0160350. [PubMed: 27500735]
17. Crepin T, Schmitt E, Blanquet S, Mechulam Y. Three-dimensional structure of methionyl-tRNA synthetase from *Pyrococcus abyssi*. Biochemistry. 2004; 43:2635–44. [PubMed: 14992601]
18. Serre L, Verdon G, Choinowski T, Hervouet N, Risler JL, Zelwer C. How methionyl-tRNA synthetase creates its amino acid recognition pocket upon L-methionine binding. J Mol Biol. 2001; 306:863–76. [PubMed: 11243794]
19. Schmitt E, Meinel T, Panvert M, Mechulam Y, Blanquet S. Two acidic residues of *Escherichia coli* methionyl-tRNA synthetase act as negative discriminants towards the binding of non-cognate tRNA anticodons. J Mol Biol. 1993; 233:615–28. [PubMed: 8411169]
20. Nureki O, Kohno T, Sakamoto K, Miyazawa T, Yokoyama S. Chemical modification and mutagenesis studies on zinc binding of aminoacyl-tRNA synthetases. J Biol Chem. 1993; 268:15368–73. [PubMed: 8340367]
21. Fourmy D, Meinel T, Mechulam Y, Blanquet S. Mapping of the zinc binding domain of *Escherichia coli* methionyl-tRNA synthetase. J Mol Biol. 1993; 231:1068–77. [PubMed: 8515465]
22. Ghosh G, Pelka H, Schulman LH, Brunie S. Activation of methionine by *Escherichia coli* methionyl-tRNA synthetase. Biochemistry. 1991; 30:9569–75. [PubMed: 1911742]
23. Palmer SO, Hu Y, Keniry M, Bullard JM. Identification of chemical compounds that inhibit protein synthesis in *Pseudomonas aeruginosa*. J Biomol Screen. 2016 pii: 1087057116679591.
24. Pistelli L, Bertoli A, Noccioli C, et al. Antimicrobial activity of *Inga fendleriana* extracts and isolated flavonoids. Nat Prod Commun. 2009; 4:1679–83. [PubMed: 20120106]
25. Angus BL, Carey AM, Caron DA, Kropinski AM, Hancock RE. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. Antimicrob Agents Chemother. 1982; 21:299–309. [PubMed: 6803666]
26. Cozzzone AJ. Stringent control and protein synthesis in bacteria. Biochimie. 1980; 62:647–64. [PubMed: 7004494]
27. Deniziak MA, Barciszewski J. Methionyl-tRNA synthetase. Acta Biochim Pol. 2001; 48:337–50. [PubMed: 11732605]

28. Schnier JB, Gadbois DM, Nishi K, Bradbury EM. The kinase inhibitor staurosporine induces G1 arrest at two points: effect on retinoblastoma protein phosphorylation and cyclin-dependent kinase 2 in normal and transformed cells. *Cancer Res.* 1994; 54:5959–63. [PubMed: 7954429]
29. Inoue K, Kumakura S, Uchida M, Tsutsui T. Effects of eight antibacterial agents on cell survival and expression of epithelial-cell- or cell-adhesion-related genes in human gingival epithelial cells. *J Periodontal Res.* 2004; 39:50–8. [PubMed: 14687228]
30. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol.* 1973; 22:3099–108. [PubMed: 4202581]
31. Nakama T, Nureki O, Yokoyama S. Structural basis for the recognition of isoleucyl-adenylate and an antibiotic, mupirocin, by isoleucyl-tRNA synthetase. *J Biol Chem.* 2001; 276:47387–93. [PubMed: 11584022]
32. Patel JB, Gorwitz RJ, Jernigan JA. Mupirocin resistance. *Clin Infect Dis.* 2009; 49:935–41. [PubMed: 19673644]

Fig. 1.

Sequence alignment of *P. aeruginosa* MetRS and close analogs. The protein sequences were downloaded from the National Center for Biotechnology Information (NCBI). Abbreviations are: Ec, *E. coli*; Pa, *P. aeruginosa*; Tth, *T. thermophiles*. Accession numbers for MetRS protein sequences of *E. coli*, *P. aeruginosa*, and *T. thermophiles* are P00959, NP_252172 and P23395, respectively. Sequence alignments were performed using Vector NTI Advance (TM) 11.0 (Invitrogen). Identical amino acid residues are white on black and similar residues are black on grey. The positions of the HIGH and KMSKS motifs are indicated. Motifs 1–4 containing zinc binding knuckles in *E. coli* and *P. aeruginosa* are indicated above the motifs using solid brackets and zinc binding knuckles in *T. thermophilus* are indicated below the motifs using dashed brackets. Amino acid residues that directly interact with Met are indicated with a cross (X), and additional residues that form the amino acid binding pocket are indicated with a bar (■). Amino acids that directly interact with ATP (★) and tRNA (●) are indicated. Amino acid residues that function in proofreading cognate tRNA are indicated by open circles (○).

Fig. 2.

Determination of activity and the kinetic parameters governing the catalytic enzymology of *P. aeruginosa* MetRS with the substrates tRNA^{Met}, ATP, and methionine. (A) Titration of *P. aeruginosa* MetRS into the aminoacylation assay to determine activity and the optimal concentration in compound screening assays. Initial velocities for the interaction of *P. aeruginosa* MetRS with (B) ATP, (C) methionine and (D) tRNA^{Met} were determined and the data were fit to a Michaelis-Menten steady-state model using XLfit (IDBS) to determine K_M and V_{max} .

Fig. 3.
The chemical structure of the hit compound BM01C11 (isopomiferin).

Fig. 4. Time-kill kinetics of the hit compound isopomiferin against (A) *H. influenzae* and (B) *S. aureus*. Isopomiferin was added to bacterial cultures at 4×MIC. Samples were analyzed by plating and determination of colony forming units (CFU) at 0, 2, 4, 6, and 24 h. Filled circles (●) represent control cultures containing no compound but an equal amount of DMSO. Filled triangles (▲) represent cultures containing isopomiferin.

Fig. 5.

Determination of the toxicity of isopomiferin in human cell cultures. MTT assays were performed for 24 hours under standard tissue culture conditions as described in 'Materials and Methods'. The control (A) staurosporine was serially diluted in assays from 1 to 0.001 $\mu\text{g/ml}$ to determine CC_{50} (0.003 $\mu\text{g/ml}$). Isopomiferin (B) was diluted in the assays from 100 to 1 $\mu\text{g/ml}$. The "% 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 data points represents an average value of 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).

Fig. 6.

Determination of the mechanism of action of isopomiferin relative to (A) ATP and (B) methionine. IC₅₀s were determined in the aminoacylation assay using the SPA format. To determine isopomiferin inhibition relative to ATP, the IC₅₀s were determined at six different ATP concentrations ranging from 25 to 1000 μM. To determine inhibition relative to methionine, the IC₅₀s were determined at five different methionine concentrations ranging from 25 to 300 μM. The data were fit to the Sigmoidal Dose-Response model using XLfit (IDBS).

Table 1

P. aeruginosa MetRS amino acid conservation (average) compared with homologs from different phyla.

MetRS	Similarity/Identity (%)
Gammaproteobacteria	73/61
Betaproteobacteria	58/48
Alphaproteobacteria	31/21
Delta-, Epsilonproteobacteria	37/24
Terrabacteria (not including Firmicutes)	35/23
Firmicutes	40/25
Spirochaetales	33/22
Chlamydiae	36/25
FCB	43/28
Human mitochondria	28/18
Human cytosolic	29/18

The kinetic parameter governing the interaction of MetRS with its substrates.

	tRNA		Methionine		ATP	
K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1} \mu\text{M}^{-1}$)	K_M (μM)	k_{cat}/K_M ($\text{s}^{-1} \mu\text{M}^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1} \mu\text{M}^{-1}$)
29	1.93	0.07	35	37.2	515	0.05

Table 3

Minimum inhibitory concentration of the hit compound against clinically important bacterial.

Bacteria	Isopomiferin
<i>E. coli</i> (ATCC 25922)	128 ^a
<i>E. coli</i> toIC (efflux mutant)	64
<i>E. faecalis</i> (ATCC 29212)	32
<i>H. influenzae</i> (ATCC 49766)	32
<i>P. aeruginosa</i> (ATCC 47085)	128
<i>P. aeruginosa</i> PAO200 (efflux mutant)	128
<i>P. aeruginosa</i> (hypersensitive)	16
<i>S. aureus</i> (ATCC 29213)	32
<i>S. pneumoniae</i> (ATCC 49619)	>128

^aThe MIC (µg/ml) against each of the bacteria was determined in three separate sets of assays.