Development of methionyl-tRNA synthetase inhibitors as antibiotics for Gram-positive bacterial infections

Omeed Faghih[a], Zhongsheng Zhang[b], Ranae M. Ranade[a], J. Robert Gillespie[a], Sharon A. Creason[a], Wenlin Huang[b], Sayaka Shibata[b], Ximena Barros-Álvarez[b,c], Christophe L. M. J. Verlinde[b], Wim G. J. Hol[b], Erkang Fan[b], Frederick S. Buckner[a]

Department of Medicine[a], and Department of Biochemistry[b], University of Washington, Seattle, Washington, USA; Laboratorio de Enzimología de Parásitos, Facultad de Ciencias, Universidad de los Andes, Mérida, Venezuela[c].

Address correspondences to Frederick S. Buckner, fbuckner@uw.edu

ABSTRACT

Antibiotic resistant bacteria are widespread and pose a growing threat to human health. New antibiotics acting by novel mechanisms of action are needed to combat this threat. The bacterial methionyl-tRNA synthetase (MetRS) enzyme is essential for protein synthesis and the type found in Gram-positive bacteria is substantially different from its counterpart found in the mammalian cytoplasm. Selective inhibitors, both previously published and new, were shown to be highly active on Gram positive bacteria with minimum inhibitory concentrations (MICs) ≤ 1.3 µg/mL against Staphylococcus, Enterococcus, and Streptococcus strains. Incorporation of radioactive precursors demonstrated the mechanism of activity was due to inhibition of protein synthesis. Little activity was observed against Gram-negative bacteria, consistent with Gram-negative species containing a different type of MetRS enzyme. The ratio of MIC to minimum bactericidal concentrations (MBC) was consistent with a bacteriostatic mechanism. Protein binding was high (>95%) for the compounds and this translated to a substantial increase in MICs when tested in the presence of plasma. Despite this, the compounds were very active when tested in the Staphylococcus aureus murine thigh infection model. Compounds 1717 and 2144 given by oral gavage resulted in 3-4-log decreases in bacterial load compared to vehicle-treated mice which was comparable to results observed with the comparator drugs, vancomycin and linezolid. In summary, the research describes MetRS inhibitors with oral bioavailability that represent a class of compounds acting by a novel mechanism with excellent potential for clinical development.

INTRODUCTION

Gram-positive bacteria such as *Staphylococcus*, *Streptococcus*, and *Enterococcus* are major human pathogens responsible for a myriad of clinical syndromes. Antibiotic resistant strains such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant

Enterococcus (VRE) are widespread and limit the effectiveness of available antibiotics. Concern about the diminishing availability of effective antibiotics has led to urgent calls for the development of new antibiotics(1). Targeting the prokaryotic protein synthesis machinery has been a highly successful strategy for developing antibiotics. Aminoglycosides, tetracyclines, macrolides, ketolides, and oxazolidinones are major classes of antibiotics that all interfere with bacterial protein translation. Inhibition of tRNA synthetases represents another possible approach to target prokaryotic protein translation. The widely used antibiotic, mupirocin, works by inhibiting the bacterial isoleucyl-tRNA synthetase (2). Mupirocin is used as an ointment to treat or decolonize patients with cutaneous infections due to *Staphylococcus* or *Streptococcus*, however, its use is limited to the topical route of administration. Another bacterial tRNA synthetase inhibitor, a boron-containing compound targeting the bacterial leucyl-tRNA synthetase (GSK2251052) made it to phase 2 trials for Gram negative infections (3). Unfortunately, its development was discontinued due to high rates of resistance occurring during treatment, which may be related to the targeting of the editing domain of the enzyme rather than the catalytic domain. Investigators at GlaxoSmithKline reported on inhibitors to the *S. aureus* methionyl-tRNA synthetase (MetRS) over a decade ago (4-7). These inhibitors had excellent antibiotic potency, but poor oral bioavailability that restricted its development (pre-New Drug Application) to topical use for skin infections and to oral use for *Clostridium difficile* infections where oral absorption is not needed (8, 9). The research in the current report also focuses on MetRS inhibitors, building on compounds that are being developed as antiprotozoan chemotherapies (10-13). The compounds have high selectivity (>1000-fold) for *Trypanosoma brucei* cells over mammalian cell lines (14) Changes to the molecules have led to improved oral bioavailability and pharmacokinetic properties (14), thus making them better candidates for antibiotic development as will be described.

With respect to the target, bacteria and all living organisms contain a complement of tRNA synthetases that are responsible for charging tRNAs with their corresponding amino acids for subsequent delivery to the ribosome. tRNA synthetases, including MetRS, catalyze a two-step reaction as follows:

$$E + aa + ATP \leftrightarrows E{\bullet}aa{\sim}AMP + PP_i \quad (1)$$

$$E{\bullet}aa{\sim}AMP + tRNA \leftrightarrows E + aa\text{-}tRNA + AMP \quad (2)$$

In the first step, a highly reactive aminoacyl adenylate (aa~AMP) is formed through condensing ATP with the carboxylate of the amino acid. The second step uses this activated species to transfer the amino acid to the 3'-end of the tRNA (aa-tRNA) (15). The bacterial MetRS enzymes are categorized in two forms (MetRS1 and MetRS2) based on sequence similarity and sensitivity to inhibitors (16). Bacteria generally have a single MetRS enzyme with most Gram positive genera containing the MetRS1 form (*Staphylococcus, Streptococcus, Enterococcus, Bacillus, Clostridium*, and others) and most Gram negative bacteria containing the MetRS2 form (*Escherichia, Klebsiella, Pseudomonas, Haemophilus, Bacteroides*, and others) (17). Exceptions include *Bacillus anthracis* and a subset of *Streptococcus pneumoniae* which contain both MetRS1 and MetRS2 isoforms (16, 18). In mammals, distinct tRNA synthetases typically operate in the cytoplasm and the mitochondria. The human mitochondrial MetRS encoded in the mitochondrial genome (19) has close sequence homology to bacterial enzymes of the MetRS1 variety, whereas the human cytoplasmic MetRS is nuclear encoded with close homology to the MetRS2 variety. As will be detailed below, the MetRS inhibitors under study in this project are active on the *S. aureus* MetRS enzyme and show broad spectrum activity on Gram positive bacteria and negligible activity on Gram negative bacteria, consistent with targeting the MetRS1 form of the enzyme. Microbiological properties, murine pharmacology, and efficacy in the murine *S. aureus* thigh infection model are described herein. The new compounds represent promising antibiotic candidates that act by a novel mechanism of action.

RESULTS

MetRS inhibitors and lead optimization. The structures and properties of compounds under study in these experiments are shown in Fig. 1. The synthetic procedures for compounds 1312-1717 were published previously (10, 14). The synthetic procedures for new compounds 1962, 2062, 2093, 2114, and 2144 are described in the supplementary materials. The starting point for these investigations was the aminoquinolone scaffold exemplified by **1312** (Fig. 1). In separate research to develop MetRS inhibitors as antiprotozoan drugs, our group introduced changes to the molecules with the goals of improving oral bioavailability while retaining potent activity on the MetRS target. The evolution of these compounds included changing the aminoquinolone group to a fluorinated-imidazopyridine (e.g. **1614**) that improved oral bioavailability in mice from <10% for **1312** to ~40% for **1614** (14). Subsequent changes to the linker region reported previously (13) and in this paper have further improved potency and pharmacological properties of the series. The results of testing the MetRS inhibitors against recombinant *S. aureus* MetRS, bacterial cultures, and mammalian cells are shown in Table 1. All compounds tested had IC_{50} values on the *S. aureus* MetRS below the level of sensitivity of the assay (25 nM). The MetRS inhibitors have potent activity on a variety of Gram positive bacterial strains, but essentially no activity on Gram negative bacteria (*E. coli* and *Pseudomonas aeruginosa*). Specifically, MIC values below 0.3 µg/mL were measured against strains of *S. aureus* (including MSSA, MRSA, and VISA), *S. epidermidis*, *E. faecalis,* and *E. faecium* (including VSE and VRE strains). The compounds with the lowest MICs were **1717, 2093,** and **2144** which were >10-times more potent than the control drugs vancomycin or linezolid against many strains. These compounds are the subject of further investigations discussed below. Higher MICs were seen against *S. pyogenes* and no activity seen on *S. pneumoniae.* The selectivity on *Staphylococci* versus

mammalian cells (comparing MIC to CC_{50}) was at least 35-fold for these three most potent compounds.

Microbiological characterization of selected compounds.

1. *Macromolecular synthesis assays:* In order to verify that the compounds are acting by the expected mechanism of action, radioisotope incorporation assays were performed (Fig. 2). Incorporation of the amino acid (^{3}H-Lysine) was inhibited by the MetRS inhibitors (**1717, 2093,** and **2144),** consistent with inhibition of protein synthesis. The findings were similar to those seen with linezolid (Fig. 2, panel A) which is known to inhibit protein synthesis by interfering with the bacterial ribosome (20). In contrast, the MetRS inhibitors had less effect on both the incorporation of ^{3}H-uridine (a measure of RNA synthesis) and the incorporation of ^{3}H-thymidine (a measure of DNA synthesis) (21). Ciprofloxacin showed selective inhibition of DNA synthesis (panel B) consistent with its mechanism as an inhibitor of DNA topoisomerases. Finally, rifampicin showed selective inhibition of RNA synthesis (slightly above protein synthesis) (panel C) consistent with its mechanism as an inhibitor of bacterial RNA polymerase.

2. *Activity on permeable E. coli strains:* The purpose of these experiments was to determine if the non-susceptibility of Gram negative strains (e.g. *E. coli* ATCC 25922, shown in Table 2) was due to inability of the MetRS inhibitors to penetrate the Gram-negative cell wall. The mutant MB4902 is an outer membrane permeable *E. coli* strain and showed no greater susceptibility to three MetRS inhibitors (**1717**, **2093**, and **2144**) than to the wild-type *E. coli* strain (MB4827). Similarly, the efflux negative strain MB5747 showed no increased susceptibility to the MetRS inhibitors, nor did the mutant containing both mutations (MB5746). The control drug, novobiocin, had increased activity in the hyperpermeable *E. coli* strains as has been previously reported (22, 23).

3. *MIC/MBC:* Measurements of minimum bactericidal concentrations (MBCs) were done with the *S. aureus* strain ATCC 29213 (Table 3). The MBC is defined as the drug concentration that reduces bacterial growth by ≥99.9%. Compounds exhibiting an MBC/MIC ratio of ≤4 are generally considered bactericidal, while an MBC/MIC ratio >4 is considered bacteriostatic (24). The data indicates that **1717**, **2093**, and **2144** have bacteriostatic activity similar to linezolid.

4. *Resistance frequency rates:* The propensity for *S. aureus* (ATCC 29213) to develop resistance to MetRS inhibitors was also studied (Table 4). This was done by plating high numbers (3.8×10^9 in Expt. 1 and 5.5×10^9 in Expt. 2) of *S. aureus* on TSA plates impregnated with compound at concentrations of 4X or 8X the MIC and incubating for 72 h. The resistance frequency rates for **1717, 2093,** and **2144** at 8X EC50 were in the range of 2×10^{-8} to 4×10^{-9}. These rates are comparable to test drug novobiocin, but higher than the rates found for ciprofloxacin or linezolid.

5. *Serum shift and protein binding assays.* Serum shift assays were done to analyze the impact of protein binding on the MICs (Table 5). The MIC shifts in the presence of 50% human serum range from 16-fold to 128-fold for the MetRS inhibitors which is consistent with high protein binding (e.g. 95.4% for **1717**). Although the shifts are much higher than the shift for vancomycin (2-fold), the absolute MICs for some compounds in serum (e.g. **1717** and **2144**) are still comparable to that of vancomycin (in the range of 1-4 µg/mL).

Metabolic stability studies:

Compounds were incubated with murine or human liver microsomes to evaluate stability to hepatic metabolic enzymes (Table 6). Metabolism rates were similar between mouse and human microsomes for individual MetRS inhibitors. Compound **2093** demonstrated the most rapid metabolism (3.2 min in mouse microsomes) whereas compound **1962** demonstrated the highest metabolic stability (27.0 min in mouse microsomes). The drug, linezolid, is more metabolically stable with half-lives of >145 min in both mouse and human liver microsomes.

Pharmacokinetics studies:

Selected MetRS inhibitors (**1614, 1717, 2093,** and **2144**) or linezolid were administered to mice in single oral or IV doses, and tail blood was sampled at time intervals out to 24 h to assess blood exposure (Table S2). The terminal half-life for the MetRS inhibitors in blood ranged from 17 - 58 minutes (compared to 35 min for linezolid). Clearance ranged from 22 - 63 mL/min/kg (compared to 20 mL/min/kg for linezolid). The maximum blood concentration (Cmax) following oral dosing ranged from 0.39 - 9.3 uM (compared to 13.7 uM for linezolid). The AUC in blood following oral dosing ranged from 117 - 615 min*uM (compared to 1707 for linezolid). Finally, the apparent oral bioavailability ranged from 24 - 46% (compared to 94% for linezolid).

Efficacy studies in mice:

Selected MetRS inhibitors were tested for *in vivo* efficacy in the neutropenic *S. aureus* thigh infection model. Compounds **1717** and **2144** resulted in a ~3-4-log decrease in CFUs compared to the vehicle group, similar to vancomycin and linezolid (Fig. 3). Note the drop is below the stasis level which was determined by harvesting a group of mice at 1 hour post-infection.

Protein sequence analysis. Using coordinates from the *T.brucei* MetRS complex with inhibitor **1312** (PDB# 4EG5), the residues in the binding site of the inhibitor were aligned for various

192 species (Table 7). The *S. aureus* MetRS (UniProtKB – A0A0H2XID2, strain USA300) has
193 extremely high sequence conservation with the *Trypanosoma brucei* MetRS with 22 of 25
194 identical amino acids (and potentially 23 identical amino acids since position 456 could either be
195 a Leu or His, but is ambiguous in the model due to loop length). This confirms that many
196 inhibitors of the *T. brucei* MetRS will likely inhibit the *S. aureus* MetRS. We also compared the
197 MetRS from the human mitochondrial MetRS with the *S. aureus* sequence and identified four
198 different amino acid residues (at positions 249, 291, 470, and 471). Three of these changes
199 occur in pocket q that binds the quinolone moiety of **1312**.
200
201 **Discussion:**
202 The essential enzyme, methionyl-tRNA synthetase, was targeted for antibiotic drug discovery
203 against Gram positive bacteria. The research capitalizes on progress to develop antimicrobial
204 agents against pathogenic protozoa including *Trypanosoma brucei* and *Giardia intestinalis* (10-
205 14, 25). In particular, challenges with poor oral bioavailability observed with early
206 aminoquinolone compounds such as **1312** (10, 22, 26) were significantly improved with the
207 fluoroimidazopyridine derivatives (**1614-2144**, Fig. 1) (14). Compounds of this scaffold were
208 optimized for activity against the *T. brucei* MetRS (a type 1 enzyme). The comparison of the
209 protein sequences of the MetRS enzymes of *T. brucei* and *S. aureus* shows identity of 22 of 25
210 amino acid residues in the inhibitor binding site (Table 7) suggesting that cross activity from
211 *T. brucei* to *S. aureus* was likely. In fact, all the compounds tested for inhibitory activity against
212 recombinant *S. aureus* MetRS enzyme (Table 1) demonstrated IC_{50} values below 25 nM, the
213 sensitivity limit of the assay. Further titration below this concentration was not possible with the
214 applied methods due to the need for 25 nM enzyme to give a suitable signal for measurement
215 (see Methods). The assays against live bacterial cultures demonstrated potency of the MetRS
216 inhibitors in the sub-microgram per milliliter range against *Staphylococcus* and *Enterococcus*
217 species, and lower potency against *Streptococci* (Table 1). Of the compounds with the “linear

linker" structure (e.g., **1312-1717**), compound **1717** was the most potent with a MIC of 0.16 µg/mL. This was the most potent compound against *T. brucei* cultures (14). Compounds with the ring sytem in the linker region of the scaffold (e.g. **1962-2144**) also had potent anti-*Staphylococcal* activity including **2093** and **2144** with MICs of 0.04 and 0.02 µg/mL (Table 1). Compound **2093** contains a single ring in the linker (an imidazole-2-one) whereas compound **2144** contains a fused imidazo[1,2-*a*]pyridine ring system in the linker region.

The spectrum of activity of the MetRS inhibitors was explored against ATCC strains of pathogenic Gram positive and Gram negative bacteria. As was predicted, the antibiotic activity is restricted to bacteria dependent upon the type 1 MetRS enzyme, i.e. Gram positive bacteria. Gram negative bacteria (i.e., *Escherichia coli* and *Pseudomonas aeruginosa*), which are known to contain the type 2 MetRS enzyme (17), were insensitive to all the tested compounds at the highest concentration of 10 µg/mL. *The selectivity for Gram positive organisms is potentially advantageous in that the MetRS inhibitor developed as a drug will not add to resistance of non-targeted Gram negative bacteria.* Sensitive Gram positive strains were *S. aureus, Enterococcus faecium, Enterococcus faecalis,* and *Staphylococcus epidermidis.* Furthermore, these included drug-resistant strains such as MRSA, VISA, and VRE whose mechanisms of resistance to semisynthetic penicillins and glycopeptide antibiotics are unrelated to the cellular processes inhibited by the MetRS inhibitors. The MICs of the MetRS inhibitors to *S. pyogenes* (ATCC 19615) was higher than to *S. aureus* and *Enterococcus* strains which we ascribe to the need to grow *S. pyogenes* in media containing lysed blood. We have already shown the effects of plasma protein binding on MICs of the compounds (Table 5) and we suspect the addition of laked horse blood to be similar. With the shift observed with blood, the MIC for **2144** (1.3 µg/mL) is about the same as MICs observed with vancomycin and linezolid (0.63 and 1.3 µg/mL). The gram positive coccus, *Streptococcus pneumoniae* (ATCC 49619), was resistant to the MetRS inhibitors (MICs >10 µg/mL). This is consistent with previous reports that ~45% of *S. pneumoniae* strains are resistant to type 1 MetRS inhibitors due to the presence of a

second (type 2) MetRS inhibitor in the genome (16). It is likely that MetRS inhibitors would need to be used with caution for treatment of pneumonia or other clinical syndromes in which *S. pneumoniae* is commonly found, at least until cultures rule out *S. pneumoniae* as the cause of the infection. Future studies will investigate a broader collection of *S. pneumoniae* isolates to assess the MIC range against this pathogen. The issue of a secondary MetRS gene has not been described in other Gram positive bacteria, so this is unlikely to be a broader concern. We expect that MetRS inhibitors will be active against many other bacteria containing the type 1 MetRS enzyme including species of *Clostridia, Corynebacterium, Bacillus, Propionibacterium, Actinomyces*, and others (16). Various species of these are, of course, pathogenic in humans and their susceptibility will be tested in future studies. An exception to the Gram positive rule mentioned above is *Brucella* (a Gram negative rod) which is known to contain a type 1 MetRS and is susceptible to MetRS inhibitors (27).

In order to address the question about target of action in living bacteria, macromolecular synthesis assays were run with MetRS inhibitors and various control drugs (Fig. 2). As was expected, MetRS inhibitors resulted in rapid dose-dependent decreases in uptake of radiolabeled amino acid (Lys) consistent with disruption of protein synthesis. The changes were similar to those seen with the protein synthesis inhibitor, linezolid. At the same time, RNA and DNA synthesis was unaffected by MetRS inhibitors (**2093** and **2144**) until concentrations above the MIC were used while the control drugs, rifampicin and ciprofloxacin, caused selective inhibition of these pathways, respectively, in the anticipated manner. Compound **1717** showed mild suppression (20-40%) of RNA and DNA suppression at the MIC which could suggest an undefined secondary target or perhaps different binding kinetics to MetRS than for **2093** and **2144**. These studies provide assurance that the compounds are likely to be mediating their antibiotic effects through inhibiting the MetRS target in vivo.

In a similar vein, selected MetRS inhibitors were tested on strains of *E. coli* with defects in cell wall permeability and/or efflux (Table 2). The purpose of these experiments was to show

that resistance of *E. coli* was not due to exclusion of the MetRS inhibitors by the Gram negative cell wall or efflux, but rather due to inherent resistance. The findings that the cell permeable strains were resistant to the three most potent MetRS inhibitors (**1717, 2093,** and **2144**) is consistent with the understanding that *E. coli* contains a type 2 MetRS enzyme (17) which is not inhibited by the compounds under development. Furthermore, it indicates that off target mechanisms of action are not at play, at least with this species of bacteria.

Minimum bactericidal concentrations of **1717, 2093,** and **2144** were determined against the *S. aureus* ATCC 29213 strain. The MBC/MIC ratio was between 16 and 32 for these three compounds. A ratio of 4 or less is considered bactericidal (28), thus the MetRS inhibitors would be considered bacteriostatic against this strain of *S. aureus.* An MBC/MIC ratio of 64 was observed with the clinical drug linezolid (known to be bacteriostatic), whereas the ratio for vancomycin was 4, consistent with its bactericidal mechanism.

Resistance frequency rates of *S. aureus* (ATCC 29213) to MetRS inhibitors were determined on agar plates containing MetRS inhibitors at concentrations 4 or 8-times times their MICs. The resistance frequency rates for MetRS inhibitors (at 8X MIC) were between $2x10^{-8}$ and $4x10^{-9}$ which is higher than observed for ciprofloxacin and linezolid (Table 4). Resistance frequency rates in the 10^{-6} to 10^{-9} range are indicative of a single drug target within the cell (28) which is consistent with the understanding of the mechanism of action of these compounds. Drugs such as rifampicin have even higher rates ($2x10^{-7}$) (28), but are generally used in combination with other drugs to avoid generating resistance. Vancomycin and linezolid are known to have low resistance frequency rates ($<10^{-11}$) and, along with this characteristic, relatively little resistance (at least from *Staphylococci*) has developed in the clinic. Further research will be necessary to find out if the rates of resistance to MetRS inhibitors are problematic for their clinical development as monotherapy agents. If the risk for resistance developing appears high, then developing the compounds with a partner antibiotic may be an attractive option to mitigate the problem.

296 The MetRS inhibitors characterized in this report exhibit high protein binding properties
297 (95-99.9%). The low unbound concentration of compounds translates to substantial effects
298 when MICs are measured in the presence of serum (Table 5). Serum shifts ranging from 16-fold
299 to 128-fold were observed with the series of tested compounds. For perspective, vancomycin
300 only demonstrates about a 2-fold serum shift (Table 5) whereas fusidic acid is reported to have
301 97% protein binding and a 130-fold increase in MIC to *S. aureus* in the presence of 50% serum
302 (28). Due to the high potency of the MetRS inhibitors, the MICs of compounds **1717** and **2144**
303 in the presence of 50% serum (1 and 4 µg/mL, respectively) are comparable to the MIC for
304 vancomycin (2 µg/mL).
305 Incubation of the MetRS inhibitors with mouse or human liver microsomes showed
306 variable rates of metabolism, although generally half-lives were relatively short (<20 min for
307 human microsomes and <10 min for mouse) (Table 6). The pharmacokinetic studies in mice
308 showed clearance values for MetRS inhibitors ranging from 18-63 mL/min/kg compared to a
309 value of 20 ml/min/kg for linezolid. The fact that clearance of MetRS inhibitors is similar to
310 linezolid despite the more rapid microsome metabolism is likely attributable to the high plasma
311 protein binding which can protect compounds from liver cytochrome P450 metabolism (29).
312 Oral bioavailability for the MetRS inhibitors ranged from 24-46% which is substantially higher
313 than the original aminoquinolone compounds such as **1312** (oral bioavailability <10%) (14), but
314 lower than linezolid (94%). This difference in oral bioavailability is probably responsible for the
315 higher AUC of linezolid (1707 min*µM) following oral dosing than observed with the MetRS
316 inhibitors (117 – 615 min*µM). As will be discussed with the efficacy results, the combined
317 properties of the compounds (particularly **1717** and **2144**) appear to be sufficient to clear
318 bacteria from infected mice with similar efficiency as vancomycin or linezolid.
319 The results of the efficacy experiments are very encouraging towards the prospects of
320 developing MetRS inhibitors as antibiotics. A pilot experiment (not shown) and two independent
321 experiments showed the reproducibility of the *S. aureus* thigh infection model in mice made

neutropenic by cyclophosphamide pre-treatment. The model represents a soft tissue infection which resembles the disease process (skin and skin structure infection) for which clinical development of the compounds would initially be targeted. Both compounds **1717** and **2144** demonstrated significant reduction of bacterial load below the stasis level at least as effectively as the comparator drugs vancomycin and linezolid. The approximately 2-log reduction below stasis levels in a neutropenic mouse is noteworthy in light of the bacteriostatic activity observed in vitro (Table 3). This shows that tissue levels at the site of infection were sufficient to substantially reduce bacteria levels even in the absence of neutrophils. Many bacteriostatic antibiotics (including linezolid) are widely and successfully used in the clinic, so the bacteriostatic characteristic of the MetRS inhibitors may not be a significant liability. Interestingly, compound **2093** was found to have weaker activity than the other MetRS inhibitors evaluated. The explanation probably relates to the particularly high protein binding of this compound that presumably reduce the levels of unbound compound below the threshold needed to exceed the MBC at the site of infection. Future dose response experiments will help us determine the relative potency of **1717** and **2144** compared to each other and additional MetRS inhibitors under development.

To date, we have observed no apparent side-effects of the MetRS inhibitors given to mice. In this study, uninfected mice received single dose (50 mg/kg) of compounds for PK analysis had no acute reactions during the 24 hour observation period. In a previous publication, compounds **1614** and **1717** were administered to mice infected with *T. brucei* for 10 days at 50 mg/kg PO twice-per-day with no deleterious effects on weight, grooming, or body condition (14). Cytotoxicity against mammalian cells was low for the MetRS inhibitors (Table 1). For example, the ratio of CC_{50} to MIC for compounds **2093** and **2144** were >500 demonstrating a wide therapeutic window. A potential toxicity concern for the MetRS inhibitors is inhibition of the mammalian mitochondrial MetRS enzyme which bears close homology to the *S. aureus* MetRS (Table 7). Manifestations of this potential toxicity have not been evident with in vitro

cytotoxicity testing (the 48 h assay against a lymphocyte and hepatocyte cell lines) nor in mice as described above. Many antibiotics acting as protein synthesis inhibitors are known to inhibit mitochondrial protein synthesis as an off-target effect (30, 31). These include widely used drugs such as tetracycline, erythromycin, aminoglycosides, and linezolid. Instead of directly affecting mitochondrial oxidative phosphorylation, these drugs interfere with mitochondrial biogenesis and are relatively slow to result in clinical problems, often with tissue specific toxicity depending on the particular drug. Linezolid for example is known to cause hematologic disturbances, peripheral neuropathy, and metabolic acidosis when it is administered for more than a 28-day period (32). The fact that these side effects are slow to manifest makes them more tolerable for antibiotics since treatment course are typically relatively short (<10 days). Studies of the effects on MetRS inhibitors on mammalian mitochondrial function will be part of future investigations.

In summary, with the aid of structure-based drug design (14) new MetRS inhibitors have been developed with potent and broad spectrum activity against Gram positive bacteria. Macromolecule labeling studies demonstrate the inhibition of protein synthesis, consistent with the designed mechanism of action. As with other protein synthesis inhibitors such as oxazolidinones, tetracyclines, and lincosamides, the MetRS inhibitors have bacteriostatic properties against *S. aureus* in vitro. The compounds are highly protein bound which may help sustain plasma levels in vivo by limiting availability to cytochrome P450 metabolism. At least two MetRS inhibitors displayed activity in the neutropenic mouse thigh infection model (comparable to linezolid) which indicates that the free fraction of compound is sufficient to inhibit bacterial growth. In fact, the bacterial load decreased by 1-2 logs below the status level indicating that the in vivo activity was not just bacteriostatic, but bactericidal. These studies and previous reports (10, 14) have shown that the MetRS inhibitors have little in vitro toxicity and appear well-tolerated when dosed to mice for up to 10 days. Additional preclinical toxicology studies are planned to further investigate the potential for adverse effects from inhibition of the mitochondrial MetRS or other off-target activities. In total, these MetRS inhibitors with oral

bioavailability represent a class of compounds acting by a novel mechanism with excellent potential for clinical development.

MATERIALS AND METHODS

Media and culture conditions. Mueller Hinton broth (MHB), cation adjusted Mueller Hinton broth (CA-MHB), and Brain Heart Infusion broth (BHI) were purchased from Becton Dickinson (Franklin Lakes, NJ). Tryptic soy agar (TSA) plates, TSA with 5% sheep blood plates, and CA-MHB with 3% laked horse blood were purchased from Remel (San Diego, CA). MHB was used to assay all *Staphylococcus aureus* strains. CA-MHB was used for *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, and *Pseudomonas aeruginosa*. CA-MHB supplemented with 3% laked horse blood was used for *Streptococcus pneumoniae* and *Streptococcus pyogenes*. *Staphylococcus*, *Enterococcus*, *Escherichia*, and *Pseudomonas* strains were cultured at 37ºC with ambient air. *Streptococcus* strains were cultured in 37ºC with 5% CO_2. (24, 33). Separate conditions for radiolabeled precursor uptake assays are described below.

Compounds, reagents, and radiochemicals. The synthesis methods for the following compounds have been previously described: **1312** (10), **1575** (14), **1614** (14), and **1717** (14). The synthesis methods for the additional compounds are described in the supplementary materials: **1962**, **2062**, **2093**, **2114**, and **2144**. The following antibiotics were purchased commercially: vancomycin (Sigma-Aldrich, St. Louis, MO), linezolid (Chem-Impex International, Wood Dale, IL), rifampicin (Chem-Impex International, Wood Dale, IL), ciprofloxacin (Acros Organics, Geel, Belgium), and novobiocin (Promega, Madison, WI). Ketoprofen was purchased from Sigma Aldrich (St. Louis, MO). Human pooled serum was purchased from Thermo Fisher

Scientific (Waltham, MA). Dulbecco's phosphate buffered saline with calcium and magnesium (dPBS) was purchased from Lonza (Basel, Switzerland). [Methyl-3H]-thymidine (2% EtOH, 69.7 Ci/mmol) and [5,6-3H]-uridine (sterile water, 60 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). L-[4,5-3H(N)]-lysine (2% EtOH, 82.4 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). The logarithm of the partition coefficient values (logP) were calculated with ChemAxon software (Cambridge, MA).

Production of recombinant *S. aureus* MetRSs. The SaMetRS gene (UniProtKB – A0A0H2XID2) was PCR amplified (Sense 5'-GGGTCCTGGTTCGGCTAAAGAAACATTCTATATAACAACCCCAATATAC-3' and Antisense 5'-CTTGTTCGTGCTGTTTATTATTTAATCACTGCACCATTTGGAATTG-3') from genomic DNA isolated from *S. aureus* (ATCC 29213) cultures. The PCR product was then cloned into the AVA0421 plasmid and sequence verified. The expression of recombinant protein was performed as previously described (34). The N-terminal 6-His fusion proteins were purified by nickel affinity chromatography followed by size exclusion gel chromatography (Superdex 75 26/60; GE Biosciences, Piscataway, NJ).

Enzyme assays. Inhibition of SaMetRS was measured using the ATP depletion assay as previously described (34) with some modifications. Compounds were pre-incubated for 15 minutes at room temperature in a 96-well plate with 400 µg/mL bulk *E. coli* tRNA, 25 nM *Sa*MetRS, 0.1 U/mL pyrophosphatase, 0.2 mM spermine, 0.1 mg/mL bovine serum albumin, 2.5 mM dithiothreitol, 25 mM HEPES-KOH pH 7.9, 10 mM $MgCl_2$, 50 mM KCl, and 2% DMSO. Reagents were purchased from Sigma-Aldrich or Roche. The reaction was started with the addition of 150 nM ATP and 20 µM L-methionine and after 120 minute incubation was stopped by the addition of an equal volume (50 µL) of Kinase-Glo® (Promega). Percent inhibition = 100 X (test compound – AVG low control) / (AVG high control – AVG low control) where the low

control is all reagents except the compound and the high control is all reagents except the compounds and L-methionine. IC_{50} values were calculated by non-linear regression, sigmoidal-dose response, in Prism 3.0.

Bacterial strains. Strains with ATCC designations were either obtained directly from the American Type Culture Collection (Manassas, VA) or were kindly provided by the University of Washington Clinical Microbiology laboratory. *Escherichia coli* permeability mutants (properties shown in Table S1 with supplementary data) were provided as a gift from Dr. Katherine Young at Merck (Rahway, NJ).

Macromolecular synthesis assays: Methods for measuring uptake of radiolabeled precursors by *S. aureus* (ATCC strain 29213) were adapted from previous publications (21, 35, 36). For these assays, bacteria were grown in defined media (DM): RPMI-1640 pH 7.3 ± 0.1 without phenol red or L-glutamine (Lonza, Basel, Switzerland) supplemented with 4mM L-glutamine (Lonza, Basel, Switzerland), 10mM HEPES (Lonza, Basel, Switzerland), and 1% (w/v) D-glucose (Sigma Aldrich, St. Louis, MO). Fresh overnight cultures grown in DM at 37ºC were diluted 1:50 in pre-warmed DM and grown at 37ºC with shaking (150 rpm) until reaching an OD_{600} of 0.420 correlating to ~$1*10^9$ CFUs/mL in mid-log phase. Each compound was assayed in quadruplicate with an 11-point three-fold serial dilution per radioisotope. A pre-warmed 96-well V-bottom plate (Corning 3894; Corning, Corning, MA) containing 25 µL of 4X final concentration of test compound was inoculated with 65 µL of mid-log phase bacteria (OD_{600} of 0.420). Both positive and negative control wells received 25 µL untreated DM and 65 µL of inoculum at the same time. After one minute, 10 µL of radiolabeled precursor (10X final concentration in DM) was added to samples and positive control wells. Final isotope concentrations for assay of [3H]-lysine (protein), [3H]-thymidine (DNA), and [3H]-uridine (RNA) were 10 µCi/mL, 2 µCi/mL, and 2µCi/mL respectively. The plates were incubated at 37ºC for 25

minutes and terminated by the addition of 50 µL of 30% trichloroacetic acid (TCA)/70% ethanol to all test and control wells. After termination, 10 µL of 10X radiolabeled precursor was added to negative control wells. The negative control consisted of adding radiolabeled precursors *after* termination of the bacterial incubation in order to represent background measurement of the isotope. Plates were sealed with plate tape (Thermo Fischer Scientific, Waltham, MA) and shaken at 250 rpm for one hour at room temperature. Aliquots of 125 µL were transferred from the 96-well V-bottom plates to 96-well filter plates (Merck Millipore, Billerica, MA). To bind macromolecules, the samples were passed through the filter membrane (0.45µM hydrophilic Durapore PVDF membrane) with a vacuum manifold, then the filter was washed with 4 x 200 µL 10% TCA and 1 x 150 µL of 95% ethanol, and dried overnight in vacuum at room temperature. 25 µL Ultima Gold scintillation fluid (Perkin Elmer, Waltham, MA) was added to each well and DPM was quantified using a MicroBeta^2-2450 (Perkin Elmer, Waltham, MA) scintillation counter. The percent incorporation was determined by subtracting each well by the average negative background and dividing by the average positive incorporation x 100. Error bars represent SEM between replicates. The assay was run twice with similar results.

Susceptibility testing. MIC determinations were performed in triplicate in 96-well round bottom microtiter plates (Corning, Corning NY) as described by the Clinical and Laboratory Standards Institute (CLSI) (24, 33). Serial two-fold dilutions of compounds were added to plates in 50 µL volumes. An additional 50 µL of media containing bacterial cells (1x10^6 CFUs/mL) was then added to each well. Maximum DMSO concentrations were 0.5%. Plates were incubated at 37˚C for at least 18 h before reading the susceptibility result by optical absorbance (OD_{600}) using a BioTek ELx800. The lowest concentration causing ≥90% growth inhibition compared to the untreated control was recorded as the MIC (and also corresponded to the visual MIC). MICs were measured at least twice and the higher value (if different) was recorded herein.

MBC determinations (defined as the concentration killing 99.9% of the inoculum) were performed according to published methods (36,37). Using glass tubes (16 x 100-mm), serial twofold dilutions of 2x compound were generated from DMSO stocks in singlicate 1mL volumes. Maximum DMSO concentrations were 0.5%. An additional 1 mL of media with $1*10^6$ CFUs/mL was added per sample. Each experiment's inoculum was serially diluted and plated on TSA to count competent cells. Cultures were incubated at 37ºC for at least 20 h and plated on TSA for CFU determination. Additionally, each concentration was sampled after the 20 h incubation for MIC determination as above.

Cytotoxicity testing on mammalian cells: Human cell lines were acquired from the American Tissue Type Collection: CRL-1855 (lymphoblasts) and HepG2 (hepatocellular carcinoma cells). The cultures were grown in RPMI medium with 10% fetal bovine serum, penicillin, and streptomycin at 37°C with 5% CO2. In 96-well plates, the cells were exposed to serial dilutions of compounds for 48 hours and toxicity was quantified using AlamarBlue (ThermoFisher Scientific, Waltham, MA) (37). Assays were performed in quadruplicate and EC_{50} values were calculated with non-linear regression methods using software by the Collaborative Drug Database (Burlingame, CA. www.collaborativedrug.com).

Resistance frequency rates determination: The spontaneous resistance frequency rates to test compounds was determined according to published methods (35, 38). Agar selection plates were made by adding compound from DMSO stocks into molten Mueller Hinton agar in a 55ºC water bath. Each compound used four plates (P5981-100EA, 150 x 15mm; Sigma Aldrich, St. Louis, MO) containing 4X or 8x the MIC of the compound. The final DMSO concentration was <0.1% per plate. Plates were dried in a sterile hood for 30 minutes prior to overnight storage at 4ºC, and pre-warmed in the 37ºC incubator for 1 hour prior to assay.

A fresh overnight culture was diluted 1:50 in MHB and grown at 37ºC with shaking (150 rpm) until reaching an OD_{600} of 0.4 correlating to ~$2x10^9$ CFUs/mL. Approximately 3 mL for a total of $6*10^9$ CFUs were distributed onto 4 plates for each compound. Plates were incubated at 37ºC for 72 h prior to counting of colonies. The starting inoculum was also serially diluted and plated to quantify initial bacterial load. The resistance frequency was determined as the number of compound-resistant colonies divided by the total colonies plated.

Serum shift assays: To assess the role of protein binding on compound susceptibility, MIC determinations were performed in triplicate in the presence and absence of 50% human serum (39, 40). Serial twofold dilutions of 2x compound were generated in MHB and 50 microliters were aliquoted onto 96 well plates with a DMSO limit of 0.5%. Bacteria were adjusted to $1*10^8$ CFUs/mL in MHB, then separately further diluted 1:100 in MHB and 100% heat deactivated filter sterilized pooled human serum. Fifty microliters were added to each well of the corresponding plates, and the plates were incubated at 37ºC for ~20 h. The lowest concentration causing ≥90% growth inhibition was recorded as the MIC. The median value of three independent assays is reported.

Protein binding assays: Compound binding to mouse plasma proteins was determined using 96-well equilibrium dialyzer plates (SDIS 9610EN, Nest Group, Inc.). Mouse plasma (MSEPLLIHP-SW-F, BioreclamationIVT, Westbury, NY) containing compound (final concentration 1 µM) was added to a donor chamber as a 150 µL volume. The buffer solution (0.2 mM phosphate buffer, 150 µL) was added to the reciprocal acceptor chamber. Each compound was tested in triplicate. To prepare calibration solution for compound quantifications, blank wells were prepared containing only mouse plasma in a donor well and buffer solution in its acceptor well. The equilibrium dialysis was carried out by rocking the plate for 22 hours in 37 °C. Once equilibrium was reached, the plasma and buffer solution from both wells were

carefully removed for further analysis with liquid chromatography-tandem mass spectrometry. Plasma solution and internal standard were mixed in the presence of 80% acetonitrile. After centrifuging the solution, the supernatant was transferred to an insert. Similarly, the buffer solution from the acceptor side was prepared containing 40% acetonitrile. Calibration standards for donor and acceptor sides were prepared with compound concentrations of 50 nM, 100 nM, 250 nM, 500 nM, and 1µM. The compound concentrations from each well were calculated from the calibration curves using Microsoft Excel. The percentage of the test compound bound was determined as follows:

% Free = (Concentration buffer chamber/Concentration plasma chamber) × 100%

% Bound = 100% - % Free

Metabolic stability. Liver microsome stability assays were done by contract research laboratory, Wuxi AppTec Co. (Hubei, China). Briefly,compounds at 1 µM concentration were incubated in singlet with human or CD-1 mouse liver microsomes for 6 time points (0, 5, 10, 20, 30, and 60 min). Loss of parent compound was quantified by liquid chromatography/tandem mass spectrometry. The measured half-lives for control compounds (testosterone, diclofenac, and propafenone) were within the expected ranges.

Murine pharmacokinetics studies. Non-fasted female Swiss Webster mice (6-8 weeks) in groups of thee were administered compounds by oral gavage (10 mg/kg PO) or by retro-orbital injection (5 mg/kg IV). MetRS inhibitors were dosed in vehicle consisting of 7% Tween 80, 3% ethanol, and 0.9% saline. Linezolid was dosed in vehicle consisting of 0.5% methylcellulose (cP400), 0.5% Tween 80, and 0.9% saline. Time points for the blood collections were as follows. Oral: 30, 60, 120, 240, 360, 480, and 1440 min. IV: 5, 15, 30, 60, 240, 360, 480, and 1440 min. Tail blood was collected in heparinized capillary tubes and 20 uL was spotted onto Whatman FTA DMPK-C Cards (GE, Faairfield, CT). The whole blood samples were extracted

553 with acetonitrile for measurement of compound concentrations by liquid
554 chromatography/tandem mass spectrometry. Pharmacokinetic parameters were calculated
555 using Phoenix WinNonlin Version 6.3 software (Certara, Princeton, NJ).
556
557 **Murine thigh infection model.** Animal studies were approved by the Institutional Animal Care
558 and Use Committee at the University of Washington, Seattle. The methods were based on
559 published literature (41-44). Female specific pathogen free CD1 mice were obtained from
560 Charles River (Wilmington, MA) weighing 23-27 grams and allowed at least 3 days to acclimate
561 prior to study. Mice had access to food and water *ad libitum*. Neutropenia was induced by
562 administering cyclophosphamide monohydrate (Sigma Aldrich C7397; St. Louis, MO) via IP
563 injection 4 days (at 150 mg/kg) and 1 day (at 100 mg/kg) prior to infection. Neutropenic status
564 was confirmed by neutrophil count < 100 cells/mm^3. Overnight culture of *S. aureus* (ATCC strain
565 29213) was diluted 1:100 in MHB and incubated until reaching mid-log phase (OD_{600} < 0.750).
566 The inoculum was prepared by pelleting log-phase culture and re-suspending in sterile dPBS.
567 The culture was adjusted to OD_{600} of 0.200 and diluted 1:100 in sterile dPBS correlating to an
568 inoculum of ~5*10^5 CFU/50 µL. The mice were infected by an intramuscular injection of 50 µL in
569 the right posterior thigh while under isoflurane gas anesthesia. At 1 h post infection, one vehicle
570 group was sacrificed for determination of initial inoculum (status level of infection). Mice were
571 dosed at 2 and 12 h post-infection with test compounds as follows. Vancomycin was given at
572 100 mg/kg SC in 100 µL in a 0.9% saline solution. Linezolid was administered at 75 mg/kg PO
573 in 200ul of 0.5% methylcellulose, cP400 (Sigma Aldrich, St. Louis, MO), 0.5% Tween80 (Sigma
574 Aldrich, St. Louis, MO) in distilled water (45). MetRS inhibitors were administered at 75 mg/kg
575 PO in 200µL of vehicle containing 60% Phosal 53 MCT (Lipoid, Ludwigshafen Germany), 30%
576 PEG400 (Sigma Aldrich, St. Louis, MO), and 10% EtOH. Mice were sacrificed at 24 h post-
577 infection; the thigh muscle was sterilely removed, weighed, homogenized in 5mLs dPBS,

serially diluted, plated on tryptic soy agar in duplicate, and incubated overnight at 37ºC. Colonies were counted to quantify the bacterial load in CFUs per gram of thigh tissue.

Sequence alignments. Global pairwise amino acid sequence alignments were generated with NCBI alignment tool, CLUSTAL omega (46).

Acknowledgments

The research was supported by a grant from the Comotion Innovation Fund at University of Washington and by the National Institutes of Health grant AI097177. The authors wish to acknowledge Jennifer McCullar and Lynn Silver for consulting on the project direction and experimental design. The authors would like to thank Nicole Duster and Stephen Nakazawa Hewitt for cloning and purifying the SaMetRS, and Uyen Nguyen for technical assistance with the microbiology work.

Figure legends.

Figure 1. Chemical structures of MetRS inhibitors

Figure 2. Macromolecular synthesis experiments. Incorporation of radiolabeled precursors into *S. aureus* (ATCC 29213) in 30 minute incubations in the presence of established antibiotics or MetRS inhibitors. The dashed vertical line indicates the MIC.

Figure. 3. Efficacy of MetRS inhibitors in neutropenic mouse *S. aureus* thigh infection model. Error bars are SEMs. Stasis level was determined from untreated mice sacrificed 1 h post-infection (p.i.).

606 TABLE 1. Assay results of representative MetRS inhibitors. The second column shows the IC_{50}
607 values against recombinant *S. aureus* MetRS enzyme.

Cmpd Name	MetRS *S. aureus* (enzyme) IC50 (μM)*	*S. aureus* MSSA (ATCC 29213) MIC (μg/ml)	*S. aureus* MSSA (ATCC 19636) MIC (μg/ml)	*S. aureus* MRSA (ATCC 43300) MIC (μg/ml)	*S. aureus* MRSA (ATCC 33591) MIC (μg/ml)	*S. aureus* VISA (ATCC 700699) MIC (μg/ml)	*E. faecalis* (ATCC 29212) MIC (μg/ml)	*E. faecium* (ATCC 19434) MIC (μg/ml)	*E. faecium* VRE (ATCC 51559) MIC (μg/ml)
1312	<0.025	2.5	ND	5	ND	ND	0.31	0.16	0.16
1575	<0.025	10	ND	>10	ND	ND	1.3		0.63
1614	<0.025	2.5	2.5	5	5	10	1.3	0.63	0.63
1717	<0.025	0.16	0.08	0.16	0.08	0.16	0.08	0.04	0.16
1962	<0.025	2.5	ND	5	ND	ND	5	1.3	1.3
2062	<0.025	0.08	ND	ND	ND	ND	1.3	0.31	0.31
2093	<0.025	0.04	0.31	0.31	0.31	0.31	0.16	0.08	0.08
2114	<0.025	0.31	ND	ND	ND	ND	0.63	0.08	0.16
2144	<0.025	0.02	0.08	0.04	0.04	0.04	0.02	0.02	0.02
VAN	>10.0	1.3	1.3	1.3	1.3	10	5	0.63	>10
CIP	>10.0	0.16	0.08	0.16	0.31	>10	1.3	5	5
LNZ	>10.0	2.5	1.3	2.5	1.3	1.3	2.5	2.5	1.3

608
609 TABLE 1 (cont.)

Cmpd Name	*S. epi-dermidis* (ATCC 49134) MIC (μg/ml)	*S. epi-dermidis* (ATCC 12228) MIC (μg/ml)	*S. pyogenes* (ATCC 19615) MIC (μg/ml)	*S. pneumoniae* (ATCC 49619) MIC (μg/ml)	*E. coli* (ATCC 25922) MIC (μg/ml)	*P. aeruginosa* (ATCC 27853) MIC (μg/ml)	Mamm. cells (CRL8155) CC50 (μg/ml)	Mamm. cells (HepG2) CC50 (μg/ml)
1312	ND	ND	ND	ND	ND		>8	>8
1575	ND	ND	>10	>10	>10	>10	12	>18
1614	10	5	>10	>10	>10	>10	15	>18
1717	0.31	0.16	2.5	>20	>10	>10	5.8	10
1962	ND	ND	ND	ND	ND	ND	>20	>20
2062	1.3	ND	ND	ND	ND	ND	>21	>21
2093	1.3	0.63	1.3	>20	ND	ND	>22	>22
2114	1.3	1.3	ND	ND	ND	ND	>21	>21
2144	0.31	0.16	1.3	>20	ND	ND	>11	>11
VAN	2.5	2.5	0.63	0.31	ND	ND	>145	>145
CIP	0.31	0.16	0.63	1.3	0.04	0.31	>33	>33
LNZ	0.63	0.63	1.3	1.3	ND	ND	>34	>34

610
611 *Lower limit of detection for assay is 0.025 □M. (All the listed inhibitors have low- or subnanomolar
612 activity on the SaMetRS enzyme). Abbreviations: CIP (ciprofloxacin), LNZ (linezolid), MSSA (methicillin
613 sensitive *Staphylococcus aureus*), MRSA (methicillin resistant *Staphylococcus aureus*), ND (not done),
614 VAN (vancomycin), VISA (vancomycin intermediate *Staphylococcus aureus*).
615

TABLE 2. MICs (µg/mL) of selected MetRS inhibitors against *E. coli* strains with increased permeability.

Compound	MB4827 (wild-type)	MB4902 (*lpxC*)	MB5747 (*tolC*)	MB5746 (*lpxC, tolC*)
1717	>20	>20	>20	>20
2093	>20	>20	>20	>20
2144	>20	>20	>20	>20
CIP	0.016	0.008	0.008	0.008
NOV	160	20	0.5	0.5

TABLE 3. MICs and MBCs against *S. aureus* (ATCC 29213)

Molecule name	MIC (µg/mL)	MBC (µg/mL)	MBC/ MIC ratio	Published mechanism (48)
1717	0.16	5	32	
2093	0.06	1.3	16	
2144	0.010	0.31	32	
LNZ	2.5	160	64	Static
VAN	1.3	5	4	Cidal
NOV	0.16	5	32	Static
CIP	0.31	1.3	4	Cidal

TABLE 4. Frequency of spontaneous resistance for *S. aureus* (ATCC 29213) (two independent experiments and average)

Compound (fold over MIC)	Resistance frequency (Expt. 1)	Resistance frequency (Expt. 2)	Resistance frequency (average or greater #)
NOV (4)	ND	1.0×10^{-7}	1.0×10^{-7}
NOV (8)	ND	1.6×10^{-8}	1.6×10^{-8}
CIP (4)	1.0×10^{-8}	7.6×10^{-9}	8.9×10^{-9}
CIP (8)	$<2.6 \times 10^{-10}$	$<1.8 \times 10^{-10}$	$<2.6 \times 10^{-10}$
LNZ (4)	$<2.6 \times 10^{-10}$	$<1.8 \times 10^{-10}$	$<2.6 \times 10^{-10}$
LNZ (8)	$<2.6 \times 10^{-10}$	$<1.8 \times 10^{-10}$	$<2.6 \times 10^{-10}$
1717 (4)	5.3×10^{-9}	2.6×10^{-8}	1.6×10^{-8}
1717 (8)	2.89×10^{-9}	5.43×10^{-9}	4.16×10^{-9}
2093 (4)	5.0×10^{-8}	1.5×10^{-7}	9.8×10^{-8}
2093 (8)	1.6×10^{-8}	2.6×10^{-8}	2.1×10^{-8}
2144 (4)	2.0×10^{-8}	8.1×10^{-8}	5.0×10^{-8}
2144 (8)	3.15×10^{-9}	2.45×10^{-8}	1.38×10^{-8}

TABLE 5. MIC values for *S. aureus* (ATCC 29213) grown in MHB or MHB with 50% human serum. The table also shows experimental or published values for plasma protein binding (reference in parentheses).

Compounds	# expts	Median MIC (no serum) µg/mL	Median MIC (+50% serum) µg/mL	Fold change of MIC	% binding to mouse plasma	% binding to human plasma
VAN	3	1	2	2	25 (49)	55*
NOV	3	0.13	16	128	ND	95*
LNZ	3	2	2	1	39.5	31*
CIP	3	0.13	0.25	2	ND	20-40*
1614	3	2	32	16	96.5	ND
1717	3	0.06	1	16	97.6	95.4
2069	3	0.25	16	64	99.6	ND
2093	3	0.06	8	128	99.9	ND
2144	3	0.06	4	64	98.4	ND

*Data from Drugbank (www.drugbank.ca)

TABLE 6. Liver microsome stability half-lives.

Molecule Name	Microsome stability: Human T1/2 (minutes)	Microsome stability: Mouse T1/2 (minutes)
1614	9.8	6.6
1717	10.3	10.3
1962	25.9	27.0
2062	12.6	8.4
2093	8.3	3.2
2114	15.2	7.7
2144	16.4	6.5
Linezolid	>145	>145
Testosterone	17.4	3.1
Diclofenac	10.7	82.8
Propafenone	7.1	1.7

634 TABLE 7. Inhibitor binding site amino acid residues for MetRS enzymes

Seq. No. [a]	247	248	249	250	287	289	290	291	292	456	460	461
Pocket [b]	*b*	*b*	*b*	*l*	*q*	*q*	*q*	*q*	*q*	*q*	*q*	*q*
T.brucei	Pro	Ile	Tyr	Tyr	Asp	His	Gly	Gln	Lys	Leu	Ala	Ile
S. aureus	Pro	Ile	Tyr	Tyr	Asp	His	Gly	Gln	Lys	¶	Gly	Val/Ile*
H.sapiens - mitochondrial	Pro	Ile	Phe	Tyr	Asp	His	Gly	Leu	Lys	¶	Gly	Ile

635

Seq. No. [a]	470	471	472	473	474	476	477	478	480	481	519	522	523
Pocket [b]	*q*	*q*	*q*	*q*	*b*	*q*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
T.brucei	Cys	Val	Tyr	Val	Trp	Asp	Ala	Leu	Asn	Tyr	Ile	Phe	His
S. aureus	Val	Val	Tyr	Val	Trp	Asp	Ala	Leu	Asn	Tyr	Ile	Phe	His
H.sapiens/mito	Thr	Ile	Tyr	Val	Trp	Asp	Ala	Leu	Asn	Tyr	Ile	Phe	His

636 [a] Sequence numbers refer to the *T.brucei* sequence; [b] *l* = linker zone, *b* = benzyl pocket, *q* = quinolone
637 pocket (13). * This residue is Val in all S.aureus sequences in UNIPROT except for A0A033UAT9 (strain
638 C0673), where it is an Ile. ¶ Due to a different loop length it is unclear what the equivalent residue is.

639

640

References

1. Infectious Diseases Society of A. 2010. The 10 x '20 Initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. Clin Infect Dis 50:1081-3.
2. Nakama T, Nureki O, Yokoyama S. 2001. Structural basis for the recognition of isoleucyl-adenylate and an antibiotic, mupirocin, by isoleucyl-tRNA synthetase. J Biol Chem 276:47387-47393.
3. O'Dwyer K, Spivak AT, Ingraham K, Min S, Holmes DJ, Jakielaszek C, Rittenhouse S, Kwan AL, Livi GP, Sathe G, Thomas E, Van HS, Miller LA, Twynholm M, Tomayko J, Dalessandro M, Caltabiano M, Scangarella-Oman NE, Brown JR. 2015. Bacterial resistance to leucyl-tRNA synthetase inhibitor GSK2251052 develops during treatment of complicated urinary tract infections. Antimicrob Agents Chemother 59:289-298.
4. Jarvest RL, Berge JM, Berry V, Boyd HF, Brown MJ, Elder JS, Forrest AK, Fosberry AP, Gentry DR, Hibbs MJ, Jaworski DD, O'Hanlon PJ, Pope AJ, Rittenhouse S, Sheppard RJ, Slater-Radosti C, Worby A. 2002. Nanomolar inhibitors of Staphylococcus aureus methionyl tRNA synthetase with potent antibacterial activity against gram-positive pathogens. J Med Chem 45:1959-1962.
5. Jarvest RL, Berge JM, Brown P, Houge-Frydrych CS, O'Hanlon PJ, McNair DJ, Pope AJ, Rittenhouse S. 2003. Conformational restriction of methionyl tRNA synthetase inhibitors leading to analogues with potent inhibition and excellent gram-positive antibacterial activity. Bioorg Med Chem Lett 13:1265-1268.
6. Jarvest RL, Berge JM, Brown MJ, Brown P, Elder JS, Forrest AK, Houge-Frydrych CS, O'Hanlon PJ, McNair DJ, Rittenhouse S, Sheppard RJ. 2003. Optimisation of aryl substitution leading to potent methionyl tRNA synthetase inhibitors with excellent gram-positive antibacterial activity. Bioorg Med Chem Lett 13:665-668.
7. Jarvest RL, Armstrong SA, Berge JM, Brown P, Elder JS, Brown MJ, Copley RC, Forrest AK, Hamprecht DW, O'Hanlon PJ, Mitchell DJ, Rittenhouse S, Witty DR. 2004. Definition of the heterocyclic pharmacophore of bacterial methionyl tRNA synthetase inhibitors: potent antibacterially active non-quinolone analogues. Bioorg Med Chem Lett 14:3937-3941.
8. Critchley IA, Ochsner UA. 2008. Recent advances in the preclinical evaluation of the topical antibacterial agent REP8839. Curr Opin Chem Biol 12:409-417.
9. Critchley IA, Green LS, Young CL, Bullard JM, Evans RJ, Price M, Jarvis TC, Guiles JW, Janjic N, Ochsner UA. 2009. Spectrum of activity and mode of action of REP3123, a new antibiotic to treat Clostridium difficile infections. J Antimicrob Chemother 63:954-963.
10. Shibata S, Gillespie JR, Kelley AM, Napuli AJ, Zhang Z, Kovzun KV, Pefley RM, Lam J, Zucker FH, Van Voorhis WC, Merritt EA, Hol WG, Verlinde CL, Fan E, Buckner FS. 2011. Selective Inhibitors of Methionyl-tRNA Synthetase Have Potent Activity against Trypanosoma brucei Infection in Mice. Antimicrob Agents Chemother 55:1982-1989.
11. Shibata S, Gillespie JR, Ranade RM, Koh CY, Kim JE, Laydbak JU, Zucker FH, Hol WG, Verlinde CL, Buckner FS, Fan E. 2012. Urea-based inhibitors of Trypanosoma brucei methionyl-tRNA synthetase: selectivity and in vivo characterization. J Med Chem 55:6342-6351.
12. Ranade RM, Zhang Z, Gillespie JR, Shibata S, Verlinde CL, Hol WG, Fan E, Buckner FS. 2015. Inhibitors of Methionyl-tRNA Synthetase Have Potent Activity against Giardia intestinalis Trophozoites. Antimicrob Agents Chemother 59:7128-7131.
13. Huang W, Zhang Z, Barros-Alvarez X, Koh CY, Ranade RM, Gillespie JR, Creason SA, Shibata S, Verlinde CL, Hol WG, Buckner FS, Fan E. 2016. Structure-guided design of novel Trypanosoma brucei Methionyl-tRNA synthetase inhibitors. Eur J Med Chem 124:1081-1092.

14. Zhang Z, Koh CY, Ranade RM, Shibata S, Gillespie JR, Hulverson MA, Nguyen J, Pendem N, Gelb MH, Verlinde CL, Hol WG, Buckner FS, Fan E. 2016. 5-Fluoro-imidazo[4,5-b]pyridine is a privileged fragment that conveys bioavailability to potent trypanosomal methionyl-tRNA synthetase inhibitors. ACS Infect Dis 2:399-404.
15. Ibba M, Soll D. 2000. Aminoacyl-tRNA synthesis. Annu Rev Biochem 69:617-650.
16. Gentry DR, Ingraham KA, Stanhope MJ, Rittenhouse S, Jarvest RL, O'Hanlon PJ, Brown JR, Holmes DJ. 2003. Variable sensitivity to bacterial methionyl-tRNA synthetase inhibitors reveals subpopulations of Streptococcus pneumoniae with two distinct methionyl-tRNA synthetase genes. Antimicrob Agents Chemother 47:1784-1789.
17. Green LS, Bullard JM, Ribble W, Dean F, Ayers DF, Ochsner UA, Janjic N, Jarvis TC. 2009. Inhibition of methionyl-tRNA synthetase by REP8839 and effects of resistance mutations on enzyme activity. Antimicrob Agents Chemother 53:86-94.
18. Brown JR, Gentry D, Becker JA, Ingraham K, Holmes DJ, Stanhope MJ. 2003. Horizontal transfer of drug-resistant aminoacyl-transfer-RNA synthetases of anthrax and Gram-positive pathogens. EMBO Rep 4:692-698.
19. Dirheimer G, Keith G, Dumas P, Westhof E. 1995. Primary, secondary, and tertiary structures of tRNAs., p 93-126, tRNA: Structure, biosynthesis and function. ASM Press, Washington, DC.
20. Shinabarger D. 1999. Mechanism of action of the oxazolidinone antibacterial agents. Expert Opin Investig Drugs 8:1195-1202.
21. Cunningham ML, Kwan BP, Nelson KJ, Bensen DC, Shaw KJ. 2013. Distinguishing on-target versus off-target activity in early antibacterial drug discovery using a macromolecular synthesis assay. J Biomol Screen 18:1018-1026.
22. Critchley IA, Young CL, Stone KC, Ochsner UA, Guiles J, Tarasow T, Janjic N. 2005. Antibacterial activity of REP8839, a new antibiotic for topical use. Antimicrob Agents Chemother 49:4247-4252.
23. Kodali S, Galgoci A, Young K, Painter R, Silver LL, Herath KB, Singh SB, Cully D, Barrett JF, Schmatz D, Wang J. 2005. Determination of selectivity and efficacy of fatty acid synthesis inhibitors. J Biol Chem 280:1669-1677.
24. Clinical and Laboratory Standards Institute. 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 7th edition. Approved standard M7-A7., vol 26 No. 2. Clinical and Laboratory Standards Institute, Wayne, PA.
25. Koh CY, Kim JE, Wetzel AB, WJ dvdS, Shibata S, Ranade RM, Liu J, Zhang Z, Gillespie JR, Buckner FS, Verlinde CL, Fan E, Hol WG. 2014. Structures of Trypanosoma brucei methionyl-tRNA synthetase with urea-based inhibitors provide guidance for drug design against sleeping sickness. PLoS Negl Trop Dis 8:e2775.
26. Guiles J, Critchley I, Sun X. 2008. New agents for Clostridium difficile-associated disease. Expert Opin Investig Drugs 17:1671-1683.
27. Ojo KK, Ranade RM, Zhang Z, Dranow DM, Myers JB, Choi R, Nakazawa HS, Edwards TE, Davies DR, Lorimer D, Boyle SM, Barrett LK, Buckner FS, Fan E, Van Voorhis WC. 2016. Brucella melitensis Methionyl-tRNA-Synthetase (MetRS), a Potential Drug Target for Brucellosis. PLoS One 11:e0160350.
28. O'Neill AJ, Chopra I. 2004. Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. Expert Opin Investig Drugs 13:1045-1063.
29. Kerns EH and Li D. 2008. Drug-like Properties: Concepts, Structure Design and Methods. Academic Press, Burlington, MA.
30. Cohen BH, Saneto RP. 2012. Mitochondrial translational inhibitors in the pharmacopeia. Biochim Biophys Acta 1819:1067-1074.

31. Moren C, Juarez-Flores DL, Cardellach F, Garrabou G. 2016. The Role of Therapeutic Drugs on Acquired Mitochondrial Toxicity. Curr Drug Metab 17:648-662.
32. Garrabou G, Soriano A, Lopez S, Guallar JP, Giralt M, Villarroya F, Martinez JA, Casademont J, Cardellach F, Mensa J, Miro O. 2007. Reversible inhibition of mitochondrial protein synthesis during linezolid-related hyperlactatemia. Antimicrob Agents Chemother 51:962-967.
33. Clinical and Laboratory Standards Institute. 2007. Performance standards for Antimicrobial susceptibility testing; Approved standard M100-S17, vol 27 No. 1. Clinical and Laboratory Standards Institute, Wayne, PA.
34. Pedro-Rosa L, Buckner FS, Ranade RM, Eberhart C, Madoux F, Gillespie JR, Koh CY, Brown S, Lohse J, Verlinde CL, Fan E, Bannister T, Scampavia L, Hol WG, Spicer T, Hodder P. 2014. Identification of Potent Inhibitors of the Trypanosoma brucei Methionyl-tRNA Synthetase via High-Throughput Orthogonal Screening. J Biomol Screen 20:122-130.
35. Ochsner UA, Young CL, Stone KC, Dean FB, Janjic N, Critchley IA. 2005. Mode of action and biochemical characterization of REP8839, a novel inhibitor of methionyl-tRNA synthetase. Antimicrob Agents Chemother 49:4253-4262.
36. Cherian PT, Wu X, Maddox MM, Singh AP, Lee RE, Hurdle JG. 2014. Chemical modulation of the biological activity of reutericyclin: a membrane-active antibiotic from Lactobacillus reuteri. Sci Rep 4:4721.
37. Kraus JM, Verlinde CL, Karimi M, Lepesheva GI, Gelb MH, Buckner FS. 2009. Rational modification of a candidate cancer drug for use against Chagas disease. J Med Chem 52:1639-1647.
38. Young K. 2006. In vitro antibacterial resistance selection and quantitation. Curr Protoc Pharmacol Chapter 13:Unit13A.
39. Mani N, Gross CH, Parsons JD, Hanzelka B, Muh U, Mullin S, Liao Y, Grillot AL, Stamos D, Charifson PS, Grossman TH. 2006. In vitro characterization of the antibacterial spectrum of novel bacterial type II topoisomerase inhibitors of the aminobenzimidazole class. Antimicrob Agents Chemother 50:1228-1237.
40. Laue H, Valensise T, Seguin A, Hawser S, Lociuro S, Islam K. 2007. Effect of human plasma on the antimicrobial activity of iclaprim in vitro. J Antimicrob Chemother 60:1388-1390.
41. Hegde SS, Okusanya OO, Skinner R, Shaw JP, Obedencio G, Ambrose PG, Blais J, Bhavnani SM. 2012. Pharmacodynamics of TD-1792, a novel glycopeptide-cephalosporin heterodimer antibiotic used against Gram-positive bacteria, in a neutropenic murine thigh model. Antimicrob Agents Chemother 56:1578-1583.
42. Wang J, Shan Q, Ding H, Liang C, Zeng Z. 2014. Pharmacodynamics of cefquinome in a neutropenic mouse thigh model of Staphylococcus aureus infection. Antimicrob Agents Chemother 58:3008-3012.
43. Keel RA, Tessier PR, Crandon JL, Nicolau DP. 2012. Comparative efficacies of human simulated exposures of tedizolid and linezolid against Staphylococcus aureus in the murine thigh infection model. Antimicrob Agents Chemother 56:4403-4407.
44. Griffith DC, Harford L, Williams R, Lee VJ, Dudley MN. 2003. In vivo antibacterial activity of RWJ-54428, a new cephalosporin with activity against gram-positive bacteria. Antimicrob Agents Chemother 47:43-47.
45. Williams KN, Stover CK, Zhu T, Tasneen R, Tyagi S, Grosset JH, Nuermberger E. 2009. Promising antituberculosis activity of the oxazolidinone PNU-100480 relative to that of linezolid in a murine model. Antimicrob Agents Chemother 53:1314-1319.
46. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7:539.

782