AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

# Targeting Multiple Aminoacyl-tRNA Synthetases Overcomes the Resistance Liabilities Associated with Antibacterial Inhibitors Acting on a Single Such Enzyme

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**Bacterial aminoacyl-tRNA synthetases (aaRSs) represent promising antibacterial drug targets. Unfortunately, the aaRS inhibitors that have to date reached clinical trials are subject to rapid resistance development through mutation, a phenomenon that limits their potential clinical utility. Here, we confirm the intuitively correct idea that simultaneous targeting of two different aaRS enzymes prevents the emergence of spontaneous bacterial resistance at high frequency, a finding that supports the development of multitargeted anti-aaRS therapies.**

The aminoacyl-tRNA synthetase (aaRS) enzymes possess several features that render them promising prospects as broad-spectrum antibacterial drug targets; they are essential for viability, are found in all bacterial pathogens, and are in many cases sufficiently structurally distinct from their eukaryotic counterparts to allow selective targeting (1, 2). Furthermore, chemical and clinical validation exists for these enzymes as useful targets for antibacterial chemotherapy (1). However, despite the potential promise of this family of targets, only a single aaRS inhibitor with a relatively limited indication has been approved for the management of bacterial infection. Mupirocin (MUP), an inhibitor of isoleucyl-tRNA synthetase, is a topical agent deployed for nasal decolonization of *Staphylococcus aureus* and for the treatment of superficial skin infection (3).

Unfortunately, as with other antibacterial agents that act on a single enzyme target, aaRS inhibitors possess an intrinsic resistance liability (4). Mutants resistant to aaRS inhibitors are selected at a high frequency in bacterial populations ($\sim 10^{-7}$), typically as a result of point mutations within the gene encoding the drug target that lead to alteration of the latter in a manner that negatively impacts inhibitor binding (1). This liability, while manageable in the context of aaRS inhibitors such as MUP that are applied topically at concentrations sufficiently high to prevent or mitigate resistance, presents a problem for the development of aaRS inhibitors for systemic treatment of more serious bacterial disease. Indeed, GlaxoSmithKline halted phase II clinical trials of the leucyl-tRNA synthetase inhibitor GSK2251052 for the treatment of complicated urinary tract infections in adults following the emergence of mutants of *Escherichia coli* that were resistant to the drug in 3 of 14 patients within 2 days of administration (5).

It has been proposed that the resistance liabilities associated with aaRS inhibitors could be overcome with an inhibitor capable of targeting two or more aaRS enzymes simultaneously (1, 2, 6); an equivalent effect could be achieved with a cocktail of two or more aaRS inhibitors delivered in combination. This proposal is supported by the multitarget hypothesis, which states that antibacterial agents for which resistance is not readily selected by mutation usually act on more than one cellular target (7). By targeting two or more aaRS enzymes simultaneously, a situation is created in which the likelihood of resistance arising as a consequence of mutation in multiple targets becomes extremely low; for two aaRS enzymes, the frequency of mutation to resistance would be predicted to drop to $\sim 10^{-14}$ ($\sim 10^{-7} \times \sim 10^{-7}$). While this idea seems intuitively correct, it is possible to conceive of reasons why it might not hold true (e.g., a single mutation at a site other than the target genes may confer resistance to inhibition of multiple aaRS enzymes), and to our knowledge, it has not been tested. Here, we sought to evaluate the potential utility of such an approach by studying the *in vitro* emergence of resistance to combinations of aaRS inhibitors in *S. aureus*.

The antibacterial aaRS inhibitors used in this study were MUP (Sigma-Aldrich, Poole, United Kingdom); GSK2251052 (GSK), which was synthesized as described previously (8, 9); and the methionyl-tRNA synthetase inhibitor REP8839 (REP; Axon Medchem, Groningen, Netherlands). MICs of each compound against *S. aureus* SH1000 (10, 11) were determined by broth microdilution in Mueller-Hinton II (MHII) following CLSI guidelines (12), and the frequency at which mutants resistant to each individual compound arose was measured at 4× MIC on MHII agar, essentially as described previously (13). MUP, REP, and GSK inhibited growth of *S. aureus* SH1000 at concentrations of 0.25, 0.125, and 4 μg/ml, respectively, and at 4× MIC, all three compounds selected resistant mutants at frequencies of $10^{-7}$ to $10^{-8}$ (Table 1). For MUP and REP, these frequencies are comparable to those previously reported for *S. aureus* (14, 15); for GSK, mutation frequencies to resistance have not been reported for *S. aureus*, but the values obtained here are comparable to those reported for *E. coli* (5). To confirm that the colonies recovered were indeed mutants exhibiting reduced susceptibility to the corresponding aaRS in-

**Received** 24 March 2016 **Returned for modification** 18 April 2016
**Accepted** 12 July 2016

**Accepted manuscript posted online** 18 July 2016

**Citation** Randall CP, Rasina D, Jirgensons A, O'Neill AJ. 2016. Targeting multiple aminoacyl-tRNA synthetases overcomes the resistance liabilities associated with antibacterial inhibitors acting on a single such enzyme. Antimicrob Agents Chemother 60:6359–6361. doi:10.1128/AAC.00674-16.

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**TABLE 1** Selection and characterization of *S. aureus* SH1000 mutants resistant to aaRS inhibitors

| Strain | Competitive fitness[a] | Mutation frequency of resistance to[a]: | | |
|---|---|---|---|---|
| | | Mupirocin | REP8839 | GSK2251052 |
| SH1000 | 1 | $(5.3 \pm 0.6) \times 10^{-8}$ | $(7.1 \pm 0.2) \times 10^{-7}$ | $(2.5 \pm 0.5) \times 10^{-7}$ |
| SH1000 $IleRS_{V588F}$ | 0.93 ± 0.04 | ND[b] | $(7.3 \pm 0.1) \times 10^{-7}$ | $(2.7 \pm 0.3) \times 10^{-7}$ |
| SH1000 $IleRS_{V631F}$ | 0.98 ± 0.04 | ND | $(7.9 \pm 0.5) \times 10^{-7}$ | $(3.1 \pm 0.5) \times 10^{-7}$ |
| SH1000 $MetRS_{I57N}$ | 0.90 ± 0.08 | $(7.8 \pm 0.4) \times 10^{-8}$ | ND | $(3.1 \pm 0.1) \times 10^{-7}$ |
| SH1000 $MetRS_{V242F}$ | 0.87 ± 0.02 | $(7.3 \pm 0.1) \times 10^{-8}$ | ND | $(3.4 \pm 0.5) \times 10^{-7}$ |
| SH1000 $LeuRS_{G303V}$ | 0.86 ± 0.02 | $(7.4 \pm 0.4) \times 10^{-8}$ | $(7.6 \pm 0.4) \times 10^{-7}$ | ND |
| SH1000 $LeuRS_{D346N}$ | 0.91 ± 0.01 | $(7.8 \pm 0.3) \times 10^{-8}$ | $(7.2 \pm 0.1) \times 10^{-7}$ | ND |
| SH1000 $IleRS_{V588F}$ $MetRS_{V242F}$ | 0.62 ± 0.02 | ND | ND | ND |
| SH1000 $IleRS_{V588F}$ $LeuRS_{G303V}$ | 0.68 ± 0.07 | ND | ND | ND |
| SH1000 $IleRS_{V631F}$ $MetRS_{V242F}$ | 0.58 ± 0.04 | ND | ND | ND |
| SH1000 $IleRS_{V631F}$ $LeuRS_{G303V}$ | 0.65 ± 0.01 | ND | ND | ND |
| SH1000 $MetRS_{I57N}$ $LeuRS_{G303V}$ | 0.65 ± 0.04 | ND | ND | ND |
| SH1000 $MetRS_{V242F}$ $LeuRS_{D346N}$ | 0.61 ± 0.01 | ND | ND | ND |
| SH1000 $MetRS_{I57N}$ $IleRS_{V588F}$ | 0.64 ± 0.09 | ND | ND | ND |
| SH1000 $LeuRS_{G303V}$ $MetRS_{V242F}$ | 0.69 ± 0.02 | ND | ND | ND |
| SH1000 $LeuRS_{D346N}$ $IleRS_{V588F}$ | 0.66 ± 0.05 | ND | ND | ND |

[a] Results are means ± SD of at least three independent experiments.
[b] ND, not determined.

hibitor (not break-through growth), they were subjected to MIC determinations and PCR amplification/DNA sequencing of the gene encoding the drug target (*ileS*, *metRS*, and *leuS* in strains selected with MUP, REP, and GSK, respectively). All colonies tested exhibited ≥4-fold reductions in susceptibility to the aaRS inhibitor used for their selection. DNA sequence analysis of two MUP-resistant and two REP-resistant strains identified nonsynonymous mutations in *ileS* encoding amino acid substitutions $V_{588}F$ or $V_{631}F$ and in *metRS* encoding $I_{57}N$ or $V_{242}F$, respectively; all of these mutations were reported previously in the context of resistance to these aaRS inhibitors (14-16). In two GSK-resistant mutants, nonsynonymous mutations were independently identified in *leuS* that encode the amino acid substitution $G_{303}V$ or $D_{346}N$; the latter substitution has previously been identified in a GSK-resistant mutant of *E. coli* (5).

To determine the mutation frequency for resistance to simultaneous inhibition of two aaRS enzymes, cultures of SH1000 were concentrated by centrifugation and plated onto MHII agar containing all three possible combinations of aaRS inhibitors (MUP/REP, MUP/GSK, and REP/GSK), with each inhibitor included at 4× its respective MIC. No mutants resistant to any combination were recovered (limit of detection, $\sim 1 \times 10^{-12}$) after 72 h of incubation. Since potential synergistic interactions between aaRS inhibitors could complicate interpretation of these results by dramatically enhancing the antibacterial activity of individual compounds and thereby increasing the effective level of selection from 4XMIC to higher multiples of the MIC, we determined the fractional inhibitory concentration (FIC) index for each combination to exclude such effects (17). All three combinations were found to be additive (i.e., not synergistic), yielding FIC index values between 0.8 and 1.0 (data not shown). Thus, targeting two aaRS enzymes simultaneously prevents the rapid development of resistance associated with targeting one aaRS enzyme.

While a dual-targeted aaRS inhibitor/inhibitor combination would therefore overcome the gross resistance liability associated with single-target aaRS inhibitors, it seems likely that resistance would nonetheless arise over time by stepwise accumulation of resistance mutations, as has been observed for other multitargeted antibacterials (e.g., fluoroquinolones, β-lactams [18, 19]). To assess this, the SH1000 mutants resistant to a single aaRS inhibitor described above were used to independently select resistance to each of the other two aaRS inhibitors at 4× MIC (Table 1). In all cases, resistance to the second aaRS inhibitor in these resistant mutants arose at a similar frequency to that observed for selection of resistance to the same aaRS inhibitor in the fully susceptible SH1000 strain (Table 1). Thus, it is not difficult to select resistance to multiple aaRS inhibitors when the bacterium is challenged with both agents sequentially rather than simultaneously. To further evaluate the likelihood that strains resistant to multiple aaRS inhibitors may emerge, spread, and persist in the clinical setting, we examined whether the resulting resistance genotypes were associated with a reduction in competitive fitness. Pairwise competition assays were conducted between resistant strains and SH1000 over 24 h, following an established protocol (14). Fitness costs were relatively modest for mutants resistant to a single aaRS inhibitor (7% to 14%), while more considerable fitness costs were observed for mutants concurrently resistant to two aaRS inhibitors (31% to 42%) (Table 1). Thus, even when mutants resistant to multitargeted or multiple aaRS inhibitors do arise, they incur fitness burdens that may act to limit their clinical prevalence.

In conclusion, we have demonstrated that simultaneous targeting of two aaRS enzymes overcomes the considerable resistance liabilities associated with inhibitors acting against a single aaRS enzyme. Although mutants resistant to inhibitors of two aaRS enzymes can become selected in a sequential manner, suggesting that such genotypes would emerge in the clinical setting following prolonged selection, the double mutants are less fit and may be compromised in respect to clinical spread or persistence. Our findings therefore support the idea of discovering/developing aaRS inhibitor combinations or single agents that achieve dual targeting of aaRS enzymes. The latter represents a particularly appealing prospect and, in view of the high degree of structural similarity shared by the catalytic sites of subsets of the aaRS family (1), one that may prove feasible.

## ACKNOWLEDGMENTS

This work was supported by a grant from the European Union Framework 7 (FP7) program, Health.2013.2.31-1-NABARSI (grant agreement 601725).

The funders had no role in the study design or the decision to submit the work for publication.

We declare no conflicts of interest.

## FUNDING INFORMATION

This work, including the efforts of Christopher P. Randall, Dace Rasina, Aigars Jirgensons, and Alex J. O'Neill, was funded by European Commission (EC) (601725).

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