Targeting multiple aminoacyl tRNA synthetases overcomes the resistance liabilities associated with antibacterial inhibitors acting on a single such enzyme

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Running title: Resistance to aminoacyl tRNA synthetase inhibitors

Abstract

Bacterial aminoacyl tRNA synthetases (aaRS) 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 multi-targeted anti-aaRS therapies.

Text

The aminoacyl-tRNA synthetase (aaRS) family of enzymes possess several features that render them promising prospects as broad-spectrum antibacterial drug targets; they are essential for viability, found in all bacterial pathogens, and are in many cases sufficiently structurally distinct from their eukaryotic counterparts to allow selective targeting (1, 2). Furthermore, there exists both chemical and clinical validation 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 to date been approved for the management of bacterial infection; mupirocin, 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, in common with other antibacterial agents that act upon a single enzyme target, aaRS inhibitors possess an intrinsic resistance liability (4). Mutants resistant to aaRS inhibitors are selected at 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, whilst manageable in the context of aaRS inhibitors such as mupirocin that are applied topically at concentrations sufficiently high to prevent or mitigate resistance, presents a definite problem for the development of aaRS inhibitors for systemic treatment of more serious bacterial disease. Indeed, GSK halted Phase II clinical trials of the leucyl-tRNA synthetase inhibitor GSK2251052 for the treatment of complicated urinary tract infection in adults following the emergence of mutants of *Escherichia coli* resistant to the drug in 3 of 14 patients within two 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 multi-target 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 simultaneously targeting two or more aaRS enzymes, 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 $\sim10^{-14}$ ($\sim10^{-7}$ x $\sim10^{-7}$). Whilst 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 could confer resistance to inhibition of multiple aaRS enzymes), and it has to our knowledge 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 *Staphylococcus aureus*.

The antibacterial aaRS inhibitors used in this study were mupirocin (MUP; Sigma-Aldrich, Poole, UK), GSK2251052 (GSK) which was synthesised as described (8, 9), and the methionyl-tRNA synthetase inhibitor, REP8839 (REP; Axon Medchem, Groningen, Netherlands). Minimum inhibitory concentrations (MIC) of each compound for *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 4XMIC on MHII agar, essentially as described (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 4XMIC, all three compounds selected resistant mutants at frequencies of 10^{-7}-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 colonies recovered on agar

containing these agents at 4XMIC were indeed mutants exhibiting reduced susceptibility to the corresponding aaRS inhibitor (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^R and two REP^R 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 have been reported previously in the context of resistance to these aaRS inhibitors (14, 15, 16). In two GSK^R mutants, nonsynonymous mutations were independently identified in *leuS* that encode the amino acid substitutions $G_{303}V$ or $D_{346}N$; the latter substitution has previously been identified in a GSK^R 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 4X their respective MIC. No mutants resistant to any combination were recovered (limit of detection $\sim 1 \times 10^{-12}$) after 72 hours 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 Concentrations (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 does indeed prevent the rapid development of resistance associated with targeting one aaRS enzyme.

Whilst 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 step-wise accumulation of resistance mutations as has been observed for other multi-targeted antibacterials (e.g. fluoroquinolones, beta-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 4X 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 could emerge, spread and persist in the clinical setting, we examined whether the resulting resistance genotypes were associated with a reduction in competitive fitness. Pair-wise competition assays were conducted between resistant strains and SH1000 over 24 hours, following an established protocol (14). Fitness costs were relatively modest for mutants resistant to a single aaRS inhibitor (7-14%; Table 1), whilst a more considerable fitness cost was observed for mutants concurrently resistant to two aaRS inhibitors (30-42%; Table 1). Thus, even when mutants resistant to multi-targeted 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 of 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 would appear to represent 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.

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Table 1: Selection and characterization of *S. aureus* SH1000 mutants resistant to aaRS inhibitors. Results are the means of at least three independent experiments, with numbers in parentheses representing standard deviations. ND= not determined

Strain	Competitive fitness	Mutation frequency of resistance to:		
		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	$7.3 \pm 0.1 \times 10^{-7}$	$2.7 \pm 0.3 \times 10^{-7}$
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

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