REVIEW

Drugging tRNA aminoacylation

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ABSTRACT

Inhibition of tRNA aminoacylation has proven to be an effective antimicrobial strategy, impeding an essential step of protein synthesis. Mupirocin, the well-known selective inhibitor of bacterial isoleucyl-tRNA synthetase, is one of three aminoacylation inhibitors now approved for human or animal use. However, design of novel aminoacylation inhibitors is complicated by the steadfast requirement to avoid off-target inhibition of protein synthesis in human cells. Here we review available data regarding known aminoacylation inhibitors as well as key amino-acid residues in aminoacyl-tRNA synthetases (aaRSs) and nucleotides in tRNA that determine the specificity and strength of the aaRS-tRNA interaction. Unlike most ligand-protein interactions, the aaRS-tRNA recognition interaction represents coevolution of both the tRNA and aaRS structures to conserve the specificity of aminoacylation. This property means that many determinants of tRNA recognition in pathogens have diverged from those of humans—a phenomenon that provides a valuable source of data for antimicrobial drug development.

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Introduction

All living organisms encode information in the form of deoxy-ribonucleic acid (DNA), a template that is transcribed into messenger ribonucleic acid (mRNA) and subsequently translated into a corresponding sequence of amino acids during protein synthesis [1]. The physiochemical basis of translational fidelity hinges on correct coupling of each amino acid to its cognate tRNA molecule [2] and accurate base pairing between a codon in mRNA and an anticodon of an aminoacyl-tRNA [3]. Fidelity is thus ensured by the specificity of aaRSs, which couple each amino acid to their cognate tRNA molecule and recognize both substrates with exquisite precision. For some aaRSs, proofreading mechanisms further ensure high fidelity by reversing incorrect aminoacylation reactions, e.g. leucyl-tRNA synthetase (LeuRS) [4]. Aminoacyl-tRNAs are synthesized by the 3'-esterification of tRNAs with the appropriate amino acids. The majority of aminoacyl-tRNAs accomplish direct aminoacylation of a particular tRNA with the corresponding amino acid via a two-step reaction:

In the first step of this reaction scheme, ATP is hydrolyzed to form an aminoacyl-adenylate (AA-AMP) intermediate with the concomitant release of inorganic pyrophosphate (PPi). This AA-AMP intermediate is subsequently ligated to the tRNA in the second step, releasing AMP. The high-fidelity conversion of genetic information requires precise operation of this network of aaRSs [5] with the rules governing interactions between aaRSs and tRNAs termed “the second genetic code” [6]. The reaction shown in Scheme 1 holds true for all known aaRSs, indicating a high degree of functional conservation during molecular evolution.

To date, biochemical assays have been extensively used to elucidate genetic determinants of aminoacylation within both aaRS enzymes and tRNA, as well as to identify numerous aminoacylation inhibitors. Available assays are capable of discriminating between perturbative effects on either step of the reaction shown in Scheme 1. Step (1) of this reaction has largely been studied using the ATP-PPi exchange assay, which measures the rate of conversion of radiolabeled [³²P]-PPi into ATP. In this assay, purified aaRS is added to a reaction mixture containing its cognate amino acid, Mg, and [³²P]-ATP. Aliquots are taken and quenched at different time points, ATP is filtered from the solution, and [³²P]-PPi quantified through liquid scintillation counting [7,8]. More recently a spectrophotometric assay to detect release of PPi has been developed, applicable for high-throughput screening [9]. In this assay, inorganic pyrophosphatase (PPiase) and malachite green are added to the reaction mixture; PPiase converts PPi into inorganic phosphate (Pi), which reacts with malachite green and is detected by monitoring absorbance at 620 nm⁹. While PPi exchange assays are typically conducted in the absence of tRNA [8], authors of the spectrophotometric protocol note that the addition of tRNA enables additional rounds of catalysis, thereby producing a larger signal [9].

Aminoacylation assays are typically used to measure activity of both steps shown in Scheme 1, assaying formation of AA-tRNA. Early aminoacylation assays used radiolabeled amino acid (either ³H or ¹⁴C) in a reaction mixture containing aaRS, tRNA, ATP, and corresponding aaRS buffer. Aliquots of the

Scheme 1.

reaction mix are removed at incremental time points, spotted on filter paper to quench the reaction, and quantified through liquid scintillation counting [10,11]. An improved assay was later devised enabling use of saturating concentrations of amino acid by instead radiolabeling tRNA at its 3' end with ^{32}P using CCA-adding enzyme [12]. After quenching, aminoacylated tRNA is then digested with P1 nuclease and the radioactive products are separated and quantified by thin layer chromatography (TLC) [12]. Together with ATP-PP_i exchange, aminoacylation assays comprise the most common biochemical method used to characterize aaRS enzymes.

As aminoacylation is an essential step in protein synthesis, *in vitro* translation assays have also been used for early characterization of aaRS inhibitors. When the mechanism of an aaRS inhibitor antimicrobial is unknown, *in vitro* translation assays can be used to identify the pathway affected, as was the case for the antimicrobial pentamidine [13]. In another recently described assay, termed Selective Toeprinting in PURE System (SToPS) [14], an artificially constructed gene containing codons specifying all 20 amino acids is translated in the PURE [15] *in vitro* translation system. Primer extension inhibition (toeprinting) is then used to identify the site of translational arrest [14]. This method is able to both detect aaRS inhibitor activity, and identify which aaRS enzyme is affected within a mixture [14].

Developing inhibitors of aminoacylation has proven to be an effective strategy for development of antimicrobial drugs [16]. While most aminoacylation inhibitors identified to date target the amino acid or adenylate binding pockets within aaRS enzymes, a growing literature on experimental inhibitors of aminoacylation includes drugs targeting both aaRS tRNA binding sites, and those which target the tRNA itself directly [16,17]. Notably, the aaRS-tRNA interaction represents the coevolution of both genes, which have had the opportunity to diverge across different domains of life. Thus, the atomic determinants mediating every point of intermolecular contact across each the aaRS-tRNA interaction contain valuable structure-activity information for the structure-guided design of novel aminoacylation inhibitors. Recent advancements in structure-guided drug design can greatly accelerate the process of drug development [18], however atomic-level characterization of target sites is critical for these approaches [19,20].

Here we review available data regarding known aminoacylation inhibitors, as well as key determinants in both the aaRS and tRNA macromolecules that determine the specificity and strength of the aaRS-tRNA interaction. We focus on the molecular mechanisms of binding, turnover, and specificity in the aaRS-tRNA interaction with respect to drug design, utilizing the increasing body of available of genomic data, *in vitro* mutational analysis, and structural information [21,22].

Inhibition of aaRS as an antimicrobial strategy

Antimicrobial drugs must fulfill the fundamental requirement of exhibiting toxicity against the targeted organism while leaving

human physiology unaffected. Although all living organisms perform protein translation, selective inhibition of microbial protein synthesis has been shown to be one of the most common and effective approaches in antibiotic development [23,24]. Due to their central role in protein biosynthesis, aaRS proteins are excellent targets, particularly since many microbial aaRSs have significantly diverged from their human homologs. Phylogenetic sequence analyses across the three domains of life have revealed ancient divergence between bacterial and archaeal variants of nine aaRS genes (PheRS, TyrRS, LeuRS, IleRS, GluRS, TrpRS, HisRS, ProRS, and AspRS), followed by subsequent divergence of eukaryotic sequences from the archaeal lineage—a phenomenon termed a ‘full canonical pattern’ [25]. Similarly, a systematic comparison of over 4,000 tRNA sequences across the three domains of life identified numerous kingdom-specific characteristics for certain tRNA isoacceptors [26]. However, active site residues within their corresponding aaRS catalytic domains show a higher degree of structural conservation, likely reflecting the identical biochemical requirements for amino acid and adenylate recognition across different organisms [25,27,28].

All known aaRSs are multi-domain proteins, and are subdivided into two distinct classes based on the structure of their catalytic domain [25]. Within the active site of each aaRS catalytic domain, there exist three distinct binding pockets responsible for recognition of amino acid, adenylate, and tRNA moieties (Fig. 1)[22, 27]. While some aaRS inhibitors target the separate editing domain, the majority bind one or more pockets within the catalytic domain [16, 22]. Structurally, most aaRS inhibitor antibiotics act as non-cleavable mimics of AA-AMP, which is the natural intermediate produced during aminoacylation (see Scheme 1) [16, 22]. The best described example of such an inhibitor is mupirocin, which is one of only three approved aaRS inhibitor antibiotics prescribed for human or veterinary use (alongside halofuginone and tavaborole) [29–31]. Mupirocin (marketed as Bactroban) is a broad-spectrum topical antibiotic that mimics isoleucyl-AMP binding to inhibit IleRS across several species of bacteria. This antibiotic exhibits a strong preference for bacterial IleRS, and has been shown to inhibit *Escherichia coli* (*E. coli*) IleRS protein ~8,000-fold more strongly than the corresponding protein variant isolated from rat liver [32]. Mupirocin exhibits this high degree of specificity despite binding to the highly structurally conserved region of IleRS; structural analysis of a susceptible *Thermus thermophilus* (*T. thermophilus*) protein revealed its binding site to differ by only two amino acids relative to the resistant eukaryotic homolog [33]. Apart from mupirocin, numerous experimental aaRS inhibitors have been developed that mimic amino acid and/or AMP binding, which have previously been the subject of extensive review [16,22] (For antimicrobials discussed here and their corresponding cellular targets and binding sites, see Table S3).

The aaRS tRNA recognition site is a target for antimicrobial inhibition

As mupirocin illustrates, a mere two amino-acid difference between prokaryotic and eukaryotic aaRS binding sites can alone confer inhibitor selectivity. However, divergent substrate binding determinants within amino acid and AMP

Figure 1. Druggable regions for inhibition of aminoacylation. Examples of drugs targeting each region of either the aaRS enzyme or tRNA substrate are shown. Acceptor stem and anticodon tRNA regions (highlighted in red) are frequently strong determinants for aaRS recognition. Notable residues comprising the anticodon (N34, N35, N36), CCA-tail (A76, C75, C74) and discriminator base (N73) are also shown. AaRS regions involved in tRNA recognition are also depicted, including the tRNA, ATP, and amino acid sites in the catalytic domain, as well as the anticodon recognition region (shown in red) of the anticodon binding domain.

binding sites are rare considering the universal biochemical requirements for recognition of these substrates. Conversely, tRNAs have coevolved alongside each aaRS; therefore, the binding criterion for tRNA substrates exhibits a higher degree of divergence between distantly related organisms. Thus, it is surprising that among the many characterized aaRS inhibitors relatively few occupy the tRNA binding pocket [16,22]. Nonetheless, the existing aaRS inhibitors that target the tRNA binding pocket demonstrate the efficacy of this mechanism as a therapeutic strategy.

Halofuginone, an antiprotozoal agent commonly used in livestock with multiple potential clinical indications in humans [30], has been shown to inhibit ProRS by mimicking both proline and tRNA^{Pro} within the binding site [34]. Similarly, phenyl-thiazolylurea-sulfonamides have been shown to exhibit broad-spectrum antibiotic activity by inhibiting PheRS, and a recent crystal structure revealed that member compound 1-(3-((4-pyridin-2-yl)piperazin-1-yl)sulfonyl)phenyl)-3-(1,3-thiazol-2-yl)urea occupies the tRNA binding pocket of Phe [22,35].

Co-crystal structures of borrelidin—an experimental macrolide antimicrobial and anticancer drug—with human and *E. coli* threonyl-tRNA synthetase (ThrRS) recently revealed this compound to utilize a ‘four-site’ inhibitory mechanism [36]. Borrelidin interacts with adenylate, threonine, and tRNA^{Thr} binding sites in both ThrRS protein variants, thereby inhibiting interaction for all three enzyme substrates [36]. Unusually, borrelidin was found to occupy a fourth pocket within the aaRS active site that is uninvolved in

substrate binding but essential for the inhibitory action of this antibiotic [36].

The mechanisms of halofuginone, phenyl-thiazolylurea-sulfonamides, and borrelidin indicate that targeting the tRNA binding pocket may represent an efficacious strategy for antimicrobial drugs. As each of these compounds were discovered without knowledge of their mechanism of action [22], development of novel compounds targeting this binding site would require detailed information on the divergence of human and microbial tRNA binding. Recognition of each tRNA by its cognate aaRS is determined by a relatively small number of nucleotides termed “identity elements”, while misacylation by non-cognate synthetases is prevented by anti-determinant elements [37,38]. Although the coevolution of mutual recognition between cognate aaRS and tRNA molecules is essential for protein synthesis, the molecular shapes involved in each interaction are fundamentally arbitrary [39].

tRNA identity elements determine aaRS specificity

Identity elements for all tRNAs were first biochemically elucidated for *E. coli*, and available data remains the most comprehensive for this organism [5,37,40]. Individual identity elements vary in their importance; following mutation, nucleotides resulting in a large decrease in aminoacylation efficiency (k_{cat}/K_m) are considered ‘strong’, while those leading to a smaller decrease are considered ‘weak’ [37]. Across all tRNAs, strong identity elements are typically concentrated in the distal ends of the tRNA, corresponding to the acceptor stem and

anticodon stem-loop regions [37,41,42]. Within the acceptor stem position N73 is commonly found as a strong identity element, termed the ‘discriminator base’ [37,43]. Located adjacent to the ubiquitously conserved CCA tail, the discriminator base was the first identity element identified [43], and has been found to play a role in CCA addition [44]. The anticodon is itself frequently an identity determinant, with recognition governed by a separate anticodon-binding aaRS domain; however, there are many notable instances wherein recognition is anticodon-independent [37,45].

While the tRNA regions containing identity elements are strongly conserved, numerous differences in identity element nucleotides have been observed across different domains of life [27]. These differences have been shown to prevent cross-complementation of incompatible aaRS-tRNA pairs, a characteristic used to explain the evolutionary patterns observed for many aaRS genes [27,46]. Given the critical importance of aminoacylation to protein synthesis, identifying tRNA identity elements that diverge between humans and pathogens can highlight pathogen-specific interactions that can be targeted for antimicrobial drug design.

Across different organisms containing tRNA of prokaryotic origin, tRNA regions confirmed as identity elements in *E. coli* can be readily compared. Such prokaryotic tRNAs encompass not only bacterial tRNAs but also other endosymbiotic organelles of prokaryotic origin, including mitochondrial and apicoplastic tRNAs [47]. These organelles encode many of their own tRNA•aaRS pairs that exhibit more bacteria-like characteristics [48], and further show low levels of redundancy for tRNA genes enabling ready identification of consensus sequences. Since many eukaryotic tRNA and aaRS genes are highly divergent from their bacterial counterparts, developing antimicrobials that inhibit aaRS interactions with bacterial or apicoplastic tRNA identity elements is a feasible strategy. Notably, such antimicrobials must not inhibit mitochondrial tRNA•aaRS interactions in order to limit off-target effects in the eukaryotic host.

Eukaryotic genomes show substantially higher levels of tRNA sequence redundancy compared to prokaryotes, complicating bioinformatic analysis. Notably, the human genome has been found to be unexpectedly enriched for tRNA isodecoder tRNAs, which contain the same anticodon sequence but differing tRNA bodies with respect to one another [49]. In a study using tRNA^{Ser}, different isodecoders were found to vary in their suppression efficiencies by as much as 20-fold, corresponding to differing levels of aminoacylation [49]. This variability has complicated identification of critical tRNA identity elements for each isoacceptor in human cytosolic tRNA. Nonetheless, bioinformatic analysis has been used to identify consensus sequences for a number of isoacceptors [47, while biochemical analysis was applied to determine identity elements for another subset of tRNA variants [37,50–54].

As a result, identifying divergence in identity elements regions across tRNAs of eukaryotic origin presents a greater challenge. Nevertheless, multiple aaRS inhibitors of natural origin have been shown to be efficacious against eukaryotic pathogens (recently reviewed at length [55]), including halofuginone, which has been approved for veterinary use against coccidia [55]. A recent bioinformatics survey examined aaRS genes in

Leishmania major (*L. major*), the pathogen responsible for leishmaniasis. In this study, 11 new or extended aaRS domain structures were identified, as well as a putative novel AspRS paralog [56]. Following additional structural studies, these atypical aaRS proteins could likely serve as drug design targets. Indeed, existing crystal structures of *L. major* TyrRS and MetRS complexes have revealed multiple structural features within the catalytic domain that diverge from human homologs [57,58]. This observation suggests that the parsing of metagenomic aaRS sequences could be further applied to identify potential target aaRS genes within other pathogens in the order Trypanosomatida, which includes *Trypanosoma brucei* (*T. brucei*, responsible for trypanosomiasis) and *Trypanosoma cruzi* (*T. cruzi*, responsible for Chagas disease), as these organisms are likely to share many features with *L. major*.

Post-transcriptional modifications in tRNA contribute to tRNA identity in a domain-specific manner [59]. To date, over 100 different types of post-transcriptional modifications of RNAs have been identified across all three domains of life. Among the three major species of RNA (mRNA, rRNA, and tRNA), post-transcriptional modifications are most commonly found in tRNA [60–63]. Modified nucleosides in several *E. coli*, yeast, and archaeal tRNA variants have been biochemically shown to constitute significant identity element features, with replacement by unmodified nucleosides leading to significant loss of aminoacylation activity [64]. Modifications affecting identity are most commonly found at position 34 (within the anticodon) and position 37 (adjacent to the anticodon) [65]. Modifications at these positions are thought to have evolved to further stabilize AU-rich codon-anticodon pairs, as well as to prevent mistranslation and frameshifting [65]. At these positions, modifications have been found to diverge across the three domains of life (see ref [65], Table 5), and are thought to represent evolution of differing mRNA decoding strategies [65]. Modified bases in *E. coli* tRNA^{Ile} and yeast tRNA^{Asp} have also been shown to act as identity anti-determinants, preventing misacylation by *E. coli* MetRS and yeast ArgRS, respectively [66,67]. Notably, modified nucleosides cannot be identified through DNA sequencing data alone, and it is thought that many cryptic nucleotide modifications remain unidentified [68]. While confirmation of a modified nucleoside requires detailed structural characterization, modifications can be predicted through phylogenetic analysis of known modifications [63]. Recently, the tRNAmoviz web server (<http://genesilico.pl/trnamoviz>) has been made available as an online tool to facilitate predictive analysis of tRNA modifications [63].

Identification of aaRS-tRNA interactions for antimicrobial development

To ascertain whether a given aaRS-tRNA recognition interaction may constitute a suitable drug target, it is necessary to compare critical atoms involved in both human and pathogenic organisms. While critical residues can be identified within a given aaRS, this analysis requires detailed structural and/or biochemical characterization of a given enzyme. Fortunately, identification of such critical residues is more facile in tRNA. As detailed above, the high degree of conservation of tRNA identity element positions and the trove of experimentally

confirmed identity elements in *E. coli* together enable identification of divergent prokaryotic tRNA recognition interactions within metagenomic sequencing data.

In selecting a specific interaction to target for drug design, off-target activity is best avoided by initially focusing on prokaryotic tRNA identity elements that are divergent from their mitochondrial counterparts. As proof of concept, we have chosen *E. coli* as our target bacteria species in consideration of the extensive available data regarding this organism. We have compared identity elements previously determined biochemically in *E. coli* to their corresponding residue in *Homo sapiens* (*H. sapiens*) mitochondria, shown in Table S1. This analysis identified divergent identity elements in seventeen tRNA sequences.

Before selecting a target for drug development, it is also essential to further validate each potential position by assessing possible interactions with cytosolic human aaRSs. However, there is substantially less available data regarding identity elements for human cytosolic tRNAs (see above discussion on eukaryotic consensus tRNA sequences). Thus, identity elements that diverge between *E. coli* and human mitochondrial tRNAs were compared against available biochemical data for six *H. sapiens* cytosolic tRNAs (Table 1).

This analysis identified three *E. coli* tRNA genes containing identity elements divergent from both human cytosolic and

mitochondrial genes: tRNA^{Ser}, containing divergent residues G2:C71; tRNA^{Gly}, containing divergent residue U73; and tRNA^{Phe}, containing divergent residues G27:C43, G28:C42, G44, and U45. Thus, drugs disrupting aaRS recognition of these regions would be expected to inhibit *E. coli* growth while remaining nontoxic to human cells.

Each of the nucleotides detailed above were evaluated for potential tRNA modifications using the tRNAmoviz database [63]. Although modification data was unavailable for mitochondrial tRNA^{Gly} and mitochondrial tRNA^{Phe}, available *E. coli* and mitochondrial data were not predicted to show any modifications at these positions. While human cytosolic tRNA^{Gly} and tRNA^{Ser} were also not predicted to have modifications at the sites listed above, cytosolic tRNA^{Phe} was predicted to contain pseudouridine at position 27 and position 28, with no modifications predicted at positions 42–45. Despite potential nucleotide modification, these identity element residues remain useful guides for drug development as they are divergent from the homologous positions in *E. coli* tRNA. However, to identify drugs inhibiting recognition at these positions, exact knowledge of the nucleotides governing tRNA recognition interactions is essential to guide virtual screening of drug candidates.

Although *E. coli* provides an attractive test case for tRNA target identification given the extensive biochemical data

Table 1. Identity elements in *E. coli* tRNA vs human mitochondrial tRNA vs. human cytosolic tRNA.

Amino acid	<i>E. coli</i>	Human Mitochondria	Human cytosol	Reference
Leucine	A73 U8:A14	A73 U8:A14	A73	Breitschopf (1994) [50] Breitschopf (1995) [115]
Serine	G73 C72 G2:C71 A3:U70 C11:G24 R4:Y69	A73 (Ser1) or G73 (Ser2) C72 (Ser1) or U72 (Ser2) A2:U71 G3:C70 (Ser1) or A3:U70 (Ser2) Δ11:24 (Ser1) or A11:U24 (Ser2) A4:U69	A2:U71 C11:G24	Achsel (1993) [51] Breitschopf (1995) [115]
Proline	A73 G72 G35, G36 G15:C48	A73 G72 G35, G36 A15:U48	G15:C48	Stehlin (1998) [52]
Glycine	U73 G1:C72 C2:G71 G3:C70 C35, C36	A73 A1:U72 C2:G71 U3:A70 C35, C36	A73 G1:C72 G3:C70	Hipps (1995) [53]
Phenylalanine	A73 G34 A35 A36 G27:C43 G28:C42 U20, G44 U45 U59 U60	A73 G34 A35 A36 A27:U43 U28:U42 U20 A44 G45 A59 U60	U27:A43 U28:A42 G20 A44 G45 U59 C60	Nazarenko (1992) [54]
Alanine	A73 G2:C71 G3:U70 G4:C69 G20	A73 A2:U71 or G2:C71 G3:C70 G4:C69 A20 or U20	G3:U70 D20(D= A, G, or U)	Hou (1989) [116]

Table 1. *E. coli* tRNA identity elements shown to be divergent from human mitochondrial tRNA were further screened for divergence from human cytosolic tRNA identity elements. Conserved identity elements are shown in black. Identity elements in human mitochondrial or cytosolic tRNAs that diverge from those in *E. coli* are shown in blue. *E. coli* identity elements divergent from both human mitochondrial and cytosolic identity elements are shown in red. Identity elements in *E. coli* were determined biochemically as described by Giegé *et al*³⁷. Corresponding mitochondrial tRNA residues in *H. sapien* were identified using the mitotRNAdb database (<http://trnadb.bioinf.uni-leipzig.de>) [117]. Identity elements for *H. sapien* cytosolic tRNAs were determined biochemically, as detailed in the references provided. For positions where identity element positions identified in *E. coli* were not biochemically shown to affect aminoacylation in human cytosolic protein variants, the corresponding nucleotide at the homologous position is listed.

known for this organism, there exist numerous pathogenic microbes of equal or greater clinical importance. As regions containing identity elements tend to be conserved [41], bioinformatic analysis can predict identity elements in other prokaryotic species by identifying tRNA residues homologous to confirmed identity elements in *E. coli*. We extended meta-analysis of identity element conservation between bacteria and human mitochondria to two clinically relevant pathogens: Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) and Gram-negative bacteria *Pseudomonas aeruginosa* (*P. aeruginosa*) (Table S2). These two species were selected on the basis of their differing Gram-types and their classification as 'ESKAPE' pathogens, which encompass a broad-spectrum group of bacteria against which new therapies are urgently needed [69,70].

Susceptibility profiles across bacterial species can vary widely for different antibiotics. While drugs with a broader spectrum of activity are essential for treatment when the infective agent is unknown, narrow-spectrum antibiotics are typically preferred to reduce side effects of therapy and stall the emergence of antibiotic resistance [71]. As shown in Table S2, many tRNA identity elements are not only divergent between mitochondria and bacteria, but also between different bacterial species. Consequently, the spectrum breadth of aaRS inhibitor antibiotics that function by inhibiting specific recognition interactions can be readily predicted.

Using data shown in Table S2, we have predicted activity spectra of putative antibiotics that target the specific identity elements identified in Table 1. A compound disrupting recognition of either *E. coli* tRNA^{Ser} residues G2:C71, *E. coli* tRNA^{Gly} residue U73, or *E. coli* tRNA^{Phe} residues G44 or U45 would also be expected to be efficacious against both *P. aeruginosa* and *S. aureus*, acting as a broad-spectrum antibiotic against both Gram-negative and Gram-positive bacteria. In contrast, a compound disrupting *E. coli* tRNA^{Phe} residues G27:C43 or G28:C42 would be expected to be efficacious against *P. aeruginosa*, but not against *S. aureus*, thereby acting as a more narrow-spectrum antibiotic efficacious against Gram-negative bacteria.

This approach can readily be extended to predict the spectrum of drugs that recognize identity elements in other microbial species where sequencing data is available. However, in the absence of biochemical data, an important caveat to this cross-species prediction lies in the variable strengths of predicted identity elements which have been previously observed to vary across taxons [37,72,73].

Direct targeting of tRNAs by small-molecule inhibitors

To disrupt a key aaRS-tRNA interaction, it is viable to target either the aaRS or tRNA macromolecule (see Fig. 1). Generally, RNA molecules pose a more challenging drug target compared to proteins. Nonetheless, direct drug binding to the tRNA has also been shown to be a viable strategy for inhibiting aminoacylation. Targeting the anticodon stem loop region of tRNA may prove a promising approach, as tRNA identity elements are frequently concentrated in this region. While aminoglycoside antibiotics are best known for their role in inhibiting

translation through direct interaction with the 30S ribosomal subunit, several have been shown to also bind tRNA [74,75]. Notably, aminoglycoside antibiotic Neomycin B was shown to inhibit the *in vitro* aminoacylation of *E. coli* tRNA^{Phe} 76. Co-crystallization of yeast tRNA^{Phe} with Neomycin B showed that the inhibitor directly interacts with residues G20, A44 and G45 in the anticodon stem loop region, occupying a known metal ion binding site (Fig. 2). Drug binding thus inhibits aminoacylation by blocking aaRS interaction with these major identity element determinants [37,76,77]. Notably, Trana Discovery Inc. has developed a strategy for identifying novel drug candidates binding the anticodon stem loop region. Their approach utilizes fluorescent probes mimicking the anticodon stem loop to assay for drug candidate binding and has been applied to develop a high-throughput screen for antibiotics against *S. aureus* [78].

Other compounds have been shown to bind the T or D-loop regions of tRNA. In one example, the aminoglycoside tobramycin was shown to competitively inhibit *in vitro* aminoacylation of yeast tRNA^{Asp}, yet did not affect formation of aspartate adenylate as measured by ATP-PPi exchange [79]. Fluorescence anisotropy experiments using fluorescein-labeled tobramycin revealed a direct binding interaction with tRNA^{Asp} ($K_D = 267$ nM) [79], and selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) analysis subsequently revealed this compound disrupted interactions between the T- and D-loop regions [80].

Multiple RNase P inhibitors have also been shown to bind the T- and D-loops of tRNA. While these compounds do not affect aminoacylation, they demonstrate the viability of targeting identity elements within these regions. Bis-benzimidazole compounds have been shown by UV-visible and fluorescence spectrophotometry to directly bind different tRNA species, with hydroxyl radical footprinting showing binding in the D- and T-loops [81]. Porphyrin and porphine compounds have also been shown by fluorescence spectroscopy to bind tRNA and inhibit RNase P via putative displacement of a metal ion in the D-loop/T-loop region [82].

Another strategy is to bind the acceptor stem of tRNA to prevent tRNA exit from the aaRS active site. AN2690 (also

Figure 2. X-ray crystal structure of Neomycin B bound to tRNA^{Phe}. Shown at 2.6Å^o resolution (PDB file 1I9V), Neomycin B occupies a binding cleft on tRNA^{Phe} one full helical turn above the anticodon stem-loop. Interactions within 3.5 Å include nucleotides 20, 44, and 45, which are represented by dashed yellow lines.

known as Tavaborole), a boron-containing antimicrobial, was shown in a crystal structure to bind yeast tRNA^{Leu} at the 3' terminal adenosine, causing the tRNA to become trapped inside the aaRS editing site [83]. Similarly, plant tumor control agent Agrocin 84 has been shown to be processed inside the cell to produce TM84, a Leu-AMP analog that crystal structures show to also bind to the 3' terminus of tRNA^{Leu} [84]. This binding interaction traps the aaRS-tRNA complex in an 'aminoacylation-like' conformation, preventing further enzymatic turnover [84].

Antimicrobial agents pentamidine and purpuromycin have been shown to inhibit aminoacylation through nonspecific tRNA binding mechanisms. These compounds have both been shown to inhibit *in vitro* aminoacylation of different synthetases while leaving formation of adenylate unaffected, as measured by ATP-PPI exchange [13,85]. Unusually, both compounds were also shown to exhibit a nonspecific tRNA binding mechanism. For pentamidine, free hydroxyl radical footprinting found that pentamidine protected many regions of tRNA^{Leu}, encompassing nearly the entire RNA phosphate backbone. Isothermal titration calorimetry (ITC) further revealed a large number of pentamidine binding sites, with binding driven largely through hydrophobic interactions [13]. Pentamidine has also been shown to inhibit self-splicing of the nuclear group I introns in the rRNA of multiple fungal species [86–88], consistent with a mechanism of nonspecific targeting of structured rRNA species in addition to tRNA [13]. Purpuromycin was shown by aminoacylation assay to inhibit the aminoacylation of several bacterial tRNA variants, and was further found to bind the acceptor stem region [85]. Authors further noted that the addition of a mixture of total tRNA to the *in vitro* mixture restored aminoacylation activity, concluding that purpuromycin directly interacts with many tRNA species in a nonspecific manner [85].

Taken together, these findings demonstrate multiple strategies for directly targeting regions of interest in tRNA. While nonspecific mechanisms such as those utilized by pentamidine and purpuromycin may be difficult to recapitulate through rational design, identity elements in the anticodon stem loop and D-loop/T-loop regions provide more feasible drug targets. Targeting metal ion binding pockets also appears to be a viable strategy to both occlude recognition of nearby identity element residues and further destabilize tRNA through displacement of metal ions. Metal ions can readily be identified within available structural data [89], with the importance of metal ions to RNA folding and function having previously been the topic of review [90]. However, efforts to target RNA for drug binding need to take into account the polyanionic charge of the backbone as well as the abundance of hydrogen bond donors and acceptors [91]. Despite recent advances, structure-guided drug development tools for targeting RNA remain less developed than those for proteins [92].

Inhibiting aaRS enzymes through tRNA mimics

Development of a compound designed to mimic tRNA recognition would be expected to competitively inhibit the cognate aaRS. Early experiments found that synthetic oligonucleotides mimicking recognition elements of tRNA competitively inhibit

the formation of aminoacyl-tRNA, while leaving the formation of aminoacyl-adenylate unaffected [93,94]. While these oligonucleotides themselves are not viable as drugs due to their inability to traverse cellular membranes, they do serve to validate the strategy of tRNA mimics as aaRS inhibitors. The existence of aaRS inhibitors targeting the tRNA binding site (described above) further demonstrate the capacity of small molecules to mimic tRNA binding.

As mentioned above, previous reviews of the aaRS literature have long established three distinct activities within the catalytic domain mediating aminoacylation activity: (1) the amino acid binding site, (2) the ATP binding site and (3) the tRNA binding site [28,95]. To elucidate structure-activity insights within the scope of this review, we focus on key aaRS motifs that interact with tRNA identity elements. Within the catalytic domain of all aaRS enzymes, the tRNA binding site interacts with the acceptor stem of the tRNA, comprising the canonical tRNA 3'-CCA end as well as N72 and the N73 'discriminator base' important for tRNA specificity recognition. This site buries the 3' terminal nucleotide A76 and makes contacts with other acceptor stem nucleotides between N72–76, with these nucleobases often found in flipped conformation in known x-ray crystal structures of the aaRS-tRNA complexes. The tRNA acceptor stem binding site is directly adjacent to the ATP site, suggesting that this orientation is critical for proper aminoacyl adenylate formation. In the absence of ATP, A76 has been observed to occupy the ATP site [96]. This structural homology between the A76 base and ATP may explain the existence of multiple dual-site inhibitors of aaRS (eg., borrelidin, halofuginone) and the absence of any known inhibitors that only bind the tRNA acceptor stem site [22].

All aaRS enzymes excluding SerRS, LeuRS, and AlaRS also contain a separate anticodon binding domain, recognizing anticodon nucleotides distal from the aforementioned catalytic site [97]. These anticodon residues (found at tRNA positions 34–36) mediate the interaction between tRNA and the genetic code, and consequently vary only under extremely rare circumstances [98,99]. Consequently, interactions of anticodon residues do not provide viable drug targets considering their near universal conservation between pathogenic species and humans. However, nucleotides adjacent to the anticodon (eg., positions 31–39) have been observed to act as tRNA identity elements (see Table S1). These residues comprise what has been termed the proximal 'extended anticodon', with each nucleotide in this region having evolved to mediate uniform mRNA decoding by modulating the stability of codon-anticodon pairs and the strength of the interaction between tRNA and the ribosomal A-site [65]. Divergent interactions at these positions may present viable drug targets, however care would need to be taken to avoid inhibition of anticodon recognition by human aaRS proteins.

Many aaRSs also contain an editing site, responsible for hydrolyzing amino acids which have either been incorrectly adenylated (termed pre-transfer editing) or aminoacylated (termed post-transfer editing). Although editing sites predominantly recognize amino acid moieties, post-transfer editing often involves binding to 3' terminal regions of tRNA [29,100]. As compounds AN2690 and TM84 (described above) demonstrate, it is possible to inhibit

of aaRS activity through interactions with the aaRS editing site [83,84]. However, targeting interactions between tRNA and the aaRS editing site would also necessitate consideration of the catalytic site during drug development, as both the editing and catalytic aaRS sites bind the tRNA acceptor stem.

Analysis of available crystal structures of aaRS-tRNA complexes make it clear that many tRNA identity elements mediate interactions outside of previously described binding pockets. In these cases, the aaRS-tRNA interface bears more similarity to a protein-protein interaction rather than a ligand interface. While approaches for drugging such comparatively featureless interactions continue to improve, such regions within aaRS structures nonetheless present more challenging targets for small molecule drug binding [101,102].

Towards identification of selective aaRS inhibitors targeting the tRNA binding site, eukaryotic GlyRS genes have been identified as particularly phylogenetically divergent in comparison to their prokaryotic homologs [103]. Prokaryotic GlyRS protein variants are known to form $\alpha_2\beta_2$ heterotetramers, belonging to aaRS subclass IIC; eukaryotic GlyRS protein variants conversely form α_2 homodimers, belonging to aaRS subclass IIA [104,105]. Functionally, this is reflected by the inability of eukaryotic and prokaryotic GlyRS enzymes to cross-aminoacylate tRNA^{Gly} species originating from the other domain [104]. Intriguingly, this selectivity is almost entirely explained by a single difference at the N73 discriminator base, with eukaryotic and prokaryotic tRNA^{Gly} containing A73 and U73, respectively (Table 1). While recent co-crystal structures were unable to provide ‘smoking gun’ evidence of the exact structural basis of tRNA acceptor stem recognition by GlyRS, they have provided strong evidence that the acceptor site of GlyRS is an appropriate target for the rational design of antimicrobials [106].

Prokaryotic PheRS represents another high-priority target aaRS for rational design of tRNA binding site inhibitors. Analogous to GlyRS, PheRS proteins of different domain origin also exhibit different quaternary structures, and based on sequence analysis it has been proposed that prokaryotic and human enzymes exhibit different tRNA binding modes [107,108]. As mentioned above, phenyl-thiazolylurea-sulfonamides are a novel antibiotics class highly specific for bacterial PheRS, exhibiting nanomolar inhibitory IC₅₀ values in aminoacylation assays using both Gram-negative and Gram-positive bacterial PheRS protein variants [109]. X-ray crystal structures of 1-(3-((4-pyridin-2-yl)piperazin-1-yl)sulfonyl)phenyl)-3-(1,3-thiazol-2-yl)urea bound to *P. aeruginosa* PheRS revealed that selectivity is determined by a single amino acid Met99, which is present in prokaryotic PheRS but mutated to leucine in both cytoplasmic and mitochondrial versions of mammalian PheRS [109].

Structural dynamics dictate functionality in the aaRS-tRNA complex

A growing body of evidence supports the idea that aaRSs are a highly dynamic enzyme class that undergo induced fit upon binding tRNA and during aminoacylation, with major conformational changes occurring for both macromolecules.

Structural dynamics analyses during aaRS activity have been performed through co-crystallization at various steps of tRNA binding, aminoacylation and turnover. For example, a series of high-resolution structures of *E. coli* leucyl-tRNA synthetase ternary complexes probed both the aminoacylation and editing conformations of the complex [110]. Furthermore, a co-crystallized inactive complex of *E. coli* AspRS and yeast tRNA^{Asp} supports a distinct tRNA-initiated control mechanism that initiates loop movement in the AspRS catalytic domain, opening the acceptor stem site of AspRS [96]. A serendipitous high-resolution structure of human GlyRS demonstrated two distinct conformational states of GlyRS recognition during tRNA complexation within a single crystal lattice, supporting conformational change in GlyRS loop regions to allow tRNA acceptor stem entry for the first step of aminoacylation [111]. A high degree of flexibility in this region during both tRNA binding and aminoacylation was further supported by limited molecular dynamics studies [111].

These critical structural studies have provided excellent snapshots of aaRS dynamics steps, and should further enable high-performance molecular dynamics studies. However, it remains a priority in the field to solve additional human cytosolic aaRS-tRNA complexes, as relatively few have been co-crystallized successfully with their cognate tRNAs [111–113]. Modern advancements in graphics processing computing over the past five years have further enabled microsecond-scale simulations of even large multi-chain systems including aaRS-tRNA complexes. A complete set of high-resolution mammalian aaRS-tRNA complex structures will be an invaluable tool towards the preparation of accurate dynamical models of aminoacylation for the rational design of species-specific therapeutic inhibitors.

Conclusion

Following its discovery, the genetic code has famously been described as a “frozen accident”, as it has remained largely unchanged throughout nature [114]. By contrast, the so-called “second genetic code” has proven far more malleable, with the network of interactions between aaRSs and tRNAs exhibiting substantial variation between distant phylogenies. Targeting such interactions in pathogens where they diverge from those in humans is likely to become an increasingly viable antimicrobial development strategy as structure-guided drug design tools continue to improve. Considering the efficacy of current antimicrobial drugs that target aaRS activity, as well as the abundant and growing body of data surrounding these enzymes, it is clear that this class of protein will remain valuable for future drug development.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed

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