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Review Article

Phenylalanyl-tRNA synthetase as a target for potential new antibacterial agents

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CONTENTS

Abstract

Increasing rates of bacterial resistance to known classes of antibiotics present a severe global challenge. As a consequence, the search for new chemical entities that address novel bacterial targets continues. Aminoacyl-tRNA synthetases are essential enzymes for protein biosynthesis that have emerged as an interesting target class in antibacterial research. The value of this target class is exemplified in this review by the progress achieved with inhibitors of bacterial phenylalanyl-tRNA synthetase (phenylalanine–tRNA ligase) as potential antibacterial agents.

Introduction

Since the introduction of penicillin in the 1940s, antibiotics have a history of success in controlling morbidity and mortality caused by infectious diseases. However, as a consequence of frequent use, bacterial resistance to known classes of antibiotics has become a severe global problem in recent years and presents a continuous clinical challenge (1-3). Resistance can result from modification of an antibacterial's target or from functional bypassing of that target, or it can be contingent on impermeability, efflux or enzymatic inactivation of the drug (4). Methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant *Streptococcus pneumoniae* (PRSP) are known as important pathogens in the clinic and are now increasingly encountered in community-acquired infections. Glycopeptides, especially vancomycin, have been the drugs of choice to treat such serious infections caused by resistant Gram-positive bacteria. But alarmingly, pathogens with intermediate or high-level resistance to this compound class have emerged as well. Glycopeptide-resistant enterococci (GRE) were first isolated in 1986 (5, 6). Vancomycin-intermediate resistant *S. aureus* (VISA) clinical isolates were described in 1997 (7) and fully vancomycin-resistant *S. aureus* (VRSA) in 2002 (8, 9). The World Health Organization (WHO) recently addressed the problem of bacterial resistance and issued a document entitled "WHO Global Strategy for Containment of Antimicrobial Resistance" (10).

There are serious concerns that untreatable pathogens may develop at an alarming rate in the near future. Strategies to address this challenge include the design of improved versions of antibacterial classes already in clinical use and the use of drug combinations. The application of these strategies can be quite successful, but a high risk of rapid resistance development remains. Thus, an urgent need for novel potent classes of antibiotics with new modes of action persists. New chemical entities that inhibit novel targets are less likely to be affected by existing resistance mechanisms. A potential novel target needs to fulfill various selection criteria: the cell function linked with the target should be essential for survival of the pathogen; selective inhibition of the bacterial target *versus* the human counterpart must be feasible; to insure long-term usefulness, it must be difficult for bacteria to develop resistance by mutations; and it should be possible to cover a broad spectrum of Gram-positive and/or Gram-negative pathogens. In addition, amenability to high-throughput screening would be favorable for the drug discovery process.

Aminoacyl-tRNA synthetases as novel targets

Aminoacyl-tRNA synthetases (aa-RSs) have attracted interest as potential novel targets in bacterial protein synthesis (11-15). These essential enzymes are found in all living organisms (16), and they are indispensable for the highly specific translation of the messenger RNA (mRNA) template into protein via specific transfer RNAs (tRNAs)

as adapter molecules. When one aa-RS is inhibited, the corresponding tRNA is not charged and is therefore unavailable for translation. This leads to protein synthesis inhibition, which in turn causes cell growth arrest. Consequently, compounds that inhibit any of the aa-RSs could be potential antibacterial agents.

In most organisms there are 20 synthetases, 1 for each amino acid, which are responsible for coupling the amino acid to the corresponding tRNA. The catalytic reaction proceeds in two steps (Figs. 1 and 2) and includes: first, the activation of the amino acid by adenylation, yielding aminoacyl-adenylate (aa-AMP) as an intermediate, with concomitant release of pyrophosphate, and subsequently, the transfer of the amino acid to the 2'- or 3'-ribose hydroxyl group at the 3'-end of the cognate tRNA. The charged aminoacyl-tRNA (aa-tRNA) is now able to interact with the elongation factor Tu (EF-Tu) and with the ribosomal A-site to elongate the nascent protein chain.

The aminoacyl-tRNA synthetases are classified into two structurally distinct classes on the basis of typical

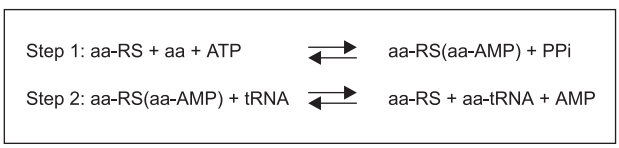

Fig. 1. Reactions catalyzed by aminoacyl-tRNA synthetases (aa-RS) during charging of tRNA with the corresponding amino acid. aa = amino acid; AMP = adenosine monophosphate; ATP = adenosine triphosphate; PPi = inorganic pyrophosphate.

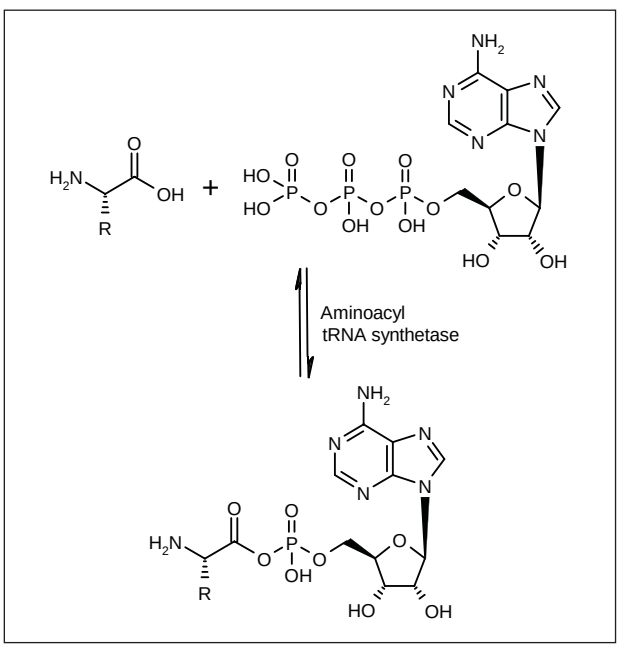

Fig. 2. Formation of aminoacyl-adenylate, the first step in the reaction catalyzed by aminoacyl-tRNA synthetases.

Table I: Classification of aminoacyl-tRNA synthetases.

Class I	Class II
Subclass Ia	*Subclass IIa*
Ile-RS	Ala-RS
Leu-RS	Pro-RS
Val-RS	His-RS
Cys-RS	Ser-RS
Met-RS	Thr-RS
Arg-RS	Gly-RS
Subclass Ib	*Subclass IIb*
Gln-RS	Asn-RS
Glu-RS	Asp-RS
Lys-RS-I	Lys-RS-II
Subclass Ic	*Subclass IIc*
Tyr-RS	Phe-RS
Trp-RS	

consensus motifs (Table I) (17-19). A database has been established which compiles the amino acid sequences of all aminoacyl-tRNA synthetases known to date (20). Each of the 2 classes consists of 10 members. The catalytic domain of class I aa-RSs is formed by the dinucleotide-binding Rossman fold, which consists of a central 5-stranded parallel β-sheet connected by α-helices. All class I proteins contain the consensus motifs HIGH and KMSKS, which have been shown to constitute a part of the ATP binding site. In contrast, the active site of class II enzymes is a core of antiparallel β-sheets surrounded by loops and α-helices in a barrel-like structure. Three characteristic motifs have been identified in the catalytic domains of the class II aa-RSs. The motif 1 amino acid residues constitute the interface between the monomers in the class II dimers, whereas the amino acid residues from motifs 2 and 3 are involved in amino acid and ATP recognition and binding. The class I and class II enzymes are further differentiated by the site of tRNA aminoacylation. The class I aa-RSs esterify the 2'-hydroxyl group of the ribose at the 3'-terminal adenosine base of the tRNA, whereas most of the class II synthetases attach the amino acid to the 3'-hydroxyl group. This difference is caused by the interaction with opposite sites of their respective substrate tRNA. Class I enzymes approach the acceptor stem of tRNA from the minor groove side, while the class II enzymes approach the major groove side. The structural and mechanistic differences between these two classes are highly conserved in prokaryotes and eukaryotes, suggesting that their divergence occurred very early in the evolution of cellular organisms, or that they did not split out of the same ancestral structure at all.

Based on their mode of binding to the tRNA acceptor stem, both classes of aa-RSs have been further subdivided into three subclasses (21, 22). In class II, the enzymes of subclasses IIa and IIb are usually homodimers. Most of the class IIa proteins possess a common *N*-terminal catalytic domain and a *C*-terminal α/β-type domain, which is probably involved in the tRNA anticodon

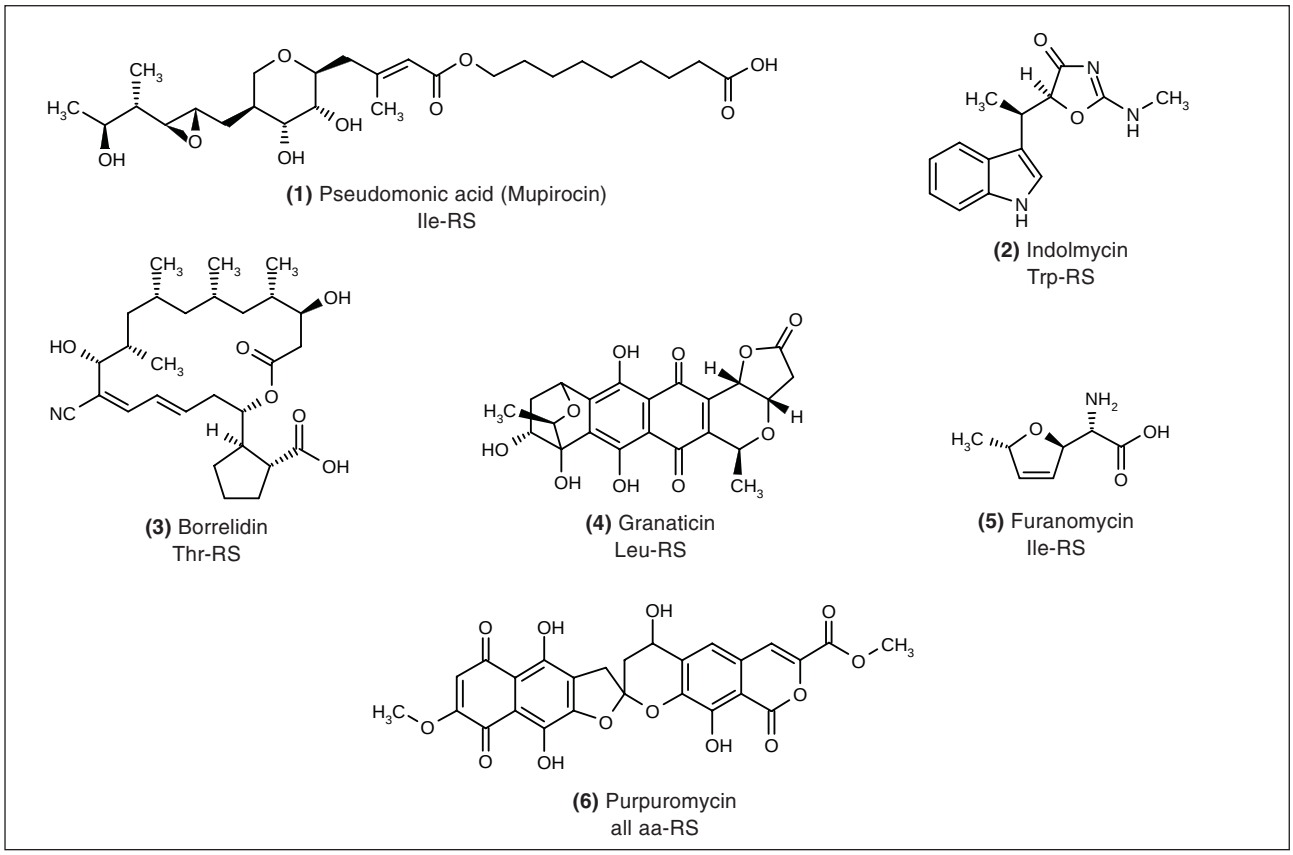

Fig. 3. Selection of natural aminoacyl-tRNA synthetase inhibitors.

stem-loop binding (Pro-RS, His-RS, Thr-RS, α_2-dimeric Gly-RS). The only exception is Ser-RS, which does not recognize the tRNA anticodons, and thus lacks the *C*-terminal domain. In contrast, the class IIb synthetases utilize a *C*-terminal catalytic domain and an *N*-terminal anticodon binding domain with a common β-barrel topology (Asn-RS, Asp-RS, Lys-RS-II). It should be noted that, while most Lys-RSs belong to class II, rare euryarcheal examples of class I Lys-RSs are known as well (23). Gly-RSs also exist in two unrelated forms, either as a class II α_2 dimer or an $\alpha_2\beta_2$ heterotetramer. Ala-RS and Phe-RS have tetrameric structures as well, but Ala-RS is a homotetramer (α_4 type), whereas Phe-RS exhibits an $\alpha_2\beta_2$ structure.

Phenylalanyl-tRNA synthetase (Phe-RS) shows some further special characteristics. Phe-RS is the only class II enzyme which attaches the amino acid to the 2'-hydroxyl group of the tRNA 3'-terminal ribose (24). Thus, functionally, it resembles class I aa-RSs, but structurally the enzyme belongs to class II, as its catalytic domain is built around an antiparallel β-sheet. The Phe-RS monomer (130,000 Da) consists of two distinct α- and β-subunits (350 and 785 amino acid residues, respectively). The crystal structure of Phe-RS alone and in complex with its substrates and a Phe-AMP analogue revealed that the α-subunit contains the catalytic center while the β-subunit is involved in binding of the tRNA (25-27). The sequences of bacterial Phe-RSs are well conserved, but differ significantly from their eukaryotic counterparts (28). Phe-RS adopts an unusual $\alpha_2\beta_2$-tetrameric structure in all prokaryotes and eukaryotic cytoplasmic sources known (29), and both subunits are required for aminoacylation of the tRNA. An exception to the tetrameric structures is the mitochondrial Phe-RS (30). The human mitochondrial Phe-RS enzyme is a small protein that is active as a monomer (31).

Inhibitors of bacterial aminoacyl-tRNA synthetases

Various chemical structures that inhibit aminoacyl-tRNA synthetases have been identified. These inhibitors have either been isolated from natural sources or have been generated synthetically. The synthetic inhibitors are modifications of natural inhibitors, derivatives of the natural synthetase substrates or reaction intermediates, or have been identified by screening of compound libraries.

Figure 3 illustrates the structural diversity of natural products that have been identified as inhibitors of aminoacyl-tRNA synthetases. Most of the compounds exhibit high specificity for only one synthetase. Pseudomonic acid **(1)** (isoleucyl-RS) (32), indolmycin **(2)**

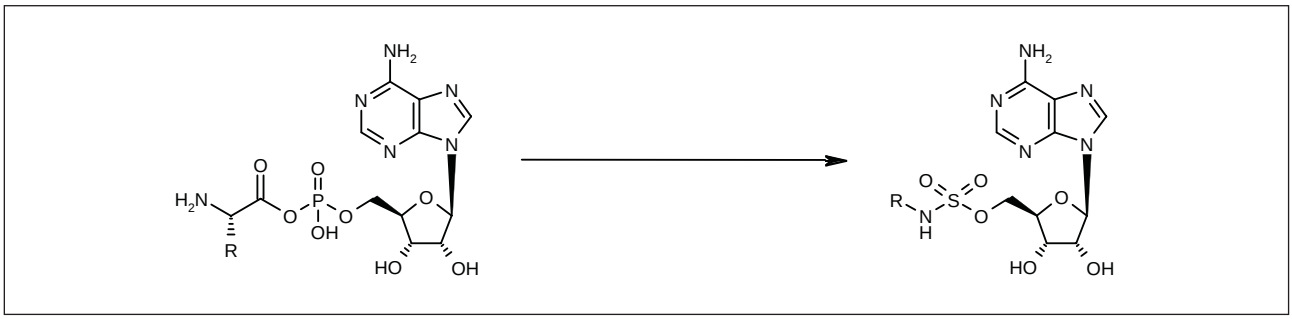

Fig. 4. Mechanism-based inhibitors by sulfamate replacement of phosphate linkage.

(tryptophanyl-RS) (33), borrelidin **(3)** (threonyl-RS) (34, 35), granaticin **(4)** (leucyl-RS) (36) and furanomycin **(5)** (isoleucyl-RS) (37) represent such specific inhibitors. Purpuromycin **(6)** is an unusual tRNA synthetase inhibitor in that it binds to all tRNAs (apparent K_d = 2.5 μM) and blocks their aminoacylation capacity (38).

In addition to extensive structural analysis aided by a number of crystal structures, much is known about the mechanism and chemistry of the aminoacylation reaction, supporting the rational design of inhibitors. In the first reaction step catalyzed by the tRNA synthetases, tightly bound aminoacyl-adenylates, with dissociation constants typically in the nanomolar range, are formed (39). Dissociation constants for amino acids and ATP are higher, generally by 2-3 orders of magnitude. Thus, analogues of the aminoacyl-adenylate reaction intermediates promise to be tight-binding inhibitors. These analogues, in which the amino acid and adenosine moieties are retained and the labile acylphosphate linkage is replaced with more stable groups like a sulfamate or sulfonamide linkage (Fig. 4), have proven to be potent synthetic inhibitors of the corresponding synthetases (40, 41). Derivatization of the adenine unit can provide additional diversity (42). A drawback of many of these inhibitors is the lack of discrimination between pathogen and human enzymes, and insufficient antimicrobial activity. The latter problem might be due to insufficient bacterial penetration caused by unfavorable physicochemical properties.

The aminoacyl-tRNA synthetases have the advantage of being amenable to high-throughput screening. They are soluble, relatively stable, easy to express from recombinant genes, and can be purified in large amounts. Robust and automated screening assays have been developed that allow the testing of large compound libraries. Most of the 20 aminoacyl-tRNA synthetases from a number of microbial pathogens have been screened and various compounds suitable as lead structures for further optimization programs have been identified (43).

Only one aminoacyl-tRNA synthetase inhibitor, the natural product Ile-RS inhibitor pseudomonic acid, is currently marketed as an antibacterial agent (Bactoban™). Pseudomonic acid, also known as mupirocin, was isolated from *Pseudomonas fluorescens*. It has approximately 8,000-fold selectivity for pathogen *versus* mammalian isoleucyl-tRNA synthetase (44). Due to its labile ester bond, the use of pseudomonic acid has been limited to the treatment of topical infections (45). No Phe-RS inhibitor useful as an antibiotic against systemic infections has been developed so far.

Inhibitors of bacterial phenylalanyl-tRNA synthetase

The first synthetic inhibitors of bacterial Phe-RS were described already in the 1970s. Danenberg and Santi reported inhibition of *Escherichia coli* Phe-RS by aromatic guanidines and amidines (Fig. 5) (46). 2-Phenylacetamidine **(7)**, benzylguanidine **(8)** and *N*-benzylbenzamidine **(9)** are competitive inhibitors with respect to phenylalanine. The K_i values for these compounds (230 μM, 77 μM and 58 μM, respectively) are similar to the K_m value of phenylalanine (50 μM) reported in this study. A variety of other aromatic amidines and guanidines showed noncompetitive inhibition, indicating a secondary binding site. Further studies by Anderson and Santi revealed *N*-benzyl-D-amphetamine **(10)** (Fig. 5) as the most potent competitive inhibitor of *E. coli* Phe-RS from a series of *N*-benzyl-2-phenylethylamine derivatives, with a K_i value of 0.14 μM (47). *N*-Benzyl-D-amphetamine shows selectivity for bacterial Phe-RS, with significantly lower activity against the corresponding enzyme from rat liver (K_i = 3.4

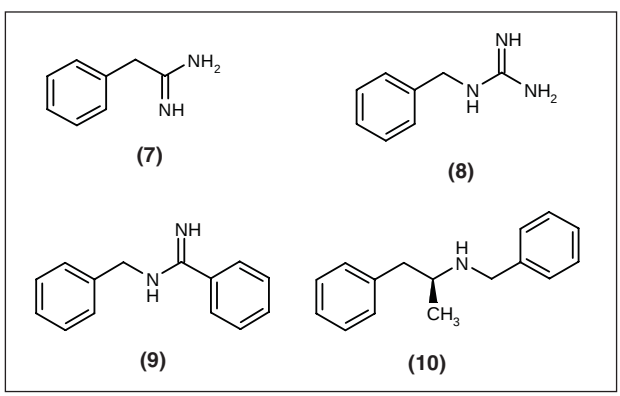

Fig. 5. Aromatic amidines, guanidines and benzylamines that inhibit bacterial phenylalanyl-tRNA synthetase.

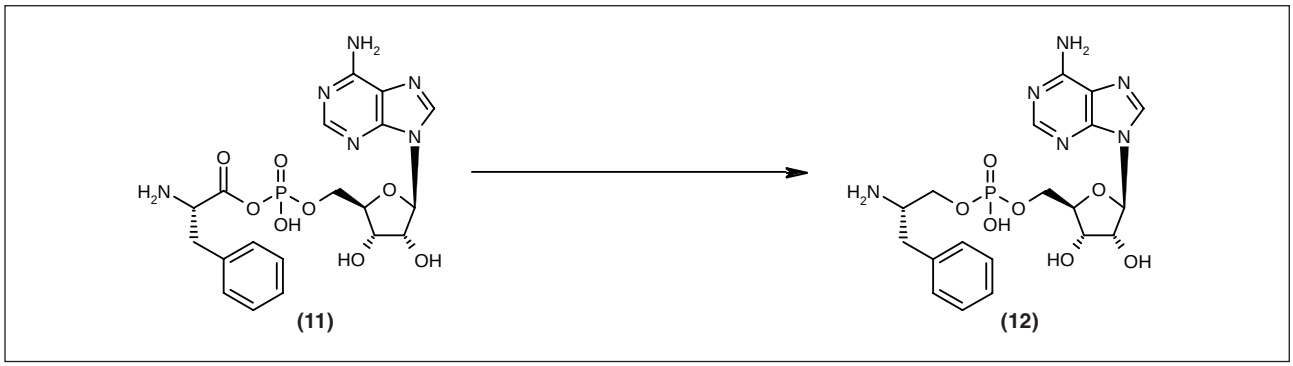

Fig. 6. Structures of phenylalanyl-adenylate **(11)** and phenylalaninyl-adenylate **(12)**.

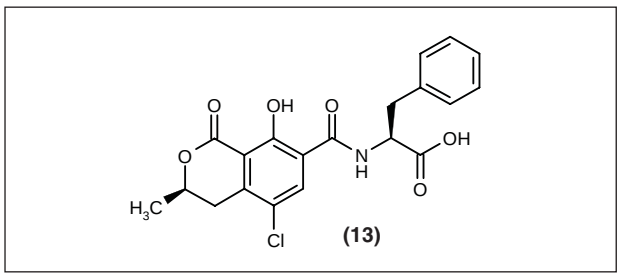

Fig. 7. Structure of ochratoxin A **(13)**.

mM) (48). In addition, this compound demonstrated antibacterial activity, inhibiting the growth of *E. coli* by 50% at a concentration of 15 μM (49). This growth inhibition was completely reversed by the addition of 2.5 mM phenylalanine, whereas other amino acids had no effect on inhibition. Further evidence that the target for *N*-benzyl-D-amphetamine was Phe-RS was obtained by demonstrating that this inhibitor reduced the intracellular levels of charged $tRNA^{Phe}$ in *E. coli* strains.

Phenylalaninyl-adenylate **(12)**, a stable synthetic analogue of the natural reaction intermediate phenylalanyl-adenylate **(11)** (Fig. 6), was shown by Lavrik *et al.* in 1978 to be a competitive inhibitor of both phenylalanine and ATP in the reaction catalyzed by Phe-RS (50).

For the food-contaminating mycotoxin ochratoxin A **(13)** (Fig. 7), inhibition of Phe-RS has been discussed as a possible mechanism of toxicity. Konrad and Röschenthaler measured the inhibition of Phe-RS from *Bacillus subtilis* by ochratoxin A and obtained an inhibition constant of 3 μM (51). These experiments were repeated in 1993 by Roth *et al*. using specifically purified *B. subtilis* Phe-RS. In this study, an inhibition constant of 4.33 mM was obtained (52). These data confirm that ochratoxin A binds weakly to moderately to Phe-RS. Nevertheless, taking into account the intracellular concentrations, the level of ochratoxin A in *B. subtilis* appears too low to significantly compete with phenylalanine for the binding site of Phe-RS. More recent modeling studies using the crystal structure of Phe-RS from *Thermus thermophilus* also did not suggest binding stronger than millimolar for various binding modes of ochratoxin A to Phe-RS (53).

Various bacterial aminoacyl-tRNA synthetases were used in a program for the identification of quality antibacterial drug discovery targets at Cubist Pharmaceuticals (43). High-throughput screening (HTS) efforts led to the discovery of various structurally distinct Phe-RS inhibitors. Novel dialkylated thiazolidinone Phe-RS inhibitors were discovered by screening versus *Enterococcus faecalis* Phe-RS (Fig. 8) (54). The HTS hit **14** inhibits *E. faecalis* Phe-RS with an IC_{50} value of 2 μM and displays broad-spectrum activity, with IC_{50} values of 1.6 μM and 0.17 μM against Phe-RS from *S. aureus* and *E. coli*, respectively. The compound is a competitive inhibitor with respect to phenylalanine and is selective for bacterial Phe-RS *versus* other *S. aureus* aminoacyl-tRNA synthetases and human Phe-RS. Mode of action studies by macromolecular labeling have shown that the compound blocks protein biosynthesis in a manner similar to the known Ile-RS inhibitor pseudomonic acid. Antibacterial activity was demonstrated for a permeable *E. coli* 37 strain, with an MIC of 50 μg/ml. No whole-cell activity could be shown for *S. aureus* and *E. faecalis* strains.

Chemical optimization yielded compound **15** with improved target and antibacterial activity (*S. aureus* Phe-RS IC_{50} = 70 nM; *E. faecalis* Phe-RS IC_{50} = 600 nM; *E. coli* Phe-RS IC_{50} = 10 nM; MIC *E. coli* 37 = 1.56 μg/ml). However, even this more potent Phe-RS inhibitor did not exhibit antibacterial activity against *S. aureus* and *E. faecalis*.

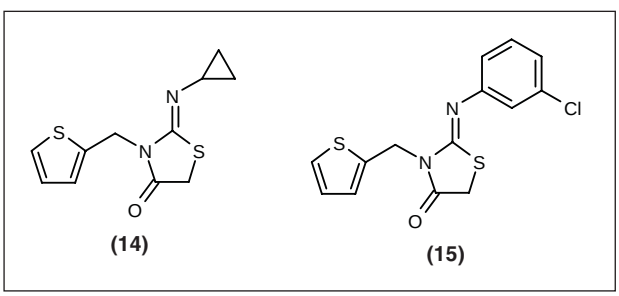

Fig. 8. Thiazolidinone inhibitors of phenylalanyl-tRNA synthetase.

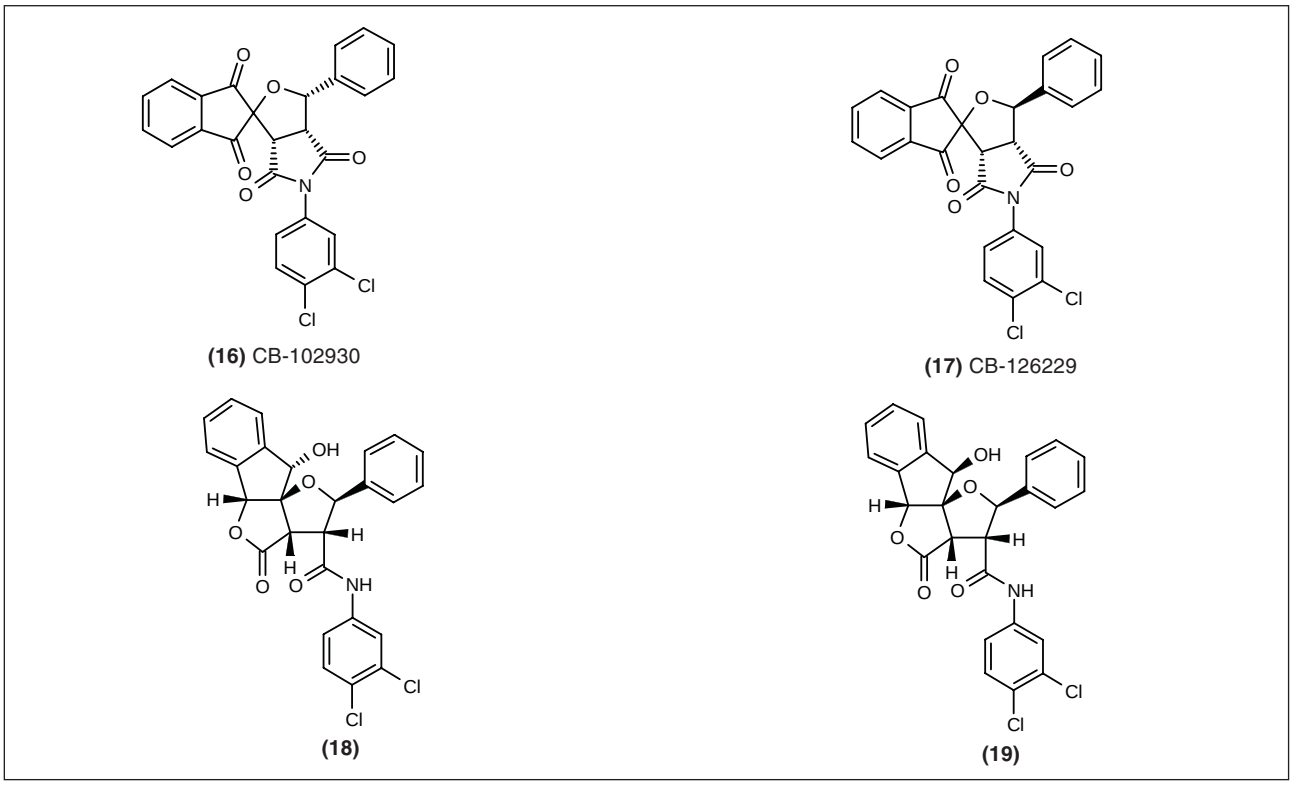

Fig. 9. Spirocyclic tetrahydrofurans and tetracyclic inhibitors of phenylalanyl-tRNA synthetase.

Spirocyclic tetrahydrofurans (Fig. 9) are another compound class identified as Phe-RS inhibitors by high-throughput screening at Cubist Pharmaceuticals (55-57). CB-102930 **(16)** is an inhibitor of bacterial Phe-RS (*S. aureus* Phe-RS IC_{50} = 600 nM; *E. faecalis* Phe-RS IC_{50} = 820 nM; *E. coli* Phe-RS IC_{50} = 3200 nM) and does not inhibit human Phe-RS. The *trans*-isomer CB-126229 **(17)** is more potent *in vitro* (*S. aureus* Phe-RS IC_{50} = 4 nM; *E. faecalis* Phe-RS IC_{50} = 5 nM; *E. coli* Phe-RS IC_{50} = 130 nM), but both compounds exhibit only weak whole-cell activity, with MIC values of 50 μg/ml against *S. aureus*. Stability assays revealed that both compounds are unstable in MIC assay media, with half-lives of 101 and 158 min, respectively. To overcome this problem, CB-126229 was reduced, yielding the stable tetracyclic derivatives **18** and **19** (Fig. 9). These compounds were still active at the target level, with IC_{50} values of 0.4-0.8 μM, and showed selectivity *versus* human Phe-RS (IC_{50} > 100 μM). The whole-cell activity of these stable compounds was more consistent with their enzymatic activity, with MIC values of 3.1 and 6.25 μg/ml against *S. aureus* for **18** and **19**, respectively.

Phenylthiazolylureasulfonamides have recently been identified as a novel class of potent inhibitors of bacterial Phe-RS by high-throughput screening and chemical variation of the screening hit at Bayer (Fig. 10) (58-60). The compounds inhibit Phe-RS derived from Gram-negative as well as Gram-positive pathogens, with IC_{50} values in the nanomolar range (**20**: *E. coli* Phe-RS IC_{50} = 8 nM; *Haemophilus influenzae* Phe-RS IC_{50} = 8 nM; *S. aureus* Phe-RS IC_{50} = 50 nM; *S. pneumoniae* Phe-RS IC_{50} = 200 nM; **21**: *E. coli* Phe-RS IC_{50} = 15 nM; *H. influenzae* Phe-RS IC_{50} = 15 nM; *S. pneumoniae* Phe-RS IC_{50} = 50 nM). The inhibitors were highly selective for the bacterial Phe-RS *versus* the corresponding mammalian cytoplasmic and mitochondrial Phe-RS. Enzyme kinetic

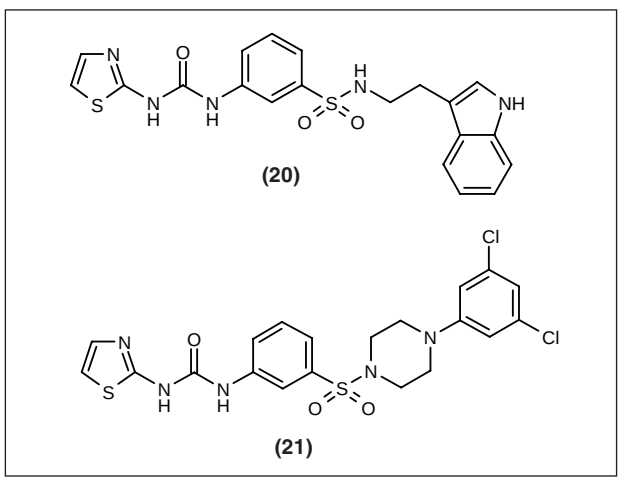

Fig. 10. Phenylthiazolylureasulfonamide inhibitors of phenylalanyl-tRNA synthetase.

measurements revealed a competitive binding mode with respect to the natural substrate phenylalanine. Antibacterial activity with submicromolar MIC values was demonstrated in a synthetic medium without phenylalanine. Compound **21** was active against *S. aureus* (MIC = 0.4 μg/ml), *S. pneumoniae* (MIC = 0.8 μg/ml), *H. influenzae* (MIC = 0.4 μg/ml) and *Moraxella catarrhalis* (MIC = 0.4 μg/ml). The antibacterial activity was partly antagonized by addition of phenylalanine to the culture medium. The MIC increase in the presence of extracellular phenylalanine provided further evidence that the antibacterial mechanism of the phenylthiazolylureasulfonamides is based on inhibition of Phe-RS. Proteome analysis with *B. subtilis* after treatment confirmed the target.

The effect of phenylalanine concentration was also evaluated *in vivo*. A single intravenous treatment with 100 mg/kg of **20** significantly reduced the bacterial titer in different organs in an *S. aureus* sepsis model in mice having an artificially reduced phenylalanine plasma concentration of 15 μM. The efficacy was reduced in mice having a normal phenylalanine blood level of 70 μM. MIC determinations in the presence and absence of phenylalanine in the culture broth suggested a less pronounced phenylalanine antagonism for *S. pneumoniae*. Thus, **21** was tested in an *S. pneumoniae* sepsis model in rats with normal phenylalanine blood levels of 63 μM. A 2-3 log decrease in colony-forming units was observed in the lung, kidney and spleen after intraperitoneal treatment with 100 mg/kg at 0.5 and 3 h after infection. These results represent the first published study in which bacterial Phe-RS inhibitors have demonstrated efficacy in *in vivo* infection models.

Conclusions

Aminoacyl-tRNA synthetases have emerged as promising targets for antibacterial therapy in recent years. However, despite a number of research programs dedicated to the identification of inhibitors of this enzyme class, no drug candidate has been discovered that is in advanced clinical evaluation. The natural product pseudomonic acid remains the only aminoacyl-tRNA inhibitor with proven therapeutic utility. The review of published literature on inhibitors of bacterial Phe-RS reveals the challenges that are encountered in the search for new drugs targeting this enzyme class. Highly potent inhibitors of bacterial Phe-RS have been identified in various structural classes. However, this target activity often does not sufficiently translate into antibacterial activity in complex media. Reaching adequate *in vivo* activity in standard animal infection models is an even more challenging goal. Problems usually encountered in antibacterial research, such as a lack of cell penetration, certainly can account for some of these difficulties and might be addressed by medicinal chemistry, *e.g.,* by optimizing the physicochemical profile of inhibitors. However, other problems appear to be unique to this target class. For most screening-derived aminoacyl-tRNA synthetase inhibitors, it has been described that they act competitively with the corresponding amino acids as the natural substrates. Since amino acids are ubiquitous in human plasma, potent inhibitors are needed to compete successfully. The clinically used isoleucyl-RS inhibitor pseudomonic acid is known to act competitively with isoleucine and ATP. A rational design approach using binding models of pseudomonic acid and the reaction intermediate Ile-AMP yielded femtomolar inhibitors (61). Such knowledge-based design and new computational virtual screening methods could support the search for novel potent compounds that inhibit bacterial aminoacyl-tRNA synthetase (62). Despite the challenges, the already proven *in vitro* activity against a broad spectrum of bacterial pathogens and the high selectivity with respect to mammalian Phe-RS enzymes support the value of aminoacyl-tRNA synthetase inhibition as a promising therapeutic principle that deserves further evaluation for future antibacterial therapy.

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