Structural insights into the specific interaction between *Geobacillus stearothermophilus* tryptophanyl-tRNA synthetase and antimicrobial Chuangxinmycin

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**Shuai Fan,‡ Guangxin Lv,‡ Xiaofeng Fei, Guangteng Wu, Yuanyuan Jin, Maocai Yan,*, and Zhaoyong Yang,*†**

*From the †Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China; ‡Research and Development Department, ArNuXon Pharm-Sci Co, Ltd, Beijing, China; †School of Pharmacy, Jining Medical University, Rizhao, Shandong, China*

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The potential antimicrobial compound Chuangxinmycin (CXM) targets the tryptophanyl-tRNA synthetase (TrpRS) of both Gram-negative and Gram-positive bacteria. However, the specific steric recognition mode and interaction mechanism between CXM and TrpRS is unclear. Here, we studied this interaction using recombinant GsTrpRS from *Geobacillus stearothermophilus* by X-ray crystallography and molecular dynamics (MD) simulations. The crystal structure of the recombinant GsTrpRS in complex with CXM was experimentally determined to a resolution of 2.06 Å. After analysis using a complex-structure probe, MD simulations, and site-directed mutation verification through isothermal titration calorimetry, the interaction between CXM and GsTrpRS was determined to involve the key residues M129, D132, I133, and V141 of GsTrpRS. We further evaluated binding affinities between GsTrpRS WT/mutants and CXM; GsTrpRS was found to bind CXM through hydrogen bonds with D132 and hydrophobic interactions between the lipophilic tricyclic ring of CXM and M129, I133, and V141 in the substrate-binding pockets. This study elucidates the precise interaction mechanism between CXM and its target GsTrpRS at the molecular level and provides a theoretical foundation and guidance for the screening and rational design of more effective CXM analogs against both Gram-negative and Gram-positive bacteria.

Antimicrobial resistance (AMR) is a global public health concern, for the misuse and overuse of antimicrobials in the past 50 years have caused and accelerated the development of drug-resistant pathogens (DRPs) (1). As AMR spreads worldwide, it is more and more difficult to treat infections and death caused by DRPs. Therefore, the urgent need has been provoked for exploring novel and more effective antimicrobials on the AMR issue. At molecular level, most antibiotics in clinical use nowadays mainly target the bio-macromolecules involved in the protein translation process, through interacting with microbial ribosomes and binding directly to rRNA or ribosomal subunit proteins. However, for drug targets, there are other macromolecular candidates beyond ribosomes within the broader scenario of protein translation process. One of such targets for existing and future antimicrobials is the aminocyl-tRNA synthetase (aaRS) family. The aaRSs are ubiquitously expressed and play a critical role in the translation of genetic codes through synthesizing aminocyl-tRNAs. Moreover, the aaRSs and tRNA are also important in the evolution of the universal genetic code and the shaping of codon assignments (2). Owing to the essential role of aaRS in maintaining the fidelity of translation, compounds that inhibit aaRSs are widely used in the treatment of infectious diseases or therapeutic applications against cancers and autoimmune diseases.

The oxaborole-antifungal agent approved by Food and Drug Administration in 2014. The structural and mechanistic differences among the different aaRS enzymes as well as orthologs of individual synthetases make it possible to selectively modulate their specific activity against different kinds of pathogens. This approach makes the aaRS enzymes attractive target candidates for developing novel anti-infective therapeutics.

Chuangxinmycin (CXM), a novel antibiotic containing an indole-dihydrothiopyran heterocyclic skeleton, was isolated from *Actinoplanes tsinannensis* CPCC 200056 (8). It exhibited *in vitro* antibacterial activity against both Gram-negative and Gram-positive bacteria, such as *Escherichia coli* and *S. aureus* (9), respectively, and showed no cross-resistance with common antibiotics, low toxicity, and few of side effects. Specifically, in a preliminary clinical trial, the CXM exhibited antibacterial effectiveness against septicemia, urinary, and biliary infections caused by *E. coli* (8). As at molecular level, CXM is an inhibitor of bacterial tryptophanyl-tRNA synthetase (TrpRS) (IC$_{50}$ value of 30 nM against *S. aureus* TrpRS) (10). However, CXM’s clinical use has been limited by its narrow spectrum of activity, the fact that it is only available in oral forms, and its relative

*These authors equally contributed to this work.

*For correspondence: Zhaoyong Yang, zhaoyongy@imb.pumc.edu.cn; Maocai Yan, yanmaocai@126.com.
lower antimicrobial activity in comparison with first-line antibiotics. Although CXM has not come into clinical use, its specific antibacterial mechanism through selectively inhibiting prokaryotic TrpRSs and its unusual heterocyclic skeleton with a sulfur have attracted the interests of pharmacologists and medicinal chemists. In addition, the biosynthesis studies of CXM had been previously reported by several groups (11, 12). A number of its analogs were also tested for their in vitro antibacterial activity (9, 10). Yet, regrettably, none of them showed higher antibacterial activity against E. coli or S. aureus than CXM. Nevertheless, if we could understand the structural basis and molecular mechanism of the TrpRS inhibition by CXM, we would be able to use structure–activity relationships in the rational design of CXM derivatives more effectively targeting at the TrpRSs of pathogens. So, a thorough understanding of the role of structure of TrpRS structure on their specificity and activity is essential in exploring CXM derivatives as antimicrobial drugs.

By far, the structural studies of TrpRSs from Geobacillus stearothermophilus (GsTrpRS) (13–16), Saccharomyces cerevisiae (ScTrpRS) (17), and Homo sapiens (hTrpRS) (18–21) have investigated the common recognition mode of aminoacyl-adenylate AMP and provided insights into the structural basis of the coupling of specificity and catalysis. Based on the architecture of the catalytic domains, aARS are naturally divided into two classes (I and II). GsTrpRS is the smallest aARS monomer and belongs with tyrosyl-tRNA synthase in subclass Ic, characterized by a central domain largely comprising of Rossmann dinucleotide-binding fold, which features the β/α/β Rossmann domains (residues M1–P198). Moreover, the helical domain consists of four α-helices (residues N199–Y260) and possesses three loops with exceptionally high mobility. The last 60 or so amino acids (residues S252–R326) form a long, discontinuous α-helix, running from one extremity to the dimer axis. Several previously structure-solved GsTrpRS were each crystallized with different ligands: ATP (15), Tryptophan/AMP (22), tryptophanyl-S’AMP (13), and adenosine tetraphosphate (23). These structures provide the structural basis for substrate binding and catalytic mechanism. Arguably, GsTrpRS is one of the most extensively characterized TrpRS enzymes (24–27). Indolmycin is a natural tryptophan analog exhibiting high selectivity for bacterial TrpRS. The structural complex of GsTrpRS/indolmycin/ATP (28) was determined to elucidate the structural basis for the high affinity inhibition of GsTrpRS by indolmycin. In contrast to the pretransition state of TrpRS from H. sapiens (hTrpRS), different transition state stabilization mechanism results in a selectivity favorite toward bacterial TrpRSs by indolmycin. Interestingly, CXM was also highly selective for the bacterial TrpRS, with no inhibition on ovine TrpRS observed at concentrations up to 30 μM. Chuangxinmycin differs from indolmycin in the following ways: (i) although the two compounds share an indole substructure in their scaffold, CXM forms a tricyclic ring system which makes its conformation very rigid; (ii) Chuangxinmycin retains a free carboxylic acid group, whereas indolmycin forms an oxazolone and loses the carboxylic acid group; (iii) the lipophilic tricyclic scaffold of CXM facilitates its hydrophobic interactions with the binding pocket of its target. Overall, the structure of CXM is different from that of existing antibiotics, as it is connected to an indole fused onto a dihydrothiopyran ring (Fig. 1).

In this work, the determination of the GsTrpRS/CXM complex structure at a resolution of 2.06 Å allowed us to probe and report the key structural elements and residues for CXM recognition and molecular mechanism of inhibition. We also present the results of molecular dynamics (MD) simulation of the GsTrpRS/CXM structure and carried out functional analyses using isothermal titration calorimetry (ITC) on a series of mutants of the GsTrpRS. These analytic results not only reveal the structural motif crucial for the recognition and binding CXM, but also provide a structural basis and guidance for the rational design of CXM analogs of high antimicrobial potential for DRPs treatment.

Results and discussion

**Overall structure of GsTrpRS/CXM complex**

The TrpRS is a conserved and ubiquitously expressed protein in prokaryotes. Specifically, the protein sequence of GsTrpRS share 55%, 57%, and 52% identity with the TrpRS protein sequences from Haemophilus influenzae, Yersinia pestis, and Vibrio cholerae, respectively (Fig. S1). Therefore, it is convenient for us to probe the structural interaction mode for CXM inhibition by determining the structure of GsTrpRS/CXM binary complex on the basis of homologous structures. The crystal structure of GsTrpRS in complex with CXM was solved using molecular replacement method and the refinement of the final structure model converged to an R-factor of 0.1694 and a free R-factor of 0.2087 to 2.06 Å resolution. The binary complex crystal grew in the hexagonal P321 space group (a = b = 91.67 Å, c = 152.37 Å, α = β = 90°, γ = 120°). The crystallographic asymmetric unit of GsTrpRS/CXM crystal contains two subunits (subunit A and subunit B) (Fig. 2). As for the functional activity, the GsTrpRS would demonstrate three regular transition states: open state, closed pretransition state (PreTS), and closed products state (13–15). Presently, the structures of the GsTrpRS in different states have been reported, such as open Trp complex (PDB: 1MB2) (15), closed PreTS complex (PDB: 1M83) (15), closed PreTS tryptophanamide-ATP complex (PDB: 1MAU) (15), and closed product complex (PDB: 116L) (14). Structural differences between the CXM complex and those reported previously for the open and closed conformations are subtle. Superposition of the subunit A and the those said reported complexes on Ca atoms yields RMSD values of 0.620 Å (1MB2), 1.168 Å (1M83),

![Figure 1. Functional equivalences of tryptophan, Chuangxinmycin, and Indolmycin.](image-url)
Specific recognition and interaction between GsTrpRS and CXM

1.163 Å (1MAU), and 1.067 Å (1I6L), respectively, whereas the superposition of the subunit B and those reported complexes with corresponding Cα atoms gives out RMSD values 0.988 Å, 0.703 Å, 0.705 Å, and 0.886 Å, respectively. So, an interesting phenomenon emerges that GsTrpRS/CXM complex contains two distinct states, in which the subunit A is with open state and the subunit B is with closed PreTS state. The crystal structure of the CXM complex shows that GsTrpRS retains an open conformation and a closed PreTS conformation when bound simultaneously to CXM and inorganic phosphate. The open conformation and a closed PreTS conformation when structure of the CXM complex shows that GsTrpRS retains an open conformation and a closed PreTS conformation when bound simultaneously to CXM and inorganic phosphate. The distance between the β–α-phosphate position and the phosphate ions bound to the GsTrpRS/CXM is 2.6 Å and 4.4 Å, respectively. Chuangxinmycin (1MAU), respectively (Fig. S2). The distance between the β–α-phosphate position and the phosphate ions bound to the GsTrpRS/CXM is 2.6 Å and 4.4 Å, respectively. Chuangxinmycin (1MAU), respectively (Fig. S2). The distance between the β–α-phosphate position and the phosphate ions bound to the GsTrpRS/CXM is 2.6 Å and 4.4 Å, respectively. Chuangxinmycin (1MAU), respectively (Fig. S2).

To summarize, there are phosphate ions bound at the active site, which mimics an intermediate state of the β- or α-phosphate group of ATP. Unfortunately, because of the disorder at the loop P177–V179 of subunit B, the three amino acids (P177–V179) were not able to model into the final structure. As shown in Figure 3, the structure of GsTrpRS/CXM displays the conserved Rossmann dinucleotide-binding fold domain (residues M1–I14, H31–R182, and E294–R326) and small domain (residues T15–Q30 and I183–M293), as mentioned earlier (13). The final structure shows a good overall quality as evidenced by the statistics presented in Table 1.

Comparisons were carried out between the subunit A and subunit B in the asymmetric unit. Superimposing the subunit A to subunit B leaves RMSD 0.667 Å between corresponding Cα atoms, revealing the conformation of subunit A is similar to subunit B. However, the binding mode of CXM in subunit A is distinguished from that of subunit B. In contrast with subunit A, subunit B exhibits a more compact structure (Fig. 3B). To further investigate the differences in the conformation of the two subunits, we performed the following analyses.

**Chuangxinmycin binding**

Our crystal structure of the GsTrpRS in complex with CXM, provides the structurally binding details, at atomic level, of CXM inhibitory mechanism for the first time. For subunit A and B, the overall structures are quite similar; however, there are several differences on the hydrogen bonding and hydrophobic interactions between the CXM and amino acid residues of GsTrpRS. Specifically, the indyl group of CXM is within the hydrogen bonding distance of the carboxyl group of the side chain of D132 in the binary complex (Fig. 4). Meanwhile, the carboxyl of CXM in subunit A forms a hydrogen bond with amino acid residue Y125 (Fig. 4A). However, surprisingly, the carboxyl group of CXM forms a salt bridge with K195 in subunit B (Fig. 4B). The hydrophobic interactions also play critical roles in the binding of CXM. The structure of binary complex suggests that there could be the hydrophobic interactions between CXM and F5, V40, I133, V141, and V143 (Fig. 4). These critical amino acid residues except F5 have been considered conserved in comparison with homologous proteins.

In contrast to the reported GsTrpRS structure conformation, the subunit A of GsTrpRS/CXM structural complex shows in an open state and subunit B shows in a closed PreTS state. The CXM occupies the Trp-binding site and interacts with D132, Y125, or K195. Importantly, no significant changes of the conformation of the D132 are observed in the structure.
of unliganded- (PDB: 1D2R), CXM- (PDB: 7CMS), Trp- (PDB: 1MB2), indolemycin- (PDB: 5DK4), and tryptophanamide-GsTrpRS (PDB: 1MAU) (Fig. 5). The carboxyl of CXM in subunit A forms a hydrogen bond with amino acid residue Y125. However, surprisingly, in subunit B, the carboxyl group of CXM forms a salt bridge with K195. In the structure of subunit B, the side chain of K195 adopts a distinct conformation from that in the unliganded GsTrpRS to accommodate binding of CXM (Fig. 5A). One observation is the loop containing 192KMSKS196 of the CXM-bound GsTrpRS, which shows an inward movement toward the CXM-binding cavity when compared to the unliganded GsTrpRS (Fig. S3). In other words, binding of CXM to GsTrpRS induces a conformational transition of loop containing 192KMSKS196 from an open state to a closed PreTS state.

Molecular dynamics simulations for interaction analysis and evaluation of binding free energy

The prediction of antibiotic activity by MD simulation would be highly valuable for the antibiotic discovery. Molecular dynamics simulation are reliable methods to explore protein–ligand interactions at the atomic and molecular levels. It can provide a deeper understanding of the intermolecular energy contributions. In this study, MD simulations were performed to predict the resistance for the CXM and to gain further insight into the contributions of the binding free energy of different GsTrpRS mutants. During MD simulation, the trajectory reached a relatively stable status when the RMSD and energy kept steady at 12 ns (Fig. S4A). After RMSD convergence of the MD trajectory, binding mode of CXM to TrpRS was analyzed. The initial structure and the structure at 20 ns were aligned with their protein Cα atoms (RMSD = 1.91 Å) and shown in Fig. S4B. It is obvious that the protein scaffold kept steady and did not change significantly in the MD simulations; the ligand-binding poses were similar before and after MD simulations, despite of a slight translation (Fig. S5).

The receptor–ligand interaction mode was displayed in Figure 5. Owing to the hydrophobic scaffold of CXM, it mainly formed hydrophobic interactions with TrpRS, including M129, Q147, V143, G7, V40, I133, etc. The indole nitrogen atom formed a hydrogen bond with D132 carboxylate anion. The carboxylate anion of CXM pointed to the ATP pocket and did not form any interactions with GsTrpRS residues.

The average Generalized Born Surface Area (GBSA) binding free energy of CXM to TrpRS of 12–20 ns period was calculated to be −17.3 kcal/mol (note that the binding entropy, which is usually a negative value, was ignored in the calculation, so the actual binding free energy is likely higher than this value). To further evaluate the contribution of each amino acid residue to the ligand binding, contribution of amino acid residues around the CXM-binding site were analyzed by decomposing the binding free energy to each residue sidechain and by virtually mutating each residue to alanine in the GBSA calculation.

Table 1

| Dataset | GsTrpRS/CXM |
|---------|-------------|
| Data collection | |
| Space group | P321 |
| a, b, c (Å) | 91.675, 91.675, 152.37 |
| α, β, γ (°) | 90.00, 90.00, 120.00 |
| Resolution (Å) | 50.00–2.06 (2.10–2.06) |
| R/ sir (°) | 23.23 (1.96) |
| Completeness (%) | 100 (100) |
| Redundancy | 18.6 (18.4) |
| Refinement | |
| Resolution (Å) | 50.00–2.06 (2.10–2.06) |
| Unique reflections | 46,939 (2350) |
| Redundancy | 18.6 (14) |
| Rwork/Rfree | 0.1694/0.2087 |
| Average B-factors | |
| Protein | 26.35 |
| Ligand/ion | 28.05 |
| R.m.s. deviations | |
| Bond lengths (Å) | 0.007 |
| Bond angles (°) | 0.903 |
| Ramachandran | |
| Favored (%) | 96.17 |
| Outliers (%) | 0 |
| Protein Data Bank Code | 7CMS |

Values in parentheses are for the highest resolution shell. Rfree calculated with 5% of all reflections excluded from refinement stages using high resolution data.

Figure 4. Schematic representation of the hydrogen-bond and hydrophobic interaction between CXM/ATP and GsTrpRS. Diagrams A and B show hydrogen-bond interactions of CXM at substrate-binding sites of subunit A and subunit B, respectively. Chuangxinmycin (green) and crucial amino acid residues of GsTrpRS (blue) are shown as sticks and labeled. The dashed lines indicate hydrogen-bond (yellow) interactions. CXM, Chuangxinmycin; TrpRS, tryptophanyl-tRNA synthetase.

Figure 5. (A) Schematic representation of the hydrogen-bond and hydrophobic interaction between CXM/ATP and GsTrpRS.
Specific recognition and interaction between GsTrpRS and CXM

Figure 5. Substrate-binding mode of GsTrpRS structural complexes. A, comparison of the substrate-binding sites of ligand-free and CXM-bound GsTrpRS; B and C, subunit A and subunit B of CXM-bound GsTrpRS; D, trp-bound GsTrpRS; E, indolemycin/ATP/Mg²⁺-bound GsTrpRS; F, tryptophanamide/ATP/Mg²⁺-bound GsTrpRS. Ligand-free, subunit A, subunit B, trp-bound, indolemycin/ATP/Mg²⁺-bound, and tryptophanamide/ATP/Mg²⁺-bound of GsTrpRS are shown in blue, magenta, cyan, orange, yellow, and light blue, respectively. Mg²⁺ (wheat) are shown as sphere model. The dashed lines indicate hydrogen-bond (yellow) interactions. CXM, Chuanxinmycin; TrpRS, tryptophanyl-tRNA synthetase.

(Table 2). This evaluation was also accomplished in AmberTools 18. It is clear that lipophilic residues including Q147, M129, V40, and I133 played very important roles in ligand binding, followed by V141, F5, V143, H43, etc. Interestingly, most of the amino acid residues (V40, M129, D132, I133, V141, V143, and Q147) were considerably conserved among the members of pathogenic bacteria. The polar residues S6, E110, and D146 showed little or negative contributions to the ligand binding. The evaluation results of free energy decomposition and alanine scanning agreed with each other roughly; however, D132, which formed an important hydrogen bond with CXM and exhibited an outstanding contribution to ligand binding in alanine scanning, displayed a negative contribution (+1.21 kcal/mol) in free energy decomposition analysis.

Charting the thermodynamic parameters of the GsTrpRS and mutants via ITC

It is generally accepted that the amino acids that participate in the substrate binding or catalytic sites are crucial for the catalysis or inhibition. The following amino acid residues of GsTrpRS, F5, I8, V40, H43, Q80, Y125, M129, D132, I133, V141, and Q147, are selected as “hot spots” to identify putative residues participating in the binding of CXM. Isothermal titration calorimetry was used to evaluate the thermodynamic parameters of GsTrpRS and mutants. We used ITC and L-alanine scanning mutagenesis to further characterize the interaction between the WT/mutants with CXM (Fig. S6). The interaction of CXM with GsTrpRS was found to be connected with a negative ΔH, so the interaction is driven by enthalpy consistent with the results of the conserved hydrogen bond formed by D132 playing a significant role in substrate binding. The results (Table 3) showed that M129A, D132A, I133A, and V141A mutants had no measurable binding affinity against CXM, which was consistent with the function of these residues as binding site residues. Similarly, the binding affinity parameters showed that the K_d value of the V40A and Q80A mutations were 10.4/14.8 times more than that of the WT, respectively. That is to say, V40A and Q80A exhibited decreased binding affinity against CXM compared with the WT. The results show that the binding affinity of K195A for CXM is not changed. Concretely, the hydrogen bond is broken by mutation of Lys195 to hydrophobic amino acid (alanine). This led to the conclusion that the mutant K195A have decreased enthalpy and increased entropy in the binding CXM. The ΔH of inhibitor with its target is essential in drug discovery and...
Specific recognition and interaction between GsTrpRS and CXM

Table 3 Determination of affinity between GsTrpRS WT/mutants and CXM

| Mutant     | $K_d$ (μM) | N | $\Delta H$ (kcal/mol) | $\Delta G$ (kcal/mol) | $-\Delta S$ (kcal/mol) |
|------------|------------|---|-----------------------|-----------------------|------------------------|
| WT         | 3.1 ± 0.1  | 0.8| −11.1 ± 0.3           | −7.6                  | 3.5                    |
| WT-Mg$^{2+}$-ATP | 4.3 ± 1.0  | 0.8| −10.4 ± 2.1           | −7.5                  | 3.1                    |
| F3A        | 3.3 ± 0.2  | 0.7| −5.9 ± 0.3            | −7.5                  | −1.6                   |
| I8A        | 16.2 ± 1.1 | 0.7| −7.8 ± 0.7            | −6.5                  | 1.3                    |
| V40A       | 32.1 ± 1.2 | 0.8| −8.0 ± 0.4            | −6.1                  | 1.9                    |
| H43A       | 5.5 ± 0.8  | 0.6| −19.8 ± 0.9           | −7.2                  | 12.6                   |
| Q80A       | 47.5 ± 3.8 | 0.8| −8.0 ± 0.5            | −5.9                  | 2.1                    |
| Y125A      | 2.1 ± 0.1  | 0.7| −14.2 ± 1.3           | −7.7                  | 6.5                    |
| M129A      | NA         | NA| NA                    | NA                    | NA                     |
| D132A      | NA         | NA| NA                    | NA                    | NA                     |
| I133A      | NA         | NA| NA                    | NA                    | NA                     |
| V141A      | NA         | NA| NA                    | NA                    | NA                     |
| Q147A      | 9.4 ± 0.2  | 0.7| −8.3 ± 0.1            | −6.9                  | 1.46                   |
| K195A      | 1.4 ± 0.3  | 0.6| −6.7 ± 0.7            | −8.0                  | −1.3                   |

Conclusions

In summary, our structural and functional studies of GsTrpRS/CXM provide a framework to elucidate the molecular basis of binding and inhibitory mechanism of CXM to GsTrpRS. The determined GsTrpRS crystal structure in complex with CXM is expected to serve as a guidance for the design and optimization of molecular structures of CXM and analogs to develop novel drug candidates against Gram-negative and Gram-positive bacteria.

Experimental procedures

Cloning and site-directed mutagenesis of GsTrpRS

The gene encoding the GsTrpRS was codon-optimized and synthesized by Beijing Genomics Institute. It was PCR-
amplified by KOD FX Neo DNA Polymerase (TOYOBO) with a set of primers GsTrpRS-F:5'-GGAATTCCATATGGGTATGAAAAACATTTTATGCG-3' and GsTrpRS-R: 5'-CCGCTCGAGCCGCGCAGACCCCAGCCATA-3', which contained an N-terminal Ndel and a C-terminal XhoI restriction site (underlined), respectively. The GsTrpRS gene was cloned into the expression vector pET-21a (+) (Novagen) by virtue of the Ndel and XhoI restriction sites, for the production of recombinant GsTrpRS protein extended at the C-terminus by a six-histidine tag. Site-directed mutagenesis of the amino acids with the GsTrpRS gene was achieved by overlapping extension PCR to make recombined pET21a-mutant plasmids. After the verification of the sequences, these plasmids containing modified-GsTrpRS genes were transformed respectively into the competent BL21 (DE3) for heterologous expression. All cloned expression constructs were checked for accuracy by DNA sequencing. All oligonucleotide primers are listed in Table S1.

**Enzyme production and purification**

Both GsTrpRS and mutants were overexpressed in E. coli strain BL21(DE3) supplemented with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside at an absorbance of 0.6 at 600 nm (A600) followed by a reduction of growth incubation temperature from 37 °C to 18 °C for 12 h. The cells were harvested by centrifugation at 6000g for 15 min at 4 °C and resuspended in 20 ml lysis buffer (20 mM phosphate buffer, 300 mM NaCl, 10 mM imidazole, pH 7.4) containing 1 mM PMSF. The cells were disrupted by high pressure homogenizer and the soluble fraction was separated by centrifugation (60,000g at 4 °C for 40 min) and passed through a 0.45 mm filter, and the cleared supernatant was immediately applied to 2 ml TALON Metal Affinity Resin (Clotech) loaded on a column which was pre-equilibrated with lysis buffer. The resin was subsequently washed with 10 ml washing buffer (20 mM phosphate buffer, 300 mM NaCl, 20 mM imidazole, pH 7.4). Elution was carried out with 10 ml elution buffer (20 mM phosphate buffer, 50 mM NaCl, 250 mM imidazole, pH 7.4). Fractions containing GsTrpRS were pooled and further purified by Superose 12 10/300 GL (GE Healthcare) in 50 mM Tris pH 7.2, 100 mM PMSF at a flow rate of 0.5 ml/min. Purity was ascertained by SDS–PAGE, and the target protein has a molecular weight of about 38 kDa, in agreement with the expected GsTrpRS size. The final protein was flash-frozen in liquid nitrogen and stored at –80 °C.

**Crystallization, X-ray diffraction, and data collection**

The initial crystallization screening was performed with Crystal Screen HR2-110 and HR2-112 (Hampton Research) as well as Wizard CRYO I and Wizard CRYO II (Rigaku), by using hanging drop vapor diffusion method at 37 °C, then, the positive hits were optimized. After optimizing the conditions, the crystals of the GsTrpRS/CXM were obtained from drops with the crystallization solution containing 1.8 M K2HPO4, pH 7.6, 1,3-Propanediol (1.5% v/v). The X-ray diffraction data were collected at BL18U1 at Shanghai Synchrotron Radiation Facility, using a CCD detector with beam at wavelength 0.97915 Å, and the data were processed and scaled by using HKL3000 (29).

**Structure determination and refinement**

The GsTrpRS/CXM complex structure was determined by using Molecular Replacement method. The program Phenix (30) was used to find an initial molecular replacement solution with a model based on the structure of GsTrpRS (PDB accession code 16M). After the apo-form structure was built, the shape of electron density within the substrate-binding pocket is consistent with the structural outline of CXM, and the molecule was fitted into the electron density. The structure was refined using data to 2.06 Å resolution. The high-resolution data allowed the structure refinement with Phenix (30), with additional rounds of manual model rebuilding in Coot (31), until the \( R \text{work} \) and \( R \text{free} \) values got to 0.1694 and 0.2087, respectively. The crystallographic and structural data statistics are listed in Table 1. The refined structure has been deposited in the Protein Data Bank with accession code 7CMS. Structural figures were drawn using the PyMOL software (http://www.pymol.org).

**Molecular dynamics simulations**

**Preparation of the system**

The cocrystal structure of TrpRS complexed with CXM (PDB entry: 7CMS) was used as initial structure of MD simulations. The system was solvated in a cuboid box with 1.5 Å water around the protein and ions were added to neutralize the system. Periodic boundary conditions were applied to the minimum box, and the initial position of the water molecules was determined by the charge of the protein. The system was solvated in 100 mM NaCl and the temperature of the system was set to 298 K. 200 ps of energy minimization followed by 100 ps of 1000 fs of equilibration simulations were performed. The system was further subjected to 100 ns of production MD simulations at a flow rate of 0.5 ml/min. Purity was ascertained by SDS–PAGE, and the target protein has a molecular weight of

**Figure 6. Binding mode of CXM to TrpRS after 20-ns MD simulations.** A, a view of the CXM binding onto GsTrpRS. Highlighted in surface view are the amino acids involved in shaping the substrate pocket. M129 (cyan), D132 (blue), I133 (magenta), and V141 (red) are shown as surface; B, receptor surface was colored with hydrophobicity. Chuangxinmycin (green) are shown as stick model. CXM, Chuangxinmycin; TrpRS, tryptophanyl-tRNA synthetase.
Specific recognition and interaction between GsTrpRS and CXM

Simulations. All MD simulations were performed with Amber 18 (32). The net charge of CXM was set to $-1$ (carboxylate anion), and the partial charges were calculated with Antechamber module in AmberTools 18. The GAFF force field parameters of CXM were generated with parmcch2 and tleap module in AmberTools 18. Amber ff14SB force field was applied to the protein, and TIP3P model was used for the water molecules. A truncated octahedron solvent box was applied to the protein, and TIP3P model was used for the water molecules. A truncated octahedron solvent box was added to dissolve the protein, and sodium cations were added to neutralize the system. The final system contains 13,524 water molecules and seven Na$^+$ ions.

Molecular dynamics simulations

For energy minimizations, firstly, all atoms in the protein and CXM were restrained, and the system was minimized for 2000 steps; then restraints on the ligand were removed and the system was further minimized for 5000 steps; finally, all the restraints were removed and another 10,000 steps minimization were performed. In the equilibration phase, the protein was restrained and NVT ensemble was used to heat the system to 300 K in 50 ps; then all the restraints were removed and the system was equilibrated with NPT ensemble for 1 ns. In the production phase, the system was simulated for 20 ns at 300 K (NPT ensemble).

Binding free energy calculation and decomposition

The MM/GBSA method, which has been implemented in AmberTools 18, was used to evaluate the binding free energy of CXM to TrpRS. The binding entropy was ignored, because the reliability of entropy calculation in GBSA or PBSA method is relatively low. The 20-ns trajectory in the production phase was analyzed, and the trajectory after RMSD convergence (12-20 ns) was selected for the binding free energy calculation and decomposition (using MM/PBSA module in AmberTools 18).

Isothermal titration calorimetry

The ITC experiments were conducted with a MicroCal PEAQ-ITC system (Malvern Instruments) Microcalorimeter. This device was connected to a computer with MicroCal PEAQ-ITC software to control the device and record data. Before every experiment, 280 μl of 30 μM GsTrpRS solution was loaded to the sample cell, and 150 μM of CXM solution was loaded to the injection syringe. After the equilibration time of the calorimeter, there was a 60 s delay before titration. The stirring speed was set to 800 rpm. The experiments were performed at least in duplicate using the following parameters: temperature, 25 °C; reference power, 5 μcal/s; injection volume, 0.2 μl first injection followed by 2 μl for the remaining 19 injections; spacing between injections, 200 s. The data were analyzed by Origin 7 software provided by the manufacturer with curves fitted with a one set of site models.

Data availability

Atomic coordinates and structure factors for the reported GsTrpRS/CXM structure have been deposited in the Protein Data Bank with PDB ID: 7CMS. All remaining data is presented in the main article and supporting information.

Supporting information—This article contains supporting information.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; AMR, Antimicrobial resistance; CXM, Chuangxinmycin; DRPs, drug-resistant pathogens; GBSA, Generalized Born Surface Area; ITC, isothermal titration calorimetry; MM, molecular mechanics; PreTS, pretransition state; TrpRS, tryptophanyl-tRNA synthetase.

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Specific recognition and interaction between GsTrpRS and CXM

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