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Binding and action of amino-acid analogues of chloramphenicol upon the bacterial ribosome

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Abstract

Antibiotic chloramphenicol (CLM) binds with a moderate affinity at the peptidyl transferase center of the bacterial ribosome and inhibits peptide bond formation. As an approach for modifying and potentially improving properties of this inhibitor, we explored ribosome binding and inhibitory activity of a number of amino-acid analogues of CLM. The L-histidyl analogue binds to the ribosome with the affinity exceeding that of CLM by 10 fold. Several of the newly synthesized analogues were able to inhibit protein synthesis and exhibited the mode of action that

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AUTHOR CONTRIBUTIONS

A.G.T. and V.A.S. performed chemical synthesis of CAM-derivatives; N.V.S. purified CAM-derivatives; A.G.T. performed 70S ribosome binding assay; A.A.S. and I.A.R. performed mass spectrometry analysis; I.A.O., E.S.K., and P.V.S. designed and performed *in vitro* translation inhibition assays; P.S.K. and A.L.K. prepared *E. coli* 70S ribosomes; J.M. and A.S.M. designed and performed toe-printing experiments; M.D.B. and Y.S.P. designed and performed X-ray crystallographic experiments. N.V.S., A.S.M., A.A.B., and Y.S.P. supervised the experiments. All authors interpreted the results. A.G.T., N.V.S., A.S.M., A.A.B., and Y.S.P. wrote the manuscript.

ACCESSION NUMBERS

Coordinates and structure factors were deposited in the RCSB Protein Data Bank with accession code 5XXX for the *T. thermophilus* 70S ribosome in complex with His-CAM, mRNA, A-, P- and E-site tRNAs; 5XXX for the *T. thermophilus* 70S ribosome in complex with D-His-CAM and protein Y; and 5XXX for the *T. thermophilus* 70S ribosome in complex with Lys-CAM and protein Y.

was distinct from the action of CLM. However, the inhibitory properties of the semi-synthetic CLM-analogues did not correlate with their affinity and in general, the amino-acid analogues of CLM were less active inhibitors of translation in comparison with the original antibiotic. The X-ray crystal structures of the *Thermus thermophilus* 70S ribosome in complex with three semi-synthetic analogues showed that CLM derivatives bind at the peptidyl transferase center, where the aminoacyl moiety of the tested compounds established idiosyncratic interactions with rRNA. Although still fairly inefficient inhibitors of translation, the synthesized compounds represent promising chemical scaffolds that target the peptidyl transferase center of the ribosome and potentially are suitable for further exploration.

Keywords

Antibiotic; ribosome; X-ray structure; protein synthesis; peptidyl transferase center

INTRODUCTION

Many antibiotics stop growth of pathogenic bacteria, and thereby cure infections, by selectively binding to the bacterial ribosomes and inhibiting protein synthesis. Antibiotics can interfere with translation by interacting with various functional centers of the ribosome and either locking of a particular conformation of the ribosome or hindering the binding of its ligands. The peptidyl transferase center (PTC), located in the large ribosomal subunit, is targeted by a particularly broad array of inhibitors belonging to several distinct chemical classes, such as phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramins A, and others [1]. From the oldest chloramphenicol (CLM) to the newest FDA-approved retapamulin, PTC-targeting drugs are known as excellent antibacterial agents.

Most of the PTC-targeting compounds inhibit protein synthesis by competing with the positioning of the amino acid side chain of the incoming aminoacyl-tRNA (aa-tRNA) in the A site [2–4]. CLM, a typical example from this family, inhibits translation in a wide range of Gram-positive and Gram-negative bacteria [5]. It binds to the A site of the PTC in a crevice formed by the bases of the conserved nucleotides U2504, A2451, and C2452 of the 23S rRNA and its nitrobenzyl ring forms a π -stacking interaction with the base of C2452 [6, 7]. The aromatic ring of the ribosome-bound CLM overlaps with the placement of the side chains of the incoming aa-tRNAs, thus efficiently preventing the aminoacyl moiety of aa-tRNA from properly accommodating into the PTC active site. CLM was originally viewed as a universal inhibitor of peptide bond formation [8]. However, this model has been recently revised because the newer data revealed CLM as a context-specific inhibitor of translation, whose activity depends on the nature of specific amino acids in the nascent chain and the identity of the residue entering the A site [9].

While CLM does not act upon the eukaryotic cytoplasmic ribosome, it readily binds to the ribosomes of the mammalian mitochondria [10–14]. The interference with mitochondrial translation, which is the cause of the major side effects of CLM, significantly curbed the medical use of this drug in many countries [5, 15, 16]. Amending the CLM structure with the additional chemical entities that would form idiosyncratic interactions specifically with

the bacterial ribosome could be one approach for the development of more selective inhibitors. In addition, the rapid spread of antibiotic resistance has significantly limited the medical utility of many available antibiotics, including the PTC-targeting drugs. One of the recently discovered but rapidly spreading resistance mechanisms operates via modification of one of the key PTC nucleotides, A2503, which is located in the binding site of several inhibitors. The rRNA methyltransferase Cfr adds a methyl group to the C8 position of the A2503 and renders bacteria resistant to a wide range of antibiotics targeting the catalytic center of the ribosome [17, 18]. The development of newer derivatives of the existing antibiotic platform, which could avoid a clash with the C8 methyl group of the Cfr-modified A2503, has proven to be a viable approach for overcoming such resistance [19].

Previously, attempts were made to prepare peptide derivatives of CLM containing peptides of up to 10 amino acids long aiming to identify interactions between the peptide chain and the elements of the nascent peptide exit tunnel (NPET) [20, 21]. In the current study, with the goal of paving the way for development newer derivatives of CLM, that would target the PTC, we have prepared several semi-synthetic analogues carrying various individual amino acids attached to the drug. Using competition binding experiments, we investigated the affinities of the newly prepared compounds for their target and found that some of them show improved binding to the bacterial ribosome. We also showed that the mode of action of the newer analogues differs from the site-specific action of CLM. By solving the X-ray crystal structures of the *Thermus thermophilus* 70S ribosome in complex with several CLM-analogues we observe specific interactions of the amino acid moiety with rRNA thereby rationalizing the improved binding. Although the higher affinity of the derivatives to the vacant *E. coli* ribosome does not directly translate into stronger inhibition of protein synthesis, the compounds described here could open new directions for improving the medical utility of amphenicol class of the ribosome inhibitors.

RESULTS AND DISCUSSION

Synthesis of CAM-derivatives

Chemical synthesis of CLM-analogues carrying amino acid residues instead of dichloroacetic moiety is based on acylation of CLM amine (CAM), an inactive CLM-derivative, with activated amino acids (Figures 1, S1) [22]. The overall synthesis scheme includes three steps: (i) acid hydrolysis of CLM to yield CAM [23]; (ii) acylation of CAM by succinimide esters of amino acids with protected D-amino group and side-chain amino groups; and (iii) de-protection of the obtained CAM-derivatives to yield aminoacyl-CAM (AA-CAM) (Figures 1, S1). Using this approach, we have prepared CLM analogues aminoacylated with different amino acids, including the N-protected ones (Table 1). Molecular weights and chemical structures of all synthesized CAM-derivatives were confirmed by mass spectrometry and ^1H - and ^{13}C -NMR. Although few individual amino-acid analogues of CLM have been studied previously [22, 24, 25], this work represents the first systematic approach to synthesis and functional assessment of more than three dozens of various AA-CAMs.

AA-CAM compounds bind tightly to the bacterial ribosome

We used the competition-binding assay, exploiting BODIPY-labeled erythromycin (BODIPYERY) to assess the affinity of the synthesized compounds for the ribosome [26, 27]. While CLM binds in the A site of the PTC [6, 28], ERY binds in the upper part of the NPET [7] but their binding sites sufficiently overlap so that even unmodified CLM and ERY compete for their binding to the ribosome [29] (Figure S2). The overlap of the AA-CAMs with ERY is expected to be even more pronounced. The apparent dissociation constant K_{Dapp} of CLM obtained using competition with BODIPY-ERY ($2.8 \pm 0.5 \mu\text{M}$) is consistent with the previously published data determined by direct [^{14}C]-CLM binding ($2.3 \mu\text{M}$ [30]). Using this approach, we found that many of the synthesized CAM derivatives exhibited considerable affinity for the ribosome (K_{Dapp} in the low micromolar range) (Table 1). Interestingly, all of the AA-CAM derivatives carrying free α -amino groups bind to the ribosome with higher affinities than the corresponding compounds in which the α -amino group was modified by acetylation, formylation or protected by the Boc group (Figure S3; Table 1). This result suggests that either the positive charge, the small size of the α -amino group, or both, contribute to the efficient ribosome binding of AA-CAMs. Importantly, one AA-CAM variant, His-CAM, binds to the ribosome with a more than 10-fold higher affinity than CLM (His-CAM, $K_{Dapp} = 0.24 \pm 0.06 \mu\text{M}$) (Figure 2A; Table 1).

AA-CAM compounds are less efficient inhibitors of translation than CLM and do not show the same context-specificity

To check whether the binding strength of AA-CAMs correlates with inhibition of translation we tested their ability to interfere with *in vitro* protein synthesis. Addition of $30 \mu\text{M}$ CLM to the cell-free transcription-translation system based on the *E. coli* cell extract resulted in a near-complete inhibition of synthesis of firefly luciferase reporter (Figure 2B). Consistent with previously published results, removal of the dichloroacetyl moiety from CLM results in nearly complete loss of its biological activity (Figure 2B, CLM vs. CAM) [23], likely due to the loss of interactions of the dichloroacetic moiety with the ribosome [6]. However, similar to the previous studies employing peptide derivatives of CLM [5, 20, 31], the inhibitory activity of CAM can be partially restored by incorporation of specific amino acids (Figure 2B), indicating that the contacts of the aminoacyl side chain of AA-CAMs with the ribosome may compensate for the interactions lost upon the removal of the dichloroacetic group. However, most of the tested AA-CAM-derivatives were less potent inhibitors of translation than CLM (Figure 2B). Even His-CAM, which exhibited higher than CLM affinity for the ribosome, reduced the yield of the functional luciferase by only 60%. Only the activity of β -Ala-CAM approached that of CLM even though this derivative demonstrated a 2-fold weaker binding to the ribosome compared to the parental compound. The modification of the α -amino group of AA-CAMs (with formyl, acetyl, or Boc groups) leads to a nearly complete loss of their inhibitory potency (Figure 2B). The inability of most of the AA-CAM compounds to efficiently inhibit translation could be potentially explained by the susceptibility of AA-CAMs to the action of peptidases present in the bacterial extract-based cell-free translation system. Therefore, we re-tested the inhibitory activity of some of the compounds in the PURE cell-free translation system reconstituted from purified components and thus, lacking peptidases (Figure 2C) [32]. Although the overall inhibitory activity of AA-CAM derivatives improved in the PURE system compared to their effect

upon translation in the cell extract, it still did not reach the level of inhibition elicited by the CLM, suggesting that stability of our compounds is unlikely to be the key issue and indicating that the equilibrium binding of the inhibitors to a vacant ribosome often does not directly correlate with their inhibitory properties. Several peptide derivatives of CAM have been prepared and tested previously in the binding and inhibition assays [20, 31]. Similar to our findings, those derivatives were found to be poor inhibitors of translation.

The inhibition of protein synthesis by CLM depends on the amino-acid sequence of the nascent peptide and the identity of the aminoacyl-tRNA with the most efficient arrests occurring after alanine (and to a lesser extent after serine or threonine) that appear at the penultimate position of the nascent chain [9]. To test whether the specificity of action of AA-CAM compounds matches that of CLM, we used primer extension inhibition assay (toe-printing) to check whether the derivatives that exhibited inhibitory activity in the PURE system (Figure 2C), would arrest the ribosome at the same codon(s) where translation is arrested by CLM. In these experiments, we used a short synthetic open reading frame (ORF) encoding the peptide MFKAFKNIIRRTL, where CLM efficiently arrests the ribosome at the 5th codon after the MFKAF peptide has been synthesized (Figure 2D, lanes 3 and 9, green arrowheads). Some translation reactions were additionally supplemented with mupirocin, an inhibitor of isoleucyl-tRNA synthetase. The depletion of isoleucyl-tRNA efficiently traps the ribosomes at the 7th codon of the ORF if they were not arrested at any of the prior codons by the action of the ribosome inhibitor (Figure 2D, lanes 1–6, blue arrowheads) helping to evaluate the efficiency of the antibiotic-dependent arrest [33]. Only a small fraction of ribosomes pre-incubated with CLM was able to reach the ‘trap’ codon (Figure 2D, lane 3) because most of the translation complexes were arrested at the 5th codon. In contrast, the intensity of the trap-codon band was only slightly diminished in samples supplemented with AA-CAM analogues and no strong arrest was observed at the 5th codon (Figure 2D, lanes 4–6). This was in spite of the fact that the concentrations of AA-CAM compounds in the toe-printing experiments (100 μ M) were several-fold higher than those used in the cell-free translation experiments (Figure 2B, C), where the same derivatives were able to inhibit expression of the luciferase reporter with a considerable activity. We noted, however, that the toe-print bands corresponding to the initiating ribosome were slightly enhanced compared to the no-drug (+/– mupirocin) control (Figure 2D, lanes 2 and 8 vs. lanes 4–6 and 10–12, respectively, black arrowheads), indicating that AA-CAM analogues weakly interfered with translation initiation, likely by inhibiting the formation of the first peptide bond. We concluded that AA-CAM derivatives do not show the same site-specificity of action as the parent compound CLM, apparently due to idiosyncratic interactions of the aminoacyl moieties of AA-CAMs with the ribosome.

Aminoacyl moieties of AA-CAMs mediate specific interactions with the ribosome

To unambiguously determine the mode of binding of AA-CAM derivatives to the ribosome, we crystallized *Thermus thermophilus* (*Tth*) 70S ribosomes in the presence of mRNA, deacylated A-, P-, and E-site tRNAs, and His-CAM and solved the structure of the complex at 2.8Å resolution. However, the other complexes prepared in this way did not diffract sufficiently well. Therefore, we amended our crystallization approach by using ribosome complexed with the protein Y (PY) as a tool to obtain structures of higher resolution [34,

35]. This approach is based on our recent finding that binding of PY to a vacant 70S ribosome stabilizes it by locking the head of the 30S subunit in an unrotated state, which leads to a better diffraction [34, 35]. Since PY binds in the mRNA channel on the 30S subunit far away from the CLM binding site in the PTC of the 50S subunit, it precludes the presence of mRNA or A- and P-tRNAs [34]. However, this does not interfere with the binding of AA-CAMs. Using this approach, we were able to solve two additional structures of D-His-CAM and Lys-CAM in complex with the PY-bound 70S ribosome at 2.7Å and 2.6Å resolution, respectively (Table S1). An unbiased difference Fourier map, calculated using the amplitudes from the crystals and phases derived from a model of the ribosome without the bound compound, revealed positive electron density resembling characteristic features of each of the compounds (Figure 3). A single binding site for each of the AA-CAMs is observed in the ribosome within the PTC of the large ribosomal subunit (Figure 4).

The binding position of the amphenicol parts of AA-CAMs is identical to those observed previously for parent CLM in the *Tth* [7] or *Eco* [28] ribosomes in the absence of mRNA and tRNAs (Figure S4). The amino acid moieties of AA-CAMs are oriented towards the NPET and exhibit unique compound-specific interactions at the PTC (Figures 4, 5). Histidine side chain of His-CAM forms tilted edge-to-face π -stacking with the nucleobase of U2506 (Figures 4D and 5A). In addition, the α -amino group of His-CAM forms hydrogen bond (H-bond) *via* a water molecule with the phosphate of nucleotide G2505 (Figure 5A). These additional interactions of the aminoacyl moiety likely account for its increased affinity to the ribosome compared to CLM.

Contrary to His-CAM and consistent with its significantly lower affinity, α -amino group of the D-His-CAM stereoisomer does not form the water-mediated H-bond with G2505 (Figure 5B). Surprisingly, the orientation of the amino acid side chain of L-Lys-CAM is similar to that of D-His-CAM (Figure 5C). The lysine moiety of Lys-CAM extends towards the wall of the NPET and, similar to D-His-CAM, forms a single H-bond with the nucleobase of A2059 explaining why their binding is less tight in comparison with His-CAM.

In the structure of the ribosome/His-CAM complex, the oxygens of the nitro group in the CAM moiety form H-bonds with the A76 ribose hydroxyls of deacylated A- and P-site tRNAs (Figure 4C). It is unclear whether such interactions are possible in the translating ribosome when the P-site tRNA is attached to the aminoacyl or peptidyl groups. Nevertheless, our structures clearly demonstrate that amphenicol part of the AA-CAMs is capable of anchoring the derivatives in the PTC active site directing the attached amino acids in the direction corresponding to the growing peptide chain.

Resistance to many peptidyl transferase inhibitors (including CLM) can be conferred by Cfr, a methyltransferase that methylates A2503 of the 23S rRNA at the C8 atom [18]. The methyl group added to A2503 by Cfr would invade the CLM binding pocket if the placement of the modified A2503 remains unchanged (Figure 6A). *In silico* modeling shows, however, that the ribosome-bound His-CAM whose placement in the PTC is somewhat shifted relative to CLM, would avoid the collision with C8-methyl group of A2503 (Figure 6B), suggesting that this compound could retain certain activity against the Cfr-modified ribosome. Likewise, the resistance to CLM conferred by the mutation of

A2503 to G [36], is likely to be less pronounced in the case of His-CAM due to a more remote position of the amino acid side chain of His-CAM relative to the site of the mutation. Therefore, the structure of the ribosome with the AA-CAM derivatives suggest that some of the amino acid analogues of CLM could be less susceptible to the action of specific resistance mechanisms. Further experiments would be required to check whether any of the synthesized CLM-analogues are active against ribosomes rendered resistant to CLM.

The structure of PTC is highly conserved among evolutionary distant ribosomes, including the ribosomes of mitochondria which are known to be targeted by CLM [10–14]. However, even minor variations in the placement of the nucleotides directly interacting with the drug, which could be influenced by the less conserved second-shell nucleotides, could account for the selectivity of the antibiotics that bind in the PTC active site as well as for the differential effect of these drugs upon ribosomes from different species. Because the compounds examined in our study have fairly low activity against the bacterial ribosome, we have not pursued studies of their effects upon mitochondrial translation. However, we believe that adding an amino acid or its analogue to the drug core could potentially influence the spectrum of action of this class of the ribosomal antibiotics opening a possibility of making them more selective inhibitors of bacterial translation.

CONCLUSIONS

The goal of the current study was to develop amino-acid analogues of CLM as an approach for exploring this line of derivatives as new inhibitors of translation. We used organic synthesis to generate a diverse set of amino-acid analogues of native CLM and examined their ribosome binding and inhibitory properties. We have demonstrated that His-CAM, a histidine analogue of CLM, exhibits 10 times higher affinity for the bacterial ribosome as compared to CLM. We have noted, however, that the inhibitory properties of the semi-synthetic CLM-analogues do not correlate with their ribosome binding affinities and the compounds with high affinities, such as His-CAM, inhibit translation less efficiently than CLM, while compounds with low affinities, such as β -Ala-CAM, demonstrated inhibitory properties similar to those of the parental drug. Our crystal structures show that amino-acid analogues of CLM establish compound-specific interactions with the nucleotides of the 23S rRNA at the PTC and orient the amino acid moiety in the direction of the upper part of the peptide exit tunnel. The idiosyncratic interactions with the ribosome of the amino acid residue attached to the CLM core open the possibility of increasing the selectivity of this class of antibiotics and possibly diminish their side effects mediated by the action upon mitochondrial translation. Moreover, the possibility that some of the amino-acid derivatives of CLM might act upon drug-resistant ribosome makes them attractive compounds for further exploration by medicinal chemists, and we expect that our findings, including the structure of the AA-CAM-ribosome complexes described here, will serve as a starting point for such studies.

MATERIALS AND METHODS

Reagents

Various amino acid derivatives used in chemical synthesis (Supplementary Methods) were from Fluka or Reanal; CLM was from Sigma; Succinimide ester of BODIPY was from Invitrogen; N-hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide (DCC) and DMAP were from Merck. BODIPY-ERY was synthesized as described previously [37].

Chemical synthesis of AA-CAM derivatives

The general scheme for the synthesis of AA-CAM derivatives is shown in Figure S1 and the details of chemical synthesis are provided in the Supplementary Methods section. CAM [(1*R*,2*R*)-2-amino-1-(4-nitrophenyl)propane-1,3-diol] was prepared as described previously [23]. Amino acids with protected α - and side-chain amino groups were activated by reaction with N-hydroxysuccinimide in the presence of DCC at 0°C. The resulting succinimide-reactive esters were used for the acylation of CAM in the presence of diisopropylethylamine as a base at room temperature. Subsequent deprotection was achieved by treatment of the obtained amino-acid CAM derivatives with trifluoroacetic acid and appropriate scavengers. Synthesized AA-CAM derivatives were purified by column chromatography on silica gel using suitable systems of solvents. For generating N-acetylated variants of AA-CAM, additional acetylation was performed by reacting the unprotected AA-CAM derivatives with the N-acetylsuccinimide. Purity and chemical structures of obtained compounds were confirmed by HPLC, LC-MS and NMR spectroscopy (see Supplementary Methods).

In vitro binding assay

Binding affinities of CAM-derivatives to *E. coli* ribosomes was analyzed by competition binding assay using fluorescently-labeled BODIPY-ERY as described before [26, 27, 37]. BODIPY-ERY (4 nM) was incubated with ribosomes (25 nM) for 30 min at 25°C in the buffer containing 20 mM HEPES-KOH (pH 7.5), 50 mM NH₄Cl, 10 mM Mg(CH₃COO)₂, and 0.05% Tween-20. The solution of CAM derivatives in a range of concentrations from 0.1 μ M to 1 mM was added to the formed complex. The mixture was incubated for 2 hours until equilibrium was reached and the values of fluorescence polarization were measured.

In vitro translation

Inhibition of firefly luciferase synthesis in cell-free translation systems by AA-CAMs was tested as described previously [38]. Briefly, the *in vitro* transcribed firefly luciferase mRNA was translated using either PURExpress system (New England Biolabs) or *E. coli* S30 extract prepared according to [39]. Reactions were programmed with 100 ng mRNA and were carried out in 5 μ L aliquots at 37°C for 30 minutes. The AA-CAMs were added to 30 μ M final concentration. The activity of *in vitro* synthesized luciferase was assessed using 5 μ L of the substrate from the Steady-Glo Luciferase Assay System (Promega).

Toe-printing analysis of compound-induced ribosome stalling

Toe-printing experiments were performed in the PURExpress cell-free translation system (New England Biolabs) following our published protocols [40, 41]. The synthetic template

encoding the amino acid sequence MFKAFKNIIRTRL was initially generated by PCR reaction using a combination of five primers: 2 μ M T7 (ATTAATACGACTCACTATAGGG), 2 μ M NV1 (GGTTATAATGAATTTTGCTTATTAAC) and 0.2 μ M of each of the following: Fv (AATACGACTCACTATAGGGCAACCTAAACCTTACACACGCCCCGGTAAGGAAATAAAAAT), inner (CCCCCGGTAAGGAAATAAAAATGTTCAAAGCATTCAAAAACATCATACGTACTCGTACTC) and Rev (GGTTATAATGAATTTTGCTTATTAACCTTGCCTGCGCTTAAAGAGTACGAGTACGTATGATGT) as described in [42]. The product was cloned into the pUC18 plasmid cut with the *Sma*I restriction enzyme and the resulting pUCMFKAFK plasmid was verified by capillary sequencing. For the toe-printing reaction, the template was PCR-amplified from the pUCMFKAFK plasmid using T7 and NV1 primers. When needed, the other antibiotics (retapamulin, CLM, and AA-CAM analogues) were present in the reaction at the final concentration of 100 μ M. In addition, all the toe-printing reactions were supplemented with the Ile-RS inhibitor mupirocin (final concentration 50 μ M), which caused translating ribosomes to pause when an isoleucine codon was encountered.

Crystallographic structure determination

Ribosome complexes with mRNA and tRNAs or with protein Y were formed as described previously [35]. CAM-derivatives were added to the pre-formed ribosome complexes to a final concentration of 250 μ M prior to crystallization. All *Th* 70S ribosome complexes were formed in the buffer containing 5 mM HEPES-KOH (pH 7.6), 50 mM KCl, 10 mM NH_4Cl , and 10 mM $\text{Mg}(\text{CH}_3\text{COO})_2$, and then crystallized in the buffer containing 100 mM Tris-HCl (pH 7.6), 2.9% (w/v) PEG-20K, 7–12% (v/v) MPD, 100–200 mM arginine, 0.5 mM β -mercaptoethanol. Crystals were grown by the vapor diffusion method in sitting drops at 19°C and stabilized as described previously [35] with the corresponding CAM-derivatives being added to the stabilization buffers (100 μ M each). Diffraction data were collected using beamlines 24ID-C and 24ID-E at the Advanced Photon Source (Argonne, IL). All crystals belonged to the primitive orthorhombic space group $\text{P2}_1\text{2}_1\text{2}_1$ with approximate unit cell dimensions of $210\text{\AA} \times 450\text{\AA} \times 620\text{\AA}$ and contained two copies of the 70S ribosome per asymmetric unit. Each structure was solved by molecular replacement using PHASER from the CCP4 program suite [43]. The search model was generated from the previously published structures of *T. thermophilus* 70S ribosome with bound mRNA and tRNAs (PDB entry 4Y4P from [35]) or with protein Y (PDB entry 4Y4O from [35]). The initial molecular replacement solutions were refined by rigid body refinement with the ribosome split into multiple domains, followed by positional and individual B-factor refinement. The final models of the 70S ribosome in complex with His-CAM and mRNA/tRNAs, or in complex with D-His-CAM/Lys-CAM and protein Y were generated by multiple rounds of model building in COOT [44], followed by refinement in PHENIX [45]. The statistics of data collection and refinement are compiled in Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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RESEARCH HIGHLIGHTS

- Aminoacyl derivatives of the antibiotic chloramphenicol (AA-CAMs) can bind to the peptidyl transferase center of the bacterial ribosome with better affinities than the parent compound;
- AA-CAM derivatives show different site-specificity of action compared to chloramphenicol and establish compound-specific interactions with the nucleotides of the 23S rRNA;
- The inhibitory properties of the semi-synthetic CLM-analogues do not correlate with their ribosome binding affinities;
- AA-CAMs represent promising chemical scaffolds that target the peptidyl transferase center and the nascent peptide exit tunnel.

Figure 1.
Schematic diagram of chemical synthesis of the histidine analogue of CLM.

Figure 2. Binding and inhibitory properties of AA-CAM-derivatives

(A) Competition binding assay to test the inhibition of BODIPY-ERY binding to the *E. coli* ribosomes in the presence of increasing concentrations of AA-CAM derivatives measured by fluorescence anisotropy. (B, C) Inhibition of *in vitro* synthesis of firefly luciferase by AA-CAM derivatives in the *E. coli* S30 cell extract (B) or in the PURE system (C). All the inhibitors were present in the reaction at 30 μM. All the reactions were performed in triplicates and error bars represent confidence interval ($\alpha = 0.05$). Inhibitory activity of AA-CAM compounds with free α -amino groups are shown as light grey bars, N-protected AA-CAM compounds – dark grey, positive control CLM – white bars. (D) Primer extension inhibition (toe-printing) analysis of site specificity of action of CLM and AA-CAMs. The synthetic mini-gene was translated in the cell-free translation ('PURE') system and sites of antibiotic-induced translation arrest were analyzed by primer extension. The reactions loaded onto lanes 1-6 contained mupirocin, an inhibitor of isoleucyl-tRNA synthetase. The sample in lane 2 (labeled 'NONE') contained no other antibiotics besides mupirocin. The control antibiotic retapamulin (RET) inhibits translation initiation and arrests the ribosome at the start codon (black arrowheads). Bands corresponding to the CLM-induced translation arrest at the 5th codon are indicated by the green arrowheads. Stalling of ribosomes at the 7th codon of the ORF due to the presence of mupirocin that causes depletion of isoleucyl-tRNA (lanes 1-6) is indicated by the blue arrowheads. U- and A-specific sequencing lanes are indicated. The nucleotide sequence of the gene and the corresponding encoded amino acids are indicated on the left. Note that the reverse transcriptase stops 15-16 nucleotides downstream from the first nucleotide of the P-site codon as indicated by the dashed arrow.

Figure 3. Chemical structures and electron density maps of CAM-derivatives

Chemical structures and difference Fourier maps of His-CAM (**A**), D-His-CAM (**B**), and Lys-CAM (**C**) in complex with the *T. thermophilus* 70S ribosome. The refined model of each compound is displayed in its respective electron density before the refinement (green mesh). Carbon atoms are colored yellow for His-CAM, orange for D-His-CAM, magenta for Lys-CAM, nitrogens are blue, oxygens are red.

Figure 4. Structure of His-CAM in complex with the 70S ribosome and A- and P-tRNAs (A, B) Overview of the His-CAM binding site (yellow) in the *T. thermophilus* 70S ribosome viewed from the PTC down the tunnel as indicated by the inset (A), or as a cross-cut section through the ribosome (B). The 30S subunit is shown in light yellow, the 50S subunit is in light blue, the mRNA is magenta and the A- and P-site tRNAs are green and dark blue, respectively. The E-site tRNA is omitted for clarity. (C, D) Close-up views of the His-CAM bound in the PTC. The *E. coli* nucleotide numbering is used throughout. Potential H-bond interactions are indicated with dashed lines. Note that side chain of His-CAM compound

forms tilted edge-to-face Π -stacking with the nucleobase of U2506 of the 23S rRNA (shown as spheres).

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Figure 5. Side-chain-specific interactions of AA-CAMs with the ribosome
Compound-specific H-bond interactions of His-CAM (A), D-His-CAM (B), or Lys-CAM (C) with the nucleotides of the 23S rRNA are indicated with dashed lines. Stacking interactions of His-CAM are shown with the black arrow.

Figure 6. Structural basis for resistance to CLM (A) and His-CAM (B)

Molecular modeling of the C8-methylation of A2503 (red sphere) catalyzed by the Cfr-methyltransferase reveals a small clash with CLM (A) but not with histidine analogue of CLM (B). Note that the path of the His-CAM in the PTC is located further away from the C8-position of A2503 (compared to CLM) that should allow it to avoid a possible steric clash with this position carrying methyl group in the Cfr-modified ribosome.

Table 1
Apparent dissociation constants (K_{Dapp}) of amino-acid CAM-derivatives with the *E. coli* 70S ribosomes

Numbers in the parenthesis correspond to the particular compound from the synthesis scheme shown in Figure S1.

N-unprotected Compounds		N-Protected Compounds	
Compound	K_{Dapp} (μ M)	Compound	K_{Dapp} (μ M)
CLM	2.8 ± 0.5	Ac-Pro-CAM (7a)	10 ± 1.5
His-CAM (1)	0.24 ± 0.06	Ac-Arg-CAM (3a)	26 ± 10
Lys-CAM (2)	1.6 ± 0.5	Ac-His-CAM (1a)	34 ± 5
Arg-CAM (3)	3.2 ± 1.4	Ac-Lys-CAM (2a)	43 ± 12
Ala-CAM (4)	5.3 ± 0.9	Ac-Ala-CAM (4a)	55 ± 13
Gly-CAM (5)	5.7 ± 0.9	Ac-Gly-CAM (5a)	69 ± 14
Gln-CAM (6)	6.3 ± 5.1	Ac-Phe-CAM (11a)	92 ± 22
Pro-CAM (7)	7.5 ± 1.6	Ac-Trp-CAM (10a)	106 ± 36
Tyr-CAM (8)	8.2 ± 1.5	Ac-Tyr-CAM (8a)	140 ± 50
Ser-CAM (9)	8.4 ± 2.4	Boc-His-CAM (1d)	19 ± 5
Trp-CAM (10)	12 ± 2	Boc-Gly-CAM (5d)	45 ± 8
Phe-CAM (11)	19 ± 8	Boc-Phe-CAM (11d)	160 ± 120
Val-CAM (12)	34 ± 8	Boc-Ala-CAM (4d)	170 ± 60
Leu-CAM (13)	36 ± 6	Boc-Pro-CAM (7d)	440 ± 230
Ile-CAM (14)	36 ± 10	f-Gly-CAM (5b)	45 ± 6
Asn-CAM (15)	40 ± 26		
D-His-CAM (1b)	2.9 ± 0.8		
D-Ala-CAM (4b)	7.9 ± 1.4		
D-Pro-CAM (7b)	13 ± 2		
β -Ala-CAM (16)	6.3 ± 1.3		