Binding and Action of CEM-101, a New Fluoroketolide Antibiotic That Inhibits Protein Synthesis

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We characterized the mechanism of action and the drug-binding site of a novel ketolide, CEM-101, which belongs to the latest class of macroline antibiotics. CEM-101 shows high affinity for the ribosomes of Gram-negative (Escherichia coli) and Gram-positive (Staphylococcus aureus) bacteria. The ketolide shows high selectivity in its inhibitory action and readily interferes with synthesis of a reporter protein in the bacterial but not eukaryotic cell-free translation system. Binding of CEM-101 to its ribosomal target site was characterized biochemically and by X-ray crystallography. The X-ray structure of CEM-101 in complex with the E. coli ribosome shows that the drug binds in the major macrolide site in the upper part of the ribosomal exit tunnel. The lactone ring of the drug forms hydrophobic interactions with the walls of the tunnel, the desosamine sugar projects toward the peptidyl transferase center and interacts with the A2058/A2509 cleft, and the extended alkyl-aryl arm of the drug is oriented down the tunnel and makes contact with a base pair formed by A752 and U2609 of the 23S rRNA. The position of the CEM-101 alkyl-aryl extended arm differs from that reported for the side chain of the ketolide telithromycin complexed with either bacterial (Deinococcus radiodurans) or archael (Haloarcula marismortui) large ribosomal subunits but closely matches the position of the side chain of telithromycin complexed to the E. coli ribosome. A difference in the chemical structure of the side chain of CEM-101 in comparison with the side chain of telithromycin and the presence of the fluorine atom at position 2 of the lactone ring likely account for the superior activity of CEM-101. The results of chemical probing suggest that the orientation of the CEM-101 extended side chain observed in the E. coli ribosome closely resembles its placement in Staphylococcus aureus ribosomes and thus likely accurately reflects interaction of CEM-101 with the ribosomes of the pathogenic bacterial targets of the drug. Chemical probing further demonstrated weak binding of CEM-101, but not of erythromycin, to the ribosome dimethylated at A2058 by the action of Erm methyltransferase.

Macrolide antibiotics bind to the large ribosomal subunit and inhibit protein synthesis by blocking the path of the nascent peptide in the exit tunnel (15, 44). The chemical structure of the prototype macrolide erythromycin A is represented by a 14-atom lactone ring decorated with C-3 cladinose and C-5 desosamine sugar residues (Fig. 1). Subsequent classes of macrolides differed in the structures of the lactone as well as the number, composition, and sites of attachment of the side chains (13, 41).

The binding site of macrolides in the ribosome is composed of 23S rRNA residues A2058, A2059, A2062, A2503, G2505, and G2611 (here and throughout the Escherichia coli numbering is used) (43). One of the main mechanisms of resistance to macrolide antibiotics is based on dimethylation of A2058 by the methyltransferase encoded in erm genes (46). Erm-catalyzed dimethylation of A2058 leads to a steric clash with the drug and reduces the affinity of erythromycin for the ribosome. Similar to several other antibiotic resistance genes, erm genes are often inducible by erythromycin and similar drugs.

In an effort to combat resistance, a newer class of macrolides, known as ketolides, was developed (5). Ketolides show improved activity against strains with inducible erm genes and are believed to exhibit a tighter binding to the ribosome compared with that of previous classes of macrolides (32, 33). In ketolides, cladinose is replaced by a keto function (hence the name of the class) and an extended alkyl-aryl side chain is attached at the C-11 and C-12 carbon atoms (in the prototype ketolide telithromycin) or at other positions of the lactone ring (Fig. 1). Early biochemical and genetic studies showed that the extended side chain of ketolides establishes important new interactions with the ribosome that might account for the increased efficacy of these drugs. Specifically, chemical probing and resistance mutations pointed to interactions of the 11,12 side chain of telithromycin with the rRNA residues in the loop of helix 35 of the E. coli 23S rRNA and with U2609 (14, 17, 49). However, subsequent crystallographic studies of the first clinically approved ketolide telithromycin bound to the bacterial...
Deinococcus radiodurans) or archaeal (Haloarcula marismortui) ribosome showed the placement of the 11,12 side chain in a position that was hardly compatible with rRNA protections and mutations observed in the E. coli ribosome (3, 43). Furthermore, the orientation of the alkyl-aryl side chain differed significantly between the reported D. radiodurans and H. marismortui structures, conforming to the general notion of idiosyncratic interactions of antibiotics with ribosomes of various bacterial species (36, 47) and therefore leaving open the question of how the drug may bind to the ribosomes of pathogenic bacteria targeted by ketolide antibiotics.

Although telithromycin, the first ketolide introduced into medical practice, in 2001, showed excellent activity against many macrolide-resistant strains of Gram-positive pathogens, the safety issues that became apparent upon the wider use of the drug have curbed its clinical use (reviewed in reference 35). The adverse effects associated with telithromycin spurred a search for newer ketolides. One of the novel promising drugs of this class is CEM-101 (Fig. 1). The structure of CEM-101 is similar to that of telithromycin, except for the chemical nature of the alkyl-aryl side chain (which in CEM-101 is represented by 11,12-carbamate-butyl-[1,2,3]-triazolyl-aminophenyl) and the presence of a fluorine atom linked to C-2 of the lactone.

In microbiological tests involving a number of clinical pathogens, CEM-101 is characterized by lower MIC values than those of telithromycin and exhibits enhanced activity against telithromycin-resistant strains of E. coli and S. aureus (26). Furthermore, in comparison with telithromycin and cladinose-containing macrolides, CEM-101 shows significantly enhanced accumulation in macrophages, which adds to its attractiveness from the medical standpoint.

Despite its favorable pharmacological properties, little is known about the mode of action and site of binding of CEM-101. In this study, we used biochemical and crystallographic approaches to characterize the mode of action and site of binding of CEM-101, a new ketolide antibiotic.

MATERIALS AND METHODS

Antibiotics, ribosomes, and reagents. CEM-101, CEM-103, telithromycin, and [14C]CEM-101 (53 mCi/mmol), synthesized by Moravek Biochemicals, Inc., were provided by Cempra Pharmaceuticals. Erythromycin and azithromycin were from Sigma. [14C]erythromycin (48.8 mCi/mmol) was obtained from PerkinElmer.

Antibiotics were dissolved in 100% ethanol at a concentration of 10 mM, and serial dilutions were made in water to obtain the concentrations needed for the competition binding studies and the cell-free transcription-translation assays. The antibiotics were diluted in ethanol for the ribosome probing experiments.

Ribosomes were prepared from E. coli strain MRE 600, Staphylococcus aureus strain ATCC 29212, or S. aureus strain N315 by standard protocols (39). Most of the chemicals were from Fisher Scientific or Sigma.

Competition binding studies. Binding of erythromycin to E. coli and S. aureus ribosomes and competition experiments were done by size exclusion chromatography using Bio-Gel P30 spin columns, as described in reference 48.

Direct antibiotic binding experiments were done by incubating ribosomes at a 100 nM concentration with various concentrations of radiolabeled drug in a total volume of 160 μl in buffer A (20 mM Tris-HCl [pH 7.5], 10 mM MgCl2, 150 mM NH4Cl, 6 mM 2-mercaptoethanol) at 37°C for 15 min and then at 20°C for 10 min. The reactions were loaded onto the spin columns and centrifuged in a swinging-bucket microcentrifuge rotor for 1 min at 1,000 × g at room temperature. The flowthrough solution was collected; 130 μl was mixed with 5 ml of the scintillation cocktail. Radioactivity was measured in a scintillation counter and used to calculate the amount of erythromycin bound to ribosomes.

For competition binding experiments, ribosomes (100 nM) were preincubated with 100 nM [14C]erythromycin (48.8 Ci/mol; PerkinElmer) in 160 μl of buffer A at 37°C for 15 min and then at 20°C for 10 min. Competing antibiotics were added at various concentrations, and the binding mixture was incubated at 20°C for 100 min. The reactions were loaded onto the spin columns and the amount of ribosome-associated radioactivity was measured as described above. Binding data were analyzed using Prism software (GraphPad).
Cell-free transcription-translation assays. The *E. coli* transcription-translation (Tnt) S30 extract system for circular DNA (catalog no. L1020; Promega) was used to evaluate the effects of antibiotics on bacterial protein synthesis. Experiments were carried out in 96-well conical-bottom plates in a final volume of 10 μl. S30 extract (3 μl), combined with 1 μl of water or antibiotic solution, was dispensed into wells of the plate and preincubated at 25°C for 5 min. The reactions were initiated by adding 6 μl of a translation mixture containing 1 μl of pBEST Luc DNA (0.7 μg), 1 μl of 1 mM amino acid mixture, and 4 μl of S30 premix. The reaction mixtures were incubated at 30°C for 40 min and then placed on ice. In another 96-well plate, 150 μl of Bright-Glo dilution reagent (catalog no. E266A; Promega) was dispensed and mixed with 1.5 μl of the translation reaction mixtures; 30 μl of the resulting solution was mixed with 30 μl of Bright-Glo luciferase assay reagent (catalog no. E2610; Promega) in a final volume of 10 μl of Bright-Glo dilution reagent. The reactions were initiated by adding 6 μl of Bright-Glo luciferase assay reagent (catalog no. E266A; Promega) was dispensed and mixed with 1.5 μl of the translation reaction mixtures; 30 μl of the resulting solution was mixed with 30 μl of Bright-Glo luciferase assay reagent (catalog no. E2610; Promega) in a 96-well white-wall plate (catalog no. 6005290; PerkinElmer). Luminescence was measured on a TopCount scintillation and luminescence counter (PerkinElmer).

The rabbit reticulocyte cell-free translation system (catalog no. LA540; Promega) was used to assay the effects of the drugs on the activity of the eukaryotic ribosome. Experiments were carried out in 96-well conical-bottom plates in a final volume of 10 μl. Rabbit reticulocyte lysate (7 μl) was mixed with 1 μl of water or antibiotics, and the mixture was preincubated at 25°C for 5 min. Polyadenylated luciferase mRNA (catalog no. L4561; Promega) was denatured before use by incubating the RNA at 65°C for 3 min and then placed on ice. The translation mixture (2 μl) was mixed with the rabbit reticulocyte lysate to start the reaction. The translation mixture contained 0.3 μl of denatured luciferase mRNA (0.3 μg), 0.2 μl of 1 mM amino acid mixture, 0.2 μl of RNase inhibitor (8 U; catalog no. 03 335 399001; Roche), 0.4 μl of 2.5 mM potassium chloride, and 0.9 μl of water. The reaction mixtures were incubated at 30°C for 30 min and then placed on ice. In another 96-well plate, 150 μl of Bright-Glo dilution reagent (catalog no. E266A; Promega) was dispensed and mixed with 1.5 μl of the translation reaction mixtures; 30 μl of the resulting solution was mixed with 30 μl of Bright-Glo luciferase assay reagent (catalog no. E2610; Promega) in a 96-well white-wall plate. Luminescence was measured on a TopCount scintillation and luminescence counter (PerkinElmer).

Ribosome chemical probing. RNA probing was done following standard protocols (27), with minor modifications. Briefly, 200 nM ribosomes was incubated with 100 μM antibiotic in 50 μl of buffer B (80 mM HEPES-KOH [pH 7.8], 20 mM MgCl₂, 100 mM NH₄Cl, 1.5 mM dithiothreitol) for 10 min at 37°C, followed by 10 min at 20°C. Modifying reagents [dimethyl sulfate (DMS), kethoxal, or 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMCT)] were added, and the modification reaction was carried out for 10 min at 37°C. After quenching of the reaction and ethanol precipitation, rRNA was frozen with liquid nitrogen. X-ray diffraction data were collected at beamline 12.3.1 of the Advanced Light Source, Lawrence Berkeley National Laboratory, using 0.1° to 0.3° oscillations at 100 K and recorded on an ADSC Q315 detector. X-ray diffraction data were reduced and scaled using the HKL2000 program package (31). The coordinates reported in Protein Data Bank entries with accession numbers 3I1M, 3I1N, 3I1O, and 3I1P were refined against the reflection data using the PHENIX software suite (1). F(obs) − F(calc) difference electron density maps (F(obs), observed amplitudes; F(calc), calculated amplitudes) were calculated using the PHENIX program, and coordinates for CEM-101 were placed into this unbiased difference density using the software Coot (12). Individual atomic displacement parameter values for the comparison between CEM-101 and telithromycin were calculated using PHENIX. Figure 4 was made using PyMol software.

Protein structure accession numbers. Coordinates of the structures are available in the Protein Data Bank with accession numbers 3OR9, 3ORA, 3ORB, and 1VT2.

RESULTS

Affinity of CEM-101 to wild-type ribosomes from *E. coli* and *S. aureus*. Binding of CEM-101 to wild-type ribosomes from Gram-negative and Gram-positive bacteria was initially analyzed by competition with [14C]erythromycin. For that, we first analyzed binding of radiolabeled erythromycin to our preparations of *E. coli* and *S. aureus* 70S ribosomes. In saturation binding experiments, [14C]erythromycin readily bound to ribosomes from both bacteria, exhibiting dissociation constants (Kd) of 66 ± 11 nM and 11 ± 1 nM for the *E. coli* and *S. aureus* ribosomes, respectively (data not shown). These values were comparable to those previously published (10⁻⁸ to 10⁻⁷ M) (8, 19). Binding of erythromycin saturated close to 1 pmol of the drug per 1 pmol of *E. coli* or *S. aureus* ribosomes, indicating that the majority of the ribosomes in the preparation were competent for binding.

In competition binding experiments, CEM-101 readily displaced erythromycin from both types of ribosomes, with the 50% inhibitory concentrations (IC₅₀) being 155 ± 8 nM for the *E. coli* ribosome and 117 ± 3 nM for the *S. aureus* ribosome (Fig. 2A and B), resulting in CEM-101 Kd(s) of 62 ± 3 nM (E. coli) and 12 ± 1 nM (S. aureus) (Table 1). In parallel experiments, another ketolide, telithromycin, as well as cladinose-containing azithromycin exhibited a comparable affinity (Table 1). When radiolabeled [14C]CEM-101 became available to us, its affinity for *S. aureus* wild-type ribosomes was reexamined by saturation binding experiments (Fig. 2C). The Kd of 50 ± 13 nM obtained with this approach was similar to that obtained by competition with erythromycin. Altogether, drug-binding studies demonstrated that the new ketolide, CEM-101, interacts with the ribosomal site that either coincides or overlaps with that of erythromycin and demonstrated that the drug binds to ribosomes of Gram-positive and Gram-negative bacteria with affinities similar to those of other macrolides.
Inhibition of bacterial protein synthesis by CEM-101. The effect of CEM-101 upon bacterial protein synthesis was assayed in an *E. coli* cell-free transcription-translation system. CEM-101 inhibited the synthesis of firefly luciferase (Lux) with an IC$_{50}$ of 1.1 μM (Fig. 3A), comparable to the inhibition afforded by azithromycin (IC$_{50}$, 0.3 μM) and telithromycin (IC$_{50}$, 0.5 μM). The specific effect of CEM-101 upon translation rather than transcription in the cell-free system was independently verified by using *lux* mRNA instead of DNA as a template (data not shown). It should be noted that the concentration of ribosomes in the bacterial cell-free translation system (600 nM) significantly exceeds the $K_a$ values of macrolide antibiotics. Therefore, IC$_{50}$s do not accurately describe the relative efficacy of the tested macrolide antibiotics in inhibition of bacterial translation but, rather, provide a qualitative indication of their ability to readily interfere with the bacterial protein synthesis.

In contrast to its effect upon the bacterial translation, CEM-101 showed no effect on the synthesis of luciferase in the eukaryotic (rabbit) cell-free translation system at concentrations up to 50 μM (Fig. 3B). Thus, CEM-101 exhibits selective and efficient inhibition of bacterial translation.

Interaction of CEM-101 with *E. coli* ribosome in a crystalline state. The chemical structure of CEM-101 is different from the structures of conventional macrolides (Fig. 1). Its structure also shows important variation from the structure of telithromycin: the imidazolyl-pyridine moiety of the telithromycin 11,12 side chain is replaced with a triazolyl-aminophenyl conjugate in CEM-101. Therefore, although binding of telithromycin was previously studied by X-ray crystallography (3, 11, 43), it was unclear how closely binding of CEM-101 to the bacterial ribosome would match that of telithromycin. This consideration prompted us to characterize the binding of CEM-101 to the *E. coli* ribosome using crystallographic analysis.

We proceeded in obtaining the high-resolution structure of the *E. coli* ribosome with the bound CEM-101 (Table 2). The general pose of CEM-101 in the ribosome is similar to that seen for telithromycin bound to the ribosome of *E. coli* (11) (Fig. 4A). The placement and configuration of the lactone ring and desosamine sugar of the two drugs are essentially indistinguishable. The triazolyl-aminophenyl head of the 11,12 side chain of CEM-101 makes a similar stacking interaction with the A752-U2609 base pair as the imidazole-pyridine moiety of telithromycin; it is located at a distance of 3.5 Å from the A752 and U2609 bases and is oriented parallel to them. Importantly, the interactions of the CEM-101 (and telithromycin) alkyl-aryl side chains that we observed in the *E. coli* ribosome are principally different from those seen previously in crystallographic

### TABLE 1. $K_a$s of binding of CEM-101 and other macrolides to *E. coli* and *S. aureus* ribosomes

| Antibiotic   | *E. coli* $K_a$ (nM) | *S. aureus* $K_a$ (nM) |
|--------------|----------------------|------------------------|
| Erythromycin | 66 ± 11              | 11 ± 1                 |
| CEM-101      | ND                   | 50 ± 13                |
| CEM-101      | 62 ± 3               | 12 ± 1                 |
| Telithromycin| 49 ± 2               | 10 ± 1                 |
| Azithromycin | 28 ± 2               | 11 ± 2                 |

*a* Determined by measuring direct binding of radiolabeled compound.  
*b* Determined by competition with radiolabeled erythromycin.  
*c* ND, not determined.

### TABLE 2. Diffraction statistics for crystals of *E. coli* 70S ribosome complexed with CEM-101

| Parameter$^a$     | Value(s) |
|--------------------|----------|
| Data collection$^b$|          |
| Space group        | P2$_1$2$_1$2$_1$ |
| Cell dimensions    |          |
| a, b, c (Å)        | 210.7, 433.2, 618.8 |
| α, β, γ (degrees)  | 90, 90, 90 |
| Resolution (Å)     | 100–3.11 (3.17–3.11)$^c$ |
| $R_{	ext{sym}}$ or $R_{	ext{merge}}$ | 11.1 (89.9) |
| I/σ(I)             | 8.27 (1.19) |
| Completeness (%)    | 93.8 (85.5) |
| Redundancy          | 3.3 (2.5) |
| Refinement         |          |
| Resolution (Å)     | 69.7–3.10 |
| No. of reflections  | 821,883 |
| $R_{	ext{work}}$/$R_{	ext{free}}$ | 0.22/0.26 |
| No. of atoms       | 284,555 |
| RMS deviations     |          |
| Bond lengths (Å)   | 0.006 |
| Bond angles (degrees) | 1.452 |

$^a$ a, b, c are the lengths of the unit cell axes; α, β, and γ are the angles of the unit cell axes; $R_{	ext{work}}$ or $R_{	ext{merge}} = \Sigma|I_o - <I>|/\Sigma|I_o|$, where $I_o$ is the intensity of the $i$th observation and $<I>$ is the mean of the reflection; $R_{	ext{work}}(R_{	ext{merge}}) = \Sigma|F_o - F_c|/\Sigma|F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively; RMS, root mean square.  
$^b$ Two crystals were used.  
$^c$ Values in parentheses are for the highest-resolution shell.

**FIG. 3.** *In vitro* inhibitory activity of CEM-101. (A) Inhibition of protein synthesis in the *E. coli* cell-free transcription-translation system; (B) inhibition of protein synthesis in the rabbit reticulocyte lysate cell-free translation system.
complexes of ketolides with the ribosomes of *D. radiodurans* or *H. marismortui* (3, 37, 43). We believe that the presence of the A752-U2609 base pair in the ribosomes of *E. coli* and many pathogenic bacteria may account for this specific mode of interaction of the ketolides’ side chain. Because of the 23S rRNA sequence differences, formation of such a base pair is impossible in either *D. radiodurans* or *H. marismortui*. This consideration makes us believe that the structures of ketolides complexed with the *E. coli* ribosome more accurately reflect interactions of the drugs with the ribosomes of pathogenic bacteria. Various ketolides differ from CEM-101 in the chemical nature of the alkyl-aryl side chain and the site of its attachment to the lactone scaffold. Nevertheless, in the RNA probing experiments, all these drugs afford a nearly complete protection of A752 in the *E. coli* ribosome from chemical modification (9, 17, 48, 49), indicating that the interaction of the alkyl-aryl side chain with the A752-U2609 base pair is important for binding of a range of clinically relevant ketolides to the ribosome.

Despite a generally similar orientation of the side chains of CEM-101 and telithromycin, the variation in their chemical structures produces an important difference in the mode of binding. The atomic displacement parameter (ADP) refinement for the CEM-101 atoms shows that the extended arm of CEM-101 exhibits a significantly lower ADP value than the side chain of telithromycin (Fig. 4B), whereas the average ADP values for all the other antibiotic atoms are very similar in both cases. This difference, which reflects better anchoring of CEM-101 in its binding site in the ribosome, likely results from additional hydrogen bonding interactions of the exocyclic amino group of aminophenyl in the side chain of CEM-101. Specifically, the amino group appears to serve as an H-bond donor to O-4/•H11032 of A752 and O-6 of G748, while it is an H-bond acceptor from N-1 of G748. None of these interactions is possible for telithromycin.

Some reports indicated that mutations in the ribosomal protein L22 could confer ketolide resistance or potentiate the effect of rRNA resistance mutations (2, 16, 18). Examination of the structure of the CEM-101–ribosome complex shows that the ε-amino group of Lys 90 of L22 is located fairly close to the

![Crystallographic structure of CEM-101 bound to the *E. coli* ribosome. (A) Position of CEM-101 (pink) within the ribosomal binding site. The neighboring 23S rRNA residues and amino acid residues of protein L22 (gray) are marked. Hydrogen bonds between the alkyl-aryl arm and 23S rRNA residues A752 and G748 are also shown. (B) Close-up of interactions involving the triazolyl-aminophenyl side chain of CEM-101. Electron density is shown as a 2Fobs − Fcalc map contoured at 2 standard deviations from the mean. (C) Difference in ADP values of the 11,12 side chains of CEM-101 and telithromycin (TEL). Bavg, ring, the average of the atomic displacement parameter values for all nonhydrogen atoms within either the imidazolyl-pyridine or the triazolyl-aminophenyl moiety following individual atomic displacement parameter refinement using the phenix.refine software. (D) Proximity of the fluorne ring of CEM-101 to rRNA in the drug-binding site. The distance between fluorine of CEM-101 and N-1 of C2611 of 23S rRNA is indicated. (E) Comparison of binding of CEM-101 (pink) and erythromycin (olive) (11) to the *E. coli* ribosome. The overlapping desosamine side chains of erythromycin and telithromycin and the cladinose residue of erythromycin are indicated. The N-6 exocyclic amino group of A2058, which is the site of Erm-catalyzed dimethylation, is indicated by an arrow.

FIG. 4.
aminophenyl moiety of CEM-101 (Fig. 4B). However, the distance (≈4 Å) appears to be too long for a direct chemical interaction, suggesting that L22 mutations may act allosterically. It is conceivable, however, that changes in the placement of the Lys 90-containing β-hairpin of L22 due to insertions or deletions of several amino acids often associated with macrolide resistance (6, 7, 50) could bring Lys 90 into prohibitively close contact with the drug, which could result in reduced affinity of CEM-101 (or telithromycin) for the ribosome.

A distinctive feature of CEM-101 compared to telithromycin and several other ketolides is the presence of a fluorine atom at the C-2 position of the lactone ring. In the structure of CEM-101 bound to the E. coli ribosome, the fluorine atom is positioned near the glycosidic bond (atom N-1) of C2611 and, thus, can potentially contribute to the drug binding (Fig. 4D). Although some reports questioned the importance of 2-F for the activity of ketolides (20), comparison of the MIC values of CEM-101 with those of its analog that lacked the fluorine atom showed that CEM-101 more readily inhibited growth of streptococci carrying the erm methyltransferase gene (Table 3). Thus, fluorination of the C-2 carbon atom may specifically contribute to a tighter binding of the drug to the ribosome dimethylated at A2058. In the structure of CEM-101 complexed to the E. coli ribosome, the fluorine atom is only 2.7 Å away from N-1 of C2611, indicating that any modifications larger than fluorine at this position would lead to a structural clash with rRNA.

**Interactions of CEM-101 with wild-type E. coli and S. aureus ribosomes in solution.** Binding of the drug to the ribosome in the crystalline state may differ from that in solution. Furthermore, biochemical, genetic, and crystallographic evidence indicates that the same compound may exhibit different interactions with the ribosomes of different species (4, 11), raising the question of whether the crystallographic structure of CEM-101 bound to the E. coli ribosome accurately reflects the drug’s interactions with ribosomes of Gram-positive pathogens. Therefore, we expanded the structural studies of CEM-101 binding by probing its interactions with the E. coli and S. aureus ribosomes using RNA footprinting (28). In this study we also included a C-3 cladinose cousin of CEM-101, CEM-103, which additionally lacks the C-2-linked fluorine atom (Fig. 1).

Similar to other investigated macrolides and ketolides, CEM-101 and CEM-103 protect A2058 and A2059 in domain V of 23S rRNA from modification with DMS (Fig. 5A and B). As can be inferred from the crystallographic structure, these protections are afforded by the C-5 desosamine sugar, which closely approaches the cleft formed by A2058 and A2059 residues. Furthermore, in excellent agreement with crystallographic structures of CEM-101 (and telithromycin) complexed with the E. coli ribosome, CEM-101 as well as telithromycin and CEM-103 strongly protects A752 from DMS modification both in E. coli and in S. aureus ribosomes (Fig. 5B and D). In contrast, erythromycin, which lacks the extended side chain, fails to protect A752 from DMS modification. Thus, the interaction of the CEM-101 side chain with the A752-U2609 base pair seen in the crystalline state appears to accurately reflect binding of the drug to the ribosome in solution. The lack of C-2 fluorine or C-3 cladinose in CEM-103 does not bring about any difference in the footprinting pattern, confirming that these moieties of the drug do not make contacts with rRNA residues accessible for DMS modification.

Importantly, the footprinting pattern of CEM-101 in the ribosome of E. coli is indistinguishable from that in the ribosome of S. aureus, indicating that the high-resolution structure of CEM-101 complexed to the E. coli ribosome that we report here likely reflects binding of the drug to the ribosomes of Gram-positive pathogens.

**Interaction of CEM-101 with S. aureus ribosomes dimethylated at A2058 by Erm methyltransferase.** Erm methyltransferase modifies A2058 in 23S rRNA by consecutively adding methyl groups to the exocyclic amino group of the adenine base. Such modification completely blocks binding of erythromycin and similar macrolides, whereas its effect on binding of ketolides is less clear (9, 23). Therefore, we investigated interaction of CEM-101 with the ribosomes isolated from a clinical strain, S. aureus N315, which carries five chromosomal copies of the constitutively expressed ermA gene present in the Tn554 transposon (22). The ribosomes isolated from the S. aureus N315 strain are extensively dimethylated at the A2058 residue, which agrees well with the lack of binding of [14C]erythromycin to ribosomes prepared from this strain (data not shown).

We used footprinting analysis to test interactions of ketol-
ides (CEM-101 and telithromycin) and cladinose-containing macrolides (CEM-103 and erythromycin) with the A2058-dimethylated ribosomes isolated from the *S. aureus* N315 strain. Ribosomes were incubated with the drugs (present at 100 μM concentration) and probed by DMS modification. Because dimethylated A2058 blocks progression of reverse transcriptase along the RNA template, a strong reverse transcriptase stop is observed at A2058 on the primer extension gel (45, 52) (Fig. 6A). Therefore, it is impossible to use footprinting to evaluate interactions of macrolides with this position in *S. aureus* N315 ribosomes (Fig. 6A). However, examination of the intensity of the A2059 band clearly showed that both CEM-101 and CEM-103, but not erythromycin, could bind to the Erm-modified ribosome, resulting in protection of A2059 from DMS modification (Fig. 6A). Telithromycin also protected A2059 but to a lesser extent than CEM-101 or CEM-103. For a more extensive analysis of binding of ketolides and CEM-103 to the *S. aureus* ribosome, we extended our footprinting studies by including two more modifying reagents, kethoxal, which modifies guanosines, and CMCT, which modifies uridines (27). Kethoxal probing showed that CEM-101 and CEM-103 partially protected G2505 from modification with kethoxal and fully protected U2609 from modification with CMCT (Fig. 6B and C). Telithromycin afforded a notably weaker protection at both positions. Erythromycin did not protect either of the two positions, consistent with the fact that dimethylation of A2058 is known to prevent erythromycin binding (46).

The overall conclusion that can be drawn from the results of the footprinting studies is that macrolides with the extended alkyl-aryl side chain, when present at a sufficiently high concentration, can bind to the ribosome dimethylated at A2058 by the action of Erm methyltransferase. It is also apparent that the side chains of CEM-101 and CEM-103, probably due to the presence of the aminophenyl moiety and its stronger interaction with the A752-U2609 base pair, exhibit more efficient binding than telithromycin.

**FIG. 5.** DMS probing of interactions of CEM-101 and other macrolides with the wild-type ribosomes of *E. coli* (A and B) or *S. aureus* (C to E). Panel E presents an enlarged segment of the gel shown in panel D. Lanes are marked as follows: A and C, sequencing lanes; control, unmodified ribosome; no drug, ribosome modified with DMS in the absence of antibiotics; CEM-101 and CEM-103, self-explanatory; Ery, erythromycin; Tel, telithromycin.
DISCUSSION

We have characterized the mechanism of action and the mode of binding of a new ketolide antibiotic, CEM-101. CEM-101 exhibited tight binding to the ribosome, showing potency similar to that of telithromycin in displacing radiolabeled erythromycin from ribosomes of Gram-negative and Gram-positive bacteria. In a cell-free translation system, CEM-101 actively inhibited bacterial but not eukaryotic translation. This demonstrated that the high selectivity, characteristic of macrolides, was retained in this new ketolide derivative.

Crystallographic and biochemical characterization of CEM-101 binding to the wild-type ribosome revealed important interactions of the drug with 23S rRNA residues that constitute its binding site. Although the positions of the lactone ring and desosamine sugar of CEM-101 closely resemble those of cladinose-containing macrolides such as erythromycin and azithromycin, an additional interaction of the 11,12 alkyl-aryl arm of CEM-101 with the A752-U2609 base pair accounts for the major difference in the binding mode of the ketolide from that of macrolides of previous classes. A specific characteristic of CEM-101 is a possibility of hydrogen bonding of the amino-phenyl moiety at the tip of the 11,12 side chain with G748 and A752 in the loop of helix 35 in domain II of 23S rRNA (Fig. 4B). These additional interactions may explain the better anchoring of the CEM-101 side chain in the ribosome than the side chain of telithromycin and may hypothetically account for the reported improved efficacy of CEM-101 (26). However, the conclusions about the contribution of the 11,12 side chain to the drug activity should be drawn only cautiously. Although original studies suggested that interactions of the side chain of ketolides with A752 in the loop of helix 35 in 23S rRNA significantly contribute to the drugs’ affinity (9, 17, 49), more recent genetic studies questioned that conclusion because mutations at and around A752 or at U2609, with which the side chains interact, had only a minor effect upon drug inhibitory action (14, 21, 30, 49). In agreement with these observations, the additional interaction afforded by the extended side chains of telithromycin and CEM-101 or, for that matter, the fluorine atom at the C-2 position of the CEM-101 lactone did not translate in our study to an improved ability of either of the ketolides compared with that of azithromycin in inhibiting erythromycin binding to the ribosome (Table 1). One possible explanation for this apparent discrepancy is that the gain in binding energy of CEM-101 attributable to the 11,12 side chain and 2-fluorine is counterbalanced by the lack of interactions that engage the cladinose sugar present in erythromycin and azithromycin but lacking in ketolides.

Several recent findings indicate that macrolides may inhibit translation in a polypeptide-specific manner (24, 25, 29, 40, 42). In this regard, it is noteworthy that although the affinity of CEM-101 for the ribosome in vitro is similar to that of the older macrolides, the drug has a clearly improved potency in inhibiting cell growth (26, 34). Direct examination of the cellular proteins that are specifically inhibited by erythromycin, azithromycin, and CEM-101 showed that the spectrum of inhibition is significantly different between cladinose-containing macrolides and ketolides (K. Kannan and A. S. Mankin, unpublished results). Thus, it is possible that the improved efficacy of CEM-101 and other ketolides is at least partly related to the spectrum of proteins inhibited by the drugs.
One of the distinctions of ketolides, including CEM-101, from macrolides of the previous classes is their improved binding to the ribosome in which A2058 has been dimethylated by Erm methyltransferase. The pioneering study by Douthwaite and coworkers (9) showed that the alky-aryl side chain of ketolides can significantly enhance binding of the drugs to the A2058(dimethylated ribosome. Our footprinting studies confirmed that the ribosomes isolated from the erm-positive S. aureus strain can bind CEM-101, at least when the drug is present at a high (100 μM) concentration. Under these conditions, none of the cladinose-containing macrolides showed any significant binding (Fig. 6 and data not shown), whereas telithromycin, a ketolide, could bind but with an apparently lower efficiency than CEM-101, as judged by incomplete protection of nucleotides from chemical modification (Fig. 6A and C). Erm methyltransferase modifies A2058 by adding two methyl groups one by one. Therefore, despite the high level of expression of ErmA methyltransferase in the S. aureus N315 strain, we cannot exclude the possibility that a fraction of the ribosomes in our preparation may have a monomethylated A2058 (10). However, since N-6 monomethylation of the adenosine base does not affect progression of reverse transcriptase on the RNA template, a strong stop observed at A2058 in the primer extension gel (Fig. 6A) clearly indicates that A2058 is dimethylated in a significant fraction (likely, in the majority) of the ribosomes in the preparation. Since the protection of residue A2059 or U2609 in these ribosomes afforded by CEM-101 is essentially complete (Fig. 6A and C), we can confidently conclude that CEM-101 is capable of binding to the ribosomes that are not only monomethylated but also dimethylated at A2058. Such improved binding of ketolides can result from three major factors. One is the interaction of the side chain with the A752-U2609 base pair, which is clearly preserved when the drug binds to the Erm-methylated ribosome, as can be judged by protection of U2609 (Fig. 6C) and A752 (data not shown) by CEM-101 or telithromycin. Second, interactions of 2-fluorine of CEM-101 with C2611 (Fig. 4D) appear to enhance the activity of the drug against the Erm-methylated ribosome (Table 3). The third factor contributing to binding of CEM-101 and telithromycin to the Erm-methylated ribosome can be the lack of C-3 cladinose. Dimethylation of A2058 should result in a steric clash with the desosamine sugar of the drug. The clash can potentially be relieved if the desosamine sugar moves away from the A2058-A2059 cleft. In cladinose-containing macrolides, the desosamine is butttressed by cladinose, whereas in ketolides, desosamine has more freedom to move away to avoid the clash with the dimethylated A2058. Altogether, it appears that specific characteristics of clinically active ketolides, the lack of cladinose, and the presence of an extended side chain may contribute more to the binding of the drugs to the Erm-modified ribosome rather than to the wild-type ribosome.

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