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The Oxazolidinone Eperezolid Binds to the 50S Ribosomal Subunit and Competes with Binding of Chloramphenicol and Lincomycin

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The oxazolidinones are a novel class of antibiotics that act by inhibiting protein synthesis. It as been reported that the drug exerts its primary activity on the initiation phase of translation. In order to study the possibility of direct interaction between the drug and the ribosome, we have developed a binding assay using ^{14}C-labelled eperezolid (PNU-100592; formerly U-100592). Eperezolid binds specifically to the 50S ribosomal subunit of *Escherichia coli*. The specific binding of eperezolid is dose dependent and is proportional to the ribosome concentrations. Scatchard analysis of the binding data reveals that the dissociation constant (K_d) is about 20 μM. The binding of eperezolid to the ribosome is competitively inhibited by chloramphenicol and lincomycin. However, unlike chloramphenicol and lincomycin, eperezolid does not inhibit the puromycin reaction, indicating that the oxazolidinones have no effect on peptidyl transferase. In addition, whereas lincomycin and, to some extent, chloramphenicol inhibit translation termination, eperezolid has no effect. Therefore, we conclude that the oxazolidinones inhibit protein synthesis by binding to the 50S ribosomal subunit at a site close to the site(s) to which chloramphenicol and lincomycin bind but that the oxazolidinones are mechanistically distinct from these two antibiotics.

The oxazolidinones are a novel chemical class of antibiotics that act against a wide spectrum of gram-positive and some gram-negative bacteria (1, 2, 4, 5, 9, 10, 18, 24). The drugs are mainly bacteriostatic and work by inhibiting protein synthesis (9, 10, 13). The oxazolidinones have been reported to block the initiation phase of translation, but no direct evidence clearly demonstrating that mechanism has yet been published (12, 13), nor have any data indicating any direct interaction with any of the components of the protein translation machinery been reported. Most of the antibiotics that inhibit protein synthesis have been demonstrated to have some direct interaction with the ribosomes that blocks the initiation, elongation, or termination phase of procaryotic translation (15, 17). In this study we examined the binding of eperezolid to ribosomes and demonstrated that eperezolid binds specifically to the 50S ribosomal subunit. A number of antibiotics were tested to determine if they compete for the oxazolidinone binding site. Among the antibiotics tested, chloramphenicol, lincomycin, and clindamycin were the only antibiotics that were found to inhibit the binding of the oxazolidinones to the 50S ribosomal subunit. However, the oxazolidinones appear to act by a mechanism that is distinct from the mechanisms of action of chloramphenicol, lincomycin, and clindamycin.

MATERIALS AND METHODS

Antibiotics. Eperezolid, linezolid, and PNU-96499 (Fig. 1) were synthesized at Pharmacia & Upjohn, Kalamazoo, Mich. Lincomycin and clindamycin were obtained from the Pharmacia & Upjohn research compound collection. Chloramphenicol, puromycin, kasugamycin, and streptomycin were obtained from Sigma, St. Louis, Mo.

Preparation of *E. coli* ribosomes. One hundred grams of *Escherichia coli* MRE600 grown in NS87 medium plus 1% yeast extract was washed with buffer LM [10 mM Tris-HCl (pH 7.8), 30 mM NH_4Cl, 1 mM $Mg(CH_3COO)_2$, 1 mM dithiothreitol] and ground with two weights of alumina for 20 min, and the paste was extracted with 100 ml of buffer LM containing 4 μg of DNase. All steps of the ribosome purification were carried out at 4°C. An S30 fraction was prepared by centrifuging the suspension at 20,000 × *g* for 20 min and recentrifuging the supernatant at 30,000 × *g* for 30 min. The resulting supernatant fraction (S30) was adjusted to contain a final NH_4Cl concentration of 1.0 M by slowly adding solid NH_4Cl. The salt-washed ribosomes were pelleted by centrifuging the S30 fraction at 150,000 × *g* for 4 h. The ribosomes thus obtained were suspended in a small volume of buffer LM and were subjected to centrifugation in a 10 to 30% sucrose gradient in buffer LM for 16 h at 18,000 rpm in an SW28 rotor. The pooled fractions containing the 30S and 50S subunits and the 70S tight couples were collected by pelleting at 100,000 × *g* for 24 h and were resuspended in buffer LM. The subunits were further purified by an additional round of sucrose gradient centrifugation. The purities of the 30S, 50S, and 70S subunits were verified by RNA agarose gel electrophoresis. Ribosomal subunit preparations were extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) and twice with chloroform-isoamyl alcohol (24:1) and were ethanol precipitated. rRNA samples were heated for 15 min at 55°C in 1× MOPS (morpholinepropanesulfonic acid) buffer (0.2 M MOPS [pH 7.0], 50 mM sodium acetate, 10 mM EDTA), 6% formaldehyde, and 50% formamide prior to electrophoresis on 1% agarose gels containing 1× MOPS buffer and 1% formaldehyde. The 16S and 23S rRNA bands were visualized at 254 nm after staining the gel in 1 μg of ethidium bromide per ml. The purity of the 30S subunits was assessed by determination of the lack of detectable 23S rRNA contamination, the purity of the 50S subunits was assessed by determination of the lack of detectable 16S rRNA, and the purity of the 70S ribosomes was assessed by determination of the presence of equal amounts of 16S and 23S rRNA.

Binding assay. Radiolabelled compounds, [^{14}C]eperezolid (59.32 μCi/mg, 23.4 mCi/mmol) and [^{14}C]PNU-96499 (142 μCi/mg, 55 mCi/mmol), were synthesized at Pharmacia and Upjohn, Inc. D-*threo*-[Dichloroacetyl-1-^{14}C]chloramphenicol (166 μCi/mg, 54 mCi/mmol) was purchased from Amersham Life Sciences, Inc. The binding studies were performed in microcentrifuge tubes that contained a total of 100 μl of reaction mixture which included 0.3 to 2.0 nmol of ribosomes, 1 to 100 μM radiolabelled compound with either 1 μl of dimethyl sulfoxide or an excess amount (100- to 1,000-fold) of unlabelled compound, 50 mM Tris-HCl (pH 7.5), 5 mM $Mg(CH_3COO)_2$, and 200 mM KCl. All other ingredients were mixed together before the addition of ribosome. The reaction mixture was allowed to incubate at 25°C for 10 min, and the reaction was terminated by the addition of 50 μl of 100% ice-cold ethanol to precipitate the ribosomes and bound drug. After incubation at 4°C for 30 to 60 min, the suspension was centrifuged at full speed in an Eppendorf microcentrifuge for 20 min. The supernatant was then carefully removed, and the radioactivity in the pellet was measured. All datum points represent the mean ± standard error of the mean (SEM) of at least three independent determinations.

For the measurement of specific binding of a compound, the total and nonspecific binding must be measured. Total binding was measured directly by adding a high concentration of radiolabelled ligand. It is assumed that at these high concentrations a very high proportion of the binding is entirely nonspecific. The specific binding sites would be completely saturated at lower concentrations, so the amount of specific binding would be small. Nonspecific binding is deter-

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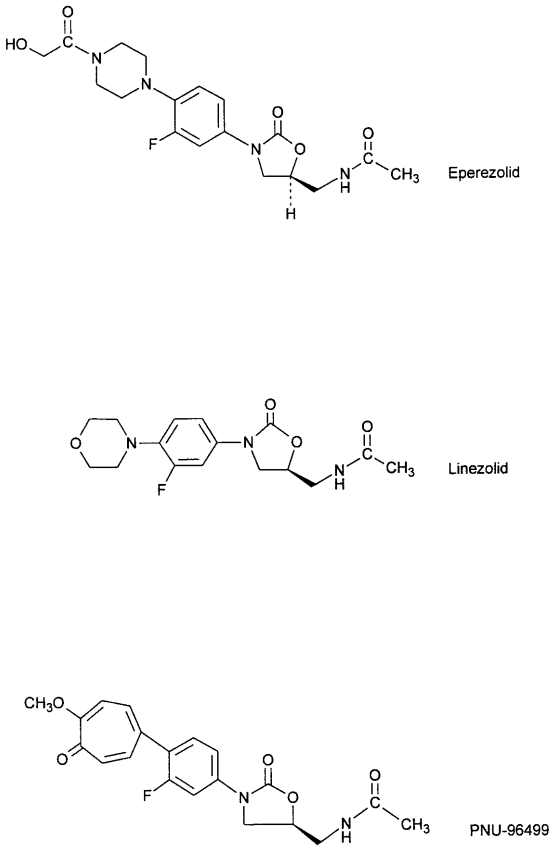

FIG. 1. Chemical structures of eperezolid, linezolid, and PNU-96499.

mined by the addition of unlabelled compound at 1,000 times the concentration of the radiolabelled compound. Virtually all of the high-affinity binding to the specific binding site will be displaced, but the nonspecific binding will not. Nonspecific binding is defined as the amount of radiolabelled compound remaining bound in the presence of an excess amount of unlabelled compound. The specific binding of the compounds is determined by subtracting the nonspecific binding from the total binding. NaCl at 1.5 M displaced the binding of the oxazolidinones, indicating that the binding was not covalent.

Statistical methods. The dissociation constant (K_d) and the maximum binding capacity (B_{max}) were estimated from a Scatchard plot (23) by the following relationship: bound compound/free compound = − bound compound/K_d + B_{max}/K_d. Hence, the bound compound/free compound was regressed against the bound compound by using the SAS statistical package (version 6; SAS Institute, Inc., Cary, N.C.). The statistical significance of the data was determined by using a one-way analysis of variance.

Puromycin reaction. For f[^{3}H]Met-tRNA-AUG-ribosome complex formation, a reaction mixture containing 0.65 nmol of total ribosomes, 0.2 nmol of f[^{3}H]Met-tRNA (32.92 μCi/mg, 9.7 Ci/mmol; New England Nuclear), and 2.5 nmol of AUG in binding buffer (20 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 150 mM NH_4Cl) in a volume of 50 μl was incubated at 30°C for 30 min.

A reaction mixture containing 3 μl of the complex described above, 3 mM puromycin, 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 150 mM NH_4Cl in a volume of 50 μl, in the presence or absence of chloramphenicol, lincomycin, linezolid, or eperezolid, was incubated at 30°C for 30 min. The reaction was terminated by the addition of 250 μl of 100 mM potassium phosphate buffer (pH 6), and then the f[^{3}H]Met-puromycin product was extracted into ethyl acetate. The radioactivity in the ethyl acetate layer was measured by liquid scintillation spectrometry (26).

Termination reaction. A reaction mixture containing 5 μl of the complex described above, 50 mM Tris-HCl (pH 7.4), 30 mM $MgCl_2$, 75 mM NH_4Cl, 80 μM UAA, and 3.5 μg of termination factors in a volume of 50 μl, in the presence or absence of chloramphenicol, lincomycin, eperezolid, or linezolid, was incubated at 22°C for 30 min. Termination factors were isolated as described by Ganoza et al. (16). Concentrated termination factors from the phosphocellulose column step were used in the assay. The reaction was halted by the addition of 250 μl of 0.1 N HCl and extraction of the f[^{3}H]Met product into ethyl acetate. The radioactivity in the ethyl acetate layer was measured by liquid scintillation spectrometry (26). If either the termination triplet codon (UAA) or the termination factors were absent, there was no release of f[^{3}H]Met.

RESULTS

In order to determine if oxazolidinones interact directly with ribosomes, radiolabelled [^{14}C]eperezolid was incubated with total ribosomes prepared from *E. coli* MRE600. Fig. 2A indicates that there is specific binding to total ribosomes, and it is dependent on the ribosome concentration. To ascertain which ribosomal subunit the oxazolidinone specifically binds to, we purified the 30S and 50S subunits from total ribosomes and examined the binding of radiolabelled eperezolid to each of the ribosomal fractions. As indicated in Fig. 2B, the specific binding of [^{14}C]eperezolid is the highest in the 50S ribosome fraction. There is no specific binding to the 30S subunits under the same conditions (Fig. 2C). Specific binding was determined by examining the ability of unlabelled eperezolid to compete off the radiolabelled compound. A dose-dependent binding curve was obtained when 50S ribosome subunits at 2.2 nmol/ml and various concentrations of [^{14}C]eperezolid were used (Fig. 3A). By using the specific binding data from Fig. 3A, the Scatchard analysis revealed that K_d is approximately 20 μM and B_{max} is 600 pmol/ml, indicating a ratio of drug to ribosome of ≤1.0 (Fig. 3B).

Since the oxazolidinones appear to exert their action by directly binding to ribosomes, a variety of antibiotics, including kasugamycin, clindamycin, lincomycin, chloramphenicol, puromycin, and streptomycin, were examined for their abilities to compete for oxazolidinone binding. Ribosomes were incubated with [^{14}C]eperezolid along with a 1,000-fold excess of each of the unlabelled antibiotics. Only chloramphenicol, lincomycin, and clindamycin inhibited the binding of [^{14}C]eperezolid to the same extent as the cold unlabelled eperezolid itself. Other

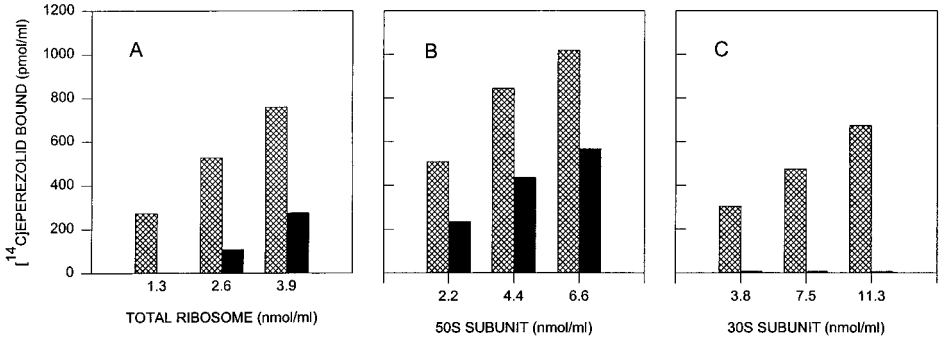

FIG. 2. [^{14}C]eperezolid binding to *E. coli* ribosomes. (A) Total ribosomes; (B) 50S subunits; (C) 30S subunits. ▨, total binding; ■, specific binding.

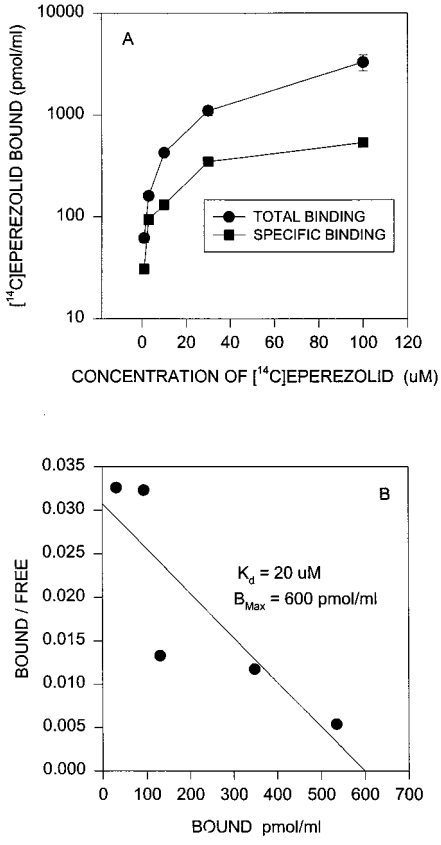

FIG. 3. [^{14}C]eperezolid binding to 50S ribosomes. (A) Dose-dependent binding; (B) Scatchard analysis.

antibiotics, such as kasugamycin, puromycin, and streptomycin, had little effect (Fig. 4).

To see if chloramphenicol inhibits only [^{14}C]eperezolid from binding to ribosomes, another oxazolidinone, [^{14}C]PNU-96499, was tested for its ability to compete with chloramphenicol binding. Figure 5 indicates that eperezolid, PNU-96499, and chloramphenicol were all capable of competing with [^{14}C] PNU-96499 binding to total ribosomes, whereas the initiation inhibitor kasugamycin was not able to compete. This result suggests that chloramphenicol inhibits oxazolidinone binding in general and is not specific to eperezolid.

By using various concentrations of cold chloramphenicol, lincomycin, and another oxazolidinone, linezolid, the binding of [^{14}C]eperezolid to total ribosomes was shown to be competitively and dose-dependently inhibited (Fig. 6A). Linezolid competed equally well with eperezolid, but both chloramphenicol and lincomycin were 1 log more potent than cold eperezolid or linezolid in competing for [^{14}C]eperezolid binding. To further illustrate the relationship between eperezolid, linezolid, and chloramphenicol, the converse experiment was done. In this experiment the binding of radiolabelled [^{14}C] chloramphenicol was allowed to compete with the binding of unlabelled eperezolid, linezolid, and chloramphenicol. Figure 6B indicates that [^{14}C]chloramphenicol binding to total ribosomes was inhibited by increasing concentrations of cold eperezolid or cold linezolid, although both oxazolidinones were about 1 log less potent than chloramphenicol itself. Due to the unavailability of radiolabelled lincomycin or linezolid, the direct binding of these two compounds could not be examined.

Since the oxazolidinones apparently bind to the 50S subunit, the question of what specific function of translation is inhibited

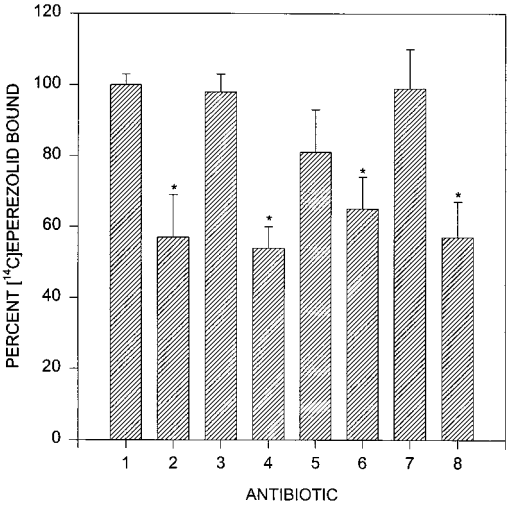

FIG. 4. Effects of other antibiotics on the binding of 10 μM [^{14}C]eperezolid to ribosomes. A 1,000-fold concentration of unlabelled antibiotic was added to compete with [^{14}C]eperezolid. The results are expressed as a percentage of control binding (mean ± SEM). 1, control; 2, unlabelled eperezolid; 3, kasugamycin; 4, chloramphenicol; 5, puromycin; 6, lincomycin; 7, streptomycin; 8, clindamycin. *, statistically significant difference compared to the results for the control ($P \leq 0.05$).

by oxazolidinone binding remains. Chloramphenicol binds to the 50S ribosomal subunit and blocks procaryotic protein translation primarily by inhibiting peptidyl transferase and thus blocking elongation (8). Lincomycin (and clindamycin) inhibits peptidyl transferase but has also been shown to inhibit translation termination (7). Since the oxazolidinones compete with chloramphenicol and lincomycin for a common or overlapping binding site on 50S ribosomes, we examined the activities of the oxazolidinones against peptidyl transferase and translation termination.

During protein synthesis, the peptide bond is formed by a displacement reaction in which the amino group of the new aminoacyl-tRNA displaces the tRNA of the preceding amino acid from the carboxyl group to yield a peptidyl-tRNA of in-

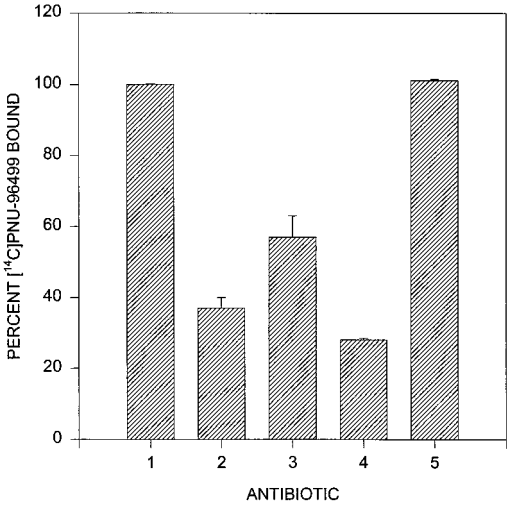

FIG. 5. Effect of other antibiotics on the binding of 3 μM [^{14}C]PNU-96499 to ribosomes. A 200-fold concentration of unlabelled antibiotic was added to compete with [^{14}C]PNU-96499. The results are expressed as a percentage of control binding. 1, control; 2, unlabelled PNU-96499; 3, unlabelled eperezolid; 4, chloramphenicol; 5, kasugamycin.

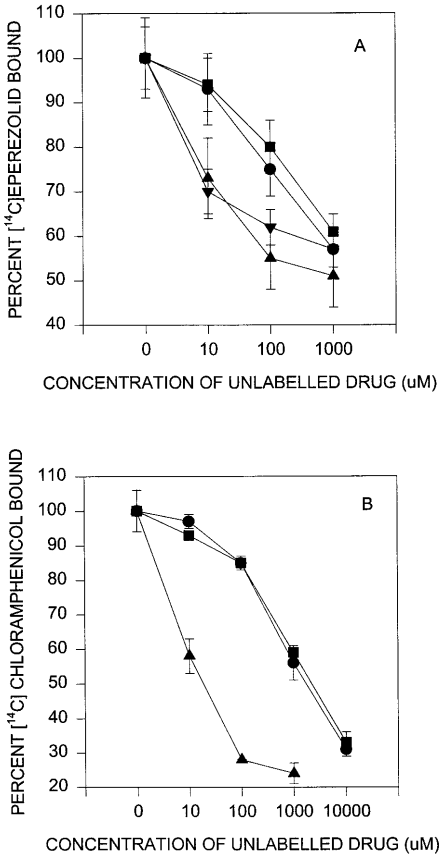

FIG. 6. Binding to ribosomes: competition by various concentrations of unlabelled antibiotics. (A) [^{14}C]eperezolid binding; (B) [^{14}C]chloramphenicol binding. ●, eperezolid; ■, linezolid; ▲, chloramphenicol; ▼, lincomycin.

creasing length. In the presence of puromycin, the growing peptide chains are prematurely released as peptidyl puromycin (20, 25). This puromycin reaction is inhibited by chloramphenicol and lincomycin, both of which inhibit peptidyl transferase (6). As summarized in Figure 7, chloramphenicol and lincomycin substantially inhibited the release of f[^{3}H]Met-puromycin from the ribosome complex, whereas eperezolid and linezolid, at the same concentrations, had no effect. Therefore, it does not appear that the oxazolidinones act by inhibiting peptidyl transferase.

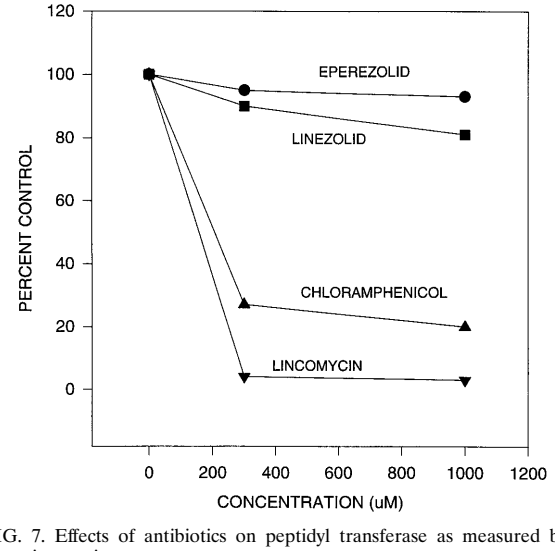

FIG. 7. Effects of antibiotics on peptidyl transferase as measured by the puromycin reaction.

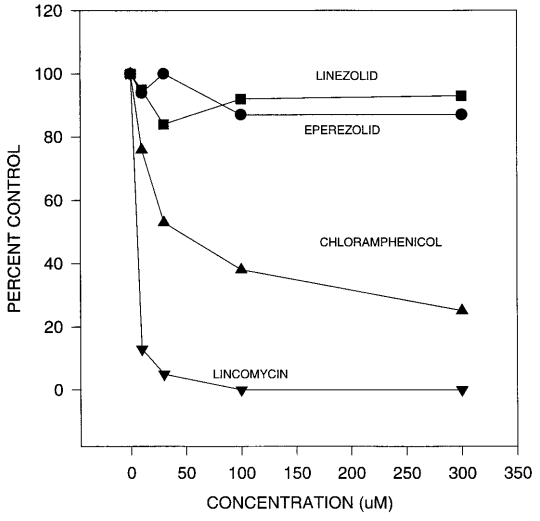

FIG. 8. Effects of antibiotics on translation termination as measured by release factor assay.

Bacterial peptide termination was assessed by using the formylmethionine release assay (26). The release of f[^{3}H]Met from an f[^{3}H]Met-tRNA-ribosome complex is an indication of the in vitro chain termination event. This reaction requires an mRNA termination template such as UAA, UGA, or UAG and the presence of release factors (RF-1, RF-2, and RF-3). The effect of oxazolidinones on the termination reaction was assessed in an in vitro termination assay and was compared to the activities of chloramphenicol and lincomycin. Figure 8 indicates that eperezolid and linezolid have no effect on the termination reaction, while lincomycin and, to a lesser extent, chloramphenicol both significantly inhibit this reaction.

DISCUSSION

It has been suggested that the oxazolidinones inhibit protein synthesis by exerting their primary action at a step preceding the interaction of fMet-tRNA and 30S ribosomal subunits with the initiator codon (12). Our results indicate that under the experimental conditions used in our assay system, eperezolid does not bind to the 30S ribosomal subunit. The drug does, however, bind to the 50S ribosomal subunit, with a K_d of 20 μM. The binding of the oxazolidinones to the ribosomes does not preclude the possibility that the drug may act on more than one aspect of protein translation. The relative weak binding property associated with this drug class is consistent with these compounds being bacteriostatic rather than bacteriocidal, although antibiotics with higher binding affinities do not necessarily have increased bacteriocidal activity.

The binding of oxazolidinones to 50S ribosomes is inhibited by chloramphenicol and lincomycin. In the binding assay, chloramphenicol and lincomycin have affinities approximately 1 log greater than that of eperezolid or linezolid (Fig. 6). These results correlate well with the previous finding that in the presence of 33% ethanol, chloramphenicol has a K_d of 2 μM (14). It had previously been demonstrated that lincomycin and chloramphenicol bind to a similar site on the 50S ribosome (11, 14), but it was somewhat unexpected that they would compete

with the binding of the oxazolidinones eperezolid and linezolid. The shared binding indicated that they might also share a common mechanism. Therefore, we examined the effect of the oxazolidinones on peptidyl transferase and translation termination, both of which can be inhibited by chloramphenicol and lincomycin.

Chloramphenicol acts on the 50S subunit to inhibit the peptidyl transferase reaction, as does lincomycin (8, 14). Studies of affinity-labeled chloramphenicol revealed that the drug preferentially binds to protein L16 of the 50S subunit, thereby preventing the attachment of the amino acid-containing end of the amino acyl-tRNA complex to the ribosome, hence inhibiting the formation of the peptide bond (21). Footprinting studies with 23S rRNA have indicated that chloramphenicol interacts moderately with nucleotides A-2058, A-2059, and A-2062 and interacts strongly with the A-2451 and G-2505 sites in the peptidyl transferase region (19). Chloramphenicol also binds to protein S3 of the 30S ribosomal subunit, which is part of the ribosomal interface between the 30S subunit and the 50S subunit in the 70S ribosomes and is located in the area of the peptidyl transferase center (22). Like chloramphenicol, lincomycin also binds to the P site of 23S rRNA and interacts with some of the same nucleotides (A-2508, A-2451, and G-2505) (3, 11).

Puromycin is an aminoacyl-tRNA analog that has been used to release nascent peptides from the ribosome. This puromycin reaction is inhibited by chloramphenicol due to its binding to the enzyme peptidyl transferase (6). Figure 7 indicates that eperezolid or linezolid at concentrations of up to 1 mM had no effect on the puromycin reaction compared to the effect of chloramphenicol or lincomycin at the same concentrations. These results indicate that although the oxazolidinones share a binding site(s) with chloramphenicol and lincomycin, they do not appear to act by inhibiting peptidyl transferase.

Translation termination is mediated by release factors and one of the termination codons. The effects of the oxazolidinones on this reaction were examined. Both lincomycin and, to a lesser degree, chloramphenicol have previously been shown to inhibit the translation termination reaction (7). Our results confirm these previous findings but indicate that eperezolid and linezolid have no effect on the termination of procaryotic translation. Thus, although it is clear that lincomycin and chloramphenicol may share a binding site with eperezolid, the oxazolidinones act to inhibit protein translation by a mechanism distinct from that for either compound.

The results of this study demonstrate rather convincingly that the oxazolidinones have no effect on peptidyl transferase or translation termination. If, in fact, the oxazolidinones act to block translation initiation, as has been suggested, it is tempting to postulate that the oxazolidinones bind to a site on the 50S subunit closely related to the chloramphenicol and lincomycin binding site and near the interface with the 30S subunit. The resulting distorted site may prevent the correct positioning of the 30S initiation complex from forming the 70S initiation complex and hence inhibit translation initiation.

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