

Mechanism of Action of Oxazolidinones: Effects of Linezolid and Eperezolid on Translation Reactions

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The oxazolidinones are a new class of synthetic antibiotics with good activity against gram-positive pathogenic bacteria. Experiments with a susceptible *Escherichia coli* strain, UC6782, demonstrated that in vivo protein synthesis was inhibited by both eperezolid (formerly U-100592) and linezolid (formerly U-100766). Both linezolid and eperezolid were potent inhibitors of cell-free transcription-translation in *E. coli*, exhibiting 50% inhibitory concentrations (IC₅₀s) of 1.8 and 2.5 μM, respectively. The ability to demonstrate inhibition of in vitro translation directed by phage MS2 RNA was greatly dependent upon the amount of RNA added to the assay. For eperezolid, 128 μg of RNA per ml produced an IC₅₀ of 50 μM whereas a concentration of 32 μg/ml yielded an IC₅₀ of 20 μM. Investigating lower RNA template concentrations in linezolid inhibition experiments revealed that 32 and 8 μg of MS2 phage RNA per ml produced IC₅₀s of 24 and 15 μM, respectively. This phenomenon was shared by the translation initiation inhibitor kasugamycin but not by streptomycin. Neither oxazolidinone inhibited the formation of *N*-formylmethionyl-tRNA, elongation, or termination reactions of bacterial translation. The oxazolidinones appear to inhibit bacterial translation at the initiation phase of protein synthesis.

The oxazolidinones are a new class of antimicrobial agents which have a unique structure and good activity against gram-positive pathogenic bacteria (Fig. 1). Early reports by researchers at E. I. du Pont de Nemours & Co., Inc., characterized the oxazolidinone S-6123 as an orally active compound with relatively weak in vitro activity (2). Further studies led to the development of the more active DuP-105 and DuP-721 compounds, which did not show cross-resistance to other clinically useful antibiotics (4). However, these compounds did not enter advanced clinical testing.

An oxazolidinone program initiated in our laboratories has yielded two compounds (1), eperezolid and linezolid, which both are active in vitro (10, 11, 13, 21) and in vivo (6) against methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant *Enterococcus faecium*. Phase I clinical trials have been completed with both antibiotics, and linezolid is currently undergoing phase II testing.

Mechanism of action studies performed with DuP-721 revealed that protein synthesis was inhibited by this compound in vivo, while RNA and DNA synthesis was not affected (4). Further studies with this oxazolidinone failed to demonstrate sensitivity of in vitro translation reactions with an *Escherichia coli* S30 extract (5). However, it was concluded that DuP-721 did not inhibit polysome-directed elongation and that the mechanism of action of oxazolidinones probably involves inhibition of an event preceding translation initiation. The present studies were conducted in order to (i) determine the mechanism of action of eperezolid and linezolid, and (ii) establish conditions to measure the effects of oxazolidinones on in vitro translation reactions.

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MATERIALS AND METHODS

Chemicals and buffers. Eperezolid (formerly U-100592), linezolid (formerly U-100766), and DuP-721 were prepared in our laboratories. Streptomycin, chloramphenicol, kasugamycin, pyruvate kinase, phenylalanyl-tRNA, and *N*-formylmethionyl-tRNA were purchased from Sigma Chemical Co. (St. Louis, Mo.). MS2 phage RNA was purchased from Boehringer Mannheim (Indianapolis, Ind.). [¹⁴C]leucine (315 mCi/mmol), [2,3,4,5,6-³H]phenylalanine (131 Ci/mmol), [³H]methionine (85 Ci/mmol), and [³⁵S]methionine (>1,000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.). *N*-[³H]formylmethionyl-tRNA was purchased from New England Nuclear/DuPont (Boston, Mass.).

Bacterial strains and phage. The membrane-permeable *E. coli* strain UC6782 and *E. coli* E1230 (*lacZ::kan*) were both obtained from the Pharmacia & Upjohn Culture Collection. *E. coli* MRE600 was obtained from the American Type Culture Collection (Rockville, Md.) as ATCC 29417. All cultures were grown in double-strength Lennox L Broth (Gibco BRL, Gaithersburg, Md.) containing 1% glucose at 37°C and aerated at 225 rpm on an orbital shaker.

MIC determination. MICs for *E. coli* UC6782 were determined by the broth microdilution method (15) using Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.).

Preparation of S30 extract from *E. coli*. *E. coli* UC6782 was grown overnight in 100 ml of double-strength Lennox L Broth. This culture was used to inoculate six 2-liter flasks containing 1 liter each of double-strength Lennox L Broth (2% inoculum). After 7 h of growth, the cultures were harvested, washed once with phosphate-buffered saline, and frozen at -80°C. The S30 extract was prepared according to the method of Pratt (16) and frozen at -80°C.

Preparation of ribosomes. Ribosomes were prepared by the low-salt wash method of Rheinberger et al. (17). Briefly, 25 g (wet weight) of *E. coli* MRE600 cells was resuspended in 45 ml of buffer A (10 mM Tris-HCl [pH 7.5], 6 mM MgCl₂, 30 mM NH₄Cl, 4 mM β-mercaptoethanol) and passed twice through a French pressure cell at 8,000 lb/in². After centrifugation for 45 min at 27,000 × *g* in an SS34 rotor, the supernatant was centrifuged at 100,000 × *g* for 18 h. The supernatant was made 10% with respect to glycerol and quick-frozen in dry ice-ethanol as a source of aminoacylating enzymes. The total ribosome pellet was gently rinsed with buffer A and finally resuspended in 1 to 2 ml of buffer B (10 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 30 mM NH₄Cl, 4 mM β-mercaptoethanol) by gentle shaking on ice for 2 h. The ribosomal suspension was clarified by centrifuging at 6,000 × *g* for 5 min, and the supernatant was frozen in a dry ice-acetone bath before being stored at -80°C (500 A₂₆₀ U/ml).

Transcription-translation assays. In vitro transcription-translation using the plasmid pGEMβGAL as a DNA template and *E. coli* UC6782 S30 extract was

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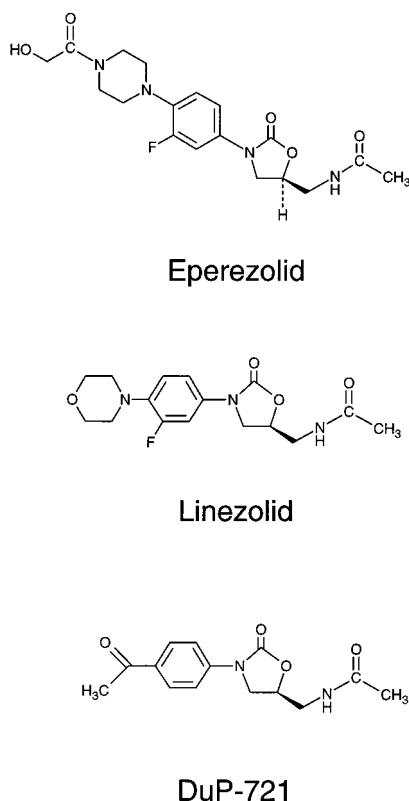


FIG. 1. Chemical structures of eperezolid, linezolid, and DuP-721.

performed as described by Promega with reagents from the *E. coli* S30 system for circular DNA. Standard assays were performed in 96-well plates with 5 μ l of S30 extract, 4 μ l of cesium chloride-purified pGEM β GAL DNA (0.7 mg/ml), 8 μ l of Promega premix, 2 μ l of amino acids mix, and 1 μ l of water, dimethylsulfoxide, or antibiotic dissolved in dimethylsulfoxide. The DNA was added to start the reaction, and the covered plates were incubated at 37°C for 30 min with shaking at 100 rpm. The rate of β -galactosidase production was measured according to the method of Miller (14) by adding 150 μ l of *o*-nitrophenyl- β -D-galactopyranoside (4 mg/ml) and reading the absorbance at 420 nm in a SpectraMax 250 microplate spectrophotometer (Molecular Devices, Inc., Sunnyvale, Calif.).

Preparation of S30 extracts from *S. aureus* and in vitro coupled transcription-translation assays were performed according to the method of Mahmood et al. (12) with the following modifications. Reaction mixtures in a final volume of 25 μ l containing 2 μ g of the *S. aureus* plasmid pSK265 were incubated at 37°C for 60 min. Incorporation of [³⁵S]methionine was measured by trichloroacetic acid precipitation of labelled protein (12).

Translation assays. The S30 transcription-translation system purchased from Promega was used according to the manufacturer's instructions to incorporate [³⁵S]methionine into protein translated from MS2 phage RNA. Samples were assayed by removing 5 μ l from each reaction mixture and adding it to 245 μ l of 1 N NaOH and incubating the mixtures at 37°C for 10 min before adding 1 ml of ice-cold 25% trichloroacetic acid containing 5% Casamino Acids. After 30 min on ice, the samples were filtered through 25-mm-diameter GF/C filters, washed three times with 5 ml of 5% cold trichloroacetic acid, and placed into vials for liquid scintillation counting.

Whole-cell protein synthesis. A cell suspension containing 89 μ l of exponentially growing *E. coli* UC6782 cells ($A_{540} = 0.2$) was mixed with 1 μ l of antibiotic solution and incubated at 37°C for 10 min. Proteins were labelled by adding 10 μ l of [U-¹⁴C]leucine (50 μ Ci/ml) and allowing the mixture to incubate for 60 min at 37°C. Samples were processed by the alkaline hydrolysis method described for the translation assay above.

Translation elongation assay. Polysomes were isolated from exponentially growing MRE600 cells and assayed for elongation by the method of Girbes et al. (8). After 5 to 60 min of incubation, [³⁵S]methionine incorporation was determined with NaOH and trichloroacetic acid as described above for the translation assay.

Poly(U)-directed translation. The low-salt wash ribosomes and S100 preparation described above were used to study the effect of antibiotics on polyphenylalanine synthesis by the basic procedure of Grise-Miron et al. (9). The reaction mixture contained in a total volume of 100 μ l 33 μ l of 3 \times translation buffer (180

mM NH₄Cl, 48 mM magnesium acetate, 189 mM Tris-HCl [pH 7.8], 48 mM β -mercaptoethanol, 3.9 mM ATP, 0.9 mM GTP, 51 mM phosphoenolpyruvate), 17 μ l of water, 1 μ l of 0.1 mM phenylalanine, 1 μ l of pyruvate kinase (5 mg/ml), 1 μ l of [2,3,4,5,6-³H]phenylalanine, 10 μ l of phenylalanyl-tRNA (4 mg/ml in 5 mM potassium acetate buffer [pH 6.0]), 4 μ l of poly(U) RNA (4 mg/ml in 5 mM potassium acetate buffer [pH 6.0]), 5 μ l of unwashed ribosomes (1 *A*₂₆₀ U/ μ l), and 5 μ l of 100,000 \times g supernatant (5 mg/ml). Polyphenylalanine polypeptide synthesis was determined as in the translation assay.

Translation termination assay. The translation termination assay was performed as described by Tate and Caskey (20). Briefly, an *N*-[³H]formylmethionyl-tRNA-AUG-ribosome complex was added to a 50- μ l reaction mixture containing 50 mM Tris-HCl (pH 7.5), 75 mM NH₄Cl, 30 mM MgCl₂, the termination codon UAA, and release factors. The reaction mixture was incubated for 30 min at 20°C and stopped by adding 250 μ l of 0.1 M HCl and 1.0 ml of ethyl acetate. The reaction was vortexed and centrifuged, and the ethyl acetate phase was assayed for *N*-[³H]formylmethionyl-tRNA release. Release factors were purified as described by Ganoza et al. (7). The termination inhibitor hygromycin was included in some reaction mixtures as a positive control (19).

In vitro N-formylmethionyl-tRNA synthesis. The effect of antibiotics on events preceding the initiation of translation was studied by measuring the formation of *N*-formylmethionyl-tRNA in *E. coli* S30 extracts. Reactions performed in the presence and absence of 100 μ M eperezolid contained 40 μ l of Promega premix without amino acids, 30 μ l of S30 extract, 2 μ l of [³H]methionine (85 Ci/mmol), and water to 100 μ l. After incubation of the reaction mixture at 37°C for 60 min, an equal volume of 0.6 M sodium acetate buffer (pH 6.0) was added and two extractions with phenol and one with chloroform were performed. The RNA extract was dried under reduced pressure and resuspended in TE buffer (10 mM Tris [pH 7.0], 1 mM EDTA). Portions of the RNA extract, representing approximately 16,000 cpm, were added to 100 μ l of 0.1 N NH₄OH and incubated at 37°C for 2 h. The hydrolysate was dried under reduced pressure and resuspended in TE buffer, and the entire sample was applied to a cellulose thin-layer chromatography plate and developed in butanol-acetic acid-water (60:15:25, vol/vol/vol). The plates were cut into 1-cm² squares and added to vials for liquid scintillation counting. Standards applied to the plates included [³H]methionine and *N*-[³H]formylmethionine prepared through alkaline hydrolysis of *N*-[³H]formylmethionyl-tRNA.

RESULTS

Unlike most gram-negative bacteria, the mutant *E. coli* strain UC6782 was sensitive to the oxazolidinones (MIC of eperezolid = 4 μ g/ml) and was therefore used in this study to investigate the antibiotic mechanism of action. Figure 2 shows the effects of the oxazolidinones DuP-721, eperezolid, and linezolid on in vivo protein synthesis in this strain. When added

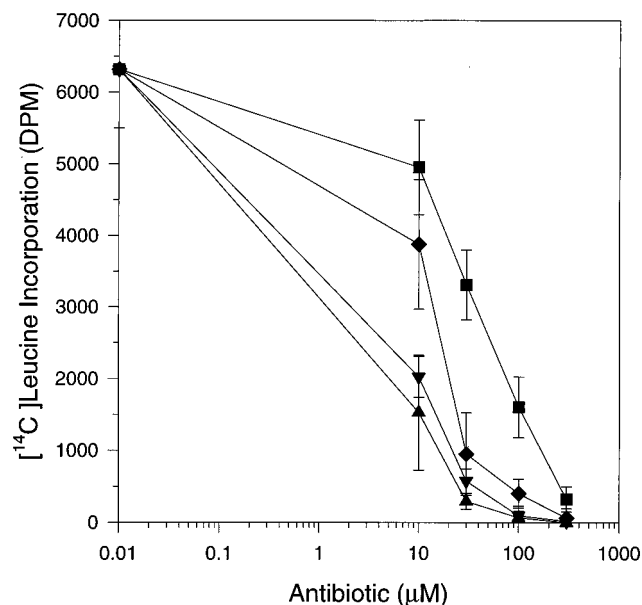


FIG. 2. Effects of oxazolidinones on protein synthesis in *E. coli* UC6782. Exponentially growing cells were labelled with [U-¹⁴C]leucine for 60 min at 37°C in the presence of various concentrations of either DuP-721 (■), eperezolid (▲), linezolid (▼), or streptomycin (◆). Error bars, standard errors of the means.

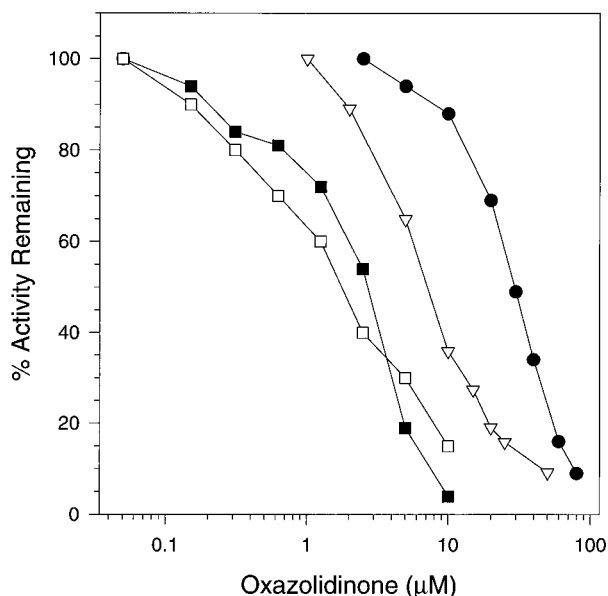


FIG. 3. Effects of oxazolidinones on coupled transcription-translation in S30 extracts from *E. coli* UC6782 and *S. aureus*. *E. coli* reaction mixtures containing either linezolid (□), eperezolid (■), or DuP-721 (●) were incubated for 30 min at 37°C, and the amount of β -galactosidase formed was measured, with *o*-nitrophenyl- β -D-galactopyranoside used as the substrate. Translation of *S. aureus* polypeptides in the presence of eperezolid (▽) was determined after 60 min at 37°C by [³⁵S]methionine incorporation and subsequent precipitation of protein with cold trichloroacetic acid.

to the cultures at a concentration of 10 μ M, both eperezolid and linezolid were at least 2.5 times more potent than DuP-721 and approximately twice as potent as streptomycin. Approximately 90% inhibition of in vivo protein synthesis occurred with either eperezolid or linezolid (30 μ M), whereas inhibition with DuP-721 at the same concentration was only 54%.

Figure 3 demonstrates oxazolidinone inhibition of coupled transcription-translation. Addition of eperezolid to S30 extracts of *E. coli* resulted in a 50% inhibitory concentration (IC_{50}) of 2.5 μ M, whereas linezolid was slightly more potent, with an IC_{50} of 1.8 μ M. Likewise, an S30 transcription-translation system from *S. aureus* was sensitive to eperezolid, exhibiting an IC_{50} of approximately 8 μ M. DuP-721 was 10-fold less active than either eperezolid or linezolid in this assay. In the *E. coli* system, translation was uncoupled from transcription by substituting MS2 phage RNA for the pGEM β GAL DNA template. Figure 4 shows that translation was inhibited by both eperezolid and DuP-721. However, 250 μ M DuP-721 was required for 20% inhibition, whereas 50% inhibition was achieved with only 20 μ M eperezolid.

Figure 5 shows that the ability of oxazolidinones to inhibit translation (uncoupled from transcription) was directly related to the amount of MS2 RNA added to the assay. With 32 μ g of MS2 RNA per ml, 25 μ M eperezolid inhibited translation by 58%, while 192 μ g of MS2 RNA per ml inhibited the reaction only 14% at the same drug concentration. The effect of RNA template concentration on oxazolidinone potency was further investigated with linezolid. Lowering the RNA to 8 μ g/ml produced a IC_{50} of linezolid of 15 μ M, compared to 47 μ M for RNA at 32 μ g/ml. This phenomenon was also demonstrated for kasugamycin (Fig. 6), an aminoglycoside inhibitor of translation initiation. However, the potency of streptomycin was not greatly affected by the MS2 concentration (data not shown).

Antibiotics which inhibit translation can exert their effect

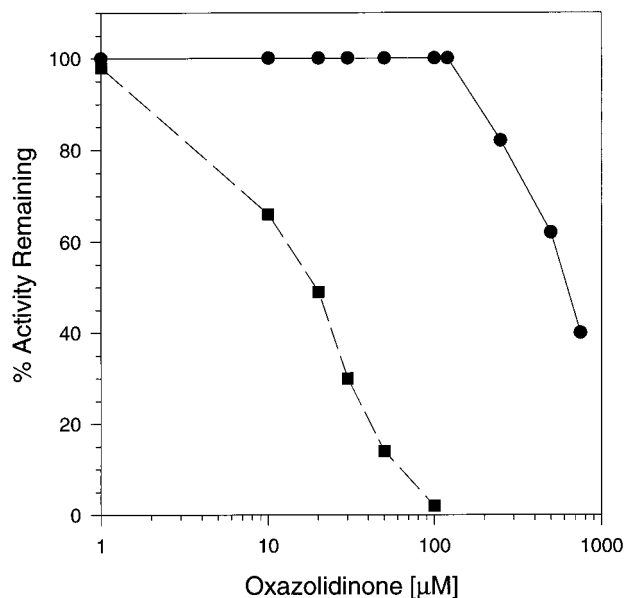


FIG. 4. Effects of eperezolid (■) and DuP-721 (●) on translation in *E. coli* UC6782 S30 extract. Translation was initiated by adding MS2 phage RNA (112 μ g/ml) to an S30 extract in the presence or absence of oxazolidinone.

upon one or more of the three basic phases, initiation, elongation, and termination. In order to begin exploring the mechanism of action of the oxazolidinones, elongation of translation was examined by using polysomes isolated from exponential-phase *E. coli* MRE600 cells. Table 1 shows that 100 μ M linezolid or eperezolid had little effect on elongation, whereas chloramphenicol was very potent. Likewise, the two oxazolidinones had little effect on termination of protein synthesis, with

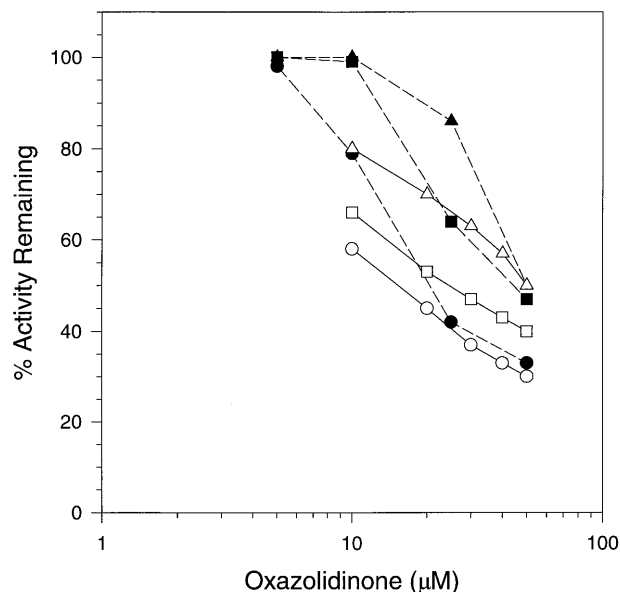


FIG. 5. Effect of the concentration of RNA used in the translation reaction mixture (*E. coli* S30 extract) on the activity of eperezolid or linezolid. Translation reaction mixtures containing MS2 phage RNA at either 32 (●), 128 (■), or 192 (▲) μ g/ml were incubated for 30 min at 37°C in the presence of various concentrations of eperezolid. In other reactions, linezolid inhibition was tested with RNA template at 8 (○), 16 (□), or 32 (△) μ g/ml.

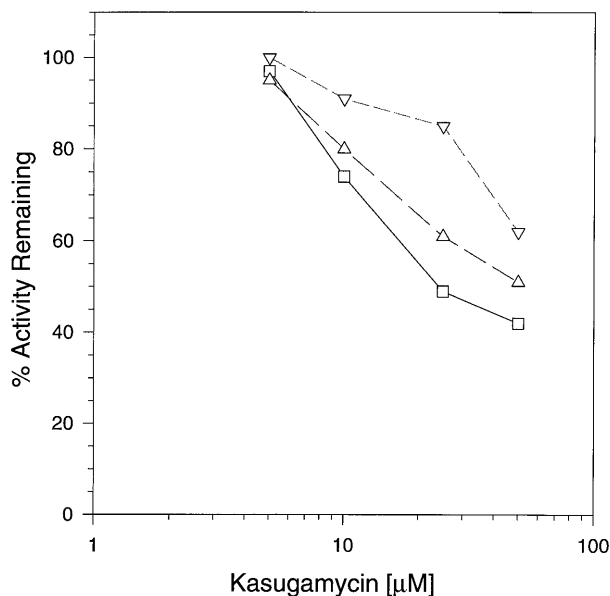


FIG. 6. Effect of the concentration of RNA used in the translation reaction mixture on the activity of kasugamycin. Translation reaction mixtures (*E. coli* S30 extract) containing MS2 phage RNA at either 32 (□), 128 (△), or 192 (▽) μg/ml were treated as described in the legend to Fig. 5.

linezolid and eperezolid at concentrations of 100 μM inhibiting only 8 and 14% of synthesis, respectively (Fig. 7).

The poly(U)-directed translation system does not require the traditional *N*-formylmethionine-tRNA that prokaryote translation initiation utilizes. Therefore, this assay is often used to determine whether a drug inhibits translation events not directly related to initiation. Table 1 shows that the two oxazolidinones were poor inhibitors of poly(U)-directed translation, whereas kasugamycin at 100 μM inhibited 66% of translation.

Initiation of translation requires the synthesis of *N*-formylmethionyl-tRNA. The effect of 100 μM eperezolid or linezolid on the *in vitro* synthesis of this initiator tRNA was measured in an *E. coli* S30 extract. Table 2 shows that neither oxazolidinone inhibited the synthesis of methionyl-tRNA or *N*-formylmethionyl-tRNA.

DISCUSSION

An earlier study by Eustice et al. (4) demonstrated that the IC₅₀ of DuP-721 for inhibition of whole-cell protein synthesis in *Bacillus subtilis* was 0.25 μg/ml (0.90 μM). The present study demonstrates an IC₅₀ of approximately 30 μM with an oxazolidinone-sensitive *E. coli* strain, a value 33-fold greater than that obtained with *B. subtilis*. The higher value obtained with this *E. coli* strain is presumably due to the lower permeability

TABLE 1. Antibiotic inhibition of *E. coli* polysome elongation and poly(U) translation

Antibiotic	Concn (μM)	% Inhibition	
		Polysome elongation	Poly(U) translation
Eperezolid	100	12	8
Linezolid	100	3	12
Kasugamycin	100	0	66
Chloramphenicol	1	90	27

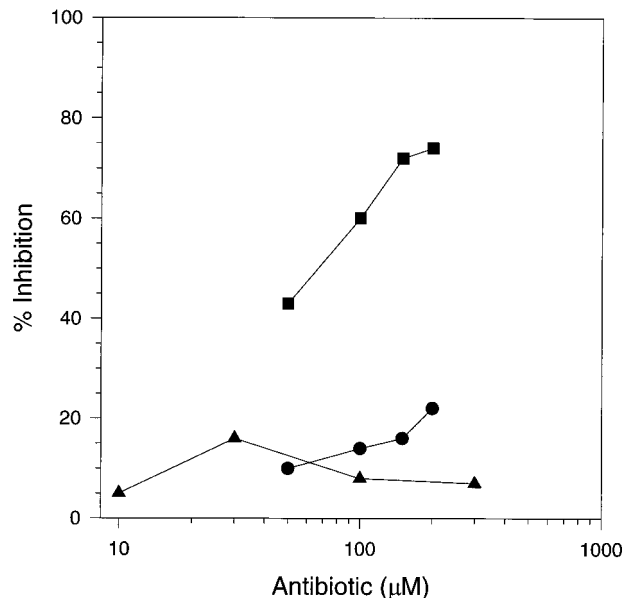


FIG. 7. Effects of linezolid (▲), eperezolid (●), and hygromycin (■) on the translation termination reaction.

of gram-negative bacteria to oxazolidinones, as reflected in the high MICs reported in the literature for DuP-721 (5). Therefore, despite the sensitivity of *E. coli* UC6782 to the oxazolidinones used in this study, the MIC of eperezolid (4 μg/ml) predicts a higher IC₅₀ for *E. coli* inhibition of whole-cell protein synthesis.

One of the goals of this study was to investigate why earlier efforts failed to demonstrate oxazolidinone inhibition of cell-free translation (4, 5), despite the potent inhibition of whole-cell protein synthesis by this unique class of antibiotics (18). In the present study, coupled transcription-translation from either *E. coli* or *S. aureus* proved to be very sensitive to linezolid and eperezolid. However, DuP-721 was 10-fold less active under these conditions, which closely mimic *in vivo* translation. The low potency of DuP-721 in the transcription-translation system is contrasted by the favorable MICs of this compound against gram-positive pathogens (18).

Initial studies in our laboratory, patterned after reports describing the insensitivity of translation to DuP-721, showed little or no effect by either DuP-721, linezolid, or eperezolid (data not shown). However, decreasing the MS2 RNA concentration in the assay from the reported 220 μg/ml to 160 μg/ml revealed slight inhibition by each oxazolidinone. Further studies demonstrated a clear effect of MS2 RNA concentration on the potency of either oxazolidinone, indicating that the mechanism of action of this class of drugs must involve the binding of mRNA to the ribosome at the initiation phase of translation.

TABLE 2. Effects of eperezolid and linezolid on *N*-formylmethionyl-tRNA synthesis

tRNA species	Methionine or formylmethionine recovered (dpm)		
	No antibiotic	Eperezolid (100 μM)	Linezolid (100 μM)
Methionyl-tRNA	3,768	3,515	3,475
<i>N</i> -Formylmethionyl-tRNA	26,447	27,100	27,014

This hypothesis was further supported by the demonstration that the potency of kasugamycin (an aminoglycoside inhibitor of translation initiation) also decreased as the amount of MS2 RNA in the assay was increased. Specificity for this mRNA effect on initiation was clarified when it was shown that the potency of the peptidyl-tRNA translocation inhibitor streptomycin was not greatly affected by increased MS2 RNA levels.

Many antibiotics inhibit bacterial translation by preventing elongation of the polypeptide chain. Chloramphenicol inhibits polysome-directed elongation by binding to a site on the ribosome near the peptidyl transferase activity region. We tested *E. coli* polysomes for elongation inhibition by either linezolid, eperzolid, or kasugamycin but did not detect an effect, whereas chloramphenicol was a potent inhibitor. The ability of the ribosome to terminate translation was also tested, but the oxazolidinones had little effect. In an effort to determine whether either oxazolidinone prevented the binding of a non-initiating message, ribosomes were programmed with poly(U) mRNA and forced to synthesize polyphenylalanine with [³H] phenylalanyl-tRNA as the only tRNA. Neither linezolid nor eperzolid inhibited this reaction, while kasugamycin and chloramphenicol were inhibitory at high concentrations. As reported previously (3), S30 extracts can provide curious results, such as the stimulation of polyphenylalanine synthesis by streptomycin. Therefore, the data obtained with the poly(U) reaction can only serve to differentiate the mechanism of action of oxazolidinones from those of other antibiotic classes.

An earlier report by Eustice et al. (5) suggested that the mechanism of action of Dup-721 involved a step preceding the interaction of fMet-tRNA_{fmet} and 30S ribosomal subunits with the initiator codon. One of the possible preceding events is the synthesis of the initiator tRNA through the aminoacylation and subsequent formylation reactions. In this study, we determined that high concentrations (100 μM) of linezolid and eperzolid did not inhibit the synthesis of *N*-formylmethionyl-tRNA in S30 extracts of *E. coli*, demonstrating that the mechanism of action of oxazolidinones is not exerted at this step.

The results of this study demonstrate that the oxazolidinones do not inhibit the formation of initiator tRNA and that they do not block the elongation or termination steps of prokaryotic translation. Members of the oxazolidinone class of antibiotics, like the initiation inhibitor kasugamycin, appear to interact with a translation component which is either directly or indirectly involved in the binding of mRNA during the initiation phase of translation. Future studies should include testing for the inhibition of initiation complex formation and developing an assay which measures the binding of oxazolidinones to the ribosome.

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