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On the target of a novel class of antibiotics, oxazolidinones, active against multidrug-resistant Gram-positive bacteria

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Abstract Oxazolidinones are a promising new class of synthetic antibiotics active against multidrug-resistant Gram-positive bacteria. To elucidate their mode of action, the effect of DuP 721 on individual steps of protein translation was studied. The drug does not interfere with translation initiation at the stage of mRNA binding or formation of 30S pre-initiation complexes. However, it inhibits the puromycin-mediated release of [^{35}S]formyl-methionine from 70S initiation complexes in a dose-dependent manner. Inhibition involves binding of the oxazolidinone to the large ribosomal subunit and is twice as high with 50S subunits from Gram-positive as with those from Gram-negative bacteria.

Key words: Antibiotic; Gram-positive bacterium; Oxazolidinone; Ribosomal subunit; Translation initiation

1. Introduction

Oxazolidinones, which were first described in 1987 [1], represent a new class of synthetic, antimicrobial agents. These drugs have attracted considerable interest as they exhibit activity against multidrug-resistant Gram-positive bacteria, which have created tremendous therapeutic problems in recent years (for a recent review see [2]). DuP 721, first synthesized by E.I. DuPont de Nemours and Co., has been analyzed in some detail as to its mode of action [3,4]. It was shown to inhibit protein synthesis in whole cells not only of Gram-positive (*Bacillus subtilis*) but also Gram-negative (*Escherichia coli*) bacteria, provided the outer membrane of the latter had been rendered permeable to DuP 721. No inhibitory activity of DuP 721 on translation elongation was found in assays derived from *E. coli* and primed with either synthetic or natural mRNAs. Preliminary evidence suggested early translation initiation steps as potential targets for DuP 721.

In order to learn more details about the molecular mechanism by which oxazolidinones inhibit bacterial protein synthesis we employed cell-free systems prepared from *E. coli* and the Gram-positive bacterium *Staphylococcus carnosus*. We show that DuP 721 exhibits a preference for Gram-positive 50S ribosomal subunits and thereby presumably interferes with a late step of translation initiation.

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2. Materials and methods

2.1. Materials

The oxazolidinone DuP 721 [1] was synthesized and kindly provided by Bayer AG, Wuppertal (Germany). Stock solutions of DuP 721 were prepared in dimethyl sulfoxide and diluted 30-fold into the reactions. Controls received an equal amount of dimethyl sulfoxide. Full-length mRNA templates were obtained by in vitro transcription of SP6 and T7 promoter-dependent bacterial genes following standard protocols. Otherwise, the following oligoribonucleotides derived from the mRNA of *S. hyicus* preprolipase [5] were used as templates: 5′-UUU *GAA GGG* ACU GGU UUA AUG AAA GAA-3′ (wild-type, the Shine-Dalgarno sequence is shown in italics) and 5′-UUU ACU UUU ACU GGU UUA AUG AAA GAA-3′ (without the Shine-Dalgarno sequence). Plasmids pCS1 (preprolipase gene of *S. hyicus*, T7 promoter [6]); pDMB (*pOmpA* gene of *E. coli*, T7 promoter [7]); pLB8000 (*lamB* gene of *E. coli*, SP6 promoter [8]) have been described elsewhere.

2.2. Cell-free protein synthesis

Previously described methods were used for in vitro transcription/translation of T7 promoter-dependent genes in extracts of *S. carnosus* [6] and *E. coli* [7].

2.3. Subfractionation procedures

An S-30 of *S. carnosus* was centrifuged for 2.5 h at $150\,000\times g_{av}$ to obtain an S-150 and a ribosomal pellet. To prepare a ribosomal salt extract, the pelleted ribosomes were resuspended in 2/3 of the original volume of 50 mM triethanolamine/CH_3COO pH 7.5, 30 mM $Mg(CH_3COO)_2$, 1 M NH_4Cl, 3.5 mM DTT, layered over 0.5 vol of 20% glycerol prepared in the same buffer, and spun overnight at $70\,000\times g_{av}$. The resulting supernatant (ribosomal salt extract) was dialyzed against storage buffer (50 mM triethanolamine/CH_3COO pH 7.5, 50 mM KCH_3COO, 5 mM $Mg(CH_3COO)_2$, 1 mM DTT) and concentrated 4-fold by ultrafiltration using Centricon 3 microconcentrators (Amicon). This extract served as source for *S. carnosus* initiation factors. The pelleted ribosomes were salt-extracted one more time and finally resuspended in storage buffer.

For separation of the ribosomal subunits, the twice salt-extracted ribosomes were resuspended in 10 mM triethanolamine/CH_3COO pH 7.5, 100 mM NH_4CH_3COO, 10 mM $Mg(CH_3COO)_2$, 1 mM DTT. The suspension was made 400 mM in NaCl using a 4 M stock solution and 80 A_{260} units of ribosomes were each applied to linear 28–35% (w/w) sucrose gradients prepared in 10 mM triethanolamine/CH_3COO pH 7.5, 10 mM $Mg(CH_3COO)_2$, 400 mM NaCl in quick seal VTi50 (Beckman) tubes, and centrifuged for 17 h at $30\,000\times g_{av}$ at 4°C. Gradients were fractionated and appropriate fractions were pooled, concentrated and exchanged against 10 mM triethanolamine/CH_3COO pH 7.5, 60 mM KCH_3COO, 10 mM $Mg(CH_3COO)_2$, 3 mM DTT by ultrafiltration.

Salt-extracted ribosomes of *E. coli* were prepared from S-30 extracts in a similar manner. Purified initiation factors 1–3 were obtained as described [9].

2.4. Initiation assays

The formation of initiation complexes was performed essentially as described [10]. Each reaction mixture of 30 μl contained 50 mM triethanolamine/CH_3COO pH 7.5, 200 mM KCH_3COO, 5 mM $Mg(CH_3COO)_2$, 1 mM DTT, 0.4 mM GTP, optimized concentrations of in vitro transcribed, full-length mRNAs or oligoribonucleotides, initiation factors, ribosomes, and $\geq 10^6$ dpm of [^{35}S]formyl-methionyl-tRNA (fMet-tRNA). Reactions were allowed to proceed for 10 min

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at 30°C, stopped by the addition of 1 ml of ice cold stop buffer (10 mM triethanolamine/CH_3COO pH 7.5, 10 mM $Mg(CH_3COO)_2$, 100 mM NH_4Cl) and filtered through Millipore HAWP 2400 nitrocellulose filters. Alternatively, incubation was continued after the addition of 1 μl of 11 mM puromycin for 30 min and [^{35}S]fMet-puromycin was extracted with ethyl acetate as described [11]. Filter-bound and ethyl acetate-extracted radioactivity was counted using 2 ml each of Ultima Gold (Packard) scintillation fluid.

When initiation was assayed using ribosomal subunits instead of 70S ribosomes, 30S pre-initiation complexes were allowed to form for 10 min at 30°C under the described conditions except that the concentration of Mg^{2+} was raised to 10 mM. Prior to use, 30S subunits (0.9 A_{260} units per 30 μl reaction) had been reactivated by incubation for 2 min at 50°C in the presence of 30 mM $Mg(CH_3COO)_2$ [12]. Subsequently, 50S subunits (1.5 A_{260} units per reaction) and 1.5 μl of 11 mM puromycin were added and incubation proceeded for another 30 min in a total volume of 45 μl with buffer, salts, DTT, and GTP adjusted to the original concentrations.

2.5. Aminoacylation and formylation of tRNA

S. carnosus tRNA was prepared from an S-150 (see above) by extraction with phenol/chloroform in the presence of 0.2 M $NaCH_3COO$ pH 4, charged with [^{35}S]methionine and formylated as described [13]. 90–150 μg of extracted RNA was incubated in 150 μl of 200 mM triethanolamine/CH_3COO pH 7.5, 20 mM $Mg(CH_3COO)_2$, 10 mM DTT, 7.5 mM ATP, 0.2 mM CTP, 20 mM creatine phosphate, 100 μg/ml creatine phosphokinase, 0.6 mM folinic acid, containing 30 μl of extensively ultra-filtered S-150 and 15 μl [^{35}S]methionine (>1000 Ci/mmol) for 10 min at 37°C. The RNA was phenol-extracted and TCA-precipitable radioactivity was determined.

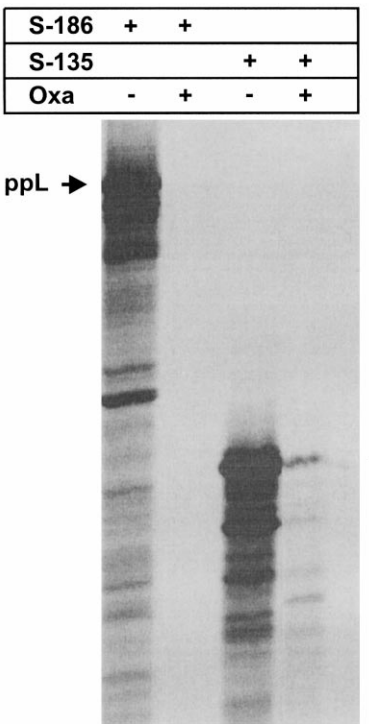

Fig. 1. DuP 721 inhibits de novo protein synthesis in cell-free systems prepared from *E. coli* and *S. carnosus*. High speed supernatants of *S. carnosus* (S-186) and *E. coli* (S-135) were used to synthesize by coupled transcription/translation the precursors of preprolipase (ppl), a secreted protein of *S. hyicus*, and the outer membrane protein OmpA of *E. coli*. Total translation products were labelled with [^{35}S]methionine and visualized by fluorography following separation by SDS-PAGE. Where indicated, DuP 721 (Oxa) was present during the reactions at 38 μg/ml.

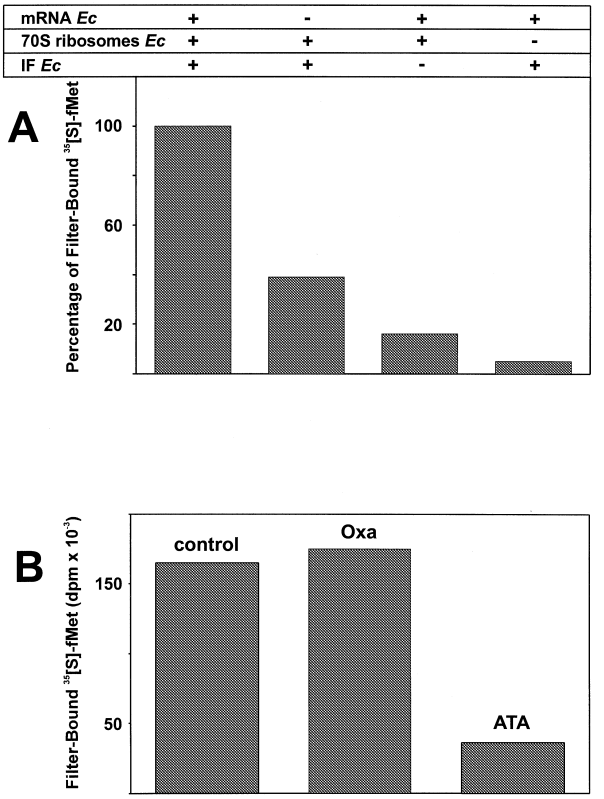

Fig. 2. DuP 721 does not inhibit the same step of translation initiation as aurin tricarboxylic acid. A: Relative binding of [^{35}S]fMet-tRNA to nitrocellulose filters. mRNA *Ec*, in vitro transcribed mRNA of the *E. coli* outer membrane protein LamB; 70S ribosomes *Ec*, salt-extracted *E. coli* ribosomes; IF *Ec*, purified initiation factors 1–3 of *E. coli*. Filter-bound radioactivity was determined by liquid scintillation counting. The dpm of the complete assays were set 100%. Depicted are the means of several independent experiments. B: Initiation reactions were performed using mRNA of the *S. hyicus* preprolipase, salt-extracted ribosomes of *S. carnosus*, and *E. coli* initiation factors. DuP 721 (Oxa) was present at 64 μg/ml (230 μM) and aurin tricarboxylic acid (ATA) at 100 μM.

3. Results

In contrast to previous results [4], we found a clear inhibition by DuP 721 of cell-free protein synthesis dependent on natural mRNAs in extracts prepared from both *E. coli* and *S. carnosus* (Fig. 1). In order to examine the proposed interference of DuP 721 with translation initiation, an initiation assay [10] was adopted for *E. coli* and *S. carnosus*. This assay measures the amount of [^{35}S]fMet-tRNA retained by nitrocellulose filters following incubation with 70S ribosomes, mRNA, and initiation factors. According to a current model of translation initiation in eubacteria [14], mRNA and fMet-tRNA bind in random order to 30S ribosomal subunits in the presence of the initiation factors 1 and 3 giving rise to a 30S pre-initiation complex. This is joined by a 50S ribosomal subunit yielding a 70S initiation complex in which finally initiation factor 2 promotes the positioning of fMet-tRNA in the ribosomal P-site. For [^{35}S]fMet-tRNA to be retained on nitrocellulose filters, only the formation of a pre-initiation complex is required. As shown in Fig. 2A for the *E. coli* system, a maximum amount of filter-bound [^{35}S]fMet-tRNA is obtained only when all three components necessary to form a 30S pre-initiation complex are present, i.e. mRNA, ribosomes, and ini-

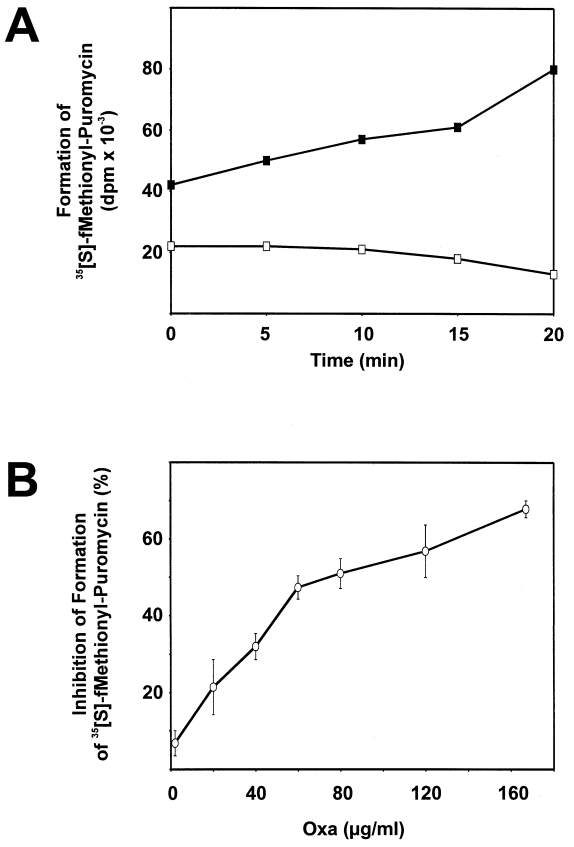

Fig. 3. DuP 721 inhibits the puromycin-mediated release of [^{35}S]fMet from a 70S initiation complex in a dose-dependent manner. A: After formation of initiation complexes from Gram-positive components, reactions were treated with 0.36 mM puromycin (■); □, controls without puromycin. At the indicated times, [^{35}S]fMet-puromycin peptide was extracted with ethyl acetate and quantitated by liquid scintillation counting. B: Initiation reactions were performed in the presence of the indicated amounts of DuP 721 (Oxa); treatment with puromycin was for 30 min. The means and standard deviation from two independent experimental series are shown.

tiation factors. Identical results were obtained with components from *S. carnosus* or mixtures of both (see below). As expected, aurin tricarboxylic acid (ATA), which is known to inhibit binding of mRNA to 30S ribosomal subunits [15], blocked formation of the 30S pre-initiation complex in our assay system. In contrast, DuP 721 had no inhibitory effect in these conditions indicating a different initiation event as target for the oxazolidinone (Fig. 2B).

When puromycin was added to these initiation assays, it caused a time-dependent release of [^{35}S]fMet-puromycin peptide measured as increase in ethyl acetate-extractable radioactivity [11]. This is depicted in Fig. 3A for the Gram-positive system. The fact that puromycin reacts with fMet-tRNA only when the latter is correctly located in the ribosomal P-site indicates that functional 70S initiation complexes had formed under these assay conditions. This puromycin-induced release of [^{35}S]fMet was clearly inhibited by DuP 721 in a dose-dependent manner (Fig. 3B). We therefore conclude that DuP 721 interferes with translation initiation at a step subsequent to the formation of a 30S pre-initiation complex.

In contrast to these results, a previous report had suggested the target of DuP 721 be the recognition of mRNA by the 30S ribosomal subunit via the Shine-Dalgarno sequence [4]. In order to examine this discrepancy we employed two oligoribonucleotides corresponding to the nucleotide sequence of *S. hyicus* preprolipase mRNA at its start codon, one containing the authentic Shine-Dalgarno sequence, the other having this deleted. Both oligos functioned equally well as templates for initiation and the inhibition of [^{35}S]fMet-puromycin release by DuP 721 reached the same level in either case (Fig. 4A). This demonstrates that the mode of action of DuP 721 does not in fact involve the Shine-Dalgarno sequence.

The extent of inhibition by DuP 721, however, turned out to be dependent on the origin of the components used for the initiation assay (Fig. 4B). Inhibition decreased when initiation factors and ribosomes of *E. coli* were used. This difference in activity of DuP 721 was independent of the amounts of initiation factors, ribosomes, and DuP 721 used (not shown). Thus, DuP 721 exhibits a higher inhibitory activity with initiation complexes from Gram-positive bacteria.

The results presented so far are consistent with an inhibition of translation by DuP 721 late during initiation. This conclusion was confirmed by the findings illustrated in Fig. 5 which reveal the 50S ribosomal subunit as target of DuP 721. Small and large ribosomal subunits of *E. coli* and *S. carnosus* were separated by sucrose gradient centrifugation in the presence of 10 mM $MgCl_2$ and 400 mM NaCl [16] (Fig. 5A). If the isolated 30S subunits were reactivated by heat treatment in the presence of 30 mM Mg^{2+}, they allowed

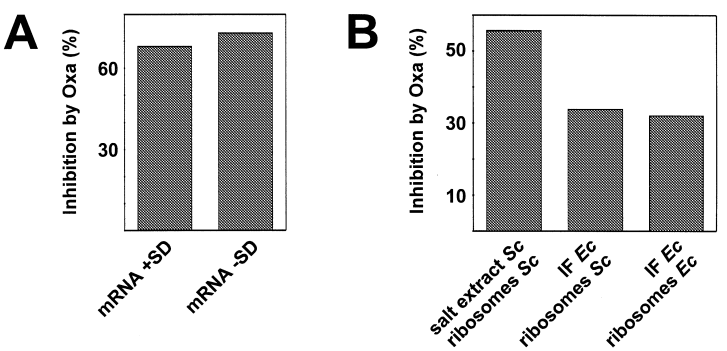

Fig. 4. Inhibition of translation initiation by DuP 721 does not involve the Shine-Dalgarno sequence and exhibits species specificity. A: Inhibition of puromycin-induced release of [^{35}S]fMet by DuP 721 (Oxa; final concentration 120 µg/ml) from Gram-positive initiation complexes. As templates, a 27-meric oligoribonucleotide comprising the first three codons of preprolipase and 18 authentic upstream nucleotides (mRNA+SD) was compared to an isomer in which the six nucleotides of the Shine-Dalgarno sequence had been deleted (mRNA−SD). B: Same as in A with the components indicated (IF, initiation factors; *Ec*, *E. coli*; *Sc*, *S. carnosus*) and preprolipase mRNA as template. DuP 721 was used at 80 µg/ml. Depicted are the means of two independent experiments.

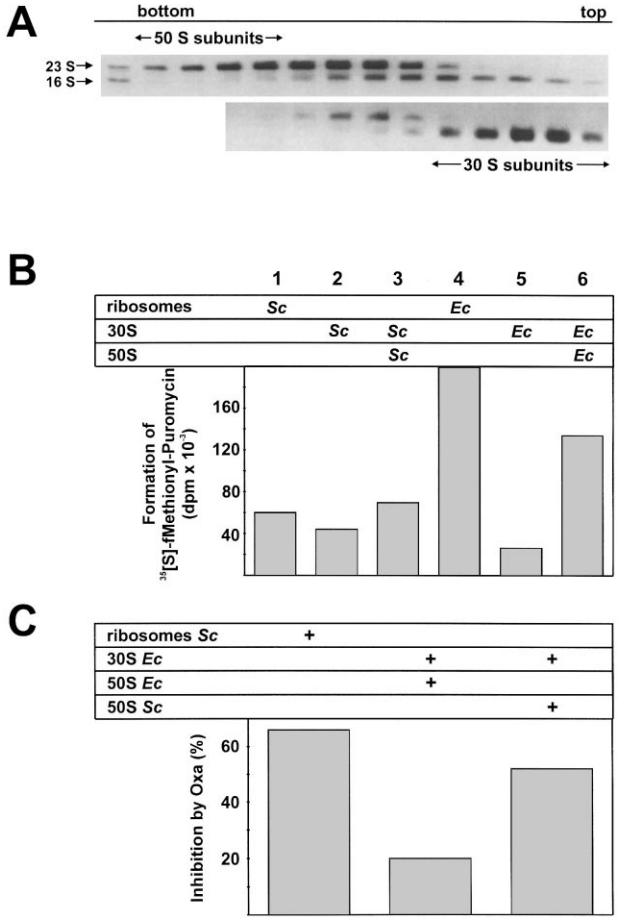

Fig. 5. Inhibition of translation by DuP 721 involves the 50S ribosomal subunit. A: Salt-washed ribosomes of *S. carnosus* (and of *E. coli*, not shown) were separated into small and large subunits by sucrose gradient centrifugation (cf. Section 2). The gradients were fractionated, aliquots were denatured in 4.7 M urea, resolved by 1% agarose gel electrophoresis and stained with ethidium bromide. The positions of 23S and 16S rRNA standards on the stained agarose gels are shown. 50S ribosomal subunits were recovered after one centrifugation run (upper panel, the pooled fractions are indicated). Purification of 30S subunits included a second run (lower panel) under identical conditions. B: Puromycin-induced release of [^{35}S]fMet from initiation complexes formed from the indicated components with oligonucleotide as template and the homologous initiation factors each. For experimental details see Section 2. Means of two sets of experiments are depicted (except lane 4). C: Inhibition by DuP 721 of the puromycin-induced release of [^{35}S]fMet from 70S initiation complexes formed from the indicated components in the presence of the oligonucleotide as template. Initiation factors were from *S. carnosus* (70S ribosomes) and from *E. coli* (30S and 50S subunits). DuP 721 (167 μg/ml) was present during the second incubation period.

formation of 30S pre-initiation complexes as reflected by filter binding of [^{35}S]fMet-tRNA (not shown). Puromycin caused release of [^{35}S]fMet from these initiation complexes (Fig. 5B, lanes 2 and 5) suggesting still some cross-contamination of the small subunits with 50S particles. The release was, however, significantly enhanced by the addition of isolated 50S subunits (Fig. 5B, lanes 3 and 6) which indicates that functional 70S initiation complexes were assembled from the isolated ribosomal subunits.

The inhibitory activity of DuP 721 was low when tested with 70S complexes reconstituted from the isolated ribosomal subunits of *E. coli* (Fig. 5C), similar to what had been observed with complete *E. coli* ribosomes (cf. Fig. 4). It increased, however, when the 50S subunits of *E. coli* were replaced by those of *S. carnosus* now reaching almost the level seen with the much more susceptible *S. carnosus* ribosomes (Fig. 5C). Obviously, the extent of inhibition by DuP 721 varied with the nature of the large ribosomal subunits. To directly demonstrate an interaction of DuP 721 with 50S ribosomal subunits, the experiments summarized in Table 1 were performed. The isolated subunits were individually incubated with DuP 721 (first incubation) and then separated from unbound material by centrifugation through cellulose membrane filters before incubation was continued in the presence of the cognate component (second incubation). In the control samples (lanes 2, 3) employing 70S ribosomes, DuP 721 caused a 35–43% inhibition compared to 64% observed without centrifugation (lane 1). No inhibition by DuP 721 was, however, obtained when the antibiotic was pre-incubated with 30S ribosomal subunits (lane 4). In contrast, when the 50S ribosomal subunits were pretreated with DuP 721 and freed from unbound drug before the remaining initiation components were provided, inhibition did occur (lane 5). The 21% inhibition found in this case compares well with the 52% seen with a non-centrifuged assay containing the same subunits (Fig. 5C, right column) taking into account that the centrifugation step caused a 50% drop of the DuP 721 activity otherwise obtained (compare lane 1 to 2 and 3). The only way how DuP 721 could have remained active during the course of this experiment was by filtration-resistant binding to the 50S ribosomal subunits.

4. Discussion

The central finding which we describe here is that an oxazolidinone prevents the puromycin-induced release of fMet from 70S initiation complexes in a dose-dependent and species-specific manner. The liberation of ribosome-associated fMet by puromycin has two prerequisites: (1) fMet-tRNA must be correctly positioned in the P-site of the 70S initiation complex and (2) the covalent linkage formed between fMet and puromycin depends on an active peptidyl transferase. Theoretically, DuP 721 could therefore interfere with the assembly of the P-site, which among other events involves a functional association of 50S subunits with 30S pre-initiation complexes. Alternatively, DuP 721, like other elongation inhibitors, could block the peptidyl transferase activity associated with 50S ribosomal subunits. Both effects would be consistent with the observed binding of DuP 721 to 50S ribosomal subunits. However, the reported elongation of in vivo assembled polysomes despite the presence of DuP 721 [4] argues against an interference of the antibiotic with the peptidyl transferase step. Furthermore, our finding that the degree of inhibition by DuP 721 drops when *S. carnosus* initiation factors are replaced by those of *E. coli* (cf. Fig. 4B) would rather support the idea that the target of DuP 721 is a late step during initiation, such as the binding of the 50S subunit or of initiation factor 2.

While this work was in progress, two papers were published describing studies on the molecular mechanisms of DuP 721 and two novel oxazolidinone derivatives [17,18]. Consistent with our finding, these authors report inhibition by oxazolidinones of in vitro transcription/translation in cell-free systems from *E. coli* and *S. aureus* [18]. They further demonstrate

Table 1
DuP 721 binds to 50S ribosomal subunits

		1	2	3	4	5
First incubation, 10 min, 30°C	30S	−	−	−	+	−
	50S	−	−	−	−	+
	70S	+	+	+	−	−
	mRNA	+	+	+	+	−
	IF	+	+	+	+	−
	Oxa	+	+	+	+	+
Isolation by ultrafiltration		−	+	+[a]	+[a]	+[a]
Second incubation, 30 min, 30°C	30S	−	−	−	−	+
	50S	−	−	−	+	−
	mRNA	−	−	−	−	+
	IF	−	−	−	−	+
	Puro	+	+	+	+	+
% inhibition by Oxa		64	43	35	<0	21

Where indicated, reactions were spun through cellulose membrane filter units (Millipore Ultrafree-MC; 5000 molecular weight cutoff) for 17 min at room temperature in a benchtop microcentrifuge. The filters were washed once ([a]) with 30 μl of 50 mM triethanolamine/CH_3COO, 200 mM KCH_3COO, 5 or 10 mM $Mg(CH_3COO)_2$ (depending on the Mg^{2+} concentration during the first incubation period, cf. Section 2), and then incubated with the reagents of the second incubation. After another spin, the ethyl acetate-extractable radioactivity of the filtrates was determined. For each lane, control reactions containing no DuP 721 were run in parallel to calculate the percentage of inhibition. DuP 721 was used at 167 μg/ml. 30S subunits were from *E. coli*, 50S and 70S particles from *S. carnosus*; mRNA and initiation factors were as in Fig. 5C.

specific binding of the radiolabelled drug to 50S ribosomal subunits of *E. coli* [17]. However, they did not observe inhibition of the puromycin-induced release of fMet from initiation complexes which were assembled from *E. coli* ribosomes and AUG as template. The tested range of inhibitor concentration was from 0.3–1 mM in which limits we have obtained a clear-cut inhibition (70–600 μM; Fig. 3B). Possibly, more natural templates than AUG are required to detect inhibition of fMet-puromycin formation by oxazolidinones. Most importantly, however, this effect of oxazolidinones might have been obscured by the use of *E. coli* ribosomes. We show here that the inhibitory activity of DuP 721 is much more pronounced with 50S ribosomal subunits from Gram-positive bacteria than from *E. coli*. Collectively, the results thus far available have led to the identification of the 50S ribosomal subunits as the molecular target for oxazolidinones. The precise mechanism of inhibition by these drugs, however, remains to be elucidated.

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