Inhibition of bacterial protein synthesis by elongation-factor-Tu-binding antibiotics MDL 62,879 and efrotomycin

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MDL 62,879 (formerly GE 2270 A) is a novel antibiotic active against Gram-positive bacteria by inhibiting protein synthesis. MDL 62,879 is not active against Gram-negative bacteria, but inhibits cell-free protein synthesis in extracts from *Escherichia coli*, and shows a high binding affinity for its elongation factor Tu (EF-Tu). We prepared ribosomes and protein-synthesis elongation factors from three sources: *E. coli*, *Bacillus subtilis*, and a strain of *B. subtilis* selected for resistance to MDL 62,879 (strain G1674). Homologous and heterologous reconstituted systems were used to compare the effects of MDL 62,879 and of efrotomycin, an EF-Tu inhibitor of the kirromycin class, which is inactive against both *B. subtilis* and *E. coli*. We showed that in cell-free protein synthesis: (a) *E. coli* was sensitive to both MDL 62,879 and efrotomycin; (b) *B. subtilis* was sensitive to MDL 62,879, but not to efrotomycin; (c) *B. subtilis* G1674 was resistant to both antibiotics. In the *E. coli* system and in the system from wild-type *B. subtilis*, inhibition by MDL 62,879 was reversed upon addition of purified EF-Tu from *B. subtilis* G1674. This demonstrates that the antibiotic acts by inhibition of EF-Tu. In contrast, extracts from *B. subtilis* failed to restore activity in an efrotomycin-inhibited *E. coli* system. Dominance or resistance to MDL 62,879 and of sensitivity to efrotomycin in heterologous cell-free protein synthesis confirms that inhibition of EF-Tu by the two antibiotics is mediated by different mechanisms of action.

INTRODUCTION

MDL 62,879 (GE 2270 A) is a recently discovered antibiotic isolated from fermentation broth of a strain of *Planobispora rosea* (Selva *et al.*, 1991). It has been characterized as an inhibitor of bacterial elongation factor Tu (EF-Tu), and its interaction with this protein has been described (Selva *et al.*, 1991; Anborgh & Parmeggiani, 1991). MDL 62,879 differs in chemical structure, in nature of interaction with EF-Tu and in spectrum of antimicrobial activity from kirromycin, another class of EF-Tu inhibitors (Selva *et al.*, 1991; Parmeggiani & Swart, 1985). MDL 62,879 appears to inhibit aminoacyl-tRNA binding to EF-Tu (Anborgh & Parmeggiani, 1991), whereas kirromycin inhibition involves stimulation of intrinsic GTPase activity and the formation of a non-dissociable ribosome-EF-Tu complex (Wolf *et al.*, 1974, 1977).

MDL 62,879 is active against Gram-positive bacteria and Gram-negative aerobes (Selva *et al.*, 1991), whereas kirromycins have no activity against some species of Gram-positive bacteria, such as staphylococci, enterococci and *Bacillus subtilis* (Frost *et al.*, 1976). Neither MDL 62,879 nor kirromycins are active against *E. coli*, but cell-free protein-synthesis systems from this micro-organism are highly sensitive to these antibiotics (Anborgh & Parmeggiani, 1991; Chinali *et al.*, 1976). We have studied the activity and mechanism of action of MDL 62,879 and of efrotomycin, a member of the kirromycin class, in different cell-free protein-synthesis systems.

MATERIALS AND METHODS

Bacterial strains

An MDL 62,879-resistant strain (G1674) of *B. subtilis* A.T.C.C. 6633 was isolated by plating heavy inocula on Penassay seed agar (Difco) containing 5 mg of MDL 62,879/l. A mutant was chosen which grew at 128 mg of MDL 62,879/l. This strain grows more slowly than the wild-type strain and was not cross-resistant to unrelated antibiotics. Both of these *B. subtilis* strains

and *Escherichia coli* K12 HfrC were used for preparation of cell-free extracts.

Chemicals

MDL 62,879 (1290 Da) was prepared in Lepetit Research Center, Gerenzano, Italy. It was dissolved in 50% *NN'*-dimethylformamide in water at 50-fold the final concentrations. Efrotomycin (1145 Da) was from Merck Sharp and Dohme, Basel, Switzerland. Solutions were made in 10% dimethylformamide in water at 10-fold the final concentrations; 1% dimethylformamide had no effect on enzymic activities.

[5,6-³H]Phenylalanine (sp. radioactivity 50 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were from Sigma, St. Louis, MO, U.S.A.

Poly(U)-directed polyphenylalanine [poly(Phe)] synthesis

Ribosomes and crude protein-synthesis factors were prepared as in Traub *et al.* (1971). Briefly, cells were washed and suspended in 20 mM-Tris/HCl, pH 7.9, containing 10 mM-MgCl₂, 5 mM-dithiothreitol and 0.2 mM-phenylmethanesulphonyl fluoride (buffer A) and broken by sonication in ice (10 cycles of 30 s with intervals of 30 s). Cell debris was removed by centrifugation at 30000 g (30 min, 4 °C). Ribosomes were pelleted by centrifugation at 100000 g for 4 h at 2 °C. The supernatant (S-100) was concentrated by (NH₄)₂SO₄ fractionation as described in Ravel & Shorey (1971). The 35–60%-(NH₄)₂SO₄ precipitate was kept and dialysed against buffer A + 0.35 M-NaCl (buffer B). This fraction (termed S-100') was used as the source of protein-synthesis elongation factors and for purification of EF-Tu. Poly(U)-directed poly(Phe) synthesis was performed in 30 mM-Tris/HCl, pH 7.9, containing 10 mM-MgCl₂, 80 mM-NH₄Cl, 0.8 mM-ATP, 1 mM-GTP, 3 mM-DTT and 0.8 µg of phenylalanine-specific tRNA from *E. coli*, 0.6 µCi of [³H]phenylalanine (sp. radioactivity 12 Ci/mmol), 3 pmol of NH₄Cl-washed ribosomes and 4 µg of protein-synthesis factors; 5 pmol of purified *B. subtilis* EF-Tu was added when required.

Abbreviations used: EF-Tu, elongation factor Tu; poly(Phe), polyphenylalanine; IC₅₀, 50%-inhibitory concentration.

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Fig. 1. Inhibition of poly(U)-directed poly(Phe) synthesis by MDL 62,879 (a) and efrotomycin (b) in cell-free systems from *E. coli* (○) and *B. subtilis* (□), and in a heterologous system with ribosomes from *E. coli* and S-100' from *B. subtilis* (△)

Values are expressed as percentages of the control. Controls incorporated (○) 7.6, (□) 5.4, and (△) 8.2 pmol of phenylalanine.

Incubation was for 30 min at 30 °C in a final volume of 0.1 ml. Samples were precipitated with ice-cold trichloroacetic acid (final concn. 5%, w/v) and heated for 10 min at 80 °C. The acid-precipitated material was collected on glass-fibre filters using a cell-harvester (LKB), and radioactivity was determined in a Beta-plate scintillation counter (Pharmacia).

EF-Tu purification

EF-Tu from *E. coli* was purified as described in Lebermann *et al.* (1980). *B. subtilis* EF-Tu was purified by loading the S-100' on to a GDP-Sepharose column (1 cm × 5 cm) pre-equilibrated with buffer B and washed with 5 vol. of the same equilibration buffer before elution of EF-Tu with 2 mM-GDP in buffer B. The purification steps were monitored by SDS/PAGE (8–25% polyacrylamide gradient gel) in the Phastgel system (Pharmacia).

Fig. 2. Poly(U)-directed poly(Phe) synthesis in cell-free extracts from *B. subtilis* G1674: effect of MDL 62,879 (□) and efrotomycin (△). Control incorporated 4.6 pmol of phenylalanine.

Fig. 3. SDS/PAGE of samples from *B. subtilis* EF-Tu purification steps

Proteins were separated on an SDS/8–25% polyacrylamide gradient slab gel and stained with Coomassie Brilliant Blue R-250. Lanes: (a) and (d), molecular-mass standards (phosphorylase *b*, 97400 Da; BSA, 66000; ovalbumin, 45000; carbonic anhydrase, 30000; trypsin soybean inhibitor, 20100); (b), *B. subtilis* ATCC 6633 S-100'; (e) GDP-Sepharose eluate; (c) *B. subtilis* G1674 S-100'; (f), GDP-Sepharose eluate; 5 µg of S-100' or 0.3 µg of GDP-Sepharose eluate was loaded on the gel.

RESULTS

Inhibition of poly(U)-directed poly(Phe) synthesis in cell-free extracts from *E. coli* and *B. subtilis*

The effects of MDL 62,879 and efrotomycin on poly(U)-directed poly(Phe) synthesis were compared by using various cell-free systems. The 50%-inhibitory concentration (IC_{50}) of MDL 62,879 was 0.12 mg/l in cell-free systems from *E. coli* and *B. subtilis* A.T.C.C. 6633, and in a mixed system in which washed *E. coli* ribosomes and *B. subtilis* S-100' were used (Fig. 1a). For efrotomycin the IC_{50} in the *E. coli* system was 0.12 mg/l, whereas poly(Phe) synthesis performed with *B. subtilis* extracts and in the

Fig. 4. MDL 62,879 inhibition of poly(Phe) synthesis in various cell-free systems

(a) Systems from *B. subtilis* A.T.C.C. 6633 (○), *B. subtilis* G1674 (□) and *B. subtilis* A.T.C.C. 6633 to which 5 pmol of EF-Tu from G1674 was added (Δ). Controls incorporated respectively 5.6, 6.2 and 7.1 pmol of phenylalanine. (b) Systems from *E. coli* (○) and *E. coli* + 5 pmol of EF-Tu from G1674 (Δ). Controls incorporated 7.4 and 8.8 pmol of phenylalanine.

mixed system was unaffected by antibiotic concentrations up to 200 mg/l (Fig. 1b). In cell-free extracts from *B. subtilis* G1674 both MDL 62,879 and efrotomycin were inactive at up to 100 mg/l (Fig. 2).

These results suggest that the lack of susceptibility of wild-type *B. subtilis* to efrotomycin is due to an intrinsically resistant EF-Tu, and that resistance to MDL 62,879 in *B. subtilis* G1674 was due to alteration of a protein-synthesis factor, presumably EF-Tu.

EF-Tu purification

To determine whether the mutation leading to MDL 62,879-resistance was indeed in the gene encoding EF-Tu, the protein

Fig. 5. Poly(Phe) synthesis performed in an *E. coli* cell-free system (3 pmol of ribosomes, 2 μ g of S-100') in the presence of 10 mg of either MDL 62,879 (a) or efrotomycin (b)/l

After 5 min at 30 °C, 2 μ g of S-100' from *E. coli* (□) or from *B. subtilis* G1674 (Δ) was added. The *E. coli* system in the absence of antibiotics served as the control (○).

was purified from both the wild-type and the resistant strains of *B. subtilis*. Purification of EF-Tu was monitored by SDS/PAGE. As shown in Fig. 3, EF-Tu represented up to 10% of total protein in the S-100' preparations from *B. subtilis* used in poly(U) assays; after GDP-Sepharose chromatography EF-Tu represented more than 90% of the protein detectable in the gel. When 5 pmol of pure EF-Tu from MDL 62,879-resistant *B. subtilis* G1674 was added to sensitive cell-free systems from either *B. subtilis* A.T.C.C. 6633 (Fig. 4a) or *E. coli* (Fig. 4b), the IC_{50} of MDL 62,879 increased from 0.12 to > 100 mg/l. Thus resistance to MDL 62,879 is mediated by an alteration of the gene encoding EF-Tu. Addition of 5 pmol of pure EF-Tu from the sensitive *B. subtilis* strain increased the IC_{50} only slightly, from 0.12 to 0.15 mg/l in the *E. coli* system and from 0.12 to 0.18 mg/l in *B. subtilis* A.T.C.C. 6633. This is probably an effect of increasing the stoichiometric ratio of EF-Tu to MDL 62,879.

Reversal of inhibition by MDL 62,879 and efrotomycin

In *B. subtilis* G1674, natural resistance to efrotomycin is coupled with acquired resistance to MDL 62,879. In order to study the dominance pattern *in vitro* of MDL 62,879- and

efrotomycin-resistance, S-100' from *B. subtilis* G1674 was added to a cell-free protein-synthesis system from *E. coli* containing 10 mg of either MDL 62,879 or efrotomycin/l. As shown in Fig. 5, the S-100' from G1674 led to a complete reversal of MDL 62,879 inhibition after a short lag period (Fig. 5a). By contrast, efrotomycin inhibition was not reversed upon addition of G1674 S-100' (Fig. 5b).

DISCUSSION

B. subtilis strain G1674, selected for resistance to MDL 62,879, also demonstrated resistance in cell-free poly(U)-directed poly(Phe) synthesis. This indicates that resistance was the result of an altered target of the antibiotic, and not due to decreased permeability. Indeed, the addition of purified EF-Tu from this strain reversed MDL 62,879 inhibition of sensitive cell-free protein-synthesis systems from *E. coli* and wild-type *B. subtilis*. This suggests that resistance is caused by mutation in the gene encoding EF-Tu and, consequently, that the antimicrobial activity of MDL 62,879 is in fact mediated by its interaction with EF-Tu.

B. subtilis is naturally resistant to kirromycins; cell-free poly(U)-directed poly(Phe) synthesis from this organism was insensitive to 200 mg of efrotomycin/l. This result supports the observation by Smith & Paress (1978) that EF-Tu from wild-type *B. subtilis* was inhibited only by high concentrations of kirromycins (100 mg/l). We are unable to explain the apparent kirromycin-sensitivity of *B. subtilis* EF-Tu reported by Woerner *et al.* (1983).

Studies on other Gram-positive bacteria (*Staphylococcus aureus*, lactobacilli) demonstrate that, in most cases, kirromycin resistance is mediated by EF-Tu (Hall *et al.*, 1989; Woerner & Wolf, 1982; Woerner *et al.*, 1983), whereas in *E. coli* resistance is due to lack of penetration into the cell. Although the *tuf* genes, which encode EF-Tu, appear to be highly conserved among bacteria (Filer & Furano, 1980), the kirromycin-binding site may be less conserved than other domains of the protein. However, the degree of heterogeneity might still be small, since a single amino acid substitution in an *E. coli* EF-Tu leads to a complete loss of affinity of kirromycins for the protein (Parmeggiani & Swart, 1985).

B. subtilis G1674 maintained the resistance to efrotomycin of the parent strain. When S-100' factor from this strain was added to a cell-free system from *E. coli* sensitive to both kirromycins and MDL 62,879, inhibition by MDL 62,879, but not by efrotomycin, was reversed. Dominance of sensitivity to efrotomycin when both a sensitive and a resistant EF-Tu are

present in a cell-free assay is probably due to stabilization of the EF-Tu-ribosome complex by kirromycins (Wolf *et al.*, 1974), and is consistent with the fact that it is necessary to mutate both of the duplicate *tuf* genes which encode EF-Tu in *E. coli* in order to obtain a kirromycin-resistant cell-free protein-synthesis system (Van der Weide *et al.*, 1980). Dominance of resistance observed for MDL 62,879 inhibition confirms that its mechanism of action differs from that of the kirromycin group, and is consistent with the mode of action proposed by Anborgh & Parmeggiani (1991). Inhibition of EF-Tu by MDL 62,879 would be mediated by preventing binding of aminoacyl-tRNA and blocking of the enzyme in its GTP-bound conformation. A MDL 62,879-bound EF-Tu should not interact with the ribosome, allowing a resistant enzyme to bind and normally support protein synthesis. This difference in mechanism of action, and the fact that some MDL 62,879-sensitive Gram-positive species have an EF-Tu which is resistant to kirromycins, suggest that the two antibiotic classes may have different binding sites.

REFERENCES

- Anborgh, P. H. & Parmeggiani, A. (1991) *EMBO J.* **10**, 779–784
- Chinali, G., Wolf, H. & Parmeggiani, A. (1976) *Eur. J. Biochem.* **75**, 55–65
- Filer, D. & Furano, A. V. (1980) *J. Biol. Chem.* **255**, 728–734
- Frost, B. M., Valiant, M. E., Weissberger, B. & Dulaney, E. L. (1976) *J. Antibiot.* **29**, 1083–1091
- Hall, C. C., Watkins, J. D. & Georgopapadakou, N. H. (1989) *Antimicrob. Agents Chemother.* **33**, 322–325
- Lebermann, R., Antonsson, B., Giovanelli, R., Guariguta, R., Schumann, R. & Wittinghofer, A. (1980) *Anal. Biochem.* **104**, 99–111
- Parmeggiani, A. & Swart, G. W. M. (1985) *Annu. Rev. Microbiol.* **39**, 557–577
- Ravel, J. M. & Shorey, R. L. (1971) *Methods Enzymol.* **20**, 306–316
- Selva, E., Beretta, G., Montanini, N., Saddler, G. S., Gastaldo, L., Ferrari, P., Lorenzetti, R., Landini, P., Ripamonti, F., Goldstein, B. P., Berti, M., Montanaro, L. & Denaro, M. (1991) *J. Antibiot.* **44**, 693–701
- Smith, I. & Paress, P. (1978) *J. Bacteriol.* **135**, 1107–1117
- Traub, P., Mizushima, S., Lowry, C. W. & Nomura, M. (1971) *Methods Enzymol.* **20**, 391–407
- Van der Meide, P. H., Borman, T. H., Van Kimmenade, A. M. A., Van de Putte, P. & Bosch, L. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3922–3926
- Woerner, W. & Wolf, H. (1982) *FEBS Lett.* **146**, 322–326
- Woerner, W., Gloeckner, C., Mierzowski, M. & Wolf, H. (1983) *FEMS Microbiol. Lett.* **18**, 69–73
- Wolf, H., Chinali, G. & Parmeggiani, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4910–4914
- Wolf, H., Chinali, G. & Parmeggiani, A. (1977) *Eur. J. Biochem.* **75**, 67–75