MYOMYCIN: MODE OF ACTION AND MECHANISM OF RESISTANCE

JULIAN DAVIES, MICHAEL CANNON† and MORRIS B. MAUER††

Unité de Génie Microbiologique, Institut Pasteur,
Paris, France
†Department of Biochemistry, King's College, University of London,
UK
††Department of Biochemistry, University of Wisconsin,
Madison, U.S.A.

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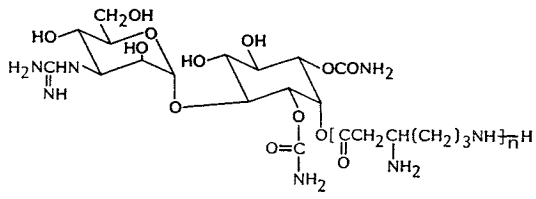
Myomycin is an unusual pseudodisaccharide antibiotic with a β-lysyl oligopeptide ester side chain that has structural similarities with kasugamycin, streptomycin and streptothricin. We show that the mode of action of myomycin *in vivo* and *in vitro* closely resembles that of streptomycin; in addition, spontaneous myomycin-resistant mutants of *Escherichia coli* are essentially indistinguishable from streptomycin-resistant mutants at the rRNA and r-protein level. Myomycin is not a substrate for the known streptomycin-modifying enzymes and could be useful in the characterization of natural streptomycin-resistant isolates and in counterselecting against the presence of streptomycin-modifying enzymes. The relationship between structure and inhibition of protein synthesis has been examined for a series of derivatives of myomycin.

The aminoglycoside-aminocyclitol antibiotics (AGACs) are a large group of important antimicrobial agents[1]. The AGACs are produced by a variety of bacterial genera, including *Actinomyces*, *Streptomyces* and *Bacillus* sp.; the primary mode of antimicrobial action of AGACs has been identified as inhibition of protein synthesis by direct interaction with the bacterial ribosome. The majority of members of this group also induce mistranslation of the genetic code and associated phenotypic suppression of bacterial mutants[2]. The most widely used AGACs, clinically, are those of the 2-deoxystreptamine type. Of the other structurally related but less well known members of the group, several have been useful in molecular studies of antibiotic mechanism of action, antibiotic resistance and target structure-activity analyses, although not employed therapeutically. AGACs have been particularly useful in analysing the mechanism of translation and the details of ribosome function both directly and indirectly (*e.g.* resistant mutants)[3].

Myomycin, a broad spectrum antibiotic, was first described in 1973[4]. It is produced by a member of the *Nocardia* genus as a mixture of three strongly-basic carbohydrate components. The major component, myomycin B, has been analyzed chemically in some detail and the structure (Fig. 1) was revised in 1981[5,6]. Myomycin is of interest since it contains the carbohydrate myoinositol; only one other AGAC, kasugamycin, possesses a similar moiety. Myomycin has a weak but broad spectrum of antibacterial activity; presumably for this reason the compound was not developed further as a clinical agent. We were interested in studying the mode of action and resistance to myomycin since it is an unusual member of the

Fig. 1. Structure of the myomycin complex[6].
Myomycin, n=2; for n=3, 4, 5 see ref 6 and Table 7.



AGAC class with (apparent) chemical similarities to kasugamycin (inositol moiety), streptomycin (carbamoyl functions), and streptothricin D (β-lysine peptide moiety). We found to our surprise, that myomycin resembles streptomycin closely in its biological activity and in its resistance mechanism at the ribosome level; the similarity in mode of action between streptomycin and myomycin implies that they have common structural and conformational aspects that allow interaction with the same site on the 30S subunit of the ribosome. Like streptomycin[7], myomycin has been shown to exert characteristic physiological changes (bleaching) in light-grown cells of *Euglena*[8].

Materials and Methods

The bacterial strains were standard *Escherichia coli* isolates and derived mutants (using CA274 as parent) taken from our laboratory collection. Spontaneous resistant mutants were obtained at a frequency of $\sim 10^{-8}$. Conjugation and transductional analysis were performed by the usual methods[9]. Methodology for the analysis of the mode of action of myomycin *in vivo* and *in vitro* has been described[10]. Myomycin was kindly provided by Parke-Davis, Ann Arbor, Michigan, and by Lederle Laboratories, Pearl River, N.Y., U.S.A.

Results

Antibacterial Action of Myomycin

Myomycin added at inhibitory concentrations (>100 μg/ml) to growing cultures of *E. coli*, rapidly arrested cell growth with concomitant decrease in viability. When macromolecular syntheses were examined in minimal medium, it was found that radioactive amino acid incorporation into protein was immediately inhibited by the drug (Fig. 2) but that incorporation of [^{14}C]uracil into nucleic acid continued unabated for at least one doubling time (Fig. 3). Myomycin thus shows the characteristic behaviour of a protein synthesis

Fig. 2. Effect of myomycin on the incorporation of [^{14}C]leucine in *Escherichia coli*.

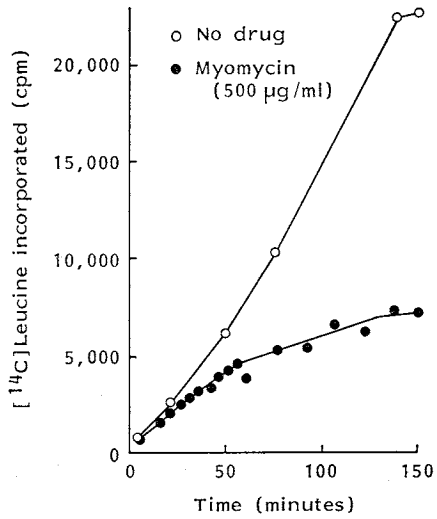
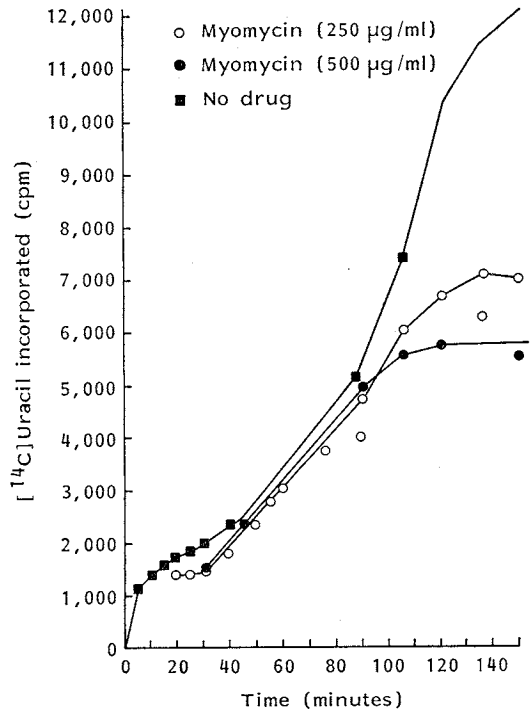


Fig. 3. Effect of myomycin on the incorporation of [^{14}C]uracil in *Escherichia coli*.



inhibitor[9]. Among *E. coli* strains tested for their response to myomycin were several streptomycin-resistant and streptomycin-dependent mutants; these were found to be generally resistant or dependent on myomycin. In addition, myomycin could replace streptomycin, paromomycin, or alcohol effectively in supporting the growth of streptomycin-dependent mutants. This suggested that myomycin had a mode of action similar to that of streptomycin and in confirmation of this, we found that myomycin could suppress phenotypically the same range of nonsense and missense mutants of *E. coli* that were susceptible to streptomycin[11].

Action of Myomycin and Cell-free Translations

The results described above strongly suggested that myomycin was a functional analogue of streptomycin at the ribosome level. When added to poly U-directed cell-free translation systems, myomycin was found to inhibit [^{14}C]phenylalanine incorporation (Tables 1 and 2) or R17-directed incorporation (Table 3) and to stimulate the incorporation of [^{14}C]leucine and other "wrong" amino acids into protein (mistranslation) (Table 4). These effects were not seen with cell-free systems derived from myomycin-resistant mutants or most streptomycin-resistant mutants tested, and were associated with ribosomes and not supernatant fractions.

Additional studies (Table 5) demonstrated that myomycin-resistance was associated with the 30S ribosomal subunit. More detailed analysis with bacteriophage R17 RNA-primed translation showed that myomycin was a potent inhibitor of this system (Tables 3 and 5) and that the antibiotic inhibited R17 RNA-directed binding of fMet-tRNA to 30S ribosomes[12] (Table 6). We conclude that myomycin is essentially identical to streptomycin in its inhibitory action on bacterial ribosome function. This conclusion was confirmed and extended by the following genetic studies.

Table 1. Incorporation of [^{14}C]phenylalanine by preincubated S30 cell-free extracts in the presence of myomycin.

S30	Drug concentration (M)	Inhibitions (%)
S (CA274)	2.5×10^{-4}	65
	5×10^{-4}	66
	5×10^{-3}	65
R-1 (CA274-14)	2.5×10^{-4}	9
	5×10^{-4}	12
	5×10^{-3}	11
R-2 (CA274-22)	2.5×10^{-4}	18
	5×10^{-4}	22
	5×10^{-3}	38

Mixtures contained 25 μl of preincubated and dialyzed S30 extract; 15 mg of poly U; incubation at 36°C for 30 minutes; total volume of 0.1 ml. CA274-14 is a high-level resistant mutant of CA274; CA274-22 exhibits intermediate-level resistance.

Table 2. Effect of myomycin on poly U-directed [^{14}C]phenylalanine incorporation on 70S ribosomes from *Escherichia coli* strains sensitive or resistant to the antibiotic.

Source of 70S	Drug concentration (M)	cpm	Inhibition (%)
Myomycin-sensitive	—	17,390	—
(CA274)	5×10^{-3}	14,360	17
	5×10^{-2}	11,670	33
	2.5×10^{-2}	11,190	35
Myomycin-resistant	—	18,910	—
(CA274-14)	5×10^{-3}	19,700	0
	5×10^{-2}	21,560	0
	2.5×10^{-2}	21,250	0

Incubation mixtures contained 0.50 OD_{260} of 70S ribosomes; ribosomes were heat activated by prior incubation at 40°C for 20 minutes; total volume 0.1 ml.

Myomycin Resistance

Streptomycin-resistant mutants (*rps* L) were almost invariably resistant to high concentrations of myomycin. Among a collection of some 100 independent str^R mutants of a single *E. coli* parent (CA274), all but one were highly resistant to myomycin; the exception, mutant 274-22, was only partially sensitive to myomycin. Independently-isolated, spontaneous myomycin-resistant mutants of *E. coli* were all found to be resistant to streptomycin and the myomycin-resistance mutations mapped to the *rps* L allele in P1 co-transduction and conjugation studies (50% linked to *aro* E; 10%

Table 3. Myomycin inhibition of R17 RNA-directed [^{14}C]valine incorporation on 70S ribosomes from drug-sensitive *Escherichia coli* CA274.

Drug concentration (M)	cpm	Inhibition (%)
—	7,480	—
3×10^{-3}	1,780	76
6×10^{-3}	1,790	76
6×10^{-2}	2,330	69
2.5×10^{-2}	1,200	85

Incubations contained 0.50 OD_{260} of 70S ribosomes; ribosomes were heat activated by prior incubation at 40°C for 20 minutes; incorporation mixtures incubated at 37°C for 30 minutes; total volume 0.15 ml.

Table 4. Translational misreading induced by myomycin.

30S	Myomycin concentration (μg/ml)	cpm	Stimulation (%)
Sensitive	—	1,250	—
(CA274)	2	7,450	600
	5	16,080	1,290
	10	16,080	1,290
	20	15,730	1,260
Resistant	—	1,120	—
(CA274-14)	2	2,600	230
	5	2,900	260
	10	5,700	500
	20	2,680	240

50S subunits from a sensitive strain were supplied to give total 70S conc per incubation at 0.50 OD_{260}; ribosomes were heat activated; mixtures incubated 30 minutes at 37°C; misreading is defined as the incorporation of labeled serine, tyrosine and isoleucine with poly U as exogenous messenger.

Table 5. Ribosomal subunit specificity of myomycin action.

30S	50S	Poly U			R17		
		Drug concentration (μg/ml)	Incorporation (cpm)	% of control	Drug concentration (μg/ml)	Incorporation (cpm)	% of control
S	S	—	17,390	—	—	2,780	—
		50	14,360	82.6	6	810	29.1
		100	11,670	67.1	12	802	28.8
		150	11,180	64.3	20	800	28.8
S	R	—	18,200	—	—	3,190	—
		50	16,230	89.0	6	1,250	39.3
		100	14,360	78.8	12	840	26.4
		150	12,200	67.0	20	500	15.6
R	R	—	18,900	—	—	740	—
		50	19,700	100+	6	870	100+
		100	21,560	100+	12	940	100+
		150	22,000	100+	20	1,230	100+
R	S	—	18,440	—	—	1,280	—
		50	19,050	100+	6	760	59.6
		100	20,550	100+	12	1,100	86.3
		150	19,640	100+	20	960	75.0

Incubation mixtures contained 0.50 OD_{260} of 70S ribosomes in a total volume of 0.1 ml; poly U incubations for 10 minutes and R17 for 30 minutes at 37°C; ribosomes were heat activated by prior incubation at 40°C for 20 minutes. S refers to subunits isolated from CA274; R to subunits from CA274-14.

Table 6. fMet-tRNA binding to 70S subunits in presence of myomycin.

Source of 30S	Drug	cpm	% of control
Sensitive	−	14,620	—
(CA274)	−	15,180	
	+	8,390	43
	+	8,860	
Resistant	−	10,840	—
(CA274-14)	−	9,120	
	+	13,770	100+
	+	16,170	

Incubations contained 0.5 OD_{260} 70S ribosomes; after addition of 30S, mix (5×buffer, initiation factors, 50S and H_2O), AUG, myomycin, and H_2O (in that order) the mixtures were preincubated for 5 minutes at 30°C; the f[^{35}S]Met-tRNA+GTP was added and the mixtures were incubated for an additional 5 minutes at 30°C; myomycin was added at 20 μg/ml (final concentration).

Table 7. Inhibition of polyphenylalanine synthesis by streptomycin, myomycin and various myomycin derivatives.

Drug	*Escherichia coli*		*Saccharomyces cerevisiae*
	Column 1	Column 2	Column 3
Streptomycin	67	62	96
A	109	95	96
B	114	104	96
Myomycin	58	56	106
D	56	54	110
E	55	57	113
F	55	57	101

Cell-free extracts were prepared from either *E. coli* or *S. cerevisiae* and assayed for poly U-directed polyphenylalanine synthesis as described[21,22]. Extracts were incubated for 60 minutes at 30°C in the presence or absence of the various drugs using drug concentrations of 5 μg/ml or 20 μg/ml for *E. coli* extracts (columns 1 and 2, respectively) and of 100 μg/ml for *S. cerevisiae* extracts (column 3). Results are expressed as % incorporation relative to 100% incorporation in control incubations. A and B are β-lysine oligomers. D, E and F are myomycin derivatives with varying length β-lysine side chain; D: n=4, E: n=5, F: n=3. See Fig. 1 and ref 6.

to *aro* B; 70% linked to *spc* R)[3]. When the ribosomes of streptomycin/myomycin-resistant mutants were examined in cell-free translation systems, they were found to be resistant to inhibition by either drug over a range of concentrations. We conclude that single amino acid changes in ribosomal protein S12 are responsible for resistance to myomycin (as with streptomycin); no other mutation to resistance to myomycin (other than low level transport defects) has been found. Furthermore, about 10% of all myomycin-resistant mutants were found to be growth dependent on the presence of the drug (or streptomycin or alcohol); all streptomycin-dependent mutants of *E. coli* tested were found to grow equally well on myomycin. Recently, MONTANDON *et al.*[13] and his co-workers have demonstrated that a C912 to U base change in *E. coli* 16S rRNA leads to a high level streptomycin-resistance phenotype[13]. Such mutants were cross-resistant to myomycin (results not shown) which confirms the identity of the binding site for the two antibiotics on the bacterial ribosome.

Many clinical bacterial isolates are resistant to streptomycin as a result of plasmid-encoded *O*-phosphorylation or *O*-adenylylation[14]; as might be expected on structural grounds, none of these resistant strains were resistant to myomycin; myomycin was not enzymatically modified by extracts of the R-plasmid containing strains tested. It should be noted that acetylation of streptothricin has been reported recently[15]; the modification does not take place on the moiety common to myomycin and strains carrying this determinant are sensitive to myomycin. To date, no plasmid-mediated resistance to myomycin has been detected by screening more than one hundred clinical isolates of bacteria. The organism that produces myomycin (a *Nocardia* sp.) has not yet been examined for enzymatic modification of the drug. Streptomycin-producing organisms (*Streptomyces griseus*, *S. bikiniensis*, *S. glaucescens*) which are known to possess streptomycin-phosphorylating enzymes[16] are sensitive to myomycin and ribosomes from other aminoglycoside-producing organisms resistant to their endogenous antibiotic (kanamycin, gentamicin, *etc.*) remained sensitive to myomycin (E.

CUNDLIFFE; personal communication).

Structure-activity Relationships

BORDERS *et al.*[5], and MCGAHREN *et al.*[6] have isolated several compounds structurally related to myomycin from the producing *Nocardia* sp., two of which had potent antimicrobial activity. The active molecules possessed the same basic pseudodisaccharide structure but differed in the length of the β-lysine chain (2~5 residues) (Fig. 1). These compounds all inhibited poly U-directed phenylalanine synthesis on *E. coli* ribosomes and all were, at the same concentrations, slightly more potent than streptomycin. This could indicate that basicity (number of amino groups) is not, alone, a determinant of activity in these compounds; the naturally-occurring β-lysine oligomers alone were devoid of antibacterial or *in vitro* activity (Table 7). As might be expected, none of these compounds (including myomycin and streptomycin) had any activity against polypeptide synthesis on yeast ribosomes, even at concentrations of 100 μg/ml.

Discussion

We describe studies on the mode of action and mechanism of resistance to the antibiotic myomycin. Although myomycin bears little obvious structural relationship to streptomycin, it has a mode of action and an altered-target-site mechanism of resistance which is essentially identical to that of streptomycin. The two antibiotics must bind to the same site on the 30S ribosomal subunit as defined by alterations in the amino acid sequence of the 30S ribosomal protein S12[17~19] shown to involve an interaction with 16S ribosomal RNA. Different interactions arising from the dissimilar structural aspects of the two drugs could be probed, presumably, by analysis of second site revertants of drug-dependent strains. In addition, single nucleotide changes in 16S rRNA led to resistance to both drugs. Myomycin and streptomycin, thus, represent an example of two structurally different ligands binding to the same biological receptor site. Not surprisingly, *E. coli* mutants resistant to streptomycin and other aminoglycosides resulting from defects in transport or energy functions[20] were also resistant to myomycin.

Thus far no plasmid-encoded modification of myomycin has been detected in screening of a large number of bacterial clinical isolates; it should be noted that myomycin has never been employed as an antibiotic product for human or animal use. It will be interesting to see if the myomycin-producing *Nocardia* (comparatively few aminoglycosides have been obtained from *Nocardia* sp.) possesses an enzymatic modification of the drug or a target-mediated mechanism for myomycin-resistance. Streptomycin-producing organisms known to possess ribosomes sensitive to streptomycin but with enzymatic mechanisms capable of mediating streptomycin-resistance, were found to be sensitive to myomycin. Myomycin provides, therefore, a convenient means for screening resistant bacterial isolates to distinguish between ribosomal alterations and the enzymatic inactivation of streptomycin.

Because of their structural differences, myomycin and streptomycin could be useful in analyses of ribosome structure and conformation at the molecular level and even more valuable if three dimensional structures of the two antibiotics could be compared, particularly in relationship to their 16S RNA binding site (the C912 region)[18,19]. Moreover, if might be instructive to use myomycin as a means of analyzing the basis of the toxic side effects of streptomycin. Since the two drugs are different in structure but identical in mode of ribosomal interaction, one could ask if the side effects of streptomycin were related uniquely to its interaction with ribosomes by carrying out a comparative toxicity study with streptomycin and myomycin. It is worth noting that myomycin and streptomycin have one other feature in common; both antibiotics are known to cause "bleaching" of chloroplasts in blue-green algae[7,8]. Finally, one might also imagine the possibility of myomycin-streptomycin hybrids that are refractory to the enzymatic modifications responsible for the existing widespread streptomycin-resistance.

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