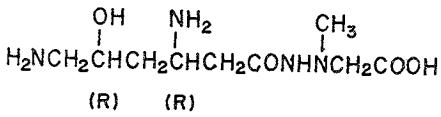
NEGAMYCIN, A MISCODING ANTIBIOTIC WITH A UNIQUE STRUCTURE

Sir:

Negamycin is a new antibiotic inhibiting Gram-positive and Gram-negative bacteria. The antibiotic is an especially promising one because of its effectiveness against both multiple-drug-resistant enteric bacteria which harbor R-factors* and *Pseudomonas*.[1] Its chemical structure[2] is shown in Fig. 1.

Fig. 1.



Using various *in vivo* and *in vitro* systems, MIZUNO *et al.*[3,4] demonstrated that negamycin is a specific inhibitor of protein synthesis with miscoding activity. This mode of action is characteristic of some aminoglycosidic antibiotics, such as streptomycin, neomycin and kanamycin.[5] Since it does not have an aminoglycosidic structure, negamycin seems to be an exception in this respect and this dissimilarity in structure prompted us to search for possible differences between negamycin and aminoglycosidic antibiotics, both in details of their miscoding spectrum and in the cross-resistance of ribosomes of resistant strains. We report in this paper the following observations: (1) the miscoding spectrum of negamycin resembled those of streptomycin and kanamycin, (2) negamycin may interact with more than one site of a ribosome, and (3) ribosomes prepared from either streptomycin- or kanamycin-resistant *Escherichia coli* retained full or slightly reduced sensitivity to negamycin.

The miscoding spectrum of negamycin:

As shown in Table 1, the miscoding spectrum of negamycin resembled those of streptomycin and kanamycin[6] in that (1) negamycin causes miscoding of only one base at a time, (2) negamycin causes miscoding of a base in only the 5′ and internal position in the pyrimidine codons, but in only the 3′ position in the purine codons,

Table 1. Miscoding spectra of negamycin, streptomycin and kanamycin

Polynucleotide and amino acid		Drug			
		None	NGM	SM	KM
		cpm	ratio		
Poly U	Phe	35,700	0.7	0.5	0.3
	Leu	1,950	1.8	1.8	1.5
	Ser	600	1.4	1.3	1.7
	Ile	400	3.3	3.1	2.4
Poly A	Lys	1,000	1.5	1.5	1.6
	Asn	370	1.4	1.6	1.7
Poly C	Pro	1,380	1.1	0.8	1.7
	Leu	490	1.1	1.1	1.7
	Thr	340	1.7	1.1	2.5
	Ser	360	1.7	1.7	3.3

A reaction mixture contained in 100 μl; 50 mM Tris HCl, pH 7.8, 160 mM NH_4Cl, 3 mM ATP, 0.2 mM GTP, 15 mM $Mg(OAc)_2$, 4 mM phosphoenolpyruvate, 5 μg of pyruvate kinase, 2 mM DTT, 0.08 μCi of a ^{14}C-labeled amino acid, 0.025 mM each of 19 amino acids, 1 mg of *E. coli* tRNA (stripped), *E. coli* S-30 containing 60 μg protein (bovine serum albumin used as standard for color assay), an mRNA (20 μg of poly U, 50 μg of poly A, or 40 μg of poly C), and an inhibitor, if added, (2.5 μg of negamycin, 5 μg of streptomycin, or 5 μg of kanamycin). The mRNA and inhibitor were added to a mixture just before initiation of incubation, which was performed at 26℃ for 30 minutes. At the end of the incubation, a 90 μl portion of each reaction mixture was transferred to a paper disc (Whatman 3 MM, 2.4 cm in diameter) and processed by the method of RUSTY *et al.*[15] to determine the trapped radioactivity. Averages of duplicate runs are shown. Sources of compounds and specific radioactivities (mCi/mmole, in parentheses) were: ^{14}C-phenylalanine (405), ^{14}C-valine (165), ^{14}C-leucine (270) and ^{14}C-isoleucine (270) from Dai-ichi Pure Chemical Co., Tokyo; ^{14}C-serine (162), ^{14}C-lysine (260), ^{14}C-asparagine (179), ^{14}C-proline (213) and ^{14}C-threonine (182) from New England Nuclear Co.; poly U and poly C from Miles; and poly A from Sigma.

The following miscodings by negamycin were negligible: poly U-directed Tyr UAU, UAC) and Val (GUU, GUC, GUA, GUG); poly A-directed Glu (GAA, GAG), Arg (AGA, CGU, CGC, CGA, CGG, AGG), Val (GUU, GUC, GUA, GUG) and Gln (CAA, CAG) poly C-directed His (CAU, CAC), Arg (CGU, CGC, CGA, CGG) and Ala (GCU, GCC, GCA, GCG). The underlined codons are included in discussin in the text.

(3) negamycin allows both transition and transversion miscoding.

Both streptamine and 2-deoxystreptamine, although lacking in antimicrobial activity, produce miscoding in cell-free extracts. Streptamine or 2-deoxystreptamine is contained in all aminoglycosides which cause miscoding such as streptomycin, kanamycin, neomycin, paromomycin, gentamicin, hygromycin B and destomycin A, but not in kasugamycin and spectinomycin, which do not cause miscoding. TANAKA *et al.*[7] and

* Infectious genetic materials, or episomes, directing synthesis of various drug-inactivating enzymes.

Fig. 2. These illustrations are based on molecular structures obtained with the use of CPK space filling models. The encircled groups of the 2 models can be superimposed.

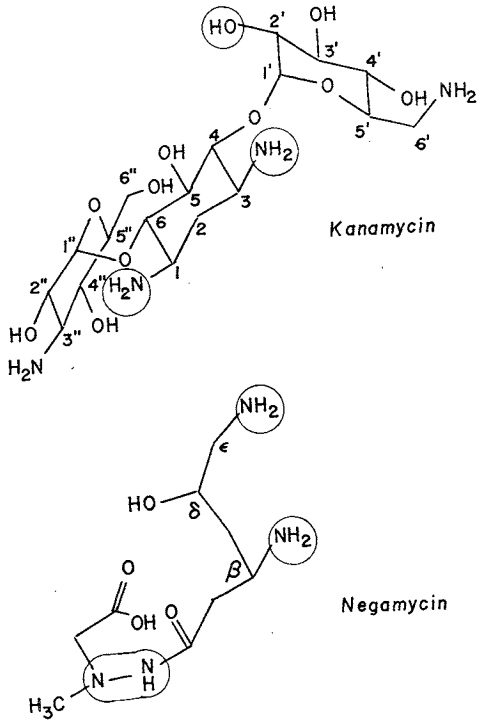
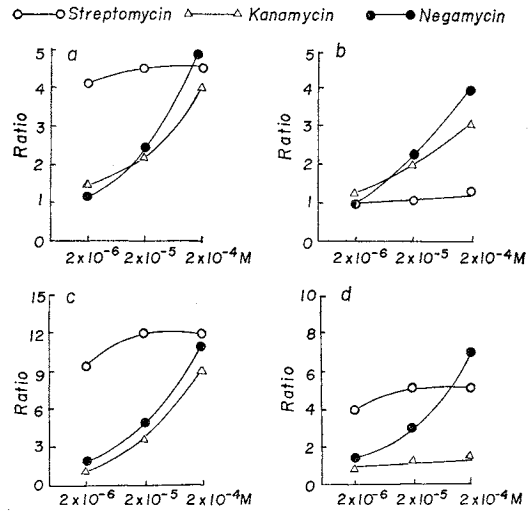


Fig. 3. Extent of miscoding and ribosomal resistance.

Miscoding (poly U-directed Ile) caused by negamycin, streptomycin or kanamycin was determined with the use of various ribosomal preparations (S-30 fractions prepared from *E. coli* strains Q13 (a) Q13-SM (b), NIHJ (c) and NIHJ-KM (d); see below) by the method described in the legend to Table 1. Strains of *E. coli* Q13 and NIHJ were successively transferred through media containing streptomycin and kanamycin, respectively, yielding corresponding resistant mutant strains abbreviated as *E. coli* Q13-SM (resistant to 400 μg/ml of streptomycin) and *E. coli* NIHJ-KM (resistant to 200 μg/ml of kanamycin). Ratio; (a) 269 cpm, (b) 415 cpm, (c) 611 cpm and (d) 413 cpm.



Davies[8] have proposed an essential structure for miscoding activity. They suggested that the structure of the 2-position of aminocyclitol residues (streptamine or 2-deoxystreptamine) of the aminoglycosides may play an important role in both miscoding and inhibition of protein synthesis. Negamycin, considering its primary structure, seems to be an exception to their proposal. However, use of CPK models (American Society of Biological Chemist, Inc.) revealed a possible similarity in the 3-dimensional structure between negamycin and the aminocyclitol antibiotics. As shown in Fig. 2, the positions of the hydrazide and β-amino groups in negamycin coincide with the 1- and 3-amino groups in the 2-deoxystreptamine moiety, and furthermore, the ε-amino group can be placed in a position similar to that of the 2′-hydroxyl group in kanamycin. In effect, the basic groups in a possible conformation of negamycin can be superimposed upon those of paromamine. For this conformation of negamycin, the existence of carbonyl and N-methyl groups and the stereochemistry of the β-amino and δ-hydroxyl groups are critical. In support of this view, an antipode of negamycin synthesized from 3-amino-3-deoxy-D-glucose[9] was shown to have much weaker antimicrobial and miscoding activities than negamycin.

The effect of negamycin on ribosomes which are resistant to aminoglycosidic antibiotics:

Streptomycin binds to a single site on the 30S subunit[10,11] containing a structure including protein P-10. In contrast, kanamycin binds to several sites on the 30S subunit,[12] in which protein P-10 is also thought to be involved.[13,14] These differences in the binding behavior can be illustrated in a dose-response relationship with the extent of miscoding plotted against increasing concentrations of the antibiotics. As shown in Fig. 3, the extent of miscoding by streptomycin reaches a plateau at a concentration as low as 10^{-6} M and stays almost unchanged

Table 2. Ribosomal resistance and inhibition of protein synthesis

Messenger	Concentration M	*E. coli* Q13		*E. coli* NIHJ	
		Sens.	SM-R	Sens.	KM-R
Poly U	SM 2×10^{-6}	61	1	39	62
	2×10^{-5}	62	9	57	71
	2×10^{-4}	62	27	59	70
	KM 2×10^{-6}	43	25	40	15
	2×10^{-5}	71	64	74	40
	2×10^{-4}	82	73	78	69
	NGM 2×10^{-6}	6	0	4	13
	2×10^{-5}	16	4	12	32
	2×10^{-4}	64	48	49	55
f2 RNA	SM 2×10^{-6}	100	2		
	2×10^{-5}	100	30		
	2×10^{-4}	100	50		
	KM 2×10^{-6}	60	35		
	2×10^{-5}	79	60		
	2×10^{-4}	90	80		
	NGM 2×10^{-6}	33	3		
	2×10^{-5}	63	34		
	2×10^{-4}	75	60		

Experimental conditions were as those described in the legend to Table 1 with a few modifications as follows: A reaction mixture for f2 RNA-directed protein synthesis contained 50 μg of f2 RNA prepared by the method of NATHANS *et al.*[16], instead of a synthetic mRNA, 1 μg of folinic acid and 300 μg of *E. coli* S-30. For f2 RNA-directed protein synthesis, the reaction mixture was incubated at 37°C for 30 minutes.

with increasing drug concentration. In contrast, negamycin and kanamycin progressively increase the extent of miscoding as their concentrations are increased from 10^{-6} to 10^{-4} M (Fig. 3-a, 3-c). In this respect, negamycin resembles kanamycin more closely than streptomycin, and indicates that several molecules of negamycin may bind to a single 30S ribosomal subunit. This is also supported by the fact that one-step mutants with high level resistance against negamycin and kanamycin are lacking while the reverse is true with streptomycin.[12] In addition, ribosomes which are resistant to either streptomycin or kanamycin are still sensitive to negamycin (Fig. 3); the miscoding ratio increased markedly with increasing concentrations of negamycin (Fig. 3-b and 3-d). In addition, protein synthesis carried out by either streptomycin- or kanamycin-resistant ribosomes was sensitive to negamycin (Table 2). Mutants with negamycin-resistant ribosomes have not been found to date. A mutant of *E. coli*, resistant to 200 μg/ml of negamycin, was isolated but its ribosomes were as sensitive to the antibiotic as those from the original strain.

In conclusion, it appears that the binding locus of negamycin on the 30S ribosomal subunit is not identical to that of the aminoglycosidic antibiotics. This is apparent from the absence of ribosomal cross resistance. Negamycin could distort the structure of ribosomes, especially the P-10 locus which controls the ambiguity in translation, in a manner similar to the aminoglycosides, because there is no basic difference between their miscoding spectra. Negamycin will provide a clue for elucidation of the structural basis for miscoding activity.

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