STRUCTURE-ACTIVITY RELATIONSHIPS AMONG NEGAMYCIN ANALOGS

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Various negamycin analogs were examined for (1) miscoding activity and (2) inhibition of the termination of protein synthesis. Since properties (1) and (2) do not correlate for the investigated compounds they may depend on different structural features of negamycin analogs. The results of biochemical and antimicrobial studies indicate that (a) the natural configuration of the carbon atom carrying the β-amino group is essential, (b) the δ-hydroxyl group is unnecessary, and (c) the acylation of the ε-amino group causes loss of activity.

Negamycin is an antibiotic effective against, among others, pseudomonas and multiple-drug resistant Gram-negative bacteria.[1] It causes miscoding in protein synthesis,[2,3,4] but unlike other antibiotics with miscoding activity which belong to a group of aminoglycoside antibiotics[5] it has a hydrazide structure.[4,6] We reported previously that negamycin inhibited the termination step in phage f2 RNA-directed protein synthesis[7] and that the termination step of protein synthesis must include a component step susceptible to negamycin action in a specific manner[8].

The present paper describes the results of further analysis of activities of various negamycin analogs and discusses the relationship between the structure and the biological activity of negamycin.

Materials and Methods

Escherichia coli S–30 extract, washed ribosomes, [^{14}C]Ile-tRNA, f[^{14}C]Met-tRNA·AUG·ribosome complex, and release factor protein were prepared as described previously.[8]

Polyuridylic acid (poly U) and UAA were purchased from Miles Laboratories. Uniformely labeled [^{14}C]methionine (250 mCi/mmole) and [^{14}C]isoleucine (279 mCi/mmole) were purchased from New England Nuclear and Daiichi Pure Chemicals, respectively.

Various negamycin analogs modified in the δ-hydroxy-β-lysine moiety were synthesized by the methods described previously.[9] Negamycin amide and (*R*,*R*)-δ-hydroxy-β-lysine amide were prepared from negamycin and (*R*,*R*)-δ-hydroxy-β-lysine methyl ester dihydrochlorides by treatment with ammonia gas in methanol. α-Ethylnegamycin, α-[2-(*R*,*R*)-δ-hydroxy-β-lysyl-1-methylhydrazino]-*n*-butyric acid, was synthesized from (*R*,*R*)-di-N,N′-benzyloxycarbonyl-δ-tetrahydropyranyloxy-β-lysine by coupling with α-(1-methyl-hydrazino)-*n*-butyric acid by the active ester method using N-hydroxy-succinimide followed by removal of the protecting groups. β-[2-(*R*,*R*)-δ-Hydroxy-β-lysyl-1-methyl-hydrazino] propionic acid was also prepared by a method similar to that described above.

Results

Miscoding Activity

Various analogs of negamycin were examined for their stimulatory effects on nonenzymatic binding of Ile-tRNA to ribosomes in the presence of poly U ("false binding"), and on the incorporation of Ile residues into peptide chains directed by poly U ("false incorporation"). These two assay systems gave similar indexes of miscoding activity of the compounds examined (Figs. 1, 2), with an exception of

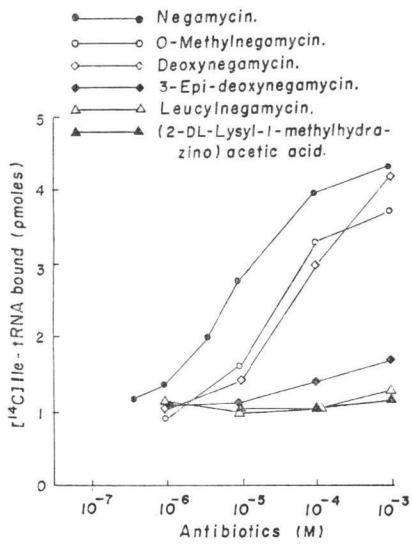
leucylnegamycin[10] which will be discussed later. Deoxynegamycin was about 1/10 as active as negamycin and 3-*epi*-deoxynegamycin, the stereoisomer of the former at the β-NH_2, was essentially devoid of stimulatory activity (Fig. 1a). The importance of correct configuration was also observed with the pair of negamycin and its antipode[4,11]. O-Methylnegamycin was somewhat more active than deoxynegamycin. (2-DL-Lysyl-1-methylhydrazino) acetic acid which has α-NH_2 instead of the β-NH_2 of negamycin, was totally inactive. Any modifications of the 1-methylhydrazinoacetic acid (MHA) moiety caused marked loss of activity. Negamycin fragments, (*R*,*R*)-δ-hydroxy-β-lysine amide and MHA showed no or slight activity (Fig. 1b). The extent of miscoding increases with increasing concentrations of negamycin, thus resembling kanamycin but not streptomycin[4,12]. The ribosomes prepared from streptomycin- or kanamycin-resistant *E. coli* retained full sensitivity to negamycin[4,8]. These characteristics suggest that there are multiple binding sites for negamycin on the ribosome.

A question arose whether the analogs with no miscoding activity lost the ability to bind to ribosome. This was tested by determining the miscoding effect of negamycin in the presence of 10 times molar concentration of a miscoding-inactive analog. As shown in Table 1 and Fig. 3, the antipode of negamycin and negamycin amide lowered the miscoding activity of negamycin, while 3-*epi*-deoxynegamycin lowered that of deoxynegamycin. The results indicate that there is competition for a common binding locus on the ribosome between these pairs and that the negamycin analogs without miscoding activity could bind to ribosomes almost as strongly as their miscoding partners. We do not know

Fig. 1. Effect of negamycin and its analogs on binding of [^{14}C]Ile-tRNA to ribosomes in the presence of poly U.

Stimulatory effect of negamycin and its analogs on binding of [^{14}C]Ile-tRNA to ribosomes in response to poly U was determined as follows. The reaction mixture contained in a total volume of 0.05 ml, 100 mM Tris-HCl, pH 7.5, 50 mM NH_4Cl, 20 mM magnesium acetate, $2A_{260}$ units of ribosomes, 10 μg of poly U, 10 pmoles ($0.44A_{260}$) of [^{14}C]Ile-tRNA and an indicated amount of a test compound. Incubation was carried out at 24°C for 20 min. and [^{14}C]Ile-tRNA bound to ribosomes was measured using nitrocellulose filters as previously described[8]. The amount of [^{14}C]Ile-tRNA bound to ribosomes in the absence of an antibiotic were 1.05 and 1.14 pmoles in (a) and (b), respectively.

(a) Modification of the δ-hydroxy-β-lysine moiety.



(b) Fragments of negamycin and modification of the 1-methylhydrazinoacetic acid moiety.

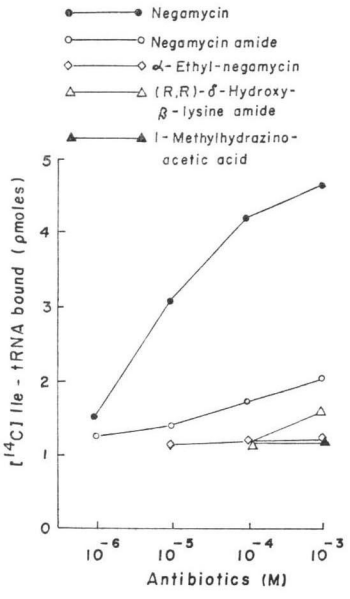
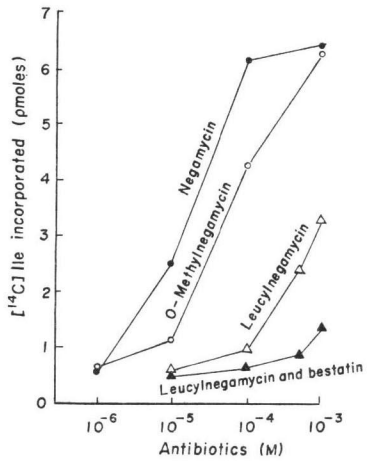


Fig. 2. Stimulation of poly U-dependent ^{14}[C]Ile incorporation into protein

The reaction mixture contained in 0.1 ml, 50 mM Tris-HCl, pH 7.8, 160 mM NH_4Cl, 3 mM ATP, 0.2 mM GTP, 15 mM magnesium acetate, 2 mM phosphoenolpyruvate, 5 μg of pyruvate kinase, 2 mM dithiothreitol, 0.4 nmole of [^{14}C] isoleucine (273 mCi /mmole), 1 nmole of each of 19 other amino acids, 30 μg of tRNA, an S-30 fraction from *E. coli* Q 13 (200 μg protein), 20 μg of poly U and an appropriate amount of an inhibitor. The mixture was incubated at 36°C for 10 minutes. At the end of incubation, a 90 μl portion of each reaction mixture was transferred on a paper disk (Whatman 3 MM, 2.4 cm in diameter) and the radioactivity in the hot trichloroacetic acid insoluble materials was determined as described previously[4]. Inhibitors and bestatin (50 μg/ml) were added just before addition of poly U. The amount of [^{14}C]Ile incorporated in the absence of an antibiotic was 0.4 pmole.



why some of these ligands cause miscoding but others not. No competition was observed with α-ethylnegamycin and other compounds (Fig. 3, Table 1).

Activity Inhibiting the Termination Process of Protein Synthesis

Release of formylmethionine (fMet) from the f[^{14}C]Met-tRNA·AUG·ribosome complex in responce to UAA and release factors (RF) was utilized as a model reaction of the termination process[18]. Negamycin at 10^{-4} inhibited this reaction by 50%. Deoxynegamycin, 3-*epi*-deoxynegamycin, O-methylnegamycin and negamycin amide at 5×10^{-4} M showed the same

Fig. 3 Miscoding activity of antibiotics in combination.

Determination of the extent of miscoding ("false binding") by (a) negamycin alone and in combination with α-ethylnegamycin, and (b) deoxynegamycin alone and in combination with 3-*epi*-deoxynegamycin. These compounds were added to each reaction mixture simultaneously. Otherwise, conditions were the same as the experiment for Fig. 1.

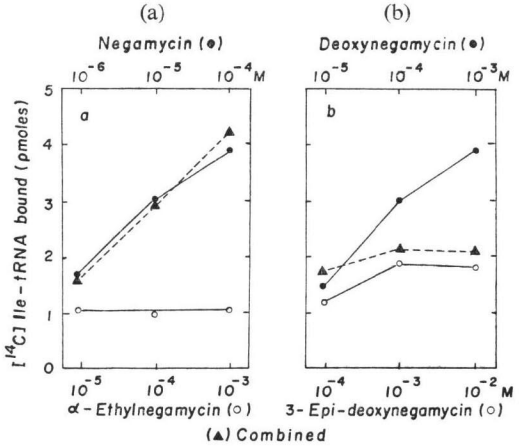


Fig. 4 Inhibition of termination reaction by negamycin and its analogs.

The termination reaction was carried out as described previously[8]. The reaction mixture contained in 0.05 ml, 50 mM Tris acetate, pH 7.2, 75 mM ammonium acetate, 30 mM magnesium acetate, 1.5 p-moles of f[^{14}C]Met-tRNA·AUG·ribosome complexes, 0.1 A_{280} unit of UAA, 70 μg of crude release factor protein, and a desired amount of an inhibitor. Incubation was conducted at 24°C for 15 minutes. The amount of fMet released in the run with no inhibitor was 0.56 pmole. The UAA independent release of fMet (0.07 pmole) was subtracted from each measurement.

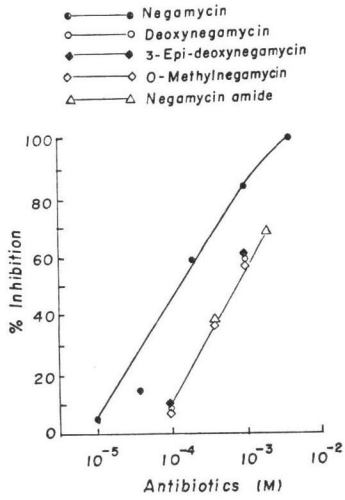
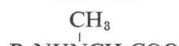
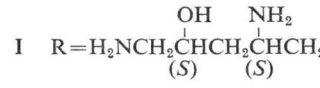
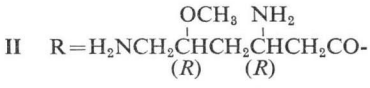


Table 1a, b. Biochemical and antimicrobial activities of negamycin and its analogs

Table 1a.

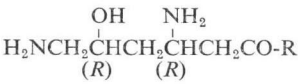


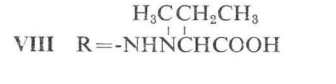


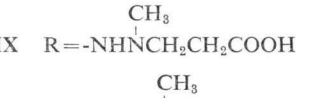


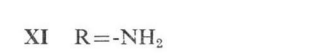
	Compound	Miscoding		Inhibition of termination reaction	Antimicrobial activity
		Activity (assay system)	Competition observed		
I	Antipode	0.1 (B)	Yes	nt	2.0
II	O-Methylnegamycin	20 (A, B)	nt	20	33
III	Deoxynegamycin	10 (A)	nt	20	29
IV	3-*epi*-Deoxynegamycin	0.2 (A)	Yes	20	2.5
V	(2-DL-Lysyl-1-methylhydrazino) acetic acid	0 (A)	No	0	<0.6
VI	Leucylnegamycin	0.1 (A), 3 (B)	nt	3	42

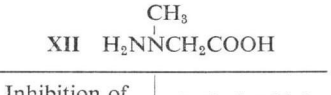
Table 1b.











	Compound	Miscoding		Inhibition of termination reaction	Antimicrobial activity
		Activity (assay system)	Competition observed		
VII	Negamycin amide	0.2 (A)	Yes	20	15~25
VIII	α-Ethylnegamycin	0 (A)	No	1	<0.6
IX	β-[2-(*R*,*R*)-δ-Hydroxy-β-lysyl-1-methylhydrazino] propionic acid	0 (A)	No	nt	<0.6
X	(*R*,*R*) δ-Hydroxy-β-lysine	0 (B)	nt	nt	<0.6
XI	(*R*,*R*) δ-Hydroxy-β-lysine amide	0.2 (A)	No	2	<0.6
XII	1-Methylhydrazinoacetic acid	0 (A, B)	No	0	3.2

Numerals indicate relative activities based on negamycin activity taken as 100(%). Miscoding activity was assayed by false binding (A) and/or false incorporation (B). Some analogs which showed no or slight miscoding activity were then tested in the same systems in combination with negamycin (or deoxynegamycin in case of analog IV). In this test, a combination of antipode (analog I) and negamycin, for example, showed a less extent of miscoding than that caused by negamycin alone (see text); this effect of antipode is indicated as "yes" under the column of "competition observed". The antimicrobial activities were determined by the cylinder agar plate method using *E. coli* K12 as a test organism.

nt: not tested

effect; they were 1/5 as active as negamycin (Fig. 4). It should be remembered that, among these analogs, 3-*epi*-deoxynegamycin and negamycin amide were only 1/500 as active as negamycin in causing miscoding (Fig. 1a, 1b). Based on these discrepancies, together with other observations, a specific effect of negamycin on the termination process was proposed[8].

Correlation with Antimicrobial Activity

The biochemical activities of negamycin analogs in cell-free systems described above, were compared with the antimicrobial activities which had been determined by the agar dilution method using 0.5% peptone-agar plates (Table 1). For each parameter the activity of negamycin was taken as 100%. There was a good correlation between biochemical and antimicrobial activities with few exceptions. For instance, 3-*epi*-deoxynegamycin was as active as negamycin amide in cell-free systems, although the former was much weaker than the latter in antimicrobial activity (see discussion). The bacteriostatic activity of MHA (unpublished data) could be due to its competitive effect on pyridoxal phosphate of glutamate-pyruvate transaminase and hence its mode of action is unrelated to that of negamycin.

Discussion

Aminoglycoside antibiotics with miscoding activity are bactericidal while those which lack this biochemical characteristics are bacteriostatic. The direct correlation between the miscoding activity and bactericidal activity is possible on the basis of the following observations; (1) miscoding has been demonstrated with natural mRNA[14] and in intact cells,[15] (2) ribosomes from an antibiotic-resistant mutant strain do not show miscoding *in vitro* in the presence of the antibiotic[4,16]. Up to now, however, the question as to how these two activities are linked is still unsettled[17]. As the miscoding activity *in vitro* can be assayed readily and free from disturbing influences due to permeation and/or metabolism, it was used as a parameter expressing the activity of a compound in structure-activity studies of some aminoglycoside antibiotics[18]. The negamycin analogs were also assayed for their ability to inhibit the termination of protein synthesis since negamycin has such an activity[7,8] as well as miscoding activity. Structure-activity studies may provide clues for producing useful derivatives of negamycin.

For miscoding activity but not for inhibition of termination, *R*-configuration of β-NH_2 is essential. *R*- and *S*-Stereoisomers showed competition with respect to miscoding activity (Fig. 3), and therefore they must share a common binding locus on a ribosome with similar affinities. Deoxynegamycin and O-methylnegamycin, both lacking the δ-OH structure, were only 1/5 as active as negamycin in cell-free system. However, these analogs may be of value since the instability of negamycin, especially at low pH, is thought to be due to the δ-OH[9].

Leucylnegamycin (Leu at ε-NH_2), which has considerable antimicrobial activity, was not active in the "false binding" and weakly active in the "false incorporation" tests (Fig. 1a, Fig. 2). The assay mixture of the "false incorporation" test included cell-free extract of *E. coli* in which some aminopeptidase, responsible for degradation of leucylnegamycin into leucine and negamycin, must be present. Evidence for this is that leucylnegamycin became inactive in the same assay system when bestatin, an inhibitor of aminopeptidase,[19] was added simultaneously (Fig. 2). The antimicrobial activity of leucylnegamycin must be exerted by enzymic release of negamycin in the cells. Leucylnegamycin is thought to be a direct precursor of negamycin in biosynthesis[10] as in the case of leucylblasticidin S[20].

The terminal carboxyl of MHA is essential, especially for miscoding activity, as its amidation (negamycin amide) leads to a 500-fold decrease in miscoding activity. However, negamycin amide is still 1/5 as active as negamycin in inhibition of the termination reaction. Negamycin amide is similar to 3-*epi*-deoxynegamycin both in miscoding activity and activity inhibiting the termination reaction, but they differ in antimicrobial activity; the former inhibits more strongly the growth of *E. coli* K12 than the latter. This discrepancy may be ascribed to different metabolism and/or permeability. Negamycin amide could be hydrolyzed by an amidase yielding negamycin in target cells. 3-*Epi*-deoxynegamycin shows about 1/2 activity of negamycin against Gram-positive bacteria such as *Staphylococcus*

aureus and *Bacillus subtilis*. (data not shown).

The loss of antimicrobial activity results from modifications of the MHA moiety, such as introduction of alkyl groups at the α-position and extension of the MHA chain with one methylene unit.

A question remains as to which one of the inhibitory effects, miscoding or inhibition of termination, is responsible for the antimicrobial activity of negamycin. On the basis of dose-response relations, the miscoding effect was about 10 times greater than inhibition of termination. However, it has not been clearly ruled out that the two biochemical effects simply reflect differences in number and/or sites of negamycin molecule bound to the ribosome. Miscoding as well as inhibition of termination showed good correlation with antimicrobial activity. Therefore, both mechanisms of action may be operative in an additive and/or synergistic way for the antimicrobial activity. The bactericidal effect of negamycin, exerted even in the presence of chloramphenicol, can be ascribed to its irreversible effect on polysomal ribosomes as well as on single ribosomes.[21]

It has been suggested that negamycin permeates into *E. coli* cells through, at least in part, the lysine permeation system (unpublished data). This permeation system must be present in a wide variety of microorganisms and hence could explain the broad antimicrobial spectrum of negamycin.[1] Permeation of the negamycin analogs remains to be studied.

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