

MECHANISM OF ACTION OF NEOTHRAMYCIN

II. INTERACTION WITH DNA

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The previous biochemical investigations (MARUYAMA, I. N. *et al.* *J. Antibiotics*, 31: 761~768, 1978) suggested that DNA is the chemoreceptor of neothramycin, a new antitumor antibiotic. Therefore, the interaction of the drug with DNA was studied in the current experiments, using UV, circular dichroism and fluorescence spectroscopies, and a [¹⁴C]neothramycin binding method. The UV absorption spectrum of neothramycin exhibited bathochromic shift and hypochromic change upon reaction with native calf thymus DNA, indicating a reaction of the antibiotic with DNA. The binding of the drug with native DNA was also demonstrated by circular dichroism. It was further observed that [¹⁴C]neothramycin interacted with native DNA.

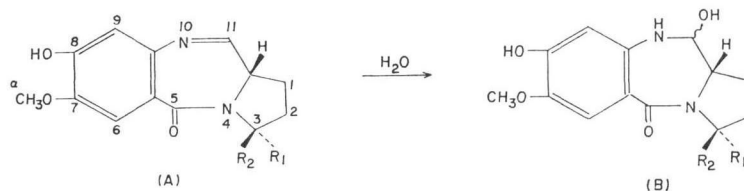
The antibiotic bound to native DNA and poly[dG·dC] more markedly than heat-denatured DNA, poly[dA·dT] and poly[dG]·poly[dC]. Fluorescence of neothramycins A and B was enhanced by native DNA, but not significantly by RNA and heat-denatured DNA. The emission maximum (420 nm) was the same in the presence and absence of DNA. Fluorospectrometric studies revealed that 10,11-dihydroneothramycins A and B, and their 3-O-butyl derivatives did not bind to DNA; and DNA reacted with 3-O-alkylneothramycin B, but not with 3-O-alkylneothramycin A. The current experiments showed that the reaction rate of neothramycin with native DNA was slower than those of other DNA-binding antibiotics, including anthramycin, tomaymycin, sibiromycin, adriamycin, and actinomycin. The time required for neothramycin to saturate DNA was several hours. The results suggested that neothramycin may recognize or interact with a highly specific portion of double helical structure of DNA, and then a covalent bond may be formed between C-11 of the antibiotic and guanine or cytosine base of DNA.

Neothramycin is a new member of pyrrolo(1,4)benzodiazepine group antibiotics. The properties, structure, and biosynthesis have been described in previous reports^{17,18,22}). The antibiotic contains two stereoisomers A and B in nearly equal amounts. Both are hydrated within 60 minutes after being dissolved in water, forming the 10-hydro-11-hydroxyl derivatives (Fig. 1). The hydroxyl groups at

Fig. 1. Structures of neothramycins (A) and their hydrated forms in water (B).

Neothramycin A: R₁=OH, R₂=H; neothramycin B: R₁=H, R₂=OH.

^aC: This carbon of [¹⁴C]neothramycin was labelled with radioisotope.



C-3 and C-11 epimerize in aqueous solution.

Neothramycin exhibits a significant activity on YOSHIDA sarcoma in rats, and sarcoma 180 and leukemia L-1210 in mice. It shows lower toxicity than other pyrrolo(1,4)benzodiazepine antibiotics: anthramycin, tomaymycin and sibiromycin²²⁾.

We have studied the mechanism of action of neothramycin as one of the basic investigations for clinical application. Moreover, since neothramycin possesses the simplest structure in the pyrrolo (1,4)benzodiazepine group of antibiotics, the elucidation of the precise mechanism of action is of interest. The effects on macromolecular syntheses of mouse tumor cells and *Escherichia coli* have been reported in a previous paper¹⁹⁾. The antibiotic shows a preferential block of RNA synthesis over DNA synthesis *in vivo* as well as *in vitro*; but does not significantly affect protein synthesis. The inhibition by neothramycin of polymerase reactions is reversed by increasing concentrations of template DNA but not by increased enzymes, suggesting that the blockage may be caused by the interference with template activity of DNA.

The current publication describes the results of experiments concerning the interaction of neothramycin with DNA, examined by means of UV, circular dichroism and fluorescence spectroscopies, and binding of [¹⁴C]neothramycin to DNA. Fluorospectrometric studies on the binding of various neothramycin derivatives have provided some information concerning the importance of groups on the antibiotic molecule.

Materials and Methods

Chemicals

Neothramycin (A and B mixture), neothramycins A and B, and their derivatives were prepared, following the procedure described previously¹⁷⁾. [¹⁴C]Neothramycin was biosynthesized in culture broth of *Streptomyces* No. MC916-C4, which was fed with L-[CH₃-¹⁴C]methionine, and purified by the method reported in a previous paper¹⁸⁾. The 7-methoxyl group was labelled with radioactive carbon. The specific activity was 230 μ Ci/mmol, and the radiochemical purity was more than 99% (by thin-layer chromatography).

Tomaymycin was generously given by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. Calf thymus DNA, *E. coli* DNA, poly[dG·dC], poly[dG]·poly[dC], and poly[dA·dT] were products of P-L Biochemicals, Milwaukee, Wis.

DNA Solution

DNA was dissolved at concentration of *ca.* 1 mg/ml in an aqueous medium, containing 50 mM NaCl and 10 mM disodium EDTA; and dialysed overnight at 7°C against 50-fold volume of the same medium with subsequent dialysis against 50 mM NaCl solution. It is stored at 2°C. DNA concentration was assayed as inorganic phosphate, following the method of AMES¹⁾. Heat-denatured DNA was prepared by heating at 100°C for 5 minutes in a water bath, and then immediately chilled in an ice-bath.

UV Absorption Spectroscopy

Absorption spectra were recorded with a Shimadzu spectromonitor UV-202. The temperature of the sample compartment was controlled to $\pm 0.5^\circ\text{C}$ by means of a circulating bath HAAKE FT. In kinetic experiments, the reaction was initiated by the addition of a small volume of antibiotic solution to a cuvette containing DNA solution. The cuvette was kept at least for 10 minutes in the temperature-controlled sample compartment, prior to the addition of antibiotics. DNA melting profiles were observed on a Gilford 2000 multiple-sample absorbance recorder, equipped with an automatic temperature programmer and a temperature-controlled circulating bath (HAAKE FT). The temperature was raised at a rate of *ca.* 0.3°C/min.

Circular Dichroism

The spectra were recorded on a Jasco J-500A spectropolarimeter, equipped with a data processor JP-500 (a light path of 0.1 cm).

[¹⁴C]Neothramycin Binding to DNA

The interaction of [¹⁴C]neothramycin with DNA was measured by radioactivity remaining on DNA, after five times rapid extraction of the free antibiotic with 3-fold volumes of water-saturated *n*-butanol. After solvent extraction, the aqueous phase of 0.5 ml was mixed with an equal of 1 M HCl, warmed at 80°C for 20 minutes, and then cooled to room temperature. The radioactivity was determined on a liquid scintillation counter (Beckmann LS-230), in toluene scintillator containing 60% Triton X-100, 0.5% PPO and 0.1% POPOP.

Fluorescence Spectroscopy

Corrected and uncorrected fluorescence spectra were recorded on a Hitachi spectrofluorometer model MPF-4, equipped with a data processor and a Hitachi 057 recorder. All measurements were performed in a cuvette with a 1-cm light path at angle of 90° to excitation beam. The temperature of the sample compartment was controlled to ± 0.5°C by a Sharp TE-12 circulating bath. The kinetic experiments were carried out by the same procedure as described in UV Absorption Spectroscopy, in which the first measurement was performed within one minute.

Results

Alterations of UV Absorption Spectrum of Neothramycin in the Presence of DNA

The UV absorption spectrum of neothramycin showed bathochromic shift and hypochromic change upon reaction with native calf thymus DNA (Fig. 2A). The difference spectrum of the drug in the presence and absence of DNA is illustrated in Fig. 2B. The greatest difference of absorbance was found at 347 nm and 312 nm, and isosbestic point at 327 nm. The spectral change needed several hours to reach the maximum, the reaction appeared to be more gradual than other pyrrolo(1,4)benzodiazepine antibiotics: anthramycin, tomaymycin, and sibiromycin (*cf.* a review⁹⁾). On the contrary, no significant change of UV spectrum was observed with heat-denatured calf thymus DNA. The results suggested that neothramycin binds to native DNA, whose double-stranded helical structure is required for the interaction.

Circular Dichroism of Neothramycin and DNA

Fig. 2. UV absorption spectra of neothramycin.

A. The spectra in the presence and absence of calf thymus DNA. (—) 0.19 mM neothramycin in 0.01 M sodium phosphate and 0.5 mM EDTA, pH 7.2; (·····) 0.19 mM neothramycin, incubated at 37°C for 60 minutes with 2.7 mM calf thymus DNA in the same buffer.

B. The difference spectra in the presence of native or heat-denatured DNA. Neothramycin 0.33 mM was incubated with 1.28 mM DNA at 37°C in the above buffer for various periods: (—) with native DNA for 5, 10, 20, 30 and 60 minutes; (·····) with heat-denatured DNA for 10 and 60 minutes.

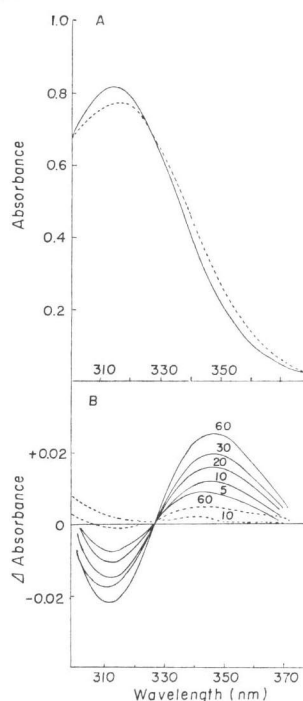
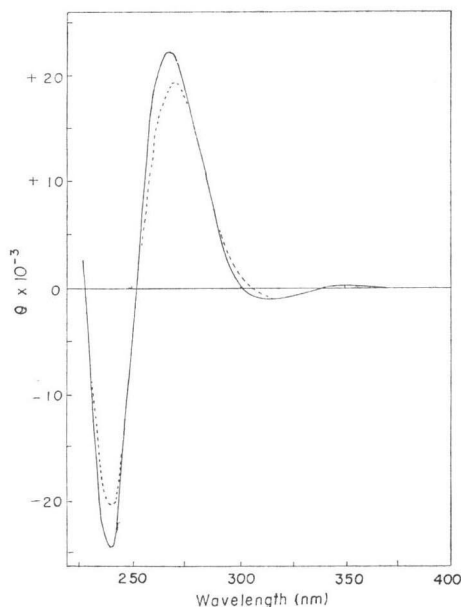


Fig. 3. Circular dichroism spectra of neothramycin and DNA.

Neothramycin 0.531 mM and calf thymus DNA 1.71 mM in 0.1 M potassium phosphate buffer, pH 7.0, were kept in a single cuvette (.....) or in separate ones (—) at room temperature for 90 minutes.



The circular dichroism spectrum of neothramycin exhibited a maximum at 264 nm and a minimum at 239 nm (data are not shown). As presented in Fig. 3, the circular dichroism of neothramycin-DNA complex showed bathochromic and hypochromic changes, indicating that the antibiotic reacts with DNA.

Binding of [¹⁴C]Neothramycin to Polynucleotides

The binding of labelled neothramycin to various forms of DNA was studied by *n*-butanol extraction method. The results are summarized in Table 1. By the method employed, the antibiotic was observed to bind to native DNA of calf thymus or *E. coli* approximately five times more than to the heat-denatured DNA. The binding of neothramycin was dependent upon concentrations of DNA and the drug. The reaction was slow and required several hours to reach the maximum.

Neothramycin was found to interact with poly[dG·dC] copolymer more effectively than poly[dA·dT] copolymer and poly[dG]·poly[dC] homopolymer, suggesting that specific base and structure of DNA is required for the antibiotic binding. Poly[dG·dC] showed less magnitude of reaction with neothramycin than native DNA did. A little binding of the drug to heat-denatured DNA, poly[dA·dT] or poly[dG]·poly[dC] might be due to non-specific binding or to incomplete extraction of the free antibiotic by the solvent.

Heating the neothramycin-DNA complex resulted in releasing a part of the drug from native DNA. However, the amount of neothramycin, remaining in the complex, was much larger than the amount bound to heat-denatured DNA, suggesting that most of the antibiotic bound to native double-stranded DNA retained its binding to single-stranded DNA even after denaturation of DNA.

Table 1. Binding of [¹⁴C]neothramycin to polynucleotides.

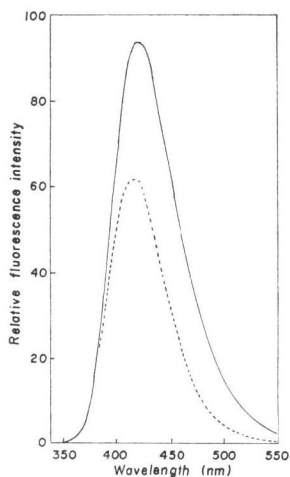
[¹⁴C]Neothramycin of 0.76 mM was reacted at 37°C for 2.5 hours with 0.29 mM DNA or polydeoxyribonucleotide in 20 mM potassium phosphate, pH 7.4 and 0.5 mM EDTA; and the radioactivity bound to polynucleotide was determined by rapid solvent extraction method (see Materials and Methods).

Polynucleotide	Neothramycin bound nmoles/100 nmoles nucleotide
Calf thymus DNA	
native	1.50 ± 0.13*
heat-denatured	0.27 ± 0.13
<i>E. coli</i> DNA	
native	1.31 ± 0.12
heat-denatured	0.32 ± 0.13
Poly[dG·dC]	0.80 ± 0.13
Poly[dG]·poly[dC]	0.22 ± 0.13
Poly[dA·dT]	0.32 ± 0.13
Calf thymus DNA**	1.04 ± 0.13

* Mean ± S.D.; n=3.

** DNA-neothramycin complex was warmed at 100°C for 5 minutes, cooled rapidly to room temperature, and then free antibiotic was removed.

Fig. 4. Fluorescence emission spectra of neothramycin A. Neothramycin A $4.0 \mu\text{M}$ in 50 mM TES-KOH buffer, pH 7.2, 5.0 mM disodium EDTA, 25 mM NaCl, and 0.1% (v/v) methanol; (—) corrected spectrum, (.....) uncorrected spectrum.



Fluorospectrometric Studies on the Active Center of Neothramycin Molecule for the Binding to DNA

The interaction of neothramycins and their derivatives to DNA was investigated by fluorescence spectroscopy, which was able to detect formation of small amounts of the antibiotic-DNA complex. The emission fluorescence spectrum of neothramycin A is illustrated in Fig. 4 (excitation at 316 nm). Fluorescence of neothramycin A or B was enhanced to a great extent by the presence of native DNA, but not significantly by RNA or heat-denatured DNA. The emission maximum (420 nm) of neothramycins was the same in the presence and absence of DNA. The fluorescence enhancement was dependent upon incubation period of the antibiotic-DNA mixture, increasing for several hours (Fig. 5). The results showed the same tendency of slow reaction rate as observed by UV absorption spectroscopy and [^{14}C]neothramycin binding, indicating that the enhancement by DNA of neothramycin fluorescence paralleled its binding to DNA. Therefore, the interaction of neothramycins and their derivatives was studied by fluorospectrometry, and the results are summarized in Table 2. Fluorescence intensity of 10,11-dihydroneothramycins A and B, and their 3-O-butyl derivatives,

Fig. 5. Time dependency of interaction of neothramycins A and B with native DNA.

The reaction mixture contained $4.0 \mu\text{M}$ neothramycin A or B, and 1.0 mM native calf thymus DNA in 50 mM TES-KOH buffer, pH 7.2, containing 5 mM disodium EDTA, 25 mM NaCl, and 0.1% (v/v) methanol. It was incubated at 25°C for indicated periods.

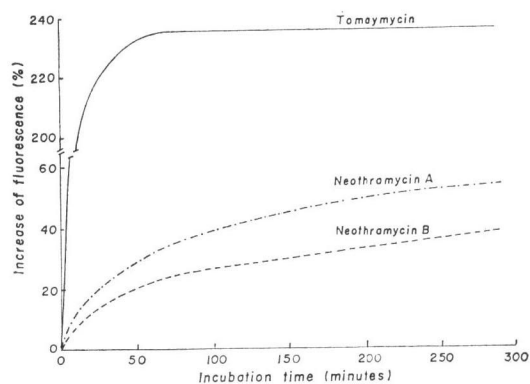


Table 2. Fluorescence enhancement of neothramycins and their derivatives by the presence of native calf thymus DNA.

The reaction mixture contained: $4.0 \mu\text{M}$ antibiotic and 1.0 mM native calf thymus DNA in a buffer of 50 mM TES-KOH, pH 7.2, 5 mM disodium EDTA, 25 mM NaCl, and 0.1% (v/v) methanol. In the case of tomaymycin, $3.3 \mu\text{M}$ drug and 0.66 mM DNA were used. It was incubated at 25°C for 90 minutes. Excitation at 316 nm and emission at 420 nm.

Antibiotics	ΔF^* %
Neothramycin (A and B mixture)	30.0
Neothramycin A	37.0
Neothramycin B	24.5
3-O-Methylnethramycin A	0.0
3-O-Methylnethramycin B	13.1
3-O-Butylnethramycin A	0.0
3-O-Butylnethramycin B	24.0
10,11-Dihydroneothramycin (A and B)	0.0
3-O-Butyl-10,11-dihydroneothramycin A	0.0
3-O-Butyl-10,11-dihydroneothramycin B	0.0
Tomaymycin	235.0

* Increase of fluorescence intensity.

which do not form the 11-hydroxyl compounds in water, was not stimulated by DNA, indicating that the C-11 of the antibiotics may react with DNA. 3-O-Alkylation of neothramycin B did not significantly affect the interaction with DNA, suggesting that the C-3 of the antibiotics does not react with DNA. 3-O-Alkyl derivatives of neothramycin A did not interact with DNA, suggesting that 3-O-alkyl groups may cause steric hindrance for the binding to DNA in the case of neothramycin A derivatives.

Discussion

The current experiments have revealed that neothramycin directly binds to DNA. DNA may be the chemoreceptor of the antibiotic, and the interaction with DNA may result in the inhibition of nucleic acid synthesis, as described in a previous paper¹⁹. However, neothramycin did not significantly affect the melting profiles of DNA, while tomaymycin raised T_m of DNA (data are not shown). Anthramycin and sibiromycin have been reported to increase T_m of DNA under similar conditions^{4,9,13,20}. The reason for the discrepancy remains open to discussion. The amount of neothramycin bound to DNA may be too small to cause T_m shift, and/or the interaction may not result in intercalation or cross-link of DNA double strands.

Other pyrrolo(1,4)benzodiazepine antibiotics, such as anthramycin, tomaymycin and sibiromycin, also react with DNA^{3,5,8,11,20,21}. However, the mode of interaction of neothramycin appears to be different from those of the other pyrrolo(1,4)benzodiazepines in various aspects: (a) slower reaction rate, (b) little shift of melting temperature of DNA, (c) little binding to poly[dG]·poly[dC], and (d) much less binding to single-stranded DNA than to double-stranded DNA. These differences may be attributed to a simple and unique structure of neothramycin, lacking in a 2-side chain and possessing a 3-hydroxyl group.

The results of the binding with various forms of polydeoxyribonucleotides suggest that neothramycin recognizes a certain specific structure of double helical DNA, forming a covalent bond with guanine or cytosine base. The reaction rate of neothramycin is slower than those of anthramycin, tomaymycin, and sibiromycin^{9,12,13}. Moreover, the pyrrolo(1,4)benzodiazepine antibiotics interact with DNA more slowly than other DNA-binding antibiotics: actinomycin, adriamycin *etc.*^{9,12,14,16}. The slow reaction rate also supports the above assumption that neothramycin may interact with a specific portion of double helical structure, and then react covalently with DNA base.

From studies on structure-activity relationship of the pyrrolo(1,4)benzodiazepine antibiotics, HURLEY⁶ has proposed following assumptions: (a) An unsaturated side chain at position 2 of the pyrrole ring may be a requirement for the activity; (b) The 9-hydroxyl group is important for the activity; (c) The carbinolamine at positions 10 and 11 is essential for the activity.

Since neothramycin is lacking in a side chain at position 2, the 2-unsaturated side chain is not essential for the activity. However, the slow reaction rate of neothramycin with DNA may be attributed to lack of the 2-side chain. The 8-hydroxyl group of neothramycin, as well as tomaymycin, seems to be equivalent to the 9-hydroxyl group of anthramycin and sibiromycin for the activity.

Anthramycin, tomaymycin, and sibiromycin have been postulated to bind covalently to DNA base, particularly guanine, at C-11^{6,7,9,10}. Unlike these antibiotics, neothramycin (hydrated form) has hydroxyl groups at C-3 and C-11, which appear to be functionally equivalent. Since 10,11-dihydrogenation of neothramycin results in loss of DNA-binding activity, DNA base may react with the C-11 of neothramycin but not with the C-3. The effect of alkylation of the 3-hydroxyl group on DNA-binding activity is different between neothramycin isomers. The reason for the discrepancy remains to be determined. However, the nucleophilic attack of DNA base to the C-11 may be prevented by steric hindrance of the 3-O-alkyl group in neothramycin A, but not in neothramycin B.

The precise association constant and stoichiometry of neothramycin binding to DNA could not be determined in the present experiments, because of the slow reaction rate and insufficient stability of the antibiotic.

The enhancement by DNA of emission fluorescence (420 nm) of neothramycin and tomaymycin

is first observed in the current experiment. The excitation maximum (316 nm) is sufficiently away from UV absorption band of DNA. The distance between the maxima of excitation and emission is longer than 100 nm. The binding of neothramycin to DNA is irreversible in comparison with reversible binding of intercalating agents, such as acridine orange, proflavine, and ethidium bromide¹⁵⁾. Moreover, the emission maximum and intensity of neothramycin fluorescence change in varying polarities and pH's in the solution. These characteristics indicate the utility of neothramycin and tomaymycin as fluorescence probes in studies on fine structure of DNA and biochemical reactions of certain drugs and proteins with DNA.

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