NMR studies on the binding of antitumor drug nogalamycin to DNA hexamer d(CGTACG)

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ABSTRACT

The interactions between a novel antitumor drug nogalamycin with the self-complementary DNA hexamer d(CGTACG) have been studied by 500 MHz two dimensional proton nuclear magnetic resonance spectroscopy. When two nogalamycins are mixed with the DNA hexamer duplex in a 2:1 ratio, a symmetrical complex is formed. All non-exchangeable proton resonances (except H5′ & H5″) of this complex have been assigned using 2D-COSY and 2D-NOESY methods at pH 7.0. The observed NOE cross peaks are fully consistent with the 1.3 Å resolution x-ray crystal structure (Liaw et al., *Biochemistry* 28, 9913 – 9918, 1989) in which the elongated aglycone chromophore is intercalated between the CpG steps at both ends of the helix. The aglycone chromophore spans across the GC Watson-Crick base pairs with its nogalose lying in the minor groove and the aminoglucose lying in the major groove of the distorted B-DNA double helix. The binding conformation suggests that specific hydrogen bonds exist in the complex between the drug and guanine-cytosine bases in both grooves of the helix. When only one drug per DNA duplex is present in solution, there are three molecular species (free DNA, 1:1 complex and 2:1 complex) in slow exchange on the NMR time scale. This equilibrium is temperature dependent. At high temperature the free DNA hexamer duplex and the 1:1 complex are completely destabilized such that at 65°C only free single-stranded DNA and the 2:1 complex co-exist. At 35°C the equilibrium between free DNA and the 1:1 complex is relatively fast, while that between the 1:1 complex and the 2:1 complex is slow. This may be rationalized by the fact that the binding of nogalamycin to DNA requires that the base pairs in DNA open up transiently to allow the bulky sugars to go through. A separate study of the 2:1 complex at low pH showed that the terminal GC base pair is destabilized.

INTRODUCTION

Nogalamycin (Figure 1) is an antitumor anthracycline antibiotic active against a number of tumor cell lines (1). It differs from other anthracycline antibiotics (e. g., daunorubicin and doxorubicin) in that it contains two sugar moieties (nogalose and aminoglucose) attached to rings A and D at the ends of the elongated aglycone chromophore (2,3). These anthracycline antibiotics bind to DNA double helix by intercalation (3). Their DNA binding affinity and sequence specificity are likely to be closely related to their biological activities. To better correlate this structure-function relationship and to design better agents based on these correlation, it is useful to have a detailed view of how drug molecules interact with their target DNA molecules by different biochemical and biophysical methods.

Nogalamycin, with bulky sugars attached at both ends of the chromophore, poses an interesting question with respect to the ways in which it inserts itself between the base pairs. These sugars are too bulky to slide through between the base pairs without breaking the hydrogen bonds between the bases. Conversely, the bound nogalamycin would be difficult to dissociate from the DNA double helix. Furthermore, there are at least two possibilities to orient the aglycone chromophore depending on whether the nogalose resides in the minor or major groove of the double helix. Another important issue related to nogalamycin binding to DNA is its nucleotide sequence specificity. DNase I footprinting experiments of nogalamycin on several DNA restriction fragments suggested that the drug binds to alternating purine-pyrimidine sequence such as TpG and GpT, though some ambiguities still remain (4).

Recently, we have determined the three dimensional structure of the complexes between nogalamycin and two modified DNA hexamers, d[CGT(pS)ACG] and d[m^{5}CGT(pS)Am^{5}CG] where pS is a thiophosphate linkage, at high resolution by x-ray diffraction (5). A similar structure of the latter complex from a different crystal form has been determined independently (6). The results of these analyses showed that the two nogalamycin molecules are intercalated between the CpG steps at both ends of a distorted B-DNA double helix. The elongated aglycone

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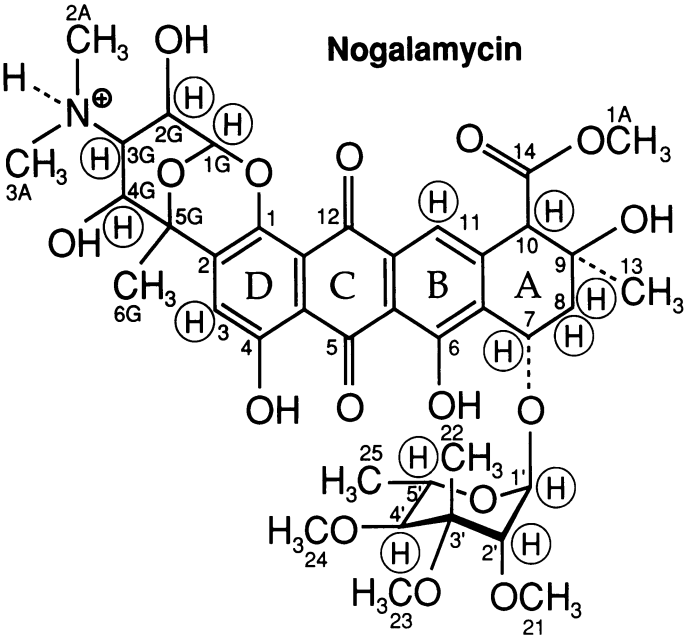

Figure 1. Molecular formula of the antitumor anthracycline antibiotic nogalamycin with the numbering system used in the paper. The molecule contains an aglycone chromophore with four fused rings (A–D). Rings B–D are unsaturated with exocyclic oxygen atoms, whereas ring A is semisaturated. Nogalamycin has two sugars attached to the aglycone with nogalose at C7 and a positively charged α-D-3,6-dideoxy-3-dimethylaminoglucose (abbreviated aminoglucose in text) at C1/C2 positions.

chromophore (rings A-D) penetrates the DNA double helix such that it is almost perpendicular to the C1′-C1′ vectors of the two GC base pairs above and below the intercalator. The drug spans the two grooves of the helix with the nogalose in the minor groove and the aminoglucose in the major groove. While these structures unambiguously reveal the detailed interactions between nogalamycin and DNA, we are interested in knowing whether the same interactions are found in complexes containing unmodified DNA sequences in solution. In addition, we also hope to understand the dynamic property of a drug that binds to both grooves of the DNA double helix simultaneously. We have carried out high resolution NMR studies of nogalamycin complexed to the unmodified DNA hexamer d(CGTACG) and compared the results with those from another NMR study between nogalamycin and a different DNA hexamer d(GCATGC) (7).

MATERIALS AND METHODS

The oligonucleotides were synthesized according to a procedure published earlier (8). Nogalamycin was a gift from Dr. Paul Aristoff of the Upjohn Co. and it was dissolved in methanol as stock solutions. The solution of d(CGTACG) for NMR studies was prepared by dissolving the ammonium salt of the DNA hexamer in 500 μl of phosphate buffer solution (50 mM sodium phosphate, pH 7.0, 0.15 M NaCl in 99.8% D_2O) to produce a final single stranded concentration of 8 mM. The solution was lyophilized twice with 99.8% D_2O, then dried in an NMR tube with a stream of argon gas and finally 500 μl of 99.96% D_2O was added to produce the sample. Nogalamycin-d(CGTACG) complexes were similarly prepared except different amounts of nogalamycin, for the 1:1 and 2:1 complexes, were added to the DNA solution at the beginning.

Both 1D and 2D NMR spectra were recorded on a GE GN500 MHz spectrometer. The chemical shifts (in ppm) are referenced to the HDO peak which is calibrated to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at different temperatures. Absorption mode 2D COSY and NOESY spectra were recorded as 512 t_1 blocks of 1024 complex points each (in the t_2 dimension) and averaged for 32 scans per block. During the recycle delay of 500 ms for the COSY and 2 s for the NOESY, the residual HDO peak was suppressed by presaturation. The mixing time for the NOESY experiments was 200 msec. The 2D data sets were processed with the program FTNMR (Hare Research, Woodinville, WA) using the Silicon Graphics workstations. For the 2D data sets, the 1024 complex points in the t_2 dimension were apodized with a 60° shifted sine-bell squared function (skew factor of 1.2). The 512 complex points in the t_1 dimension were apodized similarly and zero-filled to 1024 points prior to the Fourier transform. Double quantum COSY and phase sensitive NOESY were collected and processed in a similar way.

RESULTS

Formation of drug-DNA complexes

One dimensional ^1H NMR spectra of the aromatic and methyl regions of both DNA hexamer and the 1:1 and 2:1 complexes at 35°C are shown in Figure 2. The d(CGTACG) duplex at this temperature, which is slightly lower than the T_m at this ionic strength, is only partially dissociated (as estimated by temperature dependent changes in chemical shift; data not shown) due to the high DNA concentrations employed in these experiments. The assignments of the resonances in free DNA were carried out (*vide infra*) using the established methods (9) and they agree with the assignments published earlier (10,11). The spectrum of the 2:1 nogalamycin-d(CGTACG) complex shown in Figure 2 reveals that it is a symmetrical complex. The chemical shifts of all DNA resonances changed dramatically, as expected due to the intercalative binding of nogalamycin. For example, the thymine methyl resonance moved upfield by 226 Hz from 1.64 ppm to 1.17 ppm. The 2:1 complex is very stable as judged by the temperature-dependent study (data not shown). No appreciable changes in the chemical shifts of most of the resonances were observed up to 65°C.

An interesting phenomena occurs when only one drug per duplex is present in solution. The spectrum in Figure 2 (middle) shows that more than one molecular species is now present. For example, there are 10 distinct resonances in the methyl region. By careful analysis of the position and the area of these resonances, we believed there are three molecular species in equilibrium, namely free DNA, a 1:1 complex and a 2:1 complex, in approximately a 1:2:1 ratio. Close inspection of the 1:1 spectrum also reveals that the chemical shifts associated with the 2:1 complex remain unchanged, suggesting that it is in slow equilibrium with other species (1:1 complex) on the NMR time scale. However, many of the chemical shifts of the free DNA resonances have changed significantly, suggesting that the exchange time between the free and 1:1 complex is approaching the NMR time scale. This will be discussed in more detail later.

Resonance Assignments in the 2:1 complex

The assignment of the resonances in the 2:1 complex was carried out by using a combination of COSY and NOESY correlations (Figures 3A and 3B). The resonances of the bound nogalamycin

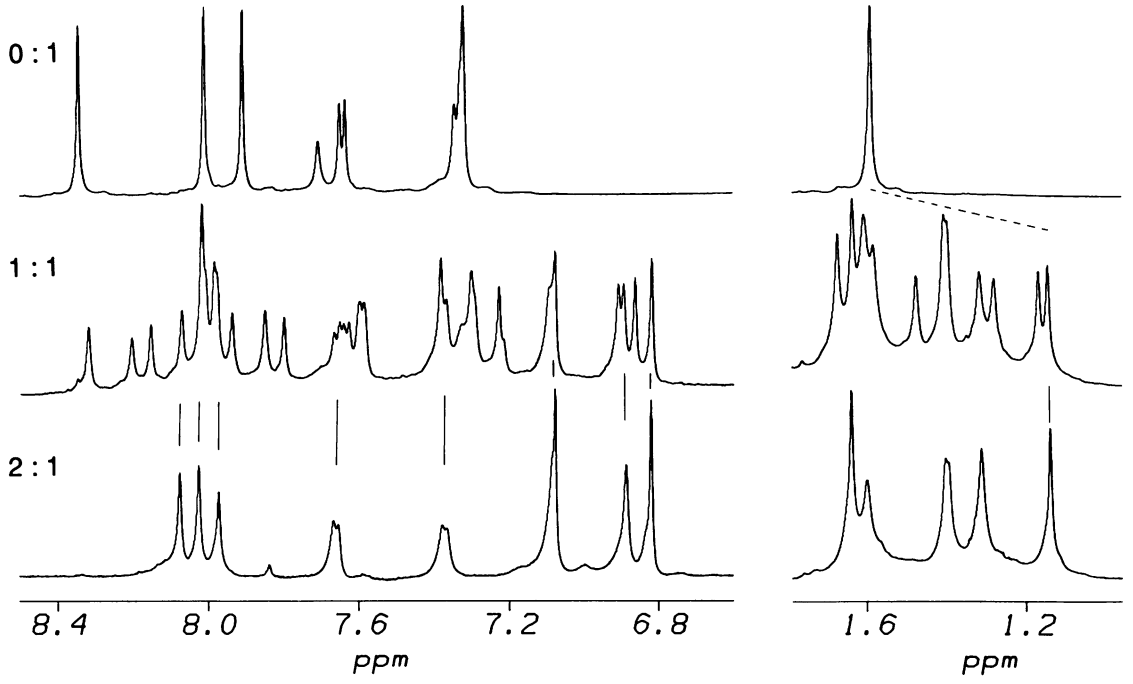

Figure 2. One-dimensional 1H NMR spectra of the aromatic and methyl regions of the complexes between nogalamycin and d(CGTACG) at different drug/DNA ratios at 35°C. Top: no drug added. Middle: one drug per DNA duplex. Bottom: two drugs per DNA duplex.

in the 2:1 complex were identified by starting with the J-correlation between N-M25 and N-H5′ (J=4 Hz) (N- stands for nogalamycin), which is the only methyl resonance that appears as a doublet (1.40 ppm). The rest of the nogalose protons were then assigned in a manner similar to the assignment sequence of Searle, et al. (7). The correlations can be followed around the nogalose sugar onto the ring A protons, ending with N-M1A and N-H11. The strong NOE between N-M6G and N-H3 (data not shown) establishes a starting point for following the correlations through the protons of the aminoglucose attached to ring D. At pH 7.0, the resonances from N-M3A and N-M2A coalesce into one broad peak with a width at half-height of 34 Hz. At pH 4.4, these two resonances become split by 95 Hz and have linewidths identical to the four other methoxy proton resonances of the drug (12.5 Hz at 35°C). This indicates exchange due to protonation/deprotonation of the dimethylamino group is fast on the NMR time scale at pH 7.0, but slow at pH 4.4. In addition, the H8 proton of the terminal guanine G6 became severely broadened and disappeared at pH 4.4. This is likely due to the protonation of the paired cytosine C1, causing the destabilization of the terminal Watson-Crick GC base pair. Whether this GC base pair now exists in equilibrium between the Watson-Crick, the Hoogsteen, or simply the open GC base pairs, remains to be determined.

With an intercalative drug separating C1 and G2, the distance between the C1H2″ and the next aromatic base proton (G2H8) on the 3′ side is too long for a detectable NOE. Therefore the sequential assignment of the DNA resonances starts with the NOE's between G2H8 and its own H2″, and T3H6's correlation to that same H2″. It then proceeds as indicated by the lines of Figure 3B, by following NOE's from the H2″ to aromatic protons as if the rest of the DNA were in a near normal B-DNA conformation. Table 1 lists the complete assignment of all the protons (except H5′ and H5″) in the 2:1 complex. Although C1 is detached from the sequential assignment via H2″ protons due to the intercalation of nogalamycin, NOEs from C1H2′ and C1H2″ can be seen to N-H11 in Figure 3B, thus completing the assignments.

Comparison of solution and crystal structures

The assignment of most of the protons, both in DNA and in nogalamycin, enables us to identify all the cross peaks in the NOESY spectrum associated with the interproton couplings between DNA and drug of the 2:1 complex. Figure 4 is a portion of the NOESY spectrum in which all significant NOE's between DNA and nogalamycin are labeled and numbered. When these cross peaks are carefully examined, it became apparent that there are two sets of them. One set (23 peaks) is associated with the protons in nogalose and ring A of the chromophore interacting with the DNA protons in the minor groove. Another set (3 peaks) is associated with protons in aminoglucose interacting with the ring protons (H5 and H6) of cytosine C1 residue in the major groove. This information defines the location and orientation of nogalamycin in the d(CGTACG) duplex. The aglycone chromophore must be intercalated between the CpG steps at both ends of the duplex with the sugars facing toward middle of the helix.

Since we have determined the high resolution (1.3 Å) crystal structure of the 2:1 nogalamycin-d[CGT(pS)ACG] complex (5), we proceeded to compare these NOE's with the distances derived from the averaged model of the crystal structure as shown in Table 2. It is clear that the agreement is remarkably good. In order to aid the visualization of these interactions, a skeletal diagram of the nogalamycin intercalated in the CpG step of a three base base helical fragment CpGpT is shown in Figure 5. It can be seen that the aminoglucose is facing toward the GC base pair in the major groove, while the nogalose nuzzles into the bottom of the minor groove. The two sugars are on the same

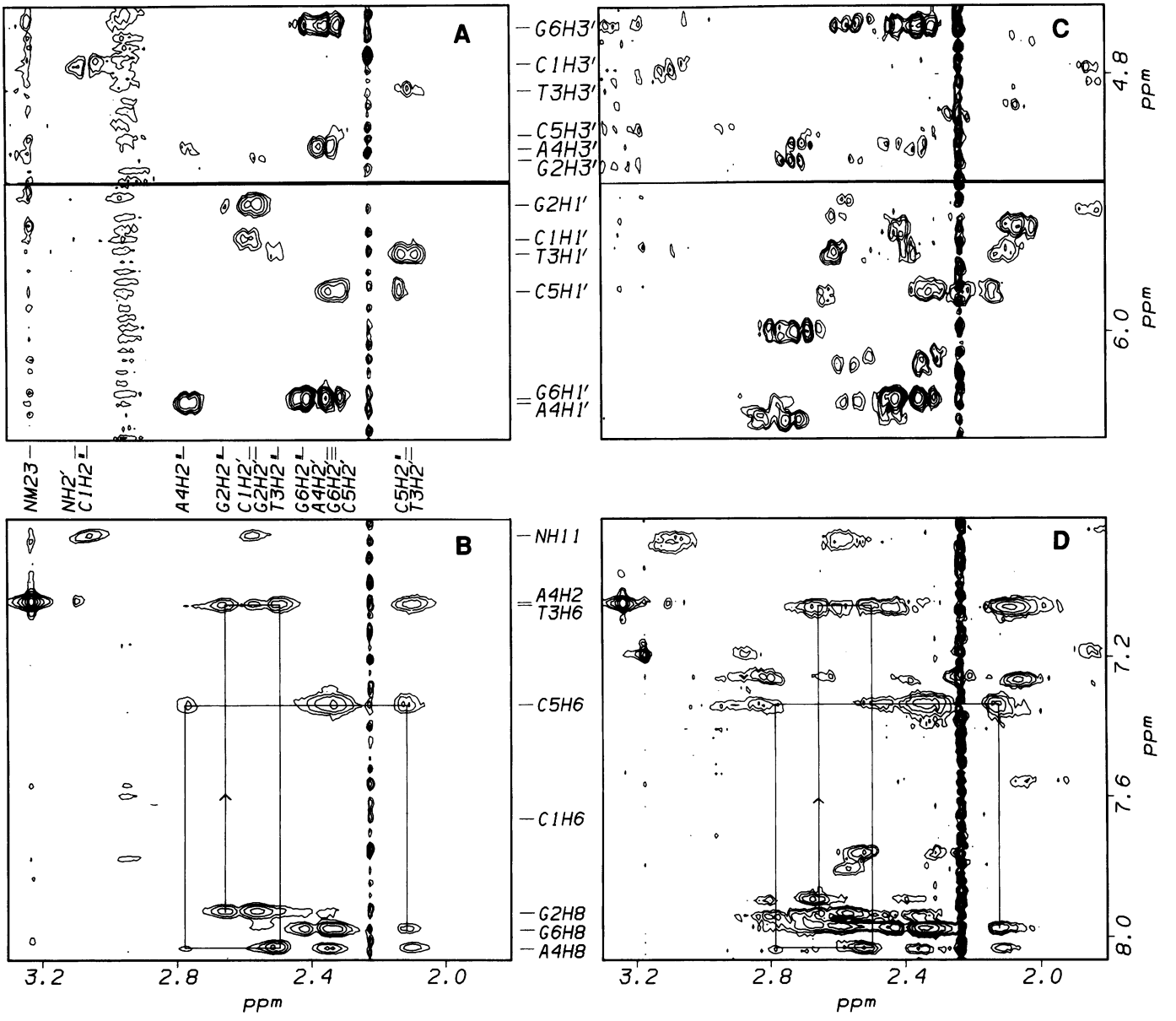

Figure 3. Portions of the two dimensional COSY and NOESY spectra of the 2:1 complex of nogalamycin and d(CGTACG) duplex which provided key information in the assignments of resonances. (A). COSY of the 2:1 complex. Cross-peaks between H2', H2" (2.0–3.2 ppm) and H3' (4.7–5.2 ppm), H1' (5.5–6.2 ppm) are shown. (B). NOESY of the 2:1 complex. Cross peaks between H2', H2" and the aromatic protons (6.6–8.1 ppm). (C). Corresponding (as in (A)) COSY of the 1:1 complex. (D). Corresponding (as in (B)) NOESY of the 1:1 complex.

side of the flat aglycone chromophore wrapping around the second (and the fourth) GC base pair and they both point toward the AT region in the middle of the helix.

A few examples are chosen to illustrate the high consistency between the crystal and the solution structure. For example, the axial C13 methyl group in ring A of the drug approaches the H1', H4' of G2 and H4' of T3 with the distances of <3.5 Å. Another substituent group in ring A, the acetic methyl ester on C10, is in the axial position almost perpendicular to the plane of the aglycone and its keto oxygen O14 atom receives a hydrogen bond from the NH_2 of G12. Its methyl group (M1A) is close to the sugar protons H1', H2" of C1 residue (~3.2 Å). In the major groove, relatively few protons are available for making the connectivity. Nonetheless, H1G of the aminoglucose is quite close to the H5, H6 protons of cytosine C1.

It is interesting to note that there is a significant NOE between the H2" of C5 residue and the H8 of G6 residue (Figure 3B), despite the fact that there is a drug intercalated between these two base pairs. This suggests that the sugar of C5 is brought close to the guanine base due to rearrangement of the DNA backbone arising from the binding of nogalamycin. One way to do this is for the C5-G8 base pair to have a large buckle such that the sugar of C5 is 'pushed' down toward the G6 residue (Figure 5). This is in agreement with the crystal structure. Based on this analysis, we believe that the 1.3 Å resolution crystal structure is highly preserved in solution.

Comparison with nogalamycin-d(GCATGC) complex

An NMR study of the 2:1 nogalamycin-d(GCATGC) complex has been carried out earlier (7). Based on 28 NOE distance

Table 1: ^{1}H NMR Chemical Shifts of Non-Exchangeable Protons in 2:1 Nogalamycin/DNA Complex

	DNA Protons (ppm)						
	H8/H6	H5,H2,Me	H1′	H2′	H2″	H3′	H4′
C1	7.66	5.36	5.74	3.07	2.59	4.78	4.36
G2	7.97		5.64	2.59	2.67	5.03	4.57
T3	7.08	1.14	5.78	2.13	2.52	4.84	4.06
A4	8.08	7.07	6.20	2.37	2.78	5.02	4.26
C5	7.37	5.26	5.88	2.35	2.14	4.94	4.17
G6	8.03		6.19	2.36	2.43	4.67	4.27

Nogalamycin Protons (ppm)					
H3 6.82	H1′ 5.37	H7 4.93	H1G 5.63	M13 1.60	M21 3.66
H11 6.89	H2′ 3.11	H8′ 2.01	H2G 4.24	M1A 3.76	M22 1.31
	H4′ 3.17	H8″ 2.95	H3G 4.83	M2A 2.97	M23 3.24
	H5′ 3.83	H10 4.29	H4G 3.91	M3A 2.97	M24 3.57
				M6G 1.63	M25 1.40

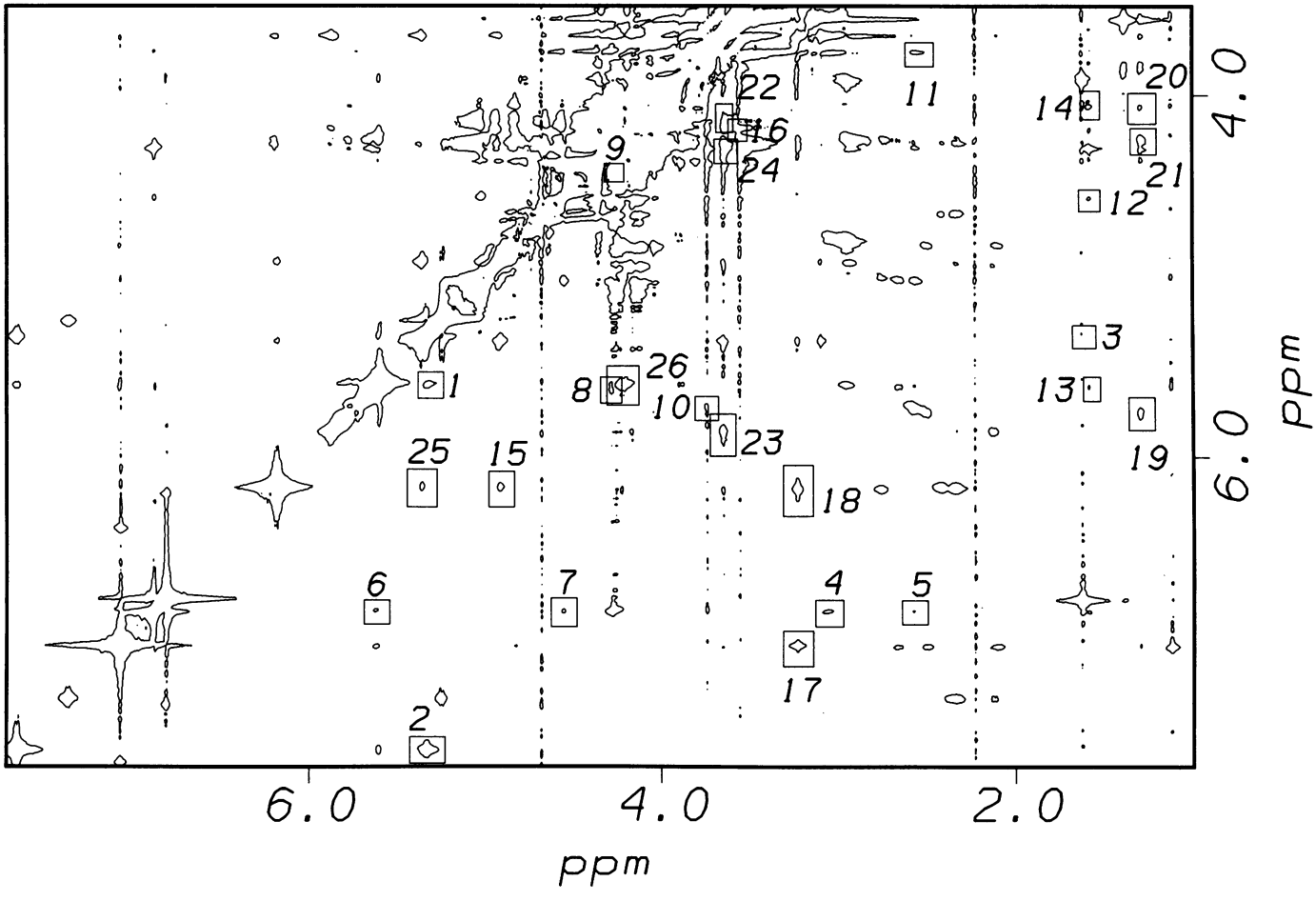

Figure 4. Portions of the 2D NOESY spectrum indicating the 26 cross peaks (numbered in diagram) associated with the protons that are proximal between nogalamycin and DNA duplex. The magnitude of those cross peaks are in excellent agreement with the ^{1}H-^{1}H distances found in the crystal structure.

constraints, that work suggested that nogalamycin intercalated at the 5′-CpA steps with the nogalose lying in the minor groove. It also correctly pointed out many features that have been observed in the 1.3 Å resolution crystal structure. For example, it suggested that the intercalation cavity is wedge-shaped (6), consistent with the nogalamycin-d[CGT(pS)ACG] crystal structure in which the GC base pairs are highly buckled (5).

These two different nogalamycin-DNA complexes in solution provide us with an opportunity to examine the sequence specificity of nogalamycin. From the crystal structure, it has been shown that the hydrogen bonding interactions between the drug and DNA in both major (i.e., the two hydroxyl groups (O2G and O4G) and N7 and O6 of guanine) and minor grooves determine the GC sequence specificity (5). Using this and other chemical footprinting information (4), we have suggested that nogalamycin has a DNA sequence preference for 5′-**NpG** or 5′-**CpN** steps.

Table 2. Correlation of the NOE Cross Peaks with the Interproton Distances between Nogalamycin and d(CGTACG)

Nogalamycin	DNA	Distance[a]	NOE[b] strength	Peak[c] number
Major Groove				
H1G	C1H5	3.4	+	1
H1G	C1H6	3.1	++	2
M6G	C1H5	3.3	+	3
Minor Groove				
H11	C1H2′	3.0	+	4
H11	C1H2″	3.5	+	5
H11	G2H1′	3.6	+	6
H11	G2H4′	3.9	+	7
H10	G2H1′	3.1	+	8
H10	G2H4′	2.6	+	9
M1A	C1H1′	3.3	+	10
M1A	C1H2″	3.2	+	11
M13	G2H4′	3.4	+	12
M13	G2H1′	2.6	++	13
M13	T3H4′	3.2	+	14
H7	G12H1′	3.0	++	15
M24	A4H4′	2.5	++	16
M23	A10H2	2.6	+++	17
M23	A4H1′	2.5	+++	18
M23	A4H4′	3.2	obscured	
M22	T3H1′	2.3	+++	19
M22	T3H4′	2.9	+	20
M22	A4H4′	3.8	+	21
M21	C11H4′	2.9	++	22
M21	C11H1′	2.1	+++	23
M21	G12H4′	3.6	++	24
H1′	G12H1′	3.9	+	25
H1′	G12H4′	3.2	+	26

[a] Average interproton distance from the orthorhombic X-ray crystal structure (5). Methyl groups were rotated to a minimum distance.
[b] NOE intensity is designated as follows: +, weak; ++ moderate; +++ strong.
[c] Peak numbers are shown in Figure 4.

More specifically, the aglycone chromophore prefers to intercalate at the 5′-side of a guanine (between **NpG**), or at the 3′-side of a cytosine (between **CpN**) with the sugars facing toward the GC base pair (12).

Based on this prediction, nogalamycin should bind to CpG steps with two possible orientations. However, in d(CGTACG) nogalamycin prefers to bind in an orientation with its sugars pointing toward the middle of the helix, because in the opposite orientation the sugars will protrude beyond the terminal CG base pair. This has been observed both in crystal structure (5) and in solution. Similarly, in the hexamer d(GCATGC) sequence, there should be two equivalent binding sites, CpA and its complement TpG. The NMR work on the 2:1 nogalamycin-d(GCATGC) complex agrees with this prediction (7).

Dynamic property of nogalamycin binding

One interesting question related to the binding of nogalamycin to DNA double helix is how nogalamycin, with two bulky sugars at two ends, inserts itself between two base pairs. Presumably, the double helix has to partially open up (pre-melt) to allow one of the sugars to go through between the base pairs. We have addressed this question by studying the solution of the 1:1 nogalamycin/d(CGTACG) complex in which three molecular species (likely to be free DNA, 1:1 complex and 2:1 complex) were noted to coexist (Figure 2). The COSY and NOESY spectra (Figure 3C and 3D) further confirm this. This is in contrast to the echinomycin-d(ACGTACGT) complex in which the binding of drug to DNA is cooperative (13), presumably due to the formation of the Hoogsteen AT base pairs adjacent to the intercalation site (14,15).

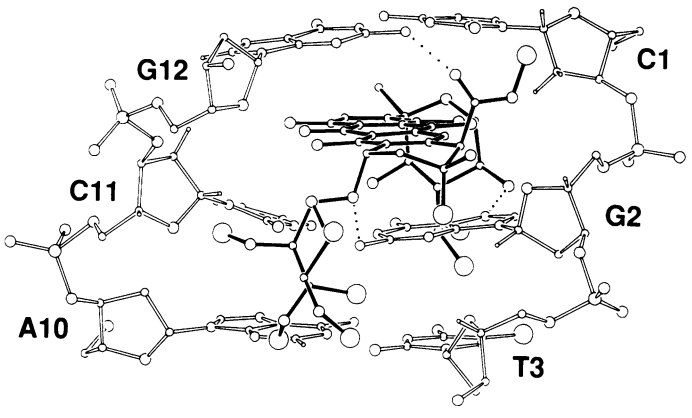

Figure 5. Skeletal diagram of the detailed surroundings of the intercalated nogalamycin. Three base pairs of the hexamer helix are shown. In solution, the other half of the complex is presumed to be identical. The methyl groups are represented as large circles. The hydrogen atoms that are in close contacts (< 3.0 Å) between nogalamycin and DNA are shown with small circles. Hydrogen bonds between the nogalamycin and DNA that are important in sequence specificity are shown as dotted lines.

An conspicuous feature in the COSY and NOESY spectra of the solution containing one drug per duplex (Figure 3C and 3D) is that all the cross peaks observed in the 2:1 complex are

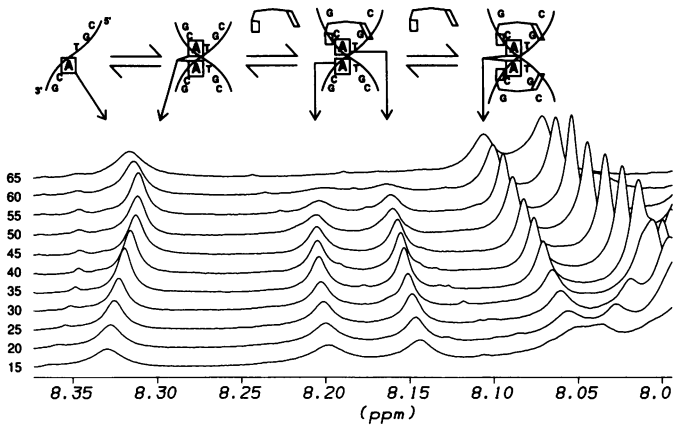

Figure 6. Temperature-dependent study of the 1:1 mixture of nogalamycin and d(CGTACG). The H8 proton of adenine residue becomes four individual resonances associated with three different molecular species as shown in the schematic diagram above the stacked 1D NMR spectra. The middle two resonances associated the 1:1 complex disappear at high temperature.

completely preserved at the same locations. The correlation connectivity can be followed in exactly the same manner in both solutions, suggesting that the structure of the 2:1 complex is retained in the solution with one drug per duplex, and that the 2:1 complex is in slow equilibrium with the 1:1 complex. However, this is not the case for the free DNA. Most of the cross peaks in the free d(CGTACG) (data not shown) do not overlap with the cross peaks in Figure 3C and 3D. This will be explained later.

This equilibrium between the three molecular species is temperature dependent as shown in Figure 6 which follows the changes of the A4H8 resonances associated with these three species. The ratio of the population of free DNA, 1:1 complex and 2:1 complex changes from 1:2:1 to 1:0:1 in going from 5°C to 65°C. The population of the 1:1 complex appears to decrease linearly above 40°C by plotting the peak area of the A4H8 resonances versus temperature, but this should be taken as tentative as extensive spectral simulation will be required to obtain accurate integration area under the resonances. The midpoint temperature at which the 1:1 population disappears was determined to be about 60°C. This temperature-dependent dynamic conversion between different complexes is rather unusual (we are not aware of other similar example) and it may be explained using the schematic diagram shown in Figure 6.

The addition of one nogalamycin per duplex allows the nogalamycin to occupy the available binding sites [CpG in d(CGTACG)] with equal probability. This would result in three molecular species at low temperature. The 1:1 complex is simultaneously in equilibrium with both the free DNA and the 2:1 complex. As these spectra were recorded at 35°C, which is near the T_m of free d(CGTACG) at the present ionic strength condition, the free DNA duplex is already in rapid equilibrium with single stranded molecule. It is reasonable to assume that the equilibrium between the free DNA and 1:1 nogalamycin-d(CGTACG) complex is still slow due to the complicated architecture of the complex, but faster than the corresponding equilibrium between the 1:1 and 2:1 complexes. At higher temperature, the free DNA duplex and the 1:1 complex are destablized and disintegrated into free single-stranded DNA and free drug. Since free nogalamycin is not soluble in aqueous solution, it will bind to the vacant CpG site of another 1:1 complex to form a 2:1 complex. The 2:1 complex is very stable; no appreciable dissociation of this complex is observed up to 65°C. This extreme stability is likely related to the slow kinetics of the binding of nogalamycin to DNA double helix. We have determined from the saturation transfer experiments on the AH8 proton that the halftime for the dissociation of one nogalamycin from the 1:1 complex is about 30 seconds.

DISCUSSION

High resolution NMR spectroscopy is the most powerful technique to study in solution the interactions between important antitumor drugs and their receptor, DNA. Numerous NMR studies have been carried out on a number of important antitumor drugs complexed non-covalently to DNA oligomers, including actinomycin D (16,17), daunorubicin (18), echinomycin (13,19), nogalamycin (7), chromomycin A3 (20,21) and several minor groove binding drugs like netropsin and distamycin (22–25). These studies provided useful structural and dynamic information of the complex in solution, which in general substantiates the crystal structure determined by x-ray diffraction analysis when available. For example, NMR studies of the echinomycin complexed to both d(TCGA) and d(ACGT) (19) and d(ACGTACGT) (13) showed that the AT base pair in their complexes is indeed in the Hoogsteen geometry, as shown previously by the single crystal x-ray diffraction analysis (14). Similarly, it was shown that the binding of distamycin to DNA minor groove is very similar in solution (22) and in crystal (26).

Our study here is the first step toward establishing *ab initio* solution structure of nogalamycin-DNA complex by NMR using COSY and NOESY data. This objective remains not straightforward for DNA-drug complexes due to various factors. First of all, the complex needs to be a stable and uniform species. Second, sufficient NOE peaks between drug and DNA, which are often quite few due to their sparse number of protons, are required to fix the relative position between drug and DNA. If those conditions can be met, it is possible to determine the solution structure by the recently developed methods, such as the combination of distance geometry and molecular dynamics refinement. While this approach has been successfully applied to many protein structures, it has only been tested in limited cases of DNA-drug complexes. An example is the structure determination and refinement of the DNA-mitomycin C adduct published recently (27).

The present results complements and significantly augments the knowledge obtained from x-ray diffraction analysis which remains the most definitive way to visualize the molecular interactions. In fact, our earlier 1.3 Å resolution structural determination of the 2:1 complex of nogalamycin and d[CGT(pS)ACG] provided exquisite detailed information regarding the geometry and conformation of both the DNA and the drug molecules (5). For example, we noted that the bound nogalamycin in the drug-DNA complex has a significant distortion relative to the free nogalamycin (12). The long aglycone chromophore appears to bend gently such that the relative position between the nogalose and aminoglucose are quite different in the bound and free nogalamycins. In addition, the two CG base pairs wrap around the aglycone such that they have large buckles. These types of subtle conformational variations are not easily detected by NMR due to the relatively few proton-proton interactions that exist in DNA molecules. While we observed 26 good intermolecular NOE cross peaks which are consistent

with distances derived from the crystal structure, the solution structure is, strictly speaking, not yet determined unequivocally. In view of the rare opportunity of having available a very high resolution (1.3 Å) crystal structure of the 2:1 nogalamycin-d(CGTACG) complex, we are interested in using it as the benchmark for comparison with the *ab initio* solution structure determined by NMR method. Toward this goal, we have collected the double quantum filtered COSY and phase sensitive NOESY data. These data, while agreeing in general with the absorption mode COSY and NOESY data used in the present work, appear to have significantly improved quality suitable for detailed structural determination and refinement. For example, we can generate the simulated 2D-NOE spectrum based on the crystal structure and make quantitative comparison with the observed data. These studies are now in progress.

In conclusion, the present NMR study does offer numerous new insights which are not attainable by x-ray diffraction. First, it shows that in solution only one molecular species exist for the 2:1 complex and its structure is in complete agreement with our crystal structure. In other words, the crystallization process did not select out a particular minor molecular species to form the crystals. Since no other species is detected, it proves that CpG is the highly prefered binding site for nogalamycin and the sugars of nogalamycin are pointing toward the middle of the helix. Further, this work shows unequivocally that the binding interactions of nogalamycin to DNA seen in the crystal is not affected by the chemical modification of thiophosphate linkage.

In addition, it provides us with the dynamic information regarding the drug binding to DNA. The temperature dependent equilibrium of the 1:1 mixture is particularly intriguing. It clearly establishes the fact that the drug binding to adjacent DNA sites is not cooperative. In the 1:1 complex, nogalamycin binds only to one of the two CpG sites, but not to any other steps (e.g., GpT, TpA or ApC). Based on this analysis, in conjunction with other data, we are able to suggest the sequence specificity of nogalamycin being **NpG** or its complement **CpN**.

Finally, it is useful to point out that many antibiotics bind exclusively in the minor groove. We have suggested that this may be due to the natural selection process for the microbes to develop secondary metabolites that attack the minor groove where few proteins bind specifically (28,29). Interestingly, nogalamycin binds to DNA with its positively charged aminoglucose in the major groove, a rare example for natural product antibiotics. Some derivatives of nogalamycin, e.g., menogaril (earlier name 7-con-O-methylnogarol), do not have nogalose at the C7 position and they have significant antitumor activity *in vivo* (2). This compound is currently in the phase II clinical trial (30). We are in the process of determining the solution structure of this derivative complexed to DNA to see in which groove the aminoglucose resides. We have also carried out preliminary NMR studies of nogalamycin with a longer DNA octamer d(ACGTACGT) which will shed some light on the influence of base pairs further away from the intercalation site on the binding of nogalamycin to DNA. More structural analyses like this would enable us to fully understand the molecular forces that govern the binding of important antitumor/anticancer compounds to DNA. This new information should be useful in designing better chemotherapeutic drugs in the future.

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REFERENCES

1. Bhuyan, B.K. and Reusser, F. (1970) Cancer Res. 30, 984–989.
2. Wiley, P.F. (1979) J. Natural Prod. 42, 569–582.
3. Crooke, S.T. and Reich, S.D. (eds) (1980) Anthracyclines, Academic Press, New York.
4. Fox, K.R. and Waring, M.J. (1985) Biochemistry 25, 4349–4356.
5. Liaw, Y.-C., Gao, Y.-G., Robinson, H., van der Marel, G.A., van Boom, J.H. and Wang, A. H.-J. (1989) Biochemistry 28, 9913–9918.
6. Williams, L.D., Egli, M., Gao, Q., Bash, P., van der Marel, G.A., van Boom, J.H., Rich, A. and Frederick, C.A. (1990) Proc. Natl. Acad. Sci. USA 87, 2225–2229.
7. Searle, M.S., Hall, J.G., Denny, W.A. and Wakelin, L.P.G. (1988) Biochemistry 27, 4340–4349.
8. van der Marel, G.A., van Boeckel, C.A.A., Willie, G. and van Boom, J.H. (1981) Tetrahedron Letters **22**, 3887–3888.
9. Hare, D.R., Wemmer, D.E., Chou, S.-H., Drobny, G. and Reid, B.R. (1983) J. Mol. Biol. 171, 319–336.
10. Gronenborn, A.M., Clore, G.M. and Kimber, B. (1984) Biochem. J. 221, 723–736.
11. Lane, A.N. and Forster, M.J. (1989) Eur. Biophys. J. 17, 221–232.
12. Gao, Y.-G., Liaw, Y.-C., Robinson, H. and Wang, A. H.-J. (1990) Biochemistry, in press.
13. Gilbert, D., van der Marel, G.A., van Boom, J.H. and Feigon, J. (1989) Proc. Natl. Acad. Sci. USA 86, 3006–3010.
14. Wang, A.H.-J., Ughetto, G., Quigley, G.J., Hakoshima, T., van der Marel, G.A., van Boom, J.H. and Rich, A. (1984) Science, 225, 1115–1121.
15. Wang, A.H.-J., Ughetto, G., Quigley, G.J. and Rich, A. (1986) J. Biomol. Struct. Dyn. 4, 319–342.
16. Patel, D.J., Kozlowski, S.A., Rice, J.A., Broka, C. and Itakura, K. (1981) Proc. Natl. Acad. Sci., USA 78, 114–117.
17. Jones, R. L., Scott, E. V., Zon, G., Marzilli, L. G. and Wilson, W. D. (1988) Biochemistry, 27, 6021–6026.
18. Chaires, J. B., Fox, K. R., Herrera, J. E., Britt, M. and Waring, M. J. (1987) Biochemistry 26, 8227–8236.
19. Gao, X. and Patel, D.J. (1988) Biochemistry 27, 1744–1751.
20. Gao, X. and Patel, D.J. (1989) Biochemistry 28, 751–762.
21. Banville, D.L., Keniry, M.A., Kam, M. and Shafer, R.H. (1990) Biochemistry 29, 6521–6534.
22. Pelton, J.G. and Wemmer, D.E. (1988) Biochemistry 27, 8088–8096.
23. Patel, D.J. and Shapiro, L. (1986) Biochimie 67, 887–915.
24. Leupin, W., Chazin, W.J., Hyberts, S., Denny, W.A. and Wuthrich, K. (1986) Biochemistry 25, 5902–5910.
25. Wang, A.H.-J., Cottens, S., Dervan, P.B., Yesinowski, J., van derMarel, G.A. and van Boom, J.H. (1989) J. Biomol. Struct. Dyn. 7, 101–117.
26. Coll, M., Frederick, C.A., Wang, A.H.-J. and Rich, A. (1988) Proc. Natl. Acad. Sci.
27. Norman, D., Live, D., Sastry, M., Lipman, R., Hingerty, B.E., Tomasz, M., Broyde, S. and Patel, D. (1990) Biochemistry 29, 2861–2875.
28. Ughetto, G., Wang, A.H.-J., Quigley, G.J. and Rich, A. (1985) Nucleic Acids Res. 13, 2305–2323.
29. Wang, A.H.-J. (1987) In Eckstein, F. & Lilley, D.M. (eds) Nucleic Acids and Molecular Biology. Springer, Berlin. Vol. 1 pp. 32–54.
30. Adams, W. J., McGovern, J. P., Dalm, E. A., Brewer, J. E. and Hosley, J. D. (1989) Cancer Res. 49, 6328–6336.