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Biochemistry

Structure of nogalamycin bound to a DNA hexamer

(anthracycline/intercalator/phosphorothioate/x-ray crystallography)

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ABSTRACT **The anthracycline antibiotic nogalamycin, which binds to DNA, is composed of a planar aglycone substituted on each end to form an unusual dumbbell-shaped molecule. At one end nogalamycin contains an uncharged nogalose sugar and a methyl ester. At the other end nogalamycin contains a positively charged bicyclo amino sugar. We report the crystal structure of nogalamycin bound to the self-complementary DNA hexamer d(^{m5}CGTsA^{m5}CG). In this complex, the cytosines are methylated at the 5 position and the DNA contains a phosphorothioate linkage at the TpA step. Two nogalamycin molecules bind to the 6-base-pair fragment of double-helical DNA. The drug has threaded between the phosphodiester backbones with three aromatic rings intercalated within the DNA. In the major groove, the bicyclo amino sugar forms two direct hydrogen bonds to span a C·G base pair and interacts indirectly with the next base pair of the duplex via a water-mediated hydrogen bond. In the minor groove, a carbonyl oxygen of nogalamycin forms a hydrogen bond directly to N2 of a guanine. The DNA base pairs are severely buckled by up to 26° and are also distorted in directions perpendicular to the Watson–Crick hydrogen bonds. This complex illustrates the deformable nature of DNA.**

Anthracyclines are DNA intercalators that constitute a widely used family of chemotherapeutic agents. The clinical properties of anthracyclines are highly dependent on their chemical structure (1, 2). For example, the anthracycline daunomycin is effective for treating acute leukemia whereas doxorubicin (former generic name, adriamycin), differing only by addition of a hydroxyl group, is more effective for treating solid tumors. Nogalamycin, a more complex anthracycline isolated from *Streptomyces nogalator* var. *nogalator*, is active against Gram-positive bacteria and experimental tumors (3–5). Nogalamycin binds to DNA and selectively inhibits DNA-directed RNA synthesis *in vivo* (5–7).

Anthracyclines such as daunomycin and doxorubicin are composed of a relatively planar aglycone chromophore substituted on one end with a positively charged amino sugar. In contrast, the aglycone of nogalamycin contains bulky substituents on both ends (Fig. 1), resulting in a molecule with the approximate shape of a dumbbell. On one end of the aglycone, nogalamycin contains both a methyl ester and a nogalose sugar. The uncharged nogalose is located at the 7 position, where daunomycin has a positively charged amino sugar. At the other end of the aglycone, nogalamycin contains a positively charged bicyclo amino sugar fused at the 1 and 2 positions, where daunomycin lacks bulky substituents.

Nogalamycin has been shown by a variety of techniques to penetrate the DNA helix and form a stable, intercalative complex (8–14) with slow association and dissociation rates

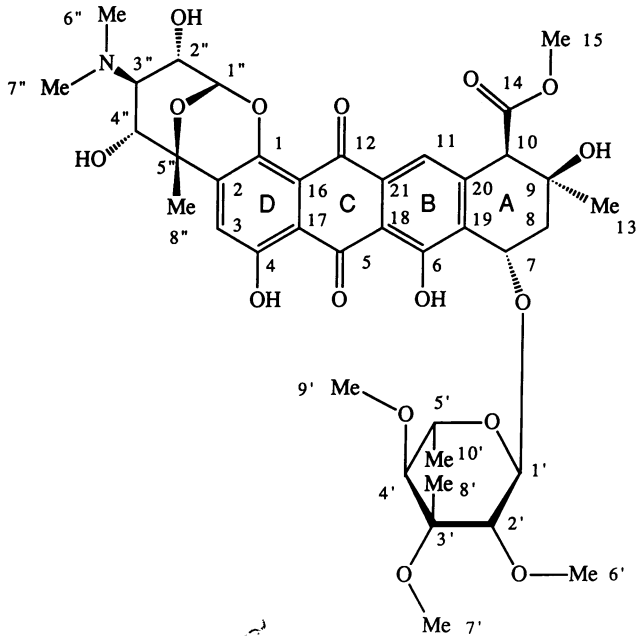

FIG. 1. Schematic diagram of nogalamycin.

(15, 16). A schematic diagram of nogalamycin intercalated in DNA is shown in Fig. 2. The dumbbell shape of nogalamycin presents a substantial mechanistic obstacle to formation of a stable DNA–drug complex, consistent with a suggestion that transient "melting" of the DNA duplex may be required for binding of nogalamycin (17).

The crystal structure and relative stereochemistry of nogalamycin were determined by Arora (18), who proposed a model DNA–nogalamycin complex. This model was based in part on the crystallographic structure of daunomycin bound to the self-complementary DNA hexamer d(CGTACG) (19). In the model proposed by Arora, nogalamycin is threaded through the duplex with the aglycone intercalated, the nogalose in the minor groove, and the fused bicyclo amino sugar in the major groove. The alternative arrangement with the drug rotated by 180° relative to the DNA was also proposed (17). However, with ^{1}H NMR spectroscopy Wakelin and coworkers (14) have shown that nogalamycin intercalates at the TpG step of $[d(GCATGC)]_2$ in the orientation proposed by Arora, with the nogalose in the minor groove and the fused bicyclo amino sugar in the major groove.

To visualize the detailed interactions of nogalamycin with DNA, we have crystallized nogalamycin bound to the self-complementary DNA hexamer d(^{m5}CGTsA^{m5}CG) (where the cytosines are methylated at the 5 position and a phosphorothioate linkage is present at the TpA step) and, with x-ray diffraction, solved the three-dimensional structure to 2.0-Å reso-

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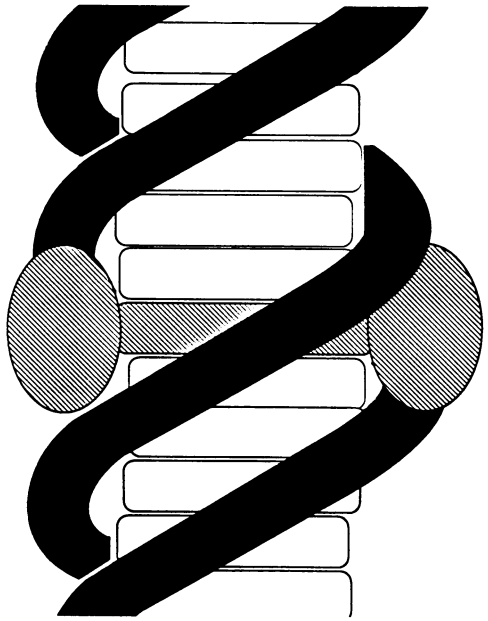

FIG. 2. Schematic diagram of a DNA–nogalamycin complex showing the dumbbell shape of nogalamycin.

lution.[§] Two drug molecules bind to the 6-base-pair fragment of double-helical DNA. The structure reveals a surprising pliability of double-stranded DNA.

MATERIALS AND METHODS

Sulfur substitution of DNA shows promise as a method to promote growth of DNA crystals that are otherwise difficult to obtain. The DNA hexamer d(^{m5}CGTsA^{m5}CG) was synthesized by the phosphotriester method (20) and the two diastereomers were separated by anion-exchange chromatography. Nogalamycin was kindly supplied by Paul Wiley (Upjohn).

Crystals were grown at room temperature in sitting drops by the vapor diffusion method. The crystallization mother liquor initially contained 1.5 mM DNA (single-strand concentration), 40 mM sodium cacodylate buffer (pH 6.0), 4 mM spermine, 13 mM $MgCl_2$, 8% 2-methyl-2,4-pentanediol, and 5% methanol saturated with nogalamycin. For stock solutions nogalamycin was dissolved in methanol, as this anthracycline is relatively insoluble in water. The sitting drops were equilibrated against a reservoir of 30% 2-methyl-2,4-pentanediol. Orange hexagonal crystals began to grow within 1 week and within 3 weeks grew to a size of 1.5 × 0.4 × 0.2 mm. The space group and approximate cell parameters were determined from precession photographs. The DNA–drug complex crystallized in space group $P6_122$ with unit cell dimensions $a = b = 26.30$ Å and $c = 100.01$ Å. Data were collected on a Rigaku AFC5 rotating-anode diffractometer using the ω scan mode. Intensities were corrected for Lorentz, polarization, and absorption effects. A total of 809 reflections with $F_{obs} > 2.0\sigma(F_{obs})$ were included in the refinement with 193 reflections between 2.0 and 2.5 Å. The structure was solved with base stacking information derived from a native Patterson map and by symmetry considerations using the daunomycin–d(CGTACG) complex, minus the solvent molecules and amino sugar, as a model. One drug molecule and one strand of the DNA duplex form the asymmetric unit. Two drug molecules bound to two base-paired strands of DNA are related by a crystallographic twofold axis. As the absolute stereochemistry of nogalamycin was unknown, refinement was attempted with both enantiomers of nogalamycin. Each enantiomer was placed in all four possible orientations at the CpG steps of the DNA, giving a total of eight different models of the complex. Electron-density ($2F_{obs} - F_{calc}$) and difference ($F_{obs} - F_{calc}$) Fourier maps were calculated and displayed on an Evans and Sutherland (Salt Lake City) PS390 graphics terminal, and manual manipulation of the models was performed with the program FRODO (21). Although the initial electron-density maps indicated with reasonable clarity that the sites of intercalation were at the CpG steps, refinement at other steps of the hexamer duplex was also attempted. Only one model refined properly. In this model the enantiomer shown in Fig. 1 intercalates at the CpG steps with the nogalose in the minor groove directed towards the center of the helix. Hydrogen bonds between the drug and the DNA were not constrained during the final stages of the refinement.

The structure was initially refined to an R factor of 24% with the Konnert–Hendrickson constrained least-squares refinement procedure (22) as modified for nucleic acids (G. J. Quigley, personal communication). The structure was further refined to a final R factor of 20.6% with the program X-PLOR (23), which makes use of molecular dynamics. The stereochemistry of the phosphorothioate remained ambiguous throughout the refinement and the sulfur was refined as an oxygen. The final refined structure of the nogalamycin–d(^{m5}CGTsA^{m5}CG) complex contained 39 water molecules per asymmetric unit and had a rms deviation in bond lengths from ideal of 0.018 Å.

RESULTS

One molecule of nogalamycin intercalates at each of the two CpG steps of the hexamer duplex [d(^{m5}CGTsA^{m5}CG)]$_2$ (Fig. 3). Nogalamycin has threaded between the phosphodiester backbones and interacts with both grooves of the DNA (Fig. 4). The long axis of the aglycone is nearly perpendicular to that of the base pairs (Fig. 5). The three aromatic rings (B–D) are stacked within the DNA, while the cyclohexene ring (A) is completely unstacked and protrudes into the minor groove. Thus, the aglycone is positioned asymmetrically relative to the grooves of the DNA.

In the major groove (Fig. 4*A*), nogalamycin forms two direct hydrogen bonds to the G(2)·C(11) base pair. The 2″-OH of nogalamycin donates a hydrogen bond to N7 of G(2) (2.63 Å) and the 4″-OH receives a hydrogen bond from N4 of C(11) (2.58 Å). Thus, nogalamycin forms hydrogen bonds to span the base pair, interacting strongly with both bases. In addition, nogalamycin interacts indirectly with the next base pair of the duplex, T(3)·A(10). The positively charged N3″ of nogalamycin forms a water-mediated hydrogen bond to N6 of A(10).

In the minor groove (Fig. 4*B*), the carbonyl oxygen of ring A forms a hydrogen bond directly to N2 of the terminal G(12) (2.94 Å), while the nogalose interacts only weakly with the DNA. This uncharged moiety does not form direct hydrogen bonds to the DNA but forms only a single water-mediated hydrogen bond from O4′ of the drug to the O3′ of T(3). In addition, the nogalose has only a single van der Waals contact with the minor groove. This 3.11-Å contact is between C7′ of nogalamycin and the ribose O4′ of A(10). We previously suggested (24) that in solution the minor-groove-binding sugar moieties of anthracyclines in DNA complexes could rapidly flip between isoenergetic conformational states. Such mobility of the nogalose would be consistent with its rather weak interactions with DNA and the inability of minor-groove-footprinting agents to detect nogalamycin bound to DNA (25).

[§]The atomic coordinates will be deposited in the Brookhaven Protein Data Bank (Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973).

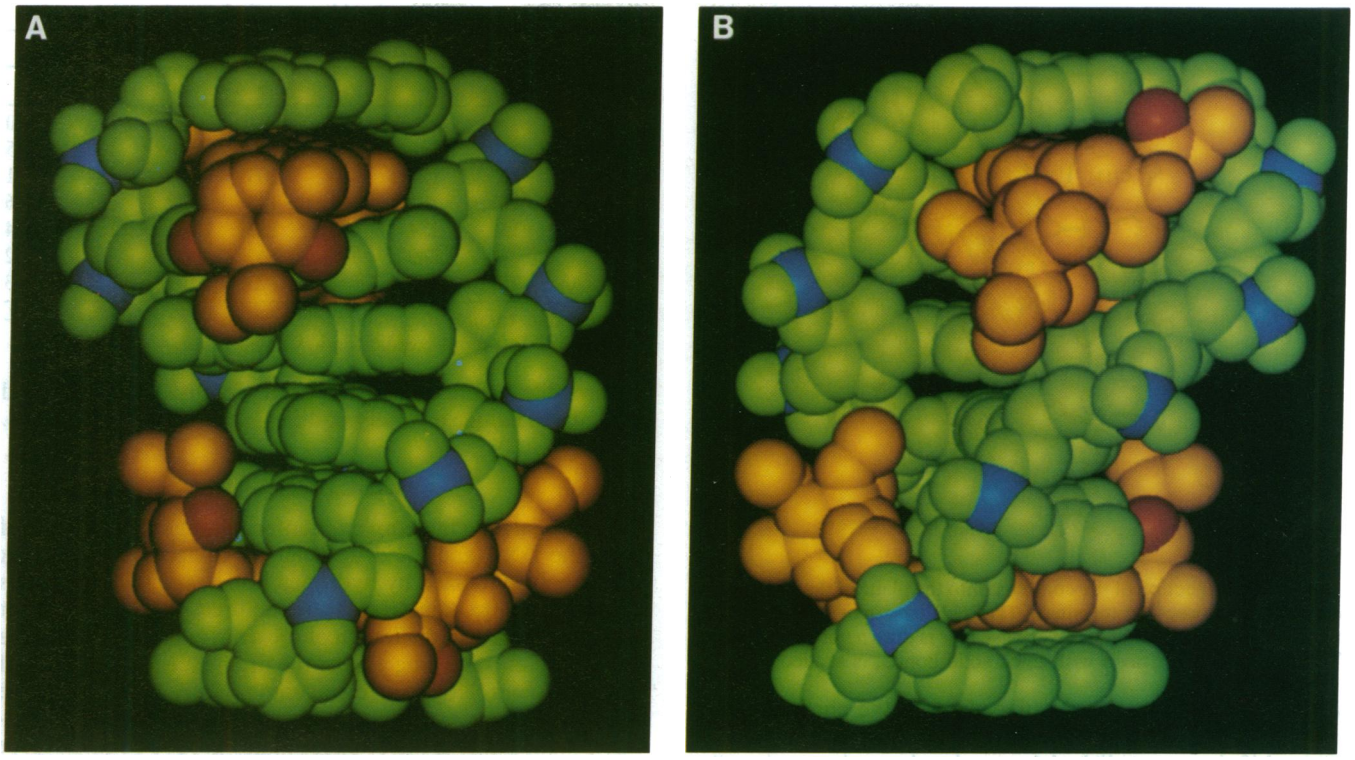

FIG. 3. Space-filling representations of the complex formed by [d(^{m5}CGTsA^{m5}CG)]$_2$ plus two nogalamycin molecules. In *A*, the major groove is at the top; in *B*, the minor groove is at the top. The van der Waals radii of the atoms are 0.9 those normally used, to facilitate visualization. The DNA is green except for the blue phosphorus atoms. Nogalamycin is gold except for the three red oxygen atoms, which form direct hydrogen bonds to the DNA. The nogalose sugars almost fill the minor groove.

The bond distances and angles of the bound nogalamycin molecule show no unusual features. However, the conformation of nogalamycin in the DNA complex is significantly different from that of nogalamycin crystallized alone. Compared with the conformation of the drug alone (18), in the complex with DNA the bicyclo amino sugar has shifted up,

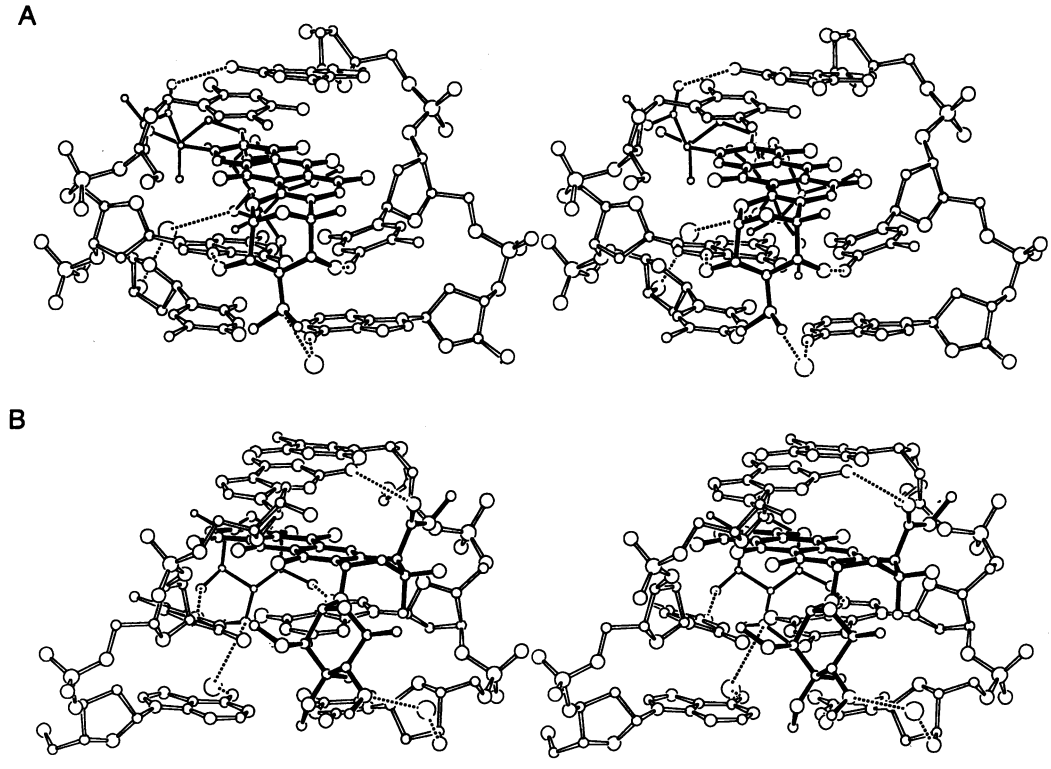

FIG. 4. ORTEP stereoview representations of the DNA–nogalamycin complex. (*A*) Looking into the major groove. (*B*) Looking into the minor groove. The DNA is drawn with hollow bonds and the nogalamycin with solid bonds. The furthest side of the nogalamycin molecule (in the opposite groove) is drawn with thin bonds. Hydrogen bonds are drawn with dashed lines. The largest spheres represent water molecules.

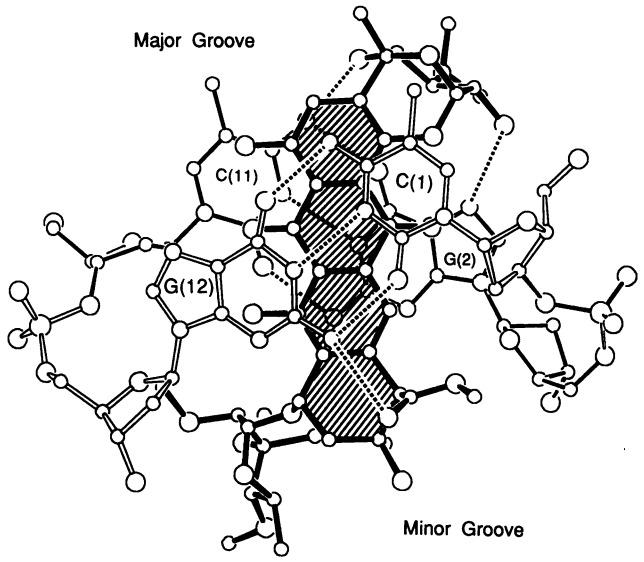

FIG. 5. Projection of the complex onto the plane of the nogalamycin chromophore. The terminal C(1)·G(12) base pair is drawn with thick hollow bonds and the lower base pair is drawn with thin bonds. The nogalamycin, drawn with thick solid bonds, is shaded.

away from the mean molecular plane of the chromophore, and is lying closer to the nogalose and methyl ester moieties. This shift is accomplished by torsional rotations primarily about three bonds, C16—C1—C2—C5″ (24°), C16—C1—O1—C1″ (17°) and C5″—C2—C3—C4 (22°).

In the nogalamycin complex, the DNA adopts a distorted right-handed helix with Watson–Crick base pairing. The sugar-phosphate backbone and glycosidic torsion angles of the DNA are listed in Table 1. The sugar-phosphate backbone torsion angles generally fall within the ranges observed in complexes formed by daunomycin-type anthracyclines bound to the same DNA sequence: daunomycin plus d(CGTACG) (26) and 11-deoxydaunomycin plus d(CGTsACG) (24). The glycosidic torsion angle χ appears to be highly constrained in intercalated complexes, although the nogalamycin complex deviates from the other two complexes in residue G(6) and to a lesser extent residue C(5).

In contrast to the phosphodiester backbone, the base pairs in the nogalamycin complex deviate from standard geometry and are distorted along their perpendiculars. The C(1)·G(12) base pair is buckled by −11° (−8°) [the corresponding values from the daunomycin–d(CGTACG) complex are in parentheses] and the G(2)·C(11) base pair by 26° (16°). The lack of planarity of the base pairs is clearly observable in Fig. 3 and 4. It appears to be a general rule of intercalation that the base pairs deform (buckle and twist) to wrap around an intercalator to maximize van der Waals contacts.

The base pairs are also distorted within their planes. Nogalamycin causes significant displacement of one base relative to its partner in the direction perpendicular to the Watson–Crick hydrogen bonds, defined as shear (27). The most pronounced shear is observed in the C(1)·G(12) base pair. As can be seen in Fig. 5, G(12) has been shifted away from optimal base-pairing geometry with C(1). It appears that the hydrogen bond from N2 of G(12) to O14 of nogalamycin forces G(12) into the minor groove. At the intercalation step, nogalamycin unwinds the DNA, decreasing the helical twist from the B-DNA value of 36°. The helical twist angles for the first, second, and third base pairs of the complex are 35.6°, 24.8°, and 39.4° (35.0°, 30.8°, and 34.5°), respectively. Extreme adjustments of sugar-phosphate torsion angles are not necessary for such distortion of the helix, confirming the deformable nature of DNA and the potential for drugs and proteins to induce a variety of different DNA conformations.

Table 1. Sugar-phosphate backbone and glycosyl torsion angles

Residue	Angle, degrees						
	α	β	γ	δ	ε	ζ	χ
1			195	93	20	173	170
2	329	96	52	126	246	169	255
3	303	120	57	103	180	287	211
4	281	181	61	103	179	299	236
5	153	174	172	117	281	180	234
6	74	240	195	144			212
B-DNA	297	171	54	123	191	252	243

Backbone torsion angles are defined as $P\overset{\alpha}{-}O5'\overset{\beta}{-}C5'\overset{\gamma}{-}C4'\overset{\delta}{-}C3'\overset{\varepsilon}{-}O3'\overset{\zeta}{-}P$; the glycosidic torsion angle is χ.

DISCUSSION

In the nogalamycin complex, conformational adjustments of both the DNA and the drug combine to achieve a complex effectively stabilized by hydrogen bonds and van der Waals contacts. These adjustments are required to compensate for the discrepancy in the length of the drug and the width of the DNA duplex. The long axis of the drug exceeds the width of the base pairs. Nogalamycin accommodates by swinging the bicyclo amino sugar in towards the DNA to maximize contacts in the major groove. These major-groove contacts force the A ring, nogalose, and methyl ester moieties away from the floor of the minor groove. A guanine must shift into the minor groove, away from optimum base-pairing geometry, to form a hydrogen bond with the methyl ester of nogalamycin.

Nogalamycin, which binds directly in both grooves, distorts the DNA to a far greater extent than the less complicated anthracyclines such as daunomycin and doxorubicin (24, 26, 28, 29). However, surprising analogies are observed between daunomycin-type complexes and the nogalamycin complex. For example, in the daunomycin-type anthracycline complexes, a solvent molecule (or Na^+ ion) that is bichelated by the drug acts as a surrogate functional group forming hydrogen bonds to the major groove. This surrogate functional group of daunomycin is replaced by a virtual functional group of nogalamycin, the bicyclo amino sugar, which forms similar hydrogen bonds to the major groove. In a second analogy, a hydroxyl group of daunomycin, with the C—O bond aligned parallel to the helical axis, forms hydrogen bonds in the minor groove to N2 and N3 of a guanine. Similarly, a carbonyl group of nogalamycin in the same alignment forms a hydrogen bond in the minor groove to the N2 of another guanine.

It is well established that intercalators unwind DNA, decreasing the helical twist. However, the helix unwinds in a very different fashion in the nogalamycin complex in contrast to daunomycin-type anthracycline complexes. The DNA is underwound by 11° in the nogalamycin complex at the site of intercalation and is not underwound elsewhere. The observed unwinding of DNA by nogalamycin in solution by 18° (8) corresponds reasonably with the decrease in helical twist observed at the intercalation site in our crystal structure. By contrast, in complexes with daunomycin-type anthracyclines there is a normal helical twist (≈36°) at the site of intercalation and a small amount of unwinding at the adjacent base pair. The total DNA unwinding in the daunomycin-type complexes correlates well with the observed unwinding by daunomycin in solution.

We can compare the general characteristics of the x-ray structure of nogalamycin plus d(^{m5}CGTsA^{m5}CG) with those of a previous NMR structure of this drug bound to d(GCATGC) (14). A detailed comparison is not possible, as the

published NMR structure was not refined with distance and geometry relationships. In both complexes the drug intercalates with the bicyclo amino sugar in the major groove and the nogalose in the minor groove. In both complexes the drug intercalates at 5′ pyrimidine-purine 3′ steps, a CpG step in the x-ray structure and a TpG step in the NMR structure. It appears that in both complexes the bicyclo amino sugar forms hydrogen bonds to span a C·G base pair, although the details are obscure in the NMR structure. The NMR interactions of nogalose with the minor groove of the DNA are consistent with the x-ray structure. However, the hydrogen bond in the x-ray structure from the carbonyl oxygen of nogalamycin to N2 of a guanine is precluded in the NMR structure, where this guanine is replaced by an adenine.

The x-ray structure allows us to examine the potential sequence specificity of nogalamycin. In the major groove of DNA, the hydrogen-bonding positions of the four base pairs, C·G, G·C A·T, and T·A, are nearly indistinguishable if one neglects proton donation or acceptance. In the major groove, nogalamycin has two hydroxyl groups that can either donate or receive hydrogen bonds. The drug could be stabilized in this position by hydrogen bonds to any base pair, although there would be some slight preference due to geometry. In the minor groove, the hydrogen bond from the carbonyl oxygen of nogalamycin to N2 of a guanine suggests the drug may bind with greater stability to the C+G-rich DNA than to A+T-rich DNA. As the amino group of guanine lies near the pseudotwofold axis of the base pair, the hydrogen bond of the N2 of a C·G base pair is nearly equivalent to that of a G·C base pair.

The replacement of an oxygen with a sulfur in the DNA backbone does not appear to significantly affect the conformation of the complex. A multiple phosphorothioated analogue of $[d(GC)_3]_2$ crystallized in the B-type conformation with no obvious structural perturbations resulting from the switch of several oxygens to sulfurs (30). In the complex of 11-deoxydaunomycin with a phosphorothioated analogue of d(CGTACG) (24), the conformation of the DNA is similar to that in related but nonphosphorothioated DNA–anthracycline complexes (26, 29). Similarly methylation at the 5 position of the cytosines is not expected to alter the conformation of the DNA–nogalamycin complex, as this modification causes no structural perturbation in other DNA–anthracycline complexes crystallized in our laboratory (unpublished observations).

The dumbbell shape of nogalamycin presents a mechanistic obstacle to formation of a stable DNA–drug complex, and it is interesting to consider how nogalamycin might obtain entry into a long DNA duplex. There are two possible mechanisms of binding, both involving DNA fluctuations. In the first mechanism, transient melting of several base pairs could create an opening in the duplex to allow entry of nogalamycin (17). In a second mechanism, unstacking of DNA base pairs without base-pair disruption could also create an opening to allow entry of nogalamycin. In this second mechanism, the fluctuation would be of greater amplitude than the opening required for binding of simple intercalators. The opening could be enlarged by full extension of the helix and by buckling the base pairs. With models, we have observed that fully unstacked but still base-paired DNA may not pose a steric block to entry of certain conformations of nogalamycin (with the bulky substituents of the A ring in an equatorial conformation). Thus, large perturbations of both DNA and the drug may be required for nogalamycin binding.

This study increases our knowledge of the interactions of a chemotherapeutic agent with its cellular target. Ultimately we hope to understand relationships among DNA sequence, drug structure, and stability and conformation of DNA–drug complexes.

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