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Molecular structure of an anticancer drug–DNA complex: Daunomycin plus d(CpGpTpApCpG)

(intercalation/x-ray diffraction/drug specificity/hydrogen bonding/anthracyclines)

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ABSTRACT The structure of the crystalline daunomycin-d(CpGpTpApCpG) complex has been solved by x-ray diffraction analysis. The DNA forms a six-base-pair right-handed double helix with two daunomycin molecules intercalated in the d(CpG) sequences. The daunomycin aglycone chromophore is oriented at right angles to the long dimension of the DNA base pairs and the cyclohexene ring rests in the minor groove. Substituents on this ring have hydrogen bonding interactions to the base pairs above and below the intercalation site. These appear to be specific for anthracycline antibiotics. The amino sugar lies in the minor groove of the double helix without bonding to the DNA. The DNA double helix is distorted in a novel manner in accommodating the drug.

An important group of antibiotics are those that interact with DNA and have antitumor activity. Daunomycin, an anthracycline antibiotic, has been found to inhibit virus multiplication and shows considerable activity against tumors; it was the first antibiotic to show activity against acute leukemia in man. It has an aglycone chromophore containing four fused rings and an amino sugar (Fig. 1). The closely related antitumor agent adriamycin (14-hydroxydaunomycin) is widely used in treating various solid tumors. These compounds have been the subject of intensive chemical and biological research since their discovery 17 years ago. More than 500 compounds of this type have been synthesized or isolated from nature and tested for activity (1, 2). These agents are believed to act by binding to DNA and inhibiting both DNA replication and transcription. Several studies suggest that the unsaturated chromophore intercalates between DNA base pairs (1, 2). However, the complexity of the molecule makes its mode of interaction with DNA a matter of considerable interest.

Here we report the crystal structure of daunomycin with a self-complementary DNA fragment, d(CpGpTpApCpG). The structure has been solved by x-ray diffraction methods. A six-base-pair fragment of double-helical DNA was found with two molecules of daunomycin bound to it, plus 80 molecules of water. The structure reveals the manner in which the stereochemistry of the antibiotic is required for DNA binding. The DNA accommodates the drug by changing its conformation in a novel manner.

EXPERIMENTAL

Materials and Methods. The deoxy hexanucleoside pentaphosphate was synthesized by a modified triester method (3); daunomycin was purchased from Sigma. A solution containing 2 mM deoxynucleotide, 2 mM daunomycin, 15 mM $MgCl_2$, 10 mM spermine, and 30 mM cacodylate buffer (pH 6.5) was equilibrated with 30% (vol/vol) 2-methyl-2,4-pentanediol. After 1 week, attractive bright red-orange, tetragonal, rod-like crystals appeared. Spectroscopic analysis of the dissolved crystal revealed a 1:1 ratio of hexanucleotide to daunomycin. X-ray diffraction studies indicated a tetragonal crystal system with space group $P4_12_12$ or $P4_32_12$, $a = b = 27.92$ Å, $c = 52.89$ Å. The asymmetric unit contained one hexanucleotide and one daunomycin molecule. Intense meridional reflections at 3.3 Å along the *c* axis strongly suggested that the bases were stacked perpendicular to that direction. Three-dimensional data were collected on a Nicolet x-ray diffractometer to a resolution of 1.5 Å. A total of 3428 reflections were collected, of which 2108 were observed at the 2σ level.

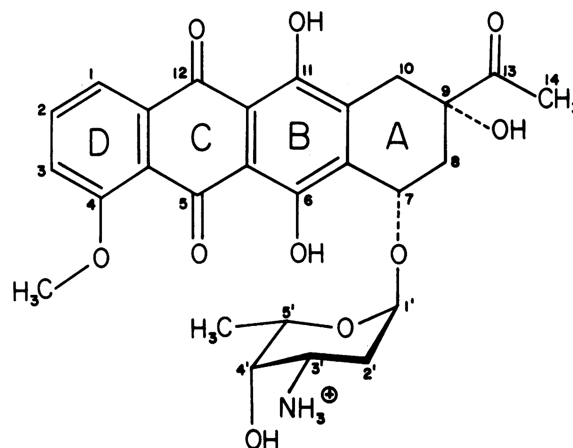

FIG. 1. Daunomycin.

Structure Determination. Dividing the 52.89 Å *c* axis by 3.3 Å gave 16. This suggested that there might be two segments of six-base-pair DNA plus four intercalating daunomycin molecules along the *c* axis. The six-base-pair duplex with two daunomyins could have two symmetry-related segments with daunomycin intercalated between d(CpG) or d(GpT). A number of intercalating structures have been found to have a pyrimidine-purine sequence (4–7). We thus made the initial assumption that the structure consisted of a fragment of B DNA in the middle with daunomycin intercalated in the d(CpG) segments at either end. We decided to attempt a solution by the method of molecular replacement, using a model and testing how well it refined against the observed diffraction data. We had solved at high resolution the structure of r(CpG) with an

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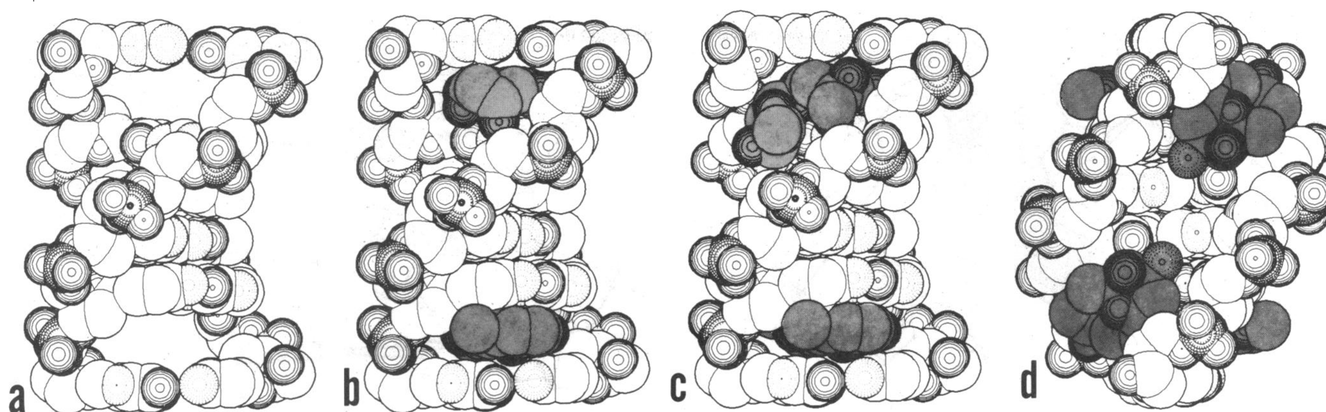

FIG. 2. Space-filling drawings of the daunomycin–d(CpGpTpApCpG) complex. In *a–c*, the molecular 2-fold axis is horizontal and in the plane of the paper. (*a*) Right-handed helical hexanucleotide duplex by itself. (*b*) Hexanucleotide and aglycone without the acetyl group on C9. Note the way O9 projects down over G2 from the aglycone at the top. (*c*) Hexanucleotide and complete daunomycin. The amino sugar extends into the minor groove. (*d*) The complex as viewed into the minor groove down the molecular 2-fold axis perpendicular to the paper. The amino sugars and ring A fill most of the minor groove. The C4 methoxy group can be seen extending through into the major groove. Heavy stippling, daunomycin atoms; open circles, carbon; dotted circles, nitrogen; solid circles, oxygen; radial-spiked circles, phosphorus. Hydrogen atoms are not shown.

acridine orange intercalator which provided a reliable set of coordinates (8). For the segment of four base pairs lying between the two intercalating compounds we used B DNA coordinates (9). The two fragments were joined by doing a least-squares superposition of a G·C base pair of the r(CpG) with the terminal base pair of the B DNA tetramer. The model was positioned in the unit cell by superimposing the molecular dyad perpendicular to the helix axis on the unit cell's 2-fold axis.

The model was initially positioned so that the helix axis coincided with the 2-fold screw axis of the unit cell along *c*. The model was refined independently in both space groups by using the constrained least-squares refinement procedure of Hendrickson and Konnert (10). As refinement proceeded, it became apparent that the correct space group was $P4_12_12$. The initial refinement reduced the R (residual) value from its starting value of 46% to 34%, at which time difference and $2F_o$-F_c electron density maps were calculated. The acridine orange had been included with its long axis oriented along the long part of the base pair. However, the difference maps revealed that the actual intercalator was oriented almost perpendicular to the acridine orange. The model was rebuilt and refined, and difference Fourier maps then revealed the amino sugar (R $\approx$ 28%). Water molecules began to appear in the difference Fourier maps and were then added.

The refinement has been carried out to an R value of 20% for the 2σ data (26% for all of the data). Even though there was a considerable reorientation of daunomycin during refinement, there was little movement of the DNA segments with the exception of the terminal cytidine, which moved approximately 2 Å, with one atom moving >3 Å. The final refined structure reveals all of the atoms of the DNA and the daunomycin plus 40 water molecules in the asymmetric unit.

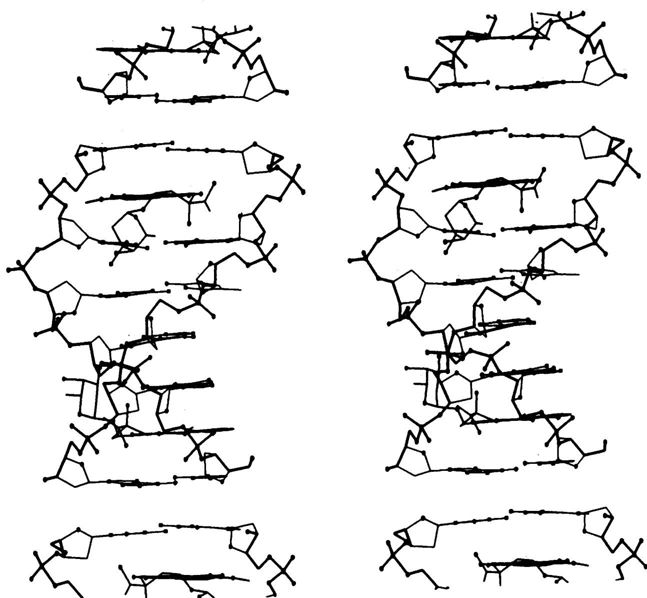

FIG. 3. Stereo drawing of the daunomycin–d(CpGpTpApCpG) complex viewed perpendicular to the 2-fold screw axis and 30° from the molecular 2-fold axis. Portions of the adjacent duplexes along the *c* axis are shown. Nitrogen and oxygen atoms are shown as dots.

RESULTS

A space-filling model of the daunomycin–DNA complex is shown in Fig. 2. The DNA double helix is shown without the drug (Fig. 2*a*) in a configuration grossly similar to that of B DNA. Fig. 2*b* shows the aglycone chromophore intercalated in the molecule. The cyclohexene ring A is shown protruding into the minor groove at the top with its axial 9-hydroxyl oxygen found below the plane of the intercalator. At the bottom, ring D protrudes through the base pair with the methoxy group at the left. Fig. 2*c* shows the complete anthracycline with the amino sugar added. Fig. 2*d* is a view down the 2-fold axis into the minor groove of the double helix, showing the extent to which the minor groove is filled by the amino sugars and ring A of the two daunomycin molecules. Only parts of the central A·T base pairs are visible.

Fig. 3 is a stereodiagram of the complex. The upper daunomycin molecule can be seen with its amino sugar extending to the left in the minor groove. The axially oriented hydroxyl group of C9 projects downward, coming close to the base pair immediately below. Several of the base pairs have a slight propeller-type twisting. A view perpendicular to the bases is shown in Fig. 4. This shows the manner in which the aglycone chromophore skewers two adjoining base pairs, lying almost at right angles to the long axis between the base pairs.

The stacking of the bases upon each other is shown in Fig. 5. The stacking of the A·T base pair over the T·A base pair at the center of the molecule is shown in Fig. 5*a*. This pair lies around the dyad axis and shows stacking typical of pyrimidine-purine sequence in B DNA. In Fig. 5*b* the stacking of the

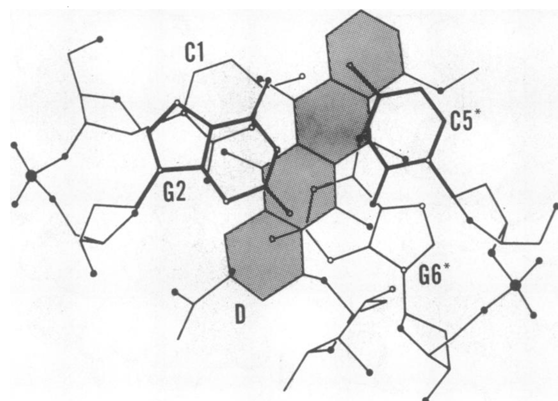

FIG. 4. View of the intercalator perpendicular to the base plane. The daunomycin (D) ring system is stippled. The adjacent G2·C5* base pair closer to the reader is shown by thick lines and the C1·G6* base pair further away is shown by thin lines. The two nucleotide backbones are different. Also note that the center of the G2·C5* base pair has moved up, toward the major groove relative to the C1·G6* pair. * designates the complementary sequence.

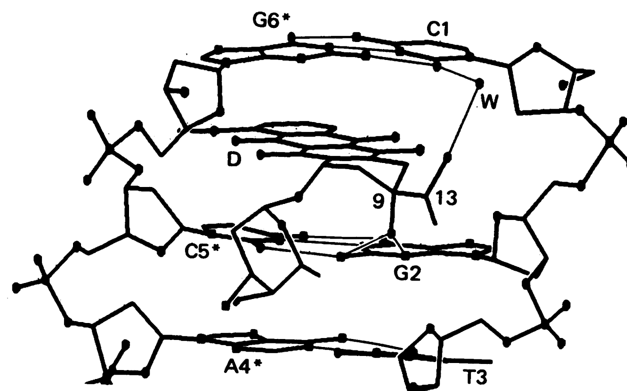

FIG. 6. Diagram of daunomycin (D) intercalated into d(CpGpTpApCpG), showing intermolecular attractions. Note two hydrogen bonds between O9 of daunomycin and N2 and N3 of G2. In addition, water (W) forms a hydrogen-bond bridge between O13 of daunomycin and O2 of C1. Oxygen atoms are shown as ellipses; nitrogen atoms are shown as squares.

T·A pair over the G·C pair is shown. This is somewhat similar to that anticipated for a B DNA purine-pyrimidine sequence but there is a significant deviation from the pseudo 2-fold axis relating the backbones of the molecules. Furthermore, the "helix axis" of the molecule is not the same in the two base pairs, and the G2·C5* appears to be translated toward the major groove by about 1.3 Å. This represents an alteration in the base stacking one removed from the intercalative site. A similar translation of the "axis" of the G2·C5* base pair is also seen around the intercalator in Fig. 4. Fig. 5*c* shows the stacking between two molecules in which the C·G end base pair of one molecule is stacking over the end G·C base pair of its neighbor along the *c* axis. The base pairs are stacked over each other in a relatively parallel fashion.

The unwinding angle is the extent to which adjacent base pairs are stacked at other than the 36° of B DNA. The unwinding angle for the two base pairs on either side of the intercalator is close to 0° (Fig. 4); the next two base pairs (Fig. 5*b*) have an unwinding angle of approximately 8°. This is associated with a distortion of the backbone which appears to be more extended in the left strand and slightly compressed in the right. An opposite effect is seen around the intercalator.

The conformation of the DNA backbone is interesting because it does not fall in into any of the classical categories such as A DNA or B DNA but rather forms a right-handed helix with significant and numerous structural variations associated with accommodation of the large intercalator. The puckers of the six deoxyribose rings are generally in the C2′-*endo* class with one exception. The actual puckers are as follows: C1, C2′-*endo;* G2, C1′-*exo;* T3, O1′-*endo;* A4, C2′-*endo;* C5, C2′-*endo;* and G6, C3′-*exo*. There is no simple mixed pucker found in the bases on either side of the intercalator. The torsion angles around the phosphodiester bonds are generally those found in a right-handed double helix, but they do not fall into regions traditionally defined as either A or B DNA. Also the χ values, which measure the rotation of the base around the sugar, vary somewhat. In B DNA, χ is about 78°; in A DNA it is 26°. Here the χ angles are: C1, 27°; G2, 87°; T3, 49°; A4, 73°; C5, 98°; and G6, 95°. The low χ angle in residue C1 may be associated with its position at the end of the chain. In surveying the backbone conformation, we see that most of the accommodations in adapting to the intercalator are in the C3′-O3′ and O3′-P bonds because these show more variation than the other torsion angles. The DNA molecule has thus changed in many ways to accommodate a rather complex intercalator with a bulky group binding in the minor groove.

Three different daunomycin derivatives have been solved (1). The conformation of daunomycin observed in the intercalative complex is similar to that observed in the three earlier structures. Atom C9 is the farthest removed from the plane of the unsaturated ring system (0.3 Å) and the C9-O9 bond is virtually perpendicular to the plane of the ring in an axial orientation. The acetyl group on C9 is in an equatorial orientation, and the carbonyl on C13 is oriented perpendicular to the ring in a direction opposite to that of the C9 hydroxyl.

One of the striking features of the anthracyclines is that changing a number of constituents produces only moderate or no influence on activity. However, some substituents seem to

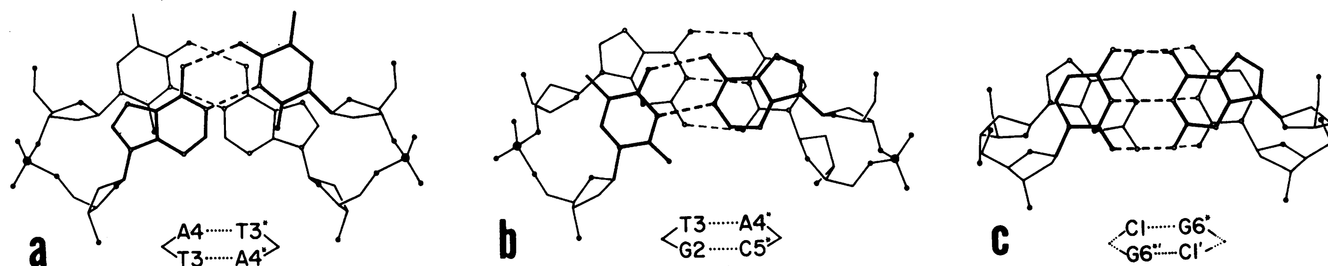

FIG. 5. Base pair stacking; the base pair closer to the reader is drawn with thicker lines. (*a*) The A·T base pair over the 2-fold-related T·A base pair at the center of the duplex. (*b*) The T·A base pair over the G·C base pair. Note the compression on the right side of the backbone. In this projection, as in Fig. 4, we are viewing the stacking from the center of the molecule toward the outside. (*c*) The terminal C·G base pair stacking over the 2-fold related G·C pair from an adjacent duplex.

be important. This is particularly true of the hydroxyl on C9, which appears to be essential for activity (11). It is thus of great interest to find that this hydroxyl oxygen is within hydrogen-bonding distance of two atoms in guanine G2 (Fig. 6). There appears to be a strong hydrogen bond between O9 and the ring nitrogen N3 of guanine G2 (2.6 Å). In addition, the amino group N2 of the same guanine is also within hydrogen-bonding distance of O9 (2.9 Å). This proton on guanine N2 is normally found with a water molecule bonding to it; there is no room for a water molecule here, and the presence of O9 within hydrogen-bonding distance suggests that two hydrogen bonds are made in this case.

Another hydrogen-bonding system is seen on the other side of the aglycone ring involving the C13 carbonyl oxygen (Fig. 6). This carbonyl oxygen is within hydrogen-bonding distance to a bridging water molecule which itself is hydrogen bonded to carbonyl O2 of C1 in the base pair above the intercalator. The angle between the carbonyl oxygen O13, the water molecule, and the carbonyl O2 of C1 is 107°, very close to that which is normally found for water molecules. This water molecule which appears strongly in the electron density map may also stabilize insertion of the unsaturated chromophore into DNA. The detailed geometry of daunomycin ring A thus appears to play an important role in its interaction with the minor groove of the double helix.

DISCUSSION

The structure refines to a reasonable, but not low, residual value (20%). This may be associated with different water molecule organizations some distance away from the double helix because only about half of the anticipated water molecules are visualized at present. The cations may be spermine, magnesium, or sodium ions, but we cannot distinguish them yet. Although there are some uncertainties associated with fine details in the molecule, we do not expect significant changes in conformation to be found on further refinement. It is possible, however, that measurements at lower temperatures will allow us to obtain data at higher resolution.

A large number of deoxyribodinucleoside- and ribodinucleoside phosphates have been studied, often with intercalators (4–8). The structure of d(pApTpApT) contains helical fragments with two base pairs (12). A left-handed DNA double helix with six base pairs has been determined (13). We have recently learned that Dickerson and colleagues (14) have solved the structure of a dodecamer in the B DNA conformation. The significance of the present study is that it shows a right-handed segment of DNA in which there are significant variations both in backbone geometry and sugar puckering associated with the accommodation of a large intercalator molecule with an amino sugar in the minor groove. The base stacking around the central two A·T pairs (Fig. 5*a*) looks similar to that seen in B DNA but there are modifications in the geometry of the backbone. This suggests that there may be various backbone conformations associated with B DNA that may be dependent on sequence or on the local environment. This suggestion has also been made by others on the basis of ^{31}P resonance studies of DNA with different base sequences (15).

The distortions to the DNA double helix associated with the daunomycin intercalator are more complex than have been anticipated (16). Up to the present, only structures of dinucleoside phosphates associated with intercalators have been determined (4–8). These generally yielded an unwinding angle but provided no information about perturbations farther away. In the present structure we see two kinds of effects. Around the intercalator we see a modification such that, instead of unwinding, the base pair G2·C5* shows in effect a lateral translation of more than 1 Å toward the major groove. There is a net dislocation of the helix axis associated with the intercalator of about 0.4 Å. There is no evidence that this translation of the axis is associated with a "kink" in the DNA (17). This translation may be associated with the presence of the amino sugar in the minor groove, or perhaps the O9 protruding below the intercalator plane induces the displacement of the G2·C5* pair. It will be interesting to see whether similar variations will be found when other intercalator complexes are solved. Instead of having unwinding associated with the bases surrounding the intercalator, there is unwinding associated with the base pair one removed from the intercalator. A 0° unwinding angle has already been observed in the r(CpG)–proflavin complex (6). The total amount of unwinding seen per daunomycin is 8°, compared with 26° observed with ethidium (5). This result correlates reasonably well with solution studies on superhelical DNA in which the unwinding associated with daunomycin has been estimated to be near 11° (18, 19). The modifications of the geometry of the double helix and the unwinding associated with this intercalator suggest that there are not likely to be general rules that can be applied for all intercalators, as had been suggested by some previous studies (4, 7). Instead, we may expect different effects to be seen, and many will be quite specific to the individual drug.

The daunomycin chromophore seems to skewer the double helix in an interesting way so that ring D actually sticks out on one side with ring A on the other. An interaction of this type had been suggested by Gabbay *et al.* (20). Ring D protrudes into the major groove with the methoxy group no longer interacting with the double helix. Removal of the methoxy group is associated with a slight increase in the effectiveness of the drug (11). On the other hand, the substituents on ring A seem to play a key role in interacting with the bases on either side of the intercalator in the minor groove. Two interactions are seen, with the hydroxyl 9 on the A ring binding to the G residue in the minor groove (Fig. 6). This suggests that there may be a slight preference for G. However, the strong interaction involving hydrogen bonding to N3 could still be there if adenine were present, even though the other interaction would be missing. Likewise, O2 of pyrimidine might play a role similar to that of N3. In a similar way, the interaction associated with bound water molecules and the carbonyl O13 would occur with various bases other than cytosine in that site.

In structure–activity studies, modifications of the groups attached to C9 produced profound effects (11). The interactions of the daunomycin substituents attached to C9 of ring A thus seem to provide specificity for the double helix. In addition, the sugar extending to the lower left (Fig. 6) with the acetyl group toward the upper right provides a natural fit into the right-handed double helix. The size of this interaction accounts for the limit, in solution, of one daunomycin per three base pairs of DNA (1). Adriamycin is 14-hydroxyldaunomycin. If a hydroxyl group were placed on daunomycin in the present conformation, it could form a hydrogen bond to O3′ of the phosphate group associated with the guanine residue. We have been able to crystallize adriamycin with the same oligonucleotide sequence but the crystals have remained small. It will be interesting to see whether adriamycin is stabilized by an additional hydrogen bonding interaction.

A number of water molecules surround the amino sugar but none of them forms single bridging water molecules back to the bases in the minor groove or to the phosphates on either side. Thus, we do not see specificity associated with the amino sugar in the minor groove. A number of modifications of the geometry of the amino sugar have been reported, many of which are associated with continued biological activity. This is compatible

with the fact that we do not see specific interactions between the amino sugar and the double helix. The positively charged amino group is not interacting with the phosphates as has been previously proposed (16, 20) but rather is sitting in the center of the minor groove, clearly separated from the phosphates.

Daunomycin has the effect of killing tumor cells by inhibiting both DNA replication and transcription (12). Does the present structure provide insight into how this may occur? The molecule has a planar chromophore which appears to be securely bound into the molecule by skewering between two base pairs. It is held in place by interactions between the substituents on ring A and the minor groove of the helix. This provides an anchor to hold the amino sugar in the minor groove, where it sits with its functional hydroxyl and amino groups facing the outside. The present structure makes one wonder whether the polymerase working its way down the double helix comes upon this amino sugar and binds to it, these functional groups securing the polymerase. This may prevent, or at least retard to a considerable extent, its further progress. A hypothesis of this type suggests that there may be some latitude for changing the conformation of the sugar and the disposition of various functional groups which will still inhibit polymerases to various extents. Such modifications have been studied (11).

The novel finding in this study is the fact that the nonplanar substituents in ring A of the daunomycin chromophore interact through hydrogen bonding with the double helix and provide an anchoring function. We can now see that there are three different functional parts in the molecule: the intercalator (rings B–D), the anchoring function associated with ring A, and the amino sugar. As mentioned above, we believe that the additional hydroxyl group in adriamycin also contributes to the anchoring function by forming an additional hydrogen bond to the nucleic acid backbone. Adriamycin is used in treating a number of tumors that are quite distinct from those against which daunomycin is active. This suggests that modifications of the anchoring function may change the manner in which the anthracycline interacts with the DNA and thereby change its activity against different types of tumors. We should be able to use the geometrical arrangements that we can see in this complex to devise further variants in anthracycline anchoring modes. Thus, we can add further substituents which will also hydrogen-bond to the backbone. By analogy with adriamycin, this may give rise to an altered specificity of these anti-tumor agents. Such studies would represent a further step toward the goal of understanding the architecture of the detailed molecular interactions between drugs and DNA so that we can make some predictions about their mode of action *in vivo*.

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