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Molecular structure of the halogenated anti-cancer drug iododoxorubicin complexed with d(TGTACA) and d(CGATCG)

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ABSTRACT

4′-Deoxy-4′-iododoxorubicin, a halogenated anthracycline derivative, is an anticancer agent currently under Phase II clinical trials. In preclinical studies, it has demonstrated significantly reduced levels of cardiotoxicity compared to currently employed anthracyclines. It also has modified pharmacological properties resulting in an altered spectrum of experimental antitumor activity. The iodine atom at the 4′ position of the sugar ring reduces the basicity and enhances the lipophilicity of this compound as compared to related anthracycline drugs. We report here single crystal X-ray diffraction studies of the complexes of 4′-deoxy-4′-iododoxorubicin with the hexanucleotide duplex sequences d(TGTACA) and d(CGATCG) at 1.6 and 1.5 Å, respectively. The iodine substituent does not alter the geometry of intercalation as compared to previously solved anthracycline complexes, but appears to markedly affect the solvent environment of the structures. This could have consequences for the interaction of this drug with DNA and DNA binding proteins in cells.

INTRODUCTION

Anthracyclines are cytotoxic DNA intercalators widely used as chemotherapeutic agents in the treatment of cancer. The biological properties of these intercalators involve ternary interactions of drug–DNA complexes with other DNA binding factors (1,2). The anticancer activity and the biological tolerance of anthracyclines depend strongly on minor modifications in chemical structure (3,4). Daunomycin, for example, is used in the treatment of acute leukemia, whereas doxorubicin (also known as adriamycin) is more effective in the treatment of solid tumors (5,6). Doxorubicin differs from daunomycin simply by the substitution of one hydroxyl group. Inversion of the stereochemistry at the 4′-position of doxorubicin yielded a drug (epiadriamycin) with improved tolerance, which is also in clinical use (3). Replacement of the 4′-hydroxyl group in doxorubicin by an iodine results in iododoxorubicin (Fig. 1), a compound which shows significantly reduced levels of cardiotoxicity in comparison with the other clinically relevant anthracyclines (7,8). In addition, iododoxorubicin has modified pharmacological properties. Ongoing Phase II clinical trials revealed that overexpression of the multi drug resistance gene (*mdr-1*), which is strongly associated with the development of a high level of resistance to classic anthracyclines such as daunomycin and doxorubicin, does not lead to resistance to iododoxorubicin, suggesting that iododoxorubicin may remain active in *mdr* cell systems (9,10).

The electron withdrawing character of the iodine atom at position 4′ of iododoxorubicin markedly influences the basicity of the adjacent amine functionality located at position 3′. Whereas the pK_a of doxorubicin and daunomycin are ~8.4, the pK_a of iododoxorubicin is reduced significantly ($pK_a = 6.4$) such that the agent is predominantly uncharged at physiological pH. Such a change in drug basicity strongly modulates important physicochemical parameters such as membrane interactions (11) and DNA binding affinity (12).

Evidence suggests that anthracycline agents act predominantly on the DNA level (13,14), and microfluorescence experiments involving doxorubicin showed that >99.8% of the drug detected in the cell nuclei is bound to DNA (15,16). We ask here whether the iodination of the sugar moiety influences the structural features of DNA binding by co-crystallizing iododoxorubicin with two DNA-hexamers, d(TGTACA) and d(CGATCG). As expected, the anthracycline formed a 2:1 complex with each B-DNA hexamer duplex. While both DNA and drug geometry show only minor variations when compared to previously reported anthracycline structures (17–24), the existence of a bulky and hydrophobic iodine atom in the minor groove affects

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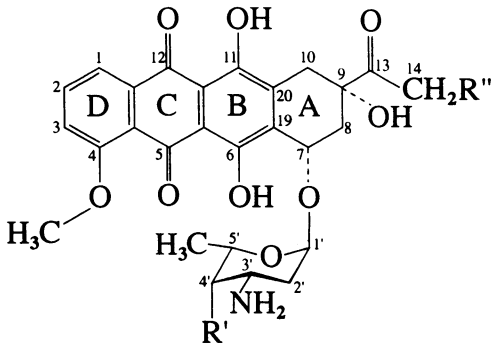

Daunomycin: R'=OH R"=H

Doxorubicin: R'=OH R"=OH

Iododoxorubicin: R'= I R"=OH

Figure 1. Molecular formula of anthracycline analogues. In the case of iododoxorubicin, the sugar is halogenated at the 4′ position. At physiological pH, iododoxorubicin is largely uncharged in contrast to daunomycin and doxorubicin.

the hydration in the complexes. This could possibly contribute to the altered pharmacological activity (7–10,12).

MATERIALS AND METHODS

Self-complementary DNA hexamers were synthesized on an Applied Biosystems DNA synthesizer and purified by reversed phase HPLC. Crystals were grown at 4°C in hanging drops over 30% 2-methyl-2,4-pentanediol (MPD) using a 24 condition crystallization matrix for nucleic acids which we developed (Berger, I., Kang, C.H., Sinha, N., Wolters, M. and Rich, A., submitted). Crystals appeared after several days in virtually every hanging drop of the matrix. The complex formed by the drug and d(TGTACA) will be referred to in the following as iodo-TGT, the complex with d(CGATCG) as iodo-CGA. In the case of iodo-TGT, a solution containing 2 mM DNA (single strand concentration), 4 mM iododoxorubicin, 10 mM $MgCl_2$, 6 mM spermine, 40 mM KCl, 20 mM potassium cacodylate buffer (pH 6.5) and 5% MPD yielded crystals shaped as tetragonal rods with the approximate dimensions 0.2 × 0.2 × 0.6 mm. The largest crystals of iodo-CGA were obtained as flat rectangular plates with the dimensions 0.5 × 0.5 × 0.2 mm from drops containing 6 mM spermine, 40 mM KCl, 20 mM potassium cacodylate buffer (pH 6.0) and 5% MPD in addition to 2 mM d(CGATCG) and 4 mM iododoxorubicin. X-ray diffraction analysis revealed that both complexes crystallized in space group $P4_12_12$. Three dimensional X-ray diffraction data were collected on a Rigaku R-axis IIC imaging plate at 4°C. Cell parameters and symmetry considerations suggested the existence of one drug molecule and one hexamer in the asymmetric unit, similar to the virtually isomorphous crystal of doxorubicin bound to d(CGATCG) (17). Coordinates for one hexamer strand of d(CGATCG) and the drug chromophore from this structure were used as a starting model in the analyses of both structures. Conventional crystallographic refinement was carried out with XPLOR (25). The iodine position was immediately and clearly visible as high contour electron density, and the halogenated sugar moiety of the drug was added to the model. At this point, the DNA sequence was adjusted in the case of iodo-TGT. Superimposed 2Fo-Fc electron density and Fo-Fc difference density maps revealed the positions of ordered water molecules which were added in groups of five until the entire clearly defined electron density was accounted for. The refinement converged at an R-factor of 17.6% for 2521 reflections above the 1σ level from 10 to 1.6 Å in the case of iodo-TGT and at an R-factor of 18.0% for 3028 reflections from 10 to 1.5 Å above 2σ in the case of iodo-CGA. Selected crystal data and refinement parameters of the iodo-TGT and iodo-CGA complexes are summarized in Table 1. The coordinates have been deposited in the Brookhaven Protein Data Base and are available from the authors upon request until released.

Table 1. Selected refinement parameters of iodo-TGT, iodo-CGA

	iodo-TGT	iodo-CGA
unit cell dimensions (Å)	$a, b = 27.96$ $c = 52.57$	$a, b = 27.73$ $c = 52.49$
crystal system	tetragonal	
space group	$P4_12_12$	
Z	8	8
V_{asym} (Å^3)	5137.2	5046.5
non-hydrogen atoms	208	211
solvent molecules	49	52
total number of variables[a]	833	845
unique data	2521 > 1σ	3028 > 2σ
overdeterminancy ratio (F_{obs}/variables)	3.0	3.6
resolution	1.6 Å	1.5 Å
r.m.s. deviations for bonds (Å)	0.016	0.019
final R-factor	17.6%	18.0%

[a] (number of atoms × 4) plus overall scale factor.

RESULTS

The complexes of iodo-TGT and iodo-CGA form distorted right handed B-DNA duplexes with, in each case, two drug molecules binding to one hexamer duplex. The asymmetric unit of the crystals contains one drug molecule and one strand of DNA, and a crystallographic 2-fold axis generates the entire molecule. Figure 2 shows the complex between iododoxorubicin and the hexamer duplex formed by d(CGATCG). The numbering scheme of the drug is indicated in Figure 1. The complex between the drug and d(TGTACA) is largely isomorphous apart from differences in DNA sequence and moderate changes in DNA conformation (r.m.s.d. value between respective drug and DNA backbone atoms is 0.26 Å).

Both the iodo-TGT and iodo-CGA complexes show a conformational pattern consistent with previously determined structures (reviewed in 18). The hexamer duplexes adopt a distorted

Table 2. Backbone torsion angles, glycosyl torsion angles and sugar puckers[a]

residue	α	β	γ	δ	ε	ζ	χ	Phase	ribose pucker
				Iododoxorubicin - d(TGTACA) Complex					
T(1) / T(7)			39.9	141.3	-126.3	-78.9	-153.6	159	C2'-endo
G(2) / G(8)	-79.6	176.6	51.1	146.1	-137.3	169.7	-86.5	147	C2'-endo
T(3) / T(9)	-61.9	140.9	56.9	130.9	-175.6	-99.6	-122.8	141	C1'-exo
A(4) / A(10)	-67.9	-178.2	51.3	135.0	-170.4	-89.1	-106.3	149	C2'-endo
C(5) / C(11)	-71.7	170.0	51.5	139.9	-106.8	171.7	-85.6	140	C1'-exo
A(6) / A(12)	-80.2	-177.1	54.3	152.5			-84.1	177	C2'-endo
				Iododoxorubicin - d(CGATCG) Complex					
C(1) / C(7)			58.0	149.7	-123.8	-76.0	-152.6	171	C2'-endo
G(2) / G(8)	-77.9	174.5	54.3	140.5	-129.3	171.0	-89.1	147	C2'-endo
A(3) / A(9)	-63.6	137.0	49.9	141.4	-170.3	-104.3	-117.9	152	C2'-endo
T(4) / T(10)	-77.1	-175.7	55.6	126.3	-167.9	-91.5	-106.9	136	C1'-exo
C(5) / C(11)	-74.5	164.5	54.7	137.4	-110.6	174.8	-84.5	135	C1'-exo
G(6) / G(12)	-81.4	-172.8	56.5	151.7			-84.1	177	C3'-exo
A-DNA	-70	172	41	79	-146	-78	-154		C3'-endo
B-DNA	-33	138	33	142	-141	-157	-139		C2'-endo

[a]The backbone torsion angles are defined as $O3'_{n-1}$ -P-α-O5'-β-C5'-γ-C4'-δ-C3'-ε-O3'-ζ-P-$O5'_{n+1}$ and χ is the glycosyl angle. Angles and phases are given in degrees.

B-DNA conformation. The torsion angles for the oligonucleotide backbone and glycosyl bonds of the individual bases are given in Table 2 together with average values for A- and B-DNA, for comparison. The individual α/ζ conformations along the backbone from 5′ to 3′ follow an identical pattern in the iodo-TGT and iodo-CGA structures, and this is in agreement with the values found in the complex of d(CGATCG) with doxorubicin (17). The values for the other backbone torsion angles, γ, δ and ε in the iodo-CGA structure are also close to the values which were determined for other anthracycline drug–DNA structures (17–24). It is noteworthy that these torsional angles in both iodo-CGA and iodo-TGT display virtually identical patterns (Table 2), despite DNA sequence differences. In this respect, the iodo-TGT complex is closer to the iodo-CGA complex than to a complex of daunomycin bound to the same hexamer sequence, d(TGTACA), solved previously by Nunn *et al.* (19).

Showing a common feature of anthracycline type intercalation, the terminal two base pairs encompassing the intercalated chromophore are considerably buckled (Table 3). The buckle angle of the second base pair in most anthracycline drug–DNA structures is more commonly founds to be ~16° (18) and does not exceed 19° as in the iodo-CGA complex (κ = 19.4°). With a helical twist of ~34°, the iodo-TGT and iodo-CGA complexes are underwound (Ω = 36°) compared to regular B-DNA, but while in the iodo-CGA complex the individual twist of the base pairs varies moderately from 29.7° to 37.2°, there is more pronounced variation of the helical twist in the iodo-TGT complex with Ω ranging from 26.3° to close to 40° at the central dinucleotide step. Most previously reported anthracycline drug–DNA complexes showed a rather limited variability in helical twist (17) similar to the iodo-CGA complex. However, the daunorubicin-d(TGTACA) complex (19) exhibited Ω values nearly identical to those found in the iodo-TGT structure.

The drug molecules intercalate between the terminal two base pairs of the individual hexamer duplexes such that the drug chromophore long axis is nearly perpendicular to the C1′-C1′ vectors of both the base pair above and that below the intercalator. The stacking of the intercalated aglycone between the two terminal base pairs in each structure can be observed in Figure 3. In addition, it can be seen that the only direct hydrogen bonds from the aglycone to the DNA involve the 9-hydroxyl group, which is essential for the biological activity of anthracyclines (26). Figure 4 displays, in a minor groove view, the interactions between one iododoxorubicin molecule and four adjacent base pair steps, including the intercalation sites as found in the structure of iodo-TGT (A) and iodo-CGA (B). Only four functional groups on the DNA are located within hydrogen bonding distance of donors or acceptors on the drug molecule in the iodo-TGT structure whereas six hydrogen bonds are possible in the iodo-CGA complex.

In addition, certain indirect hydrogen bonds via water molecules are observed in the two complexes. In the iodo-TGT complex, a single water molecule links the 3′ amino group to both O4′ and N3 of residue A(4). In the major groove, the aglycone forms indirect hydrogen bonds from oxygens O4 and O5 to A(12)N7. In most previous complexes, co-ordination by six neighboring atoms suggested that this bridging solvent molecule could be a sodium ion (17,18,21). In neither of the two complexes described here, however, could a hexa-coordination be established and therefore this solvent molecule was assigned as water

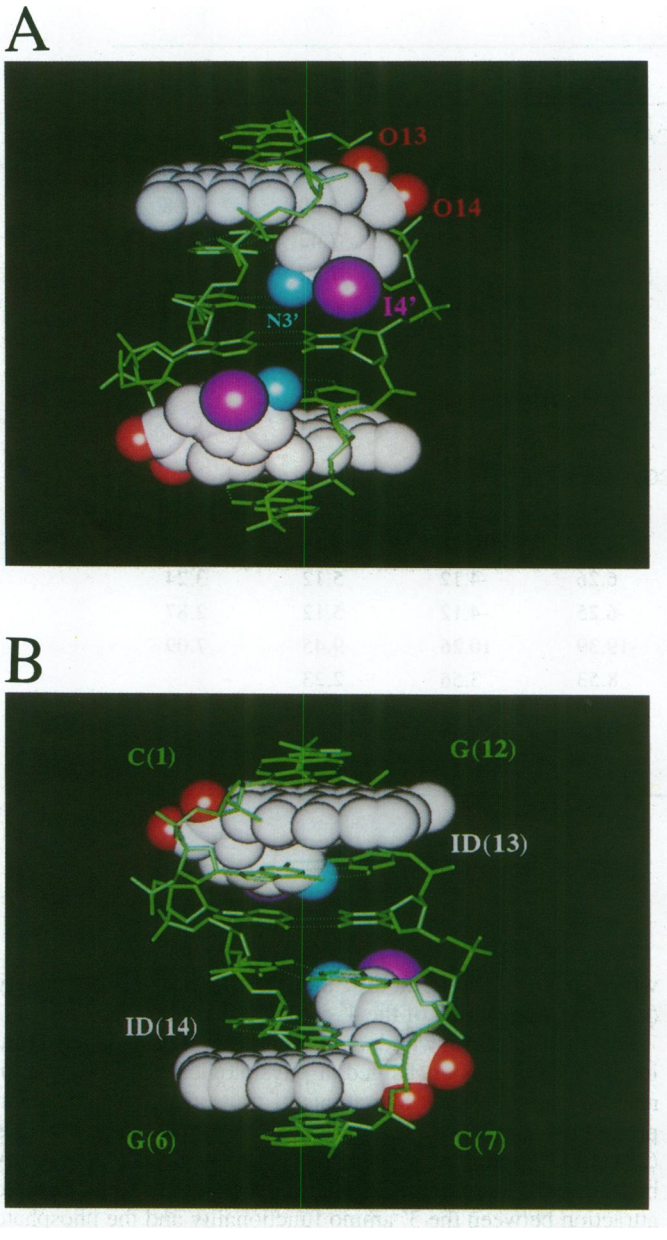

Figure 2. Two views of the complex of two molecules of iododoxorubicin (space filling representation) intercalated in the distorted B-DNA hexamer duplex formed by two strands of d(CGATCG). The DNA in this representation is drawn with green lines. Base pair hydrogen bonds are drawn with broken green lines. Looking into the minor groove (**A**) the halogenated sugar rings can be seen with colored functional groups (labelled on the upper drug molecule). In (**B**), the major groove of the hexamer duplex is rotated into view showing the protruding drug chromophores. The numbering scheme of the complex is indicated. Oxygens O13 and O14 are colored red, N3′ of the amino sugar blue and the 4′ iodine purple.

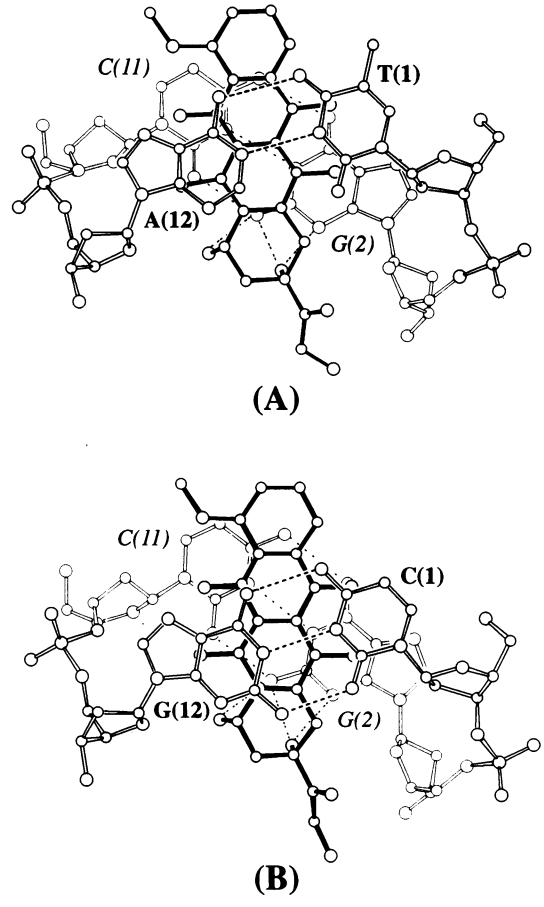

Figure 3. Iododoxorubicin chromophore and its intercalation site base pairs in the iodo-TGT complex (**A**) and the iodo-CGA complex (**B**) projected onto the chromophore plane. Terminal base pairs are drawn with thick open bonds, drug chromophore with solid black bonds and the lower base pairs are drawn with thin lines. Hydrogen bonds are drawn with broken lines.

in both cases. This has also been observed in the crystal structure of 11-deoxy-daunomycin bound to d(CGTsAGC) (24). In the iodo-CGA complex, a total of four water molecules participate in indirect interaction with the DNA in the minor groove. Two water molecules establish contacts between O4′ and O2 of residues T(4) and T(10) and the 3′ amino group of the amino sugar moiety. In addition, the 13-keto oxygen of the aglycone forms two indirect hydrogen bonds mediated by one solvent molecule each to the O2 of C(1) and N2 of G(12), respectively. In the major groove, the aglycone indirectly hydrogen bonds to G(12)N7. The interactions described for both drug–DNA structures are illustrated in an idealized schematic representation in Figure 5.

The solvent molecules assigned in the iodo-TGT and iodo-CGA complexes are within hydrogen bonding distances of drug molecule, DNA hexamer or first shell water molecules. For the iodo-TGT complex, 49 water molecules were located during the refinement with isotropic temperature factors (B) varying from 11 to 44 Å^2. In the iodo-CGT complex, 52 water molecules were included in the refinement with B values in a range of 12–44 Å^2. No cations could be unambiguously identified. The number of solvent molecules surrounding the halogenated amino sugar moiety within hydrogen bonding distance was reduced in these structures (to 3) compared to previously reported complexes (≥ 5) due to the hydrophobic nature of the covalently attached iodine. In several previously reported anthracycline drug–DNA complexes, spermine molecules were found in the structure (24). We added spermine tetrachloride to the crystallization setups and specifically monitored the electron density in the 2Fo-Fc Fourier maps for the presence of spermine in the complexes in the process of structure solution. However, we found no evidence for the existence of ordered polyamines in either crystal structure.

Table 3. Helical parameters for drug–DNA complexes[a,b]

base pairs	Tilt (τ)	Roll (ρ)	Twist (Ω)	Buckle (κ)	Propel. (ω)	Opening (σ)	Rise
			Iododoxorubicin-d(TGTACA) Complex				
T(1)-A(12)	-1.27	-4.10	39.25	-7.57	-0.24	1.44	6.99
G(2)-C(11)	-6.81	4.75	26.34	18.63	10.88	6.60	2.88
T(3)-A(10)	0.00	8.25	39.95	1.97	-6.17	7.42	3.50
A(4)-T(9)	6.81	4.75	26.34	-1.97	-6.17	7.42	2.88
C(5)-G(8)	1.27	-4.10	39.25	-18.63	10.88	6.60	6.99
A(6)-T(7)				7.57	-0.24	1.44	
av	0.00	1.91	34.23	0.00	1.49	5.15	
sd	4.89	5.67	7.20	12.78	7.74	2.90	
			Iododoxorubicin-d(CGATCG) Complex				
C(1)-G(12)	-1.80	-2.40	37.18	-8.53	3.56	2.23	7.09
G(2)-C(11)	-8.20	5.77	29.73	19.39	10.26	9.45	2.87
A(3)-T(10)	0.00	2.46	34.26	6.26	-4.12	5.12	3.24
T(4)-A(9)	8.20	5.77	29.73	-6.25	-4.12	5.12	2.87
C(5)-G(8)	1.80	-2.40	37.18	-19.39	10.26	9.45	7.09
G(6)-C(7)				8.53	3.56	2.23	
av	0.00	1.84	33.61	0.00	3.23	5.60	
sd	5.94	4.09	3.74	13.97	6.44	3.25	

[a]Obtained from the program CURVES, courtesy of H. Sklenar (33).
[b]τ, ρ, ,Ω ,κ ,ω, σ in degrees, rise in Å.

DISCUSSION

The anthracyclines are among the most effective therapeutic agents in the treatment of cancer. For instance daunomycin, a parent compound of this class of cytostatics, is widely used in the treatment of acute leukemia. Subtle chemical modifications have the potential of altering the pharmacological activity of anthracyclines in significant ways, and different derivatives of daunomycin are most effective in the treatment of specific cancer diseases. A large number of anthracycline analogues have been isolated and synthesized (3,4) with the purpose of finding more potent chemotherapeutic agents with an altered range of anticancer activity.

It is the nature of cytotoxic agents that they have severe side effects on the patients to whom they are administered. One severe side effect of many anthracycline drugs is their cardiotoxicity (27). For this reason, one aim of drug development is to identify derivatives with maintained or increased effectiveness and simultaneously reduced side effects. In this context, it is essential to gather insight into the mechanism by which this class of anticancer agents mediates its antitumor activity. However, despite intensive effort, the detailed mode of action of anthracyclines is still debated (14). Present evidence supports the idea that DNA is the main target for anthracyclines such as doxorubicin and its derivatives in the cell (15,16), and the insertion of the drug amino sugar group into the minor groove of DNA is a major factor contributing to an adequate residence time for cytotoxic activity (16). It is therefore important to investigate conformational alterations in the minor groove of drug–DNA complexes which may arise from modifications of the amino sugar moiety (e.g. the halogenation of the 4′ carbon in iododoxorubicin).

Due to the electron withdrawing character of the halogen atom, iododoxorubicin is uncharged at physiological pH (pK_a = 6.4), resulting in significant alterations of transport and metabolism properties compared to positively charged anthracyclines (28–30). In addition, it could be of importance to the DNA binding of iododoxorubicin, due to the absence of electrostatic attraction between the 3′ amino functionality and the phosphate oxygens of the DNA backbone. The iodo-TGT complex was crystallized at pH 6.5, and isomorphous crystals were obtained at pH values up to 7.0. The structure does not indicate a significant change in the intercalation geometry due to the largely reduced positive charge of the drug. Recently, a triclinic crystal structure of a similarly predominantly uncharged anthracycline derivative, methoxymorpholinyl-doxorubicin bound to DNA was reported (31). In this structure, a hydrated magnesium ion was found in the major groove opposing the amino sugar position, additionally stabilizing the complex by linking the aglycone to a base. The authors reasoned that the occurrence of this ion in the major groove constituted a possibly common pattern of stabilization of anthracycline drug–DNA complexes, which was hitherto undetected due to the electrostatic repulsion between magnesium and the positively charged 3′ amino group through the low dielectric DNA core. In the iodo-TGT structure, however, we were unable to unambigously identify a positively charged, ordered ion at that particular position in the major groove. Therefore the presence of the magnesium ion reported by Cirilli and co-workers (31) might

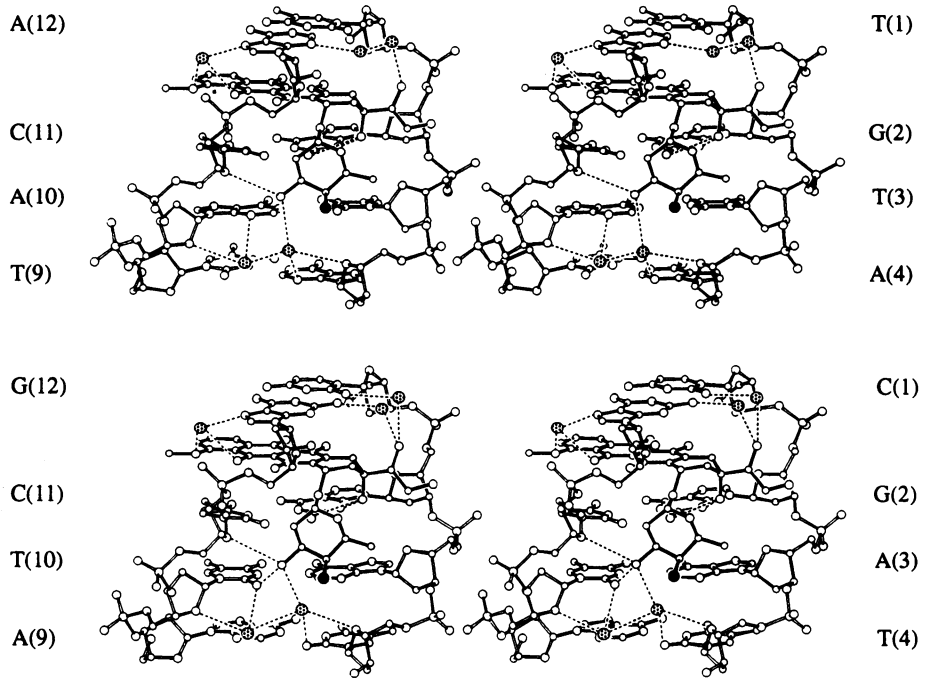

Figure 4. Stereo view skeletal representation of the detailed surroundings of the intercalated drug molecules in iodo-TGT (A) and iodo-CGA (B) looking into the minor groove. Iododoxorubicin is drawn with solid black bonds and the DNA, residues marked with labels, with open bonds. The 4′ iodine is shaded and solvent molecules involved in drug binding are highlighted by stippling. Hydrogen bonds linking iodoxorubicin to the DNA are drawn with dashed lines.

result from lattice effects, or reasons other than the absence of a positive charge on the drug.

The conformation of the amino sugar substituent is virtually identical in the iodo-TGT and iodo-CGA structures described, and very similar to the conformation of doxorubicin bound to d(CGATCG) (17) as reflected in the torsion angles of the glycosidic linkage. The N3′ amino group is within hydrogen bond distance of functional groups on the DNA molecule; however, all these hydrogen bonding distances are comparatively long ($\geq$3.3 Å) and therefore stabilize drug binding to a lesser degree, in particular in the iodo-TGT structure. The 4′ iodine is axial, and constitutes an exposed hydrophobic patch in the minor groove of the drug–DNA complexes. The presence of a bulky hydrophobic group in this position rather than a hydrophilic group constitutes the major contrast of the minor groove epitope of the iododoxorubicin complexes described compared to previously reported structures of anthracycline anti-cancer drugs with a 4′ hydroxyl group (such as daunomycin and doxorubicin) bound to DNA. Even though the geometry of intercalation is not markedly influenced by the presence of the iodine substituent, careful comparison with previously solved, less aliphatic anthracycline drug–DNA complexes (17) shows an altered pattern of hydration in the minor groove. In the crystal structures of adriamycin and daunomycin bound to DNA, numerous water mediated indirect hydrogen bonds were found linking the amino sugar moiety to phosphate oxygens of the DNA backbone, thus forming an intricate solvation network in the minor groove of the hexamer duplexes. Due to the replacement of the 4′ hydroxyl group with iodine in iododoxorubicin, this solvation network is disrupted, and hydration around the halogenated sugar moiety is markedly reduced. In particular, hydrogen bonds mediated by water molecules linking the amino sugar to the DNA were found to originate only from the N3′ nitrogen of the amino sugar in both the iodo-TGT and iodo-CGA complexes. In addition, ordered spermine molecules have been found in the solvent shell of various anthracycline drug–DNA complexes (25). Interestingly, those spermine molecules exhibited different binding geometries in otherwise isomorphous crystal structures and it was reasoned, that this was caused by the rearrangement of the hydration network due to the different functional groups of the individual drugs (for instance, the presence of a hydroxyl group at position 14 in doxorubicin). In the crystal structures we describe here, spermine molecules could not be identified despite high resolution data. This could be the result of a long range rearrangement of the water shell due to the hydrophobic nature of the iodine substituent, which prevents spermine molecules from binding in an ordered fashion or binding at all. Water molecules were found to play an important role in the interaction between DNA and various DNA binding proteins, such as the trp repressor (32). It is possible that, by affecting the interaction of DNA binding proteins, the altered hydration pattern of the drug–DNA complexes described here may contribute to the observed differences in antitumor activity with respect to the parent compounds.

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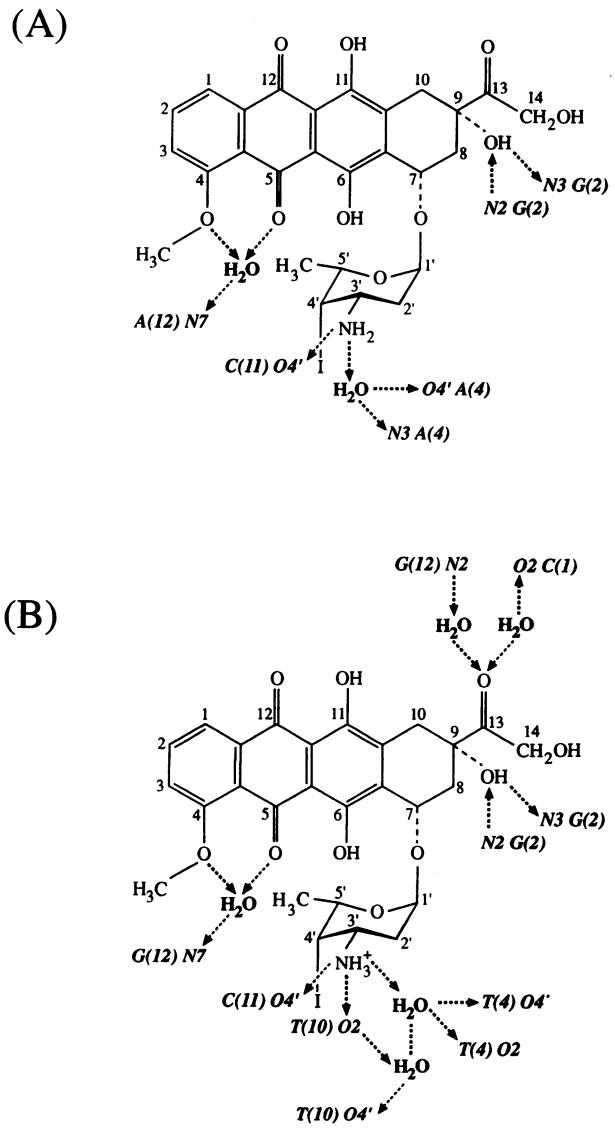

Figure 5. Idealized two-dimensional diagrams showing hydrogen bonding interactions mediated by single bridging water molecules in addition to direct hydrogen bonds between DNA and iododoxorubicin in (**A**) iodo-TGT and (**B**) iodo-CGA. The pK_a of iododoxorubicin in solution is 6.4 (11).

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