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Binding of Two Novel Bisdaunorubicins to DNA Studied by NMR Spectroscopy[†,‡]

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ABSTRACT: In the search for new generations of anthracycline drugs, lower cytotoxic side effects and higher activity against resistant cancer cells are two major goals. A new class of bis-intercalating anthracycline drugs has been designed, synthesized, and shown to have promising activity against multidrug-resistant cells. Two daunorubicins symmetrically linked together via a *p*-xylenyl group, either at their N3′ (compound WP631) or N4′ sites (compound WP652), exhibit extraordinary DNA binding affinities. We have used high-resolution NMR studies to understand the DNA binding mode of these two new bis-daunorubicin anticancer compounds. The structures of the WP631−d(ACGTACGT)$_2$ and the WP652−d(TGTACA)$_2$ complexes have been determined by NOE-restrained refinement. WP631 binds strongly to the 5′-CG(A/T)(A/T)CG hexanucleotide sequence, with the aglycons intercalated between the two CpG sites at both ends of the hexanucleotide sequence. The overall conformation of the WP631−d(CGTACG)$_2$ part is remarkably similar to the crystal structure of the 2:1 complex of daunorubicin and d(CGTACG)$_2$, as predicted previously [Gao, Y.-G., & Wang, A. H. J. (1996) *J. Biomol. Struct. Dyn. 13*, 103−117]. In contrast, the related bis-intercalator WP652 prefers the 5′-PyGTPu tetranucleotide sequence, with the aglycons intercalated between the PypG and TpPu sites. The binding of WP652 to DNA results in a severely distorted B-DNA duplex with the *p*-xylenyl tether moiety significantly protruded away from the bottom of the minor groove. While WP652 in some ways behaves similarly to other anticancer bis-intercalating antibiotics (e.g., triostine A and echinomycin), the detailed interactions between those two classes of bis-intercalators are quite different.

One of the major problems associated with the important clinical anticancer drugs doxorubicin (DOX) and daunorubicin (DNR) is their lack of activity against resistant cancer cells (Priebe & Perez-Soler, 1993; Priebe et al., 1995). This problem is associated with the interactions between the drugs and the transport proteins (P-glycoprotein and multidrug resistance associated proteins) that mediate the multiple drug resistance (MDR) processes. Extensive studies of analogs of DOX and DNR suggest that the antitumor (e.g., topoisomerase II-mediated DNA fragmentation) and the MDR properties of the anthracyclines may be partitioned into different areas of the molecular framework (Priebe, 1995). While the DNA intercalative aglycon ring is required for antitumor activity, the basic sugar moiety may be responsible for the increased affinity of anthracyclines to P-glycoprotein (Priebe & Perez-Soler, 1993). Despite earlier synthetic efforts in developing new compounds that have enhanced antitumor activities but reduced binding affinity toward the

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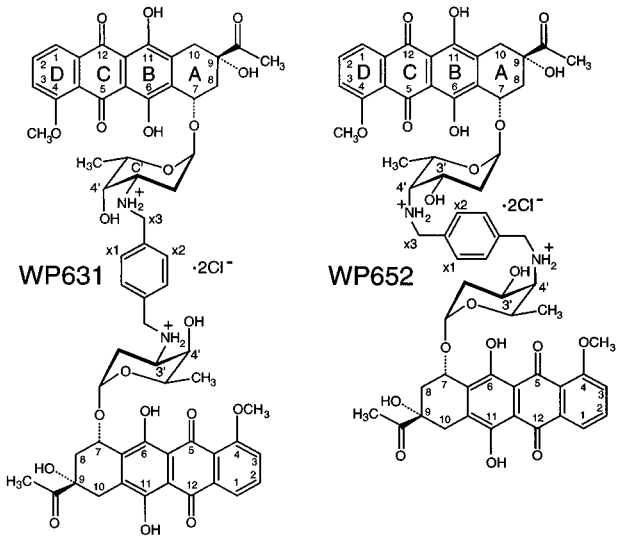

FIGURE 1: Molecular structures of WP631 and WP652.

P-glycoprotein, only limited success has been achieved (Priebe & Perez-Soler, 1993). Novel classes of derivatives involving more extensive modifications, e.g., covalently linking two DOXs together to produce a bis-anthracyclines, have been prepared previously (Henry & Tong, 1978; Seshadri et al., 1983; Brownlee et al., 1986; Skorobogaty et al., 1988), but they did not show superior pharmacological property. A possible reason for the lack of improvement in their anticancer activity may be related to the structural design of those bimolecularly linked compounds, including the type of linkers and the site of linkage on DNR/DOX. Many of the existing bis-DNR or bis-DOX, whose binding property to DNA has not been studied to any significant extent, contain a flexible tether (e.g., hexyl) attached at the C14 position on the aglycone.

In a recent study on the crystal structure of several 3′-morpholinyl-doxorubicins complexed to CGTACG (Gao & Wang, 1995), we noted that two drug molecules are intercalated at the CpG steps at both ends of the duplex and the two morpholinyl moieties are in contact to each other in the minor groove. We had proposed that it would be possible to link two DNR or DOX molecules at their N3′ sites with certain rigid linkers and such bis-DNR or bis-DOX compounds could behave as true bis-intercalators binding to a sequence like CGTACG (Gao & Wang, 1995). Indeed studies of sugar-linked anthracyclines were undertaken independently by W. Priebe in 1992 and resulted in a series of 3′ and 4′-linked bisanthracyclines. Two of them, WP631 and WP652, are bis-daunorubicins linked together at the 3′N or 4′N positions, respectively, of the amino sugar by a *p*-xylenyl tether (Figure 1). Their biological activities are being tested and appear to be promising (Chaires et al., 1997). Interestingly, both compounds are significantly more cytotoxic than DOX against MDR cells (Chaires et al., 1997).

Since the biological activity of anthracycline drugs has been shown to be related to their binding affinity and specificity toward DNA (Valentini et al., 1985), it would be desirable to gain a detailed understanding of these properties in the new class of the bis-anthracycline intercalators through biochemical and biophysical studies. While it is expected that bis-daunorubicins WP631 and WP652 would bind to DNA with significantly greater affinity (as has been borne out by the binding studies of WP631, Chaires et al., 1997), the exact mode of binding to DNA of these two compounds remains to be elucidated. In this paper, we analyze the interactions of WP631 and WP652 with a series of DNA oligonucleotides and present the three dimensional structures of two drug−DNA complexes, i.e., WP631−ACGTACGT and WP652−TGTACA, as determined by NOE-restrained refinement procedure.

EXPERIMENTAL SECTION

The oligonucleotides were synthesized on an automated DNA synthesizer at the Genetic Facility of University of Illinois. WP631 and WP652 were synthesized as described before (Priebe et al., 1996; W. Priebe, unpublished results) and dissolved in methanol as stock solutions. The solutions of various WP631−DNA and WP652−DNA complexes for NMR studies were prepared by dissolving the ammonium salt of the oligos plus the appropriate amounts of WP631 or WP652 stock solution in 0.55 mL of phosphate buffer solution (20 mM sodium phosphate, pH 7.0) to produce a final duplex concentration of 1−2 mM. For 1D H_2O spectra, the drug−DNA complex solutions were vacuum-dried in a SpeedVac at room temperature. The dry powder then was dissolved in 0.55 mL of a 90% H_2O/10% D_2O solution. 1D NMR spectra were collected using the 1−1 pulse sequence (Sklenar et al., 1987; Kochoyan et al., 1990).

For 2D NOESY spectra, each sample of the DNA oligos and their drug complexes was dried on the SpeedVac first. The dried powder was then dissolved in 0.5 mL of 99.8% D_2O and dried again on SpeedVac. This step was repeated three times after which the sample was dried in an NMR tube with a stream of dry nitrogen gas. Finally, 0.5 mL of 99.96% D_2O (Aldrich, Milwaukee, WI) was added to produce the sample. The 1D and 2D NMR spectra were recorded either on a Varian Inova 750 MHz or on a Varian VXR500 500 MHz spectrometer. The chemical shifts (in parts per million) were referenced to the HDO peak, which was calibrated to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at different temperatures. Phase sensitive NOESY spectra in D_2O were recorded at 750 MHz at 15 °C as 512 t_1 increments of 2048 complex points each and averaged for 32 scans per FID (States et al., 1987). The recycle delay was 6.9 s for the 1.1 mM WP631−DNA sample with an average T1 of 2.8 s. The recycle delay was 4.3 s for the 1.58 mM WP652−DNA sample with an average T1 of 2.2 s. The mixing times were 100 ms for the 2D-NOESYs. The 750 MHz spectra were useful in many aspects, including better resolved NOE cross-peaks, more reliable data due to increased sensitivity, and minimizing ambiguity for assignment. The 2D-NOESY spectra in H_2O were collected at 500 MHz at 2 °C using the 1−1 pulse sequence as the read pulse (Sklenar et al., 1987). The 2D data sets were processed with the program FELIX v.1.1 (formerly from Hare Research, Woodinville, WA) on Silicon Graphics workstations. All measureable NOE cross-peak integrals have been determined by the program MYLOR (Robinson & Wang, 1992) and subsequently included in the refinement.

Starting models of the DNA oligonucleotides ACGTACGT and TGTACA and their 1:1 complexes were built using MIDAS (University of California). For the ACGTACGT−WP631 complex, the initial conformation was that of the crystal structure of the entire DNR−CGTACG complex. The *p*-xylenyldiamine linker was docked between

the two symmetry-related N3′ positions. Additonal A-T base pair in the B-DNA conformation was added to both ends. In the case of the WP652−TGTACA complex, the intercalation sites for the two aglycons were deduced from the NOE data (vide infra). Models were built incorporating two adjacent DNR−dinucleotide complexes joined together with several possible tether conformations.

The structure refinement of both complexes was carried out by the procedure SPEDREF (Robinson & Wang, 1992). The procedure incorporates the full-matrix relxation theory [of that from MORASS (Post et al., 1990)] using all measurable nuclear Overhauser effect cross-peak intensities in a quatitative manner. The force field parameters (bond distance, bond angle, atom partial charge) of WP631 and WP652 were generated through standard database in X-PLOR (Brünger, 1993) and they were relaxed by energy minimization. Those of the daunorubicin part were found to conform to its high-resolution crystal structure of Wang et al. (1987). The force field parameters employed in this study are detailed in Tables 1S and 2S (Supporting Information). The program X-PLOR (Brünger, 1993) was used for the molecular dynamics simulation and energy minimization. The molecular complexes with NOE restraints were subject to conjugate gradient minimization with NOE restraints for the 40 refinement cycles. The NMR *R* factor is defined as *R* factor $= \Sigma|N_o - N_c|/\Sigma N_o$, where N_o and N_c are the experimental and calculated NOE integrals, respectively. The refinement statistics of two structures are listed in Table 3S (Supporting Information). The atomic coordinates of the refined structures of the 1:1 complexes WP631−ACGTACGT and WP652−TGTACA, NOE restraints, and X-PLOR force fields have been deposited in the Brookhaven Protein Databank (Entries 1AL9 and 1AMD).

RESULTS AND DISCUSSION

Design of DNA Binding Sequences for WP631 and WP652. It is expected that a DNA sequence such as CGTACG should be a suitable DNA binding sequence for WP631 based on our earlier prediction (Gao & Wang, 1995). We chose the DNA octamer ACGTACGT as the canonical sequence for our NMR structural study since it contains the core sequence of CGTACG and the additional base pair at both ends that helps to reduce end effects. Moreover, the crystal structure of the 2:1 complex of DNR with CGTACG has been determined at 1.2 Å resolution (Wang et al., 1987) and may thus serve as a reference structure for comparison with the NMR-refined structures.

Addition of WP631 to solutions of d(ACGTACGT) caused extensive changes in their NMR spectra as monitored in part by the imino proton resonances (Figure 2). At 0.5 (drug):1 (DNA duplex) ratio, many new resonances, resulting from the titration emerged, and they existed simultaneously with the free DNA resonances suggesting that the octamer DNA forms a stable complex with WP631 and that the binding equilibrium is in the slow exchange regime. In going from the 0.5:1 ratio to a 1:1 ratio, the spectrum simplifies so that there is only one resonance per proton, indicating that the 1:1 complex is 2-fold symmetrical.

To determine what might be the preferred DNA binding sequences for WP652, whose linker is attached at the 4′ position of the modified sugar ring of DNR, we tested several hexamer sequences, including CGTACG, TGTACA,

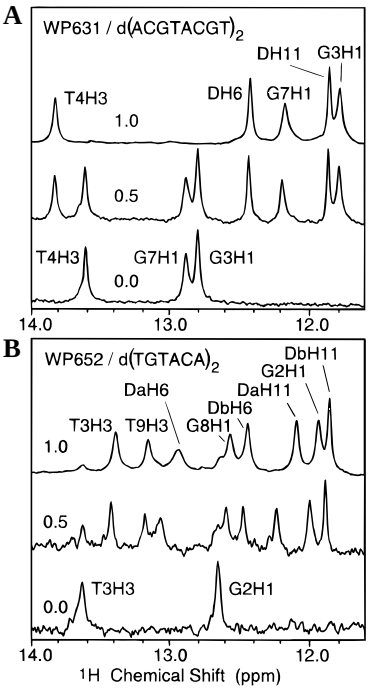

FIGURE 2: Titration of (A) WP631 with ACGTACGT and (B) WP652 with TGTACA monitored by the imino proton resonances of the proton 1D NMR spectra (2 °C) at 500 MHz. At a drug/DNA ratio of 0.5:1, resonances from both free DNA and bound DNA are present simultaneously, indicating slow exchange rate between free and bound drug. In the WP652−TGTACA complex, the two halves (one half labeled a and the other half labeled b) of WP652 are not equivalent.

ACGCGT, CGCGCG, and CGGCCG. Addition of WP652 to solutions of these sequences caused extensive changes in their NMR spectra as monitored by the imino proton resonances. Prolonged incubation of WP652 with CGTACG, CGCGCG, and CGGCCG resulted in precipitation of the complexes, preventing further NMR analysis. Binding to ACGCGT sequence appeared to stabilize the A-T base pair as evident by the emergence of a strong T imino proton at 13.75 ppm. This may suggest that the terminal base pair is enclosed by the bis-intercalator.

We chose TGTACA for the further study of WP652 binding to DNA since the complex appears to be stable indefinitively in solution and has a well-resolved NMR spectrum suitable for NMR structural analysis. At a 0.5:1 ratio of WP652 to TGTACA, new resonances coexsited with the free DNA resonances (Figure 2), suggesting again the binding is in the slow exchange regime. Further titration to a 1:1 ratio reveals that the number of new resonances is twice the sum of the drug and DNA resonances, indicating that the complex is not 2-fold symmetrical.

All resonances in the 1:1 complex of WP631−ACGTACGT and WP652−TGTACA have been assigned (vide infra). Note that some resonances continue to shift upfield on moving from the 0.5:1 complex (at 0.29 mM concentration) to the 1:1 complex (at 1.58 mM concentration) (Figure 2). This was particularly evident for the latter complex where WP652 DaH6 moves upfield from 13.1 to 12.6 ppm. Such observation may be related to increased chemical exchange at the lower concentration. Previous thermodynamic studies suggested that whereas the binding of WP631 to DNA has extremely high affinity ($K_d > 2.7 \times 10^{11}$ M^{-1}) (Chaires et al., 1997), the WP652 showed high affinity,

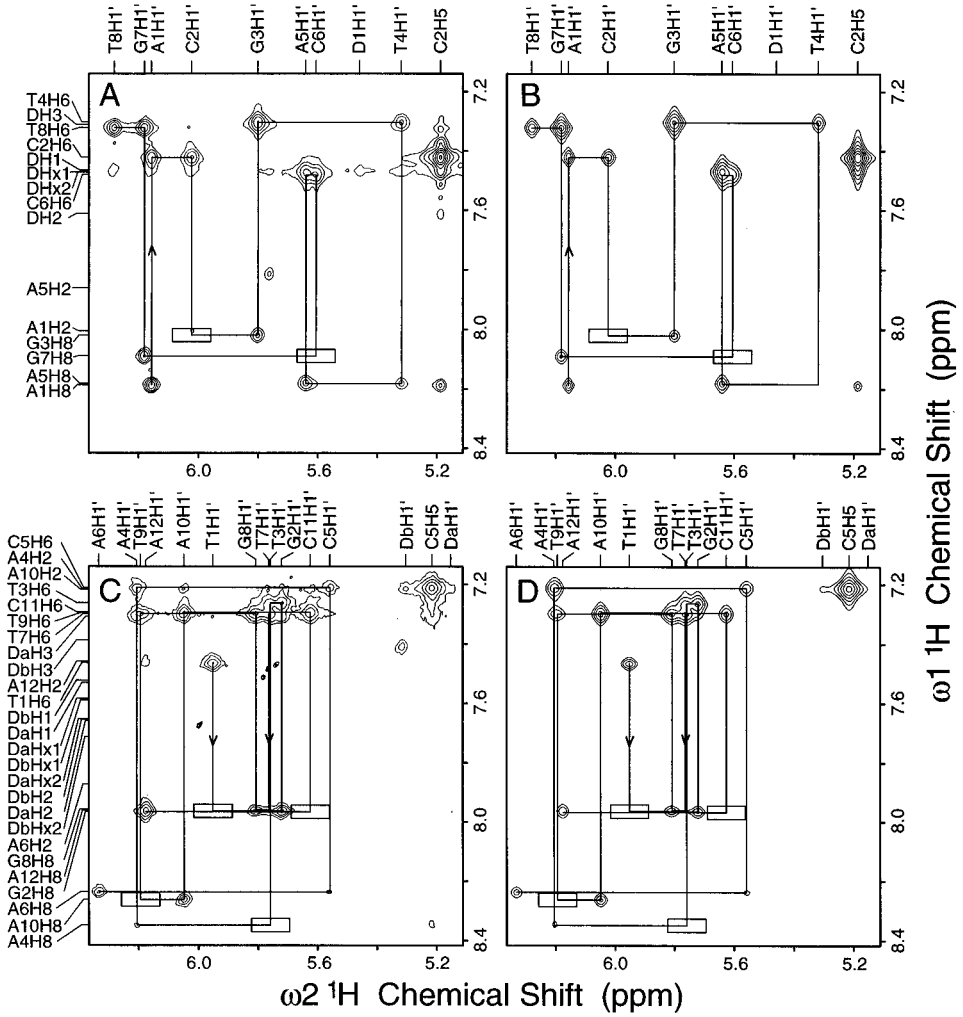

FIGURE 3: Experimental and simulated 2D-NOESY spectra of (A, B) the 1:1 complex of WP631−ACGTACGT duplex, and (C, D) the 1:1 complex of WP652−TGTACA. The 2D-NOESY data were collected on a Varian Inova 750 MHz spectrometer. The aromatic-H1′/H5 region is shown, with the sequential assignment pathway indicated. The simulated spectra were calculated using full-spin relaxation matrix based on the refined structures as described in the text. Note that in the symmetrical 1:1 WP631−ACGTACGT complex, only one set of base-to-sugar NOE connectivities is observed, whereas the 1:1 complex of WP652−TGTACA is nonsymmetrical, so each strand is observed individually in the spectrum.

although somewhat lower affinity than WP631, and the mode of binding is not clear yet. Thus, the fact that we observe chemical exchange is surprising. Additional support for the exchange property comes from the 2D-NOESY spectrum of the complex. When the ratio of WP652 to TGTACA was below 1:1, we noted many chemical exchange NOE cross-peaks between the free DNA and the bound DNA resonances. This discrepancy between the thermodynamic study (Chaires et al., 1997) and our NMR study may be related to the short length of the oligonucleotides we used.

Structure of the WP631−ACGTACGT Complex. The 1:1 complex of WP631−ACGTACGT has well-resolved 2D-NOESY NMR spectra (Figure 3A) whose resonance assignment was made by standard sequential assignment procedure and refinement carried out using the procedure SPEDREF (Robinson & Wang, 1992). The chemical shifts of all resonances are listed in Table 4S (Supporting Information). Some of the representative key NOEs are listed in Table 1. In the past, it was difficult to determine the solution structure of DNR−DNA and DOX−DNA complexes by NMR refinement method due to the rapid exchange rate of the binding of DNR or DOX to DNA. However, the tight binding of WP631 to the CGTACG sequence for the first time allows the solution structure of the drug−DNA complex to be obtained for this class of the anthracyclines.

The overall structure of the WP631−ACGTACGT complex (Figure 4A) is similar to those of the complexes of DNR or DOX with DNA hexamers (Wang et al., 1987; Gao et al., 1996 and references therein). In the complex, two DNR aglycons of WP631 are intercalated in the two symmetry-equivalent CpG steps of the distorted hexamer B-DNA duplex. The aglycon chromophores have their D ring protruding into the major groove and the two daunosamines plus the *p*-xylenyl tether moiety occupying the entire minor groove. The chemical shifts of Hx1 and Hx2 protons are identical (7.74 ppm), suggesting that the *p*-xylenyl ring rotates rapidly (on the NMR time scale), resulting in magnetic equivalency for these four *p*-xylenyl protons. It is of interest to note that the DNA backbone conformation in this complex is remarkably similar to the crystal structure of the DNR−CGTACG complex (Wang et al., 1987) with a rmsd of 0.79 Å between the two structures using the common atoms. As noted in previous crystal structures of DNR−DNA and DOX−DNA complexes, the unwinding angle (36°-twist angle) associated with the DNR or DOX intercalation is small (∼10°), and it is mostly coming from the step

Table 1: Thirty Intense NOEs between the Anthracycline Monomer Unit and DNA of the Two Complexes[a]

WP631−d(ACGTACGT)$_2$

WP631	DNA	NOE	WP631	DNA	NOE
H1	C2 H5	0.83	H1′	G7 H5″	3.17
H2	C6 H5	0.36	H1′	G7 H4′	2.16
H3	C6 H5	1.16	H1′	C6 H1′	1.82
Me21	C6 H5	3.69	H2′R	C6 H1′	3.54
Me21	C6 H6	3.42	H2′S	G7 H5″	2.92
Me21	C6 H2′	2.27	H2′S	G7 H5′	1.88
Me21	C6 H2″	2.07	H2′S	C6 H1′	1.78
H10R	G3 H1′	2.04	H3′	T4 H1′	0.68
H10S	G11 H1′	1.74	H4′	T4 H1′	0.79
Me9	T4 H4′	3.56	H5′	A5 H2	1.03
Me9	T4 H5′	2.60	Me6	G7 H4′	0.84
Me9	G3 H4′	2.67	Hx1	A5 H2	5.80
H8R	G7 H1′	0.44	Hx2	A5 H4′	2.11
H8S	G7 H1′	0.57	Hx31	A5 H2	0.90
H7	G7 H1′	1.43	Hx32	A5 H2	0.78

WP652−d(TGTACA)$_2$

WP652a	DNA	NOE	WP652a	DNA	NOE
H1	T1 Me	3.11	H1′	A12 H5′	2.14
H2	T1 Me	0.69	H1′	A12 H4′	1.31
H3	C11 H5	0.50	H2′R	C11 H1′	2.11
Me21	C11 H6	6.64	H2′R	A12 H5′	1.63
Me21	A10 H1′	4.26	H2′S	A12 H5′	4.21
Me21	C11 H5	3.55	H2′S	A12 H5″	2.22
Me21	C11 H2′	1.63	H2′S	C11 H1′	1.84
Me21	C11 H2″	1.47	H3′	C11 H1′	0.50
H10R	T1 H2″	0.35	H4′	A12 H4′	0.10
H10S	A12 H2	1.02	H5′		
Me9	G2 H4′	3.15	Me6′	A12 H1′	1.17
Me9	G2 H1′	1.29	Hx1		
H8S	A12 H2	0.92	Hx2	A12 H4′	0.20
H8R	A12 H2	0.60	Hx31	A12 H4′	0.30
H7	A12 H1′	0.82	Hx32	A12 H4′	0.30

WP652b	DNA	NOE	WP652b	DNA	NOE
H1	T9 Me	2.54	H7	A4 H2	1.22
H2	T3 Me	0.75	H1′	A4 H4′	1.65
H3	T3 Me	2.54	H1′	A4 H5′	1.31
Me21	T3 Me	6.66	H2′R	T3 H1′	1.87
Me21	T3 H6	2.32	H2′S	A4 H4′	2.32
H10R	A10 H1′	1.87	H2′S	T3 H1′	1.63
H10R	A10 H5′	1.39	H2′S	A4 H5′	1.27
H10R	A10 H4′	1.28	H3′	A10 H2	0.89
H10S	A10 H5′	1.86	H4′	A10 H2	0.18
Me9	C11 H5′	2.61	H5′	A10 H2	0.36
Me9	A10 H4′	1.52	Me6′	C5 H5′	0.81
Me9	A10 H1′	1.26	Hx1		
H8S	A4 H2	0.20	Hx2		
H8R	A4 H1′	0.30	Hx31		
H7	A4 H1′	1.26	Hx32		

[a] The thirty intense inter drug−DNA observed NOEs, including the strongest peak from each drug spin, are listed. The NOE intensities are percent of the scaled total observed NOE for a particular spin at zero mixing time at complete relaxation. The NOE is the average of the two observables. For reference, the H5-H6 NOE intensities of the cytocines C2 and C6 of the WP631−d(ACGTACGT)$_2$ complex, are 7.18 and 6.32%, respectively. Similarly, the H5-H6 NOE intensities of the C5 and C11 of the WP652−d(TGTACA)$_2$ complex, are 3.73 and 6.27%, respectively. NOE intensities are weak between 0.1 and 0.2, between 0.21 and 1.0 medium, and strong above 1.0. NOE intensities were measured by fitting with cross-peak profiles.

adjacent to the aglycon intercalated site toward the daunosamine direction. Our structure confirms this assertion and provides additional information for the step away from the daunosamine. The conformation of the A1pC2 step is similar to that of B-DNA. The exchangeable proton 2D-NOESY spectrum (Figure 1S, Supporting Information) has many drug to DNA NOE cross-peaks that are fully consistent with the refined structrue of Figure 4A.

We note that in the crystal structure of DNR−CGTACG, the two N3′ atoms are 6.9 Å apart, which is slightly shorter than the N−N distance in the fully extended *p*-xylenyldiamine tether (7.3 Å). In the complex, the drug adjusts the relative orientation of its daunosamine with the aglycon ring in order to join the two N3′ sites without strains. The pucker of the ring A of the aglycon is altered somewhat so that the daunosamine sugar is rotated slightly toward the C7′ atom side. The difference is evident from the superposition of the drug molecules derived from the solution structure of the WP631−ACGTACGT and the crystal structure of the DNR−CGTACG complexes (Figure 5).

Structure of the WP652−TGTACA Complex. Figure 3C shows the aromatic-H1′ region of the nonexchangeable proton 2D-NOESY spectrum from which the sequential assignment was deduced. It is interesting to note that the binding of a 2-fold symmetrical bis-intercalator to a palindromic DNA results in both the WP652 and DNA protons becoming nonequivalent (thus having different chemical shifts). For example, the chemical shifts of T3H1′ (5.76 ppm) and T9H1′ (6.20 ppm) are split apart by 0.44 ppm. Likewise, the C5H5 (5.23 ppm) and C11H5 (4.76 ppm) resonances are split by 0.45 ppm. This observation indicates that the complex no longer possesses a 2-fold symmetry and that its mode of binding is very different from that of WP631.

Numerous NOE cross-peaks between WP652 and DNA provide the key information about the binding mode. For example, WP652−aH11 (Figure 1S, Supporting Information) has NOEs to T1H6, T1H1′ and G2H8, G2H1′, indicating that one aglycon ring (half a) is intercalated between the T1-A12 and G2-C11 base pairs. WP652−bH11 has significant NOEs to T9H6, T9H1′ and A10H8, A10H1′, thus indicating that the other aglycon ring (half b) is intercalated between the T3-A10 and A4-T9 step.

The overall structure of the WP652−TGTACA complex (Figure 4B) is very different from those of any of the complexes of DNR or DOX with DNA hexamers (Wang et al., 1987; Gao et al., 1996 and references therein). In fact, the binding of WP652 to DNA is reminiscent of that of the other bis-intercalating antibiotics triostin A and echinomycin (Wang et al., 1984). The two aglycon rings of WP652 bracket two base pairs. Such drastically different binding modes for WP631 and WP652 are due to the respective 3′ and 4′ sites of linkage for the tether. In WP652, the N4′ group on the daunosamine ring protrudes away from the main body of the aglycon ring and thus away from the DNA base pair. The location of the xylenyl ring is 8 Å from the bottom of the minor groove (e.g., O2 of T3). This arrangement severely restricts the separation between the two aglycon rings, permitting only two base pairs to be sandwiched between the two aglycons.

In the complex, the long axes of the two DNR aglycons of the WP652 have a crossing angle of almost 90°. While the drug maintains a local 2-fold symmetry, the 2-fold axis nearly coincides with the local dyad between the G2-C11 and T3-A10 base pairs.

It is interesting to note that the conformation of the two strands of the distorted hexamer B-DNA duplex are no longer equivalent. The backbone of the T9-A10-C11-A12 strand is more extended than the T1-G2-T3-A4 strand. The difference between the two intercalation cavities in the

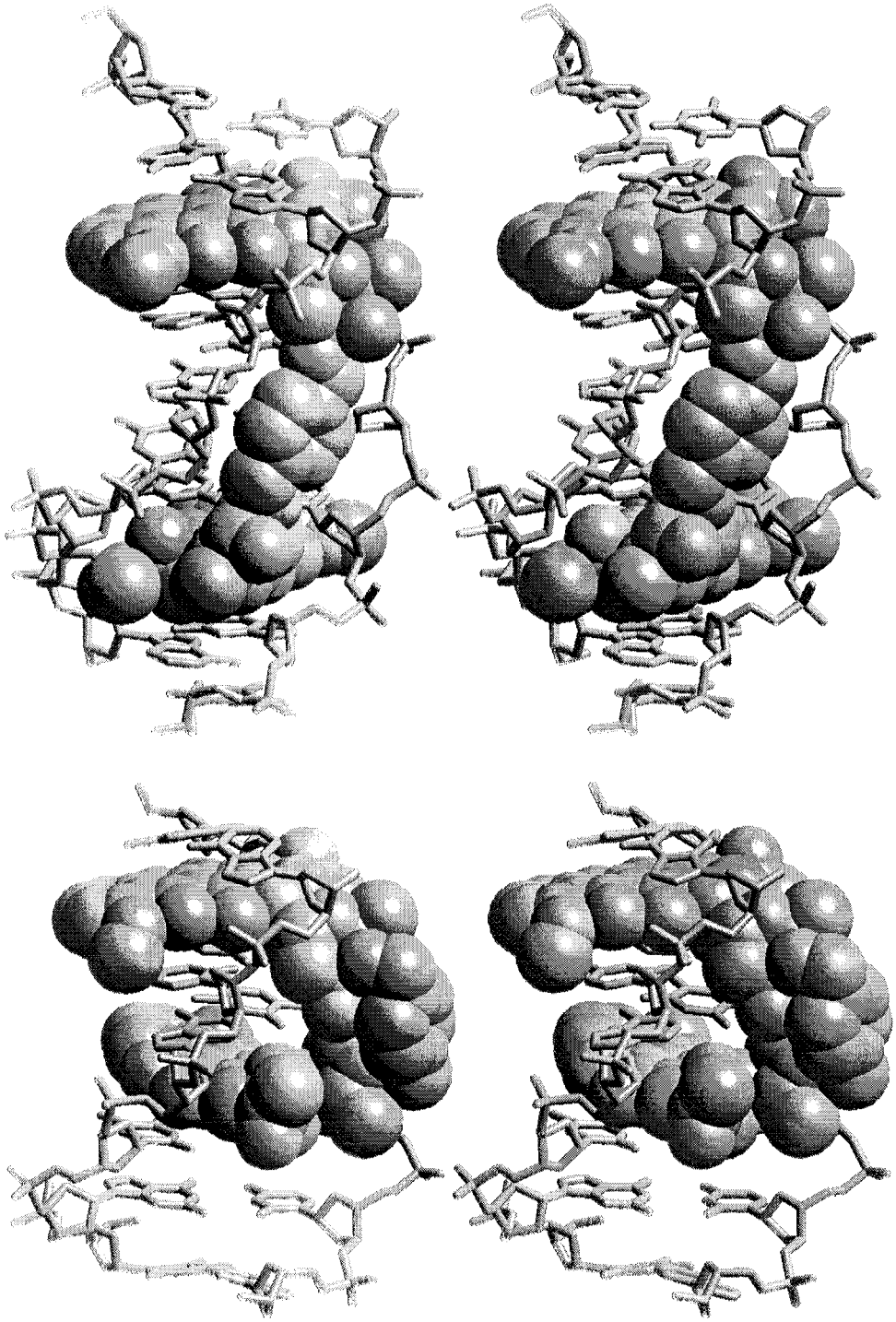

FIGURE 4: The refined structures of the 1:1 complex of (A) WP631−ACGTACGT, and (B) WP652−TGTACA as shown by stereoscopic van der Waals drawings displayed using an in-house molecular graphic program MARS (Robinson, 1988, unpublished). The view in each drawing is into the minor groove where the two linked-daunosamines are located. Near the bottom of each figure the elongated aglycone can be seen protruding toward the right into the major groove.

WP652−TGTACA complex is more evident by comparing their stacking patterns as shown in Figure 6. There we compare the stacking of the aglycon ring between two adjacent base pairs in the DNR−CGTACG crystal structure (Wang et al., 1987), the WP631−ACGTACGT complex, and the WP652−TGTACA complex. While, in all cases, the aglycon chromophores have their D ring protruding into the major groove and the aminosugar in the minor groove, the long axis of the aglycon adopts a different angle with respect to the C1′-C1′ lines of the neighboring base pairs.

Table 5S (Supporting Information) lists the chemical shifts of all nonexchangeable resonances and most of the exchangeable resonances of WP652 and DNA. A total of 1083 reliable restraints were derived for the NOE refinement, which resulted in an NMR *R*-factor of 26.6%. Some of the important intermolecular NOE cross-peaks between nonexchangeable WP652 protons and DNA are listed in Table 1.

The local structures of WP652−(T1pG2) and WP652−(T3pA4) binding sites in the complex are somewhat modified from that of the DNR−CGTACG crystal structure (Wang et al., 1987), as shown in the comparative stacking diagrams (Figure 6). Several intermolecular WP652−DNA hydrogen bonds were found, including those between the O9 of WP652a and the N3 and N2 of G2, and O9 of WP652b and

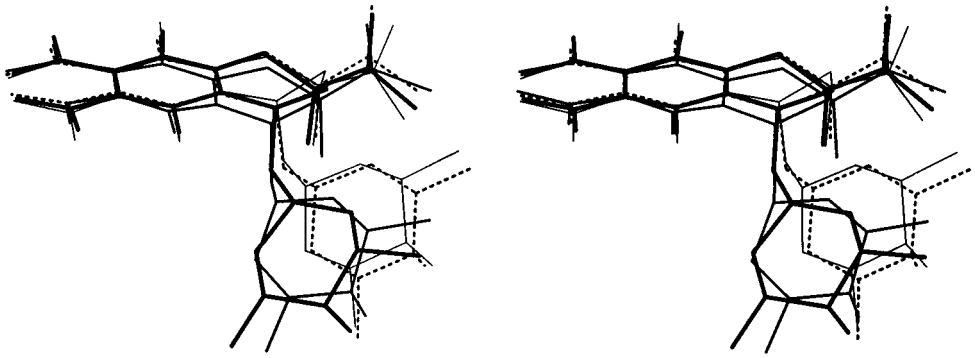

FIGURE 5: An enlarged view of the superimposition of three daunorubicins from three different drug−DNA complexes, WP631−ACGTACGT (medium line), WP652−TGTACA (thin and dotted lines, two independent DNRs), and DNR−CGTACG (thick line, from Wang et al., 1987). They are superimposed by a least-square fitting of all common atoms.

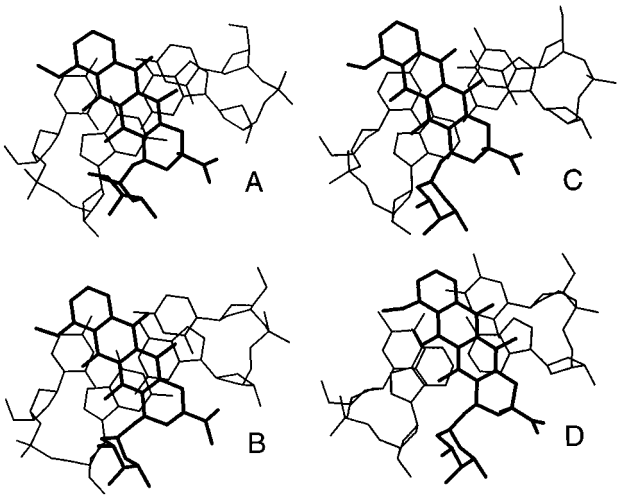

FIGURE 6: Skeletal drawing of the stacking interactions between the anthracycline drugs (thick bonds) and two base pairs (thick bonds) viewed perpendicular to the aglycone chromophore. The 5′-C1pG2 strand is at right and the 5′-C11pG12 strand is at left. (A) The crystal structure of the DNR−CGTACG complex. (B) The WP631−ACGTACGT complex. (C) The first half (half a) of the WP652−TGTACA complex. (D) The other half (half b) of the WP652−TGTACA complex.

the N3 of A10, which define the specificity for the TGTA sequence. In both complexes, the base pairs next to the aglycon have significant buckles, similar to those seen in other complexes of intercalators (e.g., daunorubicin and nogalamycin) and DNA (Wang, 1992).

Sequence Specificity of WP631 and WP652. Our structures may offer a plausible explanation for the binding preferences of WP631 and WP652. For WP631, we found, not unexpectedly, that the CGTACG sequence is a preferred sequence. Inspection of the structure revealed that the *p*-xylenyldiamine ring abuts tightly to the central A-T base pairs in the minor groove. The closest distance between the *p*-xylenyl ring and the C2 atom of the A5 base is 3.2 Å, which is consistent with the very strong NOE cross-peak between the *p*-xylenyl Hx1 and A5H2 protons (Table 1). It is predicted that if the central A-T base pairs are replaced by G-C base pairs, there will be van der Waals clashes between the *p*-xylenyldiamine tether and the N2 amino group from the guanine base. However, reversal of the TpA sequence to the ApT sequence should have no effect on the binding affinity. Thus, a sequence preference for WP631 is likely to follow the order of CGTACG ∼ CGATCG >> CGCGCG ∼ CGGCCG.

The determination of the binding sequence preference for WP652 was more complicated than for WP631. Our titration experiments suggested that at least a CpG or a TpG site is required. This could be explained by the binding preference of the triplet 5′-CGA or 5′-CGT for DNR alone. We had previously shown that the O7 and O9 atoms make hydrogen bonds with the guanine N2, N3 sites for the specificity (Wang et al., 1987). Therefore, it may be predicted that WP652 prefers sequences like CGCG or more generally PyGCPu with both aglycon rings intercalated between the two CpG steps. (It is well established that a PypPu step is a favorable intercalation step.)

In the WP652−TGTACA complex, half a of the WP652 has those similar hydrogen bonds between O9 of WP652a and G2 base. However, due to the highly folded nature of the tether moiety, there are van der Waals contacts between the aminosugar of WP652b (half b) and the A10 deoxyribose of the T3-A10 base pair, thus causing the stacking pattern to change significantly. The twist angle between the G2-C11 and T3-A10 base pairs is 19°, indicating a substantial unwinding of this helical step that may possibly have a destabilizing effect. We also noted that WP652 binds to the ACGCGT by bracketing the A1pC2 step, instead of the G3pC4 step (data not shown). This may be explained by the more flexible terminal A1-T12 base pair, which allows the DNA to adapt to the rigid WP652 frame. Our predictions here of the sequence preferences for WP631 and WP652 should be easily testable using other biochemical means such as chemical footprinting experiments.

In conclusion, our structural studies have revealed how this new class of bis-daunorubicin drugs binds DNA and provided the molecular basis for explaining sequence preference. It is gratifying to note that the knowledge derived from structural analyses of drug−DNA complex (Wang et al., 1987) can be successfully applied to the rational design of new compounds like WP631 and WP652 described in this paper. Whereas both molecules bind bis-intercalatively to DNA, their binding modes are quite different. WP631 prefers a hexanucleotide sequence like CG(T/A)(T/A)CG, and it brackets four base pairs between the two aglycons. WP652 binds to a tetranucleotide sequence like PyGTPu with its sugar plus the *p*-xylenyl tether in a folded conformation. Further studies of this type of novel bis-intercalators may yield new and improved anticancer drugs. For example, the *p*-xylenyl tether may be replaced by other kinds of compounds. Alternatively, WP652 may be modified so that an amino group at the 3′ position is restored and the new

compound may introduce the ability of these compounds to cross-link to N2 amino group of guanine in DNA mediated by formaldehyde (Gao et al., 1991, 1996; Wang et al., 1991). Experiments to test those possibilities are now underway.

SUPPORTING INFORMATION AVAILABLE

One figure of the imino/amino to aromatic/H1′/H5 cross-peak region of the proton 2D-NOESY spectra of the two complexes and five tables of force field parameters of WP631 and WP652, the refinement statistics and the chemical shifts of the proton resonances of the two complexes (25 pages). Ordering information is given on any current masthead page.

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