Formaldehyde Cross-Links Daunorubicin and DNA Efficiently: HPLC and X-ray Diffraction Studies[†,‡]

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ABSTRACT: Formaldehyde (HCHO) cross-links the anticancer drug daunorubicin (DAU) to DNA efficiently. When DAU is mixed with DNA hexamers, d(CGCGCG) and d(CGTDCG), in the presence of HCHO, stable covalent adducts of DNA are formed, as shown by the HPLC analyses. The major adducts are identical with the materials in the respective crystals which can be readily obtained from the 1:1 mixture of DAU–d(CGCGCG) and DAU–d(CGTDCG) plus HCHO, but not from the solution without HCHO. The high-resolution (1.5 Å) X-ray crystal structure of those adducts shows unambiguously that they contain a covalent methylene bridge between the N3′ of daunosamine and the N2 of the guanine or 2-aminoadenine. The perfect juxtaposition of the two amino groups in the minor groove of the complex provides a template for an efficient addition of HCHO. The methylene bridge does not perturb the conformation of the drug–DNA complex, when compared to the structure of DAU–d(CGTACG). The results suggest new approaches for synthesizing a new type of potential anticancer drug by attaching a reactive (e.g., alkylating) functional group at the N3′ amino position of daunorubicin/doxorubicin. The stable drug–DNA adduct may be useful as probes for other biological studies.

Formaldehyde is a highly reactive compound that has been shown to be a potential mutagen in humans (Report of the Federal Panel on Formaldehyde, 1982; Shauenstein et al., 1977). It has received great attention due to its widespread presence in the environment. Its toxicity is likely due to the potent cross-linking ability which causes the formation of adducts between cellular macromolecules (Krzyzanowski et al., 1990; Grafstraom et al., 1983). Among the various chemical functional groups, primary amines have been shown to be principal targets being cross-linked to DNA bases by HCHO. This was demonstrated by the preparation of covalent adducts of *n*-butylamine and poly(dG-dC) duplex by linking the amine to N2 amino groups of guanine bases with HCHO (Chen et al., 1987). However, despite extensive studies on the cross-linking reaction of formaldehyde, little structural information regarding the cross-link is known at the molecular level.

Recently, we made a novel observation that a synthetic daunorubicin (DAU)[1] derivative, MAR70, readily formed a cross-link to the DNA hexamer d(CGTDCG) due to the presence of a trace amount of HCHO in the crystallization solvent (Gao et al., 1991). The cross-linking reaction occurred because the two participating amino groups (N2 from 2-aminoadenine and N3′ from MAR70) in the complex juxtaposed perfectly (assisted by the disaccharide in MAR70) to provide a template for an efficient addition of HCHO. In order to determine whether the same reaction would occur between the clinically important daunorubicin and doxorubicin (Lown, 1988) (Figure 1) and the amino group of normal guanine base, we undertook a systematic study of the effect of HCHO on the complexes of DAU/DOX and DNA oligonucleotides by HPLC and X-ray diffraction. We found, by HPLC analyses, that adducts between drug and DNA are formed (with one major product of >90% yield) when daunorubicin is mixed with DNA hexamers, d(CGCGCG) and d(CGTDCG), in the presence of HCHO. The structures of the DAU–d(CGCGCG) and DAU–d(CGTDCG) adducts were determined by X-ray crystallography, both to 1.5-Å resolution. Our results unambiguously demonstrate a covalent methylene bridge between the N3′ of daunosamine and the N2 of guanine or 2-aminoadenine.

Experimental Procedures

The oligonucleotides d(CGCGCG) and d(CGTDCG) were synthesized according to the procedure published earlier (van der Marel et al., 1981) or synthesized on an Applied Biosystem 380B DNA synthesizer. Daunorubicin was purchased from Sigma Chemical Co. The DAU–CG complex was crystallized from a mixture containing 1.2 mM d(CGCGCG) (single strand), 4 mM $BaCl_2$, 30 mM sodium cacodylate (pH 6.0), 2.5 mM spermine, 1.2 mM DAU, and 9 mM HCHO plus 5% (v/v) 2-methyl-2,4-pentanediol (2-MPD). The solution was equilibrated with 30 mL of 1 mM HCHO in 40% 2-MPD at room temperature (~25 °C) by vapor diffusion. The DAU–TD complex was crystallized from a similar condition. Crystals in the form of tetragonal rods appeared after 3 days. They were in the space group $P4_12_12$ with respective unit-cell dimensions $a = b = 28.02$ (1) and $c = 52.47$ (2) Å for the DAU–CG adduct and $a = b = 27.94$ (3) and $c = 52.24$ (3) Å for the DAU–TD adduct. The crystal was mounted in a sealed glass capillary with a droplet of mother liquor. Data were collected on a Rigaku AFC-5R rotating-anode X-ray diffractometer at 25 °C using the ω-scan mode with Cu$K\alpha$

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[‡]The atomic coordinates have been deposited to the Brookhaven Protein Databank.

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[1] Abbreviations: A, T, G, C, and D, adenine, thymine, guanine, cytosine, and 2-aminoadenine (diaminopurine) or their corresponding nucleotides, respectively; DAU, daunorubicin or daunomycin; DOX, doxorubicin or adriamycin; DAU–TD or TD, DAU–d(CGTDCG) complex or adduct; DAU–CG or CG, DAU–d(CGCGCG) complex or adduct; DAU–TA or TA, DAU–d(CGTACG) complex.

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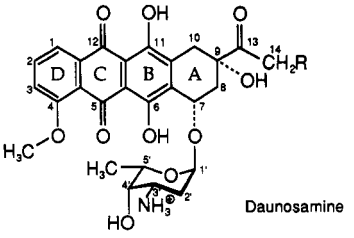

Daunorubicin R = H

Doxorubicin R = OH

FIGURE 1: Molecular formulas of daunorubicin (daunomycin) and doxorubicin (adriamycin).

radiation to a resolution of 1.5 Å for both complexes. There were 2229 (DAU–CG) and 1746 (DAU–TD) independent reflections observed at the 3.0 $\sigma(F)$ level above the background after Lp, empirical absorption, and decay corrections for the 2 datasets. The coordinates from the DAU–d(CGTACG) structure (Wang et al., 1987) were used as the starting model and refined by the Konnert–Hendrickson constrained refinement procedure (Hendrickson & Konnert, 1979). A series of Fourier maps were calculated to locate the solvent water molecules in the crystal lattice. During the refinement of both structures, the methylene bridge between N2 of D or G and N3′ of DAU appeared cleanly in the difference Fourier map, and it was included in the refinement. The DAU–CG structure was refined to a final R factor of 18.5% with the root mean square (rms) differences in bond distances of 0.016 Å from the ideal values. The final R factor is 17.6% with a rms of 0.015 Å for the DAU–TD structure. One hydrated sodium ion coordinated to the N7 of the G6 residue was located in both adducts, as in the DAU–d(CGTACG) complex (Wang et al., 1987). No other ions (Mg^{2+}, Ba^{2+}, or spermine) could be identified unambiguously. Some continuous electron densities in the solvent channels were interpreted as the hydrated formaldehyde molecules. The final atomic coordinates of both complexes will be deposited in the Brookhaven Protein Databank.

HPLC analyses were performed on a Hewlett-Packard 4500A instrument with a Vydac C4 reverse-phase column. A linear gradient, 90–60% of 10 mM triethylamine acetate (pH 7) in water and 10–40% acetonitrile (50% in water) in 90 min, with a flow rate of 0.3 mL/min was used. The samples were prepared by mixing DAU, DNA, and HCHO at a 1.25:1 (single-strand):10 and 1:1:10 molar ratio for DAU–CG and DAU–TD, respectively, and incubated for a varying amount of time.

Results and Discussion

Figure 2 shows the HPLC elution profiles of the products from the reaction between formaldehyde and the 1:1 complexes of DAU–CG and DAU–TD. DNA hexamers eluted at 22.6 min for d(CGCGCG) and 21.2 min for d(CGTDCG), while DAU eluted at 77 min. Immediately after the incubation, some adducts began to appear as indicated by peaks at 21.2, 26.0, and 29.3 min in the DAU–TD mixture. The peak at 29.3 min continued to grow during the course of the incubation and finally became the only product after 18 h. The identity of this product was shown to be that of the adduct in the crystal (see legend to Figure 2). The results suggest that this adduct, with linkage between N3′ of DAU and N2 of 2-aminoadenine, is the most stable adduct thermodynamically, though not preferred kinetically. The exact nature of the peak at 26.0 min is being determined. Our preliminary data suggest that it may be a modified DNA involving other possible DNA

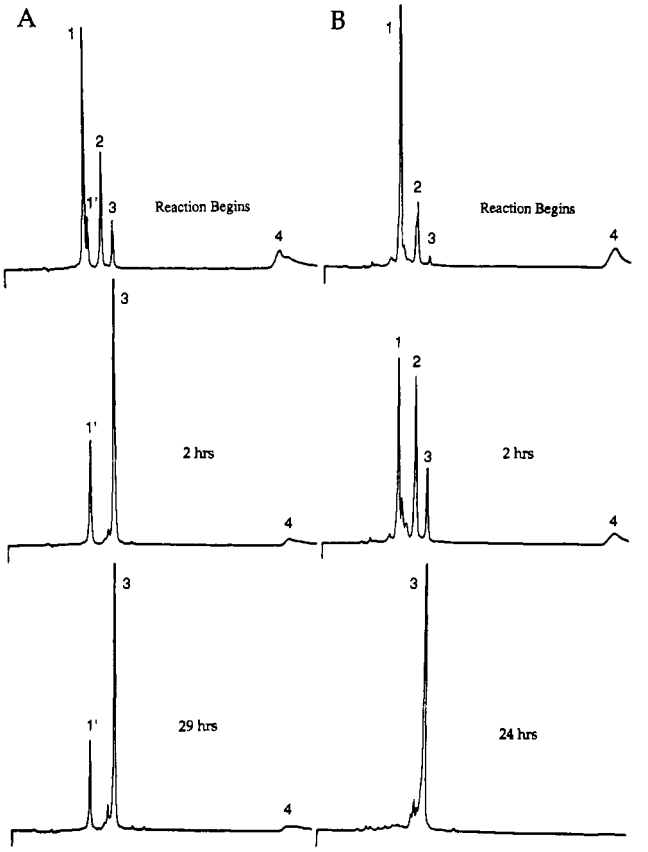

FIGURE 2: HPLC elution profiles of the formaldehyde-induced cross-linking reaction between DAU and d(CGCGCG) and d(CGTDCG). (A) DAU–d(CGCGCG) reaction. Peaks 1, 3, and 4 at 22.6, 30.6, and 77 min are due to the free DNA, DAU–d(CGCGCG) adduct (on N2 of D), and free DAU, respectively. Peak 2 at 27.4 min is a less stable DAU–DNA adduct, whose identity is being determined. Peak 3 (30.6 min) has an identical elution retention time with that of the solution from the dissolved crystals of DAU–d(CGCGCG) adduct, proving the identity of this peak. This adduct is stable, as judged by the small changes in its elution profile after several weeks. Peak 1′ (23.5 min), whose identity is still unknown, is also surprisingly stable. It can not be converted further by the excess DAU in the mixture. (B) Corresponding profiles for the DAU–TD reaction. Elution time is 21.2, 26.0, and 29.3 min, respectively, for peaks 1, 2, and 3. Peak 3 is identical with the materials from the crystals. The rate of the reaction is slower than that of the DAU–CG, suggesting that HCHO cross-links DAU to the guanine N2 more readily than to the 2-aminoadenine N2.

cross-linking sites. However, it is less stable, and consequently, in the presence of HCHO the equilibrium is gradually shifted toward adduct at the N2 binding site (of the fourth base in the hexamer) which has a geometry receptive to the methylene bridge provided by HCHO. Similar behavior was observed involving d(CGCGCG), except that the major peak at 30.6 min appeared more rapidly. This suggests that the N2 of guanine is more reactive toward HCHO than that of the 2-aminoadenine.

The unequivocal proof of the fact that HCHO indeed cross-links DAU to d(CGCGCG)/d(CGTDCG) is provided by examining the two crystal structures of DAU–CG and DAU–TD adducts. The difference Fourier ($F_o - F_c$) maps of the daunosamine moiety show the additional electron density connected to the N3′ atom in each adduct (Figure 3). The location and the geometry of the extra density are consistent with a methylene group linking the N3′ atom of daunosamine and the N2 atom of guanine or 2-aminoadenine.

The overall structure of each complex is very similar to the original DAU–TA structure (Wang et al., 1987), with re-

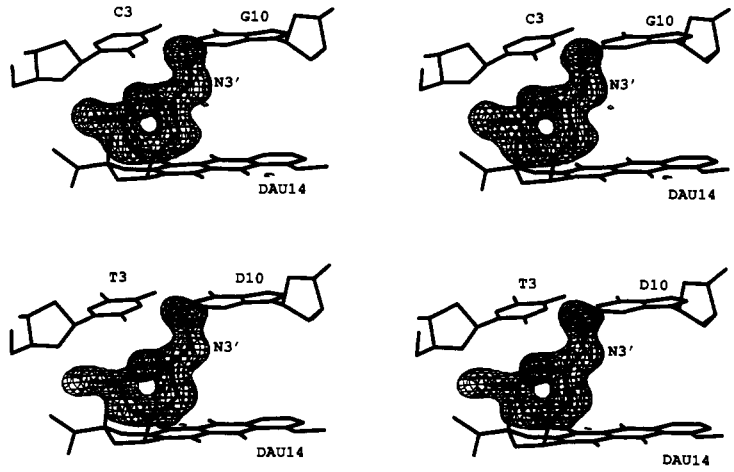

FIGURE 3: Stereoscopic views of the difference Fourier ($F_o - F_c$) electron density maps of the DAU–d(CGCGCG) (top) and DAU–d(CGTDCG) (bottom) adducts displayed by FRODO/TOM (Jones, 1978). The daunosamine sugar plus the N2 of guanine or 2-aminoadenine and the methylene group were omitted from the phase contributions. The extra electron density bridged between the N2 of G10 or D10 and the N3′ of daunosamine in both structures is evident.

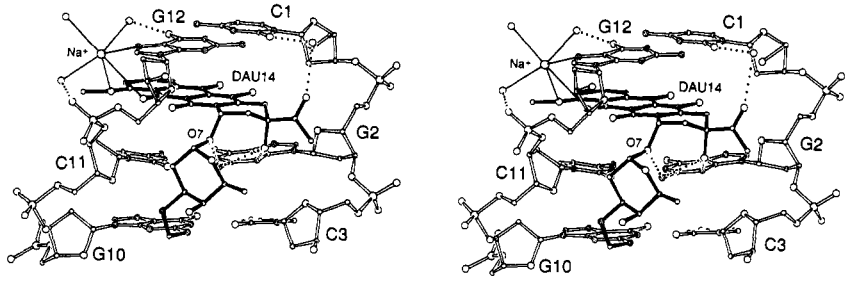

FIGURE 4: Three base pairs of the DAU–d(CGCGCG) adduct are viewed from the minor groove side. DAU (in filled bonds) is intercalated between the CpG step of the distorted B-DNA hexamer duplex (open bonds) with the aglycon chromophore penetrating through the helix. The sugar lies in the minor groove, and its N3′ amino group is near the edge of the base pairs. A hydrated sodium ion coordinates the DAU and G12 in the major groove. The tight fit of the drug to DNA results in a significant buckle in the C11-G2 base pair (14.6°). The propeller twist in C3-G10 is −7.4°. Hydrogen bonds are shown as dotted lines. The methylene carbon (CM) occupies an ideal position for bridging the N2 and N3′ atoms. The torsion angles in the bridge are 172.7° (C2′–C3′–N3′–CM), −42.9° (C3′–N3′–CM–N2), and −91.1° (N3′–CM–N2–C2).

spective root mean square differences of 0.237 Å (DAU–CG) and 0.231 Å (DAU–TD), excluding nonidentical atoms. The three-dimensional structure of the DAU–CG adduct is shown in Figure 4. The DAU molecule is intercalated between the two GC base pairs in the CpG step of a distorted B-DNA double-helical fragment. The aglycon chromophore skewers the DNA double helix with ring D reaching the major groove and the daunosamine moiety lying in the minor groove. One consequence of this binding conformation is that the N3′ amino group approaches the edge of the base pairs. For example, in the DAU–TA structure (Wang et al., 1987), N3′ is 3.29 Å from the O2 of C11 cytosine base, 3.39 Å from O4′ of C11 ribose, 3.44 Å from N3 of A10 adenine, and 3.52 Å from C2 of A10. The former three distances are consistent with weak hydrogen bonds.

When there is a guanine or a 2-aminoadenine at the fourth (and the symmetry-related tenth) sequence position (the hexamer duplex is numbered C1 to G6 in one strand and C7 to G12 in the other strand), the two amino groups (N3′ from DAU and N2 of G10 or D10) in the nonadduct complex would be in close contact (3.09 Å, shorter than the sum of the van der Waals radii of two amino groups), which may slightly destabilize the binding of DAU to a sequence of 5′-GCG in DNA. This is consistent with the footprinting data of the binding of DAU to DNA which show a sequence preference of 5′-(A/T)CG over 5′-GCG (Chaires et al., 1987). Those two amino groups are brought into close proximity and are rigidified in a somewhat hydrophobic environment of the minor groove by the intercalative binding of DAU to DNA. This affords an ideal situation for an electrophilic attack on amino groups (which become highly reactive in this shielded environment) by an agent such as HCHO. In fact, the cross-linking reaction relieves the unfavorable contact between the two amino groups existing in the noncovalently bonded complex.

The cross-linking reaction by formaldehyde in both complexes is quite effective as judged by the HPLC profile of the redissolved crystals which exhibits only a single peak associated with the adduct. The result also suggests that the reaction is sequence-specific. Only 5′-DCG and 5′-GCG have the proper drug binding conformation to place the N2 amino group of the first D or G in the triplet sequence near the N3′ of DAU. Therefore, HCHO reverses the DNA binding sequence preference of DAU from 5′-(A/T)CG to 5′-GCG. The proposed switch in sequence preference toward 5′-GCG with the aid of HCHO may significantly enhance the ability of DAU + HCHO in inhibiting the formation of Z-DNA of poly(dG-dC) (Wang et al., 1979; Chaires, 1983).

This facile cross-linking of two nucleophiles (induced by the spatial confinement) may be common in other biological systems. A number of antitumor antibiotics act by the formation of covalent adduct between the drug and DNA (Warpehoski & Hurley, 1988). For example, mitomycin C cross-links two N2 amino groups of adjacent guanines of CpG sequence in B-DNA (Tomasz et al., 1988; Teng et al., 1989; Millard et al., 1990). Similarly, CC-1065 binds to the narrow minor groove of AT-rich sequences and forms a covalent adduct at N3 of adenine in a sequence-specific manner (Hanka

et al., 1978; Warpehoski & Hurley, 1988). These cross-linking reactions require critical spatial relation between the nucleophile and the electrophile of the two molecules, as in the present case.

It should be pointed out that HCHO is an important compound in the one-carbon (C_1) metabolism in cells. The conversion of tetrahydrofolate to N^5,N^{10}-methylenetetrahydrofolate requires the participation of HCHO, which is generated during the conversion of serine to glycine catalyzed by serine hydroxymethyltransferase (Rawn, 1989). It would not be surprising that a similar cross-linking reaction caused by HCHO occurs in other cellular macromolecules, such as between proteins and DNA, which may be responsible for the toxicity of HCHO (Report of the Federal Panel on Formaldehyde, 1982; Shauenstein et al., 1977; Krzyzanowski et al., 1990; Grafstraom et al., 1983).

The present structures point out that the N3′ primary amino group of bound DAU/DOX is a functional group proximal to many nucleophilic positions (e.g., N3 of adenine, N2 of guanine) of the DNA double helix. Compounds with certain reactive functional groups, e.g., alkylating groups (Denny, 1989; Rosik & Sweet, 1990), attached at the N3′ amino position of DAU/DOX molecules may alkylate the N3 position of adenine very effectively. This new class of DAU/DOX derivatives with alkylating function may have significantly higher potency in ways similar to CC-1065, an extremely potent anticancer agent that alkylates at the N3 of adenine in some specific sequences (Hanka et al., 1978; Warpehoski & Hurley, 1988). It is also interesting to note that a new, highly potent N3′-modified derivative of doxorubicin, 3′-(3-cyano-4-morpholinyl)-3′-deaminodoxorubicin, forms a covalent adduct to DNA in vivo by the loss of the cyano group (Westendorf et al., 1989). While the exact nature of the adduct is not yet clear, the mechanism associated with the aldehyde-mediated adduct found in the present structure may be relevant. Another example is found in a highly potent anthracycline antibiotic, barminomycin, which contains an aldehyde group attached to the O4′ of the daunosamine sugar (Umezawa, 1985). This aldehyde may serve as a cross-linking functional group. Those aldehyde-mediated adducts are probably different from the irreversible drug–DNA products resulting from the reductive reaction process of DAU/DOX (Phillips et al., 1990).

Finally, it is likely there are other useful applications of the efficient formaldehyde cross-linking between DAU/DOX and DNA. For example, a stable adduct between DOX/DAU and polymer DNA can be prepared as probes for various biological studies. We have shown that daunorubicin can be cross-linked to poly(dG-dC) readily in the presence of HCHO (unpublished results). Its mode of action is likely to be the same as in the DAU–CG complex. It is interesting to note that barminomycin I complexed with poly(dG-dC) has significantly higher (10 times) anticancer activity than barminomycin alone or barminomycin complexed with poly(dI-dC) (Kimura et al., 1990). Notice that deoxyinosine (dI) nucleotide does not have the N2 amino group, which may be involved in a cross-linking reaction similar to that seen in our present work. We are testing some of those daunorubicin–DNA (oligonucleotides and polymers) adducts, cross-linked by formaldehyde, for possible antitumor/anticancer activity. Additionally, these DAU/DOX–DNA adducts may be used to produce antibodies which recognize DAU–DNA complex specifically. Those antibodies may be used in studying the interaction of drug–DNA complex with relevant proteins (repair enzymes, topoisomerase II, etc.).

In conclusion, our results demonstrate that the important anticancer drugs DAU/DOX can be cross-linked to guanine nucleotide in DNA very efficiently. Further biochemical and biophysical work on this type of reaction may lead us to design better anticancer drugs.

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