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Structure of the (+)-CC-1065–DNA Adduct: Critical Role of Ordered Water Molecules and Implications for Involvement of Phosphate Catalysis in the Covalent Reaction†

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ABSTRACT: (+)-CC-1065 is an extremely potent antitumor agent produced by *Streptomyces zelensis*. The potent effects of (+)-CC-1065 and its alkylating analogues are thought to be due to the formation of a covalent adduct through N3 of adenine in DNA. It has been previously postulated, on the basis of modeling studies, that a phosphate may be involved in stabilization of the adduct and in acid catalysis of this reaction. In this study, using ^{1}H NMR in combination with ^{17}O-labeled water and phosphate, we demonstrate the involvement of a bridging water molecule between a phenolic proton on the alkylating subunit of (+)-CC-1065 and an anionic oxygen in the phosphate on the noncovalently modified strand of DNA. In addition, a second ordered water molecule associated with one of the protons on N6 of the covalently modified adenine is also identified. This structure has important implications for catalytic activation of the covalent reaction between (+)-CC-1065 and DNA and, consequently, the molecular basis for sequence-selective recognition of DNA by the alkylating subunit of (+)-CC-1065. On the basis of the example described here, the use of ^{1}H NMR in ^{17}O-labeled water may be a powerful probe to examine other structures and catalytic processes for water-mediated hydrogen-bonded bridges that occur between small molecules and DNA or enzymes.

(+)-CC-1065 is an antitumor antibiotic with a unique structure (Hanka et al., 1984; Chidester et al., 1981) (Figure 1) and mechanism of action (Hurley et al., 1984; Warpehoski & Hurley, 1988). Previous studies have demonstrated that this antibiotic is extraordinary for both its base and DNA sequence specificity (Hurley et al., 1984, 1988, 1990; Reynolds et al., 1985). An analogue of (+)-CC-1065 designed and synthesized by Upjohn scientists was recently introduced into phase I clinical trials (J. P. McGovren, The Upjohn Company, personal communication, 1990). Structurally, (+)-CC-1065 consists of three repeated pyrroloindole subunits (A, B, and C in Figure 1) attached via amide linkages that are approximately 15° out of plane, providing the drug molecule with a right-hand twisted banana shape (Hanka et al., 1984; Chidester et al., 1981). Subunit A contains the DNA-reactive cyclopropane ring that alkylates N3 of adenine when it binds within certain reactive sequences (Lin & Hurley, 1990; Scahill et al., 1990) (Figure 1). Since only adenines in certain sequence contexts react with (+)-CC-1065, this drug has sequence selectivity (Hurley et al., 1988). Surprisingly, the A subunit alone contains sufficient structural information to encode the primary molecular basis for sequence selectivity (Hurley et al., 1988), and this subunit is also essential for antitumor activity (Warpehoski et al., 1988). However, as we have previously demonstrated, the noncovalent binding interactions of the B and C subunits with DNA can modulate or fine-tune this sequence selectivity (Hurley et al., 1988). We have previously suggested that the primary basis for sequence selectivity is through *a sequence-dependent catalytic activation and/or a sequence-dependent conformational flexibility* (Warpehoski & Hurley, 1988). In this report we provide structural evidence that is in accord with bifunctional catalysis of adduct formation in a mechanism that involves two critically positioned hydrogen-bonded water molecules at opposite ends of the covalent reaction site.

Materials and Methods

Chemicals. (+)-CC-1065 was obtained from The Upjohn Company and used without further purification. [^{17}O]Water (60 and 45 atom % ^{17}O) was purchased from Cambridge Isotope Laboratories. Reagents used to prepare the NMR buffer, sodium phosphate (99.99%), and sodium chloride (99.99%) were purchased from Aldrich. HPLC water and methanol were purchased from Baxter Scientific and Fisher, respectively. Hydroxylapatite used to purify the 12-mer duplex and the 12-mer adduct was purchased from Calbiochem. Sephadex G-25 (superfine) was purchased from Pharmacia.

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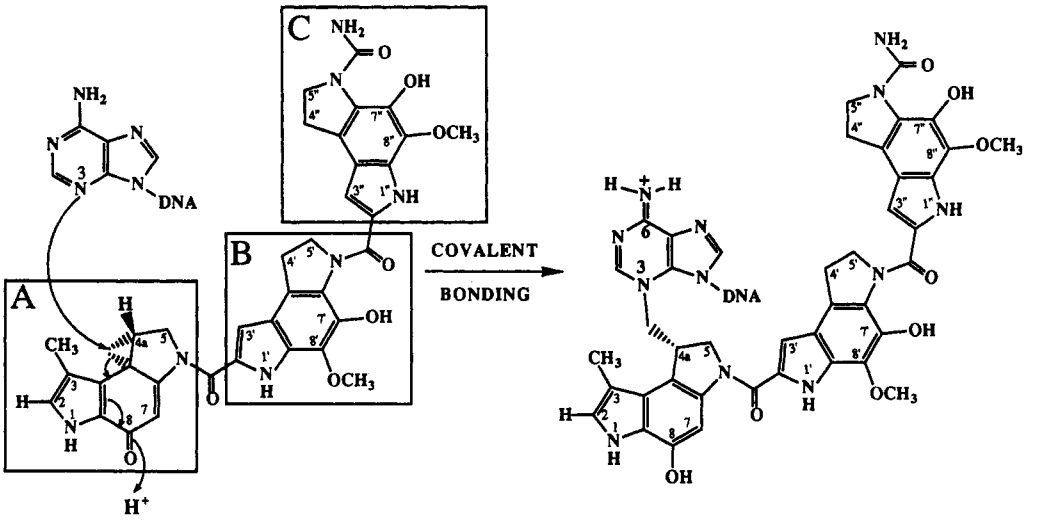


FIGURE 1: Reaction of (+)-CC-1065 with double-stranded DNA at N3 of adenine to form the (+)-CC-1065–DNA adduct. The covalently modified adenine is in the doubly protonated 6-amino form (Lin & Hurley, 1990).

Preparation and Purification of the 12-mer Duplex and (+)-CC-1065 12-mer Duplex Adduct. The non-self-complementary d(GGCGGAGTTAGG)·(CCTAACTCCGCC) 12-mer duplex for NMR studies was synthesized in-house on a 10-μmol scale by using automated solid-phase phosphotriester and phosphoramidite chemistry (Gait, 1984) on an Applied Biosystem automated DNA synthesizer, Model 381A. The general procedures for synthesis, deprotection, drug bonding, HPLC, and chromatographic purification of the 12-mer duplex and (+)-CC-1065 12-mer duplex adduct have been previously reported (Lin & Hurley, 1990).

Preparation of the ^{17}O-Labeled 12-mer Duplexes and the Corresponding (+)-CC-1065 12-mer Duplex Adducts. Oxygen-17-labeled d(GGCGGAGTTAGG)·(CCTAACTCCGCC) 12-mer duplexes were synthesized by using automated solid-phase phosphotriester and phosphoramidite chemistry (Gait, 1984). Oxygen-17 was regiospecifically incorporated into the anionic oxygen of ^{11}G-^{12}G, ^{16}A-^{17}A, and ^{17}A-^{18}C phosphate groups in three different oligomers by selectively oxidizing the appropriate phosphite intermediate with 0.1 M iodine/1 g of [^{17}O]water/10 mL of pyridine/40 mL of THF solution.

Proton–NMR Experiments. One- and two-dimensional ^1H NMR data sets were recorded on a General Electric GN-500 FT NMR spectrometer at room temperature. Chemical shifts were recorded in parts per million and referenced relative to external TSP (1 mg/mL) in D_2O. Approximately 28 mg of the (+)-CC-1065 12-mer duplex adduct in 0.5 mL of 90% H_2O/10% D_2O buffer containing 10 mM NaH_2PO_4 and 100 mM NaCl, pH 6.85, was used for ^1H NMR experiments. Suppression of the water signal was achieved with 1-$\bar{3}$-3-$\bar{1}$ pulse sequence (Hore, 1983) with a delay of 120 ms. One-dimensional NOE[1] difference experiments were performed at 23 °C.

T_1 Inversion–Recovery Experiments. T_1 measurements in 90% H_2O/10% D_2O were made on a General Electric GN-500 NMR instrument by the conventional inversion–recovery method executed with a 1-$\bar{3}$-3-$\bar{1}$ selective excitation pulse sequence. The 1-$\bar{3}$-3-$\bar{1}$ routine was optimized to provide exact 90° and 180° flip angles on the resonances of interest. The pulse repetition delay is 5 s.

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE correlated spectroscopy; CPI, cyclopropylpyrroloindole; BPDE, benzo[*a*]pyrene diol epoxide.

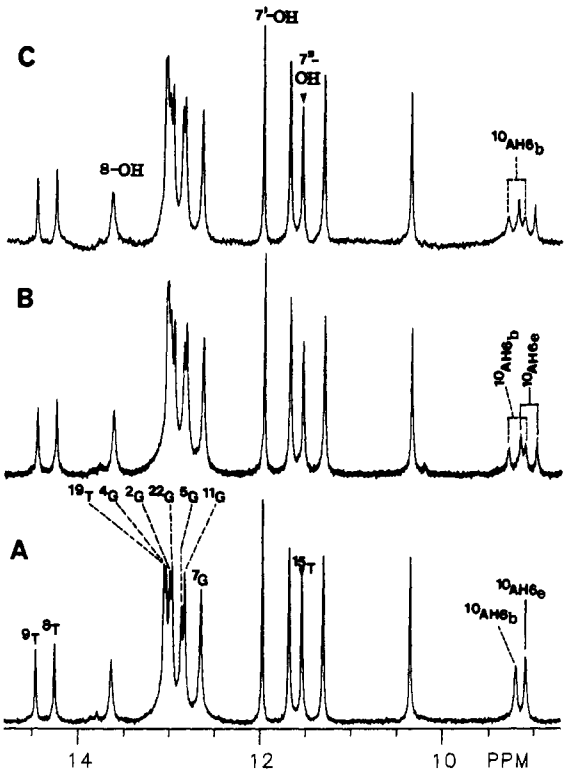
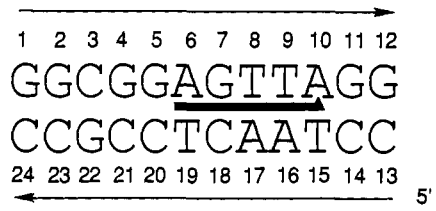


FIGURE 2: (A) Proton NMR spectrum (500 MHz; 8.5–15 ppm, downfield region) of the nonisotopically labeled (+)-CC-1065 12-mer duplex adduct (1 equiv of (+)-CC-1065 per 12-mer duplex) in 0.5 mL of 90% H_2O/10% D_2O buffer containing 10 mM NaH_2PO_4 and 100 mM NaCl, pH 6.85, at 25 °C. The assignments of the 12-mer imino and the covalently modified adenine 6-amino protons are based on one-dimensional NOE difference and two-dimensional NOESY experiments. Also shown are 500-MHz ^1H NMR spectra (8.5–15 ppm, downfield region) of the [6-^{15}N]deoxyadenosine-labeled (+)-CC-1065 12-mer duplex adduct in (B) [^{16}O]water and in (C) 40.5% [^{17}O]enriched water. The ^{15}T imino and 7″-OH proton resonances overlap.

Results and Discussion

(+)-CC-1065 was reacted with the non-self-complementary 12-mer duplex (Chart I) that contains one of the preferred bonding sequences 5′AGTTA* (where an asterisk denotes the covalently modified adenine). The duplex and its (+)-CC-1065-modified sequences were characterized by one- and

Chart I: Sequence and Numbering of the (+)-CC-1065 12-mer Duplex Adduct[a]



[a] The ^{17}O labels were specifically introduced on the anionic oxygens of phosphate backbone between ^{11}G and ^{12}G, ^{16}A and ^{17}A, and ^{17}A and ^{18}C.

two-dimensional ^1H NMR (Lin & Hurley, 1990; C. H. Lin and L. H. Hurley, unpublished results). A partial ^1H NMR spectrum (downfield region) of the nonisotopically labeled (+)-CC-1065 12-mer duplex adduct is plotted in Figure 2A. The assignments of the exchangeable protons in the (+)-CC-1065 12-mer DNA duplex adduct have been made previously (Lin & Hurley, 1990; C. H. Lin and L. H. Hurley, unpublished results) and are noted in Figure 2, panels A and C. Of particular significance are the upfield-shifted ^{10}A-^{15}T imino proton (2.07 ppm) and the downfield-shifted 6-amino protons (~2 ppm) of ^{10}A (the covalently modified adenine) that occur at 9.19 and 9.08 ppm in the duplex adduct relative to the duplex alone. These latter signals were assigned to the $^{10}AH6$ hydrogen-bonded amino proton ($H6_b$) and $^{10}AH6$ external amino proton ($H6_e$) respectively.[2] Confirmation of the assignments for the $^{10}AH6$ amino protons was made by synthesizing [6-^{15}N]-^{10}A-deoxyadenosine-labeled (+)-CC-1065 12-mer duplex adduct (Lin & Hurley, 1990) (Figure 2B). As expected, the $^{10}AH6_b$ and $^{10}AH6_e$ resonance signals are split into doublets due to coupling with the ^{15}N nucleus located at N6. Upon heating the sample to 45 °C, we found, contrary to our expectation, that the resonance signal assigned to the $^{10}AH6_b$ proton *exchanged more rapidly* than that assigned to the $^{10}AH6_e$ proton (C. H. Lin, J. M. Beale, and L. H. Hurley, unpublished results). Since the 6-amino group of adenine is in the doubly protonated form (Lin & Hurley, 1990) and the ^{10}A-^{15}T imino proton is shifted upfield, which is indicative of reduced hydrogen bonding strength, we considered the possibility that the observed rapid exchange of the $^{10}AH6_b$ proton might be due to a facile exchange with an ordered and judiciously positioned hydrogen-bonded water molecule. To evaluate this possibility, the [6-^{15}N]-^{10}A-deoxyadenosine-labeled (+)-CC-1065 12-mer duplex adduct was dissolved in ^{17}O-labeled water to attain a 40.5% overall enrichment. To our surprise, not only was the doublet for the $^{10}AH6_b$ proton broadened but the (+)-CC-1065 8-phenolic proton of the A subunit was also broadened relative to the equivalent resonance signals in ^{16}O-water (compare Figures 2B and 2C).[3] An expansion of the regions containing the broadened proton NMR signals in comparison with the ^{16}O-water sample is shown in Figure 3. Most likely, the broadening of these protons is due to their interaction with the ^{17}O nucleus. Ox-

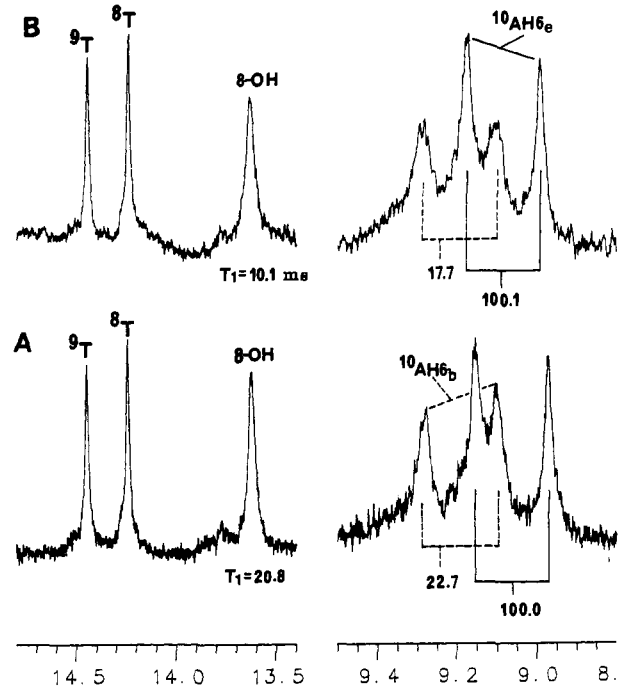


FIGURE 3: ^1H NMR spectra (500 MHz; expanded downfield regions, 8.7–9.5 and 13.4–14.8 ppm) of the [6-^{15}N]deoxyadenosine-labeled (+)-CC-1065 12-mer duplex adduct in (A) regular water (B) 40.5% ^{17}O-enriched water NMR buffer containing 10 mM NaH_2PO_4 and 100 mM NaCl, pH 6.85, at 25 °C. T_1 measurements were made on a General Electric GN-500 NMR instrument by the conventional inversion–recovery method executed with a 1-$\bar{3}$-3-$\bar{1}$ selective excitation pulse sequence. The 1-$\bar{3}$-3-$\bar{1}$ routine was optimized to provide exact 90° and 180° flip angles on the resonances of interest. Both the 8-phenolic proton of (+)-CC-1065 and $^{10}AH6_b$ of the covalently modified adenine have smaller T_1 values in ^{17}O-enriched water solution. The T_1 relaxation time of $^{10}AH6_e$ remained the same, regardless of the solvent.

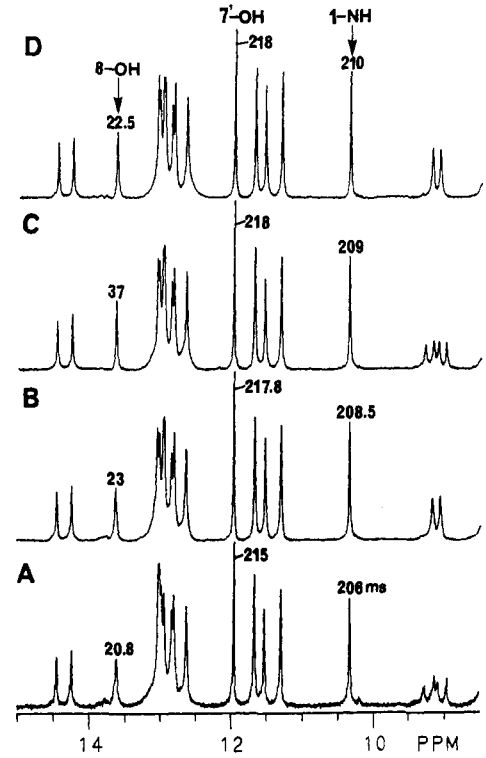


FIGURE 4: (A) ^1H NMR spectrum (500 MHz; 8.5–15 ppm, downfield region) of the ^{16}O-labeled (+)-CC-1065 12-mer duplex adduct as described in the legend for Figure 2. (B), (C), and (D) are the 500-MHz ^1H NMR spectra of the [^{17}O]phosphate-labeled ^{11}G-^{12}G, ^{16}A-^{17}A, and ^{17}A-^{18}C samples.

[2] The assignments of the $H6_b$ and $H6_e$ protons were made on the basis of comparison of coupling constants to the equivalent protons in the [6-^{15}N]-^{10}A-deoxyadenosine-labeled duplex (Lin & Hurley, 1990).

[3] The sample in [^{17}O]H_2O was prepared by lyophilizing the [6-^{15}N]-deoxyadenosine-labeled (+)-CC-1065 12-mer duplex adduct in [^{16}O]H_2O solution to complete dryness and then adding 0.45 mL of a 45% [^{17}O]H_2O/0.05 mL of D_2O mixture to the above sample. The only difference between the two samples used to provide the spectra in Figure 2, panels B and C, is the [^{17}O]H_2O content. Other factors such as DNA concentration, amount of metal, and volume of the sample are for all practical purposes the same.

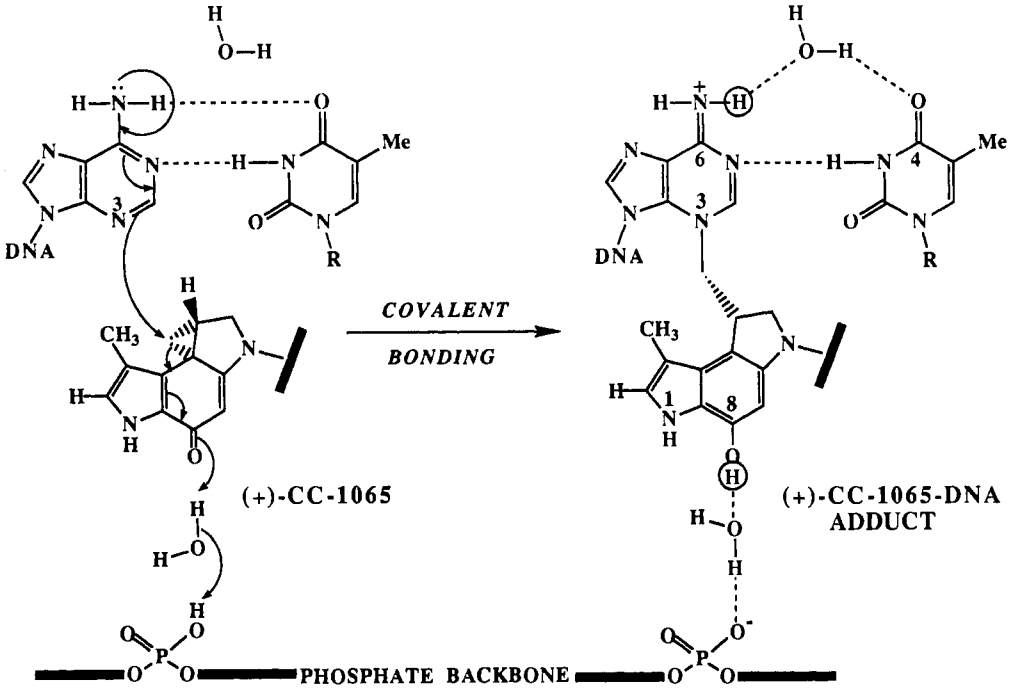


FIGURE 5: Proposed mechanism for the catalytic activation of the reaction of the (+)-CC-1065 with DNA involving two strategically placed water molecules.

ygen-17, with a spin $I = {}^5/_2$, possesses an unsymmetrical charge distribution and therefore an electronic quadrupole moment. The proximity of the 8-phenolic and $^{10}AH6_b$ protons to these fluctuating magnetic dipoles enhances their spin–lattice (and presumably also spin–spin) relaxation rates, correspondingly reducing their T_1 values. These comparative measurements in [^{16}O]water and [^{17}O]water solutions are shown in Figure 3. For the 8-phenolic proton in [^{17}O]water, a 50% reduction in T_1 relaxation time is noted in comparison with the nonisotopically labeled water, and while the T_1 of the $^{10}AH6_b$ proton in [^{17}O]water is also reduced (22%), it is not so significantly reduced as the 8-phenolic proton. For comparison we also measured the T_1 values for the two other phenolic protons and imino protons in the B and C subunits of (+)-CC-1065 and other exchangeable protons, including the H1 in the A subunits of (+)-CC-1065 in both ^{16}O- and ^{17}O-labeled water. Significant differences between T_1 values in ^{16}O- and ^{17}O-labeled water were not observed (C. H. Lin, J. M. Beale, and L. H. Hurley, unpublished results).

A two-dimensional ^{1}H NMR study of the (+)-CC-1065 12-mer duplex adduct shows that there is a discontinuity between the ^{9}T-^{16}A and ^{10}A-^{15}T base pairs, which is characterized by a highly propeller twisted ^{10}A-^{15}T (Lin & Hurley, unpublished results). The ^{10}A deoxyadenosine has a C3′-endo-like conformation and the ^{10}A-^{15}T imino proton resonance is shifted upfield by 2.07 ppm, indicative of a considerably weakened hydrogen-bonding interaction. This discontinuity may well be responsible for the bending of DNA induced by (+)-CC-1065, which appears to be at least superficially similar in magnitude, direction, and structural origin to that associated with intrinsic A-tract bending (Lin et al., 1991). In view of the discontinuity between ^{9}T-^{16}A and ^{10}A-^{15}T, it is perhaps not too surprising that a water molecule may be required to bridge the $^{10}AH6_b$ proton, and we suspect that the O4 of thymine on the opposite strand in the same base pair stabilizes the otherwise badly distorted duplex.

In previous publications, molecular modeling studies from both the French group (Zakrzewska et al., 1987) and the Upjohn–Texas group (Hurley et al., 1990) have suggested that the anionic oxygen of the ^{17}A-^{18}C phosphate on the noncovalently modified strand that is two base pairs to the 5′ side of the covalently modified adenine may be involved in a hydrogen-bonding interaction with the 8-phenolic proton of the A subunit of (+)-CC-1065 12-mer adduct. It was also suggested that this interaction might be involved in general acid catalysis of the covalent bonding reaction and be important in the observed sequence selectivity (Hurley et al., 1990). The ~2 ppm downfield shift of the 8-phenolic proton of the A subunit relative to the corresponding B and C subunit protons is in accord with this idea (Figure 2C). To further evaluate this proposal, we prepared three individual samples of the 12-mer duplex used in this study with ^{17}O labels in the anionic oxygens of the ^{11}G-^{12}G, ^{16}A-^{17}A, and ^{17}A-^{18}C phosphates.[4] The position of the ^{17}O labels in each sample was confirmed by the broadening of the corresponding phosphate resonances in the ^{31}P NMR spectrum (C. H. Lin, J. M. Beale, and L. H. Hurley, unpublished results). Unexpectedly, we found it was the ^{17}O-labeled *^{16}A-^{17}A phosphate sample* rather than the ^{17}O-labeled *^{17}A-^{18}C phosphate sample* that produced a very significant sharpening of the resonance signal of the 8-phenolic proton of the A subunit (Figure 4). A comparison of the T_1 relaxation times of the 8-phenolic proton for the three anionic oxygen ^{17}O-labeled phosphate samples is shown in Figure 4. While the ^{11}G-^{12}G and ^{17}A-^{18}C ^{17}O-phosphate labeled samples did not reveal any significant change in the T_1 relaxation time of the 8-phenolic proton, the ^{16}A-^{17}A [^{17}O]phosphate-labeled sample (Figure 4C) showed a significant increase (20.8 to 37 ms) in T_1 relaxation time in comparison to the nonisotopically enriched sample. The sharpening of the 8-phenolic proton resonance signal and associated 78% *increase* in T_1 relaxation

[4] In addition to preparing the ^{17}O label at ^{17}A-^{18}C, we decided to evaluate ^{17}O label at ^{11}G-^{12}G and ^{16}A-^{17}A steps as possible alternative phosphates involved in stabilization of the adduct because modeled results showed the distances between the anionic oxygen in each phosphate and the A-subunit quinone-oxygen were 7.35, 4.69, and 5.46 Å in the covalently bound complex and 6.81, 7.88, and 4.26 Å in the noncovalently bound complex for ^{11}G-^{12}G, ^{16}A-^{17}A, and ^{17}A-^{18}C, respectively (unpublished results).

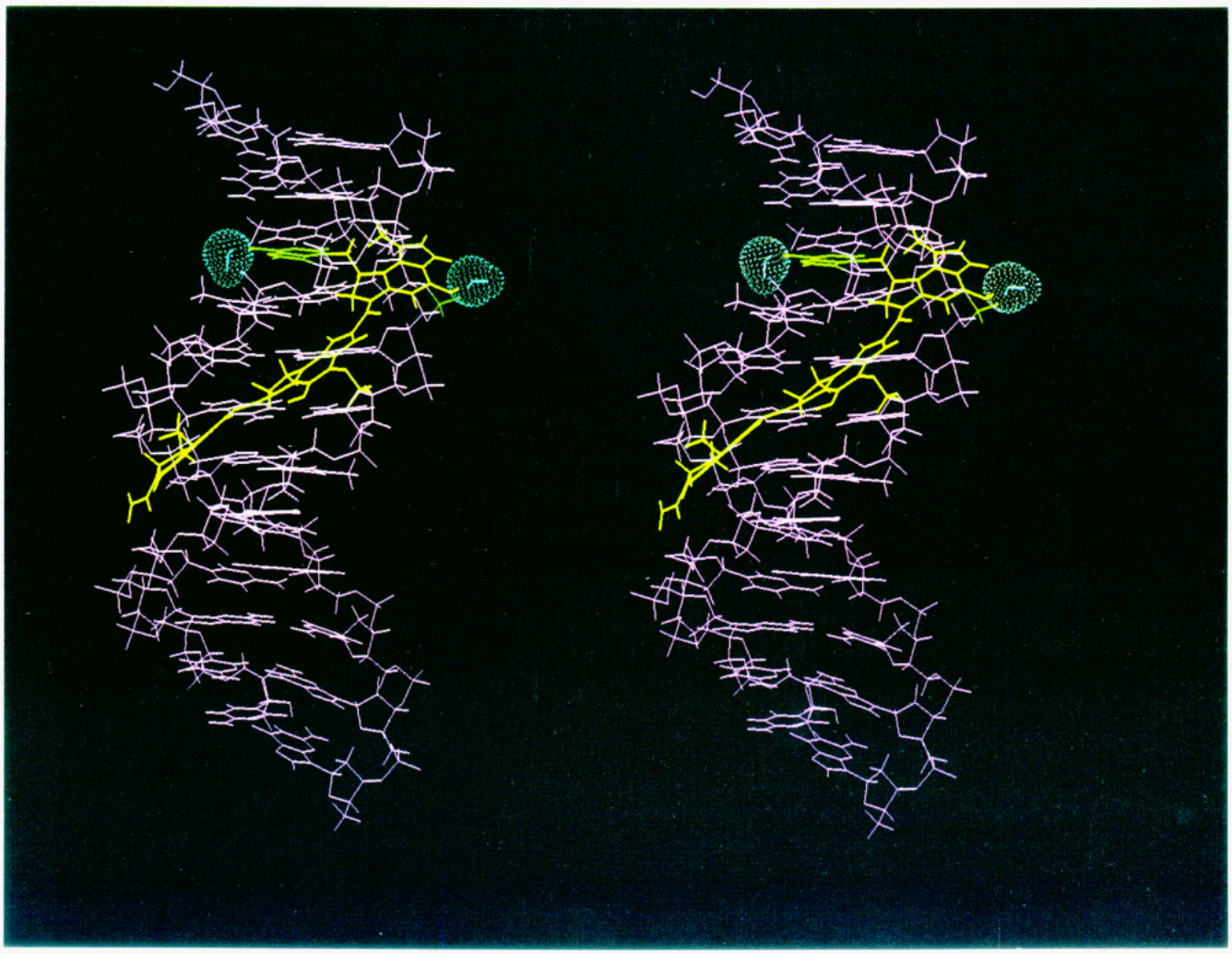


FIGURE 6: Stereoscopic diagrams of (+)-CC-1065 covalently bound to the 12-mer duplex (Chart I). The drug molecule is shown in yellow, the covalently modified adenine (A*) and the phosphate group (^{16}A-^{17}A) are shown in green, and the two bridging water molecules are shown in turquoise.

time is presumably due to the competing dipolar coupling of the ^{17}O nucleus in the phosphate for the relaxation potential of the bridging water molecule located between the anionic oxygen of the phosphate at ^{16}A-^{17}A and the 8-phenolic proton of the A-subunit of the (+)-CC-1065–DNA adduct.

On the basis of the results of this study, we are now able to further refine the proposed reaction mechanism that results in covalent modification of N3 of adenine in DNA by (+)-CC-1065 (Figure 5). The important additions are the two water molecules, one of which is demonstrated to bridge the 8-phenolic proton of (+)-CC-1065 and the phosphate between ^{16}A-^{17}A and a second that is hydrogen-bonded to ^{10}AH6$_b$ and perhaps bridges to O4 of ^{15}T, although we lack direct data that would support the O4 bridging position (Figure 6). The observation of significantly reduced T_1 relaxation times caused by quadrupole-induced relaxation of protons by specifically ^{17}O-labeled water molecules suggests these water molecules are ordered and have considerable residence times as part of the (+)-CC-1065–DNA adduct structure. To the best of our knowledge, the critical importance of ordered water molecules in relaying the catalytic activation of covalent bond formation or stabilizing the resulting DNA adducts has not previously been demonstrated. However, their importance in noncovalent complexes of drugs with DNA, such as beneril (Brown et al., 1990), and in DNA structure (Drew et al., 1981; Kopa et al., 1982) has been documented. Just as we propose here that an ordered water molecule may relay the general acid catalysis of CPI in its covalent reaction with N3 of adenine, the proposed general acid catalysis of BPDE hydrolysis by an acidic phosphate group (Gupta et al., 1987) may also involve an ordered water molecule. How general this involvement of ordered water molecules may be in providing bridging hydrogen bonds in covalent or noncovalent complexes of drug/carcinogen–DNA adducts remains to be seen. Moreover, their importance in mechanisms of molecular recognition between drug/carcinogens and DNA remains largely unrecognized. On the basis of the example described here, the use of ^{1}H NMR with ^{17}O-labeled water or phosphates may be a powerful probe for detecting such water bridging systems in complexes, as well as in catalytic processes that occur on enzymes and DNA.

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