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Determination of the Major Tautomeric Form of the Covalently Modified Adenine in the (+)-CC-1065–DNA Adduct by ^1H and ^{15}N NMR Studies†

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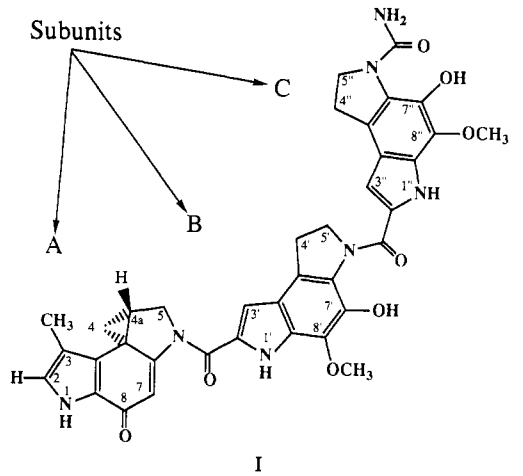
ABSTRACT: (+)-CC-1065 is an extremely potent antitumor antibiotic produced by *Streptomyces zelensis*. The potent cytotoxic effects of the drug are thought to be due to the formation of a covalent adduct with DNA through N3 of adenine. Although the covalent linkage sites between (+)-CC-1065 and DNA have been determined, the tautomeric form of the covalently modified adenine in the (+)-*CC-1065–DNA duplex adduct* was not defined. A [6-^{15}N]deoxyadenosine-labeled 12 base pair non-self-complementary oligomer, d(GGCGGAGTT*AGG)·d(CCTAACTCCGCC) (asterisk indicates ^{15}N-labeled base), containing the (+)-CC-1065 most preferred binding sequence 5′AGTTA, was synthesized and modified with (+)-CC-1065. This [6-^{15}N]deoxyadenosine-labeled 12-mer duplex adduct was then studied by ^1H and ^{15}N NMR. One-dimensional NOE difference and two-dimensional NOESY ^1H NMR experiments on the nonisotopically labeled 12-mer duplex adduct demonstrate that the 6-amino protons of the covalently modified adenine exhibit two signals at 9.19 and 9.08 ppm. Proton NMR experiments on the [6-^{15}N]deoxyadenosine-labeled 12-mer duplex adduct show that the two resonance signals for adenine H6 observed on the nonisotopically labeled duplex adduct were split into doublets by the ^{15}N nucleus with coupling constants of 91.3 Hz for non-hydrogen-bonded and 86.8 Hz for hydrogen-bonded amino protons. Parallel ^{15}N NMR experiments on the [6-^{15}N]deoxyadenosine-labeled (+)-CC-1065–12-mer duplex adduct show a triplet-like signal around −276.9 ppm and coupling constants of 91.5 and 85.6 Hz. The large downfield shift (24.05 ppm) of the ^{15}N signal of the (+)-CC-1065–12-mer duplex adduct is in accord with the formation of an extra positive charge on the exocyclic nitrogen at the N6 position and a partial double bond character between N6 and C6 atoms of the covalently modified adenine upon drug modification. We conclude that the covalently modified adenine N6 of the (+)-CC-1065–12-mer duplex adduct is predominantly in the doubly protonated form, in which calculations predict that the C6–N6 bond is shortened and the positive charge is delocalized over the entire adenine molecule.

(+)-CC-1065 (structure I) is a very potent antitumor antibiotic produced by *Streptomyces zelensis* (Hanka et al., 1978; Hurley & Rokem, 1983), which bonds covalently to double-stranded DNA through N3 of a reactive adenine, overlapping a five base pair region in the minor groove (Swenson et al., 1982; Hurley et al., 1984). An analogue of (+)-CC-1065, designed and synthesized by Upjohn scientists, was recently introduced into phase I clinical trials by J. P. McGovern (private communication, 1990). Structurally, (+)-CC-1065 consists of three repeating pyrroloindole subunits (A–C in structure I) attached via amide linkages that are approximately 15° out of plane, providing the drug molecule with a right-handed twisted banana shape (Chidester, 1981). Subunit A contains the DNA-reactive cyclopropane ring that alkylates N3 of adenine when it binds within certain reactive sequences (Figure 1) (Reynolds et al., 1985). 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, and this subunit is also essential for antitumor activity. 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 (Warpehoski & Hurley, 1988; Hurley et al., 1990). We have recently shown that in contrast to (+)-CC-1065, its synthetic enantiomer, (−)-CC-1065 has a different sequence selectivity; and this is



I

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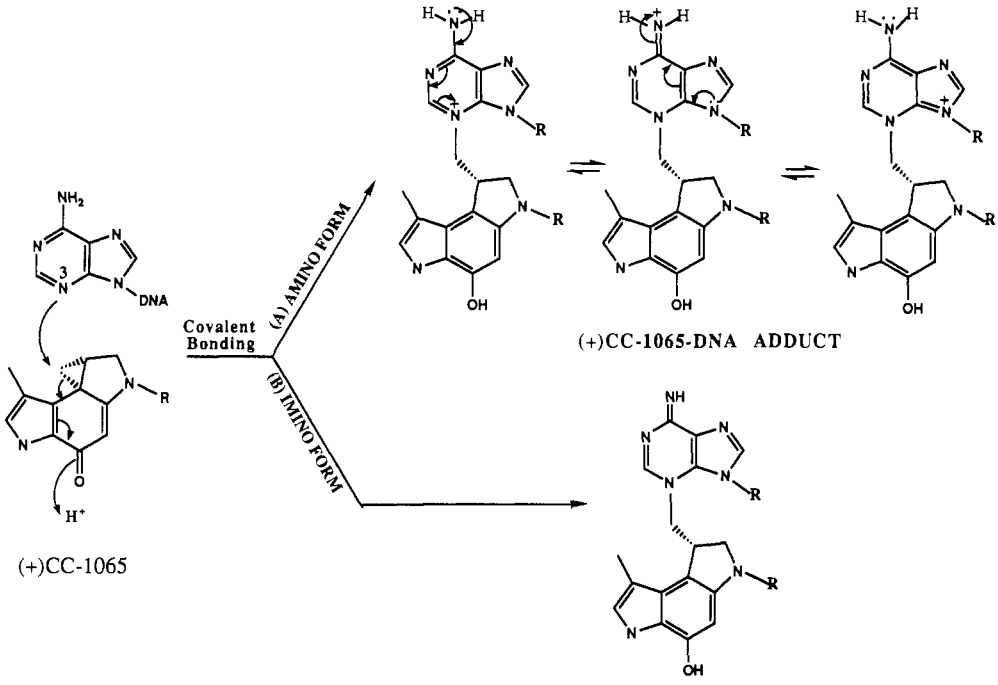


FIGURE 1: Reaction of (+)-CC-1065 with double-stranded DNA to form the (+)-CC-1065-(N3-adenine)–DNA adduct in amino form (A) and in imino form (B).

largely determined by the noncovalent binding interactions as opposed to the *covalent bonding reactivity* of (+)-CC-1065 (Hurley et al., 1990).

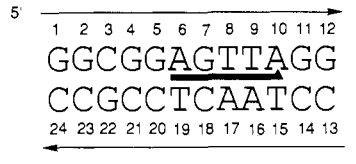
Although the covalent linkage sites between (+)-CC-1065 and DNA have been determined (Scahill et al., 1990), the tautomeric form of the covalently modified adenine was not experimentally defined. This information is critical for future molecular modeling studies and in subsequent work designed to rationalize the conformational and biochemical consequences of DNA alkylation by (+)-CC-1065. While the tautomeric form of N3-alkylated adenines such as that in *N*-(bromoacetyl)distamycin–N3-adenine (Baker & Dervan, 1989) has been determined in *isolated base adducts*, we are not aware of work that has identified the species of similar N3 adenine adducts *on DNA*. Thus this work probably has general significance to N3-alkylated adenine in DNA. A theoretical modeling study on the (+)-CC-1065–DNA adduct has been published by the French group (Zakrzewska et al., 1987) in which the positive charge was placed at N3 of the covalently modified adenine. These calculations support amino tautomeric forms of the (+)-CC-1065–DNA adduct rather than the neutral imino species we have arbitarily chosen to show in previous publications. In this paper we demonstrate using ^{1}H and ^{15}N NMR[1] that the predominant tautomeric form of the (+)-CC-1065-modified adenine in a 12-mer duplex adduct is in the N6 doubly protonated species.

Materials and Methods

Chemicals. (+)-CC-1065 was obtained from The Upjohn Co. and used without further purification. [^{15}N]Benzamide (99.5 atom % ^{15}N) was purchased from MSD Isotopes Co. Reagents used to prepare NMR buffer, sodium phosphate (99.99%), and sodium chloride (99.99%) were purchased from Aldrich Co. HPLC water and methanol were purchased from Baxter Scientific and Fisher Co., respectively. Hydroxylapatite used to purify the 12-mer duplex and the 12-mer adduct was purchased from CalBiochem. Co. Sephadex G-25 (superfine) was purchased from Pharmacia Co.

Preparation of the Nonisotopically Labeled 12-mer Duplex. The non-self-complementary d(GGCGGAGTTAGG)·d(CCTAACTCCGCC) dodecanucleotide for NMR was synthesized in house on a 10-μmol scale by use of the solid-phase cyanoethyl phosphoramidite approach on an Applied Biosystem automated DNA synthesizer, Model 381A. The oligomers were then deprotected separately with saturated ammonium hydroxide at 55 °C overnight. Solvent was evaporated at room temperature.

Preparation of the [6-^{15}N]Deoxyadenosine-Labeled 12-mer Duplex. [6-^{15}N]Deoxyadenosine (99.5% enrichment) was synthesized according to published procedures (Gao & Jones, 1987, and references cited therein). The [6-^{15}N]^{10}A-labeled 12 base pair non-self-complementary oligomer (structure II) containing the (+)-CC-1065 most preferred bonding sequence 5'AGTAA* (asterisk indicates covalently modified adenine) was synthesized on a 10-μmol scale and deprotected as described for the nonisotopically labeled oligomer.



II

Purification of 12-mer Duplex. The isotopically and nonisotopically labeled dodecamers d(GGCGGAGTTAGG) and d(CCTAACTCCGCC) were purified separately on a Macherey-Nagel nucleogen-DEAE 60-7 HPLC column with an increasing gradient from 15 mM sodium phosphate to 1 M sodium chloride and 15 mM sodium phosphate in 20% acetonitrile/water (pH 6.85). The purified single-stranded dodecamers were then desalted on four C_{18} Sep-Pak cartridges (Waters). Solvent was evaporated at room temperature. Equal amounts of salt-free single-stranded 12-mers were annealed by being heated to 65 °C for 2 h, and then the solution was allowed to cool down to room temperature slowly in 1.0 mL of NMR buffer (10 mM sodium phosphate, 100 mM

[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer.

sodium chloride, pH 6.85). The crude 12-mer duplex was applied to a hydroxylapatite column (25 cm × 3.0 cm) and eluted with a gradient from 10 to 200 mM sodium phosphate buffer (pH 6.85) at room temperature to remove the free single-stranded oligomer. The 12-mer duplex (25 mg) was then lyophilized and redissolved in 3–5 mL of HPLC-grade water and desalted on a superfine Sephadex G-25 column (50 cm × 3 cm) eluted with HPLC water. The desalted pure 12-mer duplex was lyophilized and redissolved in 0.5 mL of NMR buffer. The sample was lyophilized to dryness and redissolved in 0.5 mL of 99.996% D_2O and then transferred into a 5-mm ultrathin NMR tube for NMR studies.

Preparation and Purification of the (+)-CC-1065–12-mer Duplex Adduct. The (+)-CC-1065–12-mer duplex adduct was prepared by adding 6 mg of (+)-CC-1065 in 0.2 mL of DMF solution to 25 mg of the purified 12-mer duplex in 0.5 mL of buffer containing 20 mM sodium phosphate and 200 mM sodium chloride, pH 6.85. The reaction mixture was stirred at room temperature for 5 days in the dark, lyophilized to dryness overnight, and redissolved in 1.0 mL of HPLC-grade water. This solution was then desalted on four C_{18} Sep-Pak cartridges to remove salts and the excess undissolved drug. The desalted 12-mer duplex adduct was lyophilized, redissolved in 1.0 mL of HPLC-grade water, and applied to a hydroxylapatite column (25 cm × 3 cm) and eluted with a gradient from 10 to 150 mM sodium phosphate buffer (pH 6.85) at room temperature to remove the free drug and other impurities. The pure adduct solution was lyophilized, redissolved in 0.5 mL of NMR buffer, lyophilized, and redissolved in 0.5 mL of 90% H_2O/10% D_2O solution. This yellowish 12-mer duplex adduct solution was then transfered into a 5-mm ultrathin NMR tube for ^1H and ^{15}N NMR studies.

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 25 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 a 1–3–3–1 pulse sequence (Hore, 1983) and the delay was 120 μs. One-dimensional NOE difference experiments were performed at 23 °C.

^{15}N NMR Experiments. One-dimensional ^{15}N NMR data sets were recorded on a General Electric GN-500 FT NMR spectrometer at room temperature. Chemical shifts were referenced relative to 80% nitromethane/20% C_6D_6. Approximately 75 mg of the [6-^{15}N]deoxyadenosine-labeled (+)-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 ^{15}N NMR experiments.

Results

One-Dimensional NOE Difference Assignments of the 6-Amino Protons in the Covalently Modified Adenine of the Nonisotopically Labeled (+)-CC-1065–12-mer Duplex Adduct. A partial ^1H NMR spectrum (8.8–15 ppm, downfield region) of the nonisotopically labeled (+)-CC-1065–12-mer duplex adduct is plotted in Figure 2A. A total of 16 exchangeable protons (two signals overlap) were found in the region 10.0–14.5 ppm, of which 10 were DNA imino protons (terminal imino protons of ^1G and ^{12}G are missing at this temperature) and 6 were (+)-CC-1065 imino and phenolic protons. The imino and amino protons of the (+)-CC-1065–12-mer duplex adduct in 90% water buffer have been assigned

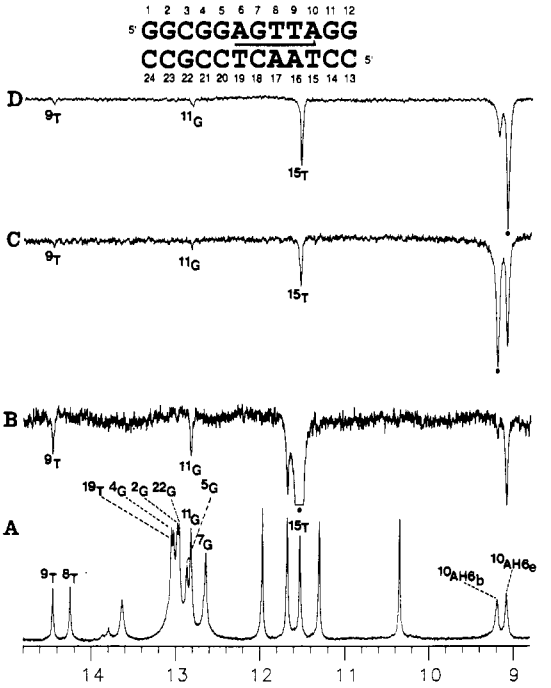


FIGURE 2: (A) 500-MHz proton NMR spectrum (8.8–15 ppm, downfield region) of the (+)-CC-1065–12-mer duplex adduct [1 equiv of (+)-CC-1065 per 12-mer duplex] in 0.5 mL of 90% H_2O/10% D_2O buffer containing 10 mM NaH_2PO_4 and 100 mM NaCl, pH 6.85 at 23 °C. One-dimensional NOE difference spectra following saturation (0.25 s) of (B) the $^{15}TH3$ imino proton of the adduct at 11.54 ppm, (C) the $^{10}AH6_b$ hydrogen-bonded amino proton at 9.19 ppm, and (D) the $^{10}AH6_e$ non-hydrogen-bonded (external) amino proton at 9.08 ppm.

by one-dimensional NOE difference (Figure 2) and two-dimensional NOESY experiments (data not shown) (Lin & Hurley, 1990). The one-dimensional NOE difference experiments leading to the assignments of the H6 amino protons on the covalently modified adenine (^{10}A) are plotted in Figure 2B–D. These experiments show that in the covalently modified adenine the H6 protons appear as two separate signals at 9.19 and 9.08 ppm, and these signals were assigned to the $^{10}AH6$ hydrogen-bonded amino proton ($H6_b$) and $^{10}AH6$ external amino proton ($H6_e$), respectively.[2,3] As expected for two protons bonded to the exocyclic nitrogen at N6, both signals show strong NOEs to the imino proton of the ^{10}A–^{15}T pair (panels C and D of Figure 2).

One-Dimensional ^1H and ^{15}N NMR Experiments on the [6-^{15}N]Deoxyadenosine-Labeled (+)-CC-1065–12-mer Duplex Adduct. The one-dimensional ^1H NMR spectrum of the (+)-CC-1065–[6-^{15}N]deoxyadenosine-labeled 12-mer duplex adduct is plotted in Figure 3B. In contrast to the nonisotopically labeled species (Figure 3A), the $H6_b$ and $H6_e$ resonance signals are split into doublets, presumably due to their coupling with the ^{15}N nucleus located at N6 (Figure 3B). This experiment confirms their assignments and reveals the expected large one-bond ^{15}N–^1H coupling constants for $H6_b$ (86.8 Hz) and $H6_e$ (91.3 Hz) (Figure 3D). These coupling constants are very close to the value (91 Hz) reported for [6-^{15}N]-deoxyadenosine (Gao & Jones, 1987). For comparison, the ^{15}N NMR spectrum of the *tert*-butyldimethylsilyl-protected

[2] In this system we found to our surprise that the $^{10}AH6_b$ proton exchanges with solvent more rapidly than the $^{10}AH6_e$ proton. This is due to a facile exchange of the $^{10}AH6_b$ proton with an *ordered and judiciously positioned hydrogen-bonded water molecule* most likely between $^{10}AH6_b$ and probably O4 of ^{15}T (Lin & Hurley, 1990).

[3] These signals normally resonate from 7 to 8 ppm.

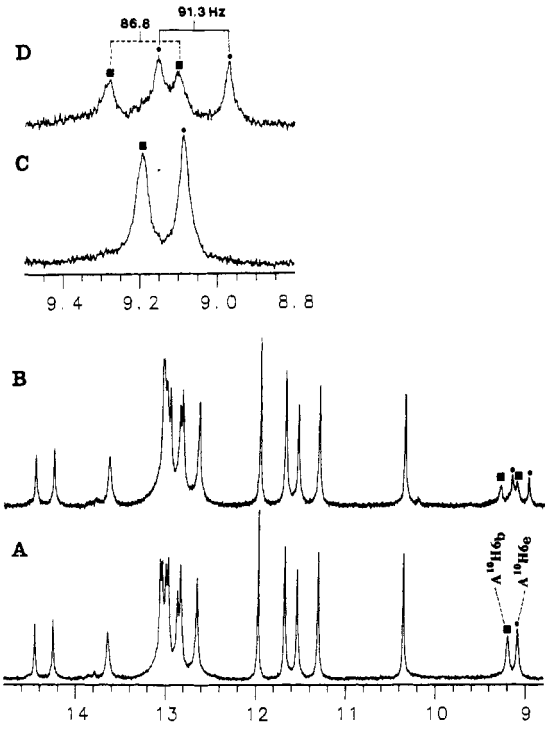


FIGURE 3: 500-MHz ^{1}H NMR spectrum (8.8–15 ppm, downfield region) of the nonisotopically labeled (+)-CC-1065–12-mer duplex adduct (A) and [6-^{15}N]deoxyadenosine-labeled (+)-CC-1065–12-mer duplex adduct (B) in 0.5 mL of 90% H_2O/10% D_2O buffer containing 10 mM NaH_2PO_4 and 100 mM NaCl, pH 6.85 at 23 °C. (C) Expanded region (8.8–9.8 ppm) of (A); (D) expanded region (8.8–9.8 ppm) of (B).

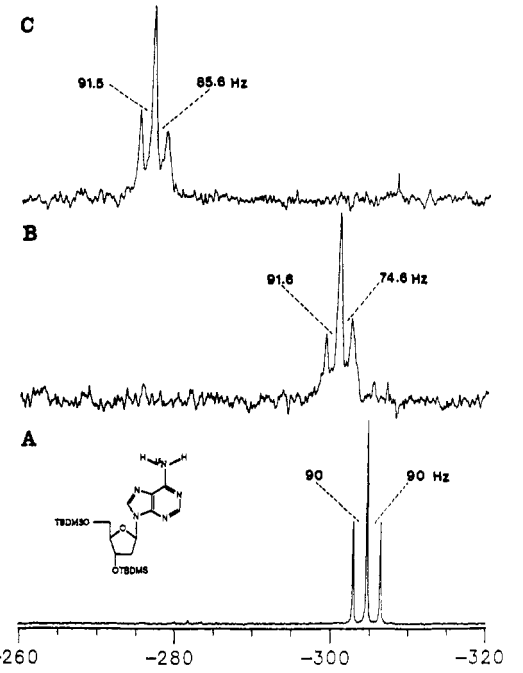
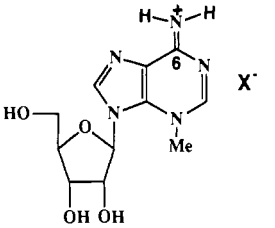


FIGURE 4: (A) 500-MH ^{15}N NMR spectrum of 3′,5′-*O*-bis(*tert*-butyldimethylsilyl)[6-^{15}N]deoxyadenosine in $CDCl_3$. 500-MHz ^{15}N NMR spectrum of [6-^{15}N]deoxyadenosine-labeled 12-mer duplex d(GGCGGAGTT*AGG)·d(CCTAACTCCGCC) (B) and [6-^{15}N]-deoxyadenosine-labeled (+)-CC-1065–12-mer duplex adduct (C) 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 23 °C. The ^{15}N chemical shifts are referenced relative to 80% nitromethane/20% C_6D_6.

[6-^{15}N]deoxyadenosine is plotted in Figure 4A. The ^{15}N NMR spectrum of the ^{15}N-labeled 12-mer duplex under the identical experimental conditions as the ^{1}H NMR spectrum is plotted in Figure 4B. This spectrum shows a triplet-like signal around −300.95 ppm with coupling constants of 91.6 and 74.6 Hz (Martin et al., 1981). This difference in coupling constants indicates that the two amino protons of ^{10}A are as expected in different chemical environments: one (74.6 Hz) is hydrogen bonded, probably to the O4 atom of thymine on the complementary strand, and the other (91.6 Hz) is extermal to the helix. The ^{15}N NMR spectrum of the [6-^{15}N]deoxyadenosine-labeled (+)-CC-1065–12-mer duplex adduct is plotted in Figure 4C. This triplet-like signal has a chemical shift of −276.9 ppm and coupling constants of 91.5 and 85.6 Hz.

Discussion

The observation of two resonance signals in the ^{1}H NMR spectrum (Figure 2A) implies that the covalently modified adenine exists in the doubly protonated form consistent with that predicted by the French group using theoretical calculation (Zakrzewska et al., 1987). In addition, the large downfield shift (24.05 ppm) of the ^{15}N signal in the (+)-CC-1065–12-mer duplex adduct can be rationalized by the formation of the extra positive charge and partial double bond character between N6 and C6 atoms on the covalently modified adenine upon drug modification. In principle, the charged nitrogen could be at the N3, N6, or N9 position (Figure 1) of the covalently modified adenine or, more likely, delocalized between two or three of these positions. A quantum mechanical calculation using AM1 (Dewar et al., 1985) on *N*3,*N*9-dimethyladenine shows that the positive charge is delocalized over the entire adenine molecule (Yuan and Hurley, unpublished results). The N6 doubly protonated nitrogen is also consistent with the computed shortened C6–N6 bond in *N*3,*N*9-dimethyladenine and with the X-ray structure of 3-methyladenosine-*p*-toluenesulfonate (structure III) that



III

also exhibits a short C6 to N6 bond and a doubly protonated nitrogen at the N6 position (Fujii et al., 1989). The smaller coupling constant between the hydrogen-bonded amino proton and 6-^{15}N atom of ^{10}A in the 12-mer duplex (74.6 Hz) relative to that in the (+)-CC-1065–12-mer duplex adduct (85.6 Hz) (Martin et al., 1981) suggests that the hydrogen-bond strength is greatly reduced in the duplex adduct. This result is consistent with the modeling studies (Seaman and Hurley, unpublished results), which show that the covalently modified base pair (^{10}A–^{15}T) is highly propeller twisted. This propeller twist is accompanied by an increase of the hydrogen-bonding distance and a corresponding reduction of the hydrogen-bond strength between $^{10}AH6_b$ and a hydrogen-bond acceptor in the (+)-CC-1065–12-mer duplex adduct.[2]

Conclusions

In this paper we have reported the first use of the combination of ^{1}H and ^{15}N NMR experiments on a [6-^{15}N]deoxyadenosine-labeled DNA to determine the predominant tautomeric form of the covalently modified adenine in the (+)-*CC-1065–DNA duplex adduct*. We conclude that the covalently modified adenine in the (+)-CC-1065–12-mer duplex adduct is in the positively charged 6-amino form, in which

calculations predict that the C6–N6 bond is shortened and the positive charge is delocalized over the entire adenine molecule.

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Articles

Stimulation of Topoisomerase II Mediated DNA Cleavage at Specific Sequence Elements by the 2-Nitroimidazole Ro 15-0216†

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ABSTRACT: The effect of the 2-nitroimidazole Ro 15-0216 upon the interaction between purified topoisomerase II and its DNA substrate was investigated. The cleavage reaction in the presence of this DNA-nonintercalative drug took place with the hallmarks of a regular topoisomerase II mediated cleavage reaction, including covalent linkage of the enzyme to the cleaved DNA. In the presence of Ro 15-0216, topoisomerase II mediated cleavage was extensively stimulated at major cleavage sites of which only one existed in the 4363 base pair pBR322 molecule. The sites stimulated by Ro 15-0216 shared a pronounced sequence homology, indicating that a specific nucleotide sequence is crucial for the action of this drug. The effect of Ro 15-0216 thus differs from that of the clinically important topoisomerase II targeted agents such as mAMSA, VM26, and VP16, which enhance enzyme-mediated cleavage at a multiple number of sites. In contrast to the previous described drugs, Ro 15-0216 did not exert any inhibitory effect on the enzyme's catalytic activity. This observation might be ascribed to the low stability of the cleavage complexes formed in the presence of Ro 15-0216 as compared to the stability of the ones formed in the presence of traditional topoisomerase II targeted drugs.

Eukaryotic topoisomerase II is an essential nuclear enzyme that is involved in central processes concerning nucleic acid metabolism, including replication (Nelson et al., 1986; Brill et al., 1987; Yang et al., 1987), transcription (Brill et al., 1987; Glikin & Blangy, 1986; Rowe et al., 1986), and chromosome segregation (DiNardo et al., 1984; Uemura & Yanagida, 1984; Holm et al., 1985). The important physiological properties of the enzyme are fundamentally embodied in its ability to catalyze a double-stranded DNA passage reaction. The catalytic process has been subject to extensive studies, and it has been elucidated that the reaction can be divided into

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