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Evidence for a Common Molecular Basis for Sequence Recognition of N3-Guanine and N3-Adenine DNA Adducts Involving the Covalent Bonding Reaction of (+)-CC-1065

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The antitumor antibiotic (+)-CC-1065 can alkylate N3 of guanine in certain sequences. A previous high-field ^{1}H NMR study on the (+)-CC-1065d[GCGCAATTG*CGC]$_2$ adduct (* indicates the drug alkylation site) showed that drug modification on N3 of guanine results in protonation of the cross-strand cytosine [Park, H.-J.; Hurley, L. H. *J. Am. Chem. Soc.* **1997**, *119*, 629.]. In this contribution we describe a further analysis of the NMR data sets together with restrained molecular dynamics. This study provides not only a solution structure of the (+)-CC-1065(N3-guanine) DNA duplex adduct but also new insight into the molecular basis for the sequence-specific interaction between (+)-CC-1065 and N3-guanine in the DNA duplex. On the basis of NOESY data, we propose that the narrow minor groove at the 7T8T step and conformational kinks at the junctions of 16C17A and 18A19T are both related to DNA bending in the drugDNA adduct. Analysis of the one-dimensional ^{1}H NMR (in H_2O) data and rMD trajectories strongly suggests that hydrogen bonding linkages between the 8-OH group of the (+)-CC-1065 A-subunit and the 9G10C phosphate via a water molecule are present. All the phenomena observed here in the (+)-CC-1065(N3-guanine) adduct at 5′-AATTG* are reminiscent of those obtained from the studies on the (+)-CC-1065(N3-adenine) adduct at 5′-AGTTA*, suggesting that (+)-CC-1065 takes advantage of the conformational flexibility of the 5′-TPu step to entrap the bent structure required for the covalent bonding reaction. This study reveals a common molecular basis for (+)-CC-1065 alkylation at both 5′-TTG* and 5′-TTA*, which involves a trapping out of sequence-dependent DNA conformational flexibility as well as sequence-dependent general acid and general base catalysis by duplex DNA.

Key words: (+)-CC-1065, N3-guanine or adenine adduct, Common molecular basis, DNA conformational flexibility, Catalysis by duplex DNA

INTRODUCTION

(+)-CC-1065 (**1**, Fig. 1) is a potent antitumor antibiotic (Hanka *et al.*, 1978; Martin *et al.*, 1981; Reynolds *et al.*, 1986) that binds with extremely high affinity to duplex DNA and predominantly alkylates N3 of adenine in the minor groove with a preference for 5′-PuNTTA* and 5′-AAAAA* sequences (Needham-VanDevanter *et al.*, 1984; Hurley *et al.*, 1984; Reynolds *et al.*, 1985). The alkylation reaction converts the cyclopropylspirocyclohexadienone structure to a *p*-hydroxyphenylethylated purine (Fig. 1B). Analogous DNA alkylation has been shown to occur with the closely related duocarmycin family of antibiotics (Takahashi *et al.*, 1988; Ichimura *et al.*, 1991; Boger *et al.*, 1991; Lin *et al.*, 1995), as well as with numerous synthetic analogues that exhibit a range of absolute and relative rates for this transformation (Hurley *et al.*, 1990; Boger *et al.*, 1995; Boger *et al.*, 1997). Rate data supports a kinetic mechanism in which the drug rapidly binds noncovalently to DNA, and, in a subsequent slower step, reacts covalently with the purine to form the stable adduct (Warpehoski *et al.*, 1995).

The substituted cyclopropylspirocyclohexadienone moiety of these analogues is remarkably inert to nucleophilic opening of the cyclopropyl ring under neutral conditions in solution, yet can react with duplex DNA in a matter of minutes. The rate acceleration of cyclopropapyrroloindole (CPI) ring-opening by DNA polymers has been estimated at 10^{10} to 10^{12} using solvolysis at pH 7 as the standard

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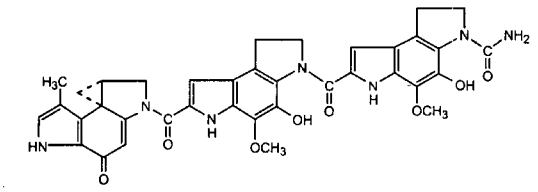
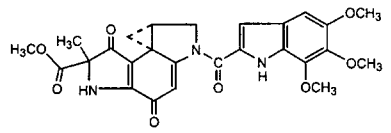
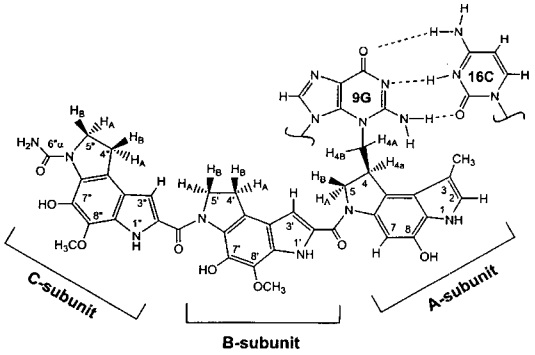


1, (+)-CC-1065



2, Duocarmycin A

(A)



(B)

Fig. 1. (A) Chemical structures of (+)-CC-1065 and (+)-duocarmycin A. (B) Chemical structure of the (+)-CC-1065(N3-guanine) adduct and protonated cross-strand cytosine in the 12-mer DNA duplex d(GCGCA ATTG*CGC)$_2$.

reaction (Warpehoski *et al.*, 1995). This is a magnitude fully comparable to rate accelerations seen in enzymatic processes. Rate or relative rate measurements made under conditions well below saturation binding clearly reflect the significant contribution made by precovalent binding affinity to the observed rates (Warpehoski *et al.*, 1995). However, precovalent binding alone cannot account for all of the rate acceleration provided by DNA. The covalent reaction itself must be catalyzed by DNA.

Detailed structural studies of the DNA N3-adenine adducts of (+)-CC-1065 and related compounds have contributed significantly to our "dynamical" (Schowen *et al.*, 1978) understanding of the transition state leading to their formation. ^{1}H NMR studies on the (+)-CC-1065DNA duplex adduct demonstrate that the alkylation of N3 of adenine results in a doubly protonated N6 and an associated positive charge that is delocalized over the covalently modified adenine (Lin *et al.*, 1990). The newly generated phenolic proton of the modified drug structure is positioned close to an anionic oxygen in the DNA ribosephosphate backbone on the nonalkylatec strand, bridged by an ordered water molecule (Lin *et al.*, 1991). As a structural consequence of N3-adenine alkylation, (+)-CC-1065 induces both DNA bending toward the minor groove and winding equivalent to about one base pair per covalent alkylation event (Lee *et al.*, 1991). These observations have strongly suggested that both sequence-dependent DNA conformational flexibility and general acid catalysis mediated by DNA contribute to transition state stabilization for the covalent reaction.

Recent studies have shown that duocarmycin A (**2**, Fig. 1A) and its synthetic analogues can also alkylate N3 of guanine in certain DNA sequences (5′-(A/T)TG*) (Mitchell *et al.*, 1993; Sugiyama *et al.*, 1993; Yamamoto *et al.*, 1993; Asai *et al.*, 1994; Sugiyama *et al.*, 1996), and the alkylation reactivity on N3-guanine is related to the electrophilicity of the alkylating subunit (Asai *et al.*, 1994). The structure of the duocarmycin A(N3-guanine) adduct has been determined by ^{1}H and ^{13}C NMR analysis of the thermal depurinated product of the duocarmycin Acalf thymus DNA adduct (Sugiyama *et al.*, 1993; Sugiyama *et al.*, 1996). It has been also reported that the duocarmycin A alkylation of N3 of guanine is induced in the presence of distamycin A, and the NMR structure of the ternary complex of [d(CAGGTG*GT)·d(ACCACCTG)](duocarmycin A/distamycin A) has been determined to explain the molecular basis of cooperative side by side binding of the two drug molecules in the minor groove of DNA (Sugiyama *et al.*, 1996).

We have observed the (+)-CC-1065 alkylation of guanine at 5′-(A/T)(A/T)TTG* and determined that the characteristics of guanine alkylation are different from those of adenine alkylation. As expected, the reaction rate for guanine alkylation by (+)-CC-1065 is much slower than that for adenine modification, but unexpectedly the resulting (+)-CC-1065-(N3-guanine) DNA adduct is more stable than the (+)-CC-1065-(N3-adenine) DNA adduct under conditions (hot 1M piperidine treatment) that favor a reverse reaction. Both of these findings suggest that there may be a fundamental structural difference between the two N3-alkylated bases. To characterize the structure of the (+)-CC-1065-(N3-guanine) DNA adduct, high-field ^{1}H NMR studies were conducted on the (+)-CC-1065d-(GCGCAA TTG*CGC)$_2$ duplex adduct (* and _ indicate alkylatec guanine and cross-strand cytosine, respectively), and the results strongly suggest that it is the cross-strand cytosine that is protonated due to the drug modification of N3-guanine (Fig. 1B) (Park *et al.*, 1997).

Herein we report the full data obtained from the ^{1}H NMR analysis coupled with rMD calculation study on the (+)-CC-1065-(N3-guanine) DNA adduct. A three-dimensional solution structure of the (+)-CC-1065-(N3-guanine) DNA duplex adduct was determined, and the molecular basis

for the sequence specificity of the interaction between (+)-CC-1065 and N3-guanine in the DNA duplex was investigated and compared with the features shown previously in the studies of the (+)-CC-1065-(N3-adenine) DNA adduct (reviewed in Hurley *et al.*, 1993; Warpehoski *et al.*, 1988; Warpehoski *et al.*, 1992). The results reveal remarkable similarities between the adenine and guanine reactions with (+)-CC-1065 in that both require sequence dependent DNA conformational flexibility and are likely to involve general acid and general base catalysis by DNA, providing sequence recognition at the covalent bonding step.

MATERIALS AND METHODS

Chemicals and enzymes

(+)-CC-1065 was a gift from The Pharmacia Upjohn Company, and reagents used for the NMR buffer, sodium phosphate (99.99%), sodium chloride (99.99%), and EDTA (99.99%), were purchased from Aldrich. HPLC water, methanol, and acetonitrile were from Fisher.

Oligonucleotide synthesis and purification for NMR studies

The self-complementary 12-mer d(GCGCAATTGCGC)$_2$ was synthesized on an Applied Biosystems Model 381A automated DNA synthesizer using solid-phase phosphoramidite chemistry. The crude oligonucleotides were deprotected with saturated ammonium hydroxide at 55°C overnight and then purified by reverse-phase HPLC on a C18 column. The purified DNA was dialyzed using Spectra/Por CE membrane (MWCO: 1000) against 2 × 2000 ml of HPLC water and evaporated to dryness. The DNA was dissolved in NMR buffer (10 mM NaH_2PO_4, 100 mM NaCl (pH 6.85), and 0.5 mM EDTA) and then examined by ^1H NMR.

Preparation and purification of the (+)-CC-106512-mer duplex adduct

Preparation of the (+)-CC-1065DNA adduct was previously described (Lee *et al.*, 1991). To remove minor side products, additional purification was conducted on a hydroxyapatite column (25 cm × 3 cm). The adduct was eluted with a gradient from 10 to 150 mM sodium phosphate buffer (pH 6.85) at room temperature and dialyzed against 1000 ml of HPLC water for 2 h at 5°C. The purified adduct solution was lyophilized, redissolved in NMR buffer, and submitted to NMR experiments.

Proton NMR experiments

One- and two-dimensional NMR experiments were recorded on a Bruker AMX 500 FT NMR spectrometer. Phase-sensitive two-dimensional NOESY spectra in D_2O were obtained (TPPI) at 27°C for 100, 250, and 350 ms, and each spectrum was collected using 1024 data points in t_2 and 512 points in t_1, with a spectral width of 5000 Hz. Two-dimensional ROESY, TOCSY, and DQF-COSY experiments were also conducted on the sample in D_2O. For the assignments of exchangeable protons, two-dimensional NOESY spectra (150, 250, and 350 ms mixing time) in H_2O buffer solution were recorded at 27°C using a 11 echo pulse sequence with 2.5 s pulse repetition time, a sweep width of 12000 Hz, and a 90° pulse width of 12 μs.

Restrained molecular dynamics

Interproton distances were determined from the measured 2D NOE intensities in D_2O-NOESY spectra using the MARDIGRAS program (Borgias *et al.*, 1989; Borgias *et al.*, 1990). MARDIGRAS was performed for the two mixing times of 100 and 250 ms and energy minimized starting structure of the B-DNA adduct to produce two sets of interproton distances. The AMBER 4.0 restraint set consisted of 380 intramolecular DNA restraints, 36 intermolecular (+)-CC-1065DNA restraints, and 6 intramolecular (+)-CC-1065 restraints in the complex. Restraints for hydrogen bonds in GC and AT base pairs, which were taken from crystallographic data, were added to maintain the WatsonCrick pairing arrangements in the adduct.

The charges for the protonated cytosine and N3-methylated guanosine ligands were calculated by semiempirical molecular orbital calculation using AMPAC (MNDO/3 Hamiltonian type) program and combined into the force-field parameters of the AMBER force-field. Parameters for (+)-CC-1065 were determined previously. The coordinates for the A- and B-DNA drug adducts were generated using SYBYL (Tripos Associates), and bad contacts in those initial models were removed by conjugate gradient minimization with the AMBER force field incorporated in SYBYL. The initial models were refined by adding the counterions and energy minimization with AMBER 4.0 and then used for *in vacuo* rMD analysis and solvated rMD calculation. All restrained molecular dynamics were performed with the SANDER module of AMBER (version 4.0) on an SGI Indigo2 XZ workstation.

For the RMSD analysis of convergent structures, four each of the A- and B-form starting structures were selected from the early period of unconstrained MD trajectories of the A- and B-form initial models, and separate *in vacuo* rMD simulations were performed on those A- and B-form starting structures. The calculations followed the temperature protocol beginning at 0 K and gradually heating up to 800 K for 65 ps. The system was equilibrated at 800 K for the next 10 ps, and then cooled down to 300 K for the

final 35 ps. The relative weights of the restraints were modulated during the course of simulation by multiplying the force constants of the parabolic regions by a scaling factor. The restraint force constants were gradually increased to a their maximum values of 30 kcal/mol·Å^2 (distance restraints), 15 kcal/mol·Å^2 (hydrogen-bonding distance restraints), and 15 kcal/mol·Å^2 (hydrogen-bonding angle restraints) at 800 K, and during the cooling period they were reduced to their final values of 20 kcal/mol·Å^2, 10 kcal/mol·Å^2, and 10 kcal/mol·Å^2, respectively. The last 30 coordinate sets of the final isothermal phase (300 K) were averaged and subjected to RMSD analysis using the CARNAL module(AMBER 4.1) (Pearlman *et al.*, 1995).

For solvated rMD, the counterions and 64 boxes of 216 Monte Carlo waters were placed around the energy-minimized B-form adduct using the AMBER EDIT module. The structure was energy-minimized until the energy gradient reached to 0.1 kcal/mol·Å^2. Belly dynamics for water only (0-300 K; 10 ps) was followed by solvated rMD. An rMD simulation was run for 100,000 × 1 fs steps (i.e., 100 ps) with periodic boundary conditions, constant pressure, and SHAKE on all bonds. The system was slowly heated up to 700 K over a period of 65 ps and equilibrated at this temperature for 10 ps. The temperature was then lowered to 300 K over the next 5 ps and maintained at 300 K to the end of the rMD simulation (20 ps). Following the temperature protocol, force constants were increased 40 kcal/mol·Å^2 (distance restraints) and 20 kcal/mol·Å^2 (hydrogen bonding restraints) at 700 K, reduced by half during cooling, and kept constant until the end of the run. After stabilization at 300 K for 20 ps, the final coordinate set was minimized without restraints to obtain the reported structure. The trajectories over the last 20 ps were used for hydrogen bonding analysis (HBOND) and measurement of distance between H2 of adenine and H1' of the 3' neighbor of the complementary residue using the CARNAL module (AMBER 4.1). The HBOND program determined the hydrogen-bonding donoracceptor pairs located within a distance of less than 4.0 Å and within an angle of less than 60°.

RESULTS

Proton NMR assignment and restrained molecular Dynamics analysis of the (+)-CC-1065 12-Mer duplex adduct

Table IA. Chemical shifts (ppm) of nonexchangeable protons in (+)CC-1065-[d(GCGCAATTGC)$_2$] adduct at 27°C

	H8	H2	H5	H6	H1'	H2'	H2"	H3'	H4'	H5'	H5"
1G	7.93 (-0.03)[a]				5.95 (-0.05)	2.56 (-0.05)	2.73 (-0.07)	5.01 (+0.15)	4.46 (+0.23)	NA[c]	NA
2C			5.33 (-0.07)	7.35 (-0.07)	5.68 (-0.08)	1.95 (-0.16)	2.31 (-0.13)	4.83 (-0.04)	4.13 (-0.06)	NA	NA
3G	7.82 (-0.05)				5.79 (-0.09)	2.31 (-0.31)	2.55 (-0.18)	4.94 (-0.05)	4.32[b]	NA	NA
4C			5.41 (+0.03)	7.35 (+0.03)	5.14 (-0.36)	2.03 (+0.11)	2.29 (-0.03)	4.81 (-0.01)	4.15 (+0.02)	NA	NA
5A	8.19 (-0.04)	7.17 (+0.02)			6.11 (+0.17)	2.79 (+0.04)	3.01 (+0.08)	5.10 (+0.05)	4.46 (+0.06)	4.14 (+0.01)	4.04
6A	8.12 (-0.03)	7.99 (+0.41)			6.16 (+0.00)	2.55 (-0.04)	2.75 (-0.17)	5.00 (+0.00)	4.47 (+0.00)	4.15 (-0.10)	4.06
7T			1.34 (+0.04)	6.93 (+0.04)	5.34 (-0.53)	1.42 (-0.53)	1.71 (-0.78)	4.30 (-0.51)	3.62 (-0.56)	3.47 (-0.85)	3.00 (-1.13)
8T			1.45 (-0.14)	7.13 (-0.14)	5.20 (-0.66)	1.70 (-0.39)	1.84 (-0.66)	4.55 (-0.33)	3.96 (-0.17)	3.61 (-0.47)	2.99
9G	7.88 (+0.04)				5.74 (-0.08)	2.69 (+0.07)	2.85 (+0.17)	4.93 (-0.05)	4.48 (+0.11)	4.05 (-0.17)	3.94 (-0.27)
10C			5.58 (+0.24)	7.23 (-0.09)	5.37 (-0.35)	1.76 (-0.21)	2.16 (-0.21)	4.58 (-0.25)	4.49 (+0.32)	NA	NA
11G	7.84 (-0.08)				5.86 (-0.10)	2.63 (+0.00)	2.72 (-0.03)	5.00 (+0.02)	4.39 (+0.05)	NA	NA
12C			5.33 (-0.31)	7.38 (+0.03)	6.15 (-0.07)	2.21 (+0.05)	2.23 (+0.05)	4.47 (-0.50)	NA	NA	NA
13G	7.91 (-0.05)				5.89 (-0.11)	2.56 (-0.05)	2.73 (-0.07)	4.83 (-0.03)	4.25 (+0.02)	3.67	3.67
14C			5.35 (-0.05)	7.40 (+0.00)	5.59 (-0.17)	2.12 (+0.01)	2.37 (-0.07)	4.83 (-0.04)	4.14	4.09	4.05
15G	7.85 (-0.02)				5.96 (+0.08)	2.52 (-0.10)	2.64 (-0.09)	4.97 (-0.02)	4.34 (-0.02)	3.95	3.95
16C			5.75 (+0.37)	7.75 (+0.47)	4.89 (-0.61)	2.25 (+0.33)	2.38 (+0.06)	4.63 (-0.19)	NA	NA	NA
17A	8.26 (+0.03)	7.30 (+0.15)			6.20 (+0.26)	2.78 (+0.03)	2.94 (+0.01)	5.06 (+0.01)	4.43	NA	NA
18A	7.99 (-0.16)	8.07 (+0.49)			5.97 (-0.19)	2.30 (-0.29)	2.72 (-0.20)	4.52 (-0.48)	4.15 (-0.32)	NA	NA
19T			1.15 (-0.15)	6.79 (-0.31)	5.34 (-0.53)	1.63 (-0.32)	2.15 (-0.34)	4.46 (-0.35)	3.80 (-0.38)	NA	NA
20T			1.43 (-0.16)	7.12 (-0.15)	5.91 (+0.05)	2.20 (+0.11)	2.34 (-0.16)	4.71 (-0.17)	3.87 (-0.26)	NA	NA
21G	7.71 (-0.03)				5.80 (-0.02)	2.42 (-0.20)	2.69 (+0.01)	4.67 (-0.31)	NA	NA	NA
22C			5.27 (-0.07)	7.29 (-0.03)	5.72 (+0.00)	2.00 (+0.03)	2.36 (-0.01)	4.89 (+0.06)	4.20 (+0.03)	NA	NA
23G	7.90 (-0.02)				5.94 (-0.02)	2.66 (+0.03)	2.75 (+0.00)	5.00 (+0.02)	4.37	NA	NA
24C			5.28 (-0.16)	7.44 (-0.15)	6.14 (-0.05)	2.21 (+0.04)	2.22 (+0.04)	4.50 (-0.47)	NA	NA	NA

[a]Chemical shift differences, δ(DNA-drug adduct) - δ(DNA), are shown in parentheses, and the chemical shift differences greater than 0.25 are uncerlined.
[b]Difference is not determined because the chemical shifts in duplex are unassigned.
[c]Not assigned due to overlap of resonance signals.

Table IB. Chemical shifts (ppm) of exchangeable DNA protons in 90%H_2O, pH6.05, at 27°C

	N3-H/N1-H	$4NH_2$
1G		
2C		8.49 (-0.02), 6.52 (-0.15)
3G	13.00 (-0.01)[a]	
4C		8.38 (+0.03), 6.28 (-0.07)
5A		
6A		
7T	13.59 (-0.06)	
8T	14.05 (+0.33)	
9G	NA[b]	
10C		8.00 (-0.35), 6.70 (-0.07)
11G	13.08 (-0.12)	
12C		
13G		
14C		8.40 (-0.11), 6.50 (-0.17)
15G	12.89 (-0.12)	
16C		10.28 (+1.93), 8.27 (+1.92)
17A		
18A		
19T	13.78 (+0.13)	
20T	12.83 (-0.89)	
21G	12.50 (-0.13)	
22C		8.28 (-0.07), 6.32 (-0.15)
23G	13.12 (-0.08)	
24C		

[a]Chemical shift differences, δ(DNA-drug adduct) - δ(DNA), and differences greater than 0.25 are underlined.
[b]Not assigned.

Table II. Proton chemical shifts (ppm) for alkylated (+)CC-1065 in the duplex adduct at 27°C.

A subunit		B subunit		C subunit	
H2[a]	7.38	HN1'	11.35	HN1"	11.35
H3Me	2.63	H3'	7.98	H3"	7.54
NH1	10.42	H4'A	3.73	H4"A	3.76
H4a	4.99	H4'B	3.33	H4"B	3.31
H4A	3.84	H5'A	5.14	H5"A	4.46
H4B	3.77	H5'B	4.96	H5"B	4.35
H5A	4.99	OH7'	12.01	OH7δ	11.08
H5B	4.95	OMe8'	3.87	OMe8"	3.87
H7	7.98	-	-	6-αNH_2	7.08
OH8	13.85	-	-	-	-

[a]For numbering of (+)-CC-1065, see Fig. 1.

The (+)-CC-1065-(N3-guanine) adduct was prepared using the self-complementary 12-mer duplex d(GCGCAA TTGCGC)$_2$. The analyses of the two-dimensional NOESY and COSY spectra of the (+)-CC-106512-mer adduct in D_2O and H_2O produced an almost complete assignment of (+)-CC-1065 and DNA protons. The ^{1}H-NMR assignments for the unmodified DNA and the drug-modified DNA along with drugDNA NOEs are given in Tables, I-III. A model for the (+)-CC-1065-(N3-guanine) adduct was obtained from rMD simulations based on 380 DNA-to-DNA NOE restraints, 36 drug-to-DNA NOE restraints, and 6 drug-to-drug NOE restraints in the complex. This model enabled the following observations to be made about the (+)-CC-1065-guanine adduct. Overall, the drug molecule

Table III. Intensities of the NOE connectivities between (+)-CC-1065 and DNA protons in the duplex adduct.

	H2	H3Me	H4a	H4A	H4B	H5A	H5B	H3'	H4'A	H4'B	H5'A	H5'B	H3"	H4"A	H4"B	H5"A	H5"B	
10CH1'		M		M	M													10CH1'
10CH4'	M																	10CH4'
9GH1'					M													9GH1'
16CH1'		M																16CH1'
17AH2			W			S	M	W										17AH2'
17AH1'		S																17AH1'
17AH2"		S																17AH2"
17AH4'		S																17AH4'
8TH1'									W									8TH1'
18AH2								W	M	W		W	W					18AH2
19TH1'												W	W	M				19TH1'
6AH2'											W	M	M	W	S	W	W	6AH2'
6AH1'															W			6AH1'
6AH2'															W			6AH2'
20TH1'														W				20TH1'
20TH2'														S				20TH2'
5AH2													W		S	M	S	5AH2
5AH2'																	S	5AH2'
5AH2"																	S	5AH2"
21GH1'														W				21GH1'
	H2	H3Me	H4a	H4A	H4B	H5A	H5B	H3'	H4'A	H4'B	H5'A	H5'B	H3"	H4"A	H4"B	H5"A	H5"B	

S = strong, M = medium, and W = weak (relative to cytosine H5 to H6 NOE intensity)

is positioned closer to the covalently modified strand; this is especially true for the A subunit, which is in close proximity to the sugar phosphate backbone between 9G and 10C. Examination of the orientation of the other subunits of the drug molecule in the minor groove revealed that the outer edge of the B subunit is closer to the nonmodified strand and that of the C subunit is in closer contact with the modified strand, in agreement with the observed NOE connectivities between DNA and the protons from the convex surface of the drug (see Table III).

(+)-CC-1065 is covalently attached to N3-Guanine (9G) in the minor groove of the 12-Mer

The location of (+)-CC-1065 in the DNA duplex adduct was determined by the NOE connectivities between protons on the concave face of the drug molecule and DNA protons in the minor groove. The intermolecular NOEs shown in Table III reveal that the drug molecule is closely associated with the minor groove of DNA, covering the regions from 5A to 10C on the covalently modified strand and from 16C to 21G on the nonmodified strand. Evidence for the alkylation of N3-guanine (9G) in the duplex comes from the two-dimensional H_2O- and D_2O-NOESY connectivities between the protons in the A subunit of (+)-CC-1065 and DNA protons of 9G, 10C, 16C, and 17A.

Proton NMR evidence supporting the protonation of the cross-strand cytosine in the N3-guanine DNA adduct was provided in a previous communication (Park *et al.*, 1997). The ^{1}H NMR data for the protonated cytosine is comparable to the NMR spectra of triplexes containing protonated cytosines (Santos *et al.*, 1989; Radhakrishnan *et al.*, 1991; Radhakrishnan *et al.*, 1993).

N3-Guanine alkylation by (+)-CC-1065 results in bending of DNA (Evidences obtained from ^{1}H NMR studies on the (+)-CC-1065(N3-guanine) DNA adduct)

For the H2 protons of 5A, 6A, and 18A in the (+)-CC-106512-mer duplex adduct, the NOE connectivities with both the 3' neighbor H1' on the same strand and the 3' neighbor of the complementary residue are observed at a mixing time of 250 ms (see Fig. 2). The H2 proton of 17A does not show any NOEs with other sugar H1' protons. In addition, after drug modification the NOEs between adenine H2s of 5A, 6A, and 18A and their own H1's have been lost. While the intrastrand cross-peaks are more intense than the interstrand cross-peaks for 5A and 6A, the interstrand cross peak (18A-H2 and 8T-H1') is stronger than the intrastrand cross-peak (18A-H2 and 19TH1') for 18A. This NOE connectivity pattern for 18A is evidence of the compression of the minor groove. A narrowing of the minor groove may result in a downfield shift of the A/T imino proton (Nadeau *et al.*, 1989). As shown in Table 1B, the imino proton of the 8T-17A base pair is shifted downfield by 0.33 ppm. The NOE pattern for 8T and the downfield shift of the 8T-17A imino proton

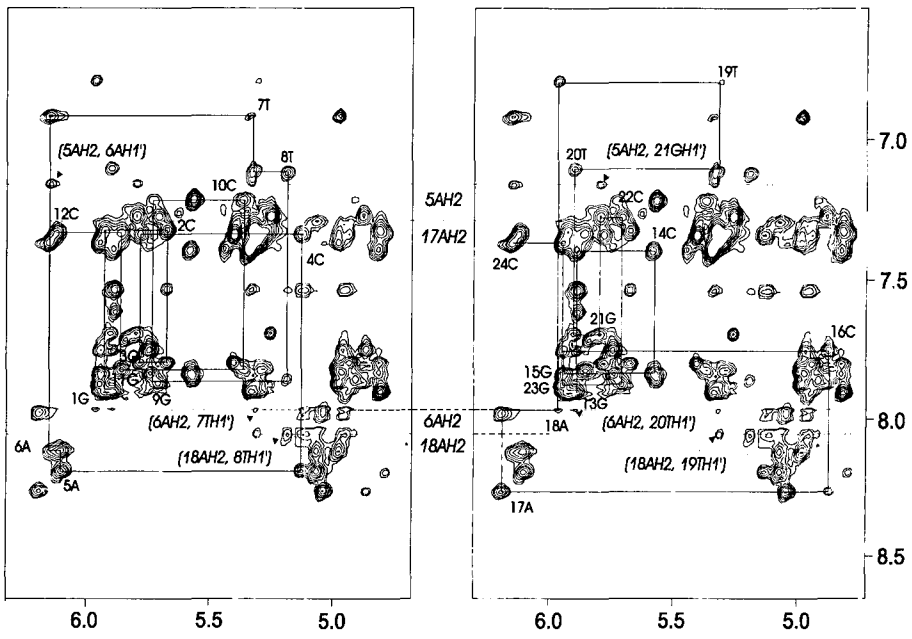


Fig. 2. Two dimensional phase-sensitive NOESY spectrum (250 ms mixing time) in D_2O solution, displaying connectivities for aromatic PuH8/PyH6 to deoxyribose H1' and to deoxyribose H1' protons of the 5'-neighbor. The NOE cross peaks between adenine H2 (dashed lines) and H1' of the 3'-neighbor on the same strand and the 3'-neighbor of the complementary residue are indicated by arrows.

suggest that (+)-CC-1065 modification narrows the width of the minor groove at the 7T to 8T step.

A notable feature revealed from the assignment of the nonexchangeable protons is that the H1' of 16C (4.89 ppm) and H6 of 19T are both shifted far upfield. The difference in H1' chemical shifts between 16C and the other residues is more than 0.25 ppm, suggesting that a structural distortion exists at the 16C17A (8T9G) dinucleotide step. This unusual upfield shift of H1' of 16C (i.e., at 5.5 ppm) was also shown in the NMR spectra of duplex DNA itself, where the difference in chemical shift was at least 0.22 ppm compared with the other residues. The upfield-shifted resonance for H1' of this residue is characteristic of a conformational discontinuity at the junction and indicates that the 16C17A dinucleotide junction may form a kink (Nadeau *et al.,* 1989; Nagaich *et al.,* 1994). A large upfield-shift of the H6 of 19T may also be related to a conformational distortion around this nucleotide (18A19T step). It is known that if a conformational aberration at the junction is adjoined with propeller-twisting of base pairs and compression of the minor groove, it induces a bending of the DNA duplex (Nadeau *et al.,* 1989; Nagaich *et al.,* 1994; Kintanar *et al.,* 1987; Katahira *et al.,* 1988). Consequently, the conformational distortion at the junctions of the 16C17A and 18A19T steps may be associated with (+)-CC-1065-induced DNA bending in the N3-guanine DNA adduct.

Furthermore, a unique NOE connectivity between the exocyclic $4NH_2$ proton of 16C and the 5-methyl protons of the cross-strand 8T was detected in the H_2O-NOESY spectra of the drug12-mer duplex adduct (150 ms mixing time), which was not observed in the spectra of the DNA duplex only. This NOE connectivity is demonstrated in the major groove view of the rMD structure shown in Figure 3A. This cross-strand strongly suggests that the width of the major groove is compressed at this step, which is consistent with the abrupt widening of the minor groove at the 8T9G step. This structural feature can be rationalized on the basis of an intrinsic property of TG steps. It is known that a TG sequence is a conformationally labile site, exhibits a large positive roll that opens the base pair step toward the minor groove, and directs the local bending toward the major groove (Nagaich *et al.,* 1994; McNamara *et al.,* 1990; Mujeeb *et al.,* 1993; Weisz *et al.,* 1994; Hassan *et al.,* 1996; Lam *et al.,* 1997; Hassan *et al.,* 1998). We propose that (+)-CC-1065 entraps this flexible step to alkylate N3 of 9G, which results in widening of the minor groove and compression of the major groove.

One of the B-form starting structures used in the RMSD studies was minimized and solvated, and the rMD simulation was conducted to further analyze the conformational properties of the drug-DNA adduct. In Fig. 3B, the

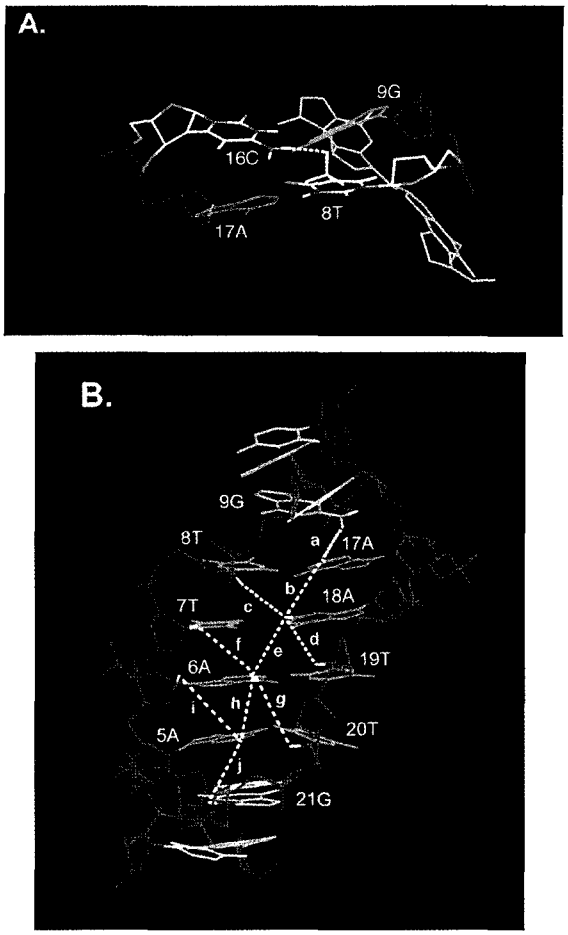


Fig. 3. (A) Expanded view of the 8T/9G (16C/17A) step from the major groove highlighting the NOE between the 4-NH_2 group of 16C and the 5-methyl group of 8T (yellow dotted line). The DNA backbone is shown in magenta and the drug is shown in cyan. (B) Internucleotide NOE connectivities of the drug-occupied region of the 12-mer duplex adduct. The NOEs are (a) 9G-2NH_2 to 17AH2, (b) 17AH2 to 18 AH2, (c) 8TH1' to 18AH2, (d) 18AH2 to 19TH1', (e) 18AH2 to 6AH2, (f) 7TH1' to 6AH2, (g) 6AH2 to 20TH1', (h) 6AH2 to 5AH2, (i) 6AH1' to 5 AH2, and (j) 5AH2 to 21GH1'. Color scheme: green (adenine), red (thymine), cyan (guanine), orange (cytosine), and magenta (backbone).

intra- and interstrand adenine H2 to deoxyribose H1' NOE connectivities are illustrated by dotted lines in the solvated rMD structure of the drugDNA complex, which was obtained after deleting the drug structure from the minor groove. The distance between the H2 proton of adenine and the H1' proton of the 3' neighbor of the cross-strand residue is a relative gauge of the minor groove width for B-like DNA in solution (Chuprina *et al.,* 1991; Kim *et al.,* 1992), and this distance is usually calculated using the volumes of NOE cross-peaks between AH2 and H1' up to mixing time of 120 ms (Chuprina *et al.,* 1991; Katahira *et al.,* 1990). In our study, measurements of the average distances between the H2 proton of adenine and the H1'

proton of the 3' neighbor for each of the cross-strand residues were conducted on the solvated rMD trajectory of the final stabilization step consisting of 200 coordinate sets (300K, 20 ps, see details in Materials and Methods) by using the CARNAL module (AMBER 4.1) (Pearlman *et al.*, 1995). The results reflect the previously described observations based on the NMR analyses. The magnitude of the average distances over the trajectory are in descending order: 9GH1'17AH2 (6.18 Å) > 6AH220TH1' (4.45 Å) ≥ 5AH221GH1' (4.44 Å) > 8TH1'18AH2 (3.94 Å). The minor groove is remarkably widened at the 8T to 9G step when compared with the corresponding distance for classical B-DNA (5.1 Å). The 5A to 6A step is also significantly widened in comparison with the corresponding distances (3.83.4 Å; calculated based on NOE intensities) in classical B-DNA, while the 7T to 8T step is partially narrowed (calculated distance, 4.2 Å) (Katahira *et al.*, 1990).

Evidence for the hydrogen bonding linkages of a triad, composed of the 8-OH of the drug, a water molecule, and the 9G10C phosphate in the (+)-CC-1065(N3-Guanine) 12-Mer duplex adduct structure

In the one-dimensional ^{1}H NMR spectra (in H_2O, 27°C) of the drug12-mer duplex adduct, two proton resonance signals were observed at 13.90 and 10.28 ppm, which can be eliminated by presaturatior of the H_2O resonance signal (Fig. 4, A and B). The signal that resonates at 10.28 ppm has already been assigned to one of the N4-amino protons of 16C by two-dimensional H_2O NOESY analysis (Park *et al.*, 1997). The 7'-OH and 7"-OH protons have relatively slow exchange rates (see atom numbers of (+)-CC-1065 in Figure 1B) and possibly exist as part of a highly ordered structure in the minor groove by being connected with the neighboring amide carbonyl oxygen and a water molecule. Consequently, these would be expected to be less affected by the suppression of the H_2O resonance. The second highly exchangeable proton resonance signal at 13.90 ppm is assigned to the phenolic proton (8-OH) in the A subunit. This proton resonance is shifted about 2 ppm downfield relative to the corresponding phenolic protons in the B and C subunits (7'-OH and 7"-OH). A similar set of assignments for the phenolic protons was made for the (+)-CC-1065 N3-adenine adduct of the 12-mer duplex, reported previously (Lin, 1991). In this case, the 8-OH proton is positioned close to an anionic oxygen in the phosphate bridged by a water molecule on the nonmodified strand, and it was proposed that this interaction may be involved in general acid catalysis of the covalent bonding reaction of (+)-CC-1065 with DNA (see later) (Lin *et al.*, 1991).

In the rMD structure of (+)-CC-1065(N3-guanine) duplex adduct (Fig. 4C), the 8-OH proton is in close contact with the anionic oxygen of the 9G10C phosphate. In contrast

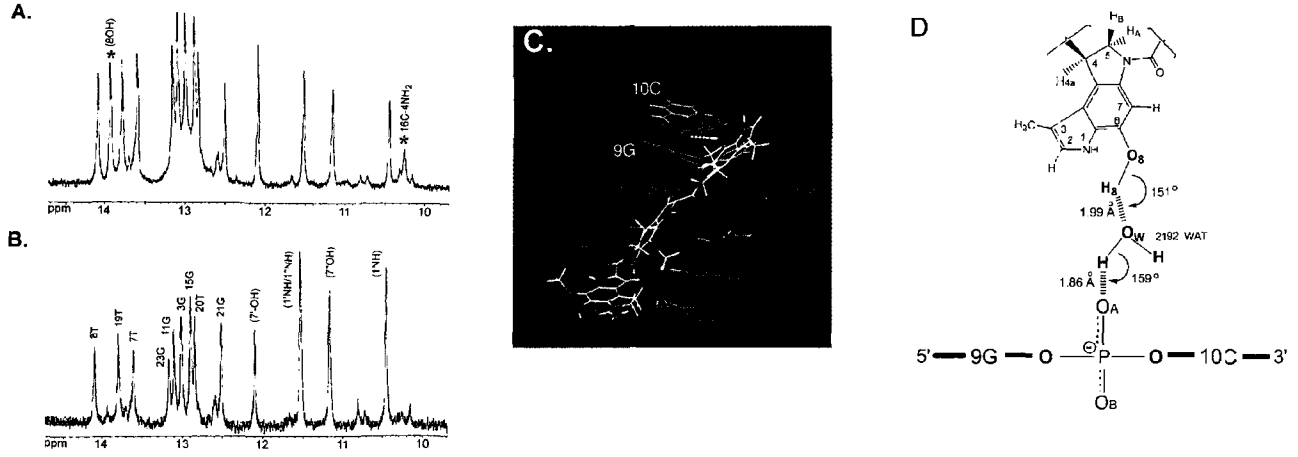


Fig. 4. Proton NMR evidence and models for the hydrogen-bonded phenolic proton via a bridging water molecule to DNA. Comparison of exchangeable one-dimensional proton NMR spectra (10.015.0 ppm) of the (+)-CC-1065[d(GCGCAATTG*CGC)$_2$] adduct in 90% H_2O:10% D_2O at 27°C obtained by two different solvent (H_2O) suppression methods. (A) The proton resonance signals (phenolic-8 OH of the drug and amino (N4) protons of 16C), which were recovered by selective excitation and detection methods (1-1 echo pulse sequence), are indicated by asterisks in spectrum A. (B) Spectrum B was obtained by the selective saturation (presaturation) method. (C) Diagram showing the relative position of the phenolic-8 OH proton of (+)-CC-1065 in the final rMD structure. The yellow dotted line indicates the proximal contact between phenolic OH (8-OH) of the (+)-CC-1065 A-subunit and anionic oxygen of the 9G-10C phosphate. (D) Diagram showing the predicted hydrogen-bonding linkages of the 8-OH group, the bridging water molecule, and the 9G-10C phosphate based upon the results of the hydrogen-bonding analysis using the HBOND option of the CARNAL module (AMBER 4.1). The distances (hydrogenacceptor) and angles (donorhydrogenacceptor) shown are the values that were averaged over the final 10 ps rMD trajectory. The residue name (2192 WAT) and atom names are from the AMBER PDB file used for the rMD studies.

to the 7'-OH and 7"-OH protons, the 8-OH proton does not show any NOE connectivities (results not shown) with DNA protons due to its rapid exchange. If a bridged water molecule between the phosphate oxygen and the 8-OH proton is present, that water molecule is more exposed to the solvent than other water molecules hydrated in the minor groove, and this may result in faster exchange of the 8-OH proton. To examine the proposed existence of a bridged water molecule between the 8-OH proton and phosphate, statistical hydrogen bonding analysis was performed on the solvated rMD trajectory of the final stabilization step consisting of a 100 coordinate set (300K, 10 ps, see details in Experimental Methods) using the HBOND option of the CARNAL module (AMBER 4.1) (Pearlman *et al.*, 1995). In one example, the A subunit of (+)-CC-1065 was first screened against 3024 water molecules in the system. As shown in Fig. 4D, the phenolic oxygen (O_8) of 8-OH is in the range of hydrogen bonding with the oxygen (O_W) of a water molecule (residue number 2192). The average $H_8\cdots O_w$ distance is 1.99 Å, the average $O_8H_8\cdots O_W$ angle is 151°, and the occurrence rate is 70% over the trajectory. Second, the phosphate residue between the 9G10C step was also screened against water molecules. At the occurrence rate of 97%, the oxygen (O_W) of the same water molecule (#2192) is consistently located in an average $H\cdots O_A$ distance of 1.86 Å and an average $O_WH\cdots O_A$ angle of 159° from one (O_A) of the anionic oxygens linked to the 9G10C phosphate. In addition, a direct hydrogen bond between the 8-OH proton and the phosphate anion was examined, but the possibility of such a bond was relatively very low (24%, 2.43 Å, 141°) over the trajectory. The combined NMR and rMD results strongly suggest that hydrogen bonding linkages between the 8-OH proton and the 9G10C phosphate via a water molecule are present in the N3-guanine adduct of (+)-CC-1065.

The differences in the conformation of the drug molecule between the N3-guanine adduct and the N3-adenine adduct are represented by the positioning of the 8-OH proton. For the adenine adduct, the A subunit of the drug has a partial face-on conformation and lies snugly in the center the minor groove, allowing the 8-OH proton to reach the phosphate backbone of the nonmodified strand (Lin, 1991). For the guanine adduct (Fig. 4C), the A subunit is positioned approximately parallel to the phosphate backbone of the modified strand, and the phenolic proton (8-OH) is positioned close to the anionic oxygen of the 9G10C phosphate.

DISCUSSION

The (+)-CC-1065N3-guanine adduct, base-paired with protonated cytosine, is itself a remarkable structure. *Ab initio* calculations suggest that cytosine is more easily protonated than guanine in double-stranded DNA, and protonation occurs mainly at N3 of cytosine (Colson *et al.*, 1992; Colominas *et al.*, 1996; Hutter *et al.*, 1996). The refined model of the (+)-CC-1065N3-guanine adduct involving the neutral drug-modified guanine and the N3-protonated cross-strand cytosine provides a simple snapshot of the model for the tautomerism and protonation of the GC base pair, which is obtained by theoretical calculation methods.

Beyond the novelty of the static structure, however, cross-strand protonation in the adduct forces consideration of *general base catalysis* of N3-guanine alkylation by the base-paired cytosine. The comparative rates of (+)-CC-1065 alkylation of the oligomers, reveals that the cross-strand cytosine plays a *kinetic* role as well in the reaction with guanine, consistent with its action as a general base. The inhibition of guanine alkylation observed when cross-strand cytosine is replaced with thymine (the results not shown) is precisely analogous to the behavior found in enzyme reactions upon mutation of, for instance, a critical active site histidine to an amino acid lacking this catalytic capability. Our NMR data show that the N3 alkylated adenine nucleobase in DNA is itself a sufficiently strong base to retain a proton upon nucleophilic reaction with (+)-CC-1065. In essence adenine appears to serve the function of both nucleophile and of general base. Thus the adenine alkylation is relatively indifferent to the "mutation" of the opposite strand nucleobase (the results not shown).

The sufficient basicity of adenine may, indeed, contribute to its faster alkylation relative to guanine, independently of its more favorable precovalent binding affinity. In contrast to the adenine reaction, the guanine alkylation would have an additional entropy cost, requiring alignment of the cross-strand cytosine as the general base. This same entropy cost, of course, would have to be exacted in the reverse reaction as well (Warpehoski *et al.*, 1992). This expectation is consistent with our observation that the (+)-CC-1065-N3-guanine adduct is significantly more stable than the corresponding adenine adduct under conditions (hot 1 M piperidine) that promote the reverse alkylation reaction (Lee *et al.*, 1993).

The involvement of general base catalysis by DNA in its alkylation with (+)-CC-1065 and analogues illuminates earlier spectroscopic experiments carried out with DNA polymers containing hypoxanthine (des-aminoguanine). Poly (dI-dC)-poly(dI-dC) (lacking the amino group which, in guanine-containing polymers, inhibits minor groove binding) showed strong noncovalent binding to (+)-CC-1065. However, this polymer underwent alkylation (followed spectrophotometrically) only slowly, relative to adenine-containing polymers. Under the experimental conditions, alkylation

occurred over about nine days. The analogous polymer with 5-bromocytosine, in contrast, formed only the strong noncovalent complex, failing to show any evidence of alkylation by (+)-CC-1065, even after several months. Krueger had also noted that replacement of uracil in poly(dA-dU)-poly(dA-dU) with 5-bromouracil did *not* significantly affect the rapid alkylation of adenine in those polymers by (+)-CC-1065 (Krueger *et al.*, 1987). We infer from Krueger's data that cross-strand cytosine must act a a general base in catalyzing the slow N3-hypoxanthine alkylation.

The evidence for general base catalysis of N3-purine alkylation by duplex DNA complements evidence for general acid catalysis by DNA. The defining chemical reactivity of the cyclopropylspirocyclohexadienone system in solution is its susceptibility to acid-catalyzed nucleophilic opening of the cyclopropyl ring (Baird *et al.*, 1963). The substituted ring systems of (+)-CC-1065, the duocarmycins, and their analogues are considerably more stable, but also show strongly acid-dependent cyclopropyl ring-opening reactivity in solution (Warpehoski *et al.*, 1988; Boger *et al.*, 1996; Boger *et al.*, 1997). This susceptibility initially prompted the suggestion of general acid catalysis of the alkylation reaction by DNA itself (Warpehoski *et al.*, 1988; Boger *et al.*, 1996; Boger *et al.*, 1997).

We have previously used NMR structural techniques to pinpoint an ordered water molecule interacting with the C-8 phenolic group of the *p*-hydroxyphenylethyl moiety in the (+)-CC-1065-N3-adenine adduct (Lin *et al.*, 1991). The water molecule bridges this end of the modified drug and a specific phosphate two base-pairs down from the modified adenine in the opposite strand. We have suggested that this adduct structure provides persuasive evidence for general acid catalysis of the alkylation reaction by DNA (Lin *et al.*, 1991). While the solvolysis of (+)-CC-1065 analogues has been shown to be specific acid catalyzed (Warpehoski *et al.*, 1994), the DNA reaction is almost certainly a general acid-catalyzed process. Although the hydronium ion concentration deep in the grooves of the DNA polyelectrolyte is calculated to be significantly greater than in bulk solvent (Lamm *et al.*, 1990), counterions associated with the highly negatively charged ribose-phosphate backbone at neutral pH, are the most probable Lewis acid catalysts at the receiving end of the electron flow. The interposition of a water molecule, which most likely provides the phenolic proton, is itself a common feature in enzymatic catalysis involving general acids and bases (Jencks, 1987).

In the (+)-CC-1065-N3-guanine adduct structure the alkylating subunit occupies a significantly altered orientation within the minor groove, relative to that of the N3-adenine adduct. Nevertheless, the C-8 phenolic group shows an analogous, ordered water-mediated positioning with respect to the DNA ribose-phosphate backbone. In this case, the G9-C10 linking phosphate on the alkylated strand, rather than a phosphate on the opposite strand, orients the product phenol. It is interesting that both adduct structures suggest a general acid-general base alignment in which one of these potential catalytic centers is located on the opposite strand, ensuring that reaction will occur most efficiently in duplex DNA. This alignment is also consistent with observations that the alkylation reversal reactions require intact, double stranded DNA (Lee *et al.*, 1993; Warpehoski *et al.*, 1992; Asai *et al.*, 1994).

The structure of the (+)-CC-1065N3-guanine adduct, with orientation of the C-8 phenolic group near the charged phosphate, and protonation of cross-strand cytosine, paints a persuasive picture of combined general acid and general base catalysis as components of transition state stabilization in the alkylation of DNA. Simultaneous general acid and general base catalysis at the active site, operating at different points on the substrate structure, has long been regarded as a classic and powerful dynamic mechanism of enzyme action (Jencks, 1987).

While these mechanisms are likely to play a large role in the rate acceleration of cyclopropyl ring opening within duplex DNA, the relative ubiquity of adenines and phosphate diesters in DNA suggests that further constraints are needed to explain the sequence selectivity of the alkylation reaction. Our earlier NMR structural studies, and experimental demonstration of DNA bending, suggested that DNA conformational flexibility, which is highly sequence-dependent, contributes significantly to transition state stabilization, and thus alkylation, at these sequences.

The concept was established in the form of a "truncated junction model" obtained from NMR studies on the (+)-CC-1065-(N3-adenine) adduct 12-mer duplex $d(\overset{1}{G}\overset{2}{G}\overset{3}{C}\overset{4}{G}\overset{5}{G}\overset{6}{A}\overset{7}{G}\overset{8}{T}\overset{9}{T}\overset{10}{A}*\overset{11}{G}\overset{12}{G})d(\overset{13}{C}\overset{14}{C}\overset{15}{T}\overset{16}{A}\overset{17}{A}\overset{18}{C}\overset{19}{T}\overset{20}{C}\overset{21}{C}\overset{22}{G}\overset{23}{C}\overset{24}{C})$ combined with gel electrophoretic analysis (Lin *et al.*, 1991; Sun *et al.*, 1993). This model suggests that the bent DNA structure formed as a consequence of the local conformational flexibility of the 9T10A and 18C19T steps (Lin *et al.*, 1992) is entrapped by (+)-CC-1065, and the existing kinks at those steps are induced (or exaggerated) after drug modification at 10A, resulting in a final bent (+)-CC-1065-DNA adduct structure.

The global DNA conformational change induced by N3-guanine alkylation is similar to that produced by N3-adenine alkylation. In the gel electrophoretic mobility analysis, both (+)-CC-1065DNA adducts at 5'-AATTG* and 5'-AATTA* induce DNA bending and winding (unpublished results). The high-field proton NMR analysis also revealed that the detailed structural features of the N3-guanine adduct at 5'-AATTG*, and that of the N3-adenine adduct at 5'-AGTTA* are similar. The minor groove width at the TT step (immediately before the drug-modified base) is

compressed and then widened on both sides, and there is also a kink at the 5'-TG (or 5'-TA*) step that is induced after drug modification. The present results suggest that similar molecular mechanisms may be involved in the (+)-CC-1065 alkylation of the 5'-TTG* and 5'-TTA* steps.

A further examination of the role of the 5'-AATTG sequence used in our study provides some important additional insights. It is known that 5'-AATT is a strong noncovalent binding site for (+)-CC-1065 (Krueger *et al.*, 1985), and that the TG motif is the most flexible dimer with low stacking enthalpy and is associated with a number of protein DNA binding sites, such as CAP and HMG domain proteins (Barber *et al.*, 1990; Schultz *et al.*, 1991; Churchill *et al.*, 1995). It has been proposed that the TG base step provides an adjustable hinge for tight protein binding (Barber *et al.*, 1990; Schultz *et al.*, 1991; Churchill *et al.*, 1995). Sites such as 5'-ATTTG* and 5'-CATATG* are also the guanine alkylation sites for (+)-CC-1065, which indicates that the 5'-TG step is an important determinant for N3-guanine alkylation of (+)-CC-1065. This study presents the case in which a small ligand molecule forms a covalent adduct in the minor groove with a flexible TG dinucleotide step. In addition to TG, the TA dimer is also found frequently in deformable regions of DNA and plays a major role in DNA bending and kinking. As mentioned before, 5'-PuNTTA* is one of the consensus sequences for (+)-CC-1065 alkylation of adenine, and this suggests that a similar molecular mechanism may be involved in the alkylation of (+)-CC-1065 at both the 5'-TA* and 5'-TG* steps. An illustration of this is that at the 5'-CATATG* guanine alkylation site, which contains both TA and TG sequences, adenine alkylation is three times greater at the 5'-TA* site than at the 5'-TG* site (a similar result was also obtained for duocarmycin A) (Sugiyama *et al.*, 1993). In each case, alkylation by (+)-CC-1065 takes advantage of the conformational flexibility of the 5'-TPu step to entrap a kink at the 5'-TPu junction. Last, we have demonstrated a parallel mechanism for recognition of the AGTTA* sequences found in the 21-bp repeat region of the early promoter region of SV40 DNA by (+)-CC-1065 and both Sp1 and T-antigen (Han *et al.*, 1996). In both cases, protein binding results in trapping out in-phase bent DNA, which results in looping structures that are proposed to be important in transcriptional activation (Sp1) and the origin of replication (T-antigen) (Sun *et al.*, 1994; Han *et al.*, 1996).

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