Covalent Modification of N3 of Guanine by (+)-CC-1065 Results in Protonation of the Cross-Strand Cytosine

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(+)-CC-1065 and duocarmycin A are structurally related antitumor antibiotics that most frequently alkylate N3 of adenine in the minor groove of duplex DNA, a reaction that results in a doubly protonated N6 of adenine and an associated positive charge that is delocalized over the covalently modified adenine.[1] Recent studies have shown that duocarmycin A[2a] and (+)-CC-1065[2b] also alkylate N3 of guanine in select DNA sequences. The structure of the duocarmycin A−N3-guanine adduct resulting from depurination of the duocarmycin A−calf thymus DNA adduct was determined by NMR analysis.[3]

The reaction rate for guanine alkylation by (+)-CC-1065 is much slower than that for adenine modification,[2b] and the resulting (+)-CC-1065−N3-guanine DNA adduct is more stable than the (+)-CC-1065−N3-adenine DNA adduct under conditions (hot 1 M piperidine treatment[4]) that favor an inverse reaction. Both of these findings suggest that there may be a fundamental structural difference between the two N3-alkylated bases, and it was these observations that inspired the structural characterization of the (+)-CC-1065−N3-guanine DNA adduct. The (+)-CC-1065−N3-guanine 12mer DNA duplex adduct (Figure 1) was prepared,[5] and high-field ^{1}H NMR studies were conducted on this product.[6] Surprisingly, a comparison of the ^{1}H NMR chemical shifts of the H8 proton of 9G between the free DNA duplex and the drug−DNA duplex adduct revealed that the chemical shift was not significantly changed upon drug modification. In contrast, the H5 and H6 protons of 16C, which is base-paired to the (+)-CC-1065-alkylated guanine, show large downfield chemical shifts (0.4 and 0.5 ppm) and the imino proton of the alkylated 9G base is absent (unpublished results). Furthermore, an examination of the cytosine amino proton region (Figure 2) shows that while the amino protons of 2C, 4C, 10C, 14C, and 22C were found in the normal range (6.0−8.5 ppm), the amino protons of 16C were found at 8.27 and 10.28 ppm. The large downfield chemical shifts of H5 and H6 and the amino protons of 16C strongly suggest that the cross-strand cytosine (16C) is in the protonated state after drug modification. This is in sharp contrast to the (+)-CC-1065−N3-adenine DNA adduct, in which the resulting positive charge is delocalized over the drug-modified adenine.[1]

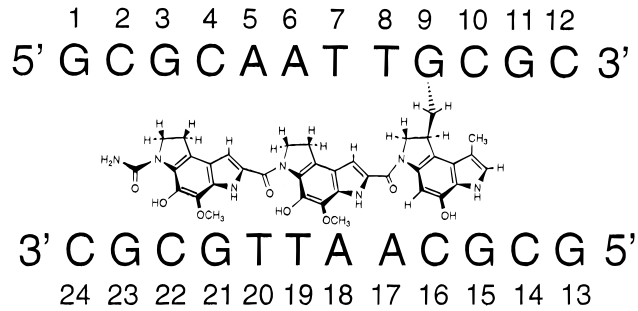

Figure 1. The 12mer DNA duplex sequences showing the site of covalent modification by (+)-CC-1065.

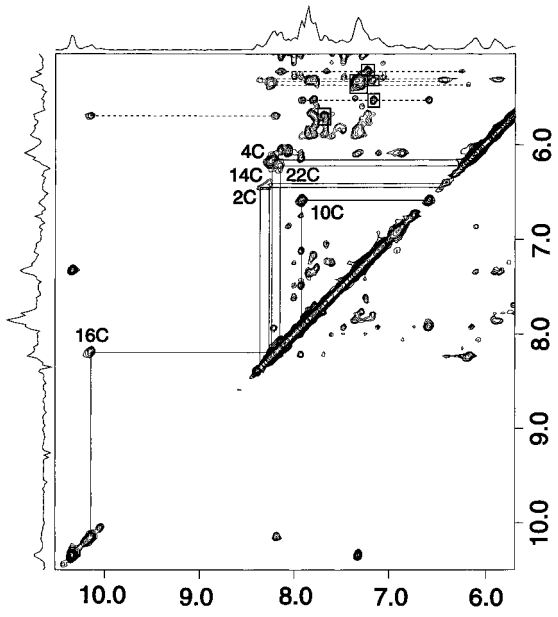

Figure 2. The 5.0−10.5 ppm region of the expanded NOESY contour plot (250 ms mixing time) of the (+)-CC-1065−[d(GCGCAATTG*CGC)$_2$] adduct (an asterisk indicates the covalent modification site) in H_2O, pH 6.05, at 27 °C. The NOEs between the geminal amino protons of cytosine are indicated. Each of these amino protons exhibits NOEs to its linked cytosine H5 protons (dotted lines), resonating between 5.2 and 5.8 ppm. NOE crosspeaks between the H5 and H6 protons of each cytosine are designated by solid boxes. The two-dimensional NOESY experiment was recorded using the 1−1 echo read pulse sequences[7] with a 2.5 s pulse repetition time and a sweep width of 24.396 ppm. For FID apodization, the 60° phase-shifted skewed sine-bell window function (skew factor 0.7) was used in both t_2 and t_1. Polynomial base line corrections were applied to ω_2, and the processed matrix size was 1025 × 1024 in real points.

To further confirm the presence of a protonated cytosine in the (+)-CC-1065−N3-guanine DNA adduct, the temperature dependency of the exchangeable protons (10−15 ppm) between 5 and 27 °C was determined (Figure 3). A resonance signal at 10.28 ppm was observed, which could be eliminated by presaturation of the H_2O resonance (shown in Supporting Information), and at 5 and 15 °C a single broad signal at 14.6 ppm appeared. Significantly, proton resonance signals with similar chemical shifts and fast exchange rates have also been observed in the NMR spectra of DNA triplexes containing

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(5) The (+)-CC-1065 adduct was prepared by adding 2.6 mg of (+)-CC-1065 in 0.1 mL of DMF solution to 10 mg of purified 12mer duplex DNA in 0.4 mL of buffer containing 10 mM sodium phosphate and 100 mM sodium chloride (pH 6.85). The reaction mixture was stirred for 5 days in the dark and centrifuged to spin down unreacted drug molecules, and the supernatant was separated and lyophilized several times to dryness. The sample was redissolved in 90% H_2O/10% D_2O (or D_2O only) and submitted to NMR experiments. One- and two-dimensional NMR data sets in H_2O and D_2O solution were recorded on a Bruker AMX 500 FT NMR spectrometer.

(6) Confirmation of complete alkylation at 9G, and therefore absence of noncovalently bound drug, was made by a heat-induced strand breakage assay. Tables of the complete ^{1}H NMR assignments for the (+)-CC-1065−12mer adduct are provided in Supporting Information. Two-dimensional NOESY experiments showed the expected NOE connectivities between protons in the alkylation unit of (+)-CC-1065 and 9G-2NH$_2$, 9G-H1′, and 10C-H1′ protons on the duplex DNA (unpublished results).

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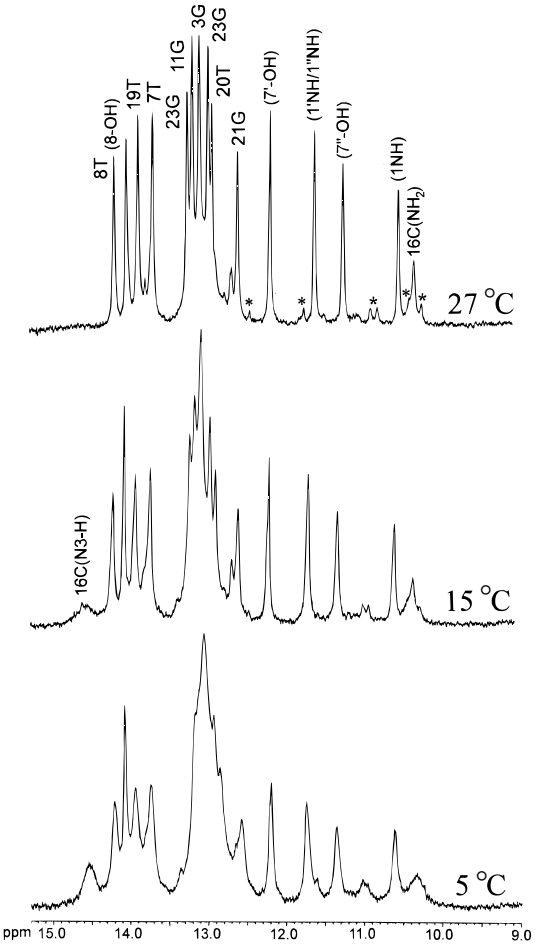

Figure 3. Exchangeable proton NMR spectra (10.0−15.0 ppm) of the (+)-CC-1065−[d(GCGCAATTG*CGC)$_2$] adduct in H_2O, pH 6.05, between 5 and 27 °C. The imino proton assignments are shown above the spectra, and the imino and phenolic-OH protons listed in parentheses are from the drug molecule. Also shown are the signals for the imino (N3) and amino (N4) protons of 16C (an asterisk indicates unassigned signals from minor side products).

protonated cytosines, and these signals were assigned to the N4 amino proton and N3 imino proton of the protonated cytosines.[8] Similar assignments are made here for the cross-strand cytosine of the (+)-CC-1065−N3-guanine DNA adduct.

The absence of the 9G imino proton, together with the protonation of 16C due to (+)-CC-1065 modification of guanine in DNA, suggests a novel mechanism for drug alkylation of N3 of guanine. It is known that tautomeric enol and imino forms of bases, which can lead to substitution mutations, occur only rarely.[9] For example, guanine in its enol form (G* in Figure 4) can be base-paired with thymine, or imino cytosine (C* in Figure 4) with adenine. Recently, the energetic provisions of the G−C tautomeric mechanism involving the concerted transfer of two protons in the interbase hydrogen bonds in DNA have been reported.[10] As shown in Figure 4 (pathway a), the most energetically favorable pathway leading to the enol-G−imino-C tautomer is via an ion pair intermediate, a product of the imino (N1) proton transfer from guanine to cytosine. The

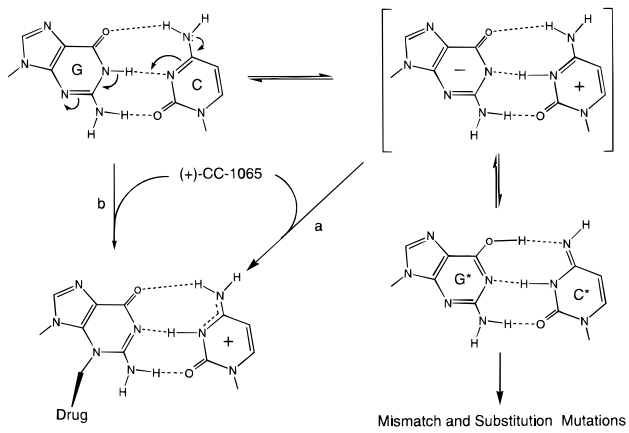

Figure 4. Proposed mechanisms for the alkylation of N3 of guanine involving either the tautomerization of the G−C base pair to give the enol-G−imino-C tautomer (pathway a) or a concerted mechanism (pathway b).

(+)-CC-1065−N3-guanine DNA adduct (9G) and the associated protonated cross-strand cytosine (C16) species demonstrated here support the transient existence of a G^-−C^+ ion pair complex, and it is possible that (+)-CC-1065 alkylation traps out this anionic state of guanine. However, alternative mechanisms, such as that involving a concerted attack at N3 of guanine with the transfer of the imino (N1) proton of guanine to N3 of the cross-strand cytosine (pathway b in Figure 4), are also possible.

The chemical properties of the (+)-CC-1065−N3-guanine DNA adduct determined in this study rationalize the enhanced chemical stability of the guanine DNA adduct in comparison to the adenine DNA adduct, and the cross-strand protonation of cytosine suggests biological consequences. First, since the alkylated guanine is a *neutral* species, the (+)-CC-1065−N3-guanine DNA adduct is, as expected, chemically stable under the inverse reaction conditions in which (+)-CC-1065−N3-adenine linkage is unstable. Since protonated cytosines are deaminated to uracil with a much higher rate than the unprotonated cytosines, a (+)-CC-1065−N3-guanine DNA adduct that results in a cross-strand protonated cytosine may, with increased frequency, produce a C → U transition mutation on the opposite strand.[11] This may contribute to the mutagenic and carcinogenic potential of drugs that alkylate N3 of guanine in DNA. Indeed, in a mutagenicity study with (+)-CC-1065,[12] G:C to A:T transitions were observed as minor mutagenic events. However, since duocarmycin A and its synthetic analogs produce higher levels of N3 guanine alkylation,[2a] these compounds may represent a higher mutagenic risk than (+)-CC-1065.

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Supporting Information Available: Expanded plots (10−15 ppm) of 1D spectra of the duplex−drug adduct at 27 °C, pH 6.05, in H_2O buffer and tables of nonexchangeable and exchangeable proton chemical shifts of the (+)-CC-1065−12mer adduct (5 pages). See any current masthead page for ordering and Internet access instructions.

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