Enediyne C1027 Induces the Formation of Novel Covalent DNA Interstrand Cross-Links and Monoadducts

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Enediyne antitumor antibiotics are believed to kill cancer cells by damaging cellular DNA.[1] These agents undergo activation by thiols, reducing compounds, or a spontaneous process to form diradical species that abstract hydrogen atoms from minor-groove-accessible carbons of deoxyribose. In the presence of O_2, the carbon-centered radicals on deoxyribose are further oxidized to form strand breaks and abasic sites. We now show for the first time that when O_2 is depleted from the reaction, the enediyne antibiotic C1027[2] produces sequence-specific covalent DNA drug adducts and DNA interstrand cross-links mediated by the drug. Interstrand cross-links may be especially important in C1027-induced cytotoxicity in central regions of large tumors, where relative anaerobic conditions prevail.[3] Further, unlike alkylating drugs, C1027-induced adducts and cross-links probably involve the deoxyribose moieties of the complementary DNA strands.

After binding to DNA by intercalation,[2f] the enediyne core of C1027 chromophore directly rearranges to a 3,6-diradical form (**2**) (Figure 1). At the target sequence GTTA1T/ATA2A3C (damaged residues are numbered), the diradical is positioned by the benzoxazonilate and sugar moieties[2g] in a manner that enables one of the diradical centers to abstract a hydrogen atom from the C4′ position of the A1 nucleotide and the other to attack either C1′ of A2 or C5′ of A3.[2c] Thus, a single binding mode of C1027 in DNA induces two types of double-stranded lesions involving either A1 and A2 or A1 and A3.[2c] In the presence of O_2, hydrogen atom abstraction from C4′ of A1 results in a 4′-hydroxylated abasic site or a strand break with a phosphoglycolate at the 3′-terminus (Figure 2, lane 3) and PO_4 at the 5′-terminus. The C5′-attack at A3 leads to a strand break with mainly a PO_4 at the 3′-end (lane 9) and a nucleoside aldehyde at the 5′-end. The C1′-attack at A2 generates an unstable abasic lesion having a 2′-deoxyribonolactone, which breaks down to form a 3′-phosphate-ended fragment (lane 9).[2c] When the drug/DNA reaction is done anaerobically,[4] strand breaks are dramatically reduced (compare lane 4 with 3), and two new sets of slowly moving bands are identified with 5′-^{32}P-end-labeled D1 (indicated by square brackets and solid arrows, lanes 4 and 6) with overall total damage being almost unchanged. The electrophoretic behaviors

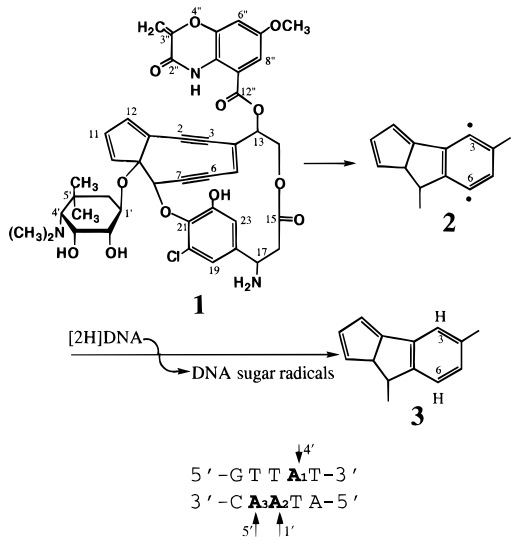

Figure 1. Proposed mechanism of activation of C1027 chromophore and C4′-, C1′, and C5′-hydrogen abstractions at A1−A3 sites of the model GTTA1T/ATA2A3C DNA sequence.

of the two new damage products suggest that DNA strand cross-links and drug adducts are produced.

To test this proposal, oligo D3, which contains two more T residues than D2, annealed to D1 was used as a substrate (Figure 2A). Lanes 12 and 14 show damage products from the 5′-^{32}P-end-labeled D1 annealed to D2 or D3, respectively. The indistinguishable mobilities of the damage products indicated by the arrows suggest that they involve only oligo D1, not complementary strand D2 or D3. In contrast, the slower mobility of the damage products in lane 14, indicated by the square bracket (compare lane 14 with 12), indicates that these products involve both strands of the DNA duplex. Lanes 15 and 16 represent drug/DNA reactions containing 5′-end-labeled D3 duplexed with D1. The slower migration of the product indicated by the arrow, when compared with the reaction involving labeled D1 (compare lanes 15 and 16 with lanes 14 and 12), and the similar mobility of the product in the square bracket (compare lane 16 with 14) strongly indicates that under anaerobic conditions C1027 produces DNA interstrand cross-links (brackets) and drug adducts (solid arrows). Quantitation of the damage at A2 and A3 under aerobic and anaerobic conditions suggests that the DNA cross-links and drug adducts on D2 mainly involve A2, since cleavage at A2 decreases most under anaerobic conditions. Sequence random fragmentation of isolated major DNA cross-link and drug adduct bands by hydroxyl free radicals[6] reveals that the connectivity of the interstrand cross-link is either A1 to A2 or A1 to A3 and that drug adduct is formed on either A2 or A3. Under aerobic conditions C1027 produces a small amount of DNA drug adduct on strand D3 but not on D1 (compare lane 15 with lanes 11

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(4) 5′-End-labeled oligomers were annealed to the complementary strands (1:1) by heating at 90 °C for 5 min and slow cooling to room temperature. A standard reaction contained 10 mM Tris·HCl, 0.5 mM EDTA, pH 8.0, and 2 μM oligos. C1027 was added to initiate DNA/drug reactions, and incubation was in the dark at 37 °C for 3 h and stopped by drying in a Speed-Vacuum. Anaerobic reactions were in a Warburg vessel with drug in the side arm and other components in the main chamber. The contents were frozen in liquid nitrogen and evacuated at $<10^{-3}$ Torr in a Kontes high-vacuum setup. Freeze−evacuate−thaw was repeated five times, and the vessel was then filled with ultrapure argon (O_2 < 1.0 ppm, Medical-Technical Gasses, Inc.). The reaction was initiated by mixing the contents of the two chambers.

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A

D1 5′-G C C **G T T A₁T** G C C G-3′
D2 3′-C G G **C A₃A₂T A** C G G C-5′

D3 3′-T C G G **C A₃A₂T A** C G G C T-5′

B

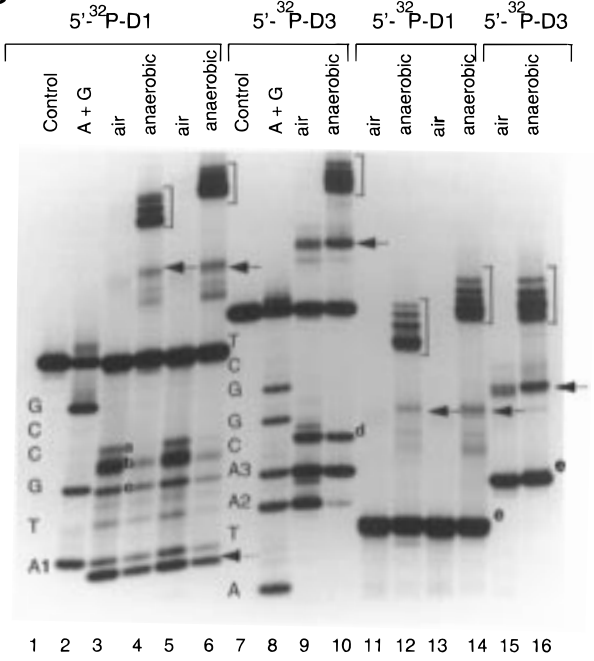

Figure 2. A. Sequences of DNA oligomers. The GTTA1T/ATA2A3C target sequences are printed in bold. B. Denaturing gel analysis of DNA damage products. The 5′-^{32}P-end-labeled D1 was annealed to D2 (lanes 1, 3, 4, 11, and 12) or D3 (lane 5, 6, 13, and 14), and the 5′-^{32}P-end-labeled D3 was annealed to D1 (lanes 7, 9, 10, 15, and 16). DNA substrates were incubated with C1027 in a standard reaction.[4] Lanes 2 and 13 are Maxam−Gilbert markers. Slow moving bands denoted by a, b, c, and d are likely products generated by decomposition of the labile abasic lesions at A1 and A2.[5] Lanes 11−14 were the same as lanes 3−6, except that electrophoresis was run 2 h longer for better resolution. Similarly, samples in lanes 15 and 16 were run longer than those in lanes 9 and 10. Arrows and square brackets indicate drug adducts and cross-linked structures, respectively. The e denotes undamaged DNA. The minor bands in the cross-linked region are probably caused by incomplete denaturation of the cross-linked DNA under this condition and/or structurally different interstrand cross-links. Although the DNA fragments with 3′-phosphoglycolate ends (dashed arrow) decrease markedly with anaerobiosis, the remaining "glycolate" band indicates that some residual oxygen is present.

and 13); interstrand cross-links, however, were not observed to any extent.

The two new damage products were purified in two steps. The major slow-moving bands containing cross-linked DNA and drug adduct (on D2) were separated on a high-resolution denaturing gel, excised, and eluted by shaking overnight. The supernatants, after centrifugation, were subjected to reverse

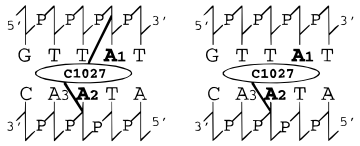

Figure 3. Proposed structures of the major DNA damage products, interstrand cross-link (**4**), and drug adduct (**5**), induced by enediyne C1027 under anaerobic conditions.

phase HPLC purification on a C-18 column using a linear gradient of 7−17% of acetonitrile in 10 mM triethylamine acetate, pH 7.0. On HPLC the duplex DNA is well denatured, and the two strands are separated. The elutions were monitored by following 260 nm UV absorbance and 435 nm fluorescence (excitation at 350 nm), which detect the postactivated C1027 (**3**). The drug adduct and cross-linked materials eluted at 31 and 17 min, respectively. Importantly, the typical fluorescence of the drug product was associated with the peak of 260 nm absorbance of the major cross-linked product (and drug adduct), which strongly suggests that the two DNA strands are covalently cross-linked by the drug itself. Preliminary mass spectrometry data confirm that the material eluting at 17 min contains drug product and both DNA strands, whereas that eluting at 31 min contains only drug and D2.[7]

Under aerobic conditions, O_2 adds to the carbon-centered radical on DNA deoxyribose to form strand breaks and abasic lesions. In the absence of O_2, the deoxyribose radical of each complementary DNA strand most likely reacts covalently instead with the bound drug to form minor-groove-based interstrand cross-links and drug monoadducts. Under similar conditions, neocarzinostatin has been shown to produce drug adducts on DNA with covalent attachment to deoxyribose at the 5′-position,[8] but interstrand cross-links have not been reported for this or other enediyne antibiotics.

The results with C1027 are best accommodated by structures **4** and **5** in Figure 3. The interstrand cross-link appears to be mediated by the drug itself, which is covalently linked with probably deoxyribose moieties on each of complementary strands. This contrasts with all other known interstrand cross-links induced by bifunctional alkylating agents, which involve the nucleobases.

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Supporting Information Available: Table of quantitation of damage at A1, A2, and A3 sites and figure of sequence random fragmentation of isolated major DNA cross-link and drug adduct (2 pages). See any current masthead page for ordering and Internet access instructions.

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