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The DNA Phosphate Backbone is Not Involved in Catalysis of the Duocarmycin and CC-1065 DNA Alkylation Reaction

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Abstract—The rates of DNA alkylation were established for the reaction of (+)-duocarmycin SA (**1**) with the native duplex d(G[1]TCAATTAGTC[11])·d(G[12]ACTAATTGAC[22]), an 11 bp deoxyoligonucleotide that contains a single high-affinity alkylation site that has been structurally characterized at exquisite resolution, and modified duplexes in which the four backbone phosphates proximal to the C4 carbonyl of bound **1** were replaced with methylphosphonates. All were found to react at comparable rates establishing that these backbone phosphates do not participate in catalysis of the DNA alkylation reaction.

CC-1065 and the duocarmycins are exceptionally potent antitumor agents that derive their properties through a sequence-selective adenine N3 alkylation of DNA (Fig. 1).[1,2] Although the DNA alkylation selectivity of the natural products, key partial structures, their unnatural enantiomers, and an extensive series of analogues have been defined, the origin of this selectivity and the source of catalysis for the reaction with DNA remain controversial. In the studies to date, two models have emerged. One, which is based on the premise that **3** and **6** exhibit identical alkylation selectivities,[3] proposes a sequence-dependent backbone phosphate protonation of the C4 carbonyl of **1**–**3** activating them for DNA alkylation accounting for both the selectivity and catalysis of the DNA alkylation reaction.[1,3,4]

Although efforts have gone into supporting the role of acid catalysis required for this model,[5] it remains undocumented for the DNA alkylation reaction. At pH 7.4, the DNA phosphate backbone is fully ionized (0.0001–0.00004% protonated) and unlikely to provide catalysis by internal delivery of a proton to the C4 carbonyl of **1**–**3**. Although increases in the local hydronium ion concentrations surrounding 'acidic domains' of DNA have been invoked to explain DNA-mediated acid catalysis,[6] nucleotide reactivity,[6] and extrapolated to **1** for alkylation site catalysis,[5] the remarkable stability of **1** and **3** even at pH 4–5[2] suggests that it is insufficient to account for catalysis. Consistent with this expectation, the DNA alkylation reaction of **1** does not exhibit a pH dependence between pH 6–8 where it has been examined.[7]

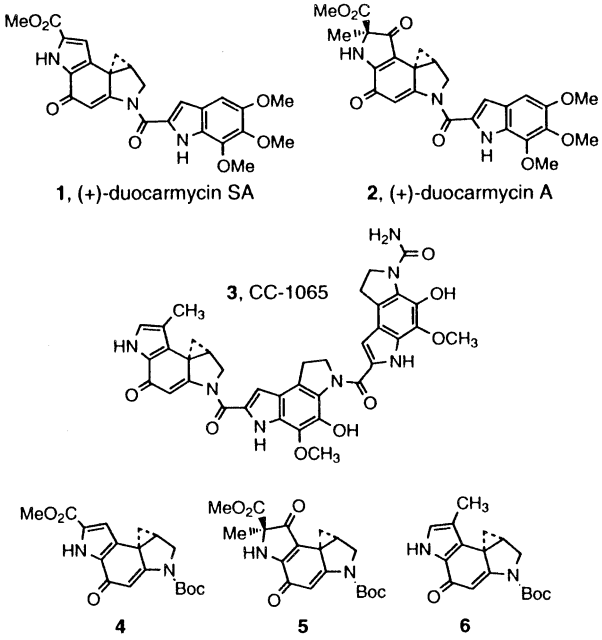

Figure 1.

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However, it is possible that Lewis acid mediated sequence-dependent catalysis could occur through delivery of a phosphate-associated metal cation to the C4 carbonyl. Consequently, we elected to establish whether the backbone phosphates participate in the catalysis of the DNA alkylation reaction of **1**–**3**.

For this purpose, we examined the rates of DNA alkylation by (+)-duocarmycin SA within the 11 bp duplex DNA **7** that contains a single high affinity alkylation site (5′-AATTA), which has been structurally characterized by NMR (Fig. 2).[8] Within this structure, the two phosphates that span the C4-carbonyl position which have been implicated in catalysis are located between G9 and T10 and A16 and A17 (Fig. 2). The rates of DNA alkylation of the native duplex (**7**) and modified duplexes in which each (**8** and **9**), as well as both (**10**) these backbone phosphates have been replaced with methylphosphonates were compared (Fig. 3). If either is involved in catalysis, the corresponding methylphosphonate modified oligos would be incapable of supporting catalysis and fail to undergo alkylation. Notably, the G9–T10 phosphate resides closest to the C4-carbonyl center in our structure of the adduct[8] and the A16–A17 phosphate is analogous to the phosphate in a structurally characterized deoxyoligonucleotide CC-1065 adduct that Hurley et al.[4] have defined as responsible for catalysis. For the sake of completeness, we examined the modified oligos **11** and **12** containing methylphosphonates between A8 and G9, and A17 and T18, that also span the alkylation site of which the A17–T18 phosphate resides closest to C4 carbonyl and could be involved in catalysis.

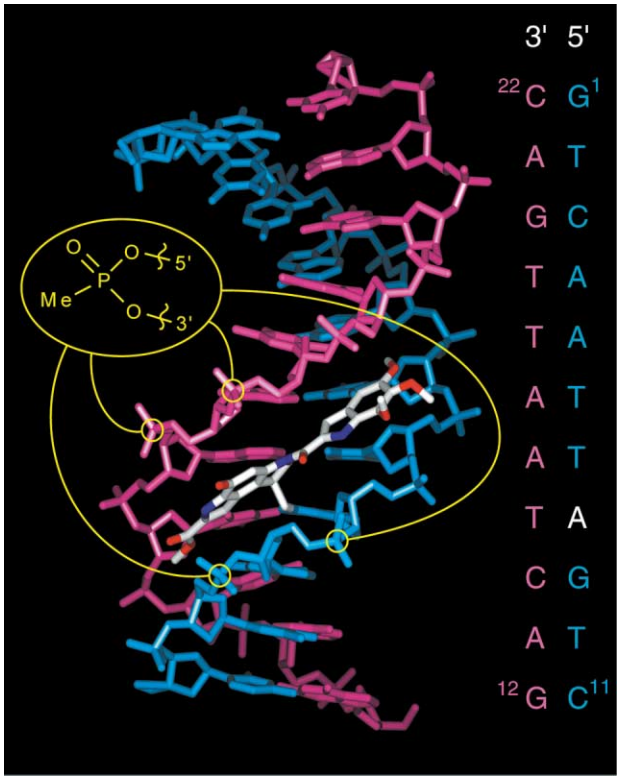
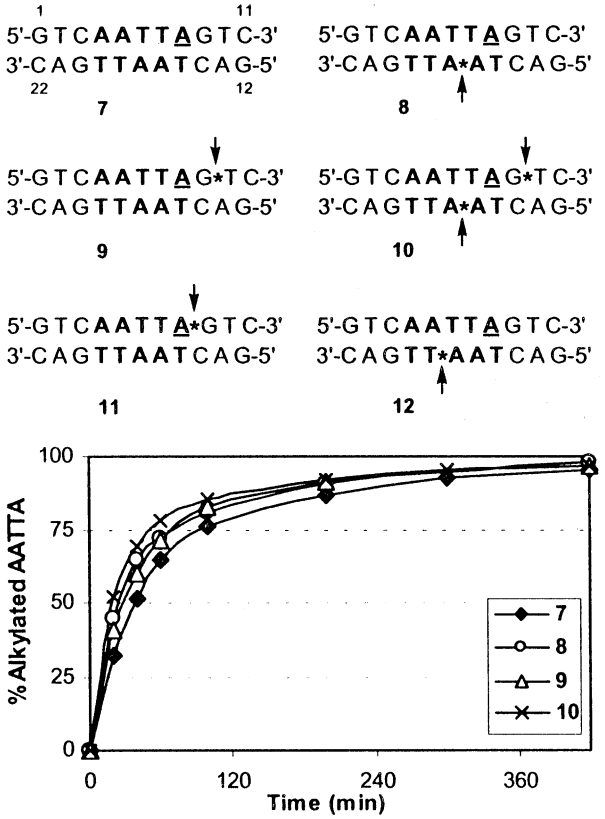


Figure 2. High-resolution NMR structure of (+)-duocarmycin SA (**1**) covalently bound in the deoxyoligonucleotide high affinity site.[8] The DNA sequence is shown and its alkylation site (adenine-8) is highlighted. Methylphosphonate-modified backbone phosphates are circled (between A8 and G9; G9 and T10; A16 and A17; A17 and T18).

The rates of DNA alkylation were monitored by HPLC of the reaction mixtures under denaturing conditions (0.05 M HCO_2NH_4/5–20% then 50% CH_3CN) measuring the disappearance of starting DNA and the appearance of the alkylated DNA strand and were run in triplicate under first order conditions (0.2 μM) enlisting excess **1** (10 equiv) at pH 7.2 (0.05 M Tris, 0.05 M NaCl). Alkylation was established to occur at the same A8 site by thermal depurination and strand cleavage (95 °C, 30 min, 20 equiv of piperidine) of the alkylated duplex DNA from reactions of **7**–**12**, each of which released a single and the same shorter oligo 5′-GTCAATT (HPLC coelution). When a single methylphosphonate modification was introduced either between A16 and A17 (**8**), G9 and T10 (**9**), both A16 and A17, and G9 and T10 (**10**), A8 and G9 (**11**), or A17 and T18 (**12**), the observed first order rate constants for DNA alkylation and kinetic profiles (Fig. 3) were found to be essentially indistinguishable ($k_{obs} = 0.016$–$0.019\ min^{-1}$)



duplex	k_{obs} (min^{-1})	duplex	k_{obs} (min^{-1})
7	0.014 ± 0.002	**10**	0.019 ± 0.002
8	0.016 ± 0.002	**11**	0.016 ± 0.002
9	0.017 ± 0.003	**12**	0.017 ± 0.001

Figure 3. Sequences of native duplex **7** and modified duplexes with a methylphosphonate between A16 and A17 (**8**), G9 and T10 (**9**), both A16 and A17 and G9 and T10 (**10**), A8 and G9 (**11**), A17 and T18 (**12**), and time dependence and rate constants for the (+)-duocarmycin SA (**1**) alkylation reaction of the six DNA duplexes (**7**–**12**). For clarity, kinetic profiles for **11** and **12** are not shown.

from that of the native DNA ($k_{obs} = 0.014\,min^{-1}$). In fact, the rates of DNA alkylation were slightly faster with **8–12** indicating that the methylphosphonate modifications are not detrimental to the rate of DNA alkylation. Importantly, the magnitude of the first order rate constants with complete reaction within 1–2 h at 23 °C is representative of a catalyzed reaction at a high affinity alkylation site and a similar first order rate constant has been disclosed for the duocarmycin A alkylation of $d(CGTATACG)_2$.[9]

These results are incompatible with a backbone phosphate participation in the DNA alkylation reaction whether it be through acid or Lewis acid (metal cation) catalysis. However, they are consistent with a second model, which is based on the premise that **1–3** and **4–6** exhibit distinct alkylation selectivities[10,11] that are controlled by the AT-rich noncovalent binding selectivity of the agents and their steric accessibility to the adenine N3 alkylation sites.[2] Superimposed on this preferential AT-rich binding is catalysis that we have suggested is derived from a binding-induced conformational change in the agents which twists the linking amide disrupting the stabilizing vinylogous amide conjugation activating the cyclopropane for nucleophilic attack.[12,13] Since the extent of this binding-induced conformational change is dependent on the shape of the minor groove, being greatest in the narrower, deeper AT-rich minor groove, we have come to refer to this as shape-dependent catalysis. This model accommodates the reverse and offset 3–4 or 5 bp AT-rich selectivity of the natural and unnatural enantiomers of **1**–**2**[11] and **3**[10] and requires that **4–6** exhibit alkylation selectivities distinct from the natural products. It offers a beautiful explanation for the identical selectivities of both enantiomers of **4–6** (5′-AA, 5′-TA), and the more extended AT-rich selectivities of **1–3** corresponds nicely to the length of the agent and the size of the required binding region surrounding the alkylation site.[10,11] Further support for this model includes the demonstrated AT-rich noncovalent binding selectivity of the compounds,[14] their preferential noncovalent binding coincidental with DNA alkylation,[15] and the observations that relocation[16] or removal[17] of the C4 carbonyl, replacement of the cyclopropane with alternative electrophiles,[17] or use of exogenous sources of catalysis[18] do not alter the characteristic alkylation selectivity. The switch in the enantiomer alkylation selectivity of reversed versus extended analogues of the natural products coupled with the different but identical alkylation selectivities of enantiomeric sandwiched analogues confirm that it is the noncovalent binding selectivity, and not the alkylation subunit or alkylation site, that is controlling the alkylation selectivity.[13,18] That catalysis is derived from a binding-induced disruption of the vinylogous amide is consistent with the requirement for a rigid, extended heteroaromatic N^2 amide substituent for catalysis,[13] the lack of catalysis when the linking amide carbonyl is removed,[19] structural correlations between vinylogous amide disruption and reactivity (10^4-fold reactivity increases),[20] and pH rate profiles of reactive alkylation subunits indicating that pH 5–8 reactivity is not hydronium ion sensitive.[20,21] Further, it explains subtle substituent effects on the rate of DNA alkylation that do not correlate with pH-dependent reactivity trends,[22] and structural studies of DNA bound adducts have defined the required twist in the linking amide and provided correlations with DNA alkylation rates.[8,23]

The studies detailed herein, enlisting a structurally characterized high affinity alkylation site for (+)-duocarmycin SA, demonstrate that the backbone phosphates proximal to the C4 carbonyl of bound **1** do not participate in catalysis of the DNA alkylation reaction. This observation is inconsistent with that expected of an alkylation site model where both the DNA alkylation selectivity and catalysis are derived from a sequence-dependent backbone phosphate protonation of the C4 carbonyl activating **1** for nucleophilic attack,[1,3–5] but is consistent with an alternative noncovalent binding model involving shape-selective binding and shape-dependent catalysis.[2,10–13]

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