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Role of the Azinomycin Naphthoate and Central Amide in Sequence-Dependent DNA Alkylation and Cytotoxicity of Epoxide-Bearing Substructures

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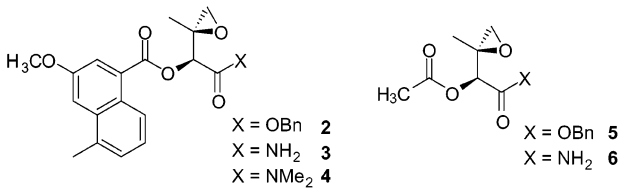
ABSTRACT



Studies report a strong correlation between duplex DNA alkylation and in vitro cytotoxicity for a series of azinomycin partial structures 2–6 bearing the biologically relevant epoxide. Compounds lacking the naphthoate ester (e.g., 5 and 6) were poorly reactive toward DNA and were biologically inactive, as were compounds bearing the naphthoate but lacking the terminal carboxamide (e.g., 2). Compounds were evaluated for cytotoxicity against two breast cancer cell lines.

The DNA cross-linking agents azinomycins A (**1a**) and B (**1b**) have generated considerable interest among the synthetic,[1] medicinal,[2] and bioorganic communities.[3] These natural products possess the unusual aziridino[1,2-*a*]pyrrolidine ring system (Figure 1).[4] These scarce and unstable agents exhibit sub-micromolar in vitro cytotoxic activity and promising in vivo antitumor activity[5] and appear to exert their effect by the formation of covalent interstrand cross-links within the major groove of duplex DNA.[6] We recently reported the first total synthesis of azinomycin A,[1] and we have evaluated the sequence selectivity, noncovalent as-

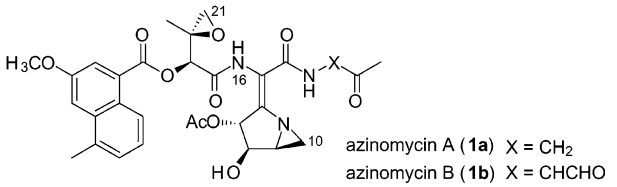


Figure 1. Structure of azinomycins A and B.

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(1) Coleman, R. S.; Li, J.; Navarro, A. *Angew. Chem., Int. Ed.* **2001**, *40*, 1736 and references therein.

(2) (a) For related structure−activity work, see: Hodgkinson, T. J.; Kelland, L. R. Shipman, M.; Suzenet, F. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 239. (b) Hartley, J. A.; Hazrati, A.; Khanim, R.; Hodgkinson, T. J.; Shipman, M.; Suzenet, F.; Kelland, L. R. *Chem. Commun.* **2000**, 2325. (c) Hartley, J. A.; Hazrati, A.; Kelland, L. R.; Khanim, R.; Shipman, M.; Suzenet, F.; Walker, L. F. *Angew. Chem., Int. Ed.* **2000**, *39*, 3467.

(3) (a) Zang, H.; Gates, K. S. *Biochemistry* **2000**, *39*, 14968. (b) Fujiwara, T.; Saito, I.; Sugiyama, H. *Tetrahedron Lett.* **1999**, *40*, 315. (c) Armstrong, R. W.; Salvati, M. E.; Nguyen, M. *J. Am. Chem. Soc.* **1992**, *114*, 3144.

(4) Nagaoka, K.; Matsumoto, M.; Oono, J.; Yokoi, K.; Ishizeki, S.; Nakashima, T. *J. Antibiot.* **1986**, *39*, 1527. Yokoi, K.; Nagaoka, K.; Nakashima, T. *Chem. Pharm. Bull.* **1986**, *34*, 4554.

(5) Ishizeki, S.; Ohtsuka, M.; Irinoda, K.; Kukita, K.; Nagaoka, K.; Nakashima, T. *J. Antibiot.* **1987**, *40*, 60.

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sociation, and orientation of azinomycin B on its duplex DNA receptor.[7] We now provide further mechanistic clarification on the critical role that the naphthoate and N16 carboxamide play in sequence selective DNA alkylation in a series of partial structures, and we demonstrate a strong correlation between the extent of covalent modification of DNA and in vitro cytotoxicity. We find that the naphthoate and the carboxamide increase alkylation yields and sequence selectivity and that both are important for effective cytotoxic activity.

Armstrong and co-workers reported that azinomycin B cross-links duplex DNA in the major groove at the N7-positions of 5′-disposed purine bases in the sequence 5′-PuNPy-3′.[3c] Saito and co-workers confirmed this result and provided evidence of the orientation of the agent on the DNA duplex.[3b] Modeling studies by Alcaro and Coleman[8] and experimental work by Coleman and co-workers[7] provided evidence that aziridine C10 alkylates the 5′ guanine in a GGC strand in the initial monoalkylation, and cross-linking ensues by epoxide C21 alkylation of the guanine in the CCG strand. Computational work was consistent with either an intercalative or non-intercalative binding mode for the naphthoate, and experimental work by Zang and Gates indicated that naphthoate-bearing fragments were weak intercalating agents.[3a] Our studies on azinomycin B point to a non-intercalative binding mode.[7] Shipman and co-workers provided the first evidence that the epoxide was important for cytotoxic activity.[2a] We now demonstrate a strong correlation between the alkylating ability of a series of epoxide-bearing partial structures (Figure 2) and in vitro cytotoxicity.

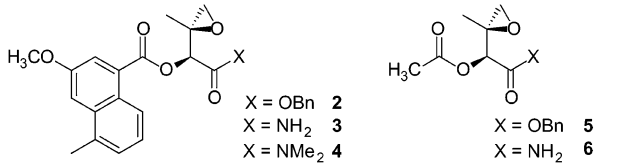

Figure 2. Azinomycin partial structures.

Using synthetic DNA oligomers containing the azinomycin recognition sequence GGC·CCG,[7] and the inverted sequence CGG·GCC, wherein both triplets were embedded within an unreactive A·T tract (Figure 3), we examined the ability of compounds **2**−**6** to alkylate guanine bases in both single-stranded and double-stranded forms of these oligodeoxynucleotides.

```
5′-d(TAT TAT GGC ATT ATT)-3′  (7)
3′-d(ATA ATA CCG TAA TAA)-5′  (8)

5′-d(ATA TTA CGG AAT ATA)-3′  (9)
3′-d(TAT AAT GCC TTA TAT)-5′  (10)
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Figure 3. DNA sequences.

Alkylation of GGC-strand **7** in duplex **7·8** by azinomycin partial structure **3** was highly effective (86%, Table 1) and occurred with 4:1 selectivity for the more nucleophilic 5′ guanine.[9] The yield was lower with the CCG strand **8** (36%), in accord with consideration of base nucleophilicity. (Combined yields for duplex **7·8** are greater than 100% because of double alkylation.) With duplex **9·10** containing an inverted azinomycin recognition triplet where the guanine bases are 3′-disposed, the CGG strand **9** underwent modestly effective alkylation with **3** (42%), again with 4:1 selectivity for the more nucleophilic 5′ guanine. The complementary GCC strand **10** was alkylated to a similar extent by **3** (52%). These results indicate that the partial structure **3** must interact with duplex DNA by multiple binding modes and with altered sequence selectivity from the natural product. In duplexes **7·8** and **9·10**, all guanine bases are alkylated to a significant but differential extent by **3**; azinomycin B reacts selectively at the two distal 5′-disposed guanines in this triplet by an apparently well-defined binding mode.[7] In the duplex **7·8**, the extent of alkylation by the epoxide of **3** correlated well with guanine nucleophilicity, but with the inverted duplex **9·10**, the less reactive guanine in the GCC strand **10** underwent more efficient alkylation.

Table 1. Yield of Covalent Adduct Formation[a] between Epoxyamide **3** and DNA Oligomers **7**−**10**[b]

	GGC (**7**)	CCG (**8**)	CGG (**9**)	GCC (**10**)
ds	86%	36%	42%	52%
ss	23%	17%	28%	12%

[a] Yields are the average of three measurements. Yields reported for double-stranded oligomers represent the results obtained when that oligomer was ^{32}P end labeled. [b] Reactions were run at 8 °C for 20 h using 100 equiv of **3** per oligodeoxynucleotides duplex.

Alkylation yields dropped significantly in single-stranded oligomers, being slightly higher for **7** than **8**, and similarly for **9** compared to **10**. With the GGC strand **7** and CGG strand **9**, there was no sequence selectivity for alkylation of the two guanines (1:1) when these oligomers were single-stranded. There was no sequence recognition of single-stranded DNA by **3**, although it is instructive to compare the alkylation yields for single- versus double-stranded

(6) For a review of the azinomycins, see: Hodgkinson, T. J.; Shipman, M. *Tetrahedron* **2001**, *57*, 4467. For a review on the synthesis of DNA cross-linking agents, see: Coleman, R. S. *Curr. Opin. Drug Discovery Dev.* **2001**, *4*, 435. For a review on DNA cross-linking agents, see: Rajski, S. R.; Williams, R. M. *Chem. Rev.* **1998**, *98*, 2723. For a review of agents that covalently modify DNA, see: Gates K. S. Covalent Modification of DNA by Natural Products. In *Comprehensive Natural Products Chemistry*; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Pergamon Press: Elsevier Science, Oxford, U.K., 1999; p 491.

(7) Coleman, R. S.; Perez, R. J.; Burk, C. H.; Navarro, A. *J. Am. Chem. Soc.* **2002**, *124*, in press.

(8) Alcaro, S.; Coleman, R. S. *J. Med. Chem.* **2000**, *43*, 2783. Alcaro, S.; Ortuso, F.; Coleman, R. S. *J. Med. Chem.* **2002**, *45*, 861.

(9) Sugiyama, H.; Saito, I. *J. Am. Chem. Soc.* **1996**, *118*, 7063.

oligomers to establish the apparent background alkylation and to understand the importance of noncovalent association in driving covalent bond formation.

In these assays, a 100-fold excess of agents was used in order to provide measurable alkylation yields in single-stranded oligodeoxynucleotides. It was important to use the same stoichiometry in all DNA systems so that a direct comparison of yields could be made. Polyacrylamide gel electrophoresis (PAGE) of the reaction of epoxyamide **3** (100 μM) and duplex **7·8** (5 μM) in Tris buffer (pH 7.5, drug/DNA ratio = 20:1) showed alkylation had proceeded in ≥75% yield after 20 h at 8 °C (Figure 4). With compound **3** and duplex **7·8** using a drug/oligonucleotide ratio of 5:1, we obtained 40−50% alkylation under the same reaction conditions (data not shown).

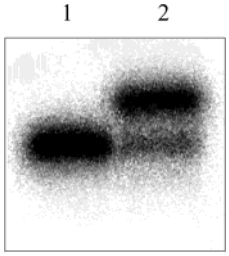

Figure 4. Alkylation of duplex **7·8** with epoxyamide **3**. Polyacrylamide gel electrophoresis autoradiogram. Lane 1: 5′ ^{32}P end-labeled DNA (strand **7**). Lane 2: 5′ ^{32}P end-labeled DNA (strand **7**) + drug (**3**) after incubation at 8 °C for 20 h.

Coleman and co-workers[7] and Saito and co-workers[3b] demonstrated that the azinomycin epoxide alkylates the purine of the 3′-PyPyPu-5′ strand, so it appears from the above results that azinomycin partial structure **3** binds to duplex DNA in a mode(s) not observed with the native agent, thus bestowing the remainder of the azinomycin molecule with either a substantial or critical portion of the sequence recognition ability. In duplex DNA the extent of alkylation of guanine bases by **3** follows the order GGC ≫ GCC > CGG > CCG, where there is a significant difference in alkylation yields between the GGC strand and the other three strands.

When the native carboxamide in epoxyamide **3** was replaced with a benzyl ester in **2**, alkylation of DNA was completely abolished. With levels of **2** as high as 10^4 equiv per oligomer **7** or duplex **7·8**, there was no observable alkylation of DNA. To determine whether this effect was due to favorable hydrogen bond donation by the primary amide of **3**, we examined *N*,*N*-dimethylamide **4** and found that alkylation of **7** in duplex **7·8** was partially restored (60%). The hydrogen bond donating C16 amide appeared important but not critical for DNA alkylation.

With structure **6** that lacks the naphthoate completely, the efficiency of DNA alkylation dropped dramatically, and both the reaction temperature (37 °C) and equivalents of agent had to be increased substantially to achieve alkylation with duplexes **7·8** and **9·10**. Under forcing reaction conditions, no distinct alkylated species was formed on PAGE, and the electrophoresis profile resembled that obtained with simple epoxides such as glycidol with less than 10% lower mobility species formed. Piperidine-induced strand cleavage showed that both guanines in the GGC or CGG strands (i.e., **7** or **9**) were alkylated equally. When the primary amide of **6** was replaced with a benzyl ester as in **5**, DNA alkylation was completely abolished.

Cytotoxic activity of azinomycin partial structures was measured against two standard breast cancer cell lines: hormone-dependent MCF-7 cells and hormone-independent MDA-MB-231 cells (Table 2), using the CellTiter 96 aqueous nonradioactive cell proliferation assay.[10] In each case the MDA-MB-231 cells were more sensitive to the effects of these agents. The lack of reactivity toward DNA by ester **2** relative to that of amide **3** paralleled the much weaker cytotoxic activity exhibited by **2**, and the corresponding increase in alkylation by dimethylamide **4** correlated with increased cytotoxicity. *In both cell lines there was a marked correlation between the extent of DNA alkylation and cytotoxicity*, with compounds **3** and **4** exhibiting the highest levels of DNA alkylation and of cytotoxicity, indicating that alkylation of DNA is a critical biological event in the mechanism of action of these agents and presumably for azinomycin B.

Table 2. Cytotoxic Activity of Azinomycin Partial Structures **2**−**6** against Breast Cancer Cell Lines MCF-7 and MDA-MB 231

compound	IC_{50} (MCF-7) (μM)	IC_{50} (MDA-MB-231) (μM)
2	>25	10.1
3	2.2	0.4
4	4.2	1.6
5	na[a]	na
6	na	na

[a] na = not active.

These results indicate the important role that the azinomycin naphthoate plays in increasing sequence selective binding affinity (as evaluated by DNA alkylation yields) and cytotoxic activity. In the absence of the naphthoate, epoxide-bearing partial structures exhibited low reactivity toward DNA and no sequence selectivity, with correspondingly weak cytotoxicity. The azinomycin "left-half" partial structure **3** exhibited altered sequence selectivity compared to that of the native agent, while retaining a substantial portion of the cytotoxic activity: epoxide **3** alkylated both guanines in the GGC sequence **7** to a significant extent, whereas azinomycin B alkylates the 5′ guanine in the GGC sequence **7** with complete selectivity.[7] Even more remarkable is the ineffectual activity of the corresponding benzyl ester **2** in DNA

(10) Barltrop, J. A.; Owen, T. C.; Cory, A. H. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 611. Cory, A. H.; Owen, T. C.; Cory, J. G. *Cancer Comm.* **1991**, *3*, 207.

alkylation assays, where it exhibited no reactivity, and in the cytotoxicity assays, where it was significantly less cytotoxic than the corresponding primary carboxamide **3** or tertiary carboxamide **4**.

Our results provide the first indication that covalent modification of DNA correlates directly with cytotoxic activity for this class of antitumor agents. These studies provide important information about structure−activity relationships[2] in the left half of the azinomycins, showing that both the naphthoate and N16 amide nitrogen are critically important for effective interaction with duplex DNA. Understanding and utilizing such information obtained from studies of partial structures will prove critical in designing more effective antitumor agents based on the azinomycin skeleton.

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Supporting Information Available: Experimental procedures and spectral characterization of **2**−**6** and experimental details for DNA alkylation and cytotoxicity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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