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Dependence of DNA Sequence Selectivity and Cell Cytotoxicity on Azinomycin A and B Epoxyamide Stereochemistry

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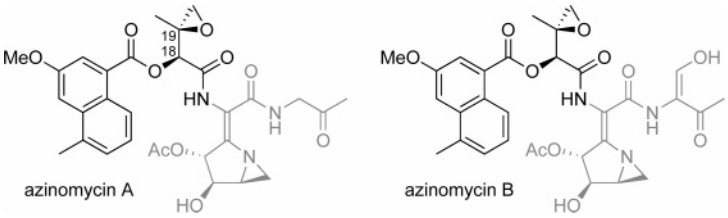
ABSTRACT



Evaluation of the importance of C18/C19 stereochemistry of azinomycin A/B epoxyamide partial structures with respect to DNA alkylation sequence selectivity is reported using a unique assay with a DNA oligomer containing imbedded normal (5′-GGC-3′/3′-CCG-5′) and inverted (5′-CGG-3′/3′-GCC-5′) azinomycin consensus cross-linking sequences. Both species were found to have unique selectivity profiles and alkylate DNA in a manner distinct from azinomycin B. Computational docking experiments support altered binding modes for the enantiomers.

The importance of natural products as lead compounds for anticancer drug development is evident in the fact that more than half of existing agents are natural products or derivatives thereof,[1] and a wide variety of bioactive natural products are currently in clinical trials as anticancer agents.[2] Many such compounds exert their action by covalent modification[3] or cross-linking of duplex DNA[4] and provide the basis for much of cancer chemotherapy.

The azinomycins[5] are *Streptomyces* metabolites isolated in 1986 that show good levels of in vitro cytotoxicity and effective, potent in vivo antitumor activity.[6] Azinomycin B was subsequently demonstrated to covalently cross-link double-stranded DNA by reaction of the N7 positions of suitably disposed purine bases with the electrophilic C10 and C21 carbons.[7,8] Recent studies have further supported this finding, demonstrating that nuclear DNA is the relevant molecular target of the natural product.[9] There has also been

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(6) Ishizeki, S.; Ohtsuka, M.; Irinoda, K.; Kukita, K.; Nagaoka, K.; Nakashima, T. *J. Antibiot.* **1987**, *40*, 60. In vitro cytotoxicity: $IC_{50} = 0.07$ μg/mL (**1a**) and 0.11 μg/mL (**1b**) against L5178Y cells. In vivo antitumor activity: 193% ILS at 16 μg/kg of **1b** (3/7 survivors) against P388 leukemia; 161% ILS at 32 μg/kg of **1b** (5/8 survivors) against Erlich carcinoma. In the same system, mitomycin C exhibited a 204% ILS at 1 mg/kg against P388 leukemia.
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a considerable body of work published on synthetic efforts toward these challenging natural products, including the total synthesis of azinomycin A.[10,11] Much of the earlier work has been reviewed.[12,13]

Because the aziridino[1,2-*a*]pyrrolidine ring system is unstable and synthetically difficult to access, a truncated analogue that exhibits similar cytotoxic effects would be of significant utility. There have been several mechanistic studies with synthetic partial structures of the azinomycins.[14,15] A recent study described the influence of stereochemistry on sequence selectivity, but the mechanistic issues surrounding differential activity were not presented.[16] We now detail DNA alkylation sequence preferences and cytotoxicity of the four C18/C19 stereoisomeric azinomycin partial structures **1**−**4**[17] (Figure 1) via a competitive DNA

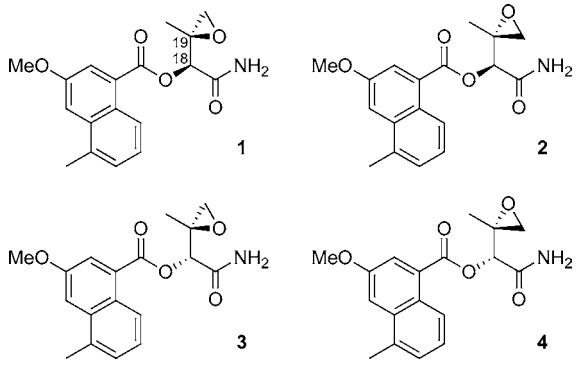

Figure 1. Stereoisomeric azinomycin partial structures.

binding assay to discern if alterations in binding orientation occur as a function of absolute configuration. The natural epoxyamide **1** has been reported previously,[7d] as has the enantiomeric compound **4**.[18]

The duplex DNA receptor used in this study (Figure 2) contained the azinomycin B consensus binding sequence, in

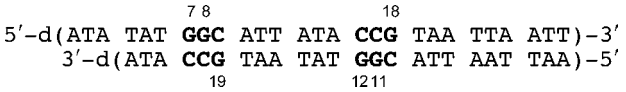

Figure 2. Sequence of DNA duplex.

both the normal 5′ → 3′ (5′-GGC-3′) and inverted 3′ → 5′ contexts (5′-CGG-3′). In alkylation studies, *both* DNA strands were 5′-end-labeled, as described.[7d] This duplex sequence was designed so that each G-alkylation event would provide a uniquely sized ^{32}P 5′-end-labeled oligonucleotide upon subsequent piperidine-induced strand cleavage,[19] indicated by numbers in Figure 2.

The oligomers were incubated at 4 °C with a 100-fold excess of drug to ∼50% completion to avoid multiple alkylations that would bias data toward shorter cleavage products. After cleavage with piperidine, the DNA fragments were separated by denaturing PAGE. The gel was visualized by Phosphorimaging, and the image was analyzed with ImageQuant software. Integrated data were normalized to correct for unequal labeling between the two oligomers. Differing percent completion across trials was accounted for by further normalization (Figure 3).[20]

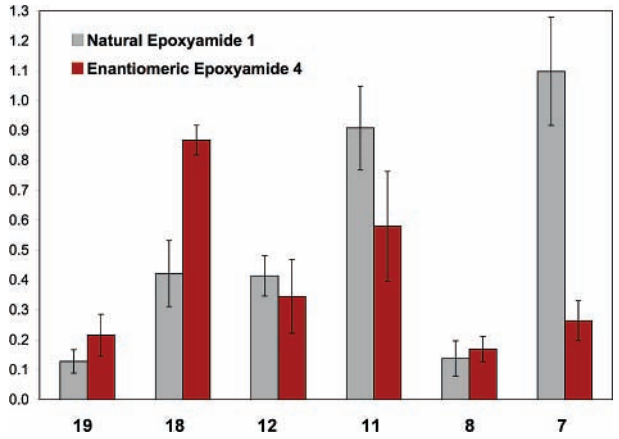

Figure 3. Relative DNA alkylation by natural **1** and enantiomeric **4** at numbered guanines.

We observed a significant alteration of sequence selectivity between the enantiomeric pair **1** and **4** (Figure 3), with intermediate patterns and less effective alkylation for diastereomers **2** and **3** (data not shown). Previous research has

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(20) Normalization to account for unequal labeling between oligomers entailed dividing the area of a peak for a given fragment by the area of the peak for its corresponding unreacted strand. Variability in percent completion was resolved by dividing the total area of a peak for a given fragment by the percent completion of alkylation × 100.

shown that azinomycin B shows selectivity for the highly nucleophilic 5′ guanine in the 5′-GGC-3′ sequence[21] (position 7 in Figure 2), which is alkylated by the electrophilic aziridine of the agent. Similarly, the natural epoxyamide **1** demonstrated a high selectivity for this position, which accounted for 35% of the total observed alkylation. However, alkylation also occurred at all other guanines to a variable extent, most notably at position G11 (29% of total alkylation).[14] This would be expected due to this position being the next most nucleophilic site on the duplex because G11 not only is 5′ disposed but also has the favorable π-stacking interaction of two sequential guanines.[19] Although this reveals an altered sequence selectivity from that of the natural product, the extent of alkylation still appears notably dependent on the nucleophilicity of the base.

Diastereomers **2** and **3** yielded an alkylation profile that was not as selective as epoxyamide **1** and suggested that stereochemistry might have an influence on sequence selectivity. Both of these compounds alkylated position G11 most readily with significant alkylation also occurring at positions G7 and G18 (data not shown). Diastereomer **2** seemed to be slightly more reactive than **3**, but both were lower in reactivity overall than natural epoxyamide **1**. Out of the four diastereomers, epoxyamide **3** was the second most reactive after **1** at positions G7 and G11. This is most likely due to **3** having the same stereochemistry as **1** at the electrophilic epoxy ring, which alkylates the DNA. This suggests that the epoxy ring prefers the more nucleophilic positions G7 and G11 when in the orientation of **1** and **3**, rather than in the orientation of **2** and **4**.

The previous statement and the significant change in selectivity of epoxyamides **2** and **3** overall imply that the nucleophilicity of the base is not the sole governing factor in these DNA−drug interactions and that stereochemistry must be important as well. This conclusion is further supported by the unique selectivity observed with the enantiomeric epoxyamide **4**. In contrast to the natural epoxyamide **1**, enantiomeric epoxyamide **4** demonstrated a low selectivity for position G7, instead alkylating most readily at position G18 (36% of total observed alkylation). Position G11 was once again alkylated to a significant extent (24%), but it is this change in preference from position G7 to position G18 that is of importance. Not only is position G18 unfavorably 3′-disposed but also it lacks the π-stacking that makes positions G7 and G11 so nucleophilic. This suggests that each of the "top-half" partial structures possesses a unique binding orientation with respect to the DNA duplex and also that the unnatural enantiomers of the azinomycins may bind in an inverted orientation.

Several investigations of the DNA binding selectivity of other natural products have also considered unnatural enantiomers. Not surprisingly, given the chirality of the DNA double helix, some of these structures exhibit DNA binding properties unique from their natural products.[22] For example, natural (+)-CC-1065 binds to DNA via a 3′-adenine-N3 alkylation event and demonstrates sequence selectivity within a three base pair region as follows: 5′-AA*A* = 5′-TT*A* > 5′-TA*A* > 5′-AT*A*. The sequence binding preference exhibited for the two 5′ bases indicates that binding occurs in the 3′ → 5′ direction. However, its unnatural enantiomer *ent*-(−)-CC-1065 exhibits sequence selectivity within a three base pair region with a strong preference that the bases 3′ from the alkylation site be A or T. This implies that a 5′-adenine-N3 alkylation event has occurred, resulting in *ent*-(−)-CC-1065 binding in the 5′ → 3′ direction.[23] Thus, inversion of stereocenters can result in an inversion in binding orientation. This is specifically the effect predicted for the azinomycins and their unnatural enantiomers given our results presented above.

In concert with their differing binding properties, compounds **1** and **4** demonstrated different cytotoxic activity (Table 1), with the more effective alkylating agent (**1**) exhibiting modestly higher levels of cytotoxicity. Activity was measured using a standard MTS/PMS assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega). Natural epoxyamide **1** was more cytotoxic than **4** in both of the cell lines tested, although the difference was small. Diastereomers **2** and **3** displayed intermediate cytotoxicity (data not shown).

Table 1. In Vitro Cytotoxicity

compd	MCF IC_{50} (μM)	MDA-MB-321 IC_{50} (μM)
1	0.8[a]	1.1[b]
4	6.9[c]	8.3[d]

[a] 95% CI 0.6−1.1. [b] 95% CI 0.8−1.3. [c] 95% CI 6.0−8.0. [d] 95% CI 7.3−9.5.

This effect is in contrast with the enantiomeric pair of (+)-CC-1065 and *ent*-(−)-CC-1065, both of which possess a cytotoxic activity of IC_{50} = 20 pM against L1210 cells.[24,25] The present compounds are similar, however, being examples of enantiomers with similar cytotoxic activity which possess apparently distinct modes of DNA binding.

Preliminary modeling studies further support these conclusions. Computational work started with the core model d(5′-TATGGCATTATACCGTAA-3′) of the DNA duplex (Figure 2). As in our previous models,[8b−d] the electrostatic net charge of the 18-mer was neutralized by including one Na^+ counterion for each phosphate. The standard B form conformation was generated by the Maestro interface of Macro-

(21) The nucleophilicity of this guanine is due to the HOMO of the stacked 5′-GG-3′ bases being localized on the 5′ guanine. For full details, see: Sugiyama, H.; Saito, I. *J. Am. Chem. Soc.* **1996**, *118*, 7063.

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Model version 7.2.[26] Charges were assigned according to the AMBER* united atom notation. The same software and force field were used for a preliminary Monte Carlo search of enantiomers **1** and **4**, resulting in nine unique minimum-energy conformations. In accordance with the MOLINE docking methodology,[27] all conformers of **1** and **4** were docked against the DNA 18-mer model. Docking was amplified in the GRID module of the MOLINE by putting negative charges on the N7 of G7 and G18 and a positive charge on the methylene C of the epoxide moiety. This electrostatic bias increased the generation of multiple configurations that were fully optimized with the same force field, removing this artificial parameterization. The most stable configuration per guanine−epoxide complex was filtered using energy and distance criteria. A stochastic molecular dynamics simulation was performed for each filtered configuration with the following conditions: thermal bath at 300 K, total simulation time equal to 1 ns, time step of 1.5 fs, AMBER* force field with united atom notation, water environment with the GB/SA implicit model of solvation. The 100 saved complex structures, one every 10 ps, were submitted to a full-energy minimization procedure in the same force field and solvation conditions. The most stable complex geometries were more than 94% populated at 300 K. The results of the molecular modeling work are reported in comparison to experimental alkylation (Table 2).

Table 2. Modeling Results with Respect to Alkylation

	1:G7	**4**:G7	**1**:G18	**4**:G18
exptl alkylation %	33	11	14	36
energy in kJ/mol	−47143	−47090	−47064	−47145
N7−C distance (Å)	3.24	3.34	5.09	2.94
N7−C−O angle (deg)	145	161	112	158
intermolecular H-bonds	2	0	2	3
Boltzmann % at 300 K	100	96	94	100

The most highly alkylated guanine bases were predicted to be the most stable complexes. The energy difference between them is not high, estimated at only 2 kJ/mol. In agreement with the experimental results, the number of intermolecular hydrogen bonds and alkylation distance slightly favored the **4**:G18 over the **1**:G7 complex. Docking details for these complexes are reported using the Pymol software (Figure 4).[28] In accordance with experimental data, both less reactive complexes were evaluated as less stable (Table 2). Although the **4**:G7/**1**:G18 relative energies did not reproduce the alkylation trend, the hydrogen bond networks were quite different in these models, stabilizing the configuration of the **1**:G18 complex in agreement with the experimental data.

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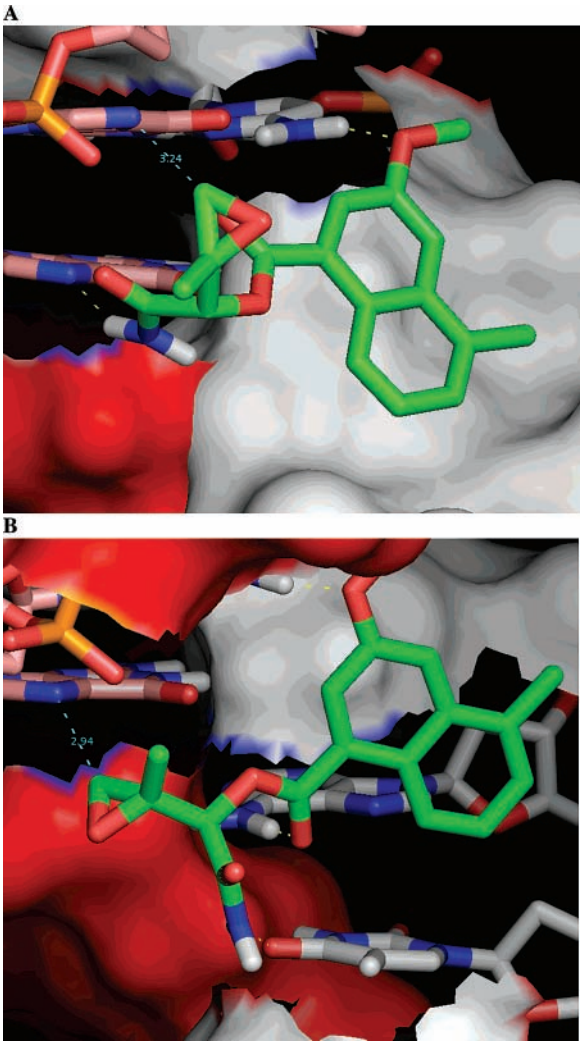

Figure 4. Energy minimum binding mode of **1** and **4** (green carbon atoms) interacting with DNA displayed in polytube style: (A) **1**:G7, (B) **4**:G18. Hydrogen bonds are shown as yellow dotted lines, and the epoxide methylene carbon−N7 distance is shown as a cyan dotted line labeled in angstroms. Nucleobases not directly involved with the interaction are reported with surface style rendering.

In conclusion, a computational model was designed to reproduce the experimentally enantioselective reactivity of compounds **1** and **4** against G7 and G18 DNA sites. The models revealed a major role for the hydrogen bonding network in stabilizing the complexes prior to alkylation.

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