Dimeric and trimeric derivatives of the azinomycin B chromophore show enhanced DNA binding†‡

Milena Balazy, Alejandra Fausto, Christina Voskanian, Bianca Chavez, Harmanpreet Panesar and Thomas G. Minehan*

To explore the utility of the azinomycin B chromophore as a platform for the development of major-groove binding small molecules, we have prepared a series of 3-methoxy-5-methyl-naphthalene derivatives containing diamine, triamine, and carbohydrate linker moieties. All bis- and tris-azinomycin derivatives are intercalators that display submicromolar binding affinities for calf-thymus DNA, as revealed by viscometry measurements and fluorescent intercalator displacement (FID) assays, respectively. Although the tightest binding ligand 1d ($K_a = 2.42 \times 10^7$ M$^{-1}$) has similar affinities for sequence diverse polynucleotides, competition binding studies with methylated phage DNA and known major and minor groove binding small molecules suggest that the tether moiety linking the naphthalene chromophores may occupy the major groove of DNA.

There are relatively few naturally occurring compounds that associate with the major groove of DNA. The vast majority of nucleic acid-binding natural products prefer to occupy the narrower minor groove, where hydrophobic and van der Waals interactions with the walls and floor of the groove are maximized. The notable exceptions to this trend are the pluramycins, aflatoxins, azinomycin, leinamycin, and neocarzinostatin i-gb. Intercalation of a planar delocalized-$\pi$ system into the backbone of DNA is a consistent binding mode among all of these natural products, and with the exception of the aflatoxins, all of these substances also possess polar major-groove binding moieties. After a detailed study of the DNA binding interaction of azinomycin B, Gates suggested that the 3-methoxy-5-methyl naphthalene chromophore is a uniquely effective small, uncharged intercalator that accurately positions appended groups in the major groove of DNA. Coleman’s subsequent studies on non-covalently binding azinomycin derivatives indicated that intercalation did not occur for these compounds. However, Searcy’s later investigation of similar naphthoate and naphthamide analogues of the azinomycin chromophore again provided evidence supporting an intercalative mode of binding. Given the synthetic accessibility of simple naphthalene derivatives, we envisioned that the azinomycin chromophore would be an ideal template for the development of sequence-specific major-groove binding small molecules. As a first step toward this goal, we have prepared a series of 3-methoxy-5-methyl naphthalene derivatives and evaluated their affinity for duplex DNA by ultraviolet and fluorescence spectroscopies.

DNA binding ligands possessing positively charged residues have a high affinity for duplex nucleic acids due to electrostatically favourable interactions with the negatively charged phosphate backbone and electronegative atoms in the major and minor grooves. Thus the primary series of naphthalene derivatives (Fig. 1, compounds 1-3) targeted for synthesis

Fig. 1 Designed ligands bearing the azinomycin chromophore.
contain basic nitrogen atoms in the tethers linking the chromophores. Furthermore, dimerization of intercalating moieties has been shown to result in high-affinity DNA-binding ligands such as ditercalinium\(^6\) and naphthalene diimides.\(^7\) It was envisioned that ligands 1a–1d, containing 2-, 3-, 4- and 5-atom tethers between the azinomycin chromophore units, would allow us to explore optimal distances between the aromatic moieties to achieve bis-intercalation.

Waring\(^8\) and McGee\(^9\) have previously demonstrated that the presence of a carbohydrate moiety on an intercalating chromophore contributes positively and significantly to the binding association with DNA. It is also likely that carbohydrate moieties directed into the major groove would be able to participate in non-covalent interactions with functional groups on the edges of the AT and GC base pairs.\(^10\) Ligand 3b was therefore designed as an initial candidate for assessing high-affinity binding in the major groove of DNA.

Ethyl 3-methoxy-5-methylnaphthalene-1-carboxylate, readily available from 1-(2-methylphenyl)-2-propanone by Shibuya’s protocol,\(^11\) was our starting material for the synthesis of azinomycin chromophore derivatives (Scheme 1). LiAlH\(_4\) reduction of the ester gave the corresponding alcohol, which was oxidized with PCC to give the corresponding aldehyde 5 in 91% overall yield. Compounds 1a–1c were prepared in 62–86% yields by combining 2 equivalents of naphthaldehyde 5 with one equivalent of ethylene diamine, propylene diamine, or butylene diamine, respectively, in CH\(_2\)Cl\(_2\) (Et\(_3\)N, MgSO\(_4\), rt, 12 h) followed by addition of CH\(_3\)OH and NaBH\(_4\) (5 equiv.) and stirring for 2 hours. Similarly, compound 1d was prepared in 52% yield by combining 3 equivalents of naphthaldehyde 5 with one equivalent of tris(2-aminoethyl) amine in methanol (rt, 18 h), followed by addition of NaBH\(_4\) (6 equiv.) and stirring at rt for 2 hours. Combining instead N-Boc-N-methylene diamine with one equivalent of 5 in methanol (rt, 18 h), followed by addition of NaBH\(_4\) (6 equiv., rt, 2 h) gave an intermediate carbamate, which was then treated with 1:1 TFA:DCM for 30 minutes and concentrated in vacuo to afford 2a in 68% yield after purification.

Compound 3b was derived from C-allyl pyranoside 6, which was prepared in turn according to the protocol of Trauner\(^12\) by TMSOTf-promoted ring opening of 1,6-anhydro-2,3,4-tri-O-benzyl-\(\beta\)-D-glucose in the presence of allyl trimethylsilane (Scheme 2). Oxidative cleavage (cat. OsO\(_4\), NMO, acetone/H\(_2\)O; KIO\(_4\), dioxane/H\(_2\)O) of the olefin and reduction (NaBH\(_4\), MeOH, 2 h, rt) provided an intermediate diol that was then treated with excess TsCl (Et\(_3\)N, DMAP, CH\(_2\)Cl\(_2\), 2 h, rt). The crude bis-tosylate was immediately combined with NaN\(_3\) (6 equiv.) in DMF (50 °C, 48 h) to provide bisazide 8 in 60% overall yield from 6. Stirring 8, PPH\(_3\) (2 equiv.) and naphthaldehyde 5 (2 equiv.) in benzene (1 M, 80 °C, 2 h), followed immediately by evaporation, dissolving in methanol, and treatment with NaBH\(_4\) (4 equiv., 2 h, rt) provided the intermediate bisamine 9 in 87% yield from 8. Boc protection (3 equiv. Boc\(_2\)O, Et\(_3\)N, DMAP, rt, 12 h) of the secondary amines, followed by catalytic hydrogenation (H\(_2\), cat Pd(OH)\(_2\), EtOH, rt, 12 h) and acidification (1:1 TFA/CH\(_2\)Cl\(_2\), 30 min, rt) then gave rise to ligand 3b in 52% yield from 9.

To compare the strengths of binding of compounds 1a–1d, 2a, and 3b to calf thymus (CT) DNA, the competitive ethidium displacement technique was employed to obtain apparent association constants (K\(_{app}\))\(^13\) and binding ratios (r\(_{bd}\)).\(^14\) From this analysis (Table 1) the tightest binding synthetic ligand was the trisazinomycin derivative 1d (K\(_{app}\) = 2.42 ± 1.5 × 10\(^7\) M\(^{-1}\)), which has an apparent association constant more than 50-fold greater than that obtained for monoazinomycin derivative 2a (K\(_{app}\) = 4.7 ± 0.3 × 10\(^6\) M\(^{-1}\)). In addition, the bisazinomycin derivative 1a (K\(_{app}\) = 7.2 ± 0.9 × 10\(^6\) M\(^{-1}\)) has a 15-fold higher binding constant for CT DNA than 2a, and since both

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**Scheme 1** Synthesis of ligands 1a–d and 2a.

**Scheme 2** Synthesis of ligand 3a.
ligands have two positively charged protonated nitrogen atoms at pH = 5.48 and bind approximately the same number of base pairs ($n_{bd} \approx 4.0$), these results are suggestive of a bis-intercalative mechanism of association for the bis- and trisazino derivatives with DNA (vide infra). Interestingly, ligand 3b ($K_{app} = 6.5 \pm 0.7 \times 10^6 \text{M}^{-1}$) showed no significantly enhanced binding relative to 1a–1c, perhaps indicating that any stabilization due to hydrogen-bond interactions between the glucosyl moiety linking the azinomycin chromophores and the DNA bases is offset either by steric crowding or entropic restriction of bond rotation in the intercalated complex.

To investigate the importance of electrostatic interactions$^5$ in the binding of the azinomycin chromophore derivatives to DNA, we evaluated the association of both 1a and 1d with CT DNA under high- and low-salt conditions using the ethidium displacement assay (Table 2). Increasing NaCl concentrations in the buffer (10 μM CT DNA in 10 mM Tris-EDTA, pH = 5.48) from 0.01–1.0 M results in an approximately four-fold drop in 1d’s DNA binding affinity; a similar lowering of DNA affinity was observed for 1a between the concentrations of 0 mM and 100 mM NaCl. These data suggest that the positively charged protonated nitrogen atoms may be involved both in polar hydrogen bonding interactions in the grooves of DNA and in ionic interactions with the phosphate backbone.

To evaluate the groove occupancy of the aliphatic tethering moiety connecting the intercalating naphthalene chromophores of our ligands, we investigated the displacement of ethidium bromide from CT DNA by 1d in the presence of either the minor groove binding agent netropsin (NP, 30 μM) or the major groove binding agent methyl green (MG, 30 μM).$^{18,19}$ The addition of netropsin resulted in an increase in the $C_{so}$ value (concentration of 1d required to achieve a 50% decrease in the fluorescence of ethidium) from 0.85 μM to 1.5 μM, corresponding to a 1.7-fold drop in the binding affinity of 1d for CT DNA (Fig. 2). The addition of methyl green instead resulted in a more pronounced increase in the $C_{so}$ value from 0.85 μM to 2.0 μM, corresponding to an approximately 2.4-fold drop in the binding affinity of 1d for CT DNA. These data suggest that the tethering moiety of 1d may occupy either the major or minor grooves of DNA. Subsequent experiments (see below) provided additional evidence supporting major groove occupancy by 1d.

The binding of 1d to different DNA polynucleotides was also explored using the ethidium displacement assay (Table 2). The $K_{app}$ values for the association of 1d with poly(G-poly(dC), poly(dA)-poly(dT), and poly[d(a-T)]$_2$ were in the range of 18–20 × 10$^6$ M$^{-1}$, indicating similar affinities for these binding sites. The higher value obtained for calf thymus DNA, however, likely indicates that 1d prefers to bind heterogeneous sequences of natural B-form DNA. Since lambda phage DNA$^{20}$ is methylated in the major groove (N$_6$-methyl adenine, C$_7$-methyl cytosine), the three-fold lower binding constant observed for the association of 1d with phage DNA vs. calf thymus DNA suggests that the aliphatic tethering moiety may occupy the major groove of DNA. In contrast, it was found that the minor groove binder netropsin binds to lambda phage DNA with a slightly higher apparent affinity than to calf thymus DNA ($3.79 \pm 0.26 \times 10^5$ M$^{-1}$ and 2.11 ± 0.79 × 10$^5$ M$^{-1}$, respectively; see ESI†).

Monitoring the UV absorbance of 1d at 228 nm upon titration with CT-DNA revealed a distinct hypochromic shift along with a slight redshift to 236 nm, observations again suggestive of an intercalative mode of binding (Fig. 3).$^{15,16}$ To confirm this, we performed viscometry studies for all synthetic

| Table 1: Estimation of CT-DNA association constants and stoichiometry for ligands 1a–d, 2a and 3b by ethidium displacement assays |

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_{app}$ ($\times 10^6$ M$^{-1}$)</th>
<th>$n_{bd}$</th>
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<tbody>
<tr>
<td>1a</td>
<td>7.19 ± 0.92</td>
<td>4.2</td>
</tr>
<tr>
<td>1b</td>
<td>3.15 ± 0.60</td>
<td>6.2</td>
</tr>
<tr>
<td>1c</td>
<td>5.00 ± 0.46</td>
<td>7.0</td>
</tr>
<tr>
<td>1d</td>
<td>24.2 ± 1.5</td>
<td>9.1</td>
</tr>
<tr>
<td>2a</td>
<td>0.47 ± 0.03</td>
<td>4.0</td>
</tr>
<tr>
<td>3b</td>
<td>6.47 ± 0.65</td>
<td>10.0</td>
</tr>
</tbody>
</table>

$^a K_{app}$ values obtained by the competitive ethidium displacement method (10 mM Tris-EDTA, pH = 5.48), where $K_{app} = K_a \times C_a/C_{so}$ and $K_a = 2.1 \times 10^6$ M$^{-1}$. $^b n_{bd}$ (ratio of CT DNA (bp) : ligand) values were determined from the breakpoint of the curve in a plot of Δ fluorescence vs. CT DNA : ligand ratio, with the data obtained from the competitive ethidium displacement method.$^{14}$

| Table 2: Evaluation of the binding of 1d to different DNA polynucleotides by ethidium displacement assay |

<table>
<thead>
<tr>
<th>DNA</th>
<th>$K_{app}$ ($\times 10^6$ M$^{-1}$)</th>
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<tbody>
<tr>
<td>PolyG-polydC</td>
<td>18.4 ± 0.6</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>24.2 ± 1.3</td>
</tr>
<tr>
<td>Poly[d(a-T)]$_2$</td>
<td>17.7 ± 1.5</td>
</tr>
<tr>
<td>PolyA polydT</td>
<td>20.0 ± 2.5</td>
</tr>
<tr>
<td>Lambda phage</td>
<td>8.02 ± 0.7</td>
</tr>
</tbody>
</table>
Increasing concentrations of 1d (Fig. 4) in the presence of CT-DNA resulted in an increase in solution viscosity comparable to that obtained with reference compound ethidium bromide, indicative of the helix lengthening and rigidification that occurs upon ligand intercalation. Similar results were obtained for ligands 1a–1c, 2a, and 3b (see ESI†). By comparison, the non-intercalative minor groove binder netropsin showed a relatively minimal increase in solution viscosity under the same conditions.

CD spectra of CT DNA recorded in the 220–310 nm region in the presence of increasing concentrations of 1d revealed an initial increase in the intensities of both the helicity band at 240 nm and base-stacking band at 280 nm at low 1d : DNA ratios, followed by a decrease in both bands at higher ligand concentrations (Fig. 5). These observations, which mirror data previously recorded for the titration of CT DNA with ethidium bromide, are indicative of an intercalative mode of DNA binding.

Molecular docking studies of ligand 1d with 5′-ATGCAT-3′ (PDB 1×95) were performed using Autodock vina. The energy-minimized bis-intercalation complex shows the chromophore tethering moiety occupying the major groove of DNA, with the ligand amino groups engaging in hydrogen bonding contacts with the edges of the sandwiched GC base pairs (Fig. 6).

Conclusions

We have prepared a series of dimeric (1a–1c, 3b) and trimeric (1d) derivatives of the azinomycin chromophore that show submicromolar binding affinities for duplex DNA. Viscometry data...
indicate that each of these ligands intercalate the backbone of DNA. Although the tightest binding derivative, 1d, was shown to have low sequence selectivity, competitive binding studies with methyl green and lambda phage DNA indicate that association of this ligand with DNA may take place via the major groove. Since the CD and viscometry data for the interaction of 1d with CT-DNA are very similar to that for ethidium bromide,22 a bis-intercalation model for the association of 1d with DNA is proposed, with the tethering moiety occupying the major groove.

We are currently exploring the cell permeability and cytotoxicity of these compounds (via MTT/MTS assays)23 and the results of these studies will be reported in due course.

Acknowledgements

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Notes and references

16 Thermal denaturation studies of calf thymus (CT) DNA in the presence and absence of 1d were complicated by overlap of absorbances of drug and DNA in the 260–280 nm region of the UV spectrum (Fig. 2): W. D. Wilson, F. A. Tanious, M. Fernandez-Saiz and C. T. Rigl, Methods Mol. Biol. Drug, 1997, 90, 219.
23 Minimized using Spartan 14 for Macintosh (Wavefunction, Inc., Irvine, CA).
26 Additional binding modes for the association of 1d with 5′-ATGCAT-3′ were apparent from the molecular docking studies, and these included mono-intercalation from the major groove and non-intercalative major groove binding (see ESI‡). Though the DNA search space included both major and minor grooves, none of the computed binding modes showed the ligand associating with the minor groove of DNA.