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Binding mode and affinity studies of DNA-binding agents using topoisomerase I DNA unwinding assay

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Abstract—A topoisomerase I DNA unwinding assay has been used to determine the relative DNA-binding affinities of a model pair of homologous naphthalene diimides. Binding affinity data were corroborated using calorimetric (ITC) and spectrophotometric (titration and T_m) studies, with substituent size playing a significant role in binding. The assay was also used to investigate the mode of binding adopted by several known DNA-binding agents, including SYBR Green and PicoGreen. Some of the compounds exhibited unexpected binding modes.

The interaction of small molecules with DNA continues to be a prolific area of study, in part because a number of therapeutically important compounds bind reversibly to nucleic acids.[1–6] Determination of the binding thermodynamics, relative binding affinity, as well as the exact mode of binding has often been instructive in deciphering the molecular interactions involved.[4,7] Assessment of the relative contributions to binding from substituent groups has also been important. Contributions from substituent groups can be reliably assessed by comparing binding of homologous compounds. It has been suggested that some DNA-binding drugs, especially in the minor groove class, exhibit multiple binding modes while binding in a sequence specific manner.[8–11] In fact, some researchers have proposed that the anticancer efficacy of these drugs may be linked to their ability to exhibit mixed binding modes.[12,13] The existence of additional binding modes for minor groove binders has however been difficult to grasp given the widely accepted view that the crescent-shaped structure typical of these compounds is isohelical with the DNA minor groove.

In the present work, we have used a topoisomerase I DNA unwinding assay (Topo I assay)[14] to investigate the relative DNA-binding affinities of a model homologous pair of naphthalene diimides (NDI) (Fig. 1), as well as the binding mode adopted by several DNA-binding agents with known therapeutic activities [netropsin, distamycin A, berenil, Hoechst 33258, and 4′,6-diamidino-2-phenylindole (DAPI)] and which are generally regarded as typical minor groove binders. Additionally, we report a preliminary investigation of the binding mode adopted by SYBR Green I and PicoGreen, two fluorescent cyanine dyes widely used in double-stranded DNA detection and quantification. We have employed a combination of isothermal titration calorimetry (ITC),[15] spectrophotometric titrations,[16] and UV-thermal melting[17] studies in order to corroborate our binding affinity data.

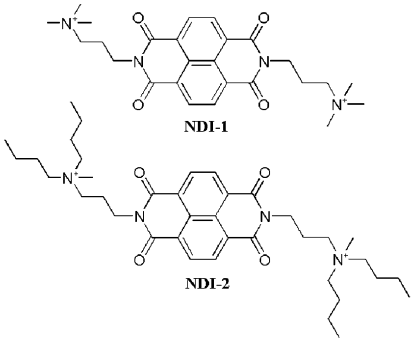

Figure 1. Homologous pair of naphthalene diimides studied. A quaternary amino group is incorporated into each side chain to provide electrostatic interaction with the negatively charged DNA backbone.

The Topo I assay exploits the ability of Topo I to relax supercoiled DNA. Briefly, intercalators will cause re-supercoiling of plasmid DNA after initial relaxation by

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Topo I and subsequent removal of the compound and enzyme.[18,19] Re-supercoiling is due to the change in DNA linking number that accompanies relaxation by the enzyme.[18] Conversely, minor groove binders should not induce re-supercoiling due to negligible DNA unwinding on binding. To assess the usefulness of the Topo I assay in determining relative DNA-binding affinity, we have studied a pair of homologous NDI intercalators. The NDI compounds were synthesized as previously described.[20] These symmetrical molecules have two substituents (trimethyl- or dibutylmethyl-propylamino) on either side of the intercalating moiety and differ in substituent size and hydrophobicity. The threading molecular geometry also requires that the side chains are involved in binding.[11] As expected, we have observed re-supercoiling of our plasmid DNA by the NDI compounds consistent with an intercalative binding mode (Fig. 2). It should be noted that complete re-supercoiling (compared to the control lane with only DNA) by **NDI-1** occurred at lower drug concentrations (3 μM) than **NDI-2** (5 μM) suggesting a higher binding affinity for **NDI-1**. Initial DNA unwinding will be dependent only on the extent to which binding occurs, and not on intercalator structure because the intercalating moiety in both molecules is the same. Therefore, the minimum concentration (or drug/DNA_{bp} ratio) of each compound required to cause complete re-supercoiling of the DNA will be indicative of how much compound was initially bound and thus indicated the relative binding affinity.

The higher binding affinity of **NDI-1** was also consistent with results we obtained using ITC (Fig. 3), spectrophotometric titration, and thermal melting (Table 1). These approaches all yielded binding affinity data (K or ΔT_m) that showed **NDI-1** having a greater DNA-binding affinity than **NDI-2**, thus supporting the utility of the Topo I assay in determining the relative binding of homologous intercalators. The stronger binding exhibited by **NDI-1** can only be due to contributions from either molecular size and/or hydrophobicity given that the two compounds differ only in these features. We have measured experimental octanol/water partition coefficients ($\log P$)[21] for the two compounds and have found that **NDI-2** ($\log P = -0.9$) is over 40 times more hydrophobic than **NDI-1** ($\log P = -2.5$). Hydrophobicity has been known to be a major determinant of DNA binding, with binding affinity generally increasing with the addition of hydrophobic groups.[2,4] However, given that **NDI-2** is significantly more hydrophobic, but still shows less

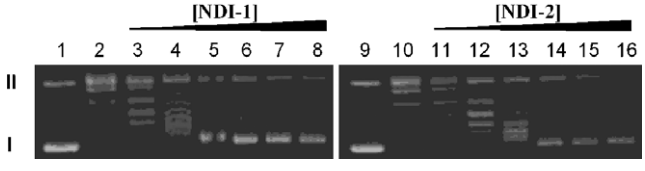

Figure 2. Topo I assay of **NDI-1** (left) and **NDI-2** (right) using 5 U of Topo I. Lanes 1 and 9 contain only DNA (neither compound nor Topo I) and serve as controls. Lanes 2 and 10 contain DNA and Topo I, but no compound. Lanes 3–8 and 11–16 contain DNA, Topo I and increasing concentrations of compound (1, 2, 3, 5, 6, and 7 μM).

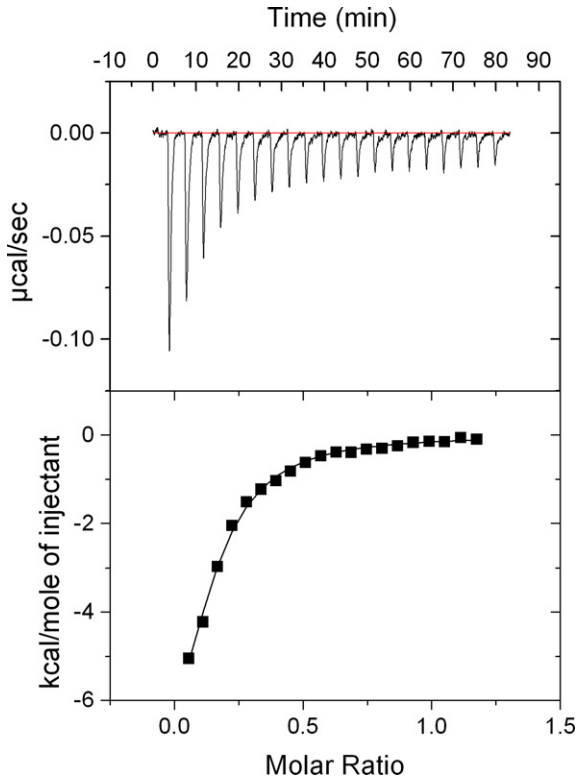

Figure 3. Calorimetric data (raw) for the titration of **NDI-1** (75 μM) into calf thymus DNA (9.5 μM) at 25 °C (top). Binding isotherm (heat change vs drug/DNA molar ratio) was obtained from the integration of raw data and fitted to a 'one-site' binding model (bottom).

binding to DNA than **NDI-1**, argues for molecular size playing a greater role during binding in our system. We have previously reported a similar DNA binding trend as a function of molecular size using a homologous series of anthraquinones.[22]

Of the other compounds studied using the Topo I assay, only berenil, distamycin A, and DAPI exhibited the expected result for minor groove binders (Fig. 4), that is, no re-supercoiling even at drug/DNA_{bp} ratios (r-values) as high as 2. However, netropsin, regarded as a classical minor groove binder, and Hoechst 33258 (H33258), another compound viewed as a typical minor groove binder, caused varying extents of re-supercoiling. In the case of netropsin, re-supercoiling was not as pronounced as that observed for the known intercalator, ethidium bromide (EtBr), which was included for comparison. In fact, only a few partially re-supercoiled topoisomers were observed even at high r-values, and complete re-supercoiling was never attained. The extent of re-supercoiling was unchanged after $r = 0.27$ and is consistent with viscometric results obtained by Breslauer and coworkers who observed a slight increase in DNA solution viscosity with no further increase after $r = 0.34$.[13] Although this result seems to imply an intercalator-like binding mode, according to these researchers, it may be due to non-specific exterior binding by netropsin resulting in increased stiffness and thus changes in the hydrodynamic mobility of the DNA. Other groups have also reported that netropsin is capable of introducing supercoils into plasmid DNA via non-inter-

Table 1. Binding affinity data for **NDI-1** and **NDI-2**

Compound	log P[a]	ΔT_m[b] (°C)	K (ITC)[c] (10^5 M^{-1})	K (Spec. tit.)[d] (10^5 M^{-1})	Topo I assay[e] (μM)
NDI-1	−2.5	7.6	12.2 ± 1.6	5.0 ± 0.6	3
NDI-2	−0.9	4.6	5.7 ±1.7	1.0 ± 0.1	5

[a] Experimental *n*-octanol/water partition coefficient.
[b] Na_2HPO_4 buffer (20 mM), pH 7.0.
[c] MES00 buffer, pH 6.25.
[d] Spectrophotometric titration, BPES buffer, pH 7.0.
[e] Minimum concentration of compound required for complete re-supercoiling.

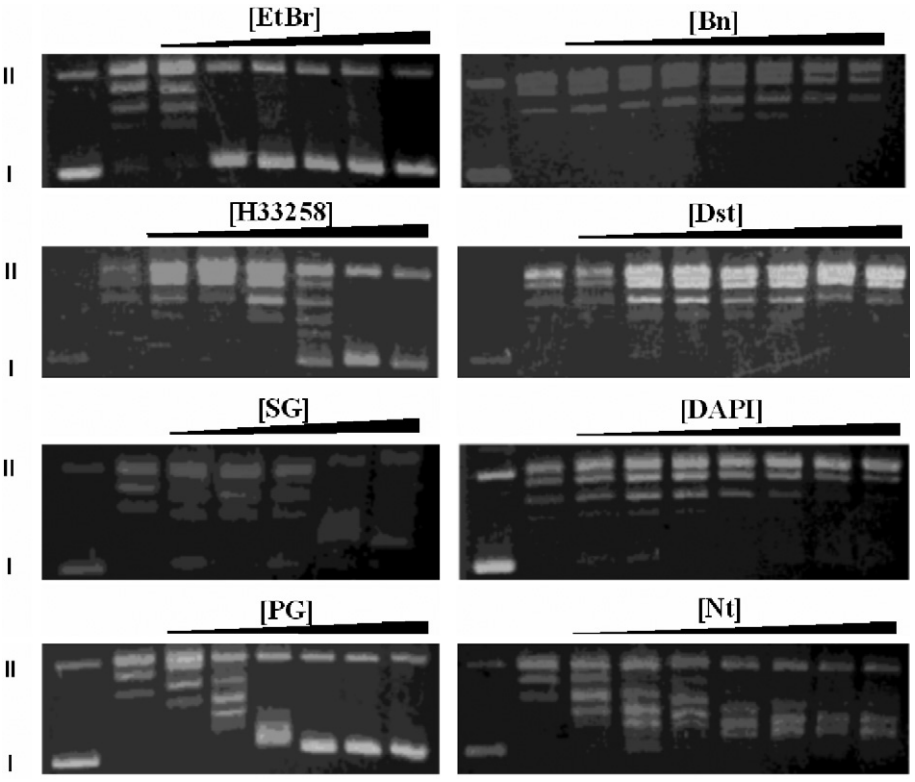

Figure 4. Topo I assay of EtBr, berenil (Bn), H33258, distamycin A (Dst), SYBR Green 1 (SG), DAPI, PicoGreen (PG), and netropsin (Nt) using 5 U of Topo I. In each case, the first lane (from left) contains only DNA and serves as a control, while the second lane contains DNA and Topo I, but no compound. Remaining lanes contain DNA, Topo I, and increasing concentrations of compound (0–20 μM).

calative binding.[23] H33258, on the other hand, exhibited features that were not unlike those of the classical intercalator, EtBr. Much like EtBr, H33258 caused complete re-supercoiling of the DNA albeit at higher *r*-value ($r = 0.38$, as opposed to $r = 0.11$ for EtBr). The higher *r*-value for H33258 required for re-supercoiling may indicate an initial groove binding mode at low drug/DNA ratios, followed by intercalation as drug concentration increases. This bimodal characteristic was also implied by our spectrophotometric titration results which revealed two non-equivalent binding constants (K); an initial high affinity K ($1.8 \pm 0.4 \times 10^7$ M^{-1}), followed by a lower affinity K ($6.7 \pm 0.7 \times 10^4$ M^{-1}). Since groove binding is often associated with greater binding affinity than intercalation,[4,11] the order of binding constant is consistent with groove binding followed by intercalation. This result for H33258 is in accordance with published work,[24] however, it is interesting to note that, like H33258, DAPI and berenil have also been reported to exhibit both minor groove and intercalative binding at AT- and GC-rich sequences, respectively.[9,13] These studies have found evidence for intercalation of DAPI and berenil at drug/DNA_{bp} ratios between ~0.2 and 0.4, well within the range of *r*-values ($\leqslant 0.8$) studied in our experiments. However, we have not observed intercalation of either compound. We believe this is due to two main factors. First, many of the studies in which evidence for intercalation of DAPI or berenil was observed involved using short specific DNA sequences such as poly[d$(GC)_2$]. However, in our studies, we used a 2686 base pair long heterogeneous plasmid, pUC19. Heterogeneity in potential binding sites will presumably present the drug molecules with additional binding site options. It is therefore expected that it would be more difficult for the drug molecule to 'find' the requisite GC-rich sequence in our system given the higher level of sequence diversity. Wilson and coworkers have reported a viscometric study using a natural plasmid DNA, and showed that DAPI caused only a slight increase in solution viscosity (~16%) as compared to

ethidium, a classical DNA intercalator, which caused a ~100% increase in viscosity.[9] This suggests that DAPI does not cause significant DNA unwinding in plasmid DNA, probably due to only partial intercalation of the amidinophenyl moiety,[11] and is consistent with the lack of re-supercoiling observed in our Topo I assays. Interestingly, berenil is also expected to only partially intercalate since it also contains amidinophenyl groups. Second, the ability of berenil to cause viscosity increases in DNA was reported to be extraordinarily sensitive to salt concentration.[13] In fact, the viscosity studies revealed that berenil exhibit noticeable intercalation only at very low salt concentration (2 mM Na^+), which is significantly lower than the salt concentration (150 mM Na^+) used in our Topo I assays. The high salt dependency of the binding mode adopted by berenil was also observed by Barcelo and coworkers in their ITC and circular dichroism studies.[8] We therefore propose that under the conditions of our Topo I assay, only 'mixed-mode' drug molecules with sufficiently strong intercalative binding abilities, such as H33258, will lead to DNA re-supercoiling. It is also worth noting that some of the compounds studied herein (particularly distamycin A and H33258) have been linked to Topo I inhibition,[25–27] however, given that our plasmid was first relaxed by excess Topo I before any compound was added, the presence of re-supercoiling can only be due to binding of the compound and not as a result of enzyme inhibition.[28]

SYBR Green I (SG) and PicoGreen (PG) are two highly sensitive fluorescent dyes routinely used in a variety of double-stranded DNA assays and real-time PCR protocols.[29–31] To date, information on the exact binding mode adopted by SG and PG have been scarce. Such information could have implications with regard to drug design and molecular biological applications given the highly sensitive diagnostic and bioanalytical applicability of these compounds. Vitzthum and coworkers have recently proposed structures and approximate extinction coefficients for both PG and SG.[32] These researchers have proposed a biphasic DNA-binding mode for SG, with an initial intercalative mode followed by groove binding at high drug/DNA ratios. This biphasic feature is presumably also implied for PG given the distinct structural similarities reported. Our Topo I assay clearly shows that PG binds via intercalation since the compound (a 500 times diluted stock solution) caused re-supercoiling of the plasmid DNA that was similar to the standard intercalator (EtBr) (Fig. 4). Although the Topo I assay for SG also suggests intercalation, re-supercoiling was not as pronounced as that shown by PG and occurred at higher (~5 times) drug concentrations. This may imply a difference in the intercalative abilities of the two compounds. Since the exact stock concentrations of PG and SG have not been disclosed by the manufacturer, we will not attempt to report the drug/DNA ratio that elicited re-supercoiling. Of note, we observed that SG induced a gel mobility shift at high drug amounts (a 60 times diluted stock solution) and may indicate a change in the nature of the interactions. If the structures of SG and PG proposed by Vitzthum and coworkers are correct, it could be envisaged that the fused bicyclic rings could potentially slide between adjacent DNA base pairs to form an intercalation complex, although not completely filling the intercalation site.[11] This may be the case for H33258, which also contains bicyclic ring systems and has been shown to intercalate even though it possesses several features typical of minor groove binders.

In summary, we have shown the usefulness of the Topo I assay in determining the relative DNA-binding affinities of homologous intercalators. Our data support the importance of substituent size during DNA binding and it may be of interest to determine whether this is a common trend amongst other similar series. The Topo I assay was also used to provide evidence for the involvement of an intercalative binding mode for PG and possibly SG. To our knowledge, this is the first report that has utilized a Topo I assay approach to investigate the mode of binding adopted by SG and PG. H33258 and the NDI derivatives were also shown to bind via intercalation with the remaining compounds binding primarily via the DNA minor groove.

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14. Typically, 0.24 μg of supercoiled pUC19 plasmid DNA (Bayou Biolabs, Harahan, LA) was incubated with 5 U of human topoisomerase I enzyme (TopoGEN Inc., Port Orange, FL) for 5 min at 37 °C in 1× Topo I reaction

buffer. The appropriate amount of compound was then added and the reaction mixture incubated for a further 1 hr at 37 °C. The reaction was terminated using 0.5% SDS and 0.5 mg/mL proteinase K. Subsequent incubation for an additional 15 min was followed by enzyme and compound extraction using a mixture of phenol/chloroform/isoamyl alcohol (25:24:1). The remaining DNA sample was then run on an agarose gel (1%) at 75 V for 3 h, stained with ethidium bromide, and photographed (type 667 film).

15. Calorimetric titrations were carried out on a MicroCal VP-ITC (MicroCal Inc., Northampton, MA). The data were analyzed using the Origin 7.0 software provided by the manufacturer. All experiments were run at 25 °C in MES00 buffer (1×10^{-2} M MES (2(*N*-morpholino)ethanesulfonic acid) containing 1×10^{-3} M EDTA, with the pH adjusted to 6.25 with NaOH). Exactly 12 μL of the drug solution (7.5×10^{-5} M) was injected into a buffered solution of DNA (calf thymus DNA, 9.5×10^{-6} M in bp, 1.4 mL) over 24 s at 300 s intervals using a 250 μL syringe rotating at 290 rpm. Samples were degassed at 20 °C using a ThermoVac apparatus (MicroCal) before use. Each peak corresponded to the decrease in the power supplied to keep the temperatures of the sample and reference cells the same for each injection and represents the heat given off. In each case, response signals were corrected for the small heat of dilution associated with titrating the drug into the buffer. The heat of dilution for titrating buffer into DNA was found to be negligible. The heat released on binding was directly proportional to the amount of binding. A binding isotherm of heat released versus the molar ratio was constructed and the data fitted by non-linear least square fitting analysis to a model based on a single set of identical binding sites.
16. Typically, a concentrated solution of calf thymus DNA ($\sim 2 \times 10^{-4}$ M bp) was titrated into 2300 μL of a fixed concentration of compound solution in buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl, pH 7.0), by adding 50–100 μL increments with constant stirring. Absorbance values (A_{obs}) were monitored at the λ_{max} of the compound using a Varian Cary 100 Bio UV–visible spectrophotometer equipped with a temperature controller set at 25 °C. A_{obs} were used to determine the apparent extinction coefficient ($E_a = A_{obs}$/[compound]), and the intrinsic binding constant (K) determined from a plot of $[DNA]_{bp}/(E_a - E_f)$ versus $[DNA]_{bp}$, where E_f is the extinction coefficient of the free compound. K was given by the ratio of the slope to the y-intercept.[33]
17. Thermal denaturation (T_m) studies were conducted on a Varian Cary 100 Bio UV–visible spectrophotometer attached to a temperature controller. Experiments were performed in 20 mM sodium phosphate buffer, pH 7.0. Melting profiles were obtained by measuring the absorbance at 260 nm as a function of temperature (25-95 °C) at a ramp rate of 0.5 °C/min. The concentration of DNA (calf thymus) was generally 10 μM (bp) with a DNA/drug ratio of 3:1.
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21. Experimental partition coefficient (log *P*) for each NDI compound was determined by the *Shake-flask* method by preparing a saturated solution in water, followed by thorough mixing with an equal volume of *n*-octanol. The mixture was then allowed to partition and equilibrate overnight. Subsequent spectrophotometric determination of the relative NDI concentration in *n*-octanol versus water layers yielded the *n*-octanol/water log *P*.
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