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A Circular Dichroism Spectroscopy and Isothermal Titration Calorimetry Approach to Effectively Characterize the DNA Binding Mode of Compounds

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Abstract

Isothermal titration calorimetry (ITC) and circular dichroism spectroscopy (CD) were used to investigate the binding of eight compounds [quinacrine, daunomycin, ethidium bromide, a naphthalene diimide, distamycin, 4',6-diamidino-2-phenylindole (DAPI), berenil and Hoechst 33258] to two 20-bp DNA duplexes, $[poly(dAdT)]_2$ and $[poly(dGdC)]_2$. A primary goal was to illustrate the utility, ease and convenience of using ITC and CD in tandem to characterize the DNA binding mode of potential compounds, based on their preference for AT-rich versus GC-rich DNA sequences. Our CD studies showed that the four compounds (distamycin, DAPI, berenil, Hoechst 33258) known to bind DNA via the minor groove, elicited strong induced CD (ICD) signals between 300 - 400 nm when binding to the AT-rich DNA sequence. No ICD signal was observed for distamycin, DAPI and berenil when titrated into the GC-rich DNA sequence. Hoechst 33258, however, showed strong binding to both the AT-rich and GC-rich DNA sequences, indicating a mixed binding mode, likely involving DNA intercalation. All four of the compounds known to bind via intercalation displayed significant binding to both DNA sequences, with perturbations at the signature bands for DNA (~245 and 275 nm). Our ITC thermodynamic studies corroborated the CD results showing strong AT-DNA sequence binding for distamycin, DAPI and berenil (K~$10^{6\text{-}7}$ M^{-1}) with only background binding (associated with the heats of dilution) to the GC-DNA sequence, while Hoechst 33258 showed binding to both DNA sequences, indicating an additional binding mode. ITC showed that the four intercalators bound strongly to both DNA sequences. Binding of the compounds to the DNA sequences were enthalpically driven with large negative

enthalpy changes accompanied by generally small negative entropy changes.

Keywords

Isothermal Titration Calorimetry, Circular Dichroism Spectroscopy, DNA Binding Mode, Intercalation versus Minor Groove Binding, AT versus GC DNA

1. Introduction

It is now well established that several known therapeutic drugs interact with DNA as a means of carrying out their actions [1]-[5]. The targeting of DNA by therapeutic compounds is unsurprising given the critical role played by DNA, serving as the blueprint for essentially all biomolecules and biological processes [3]. The availability of binding sites in DNA makes it a prime target for many therapeutic drugs which includes drug categories from anticoagulants and antihistamines, to antimicrobials and anticancer as cell-cycle or signal-transduction blockers and inhibitors of the central dogma [6]-[12]. The two principal binding sites in DNA are via intercalation, where planar molecules slide between adjacent DNA base pairs, and/or minor-groove binding, where molecules with the requisite isohelicity with the DNA minor groove fit snugly into this DNA groove [13]-[15]. Some of the most notable members of the DNA binding therapeutic drug class include intercalators such as the anticancer anthracyclines (e.g., daunomycin) and the acridines (e.g., quinacrine), while notable minor groove binders include distamycin, DAPI (4',6-diamidino-2-phenylindole), berenil and their analogues [5] [15]. These therapeutic compounds have been found to bind to DNA while interfering with the activities of many vital enzymes and protein factors involved in DNA metabolism. For example, early work showed that daunomycin exerts its cytotoxic effect by trapping the ternary complex formed between daunomycin and DNA-topoisomerase II, slowing proliferation in cancer cells [16]-[18], while quinacrine and other acridines have been found to bind DNA via intercalation, followed by inhibition of topoisomerase [19] [20], or the epigenetic enzyme, DNA methyl transferase, responsible for maintaining the gene repressive marker, 5-methylcytosine, thus reactivating key tumor suppressing genes such as p16 [21]. Other DNA binding ligand of interest include the naphthalene diimides (NDIs), known for their versatility as a tunable scaffold in the synthesis of many therapeutic derivatives especially in the targeting of G-quadruplex DNA in telomeric regions, while blocking telomerase activity, and more recently, targeting the k-RAS oncogene in treating pancreatic cancer [22]-[25]. It is therefore clear that effective detection of the preferred DNA binding mode/mechanism is critical to our understanding and characterization of these drug-DNA interactions.

Today, some researchers within the field utilize high-end approaches such as NMR and X-ray crystallography studies in an attempt to decipher the structure of

these drug-DNA complexes [26]-[30]. Of course, these approaches have the potential to yield detailed structural data, but only if they are in-house, accessible and accompanied by the requisite expertise to interpret such data [31]. NMR and X-ray studies are typically more expensive and requires resource intensive instrumentation (e.g., NMR requires high magnetic fields) relative to other applicable biophysical techniques such as circular dichroism (CD) spectroscopy and isothermal titration calorimetry (ITC), which can be successfully utilized to solve drug-DNA interactions [6]-[9] [32]-[34]. For example, CD and ITC are fast, less expensive and convenient approaches requiring small sample sizes/concentrations and are conducted exclusively in solution, so they do not require crystallization (such as for X-ray) which could select for a specific less prominent conformations [35]-[37]. NMR and X-ray experiments are typically done in more restrictive experimental conditions, while CD and ITC offer a wide range of tunable experimental milieu, where conditions of study can be optimized [38]. Furthermore, NMR interpretations of large biomolecular complexes containing numerous atoms/nuclei in proximity can prove to be very tedious, while X-ray crystallography is limited to static structures, thus dynamic analyses of biomolecular complexes are mostly absent. Although NMR and X-ray crystallography still remain the gold standards for biomolecular structural analyses, in this study, we show that CD spectroscopy and ITC can be combined to serve as efficacious preliminary (*first-step)* biophysical methods to characterize the preferred DNA binding mode adopted by therapeutic compounds. To illustrate the utility, ease and convenience of using CD spectroscopy and ITC to characterize drug-DNA interactions, we have investigated *four* known DNA intercalators (daunomycin, quinacrine, ethidium bromide and a NDI) and *four* groove binders (distamycin, DAPI, berenil, Hoechst 33258) (**Figure 1**) binding to $[poly(dAdT)]_2$ compared to $[poly(dGdC)]_2$ DNA duplexes. In this study, we show that the preferred DNA mode of a drug (or drug candidate) can be conveniently determined using the two techniques (CD and ITC) in tandem and two short DNA sequences ($[poly(dAdT)]_2$ and $[poly(dGdC)]_2$ 20-mer DNA duplexes), **Figure 2**.

It has been established that known groove binding compounds (e.g., distamycin and berenil) show a strong preference (an order of magnitude or greater) for binding to AT-rich DNA sequences relative to GC-rich DNA [32] [39]-[41]. The lower affinity for GC-rich DNA sequences exhibited by groove binders is largely due to (1) their restricted access to the minor groove of GC-rich sequences caused by the protruding 2-NH_2 group of guanine [40] [42]. Furthermore, it has been reported that the AT-rich minor groove is narrower and possesses a more electronegative potential relative to its GC-rich counterpart, thus providing a more conducive and snug fit for a positively charged drug to bind [42] [43]. On the other hand, DNA intercalators are expected to bind both sequences with similar affinity and are only expected to be affected if a substituent is placed into the DNA minor groove during formation of the intercalation complex. It is also expected that compounds that exhibit mixed binding mode (*i.e.*, intercalation and groove binding) will exhibit less of a preference for the AT-rich sequence [32] [41] [44]-[46].

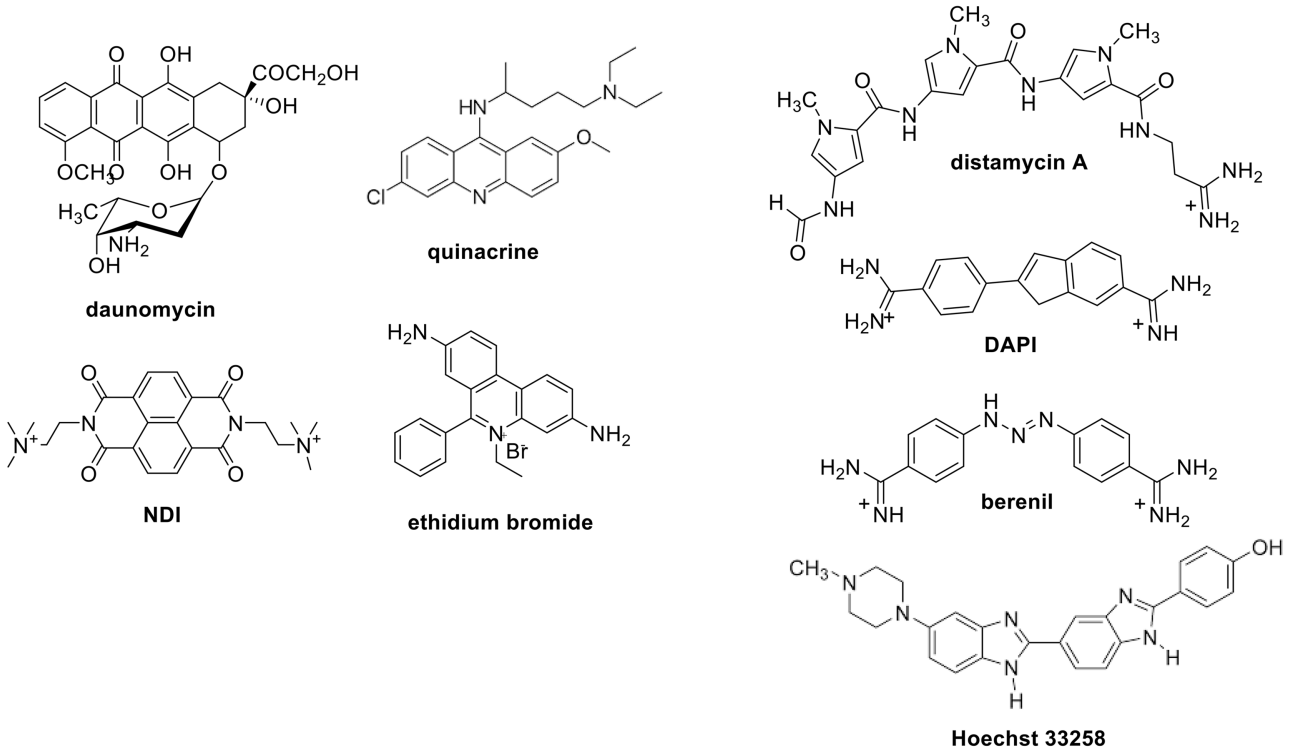


Figure 1. Structures of eight compounds in this study.

CD spectroscopy is a biophysical technique that can be used to detect structural changes in DNA as a compound binds. For double-stranded DNA intercalation, these changes typically result in perturbation of one or both of the signals/bands at the signature wavelength for DNA, *i.e.*, a minimum ~245 nm and maximum around 275 nm, corresponding to the overall DNA helicity and base-stacking, respectively [47]. Structural perturbations in DNA occur because during intercalation, there is a requirement for the DNA to unwind and unstack in creating the intercalation site. Since the DNA groove is a pre-existing site, groove-binding does not typically require significant changes in the structure of the DNA. As a consequence, CD spectra for a DNA groove binder binding to DNA is not expected to result in significant changes at these signature DNA bands. However, if there is overlap between the CD bands associated with the drug itself and that of DNA (*i.e.*, at 245 and/or 275 nm), there may be perturbation at these wavelengths, even for a groove binder. This is especially true if the drug is able to be involved in self-stacking interactions, in which case, transition moments for one drug molecule affect another molecule in close proximity [32] [35] [48]. It is also well-documented that a DNA minor groove binder generally elicits a strong positive induced CD (ICD) signal in the wavelength region where the minor groove binder itself exhibits strong spectrophotometric absorption [32] [41]-[43] [48]-[53]. This results from the close proximity of the drug and DNA bases while in the DNA minor groove. For most minor groove binders, these ICD signals are typically between 300 - 400 nm.

2. Materials and Methods

2.1. DNA

Poly(dAdT) and poly(dGdC) DNA sequences (**Figure 2**) were obtained from Midland Certified Reagent Company (Midland, TX) as HPLC-purified and desalted products. These 20-mer sequences are self-complementary and readily form the appropriate 20-bp duplex DNA in solution. To ensure optimal duplex formation, a buffered solution of each DNA sequence was heated to 90˚C and slowly cooled to room temperature (22˚C - 23˚C) before use. The resulting 20-bp DNA duplexes ($[poly(dAdT)]_2$ and $[poly(dGdC)]_2$) were equilibrated in the appropriate buffer by dialyzing for at least 24 hr before being used. DNA duplex concentrations (15 μM in terms of base pairs) were determined spectrophotometrically using ε_{262} = 13,200 $M^{-1}{\cdot}cm^{-1}$ for $[poly(dAdT)]_2$, ε_{256} = 16,800 $M^{-1}{\cdot}cm^{-1}$ for $[poly(dGdC)]_2$.

2.2. Compounds

All compounds (generally ≥90 purity) for the study (except for the NDI) were obtained commercially. Daunomycin hydrochloride, distamycin A hydrochloride, 4',6-diamidino-2-phenylindole dihydrochloride, quinacrine dihydrochloride, ethidium bromide and diminazene aceturate (berenil) were obtained from Sigma-Aldrich. Hoechst 33258 (~98% purity) was obtained from Acros Organics (now ThermoFisher). The NDI dihydrochloride was synthesized as previously reported [41]. Compound solutions were prepared in a low salt phosphate buffer (pH 7.0, 10 mM phosphate) from a concentrated stock solution that was either in water or methanol. Final solutions were in 95% - 100% aqueous buffer. Concentrations were determined spectrophotometrically using the extinction coefficient for each compound.

2.3. Isothermal Titration Calorimetry (ITC)

Calorimetric titrations were carried out on a MicroCal VP-ITC. The data was analyzed using the Origin 7.0 software provided by the manufacturer. All experiments were run at 30˚C in MES40 buffer (1×10^{-2} M MES (2(N-morpholino)ethanesulfonic acid) containing 1×10 M^{-3} EDTA, 40 mM NaCl, with the pH adjusted to 6.3 with NaOH) using $[poly(dAdT)]_2$ or $[poly(dGdC)]_2$ DNA (15 μM in bp). Exactly 12 μL of the compound solution (~75 μM) was injected into a buffered solution of DNA over 24 s, at 300 s equilibration intervals using a 250 μL syringe rotating at 300 rpm. DNA was generally titrated until only background signals associated with the heats of dilution (especially for drug titrated into buffer; buffer titrated into drug was negligible) were observed. This typically required 25 - 35 independent titrations. Samples were degassed at 20˚C before use. A binding isotherm of heat released versus the molar ratio was constructed and the data fitted by nonlinear least square fitting analysis to an appropriate model (either a one or two site binding model). Reported errors were associated with fitting analysis.

2.4. Circular Dichroism (CD) Spectroscopy

Studies were carried out using a JASCO J-815 spectrometer at 20˚C - 25˚C in MES40 buffer, using a 1 cm quartz cuvette. Baseline corrections associated with the buffer only were done for all runs. DNA ($[poly(dAdT)]_2$ or $[poly(dGdC)]_2$, 15 μM in terms of base pairs) was titrated with the compound of interest. DNA was titrated until a compound:DNA ratio of 2:1 (in terms of concentrations) was obtained.

5'-ATATATATATATATATATAT-3'
3'-TATATATATATATATATATA-5'

5'-GCGCGCGCGCGCGCGCGC-3'
3'-CGCGCGCGCGCGCGCGCG-5'

Figure 2. AT-rich and GC-rich DNA sequences used in this study.

3. Results and Discussion

Our CD studies clearly showed that the four compounds (distamycin, DAPI, berenil, Hoechst 33258) known to bind DNA via the minor groove elicited strong ICD signals between 300 - 400 nm when binding to the AT-rich sequence (**Figure 3(A)**). This ICD signal increased as more compound was titrated into the AT-rich DNA. No such ICD signal was observed for distamycin, DAPI and berenil when they were titrated into the GC-rich DNA sequence. This indicates a preference by these three minor groove binders for AT-rich DNA sequences. There was also no significant perturbation in the signature bands for DNA (*i.e.*, 245 and 275 nm), consistent with a non-intercalative mode of binding for the three compounds. On the other hand, Hoechst 33258 (a known DNA binding fluorescent dye) showed binding to both the AT- and GC-rich DNA sequences as is indicated by the strong ICD signal around ~330 nm induced for both DNA sequences (**Figure 3(A)**). This suggests that although Hoechst 33258 is widely considered as a minor groove binder, it does not exhibit the strong (exclusive) preference for AT-rich sequences generally observed for the classical minor groove binder, and in fact, exhibited strong binding to both the AT- and GC-rich sequences (**Figure 3(A)**). It is also important to note that Hoechst 33258 was also able to elicit significant perturbations at and around the signature bands for DNA (*i.e.*, 245 and 275 nm) showing strong hyperchromic shifts at both bands. This suggests that not only is Hoechst 33258 able to bind to both AT- and GC-rich DNA sequences but is also able to cause significant perturbations in the structure of DNA, consistent with a DNA intercalation binding mode. That is, Hoechst 33258 is a mixed binder when interacting with DNA.

The involvement of an intercalative DNA binding mode for Hoechst 33258 has been reported by our group and others [47] [52] [54]. Noren and coworkers reported that Hoechst 33258 was able to bind an AT-rich sequence via two sequential binding modes; a higher affinity site, followed by a low affinity site [43]. In earlier reports, it was also observed that Hoechst 33258 bound a purely GC-sequence DNA presumably via an intercalative mode [54] [55].

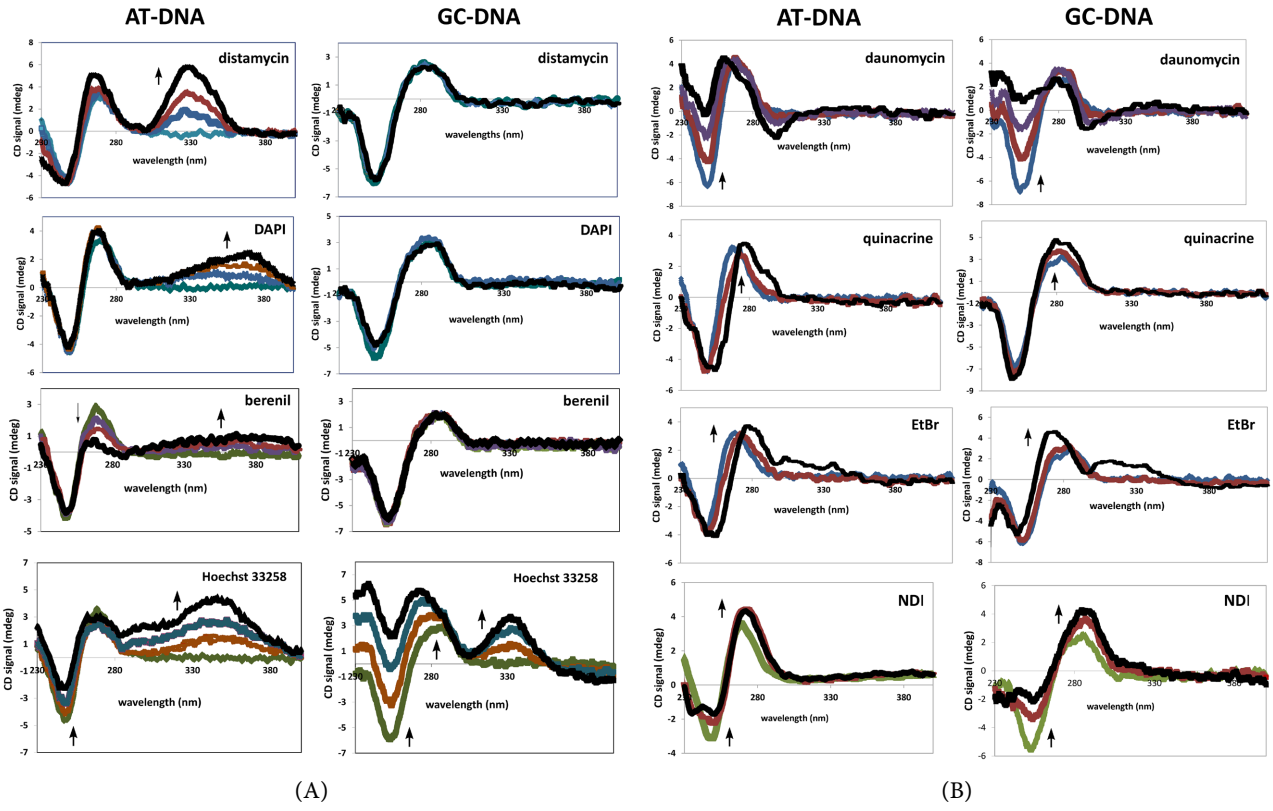


Figure 3. (A) CD spectra for distamycin, DAPI, berenil, and Hoechst 33258 binding to AT-rich (left column) versus GC-rich (right column) DNA sequences. Each compound was titrated into the DNA (15 μM in base pairs) until a compound:DNA ratio of 2:1 was obtained. Arrows indicate the direction of increasing [compound]. (B) CD spectra for daunomycin, quinacrine, ethidium bromide (EtBr) and NDI binding to AT-rich (left column) versus GC-rich (right column) DNA sequences. Each compound was titrated into the DNA (15 μM in base pairs) until a compound:DNA ratio of 2:1 was obtained. Arrows indicate the direction of increasing [compound].

Classical DNA intercalators generally do not show strong sequence selectivity (*i.e.*, are able to bind to both AT- and GC-rich sequences with roughly the same affinity) and generally cause significant structural changes in DNA upon binding, as the intercalation site is created. This can clearly be seen in the CD spectra for the four compounds (daunomycin, quinacrine, ethidium bromide and the NDI derivative) known to bind DNA via intercalation. As can be seen in **Figure 3(B)**, all four DNA intercalators were able to bind to both the AT- and GC-rich DNA sequences. Daunomcyin and the NDI even showed a slight preference for the GC-rich DNA, and this has been reported in the literature [30] [45] [56] [57]. Both daunomycin and the NDI showed stronger hyperchromic shifts around 245 nm for the GC-rich sequence. As expected for DNA intercalation, all four compounds were able to elicit perturbations in one or both of the signature bands for DNA. Probably more strikingly obvious for the DNA intercalators, was the lack of a *strong* positive ICD signal between 300 - 400 nm. This is especially true for quinacrine and the NDI derivative. Both daunomycin and ethidium bromide did show a small but noticeable perturbation above 300 nm, however, this is presumably due to the fact that the substituent (daunosamine ring in the case of daunomycin and phenyl ring for ethidium bromide) on both compounds interacts with the

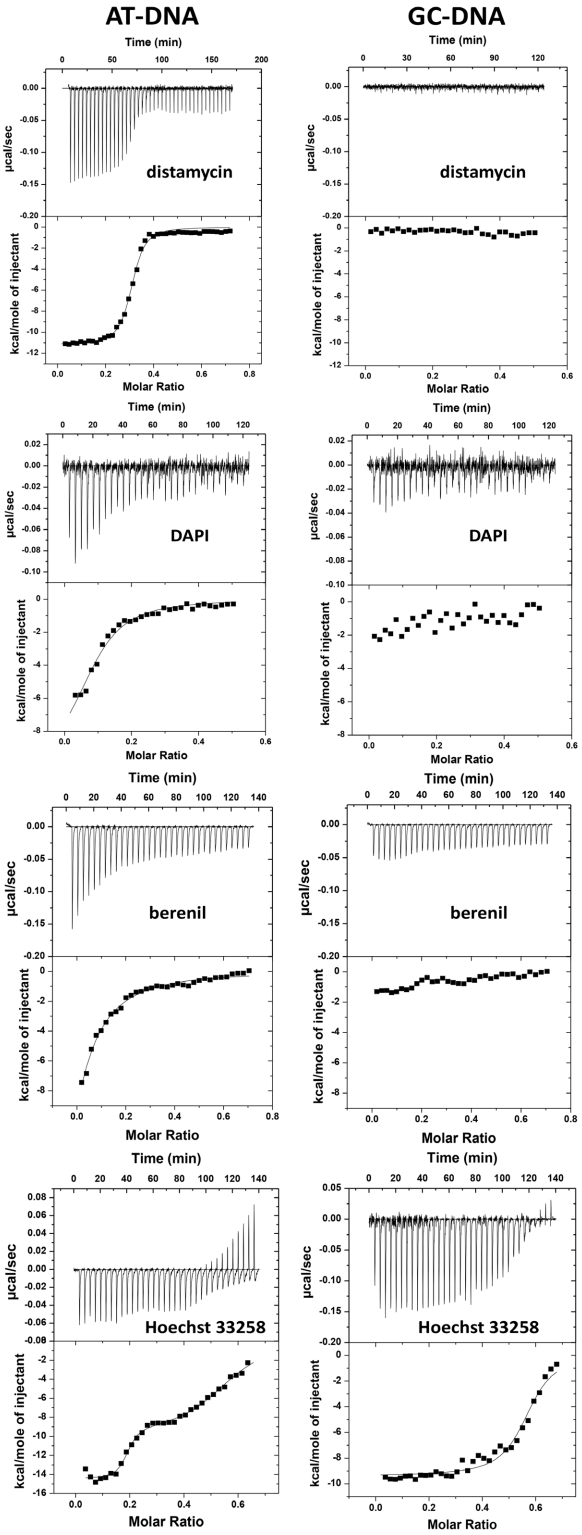
DNA minor groove upon intercalation [30] [58] [59].

The ICD signals exhibited by daunomycin and ethidium bromide are however small in comparison to that elicited by the known minor groove binders. It should also be noted that although berenil displayed the generally expected behaviour for a compound binding via the minor groove, the compound did cause an apparent perturbation (hypochromicity) around 270 nm, implying changes in DNA base stacking. However, it should be noted that this absorption band overlaps with the intrinsic absorption of berenil itself, which would result in signal perturbation ~275 nm due to the transition moments of one berenil molecule stacking with another in close proximity. Therefore, we do not attribute this perturbation to conformational changes in DNA.

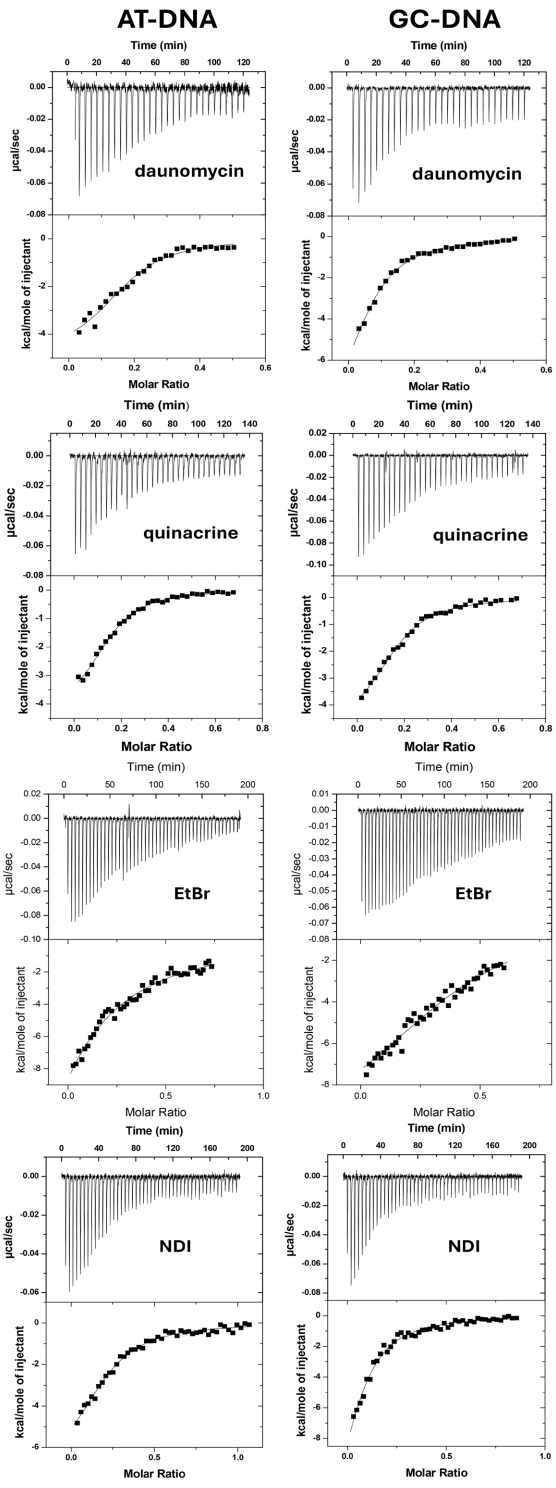
Although CD spectroscopy studies are generally powerful, they primarily give reliable empirical, qualitative and global views of drug-DNA interactions. As a result, ITC studies were also done in order to corroborate our CD data. ITC is a biophysical technique that is able to provide robust quantitative data on the relative strength of biomolecular interactions. In fact, ITC is regarded as the gold standard and method of choice when parsing the thermodynamic parameters associated with such interactions, since it can quickly and reliable provide a complete set of thermodynamic binding data [6]-[9] [32]-[34] [60] [61]. The ITC raw data for the minor groove compounds distamycin, DAPI, berenil and Hoechst 33258 were compared to that for the intercalators daunomycin, quinacrine, EtBr and NDI binding the AT-rich vs GC-rich DNA sequences. As can be seen in **Figure 4(A)** and **Figure 4(B)**, there is a distinctive difference in binding characteristics of the minor groove binders as compared to the intercalators for the two types of DNA sequences. For the minor groove binders distamycin, DAPI, and berenil, the difference in binding to AT-rich vs GC-rich DNA was striking. Distamycin, DAPI and berenil exhibited a preference for the AT-rich DNA ($K = 2.20 \pm 3.73 \times 10^7\ M^{-1}$, $1.65 \pm 0.32 \times 10^6\ M^{-1}$ and $2.15 \pm 0.41 \times 10^6\ M^{-1}$, respectively), with only background signals (associated with to the heats of dilution) for the GC-rich DNA sequence which were unfittable due to the lack of binding characteristics. This preference for the AT-rich DNA sequence is completely consistent with what we observe in the CD studies for the minor groove binders distamycin, DAPI and berenil and is consistent with what has been reported in the literature [40].

The ITC data (**Table 1**) showed that the binding of distamycin, DAPI, berenil and Hoechst 33258 were enthalpically driven with large negative enthalpy changes ($\Delta H = -0.987 \times 10^4$ cal/mol, -1.031×10^4 cal/mol, -1.735×10^4 cal/mol and −1.445/−0.963 cal/mol, respectively) accompanied by a small negative or very small positive change in entropy as expected for small molecules binding to DNA. While the minor groove binders showed an AT-DNA preference, the intercalators daunomycin, quinacrine, ethidium bromide and the NDI were able to bind strongly to both DNA sequences (**Figure 4(B)**). In fact, daunomycin not only bind to both AT- and GC-rich sequences but also showed slighter stronger binding to the GC-rich sequence ($K = 3.43 \pm 0.29 \times 10^6\ M^{-1}$ for GC sequence versus $2.31 \pm 0.37 \times 10^6\ M^{-1}$ for the AT sequence). This slight preference for the GC-rich DNA

was also observed during our CD studies and has been reported [30] [40] [45] [56]-[58]. As was the case for the groove binders, the binding of the intercalators were enthalpically driven.



(A)



(B)

Figure 4. (A) ITC calorimetric data for distamycin, DAPI, berenil and Hoechst 33258 binding to AT-rich versus GC-rich DNA sequences. Calorimetric titrations were carried out by titrating the compound into the DNA (15 μM in base pairs). (B) ITC calorimetric data for daunomycin, quinacrine, ethidium bromide (ETBr) and NDI binding to AT-rich versus GC-rich DNA sequences. Calorimetric titrations were carried out by titrating the compound into the DNA (15 μM in base pairs).

Table 1. Thermodynamic data (obtained from ITC) for the compounds of this study binding to the AT versus GC DNA sequences.

Compound	K (10^6 M^{-1}) (AT)	K (10^6 M^{-1}) (GC)	ΔH (10^4 cal/mol) (AT)	ΔH (10^4 cal/mol) (GC)	T*ΔS (10^4 cal/mol) (AT)	T*ΔS (10^4 cal/mol) (GC)
Distamycin	22.0 ± 3.73	-	−0.987	-	0.032	-
DAPI	1.65 ± 0.32	-	−1.031	-	−0.17	-
Berenil	2.15 ± 0.41	-	−1.735	-	−0.86	-
Hoechst 33258	39.2 ± 12.6	1.15 ± 0.27	−1.445	−2.042	−0.39	−1.20
	2.32 ± 0.51		−0.963		−0.080	
Daunomycin	2.31 ± 0.37	3.43 ± 0.29	−1.135	−0.941	−0.25	−0.040
Naphthalene diimide	0.64 ± 0.11	0.77 ± 0.13	−1.228	−0.831	−0.43	−0.02
Quinacrine	0.98 ± 0.09	0.86 ± 0.08	−0.434	−0.498	0.40	0.32
Ethidium bromide	0.62 ± 0.13	0.47 ± 0.90	−1.007	−1.059	−0.20	−0.28

The minor groove binders distamycin, DAPI and berenil showed mostly background signals when binding to the GC-duplex DNA. These background signals were associated with the heats of dilution and was unfittable; therefore did not yield any binding parameters.

Similar to the CD spectroscopy studies, our ITC data showed that Hoechst 33258 displayed binding to both the AT-rich and GC-rich DNA, with a very strong binding constant (K) for the AT-rich sequence. The binding of Hoechst 33258 to the AT-rich DNA sequence was fitted best by a model that assumes two different binding sites, a higher affinity site ($K_1 = 3.92 \pm 1.26 \times 10^7$ M^{-1}) followed by a lower affinity site ($K_2 = 2.32 \pm 0.51 \times 10^6$ M^{-1}), consistent with the two binding site model proposed by Noren and coworkers [43]. On the other hand, the binding of Hoechst 33258 to the GC-rich sequence was defined by a one-site model ($K = 1.15 \pm 0.27 \times 10^6$ M^{-1}), which makes sense since only DNA intercalation is expected to be involved when Hoechst 33258 binds to the GC-sequence. It is important to note that the binding constant (K) for Hoechst 33258 binding to the GC sequence was similar to the K_2 for the AT-rich DNA, arguing for these two binding modes being similar, *i.e.*, DNA intercalation. We suggest that while Hoechst 33258 binds to the AT-rich DNA via minor groove binding (higher affinity site) followed by intercalation (lower affinity site), binding to the GC-DNA occurs via intercalation. Minor groove binders are known to have generally higher binding constants (10x or greater) relative to DNA intercalation since there is no need for free energy input to un-stack adjacent base pairs as is the case for intercalation [61] [62]. Other studies have also found multiple binding modes for Hoechst 33258, with a higher (~10^7 M^{-1}) and lower (~10^6 M^{-1}) binding constant consistent with DNA minor groove binding and intercalation, respectively (Guan *et al.* 2007). Furthermore, an earlier report by our group also showed evidence of an intercalative binding mode for Hoechst 33258 during our DNA unwinding topoisomerase gel-based assay [52]. **Figure 5** shows a side-side comparison of Hoechst 33258 with that of the classical DNA intercalator EtBr, both binding to

supercoiled plasmid DNA in our DNA unwinding topoisomerase assay. Distamycin, with only a DNA minor groove binding mode, is also shown for comparison [52]. As can be seen in **Figure 5**, Hoechst 33258 was able to elicit DNA re*supercoiling* (albeit at higher concentrations), akin to the classical DNA intercalator, ethidium bromide. Distamycin, on the other hand, was unable to elicit DNA re*supercoiling*, consistent with a compound that binds DNA exclusively via the minor grove. In that same report, spectrophotometric titration studies of Hoechst 33258 binding to DNA revealed two non-equivalent binding constants (K); an initial high affinity K (~10^7 M^{-1}) followed by a lower affinity K (~10^5 M^{-1}), consistent with an initial minor groove binding mode at low drug/DNA ratios, followed by intercalation as drug concentration increases. Therefore, our ITC result is consistent both with our earlier report and what we observe in the current CD spectroscopy studies for Hoechst 33258 (discussed above), confirming Hoechst 33258 is not a classical minor groove binder and in fact binds to both AT-rich and GC-rich DNA sequences, *i.e.*, a mixed-binder.

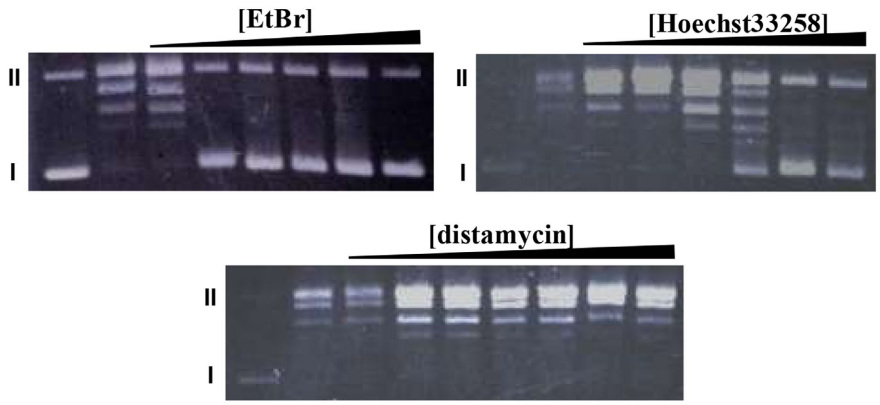


Figure 5. Topoisomerase DNA unwinding assay of the DNA minor groove binders Hoechst 33258 and distamycin compared to the classic DNA intercalator, ethidium bromide (ETBr). Experiment was performed as previously reported [52]. Form **I** and **II** indicate supercoiled and relaxed plasmid DNA, respectively. Notice how Hoechst 33258 is able to elicit re*supercoiling* (form **I**) similar to EtBr, while distamycin (a classical minor groove binder) does not.

A mixed binding mode is not unusual in drug-DNA interactions. In fact, some researchers have even proposed that there may be a correlation between the therapeutic potential of a drug and their ability to exhibit mixed-binding modes [46] [63].

4. Conclusions

In conclusion, our CD spectroscopy and ITC studies show that the classical DNA minor groove binders of this study (distamycin, DAPI and berenil) bound strongly to an AT-rich DNA sequence but not to a GC-rich sequence, while the DNA intercalators (daunomycin, quinacrine, ethidium bromide and NDI) displayed binding to both DNA sequences. Although not stand-alone conclusive

proof of binding mode, our CD and ITC results are compatible with the proposed binding modes of the compounds in this study. Our studies also show that Hoechst 33258 is not a classical minor groove binder and in fact binds to both AT-rich and GC-rich sequences, consistent with a mixed-binder, adopting both DNA minor groove and intercalative binding modes. This report illustrates the efficacy and convenience of combining CD spectroscopy and ITC studies in tandem to investigate the preferred mode of binding adopted by therapeutic small molecules (*aka* drugs) when interacting with DNA. These results therefore show that the biophysical techniques of CD spectroscopy and ITC, when used in tandem, can serve as viable, efficacious and relatively expedient preliminary methods to characterize the preferred DNA binding mode adopted by therapeutic drugs or potential drug candidates.

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Author Contributions

Conceptualization, Ruel Earl McKnight; Methodology, Ruel Earl McKnight, Luke Timothy Marr, Gavin Smiley Gullickson, Kevin David Siegenthaler; Investigation, Luke Timothy Marr, Gavin Smiley Gullickson, Kevin David Siegenthaler; Writing-original draft preparation, Ruel Earl McKnight; Writing-review and editing, Ruel Earl McKnight; Supervision, Ruel Earl McKnight; All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Sun, X., Setrerrahmane, S., Li, C., Hu, J. and Xu, H. (2024) Nucleic Acid Drugs: Recent Progress and Future Perspectives. *Signal Transduction and Targeted Therapy*, **9**, Article No. 316. https://doi.org/10.1038/s41392-024-02035-4

[2] Watanabe, K. and Seki, N. (2024) Biology and Development of DNA-Targeted Drugs, Focusing on Synthetic Lethality, DNA Repair, and Epigenetic Modifications for Cancer: A Review. *International Journal of Molecular Sciences*, **25**, Article 752. https://doi.org/10.3390/ijms25020752

[3] Kulkarni, J.A., Witzigmann, D., Thomson, S.B., Chen, S., Leavitt, B.R., Cullis, P.R., *et al.* (2021) The Current Landscape of Nucleic Acid Therapeutics. *Nature Nanotechnology*, **16**, 630-643. https://doi.org/10.1038/s41565-021-00898-0

[4] Waring, M.J. (2018) DNA-Targeting Molecules as Therapeutic Agents. Royal Society of Chemistry (Great Britain).

[5] Wilhelm, M., Mukherjee, A., Bouvier, B., Zakrzewska, K., Hynes, J.T. and Lavery, R. (2012) Multistep Drug Intercalation: Molecular Dynamics and Free Energy Studies

of the Binding of Daunomycin to DNA. *Journal of the American Chemical Society*, **134**, 8588-8596. https://doi.org/10.1021/ja301649k

[6] Lavanya, K., Saranya, J., Bodapati, A.T.S., Reddy, R.S., Madku, S.R. and Sahoo, B.K. (2023) Biophysical Insights on the Interaction of Anticoagulant Drug Dicoumarol with Calf Thymus-DNA: Deciphering the Binding Mode and Binding Force with Thermodynamics. *Journal of Biomolecular Structure and Dynamics*, **42**, 1392-1403. https://doi.org/10.1080/07391102.2023.2199872

[7] Bodapati, A.T.S., Reddy, R.S., Lavanya, K., Madku, S.R. and Sahoo, B.K. (2024) Minor Groove Binding of Antihistamine Drug Bilastine with Calf Thymus DNA: A Molecular Perspective with Thermodynamics Using Experimental and Theoretical Methods. *Journal of Molecular Structure*, **1301**, Article 137385. https://doi.org/10.1016/j.molstruc.2023.137385

[8] Pavithra, K., Durga, P.R., Vennila, K.N. and Elango, K.P. (2023) Multi-Spectroscopic, Calorimetric and Molecular Dynamics Evaluation on Non-Classical Intercalation of Antiviral Drug Molnupiravir with DNA. *Journal of Biomolecular Structure and Dynamics*, **42**, 8897-8905. https://doi.org/10.1080/07391102.2023.2249994

[9] Alniss, H.Y., Al-Jubeh, H.M., Msallam, Y.A., Siddiqui, R., Makhlouf, Z., Ravi, A., *et al*. (2024) Structure-Based Drug Design of DNA Minor Groove Binders and Evaluation of Their Antibacterial and Anticancer Properties. *European Journal of Medicinal Chemistry*, **271**, Article 116440. https://doi.org/10.1016/j.ejmech.2024.116440.

[10] Amir, M., Qureshi, M.A., Khan, A., Nayeem, S.M., Malik, W.A. and Javed, S. (2024) Exploring the Interaction of Tepotinib with Calf Thymus DNA Using Molecular Dynamics Simulation and Multispectroscopic Techniques. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **308**, Article 123678. https://doi.org/10.1016/j.saa.2023.123678

[11] Sarkar, E., Kotiya, A., Bhuyan, R., Raza, S.T., Misra, A., Ahmad, R., *et al*. (2025) Curcumin Chemo-Sensitizes Intrinsic Apoptosis through ROS-Mediated Mitochondrial Hyperpolarization and DNA Damage in Breast Cancer Cells. *Cellular Signalling*, **128**, Article 111637. https://doi.org/10.1016/j.cellsig.2025.111637

[12] Azimi, S.G., Shakour, N., Bagherzade, G., Saberi, M.R., Azimi, H. and Moosavi F., M. (2025) A Comprehensive Review of the Biological Activities of Medicinal Metal Complexes Synthesized from Quinoline Scaffolds. *Bioinorganic Chemistry and Applications*, **2025**, Article 3133615. https://doi.org/10.1155/bca/3133615

[13] Shahabadi, N., Shiri, F., Hadidi, S., Farshadfar, K., Darbemamieh, M. and Mark Roe, S. (2021) The Role of Both Intercalation and Groove Binding at At-Rich DNA Regions in the Interaction Process of a Dinuclear Cu(I) Complex Probed by Spectroscopic and Simulation Analysis. *Journal of Molecular Liquids*, **335**, Article 116290. https://doi.org/10.1016/j.molliq.2021.116290

[14] Bhaduri, S., Ranjan, N. and Arya, D.P. (2018) An Overview of Recent Advances in Duplex DNA Recognition by Small Molecules. *Beilstein Journal of Organic Chemistry*, **14**, 1051-1086. https://doi.org/10.3762/bjoc.14.93

[15] Chaires, J.B. (2006) A Thermodynamic Signature for Drug-DNA Binding Mode. *Archives of Biochemistry and Biophysics*, **453**, 26-31. https://doi.org/10.1016/j.abb.2006.03.027

[16] Bodley, A., Liu, L.F., Israel, M., Seshadri, R., Koseki, Y., Giuliani, F.C., Kirschenbaum, S., Silber, R. and Potmesil, M. (1989) DNA Topoisomerase II-Mediated Interaction of Doxorubicin and Daunorubicin Congeners with DNA. *Cancer Research*, **49**, 5969-5978.

[17] Tewey, K.M., Rowe, T.C., Yang, L., Halligan, B.D. and Liu, L.F. (1984) Adriamycin-

Induced DNA Damage Mediated by Mammalian DNA Topoisomerase II. *Science*, **226**, 466-468. https://doi.org/10.1126/science.6093249

[18] Crow, R.T. and Crothers, D.M. (1994) Inhibition of Topoisomerase I by Anthracycline Antibiotics: Evidence for General Inhibition of Topoisomerase I by DNA-Binding Agents. *Journal of Medicinal Chemistry*, **37**, 3191-3194. https://doi.org/10.1021/jm00045a026

[19] Marques, D.S.C., da Silva Lima, L., de Oliveira Moraes Miranda, J.F., *et al.* (2025) Exploring the Therapeutic Potential of Acridines: Synthesis, Structure, and Biological Applications. *Bioorganic Chemistry*, **155**, Article 108096. https://doi.org/10.1016/j.bioorg.2024.108096

[20] Rupar, J., Dobričić, V., Aleksić, M., Brborić, J. and Čudina, O. (2018) A Review of Published Data on Acridine Derivatives with Different Biological Activities. *Kragujevac Journal of Science*, **40**, 83-101. https://doi.org/10.5937/kgjsci1840083r

[21] Hossain, M.Z., Healey, M.A., Lee, C., Poh, W., Yerram, S.R., Patel, K., *et al.* (2013) DNA-Intercalators Causing Rapid Re-Expression of Methylated and Silenced Genes in Cancer Cells. *Oncotarget*, **4**, 298-309. https://doi.org/10.18632/oncotarget.863

[22] Zou, T., Sato, Y., Kaneyoshi, S., Mano, K., Yasukawa, R., Nakano, Y., *et al.* (2022) Naphthalene Diimides Carrying Two *β*-Cyclodextrins Prefer Telomere RNA G-Quadruplex Recognition. *Molecules*, **27**, Article 4053. https://doi.org/10.3390/molecules27134053

[23] Platella, C., Napolitano, E., Riccardi, C., Musumeci, D. and Montesarchio, D. (2021) Disentangling the Structure–Activity Relationships of Naphthalene Diimides as Anticancer G-Quadruplex-Targeting Drugs. *Journal of Medicinal Chemistry*, **64**, 3578-3603. https://doi.org/10.1021/acs.jmedchem.1c00125

[24] Ahmed, A.A., Chen, S., Roman-Escorza, M., Angell, R., Oxenford, S., McConville, M., *et al.* (2024) Structure-Activity Relationships for the G-Quadruplex-Targeting Experimental Drug QN-302 and Two Analogues Probed with Comparative Transcriptome Profiling and Molecular Modeling. *Scientific Reports*, **14**, Article No. 3447. https://doi.org/10.1038/s41598-024-54080-2

[25] Marchetti, C., Zyner, K.G., Ohnmacht, S.A., Robson, M., Haider, S.M., Morton, J.P., *et al.* (2018) Targeting Multiple Effector Pathways in Pancreatic Ductal Adenocarcinoma with a G-Quadruplex-Binding Small Molecule. *Journal of Medicinal Chemistry*, **61**, 2500-2517. https://doi.org/10.1021/acs.jmedchem.7b01781

[26] Thomos, D., Tsolis, T., Plakatouras, J.C., Chronakis, I.M., Magklara, A. and Garoufis, A. (2025) DNA Noncovalent Interactions of Dinuclear η6-Arene Ru(II) Complexes: Influence of Complex Charge and Bridging Ligand Length on DNA Binding Mode and Cytotoxic Activity. *Chemistry*, **31**, e02680. https://doi.org/10.1002/chem.202502680

[27] Sifnaiou, E., Tsolis, T., Ypsilantis, K., Roupakia, E., Kolettas, E., Plakatouras, J.C., *et al.* (2023) Synthesis and Characterization of Ruthenium-Paraphenylene-Cyclopentadienyl Full-Sandwich Complexes: Cytotoxic Activity against A549 Lung Cancer Cell Line and DNA Binding Properties. *Molecules*, **29**, Article 17. https://doi.org/10.3390/molecules29010017

[28] Buric, A.J., Dickerhoff, J. and Yang, D. (2021) Novel DNA Bis-Intercalator XR5944 as a Potent Anticancer Drug-Design and Mechanism of Action. *Molecules*, **26**, Article 4132. https://doi.org/10.3390/molecules26144132

[29] Emwas, A.H., Szczepski, K., Poulson, B.G., Chandra, K., McKay, R.T., Dhahri, M., Alahmari, F., Jaremko, L., Lachowicz, J.I. and Jaremko, M. (2020) NMR as a "Gold Standard" Method in Drug Design and Discovery. *Molecules*, **25**, Article 4597.

https://doi.org/10.3390/molecules25204597

[30] Qu, X., Wan, C., Becker, H., Zhong, D. and Zewail, A.H. (2001) The Anticancer Drug-DNA Complex: Femtosecond Primary Dynamics for Anthracycline Antibiotics Function. *Proceedings of the National Academy of Sciences*, **98**, 14212-14217. https://doi.org/10.1073/pnas.241509698

[31] Suh, D. and Chaires, J.B. (1995) Criteria for the Mode of Binding of DNA Binding Agents. *Bioorganic & Medicinal Chemistry*, **3**, 723-728. https://doi.org/10.1016/0968-0896(95)00053-j

[32] McKnight, R.E., Gullickson, G.S., Kasper, B., Siegenthaler, K., Biggs, D.A.C. and Porter, R.B. (2025) DNA Binding Studies of Abietane Diterpenes Natural Products Using Isothermal Titration Calorimetry, Circular Dichroism, Fluorescence and Gel Assays. *Bioorganic & Medicinal Chemistry Letters*, **123**, Article 130216. https://doi.org/10.1016/j.bmcl.2025.130216

[33] Durga, P.R., Jeyashree, R., Preethi, R., Vennila, K.N. and Elango K.P. (2024) Intercalation of Anticancer Drug Palbociclib with Calf-Thymus DNA: New Insights from Molecular Spectroscopic, Molecular Dynamic Simulations and Cleavage Studies. *Journal of Biomolecular Structure and Dynamics*, **42**, 1932-1939. https://doi.org/10.1080/07391102.2023.2212787

[34] Prasad, T.K., Bairy, S. and Kamariah, N. (2025) Characterization of FOXO3-14-3-3 Interaction by Isothermal Titration Calorimetry. In: *Methods in Molecular Biology*, Springer, 107-114. https://doi.org/10.1007/978-1-0716-4217-7_10

[35] Garbett, N.C., Ragazzon, P.A. and Chaires, J.B. (2007) Circular Dichroism to Determine Binding Mode and Affinity of Ligand-DNA Interactions. *Nature Protocols*, **2**, 3166-3172. https://doi.org/10.1038/nprot.2007.475

[36] Thanassoulas, A. and Nounesis, G. (2019) Isothermal Titration Calorimetry: A Powerful Tool for the Characterization of Molecular Interactions. In: Demetzos, C. and Pippa, N., *Thermodynamics and Biophysics of Biomedical Nanosystems*. *Series in BioEngineering*, Springer, 63-103.

[37] Chang, Y.M., Chen, C.K. and Hou, M.H. (2012) Conformational Changes in DNA upon Ligand Binding Monitored by Circular Dichroism. *International Journal of Molecular Sciences*, **13**, 3394-3413. https://doi.org/10.3390/ijms13033394

[38] Kypr, J., Kejnovská, I., Renciuk, D. and Vorlícková, M. (2009) Circular Dichroism and Conformational Polymorphism of DNA. *Nucleic Acids Research*, **37**, 1713-1725. https://doi.org/10.1093/nar/gkp026

[39] Kim, H.K., Kim, J.M., Kim, S.K., Rodger, A. and Nordén, B. (1996) Interactions of Intercalative and Minor Groove Binding Ligands with Triplex Poly(dA).[Poly(dT)]2 and with Duplex Poly(dA).Poly(dT) and Poly[d(A-T)]2 Studied by CD, LD, and Normal Absorption. *Biochemistry*, **35**, 1187-1194. https://doi.org/10.1021/bi951913m

[40] Ren, J. and Chaires, J.B. (1999) Sequence and Structural Selectivity of Nucleic Acid Binding Ligands. *Biochemistry*, **38**, 16067-16075. https://doi.org/10.1021/bi992070s

[41] McKnight, R.E., Reisenauer, E., Pintado, M.V., Polasani, S.R. and Dixon, D.W. (2011) Substituent Effect on the Preferred DNA Binding Mode and Affinity of a Homologous Series of Naphthalene Diimides. *Bioorganic & Medicinal Chemistry Letters*, **21**, 4288-4291. https://doi.org/10.1016/j.bmcl.2011.05.069

[42] Stojković, M.R., Škugor, M., Dudek, Ł., Grolik, J., Eilmes, J. and Piantanida, I. (2014) Molecular Recognition of AT-DNA Sequences by the Induced CD Pattern of Dibenzotetraaza[14]annulene (DBTAA)-Adenine Derivatives. *Beilstein Journal of Organic*

Chemistry, **10**, 2175-2185. https://doi.org/10.3762/bjoc.10.225

[43] Fornander, L.H., Wu, L., Billeter, M., Lincoln, P. and Nordén, B. (2013) Minor-Groove Binding Drugs: Where Is the Second Hoechst 33258 Molecule? *The Journal of Physical Chemistry B*, **117**, 5820-5830. https://doi.org/10.1021/jp400418w

[44] McKnight, R.E., Onogul, B., Polasani, S.R., Gannon, M.K. and Detty, M.R. (2008) Substituent Control of DNA Binding Modes in a Series of Chalcogenoxanthylium Photosensitizers as Determined by Isothermal Titration Calorimetry and Topoisomerase I DNA Unwinding Assay. *Bioorganic & Medicinal Chemistry*, **16**, 10221-10227. https://doi.org/10.1016/j.bmc.2008.10.051

[45] Liu, Z.R., Hecker, K.H. and Rill, R.L. (1996) Selective DNA Binding of (N-Alkylamine)-Substituted Naphthalene Imides and Diimides to G+C-Rich DNA. *Journal of Biomolecular Structure and Dynamics*, **14**, 331-339. https://doi.org/10.1080/07391102.1996.10508128

[46] Pilch, D.S., Kirolos, M.A., Liu, X., Plum, G.E. and Breslauer, K.J. (1995) Berenil [1,3-Bis(4'-Amidinophenyl)triazene] Binding to DNA Duplexes and to a RNA Duplex: Evidence for Both Intercalative and Minor Groove Binding Properties. *Biochemistry*, **34**, 9962-9976. https://doi.org/10.1021/bi00031a019

[47] Moon, J.H., Kim, S.K., Sehlstedt, U., Rodger, A. and Nordén, B. (1998) DNA Structural Features Responsible for Sequence-Dependent Binding Geometries of Hoechst 33258. *Biopolymers*, **38**, 593-606. https://doi.org/10.1002/(sici)1097-0282(199605)38:5<593::aid-bip5>3.0.co;2-n

[48] Eriksson, M. and Nordén, B. (2001) Linear and Circular Dichroism of Drug-Nucleic Acid Complexes. In: *Methods in Enzymology*, Elsevier, 68-98. https://doi.org/10.1016/s0076-6879(01)40418-6

[49] Wang, S., Nanjunda, R., Aston, K., Bashkin, J.K. and Wilson, W.D. (2012) Correlation of Local Effects of DNA Sequence and Position of B-Alanine Inserts with Polyamide-DNA Complex Binding Affinities and Kinetics. *Biochemistry*, **51**, 9796-9806. https://doi.org/10.1021/bi301327v

[50] Lying, R., Rodgers, A. and Norden, B. (1991) The CD of Ligand-DNA Systems. I. Poly(dG-dC) B-DNA. *Biopolymers*, **31**, 1709-1720. https://doi.org/10.1002/bip.360311405

[51] Lying, R., Rodgers, A. and Norden, B. (1992) The CD of Ligand-DNA Systems. I. Poly(dA-dT) B-DNA. *Biopolymers*, **32**, 1201-1214. https://doi.org/10.1002/bip.360320910

[52] McKnight, R.E., Gleason, A.B., Keyes, J.A. and Sahabi, S. (2007) Binding Mode and Affinity Studies of DNA-Binding Agents Using Topoisomerase I DNA Unwinding Assay. *Bioorganic & Medicinal Chemistry Letters*, **17**, 1013-1017. https://doi.org/10.1016/j.bmcl.2006.11.038

[53] McKnight, R.E., Ye, M., Ohulchanskyy, T.Y., Sahabi, S., Wetzel, B.R., Wagner, S.J., *et al*. (2007) Synthesis of Analogues of a Flexible Thiopyrylium Photosensitizer for Purging Blood-Borne Pathogens and Binding Mode and Affinity Studies of Their Complexes with DNA. *Bioorganic & Medicinal Chemistry*, **15**, 4406-4418. https://doi.org/10.1016/j.bmc.2007.04.033

[54] Guan, Y., Shi, R., Li, X., Zhao, M. and Li, Y. (2007) Multiple Binding Modes for Dicationic Hoechst 33258 to DNA. *The Journal of Physical Chemistry B*, **111**, 7336-7344. https://doi.org/10.1021/jp066344e

[55] Norden, B., Kubista, M. and Kurucsev, T. (1992) Linear Dichroism Spectroscopy of Nucleic Acids. *Quarterly Reviews of Biophysics*, **25**, 51-170.

https://doi.org/10.1017/s0033583500004728

[56] Chaires, J.B., Fox, K.R., Herrera, J.E., Britt, M. and Waring, M.J. (1987) Site and Sequence Specificity of the Daunomycin-DNA Interaction. *Biochemistry*, **26**, 8227-8236. https://doi.org/10.1021/bi00399a031

[57] Chen, K.X., Gresh, N. and Pullman, B. (1985) A Theoretical Investigation on the Sequence Selective Binding of Daunomycin to Double-Stranded Polynucleotides. *Journal of Biomolecular Structure and Dynamics*, **3**, 445-466. https://doi.org/10.1080/07391102.1985.10508434

[58] Chaires, J.B., Dattagupta, N. and Crothers, D.M. (1982) Studies on Interaction of Anthracycline Antibiotics and Deoxyribonucleic Acid: Equilibrium Binding Studies on the Interaction of Daunomycin with Deoxyribonucleic Acid. *Biochemistry*, **21**, 3933-3940. https://doi.org/10.1021/bi00260a005

[59] Chaires, J.B. (1996) Molecular Recognition of DNA by Daunorubicin. In: Hurley, L.H. and Chaires, J.B., *Advances in DNA Sequence-Specific Agents*, Elsevier, 141-167.

[60] Haq, I., Jenkins, T.C., Chowdhry, B.Z., Ren, J. and Chaires, J.B. (2000) Parsing Free Energies of Drug-DNA Interactions. In: *Methods in Enzymology*, Elsevier, 373-405. https://doi.org/10.1016/s0076-6879(00)23374-0

[61] Chaires, J.B. (1997) Energetics of Drug-DNA Interactions. *Biopolymers*, **44**, 201-215. https://doi.org/10.1002/(sici)1097-0282(1997)44:3<201::aid-bip2>3.0.co;2-z

[62] Ridge, G.S., Bailly, C., Graves, D.E. and Waring, M.J. (1994) Daunomycin Modifies the Sequence-Selective Recognition of DNA by Actinomycin. *Nucleic Acids Research*, **22**, 5241-5246. https://doi.org/10.1093/nar/22.24.5241

[63] Trieb, M., Rauch, C., Wibowo, F.R., Wellenzohn, B. and Liedl, K.R. (2004) Cooperative Effects on the Formation of Intercalation Sites. *Nucleic Acids Research*, **32**, 4696-4703. https://doi.org/10.1093/nar/gkh788