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Investigations into the DNA-binding mode of doxorubicinone†

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Cancer treatment is one of the major challenges facing the modern biomedical profession. Development of new small-molecule chemotherapeutics requires an understanding of the mechanism of action for these treatments, as well as the structure–activity relationship. Study of the well-known DNA-intercalating agent, doxorubicin, and its aglycone, doxorubicinone, was undertaken using a variety of spectroscopic and calorimetric techniques. It was found that, despite conservation of the planar, aromatic portion of doxorubicin, the agylcone does not intercalate; it instead likely binds to the DNA minor-groove.

Background and introduction

Despite an enormous amount of research that has been focused on understanding and treating cancer, the United States National Cancer Institute anticipates over 1.7 million new cases of will be diagnosed in the US alone in 2018, with 600 000 deaths attributed to cancer and related complications.[1] While many advances have been made, cancer remains one of the major challenges for the biomedical field,[2] in part due to the multifaceted nature of the disease, which in turn has given rise to multifaceted approaches to treatment. One such approach is the use of small-molecule ligands, which interact with DNA by a variety of mechanisms and prevent transcription, ultimately leading to cell death. Since the discovery of cisplatin, one of the most widely-known DNA-binding chemotherapeutic agents,[3] a number of other natural and synthetic drug-molecules have been discovered that bind to DNA.[4] However, the complexity of drug–DNA interactions has made it challenging to understand fully how this binding occurs, which, in turn, has hampered efforts to develop more selective small-molecule chemotherapeutics.

For this reason, we became interested in studying the details of DNA–ligand interactions and how those interactions change with subtle modifications to the molecular structure of known small-molecule ligands. Our previous work[5] with doxorubicin (DOX, Fig. 1) had provided insight into the entropic and enthalpic influences for DNA-binding but also left a number of questions unanswered. DOX, a member of the anthracycline-family of natural products, was first isolated in 1969 from *Streptomyces peucetius*[6] and has been used to treat various forms of cancer since 1974. DOX is known to interrupt DNA transcription by intercalation between DNA base pairs, which inhibits topoisomerase II.

However, the mechanism by which intercalation occurs has been vigorously debated.[7] While it is generally accepted that DOX first pre-coordinates weakly to the DNA minor-groove before intercalation, both computational and experimental efforts have put forward a number of more complex mechanisms and have measured binding constants that, when normalized for salt concentrations, vary by several orders of magnitude.

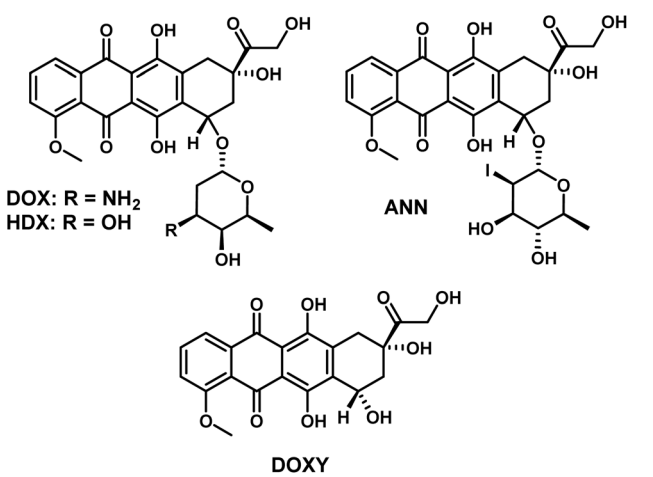

Fig. 1 The structures of naturally occurring doxorubicin (DOX) and several synthetic analogues; hydroxyrubicin (HDX), annamycin (ANN), and doxorubicinone (DOXY).

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Even less clear is the importance of the daunosamine sugar for DOX–DNA binding. This sugar, which is protonated under physiological conditions, presumably interacts with the negatively-charged phosphate backbone of DNA. What is not clear is whether this electrostatic interaction is necessary for the final intercalated state of DOX. Hydroxyrubicin (HDX), which lacks the 3′ amino-moiety, still inhibits topoisomerase II and it has been assumed that the activity of HDX is also due to DNA intercalation, albeit to a lesser degree than DOX based on DNA unwinding assays.[8,9] Similarly, annamycin (ANN) actually exhibits enhanced topoisomerase activity, despite significant modification to the sugar.[10] Considering the importance of DOX as a chemotherapeutic and the uncertainty about the role that the sugar plays in DNA-binding, it is puzzling that the doxorubicin-aglycone (doxorubicinone, adriamycinone, DOXY) has received very little scholarly attention.[7a]

Given the proposed pre-coordination of DOX to the minor-groove, the absence of the amino-sugar on DOXY would be expected to lead to at least one of the following outcomes: (1) slower DNA–ligand binding kinetics but the same final bound state, (2) a similar final intercalative state, albeit with a weaker binding constant, or (3) an entirely different final binding mode (*i.e.* DOXY could remain bound in the minor-groove or only intercalate slightly).

To differentiate between those three potential outcomes, we undertook a side-by-side comparison study of DOX and DOXY, looking specifically at conformational changes to DNA and the relative strengths of the DNA–ligand binding. Our methods focused on DNA melting temperature analysis, circular dichroism (CD), and fluorescence titration, although additional methods were also examined.

Methods and experimental

General experimental

The mono-HCl salt of doxorubicin and CT DNA was purchased from Sigma-Aldrich. Silica gel used for chromatographic separation (60 Å, 230–400 mesh) was purchased from Sigma-Aldrich. Thin-layer chromatographic (TLC) analysis was conducted using glass-backed EMD/Millipore silica-gel plates (60 Å, 230–400 mesh). All other reagents were purchased from commercial vendors and used as received, without further purification. ^{1}H NMR spectra were collected using a 300 MHz JEOL-Eclipse NMR spectrometer (75 MHz on the ^{13}C channel) and referenced using the residual $CHCl_3$ solvent peak (7.26 ppm for ^{1}H, 77.16 ppm for ^{13}C).

Hydrolysis of doxorubicin. Using a modification of the conditions reported by Menna *et al.*,[11] doxorubicin-mono-HCl salt (50 mg, 0.086 mmol) was dissolved in methanol (3.0 mL), topped with a water condenser, then heated to 80 °C with stirring. A 1 M aqueous HCl solution was added (0.6 mL, resulting in 0.2 M overall HCl concentration) and heating was continued for 90 minutes. Upon complete cleavage of the sugar, as judged by TLC analysis, the reaction volume was reduced *in vacuo* to approximately 25% of the original volume. H_2O (0.5 mL) was added, after which the reaction mixture was extracted with CH_2Cl_2 (3 × 1 mL). The organic extracts were dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*, and the resulting dark-red solid was purified by column chromatography (SiO_2, 19 : 1 $CHCl_3/CH_3OH$, R_f = 0.13, UV) to afford doxorubicinone as a bright-orange solid (24 mg, 0.058 mmol, 67% yield) that matched reported spectroscopic values by ^{1}H NMR analysis.[12] This characterization was confirmed by HRMS data.

DNA melting curves. Samples contained 30 μM calf thymus DNA (CT DNA) and 2 μM of DOX or DOXY in a low ionic strength phosphate buffer (3 mM phosphate buffer + 2.0 mM NaCl). They were heated at 5 °C increments and the absorbance of CT DNA was monitored at 258 nm. T_m values were determined from the first-derivative of the melting profile.

Circular dichroism (CD) spectra. The CD spectra were recorded on a Jasco spectrometer with the following parameters: 1000 mdeg sensitivity, 0.1 nm resolution, 1.0 nm bandwidth, 1.0 s response, 50 nm min^{-1} scan speed, 1.0 cm path-length cell at 25 °C. A fixed concentration of CT DNA (10 μM BP in 10 mM phosphate buffer + 50 mM NaCl + 1 mM cacodylate) was titrated with DOX and DOXY solutions to provide [drug] : [DNA] ratios typically in the range of 0.1 to 10.

Fluorescence titrations and the osmotic stress method. Fluorescence spectroscopy was used to measure the binding constants (K_b) of DOX and DOXY with CT DNA. A fixed concentration of 2 μM DOX or DOXY was titrated with DNA solution in order to provide DNA/drug ratios typically in the range of zero to 18. The titration solutions were mixed well and were allowed to equilibrate to room temperature. The fluorescence spectrum of each titration solution was collected using a Horiba Jobin Yvon Fluoromax-3 fluorimeter with the following parameters: $\lambda_{excitation}$ = 480 nm and $\lambda_{emission}$ = 592 nm, increment of 0.5 nm, integration time of 0.1 s, excitation slit of 1.00 nm, emission slit of 3.00 nm, and room temperature. After generating the binding curve in the absence of osmolyte, osmolyte solution was added to each titration solution, allowed to equilibrate, and a new binding curve was generated. Typically, five additional binding curves were collected, with osmolalities ranging from approximately 0–3.3 osm. Triethylene glycol (TEG) was used as the osmolyte. Origin© data analysis and graphing software was used to determine the binding constant at each osmolality. The raw fluorescence data were fit with an independent, non-site-specific binding function:[13]

$$I_0 = \frac{\left[\left(K_b[S]_0 + K_b[D]_0 + 1\right) - \sqrt{\left(K_b[S]_0 + K_b[D]_0 + 1\right)^2 - 4K_b{}^2[S]_0[D]_0}\right][I_b - I_f]}{2K_b[D]_0} + I_f. \quad (1)$$

In eqn (1), I_0 is the fluorescence intensity at a given DNA/drug ratio, I_f is the fluorescence intensity of the drug in the absence of DNA, I_b is the fluorescence intensity of the drug in its bound state, $[D]_0$ is the total concentration of drug in solution, and $[S]_0$ is the total concentration of DNA in solution (in units of moles of base pairs per liter of solution). The absolute fluorescence response, I_0, was plotted as a function of total DNA concentration, $[S]_0$. The total drug concentration, $[D]_0$, was treated as a constant, and K_b treated as a parameter.

After calculating the binding constant at each osmolality in a titration series, ln(K) was plotted against solution osmolality to obtain a linear relationship. The number of water molecules exchanged during the binding event, Δn_w, was calculated using the slope of this line and eqn (2). The binding constants were then plotted against solution osmolality. A form of the Gibbs–Duhem equation[14] tells us that the slope of this line is directly proportional to Δn_w, the net uptake or release of water molecules that accompanies the binding event:

$$\frac{\partial \ln K_b}{\partial[\mathrm{Osm}]} = \frac{-\Delta n_w}{55.5}. \quad (2)$$

A van' t Hoff analysis (eqn (3)–(5)) was used to calculate enthalpy (ΔH) and entropy (ΔS) values. Data was collected over a temperature range (T) of 15 °C to 60 °C in 5 °C increments.

$$\Delta G = -RT \ln K \quad (3)$$

$$\ln K_1 - \ln K_2 = \frac{-\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad (4)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T}. \quad (5)$$

Stopped flow kinetics. A Hi-Tech Scientific SFA-20 Rapid Kinetics stopped flow accessory was used to mix solutions for kinetic experiments. Fluorescence parameters were: $\lambda_{excitation}$ = 480 nm and $\lambda_{emission}$ = 592 nm, integration times 0.005 s for DOX and 5.0 s for DOXY, and T = 30 °C. Samples for the association kinetics were prepared with DNA in excess to assure complete binding: [DOX] = 4 μM + [CT DNA] = 160 μM and [DOX] = 1 μM + [CT DNA] = 10 μM. For the dissociation kinetics measurements, one syringe contained CT DNA and DOX or DOXY at the same concentrations used for the association experiments. Samples were left overnight to assure binding equilibrium. The second syringe contained 2% sodium dodecyl sulphate (SDS), which sequesters bound DOX or DOXY upon mixing in the cuvette. Data was analysed using exponentials to obtain florescence lifetimes, τ_n, and weighting coefficients. In addition to goodness of fit, R^2, several criteria were used to determine the order of the best fit exponential. The weighting coefficients, A_n, had to be physically significant (>1%), the difference between lifetime values had to be at least an order of magnitude, and fits were robust, allowing for a range of initial parameters. Using these criteria, binding by DOX and DOXY were determined to be second order according to eqn (6), where t is time, y is fluorescence intensity, and y_0 is the baseline fluorescence intensity.

$$y = y_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} (6) \quad (6)$$

Results and discussion

After some optimization, acidic hydrolysis of the sugar moiety on DOX, followed by chromatographic purification, provided DOXY in high purity and sufficient quantities for our studies.

As the first of our three study questions dealt with the kinetics of DNA-binding and how the removal of the amino-sugar would impact binding-rate, we used a stopped-flow fluorescence experiment with each drug and CT DNA. From this method, the binding event was fit using a second order exponential, which yielded two lifetimes for DOXY, τ_{1a} = 620 ± 100 s (A_{1a} = 0.11) and τ_{2a} = 6700 ± 500 s (A_{2a} = 0.89). This means that there are at least two steps involved for binding. Similarly, fits of DOX-DNA binding data also yielded two lifetimes τ_{1a} = 0.019 ± 0.002 s (A_{1a} = 0.98) and τ_{2a} = 0.87 ± 0.08 s (A_{2a} = 0.02). The difference in association binding rates was significant, though not entirely surprising considering the uncharged DOXY could not benefit from a strong electrostatic attraction to the DNA backbone. Dissociation kinetics measurements for DOX were also measured and yielded lifetime values of τ_{1d} = 0.43 ± 0.005 s (A_{1d} = 0.57) and τ_{2d} = 1.7 ± 0.008 s (A_{2d} = 0.02). The dissociation lifetime of DOXY appears to be <100 ns and, therefore, beyond the detection limits of stopped-flow. Again, the kinetic behavior of DOXY differs significantly from that of DOX. These kinetics are summarized in Fig. 2. Because the rate of association was so much slower than known DNA-intercalating agents,[15] the question about whether a different binding mode might be operative was reinforced.

We therefore proceeded to examine the drug–DNA complex for structural changes that might point towards either intercalation or an alternate binding mode. Measurement of DNA-melting temperature (T_m) is a widely used method for recognizing structural changes in DNA.[16] Specifically, DNA denaturation has been used to extensively characterize whether or not anthracycline small-molecules have specificity for certain DNA sequences.[17] In addition, it has been shown that intercalators stabilize the DNA double-helix through pi-stacking interactions, raising the T_m,[18] while minor-groove binding has little effect on the melting point.[19] It should be noted that neomycin, a known major-groove binder, is reported to have no effect on the T_m,[20] although there is a paucity of melting data about other major-groove-bound DNA–drug complexes.

The results of our DNA melting-point study are shown in Table 1. In agreement with previously published studies, known intercalators (ethidium bromide and DOX) increase T_m for CT DNA significantly (9.1 °C and 11.4 °C, respectively.) Hoechst, a known minor-groove binder, had little effect on T_m, lowering the value by 0.8 °C. DOXY raised the temperature but

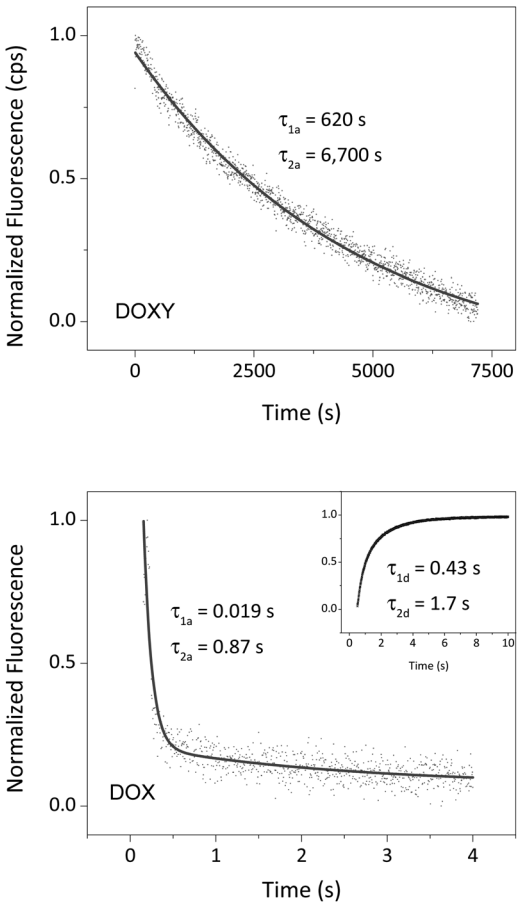

Fig. 2 Association kinetics of DOXY (top) and DOX (bottom) with CT DNA. SDS induced dissociation kinetics of DOX from CT DNA (inset). Solid lines are the best fit curves used to calculate fluorescence lifetimes. Dissociation kinetics of DOXY was too fast to be measured using the same method. While association kinetics is second order for both, DOXY binds several orders of magnitude more slowly than DOX.

Table 1 The difference in melting points of DNA binding molecules compared to the melting temperature of CT DNA alone

	Ethidium bromide	DOX	Hoechst	DOXY
ΔT_m (°C)	+9.1	+11.4	−0.8	+2.5

only by 2.5 °C, suggesting that if intercalation is occurring, it is only occurring to a small extent.

To clarify the results of the melting point study, we next attempted viscosity titrations using methods published in previous work.[21] Despite the general reliability of this method to determine DNA binding mode,[22] the low solubility of DOXY prevented us from obtaining meaningful results. Because CD is such a widely used tool for analyzing structural changes in DNA, particularly in the context of DOX binding to DNA,[7c,23] it seemed natural to undertake a similar analysis with DOXY.

When considering our experimental conditions, there were several potential challenges, of which we needed to be cognizant. DOX exhibits significant aggregation at concentrations above 50 μM in a solution with ionic strength of 2.5 mM.[7b,24] Furthermore, DOX may form higher-order complexes with DNA at high concentrations, further complicating analysis. DOXY, which lacks the hydrophilic sugar-moiety, could be expected to be even less soluble and, indeed, self-aggregation was observed at concentrations of 15–20 μM. Thus, the range of concentrations that we were able to analyze for DOXY fell outside the ranges of many previously published studies on DOX.

Therefore, to achieve the desired DOXY/DNA ratios (ranging from 0 to 10), with DOX/DNA ratios in the same regime, we found it expedient to fix the CT DNA concentration at 10 μM, while adding variable amounts of DOX or DOXY. The results can be seen in Fig. 3. The spectrum of CT DNA shows a negative band at 245 nm and a positive band at 275 nm, which are measures of right-handed helicity and ππ* stacking, respectively.[25] It had previously been reported by Garcia and coworkers that intercalation of DOX with CT DNA could be observed in the DOX CD spectrum by a red-shift in the spectral band at 300 nm, accompanied by a decrease in intensity. They observed a maximal shift at a DOX/DNA ratio of 0.35, which

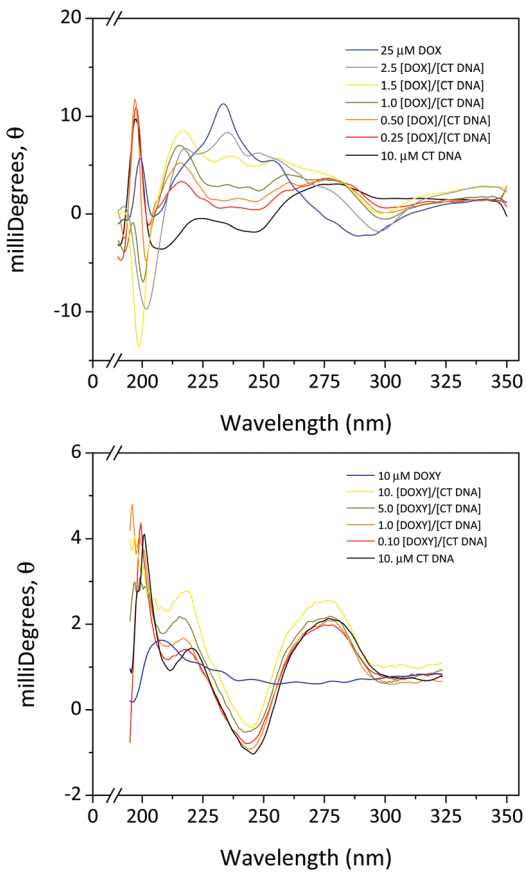

Fig. 3 CD spectra of CT DNA, DOX, and DOX/CT DNA ratios between 0 and 2.5 (top). CD spectra of CT DNA, DOXY, and DOXY/CT DNA ratios between 0 and 10 (bottom). DOX is a known intercalator and induces band shifts at 195 nm, 225 nm, and 300 nm. These shifts are not observed in the spectra for DOXY bound to CT DNA.

agrees with the accepted binding of DOX at roughly every third DNA base pair. We also observed this shift in the spectrum for DOX/DNA ratios between 0.25–1.0.

Garcia also pointed out the positive spectral band at 293 nm and assigned that as a shift of the DOX band from 250 nm. However, CT DNA has a broad positive band centered at 275 nm and it seems likely that in the regime examined by Garcia (with a 5-fold excess of CT DNA relative to DOX), the DOX band at 250 nm was simply buried under the larger CT DNA band. We did observe a blue-shift in the CT DNA spectral band, from 225 nm to 215 nm, suggesting structural changes in the DNA helix. This same shift was noted by Giustini and was attributed to DOX binding to poly-GC DNA sequences.[7c] This shift was observed with as little as 0.1 DOX/DNA and became more pronounced up to a 1 : 1 ratio, after which the DOX band at 234 nm started to predominate.

The final noteworthy spectral shift was observed in the CD spectrum of CT DNA at 195 nm. The positive band was remained largely unchanged up to a DOX/DNA ratio of 0.50. However, at and above concentrations of 10 μM DOX, this band was completely replaced by a large, negative band at 197 nm. The fact that DOX has a positive spectral feature centered at 200 nm suggests that this shift represents a binding event between DNA and DOX; possibly through a secondary binding mode, as postulated by Garcia.

By contrast, the CD spectrum for DOXY with CT DNA exhibits no such shifts, even when DOXY was added in great excess. The lack of a strong signal from the DOXY is not surprising, considering the cleavage of the amino-sugar significantly decreases (but does not entirely negate) the chiral character of DOXY. However, the complete lack of change to the CT DNA bands, particularly the band at 225 nm, supports the theory that DOXY is not intercalating.

Considering the mounting evidence that DOXY does not intercalate, but instead stays in the DNA minor-groove, by analogy with the DOX pre-intercalative state, DOXY should also bind less strongly to DNA than does DOX. Granted, the lack of the protonated amino-sugar on DOXY removes the electrostatic attraction to the phosphate backbone, which would automatically decrease the binding affinity. Fortunately, Chaires *et al.*[7a] had already shown that, by using salt back-titration[26] and applying the Manning–Record equation,[27] the electrostatic component of the binding constant can be isolated. They found that HDX, which possess the sugar-moiety but lacks the charged ammonium, binds to CT DNA 100-times less strongly than DOX, corresponding to a $\Delta\Delta G$ of 2.5 kcal mol^{-1}, of which 1.9 kcal mol^{-1} were electrostatic. Taken together, the non-electrostatic $\Delta\Delta G$ between DOX and HDX was only about 0.6 kcal mol^{-1}. By contrast, after accounting for electrostatic differences, the $\Delta\Delta G$ between DOX and DOXY was 2.0 kcal mol^{-1}, which makes up over 1/5th of the total binding free energy. He attributes this difference in binding energy to the importance of the sugar rather than a shift in DNA binding mode.

Under our titration conditions ([Na^+] = 63 mM), DOX had a binding constant of 1.3 × 10^7 ± 0.2, while for DOXY, K = 2.9 × 10^4 ± 0.2 (Fig. 4). When corrected for the difference in ionic strength, the relative magnitudes match well with Chaires' values. If, as we think, DOXY is binding to the minor-groove of DNA, it is additionally worth comparing with Garcia's value for pre-intercalative binding of DOX, which was measured as K = 1 × 10^4 when I = 100 mM. After correcting for ionic strength, that number is almost identical to our DOXY binding constant.

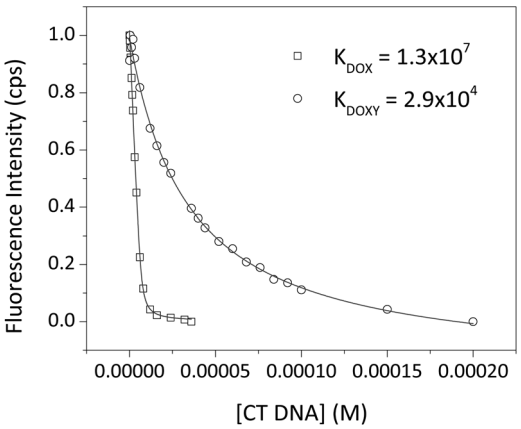

Fig. 4 Binding isotherms for the interactions of CT DNA with DOX and DOXY. Solid lines are the best fit curves used to calculate equilibrium constants: K_{DOX} = 1.3 × 10^7 ± 0.2 and K_{DOXY} = 2.9 × 10^4 ± 0.2. The fluorescence signal is quenched to a greater degree at low concentrations of DNA for DOX, which is indicates more efficient binding by DOX.

In previous work in our laboratory, we found that the number of water molecules exchanged during the binding process of small molecules with DNA depended on the binding mode. In the case of DOXY, the water exchange value was +66, which is a large increase in the number of coordinated waters, consistent with other groove binding drugs (Fig. 5). For example, netropsin binding to DNA takes up +50–60 water molecules, while Hoechst uptakes +74 water

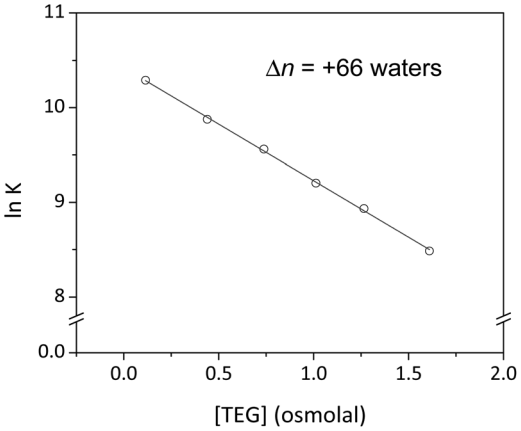

Fig. 5 Eqn (2) and the equilibrium constants obtained from titrations of DOXY and CT DNA in the presence of various concentrations of TEG were used to make a water exchange plot. From the linear regressions of the data, a net uptake of water, Δn_{DOXY} = +66 waters (R^2 = 1.00), was calculated.

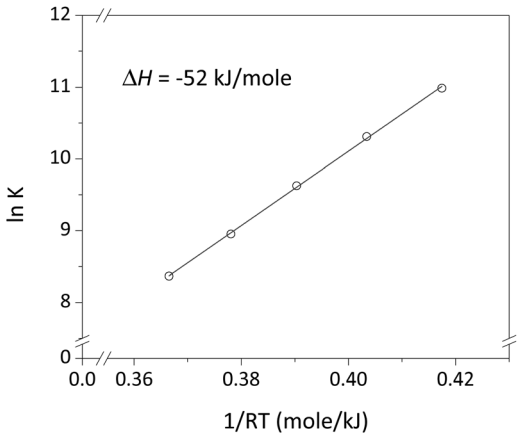

Fig. 6 Van't Hoff plot to determine the enthalpy of binding of DOXY with CT DNA. The equilibrium constants were measured at five different temperatures. From the linear regression of the data, an exothermic enthalpy, $\Delta H_{DOXY} = -52$ kJ mol^{-1} ($R^2 = 1.00$), was calculated.

molecules.[28] Intercalating molecules typically uptake a significantly smaller number of waters (0 to +30),[14a] as exemplified by DOX (+13 waters). It is interesting to note that, in this experiment, DOXY more closely resembles the charged, structurally dissimilar groove binders than it does the anthracycline, DOX.

The thermodynamic data from the van't Hoff analysis in Fig. 6, also supports a groove-bound state for DOXY. While DOX displays an entropic increase for binding ($\Delta S = +21$ J K^{-1} mol^{-1}), indicative of a more disorder in the intercalated state, DOXY has a negative entropy of binding ($\Delta S = -88$ J K^{-1} mol^{-1}). The large uptake of water molecules by DOXY likely contributes to the large, negative entropic cost of binding. Similarly, the increase in hydrogen-bonding from a more extended water network is consistent with a large negative enthalpy for DOXY ($\Delta H = -52$ kJ mol^{-1}). DOX ($\Delta H = -31$ kJ mol^{-1}), by comparison, is significantly less exothermic than DOXY. Chaires *et al.*[29] reviewed thermodynamic data in the literature and found entropy changes for various DNA-binding small-molecules to range from −40 to +220 J K^{-1} mol^{-1}, while enthalpy changes were between −38 to +18 kJ mol^{-1}. The ΔS and ΔH values calculated for DOXY both extend beyond the ranges for either typical minor-groove binders or intercalators, indicating either another binding mode (such as quasi-intercalation)[30] or a unique thermodynamic profile due to the neutral charge (the thermodynamic data surveyed by Chaires all came from cationic DNA-binding molecules.)

Conclusions

Despite conservation of the entire planar, aromatic tricyclic portion of DOX – in short, the entire portion of the molecule that intercalates between DNA base pairs – DOXY has a completely different DNA-binding mode with CT DNA. It is presumed that the mode involves binding to the DNA minor-groove based on the precedence of other non-intercalating, DNA-binding small-molecules. This is supported by the evidence obtained from CD, DNA melting temperature, the shift in binding constant, and water uptake studies. The possibility of quasi-intercalation cannot be discounted, however, based on the unusual entropy and enthalpy values for DNA binding. There is no obvious structural reason that DOXY could not intercalate and this, therefore, invites a more careful study of all new proposed intercalating agents, rather than basing assumptions on structural analogy.

It is, furthermore, clear from this study that the protonated amino-sugar moiety plays a role, not only in rate of pre-coordination between the drug-molecule and DNA, but also in determining the ultimate bound state of the small-molecule ligand. The implications of these results for chemotherapeutics research warrants further study, as understanding the specifics of molecular binding modes may prove useful in determining other cellular outcomes, which would have implications for cell physiology and eventual patient outcomes.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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