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Substituent control of DNA binding modes in a series of chalcogenoxanthylium photosensitizers as determined by isothermal titration calorimetry and topoisomerase I DNA unwinding assay

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ABSTRACT

The DNA binding efficacy and preferred mode of binding of a series of rhodamine-related chalcogenoxanthylium dyes was investigated by isothermal titration calorimetry (ITC) using ctDNA, $[poly(dCdG)]_2$ and $[poly(dAdT)]_2$, and by a topoisomerase I DNA unwinding (Topo I) assay. The dyes of this study showed tight binding to ctDNA with binding constants, K_b, on the order of 10^6–10^7 M^{-1}. The ITC and Topo I assay studies suggested that the 9-substituent has a strong impact on binding modes ranging from an apparent preference for intercalation with a 9-2-thienyl substituent (similar binding to $[poly(dCdG)]_2$ and $[poly(dAdT)]_2$, re-supercoiling of DNA in the Topo I assay at $<10^{-5}$ M dye), to mixed binding modes with 9-phenyl derivatives (2- to 3-fold preference for binding to $[poly(dAdT)]_2$, re-supercoiling of DNA in the Topo I assay at $\sim 2 \times 10^{-5}$ M dye), to minor groove binding in a 9-(2-thienyl-5-diethylcarboxamide) derivative (strong preference for binding to $[poly(dAdT)]_2$, did not show complete re-supercoiling in the Topo I assay). No binding to ctDNA was observed in one derivative with a 9-(3-thienyl-2-diethylcarboxamide) substituent, which cannot be co-planar with the xanthylium core. In series of dyes where the chalcogen atom was varied, the selenoxanthylium derivatives had 2- to 3-fold higher values of K_b than the corresponding xanthylium, thioxanthylium, or telluroxanthylium derivatives, which all showed comparable values of K_b. The chalcogen atom appeared to have little influence on binding mode.

1. Introduction

Photodynamic therapy using a photosensitizer (**PS**), oxygen, and light is currently being evaluated as a method to rid blood samples of viral and bacterial pathogens.[1] Since mature red blood cells lack genomic material, the nucleic acids of viral and bacterial pathogens become specific targets for the **PS**. In addition, a viable **PS** for this application needs to have little affinity for red cells (specific or non-specific), must permeate both viral and cellular membranes, absorb red light to avoid light attenuation by hemoglobin absorption, and display little dark toxicity toward either red cells or transfusion recipients. While many **PS**s have been examined, most have failed due to non-specific actions of the **PS** including low efficacy against pathogens and unwanted background hemolysis of red blood cells.[2,3]

To circumvent these problems, **PS**s that target the pathogenic genomic material are currently being investigated.[4–7] Among these, chalcogenoxanthylium compounds have exhibited potency against several types of viral and bacterial pathogens that can infect the blood supply.[8] These dyes do not show appreciable spectral shifts when bound to DNA; which limits conventional approaches to measuring DNA binding constants with spectrophotometry. In the present study, the DNA binding efficacy of the series of chalcogenoxanthylium dyes of Chart 1 is investigated by the non-spectrophotometric methods of isothermal titration calorimetry (ITC) and a topoisomerase I DNA unwinding assay (Topo I assay). ITC is an established biophysical technique used to study drug–DNA interaction.[9,10]

The utility of the Topo I assay to determine both the relative DNA binding strengths and DNA binding mode has been described.[6,11] Briefly, the Topo I assay exploits the ability of topoisomerase I to relax supercoiled DNA.[12,13] Under the conditions of the assay, plasmid DNA is first relaxed by the topoisomerase I enzyme and then is exposed to the compound under study. After removal of the compound and enzyme, intercalators will cause re-supercoiling of the plasmid DNA. Re-supercoiling is due to the change in DNA linking number that accompanies relaxation by the enzyme and occurs to the extent to which the intercalator molecule was bound.[6,11–13] Initial DNA unwinding will be dependent only upon

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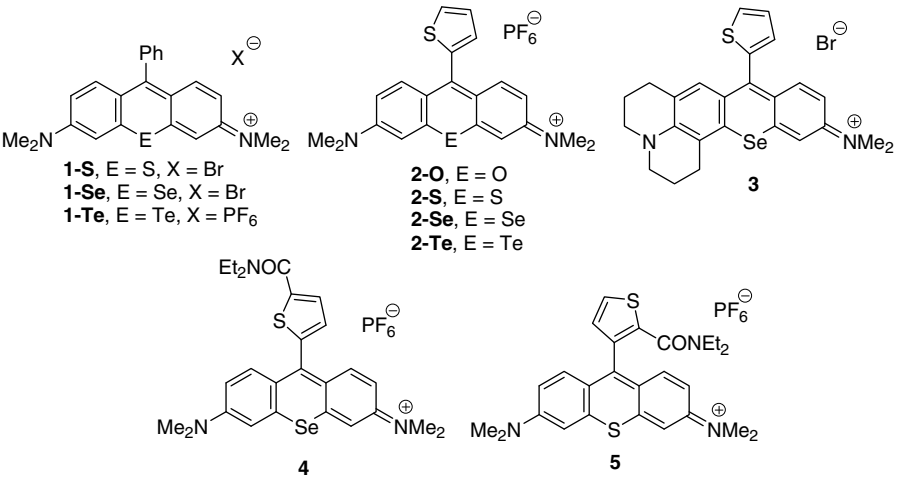

Chart 1. Chalcogenoxanthylium dyes evaluated in this study.

the extent to which binding occurs, thus the minimum concentration needed to cause complete re-supercoiling will be indicative of how much compound was initially bound and thus the relative binding affinity.[6,11] Conversely, minor groove binders should not induce appreciable re-supercoiling due to negligible DNA unwinding upon binding.

2. Results and discussion

2.1. Selection and synthesis of chalcogenoxanthylium dyes

The dyes of Chart 1 were selected for evaluation in order to compare (1) the influence of the chalcogen atom (O, S, Se, Te) on binding to DNA, (2) the difference between 9-aryl substituents that are essentially orthogonal to the xanthylium core (the phenyl substituent of series **1-E**[14,15] and the *N*,*N*-diethyl 3-thienyl-2-carboxamide substituent of compound **5**) and 9-aryl substituents that can be rigorously co-planar with the chalcogenoxanthylium core (the 2-thienyl substituents of series **2-E**[14,15] and compound **3**[16] and the *N*,*N*-diethyl 2-thienyl-5-carboxamide substituent of compound **4**[16]), and (3) the difference between a 'flat' chalcogenoxanthylium core (series **2-E**) and the xanthylium core in **3** where the pentacylic structure is non-planar in the julolidyl-containing half.

2.2. Determination of DNA binding affinity using ITC

The DNA binding constants for the binding of the chalcogenoxanthylium compounds of Chart 1 to ctDNA were determined using ITC. Representative ITC data for this binding to ctDNA is shown in Figure 1 and all ITC data are summarized in Table 1 as the binding constant, K_b, to ctDNA. In comparing series **1-E** and **2-E**, the chalcogenoxanthylium dyes with a 9-phenyl substituent bound 2–3 times more tightly to ctDNA than dyes with a 9-(2-thienyl) substituent. Within each of these series, the xanthylium, thioxanthylium, and telluroxanthylium dyes displayed comparable binding constants to ctDNA while the selenoxanthylium dyes in each series bound two to three times more tightly to ctDNA than other chalcogen analogues ($K_b \sim 1 \times 10^7\ M^{-1}$ for **1-Se** and $K_b \sim 5 \times 10^6\ M^{-1}$ for **2-Se**, Table 1). The bulkier selenoxanthylium core of compound **3** (with the two additional fused rings on one side) decreased binding to ctDNA by a factor of 3 relative to **2-Se**. The binding of compound **4** to ctDNA ($K_b \sim 1 \times 10^7\ M^{-1}$, Table 1) was quite similar to that for **1-Se**. In our earlier work,[8] the selenoxanthylium derivatives **1-Se** and **2-Se** were the most effective **PS**s toward both viral and bacterial pathogens relative to lighter chalcogen analogues. This was originally attributed to higher quantum yields for the generation of singlet oxygen,[8] but the increased binding to DNA may also contribute to their effectiveness.

The weakest binding among these dyes was observed with compound **5**, which showed essentially no binding to ctDNA. The lack of binding shown by **5**, which is electronically similar to **4**, is likely due to the restricted rotation of the 9-thienyl ring substituent resulting from steric interactions between the diethylcarboxamide group and the *peri*-protons of the thioxanthylium ring system. These steric interactions would be expected to be greater than the steric interactions between the *ortho*-protons of the phenyl substituent and the *peri*-protons of the xanthylium core in the **1-E** series. Thus, the 9-(3-thienyl-2-diethylcarboxamide) substituent is unable to approach a co-planar configuration with the xanthylium core limiting the conformational degrees of freedom of **5**. While compounds **4** and **5** are electronically similar, the diethylcarboxamide group in **4** is found at the 5-position of the 2-thienyl substituent and has minimal steric interaction with the xanthylium core. Thus, compound **4** has far fewer conformational restrictions than **5** and binds ctDNA tightly.

In both the **1-E** and **2-E** dyes, binding affinity increases when S is replaced by Se in the xanthylium core (Table 1). Why should such a simple substitution have such a dramatic impact on binding to DNA? This substitution has minimal impact on values of log *P* for the dyes (log *P* of 0.07 for **1-S** vs 0.09 for **1-Se** and log *P* of –0.49 for **2-S** vs –0.31 for **2-Se**.)[8] This substitution also has minimal impact on the dimensions of the molecules with the N1–N2 distance only increasing from 10.007 Å in **1-S** to 10.155 Å for **1-Se**.[16] Consequently, changes in these parameters do not appear to be responsible for the increased binding.

The Se atom is slightly more electropositive than the S atom and the atomic orbitals involved in π-bonding with the 2p orbitals of the carbon framework are 4p orbitals for Se and 3p orbitals for S. These differences have a more pronounced impact on the electronic structure of the chalcogeoxanthylium dyes. As shown in Chart 2, two types of resonance are found in the chalcogenoxanthylium dyes.[15] The heteroatom can interact directly with the carbon π-framework to give benzenoid-type resonance as shown for resonance forms **IA** and **IB** in Chart 2. In resonance forms where the heteroatom lonepair does not contribute, cyanine resonances as shown for resonance forms **IIA** and **IIB** will predominate. The substitution of Se for S decreases the benzenoid-type resonance from the heteroatom, which consequently increases the contributions from the cyanine-like resonances. The net result is an increase in the net positive charge on the two N atoms, which

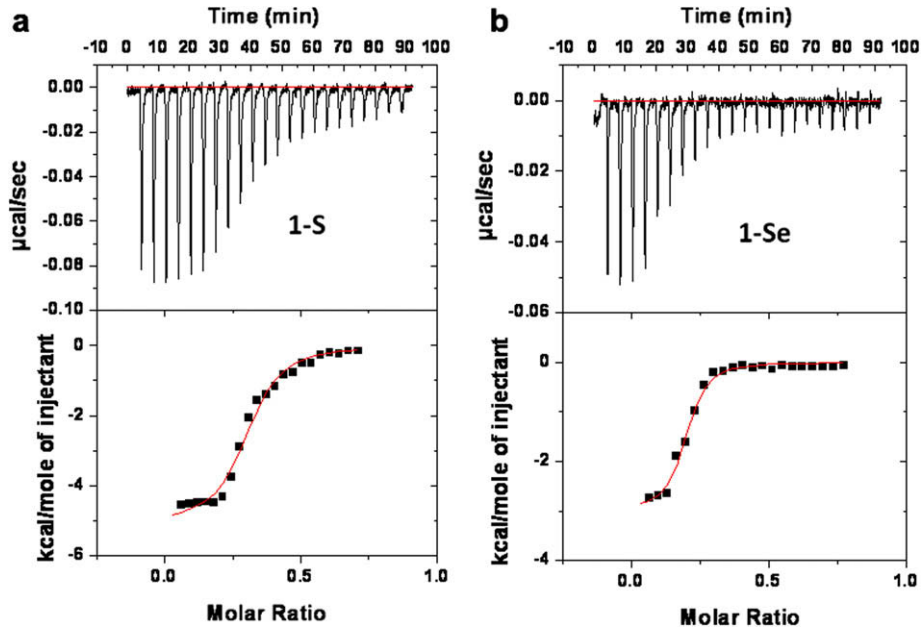

Figure 1. Calorimetric data (raw) for the titration of (a) **1-S** (4.5×10^{-5} M) and (b) **1-Se** (5.1×10^{-5} M) into ctDNA (1.2×10^{-5} M and 1.3×10^{-5} M in bp, respectively) at 30 °C (top). Binding isotherm (heat change vs drug/DNA molar ratio) was obtained from the integration of raw data and fitted to a 'one-site' binding model (bottom).

Table 1
DNA binding affinity data (K_b), and concentrations for complete re-supercoiling in a topoisomerase I DNA unwinding assay (Topo I assay) for the chalcogenoxanthylium dyes of this study.

Compound	K_b (ctDNA) (10^6 M^{-1}) (ITC)[a]	K_b (AT) (10^6 M^{-1}) (ITC)[b]	K_b (GC) (10^6 M^{-1}) (ITC)[c]	Topo I assay (10^{-6} M)[d]
1-S	5.2 ± 1.1	2.2 ± 0.3	1.3 ± 0.2	20
1-Se	10.3 ± 2.1	2.8 ± 0.2	0.9 ± 0.2	23
1-Te	6.4 ± 2.3	—	—	—
2-O	2.1 ± 0.3	2.2 ± 0.3	2.1 ± 0.3	5.1
2-S	1.6 ± 0.2	1.5 ± 0.2	2.7 ± 0.5	9.7
2-Se	4.7 ± 1.0	1.6 ± 0.3	3.7 ± 0.7	5.1
2-Te	2.1 ± 0.4	—	—	—
3	1.4 ± 0.3	—	—	—
4	12.4 ± 2.2	2.3 ± 0.4	~0	—
5	~0	—	—	—

[a] MES00 buffer, pH 6.25, using ctDNA.
[b] MES buffer containing 40 mM NaCl, pH 6.25, using $[poly(dAdT)]_2$.
[c] MES buffer containing 40 mM NaCl, pH 6.25, using $[poly(dCdG)]_2$.
[d] Minimum concentration of compound required for complete re-supercoiling.

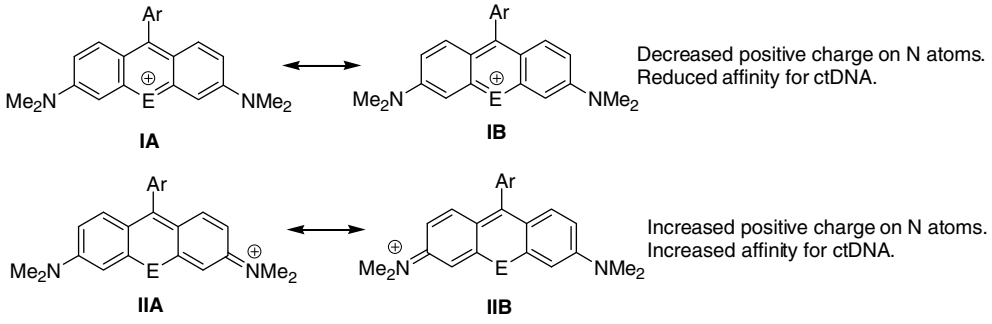

Chart 2. Two types of resonance contributions in chalcogenoxanthylium dyes.

would lead to tighter binding to ctDNA if the amino functionality is involved in direct contact with the DNA.

Binding constants to ctDNA are quite similar for **2-O** and **2-S** and one can argue that resonance contributions are similar in both compounds. The telluroxanthylium analogues **1-Te** (K_b ~6×10^6 M^{-1}, Table 1) and **2-Te** (K_b ~2×10^6 M^{-1}, Table 1) displayed reduced binding affinity for ctDNA relative to the corresponding selenoxanthylium analogues **1-Se** and **2-Se**, respectively. Even though the cyanine contributions should be more pronounced in the telluroxanthylium dyes, the Te atom is significantly more electropositive than the Se atom and is actually electropositive relative to C. The net result is an inductive increase in electron density in the carbon framework,

which would diminish the net delocalized positive charge at the two N atoms and decrease affinity for ctDNA. The Te atom also has a much larger covalent radius than the other chalcogen atoms and the central ring of the chalcogenoxanthylium core is distorted (Te–C bond lengths of 2.07 Å and a C–T e–C bond angle of 95.3°),[15] which might also contribute to decreased interactions with ctDNA.

Determination of the binding constants for the telluroxanthylium derivatives was complicated by the fact that these analogues are more reactive at a pH $\geqslant$7 in the presence of light and oxygen than the lighter chalcogen analogues. The telluroxanthylium chromophore was rapidly 'bleached' as the tellurium atom was oxidized or the chromophore reacted with hydroxide. As a result, studies with the telluroxanthylium derivatives were carried out in the dark in degassed buffer at lower pH (~5.5).

Overall, the **1-E** series with 9-phenyl substituents exhibited higher binding affinity compared to the **2-E** series with 9-(2-thienyl) derivatives. The higher binding exhibited by the 9-phenyl substituted compounds is likely due to favorable hydrophobic contributions from the phenyl ring relative to the 2-thienyl ring as indicated by the more positive values of log*P* (see above). Favorable contributions to drug–DNA interactions from hydrophobicity have been reported.[17]

In order to gain more insight into the possible involvement of a minor groove vs an intercalative binding mode, the binding of representative compounds (**1-S, 1-Se, 2-O, 2-S, 2-Se, 4**) to $[poly(dAdT)]_2$ and $[poly(dCdG)]_2$ sequences was also studied using ITC. Figure 2 shows typical ITC data for the binding of **2-O** to $[poly(dAdT)]_2$ and $[poly(dCdG)]_2$ sequences while binding constants, K_b, for binding of the chalcogenoxanthylium dyes to $[poly(dAdT)]_2$ and $[poly(dCdG)]_2$ sequences are compiled in Table 1. It is known that compounds that bind solely to the DNA minor groove generally show a strong preference (a factor of 10 or greater) for binding to AT-rich sequences relative to GC-rich sequences[18] due to the occlusion from the GC-rich minor groove by the protruded 2-NH_2 group of guanine.[19] Only compound **4**, which showed high ctDNA affinity, exhibited a strong relative preference for the AT-mer ($[poly(dAdT)]_2$) strongly suggesting the prevalence of a minor groove binding mode. In fact, our ITC studies showed that the binding of **4** to $[poly(dCdG)]_2$ was negligible (K_b ~0), showing only background signal associated with titration of the drug into buffer. Evidence for a minor groove binding mode for **4** was also shown in our Topo I assay described below.

Both **1-S** and **1-Se** showed binding to both the AT- and GC-rich sequences, but with a slight preference for the AT-mer (1.7/1 and 3.1/1 AT/GC selectivity, respectively, Table 1) suggesting a possible mixed binding mode, that is, contributions from both intercalative and minor groove binding for these compounds. The existence of mixed DNA binding modes is not uncommon and, in fact, some researchers have proposed that the anticancer efficacy of some therapeutic drugs may be linked to their ability to exhibit mixed binding modes.[20,21]

Compound **2-O** had identical binding constants within experimental error to both AT- and GC-rich sequences (Table 1), suggesting that a minor groove mode does not predominate. The sulfur (**2-S**) and selenium (**2-Se**) analogues showed a preference for the GC-rich sequence (AT/GC selectivity of 0.56/1 and 0.43/1, respectively, Table 1). It is likely that the primary mode of binding of **2-O, 2-S**, and **2-Se** to ctDNA is intercalation since no AT preference was observed.

We see here an overall increase in the AT preference as the 9-substituent is changed from 2-thienyl to -phenyl to 2-thienyl-5-diethylcarboxamide, suggesting an increase in minor groove binding. This trend is even more evident when we look at the relative AT vs GC preference for analogous members of the three series, which differ only in their 9-substituent (e.g., **2-Se**, **1-Se**, and **4**). With the 9-(2-thienyl) substituent (compound **2-Se**), there is no AT preference. When the 9-phenyl substituent is present (compound **1-Se**), there is a ~3-fold higher binding affinity for the AT-rich sequence compared to the GC-rich sequence. However, when the 9-(2-thienyl-5-diethylcarboxamide) group is present (compound **4**), a significant AT preference is observed since there is essentially no binding to the GC-rich sequence. A similar (but less pronounced) trend is observed when we compare the 9-(2-thienyl) thioxanthylium derivative (**2-S**) with the 9-phenyl thioxanthylium derivative (**1-S**).

The preference for intercalation vs minor groove binding exhibited by the 9-(2-thienyl)-substituted relative to the 9-phenyl-substituted dyes is plausible given the large free energy

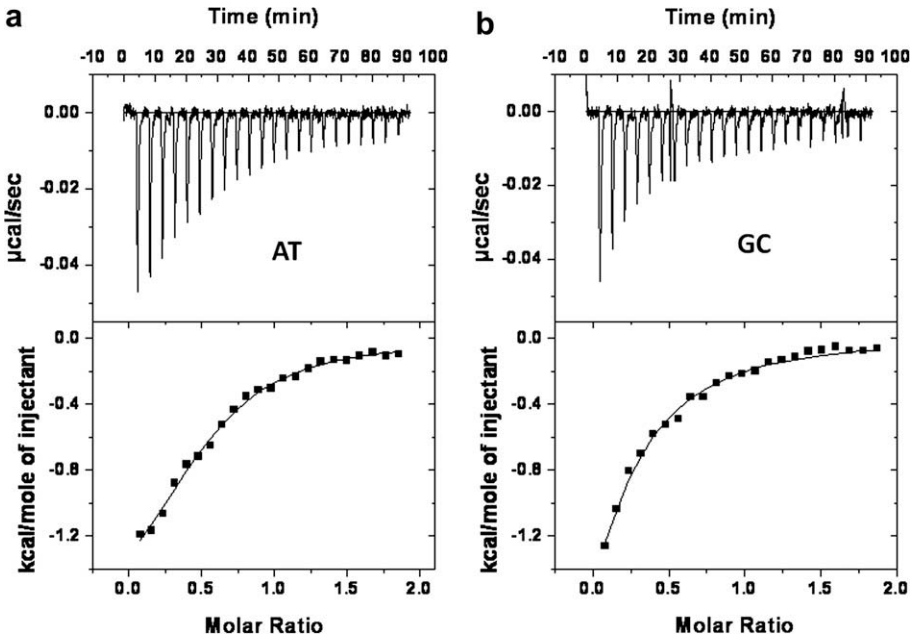

Figure 2. Calorimetric data (raw) for the titration of 1.13×10^{-4} M **2-O** into 1.2×10^{-5} M of (a) $[poly(dAdT)]_2$ and (b) $[poly(dCdG)]_2$ at 30 °C (top). Binding isotherm (heat change vs drug/DNA molar ratio) was obtained from the integration of raw data and fitted to a 'one-site' binding model (bottom).

difference associated with placing a 9-(2-thienyl) substituent and a 9-phenyl substituent co-planar with the xanthylium core. The steric interaction between a 9-phenyl substituent and the xanthylium core in the rhodamines should be quite similar to the steric interactions within 9-phenylanthracene, where there is a 20 kcal mol^{-1} cost for co-planarity.[22] In earlier work,[15] we determined that the smaller 9-(2-thienyl) substituent has roughly a 5 kcal mol^{-1} barrier to co-planarity in the 2,7-bis(dimethylamino)xanthylium system compared to 20 kcal mol^{-1} for the 9-phenyl substituent using optimized molecular geometries at the DFT level using the B3LYP hybrid functional.[15] In other words, it is much easier for the 9-thienyl dyes to become flat enough to slide between adjacent base pairs of DNA during intercalation.

The increased preference for a minor groove binding mode shown by compounds **4** and **1-Se** may also be contributing to their higher overall binding constants for ctDNA. Greater contributions from minor groove binding over intercalation generally increases the DNA binding constant since during minor groove binding, no additional free energy is required to open up the DNA binding site and unstack adjacent base pairs, as is the case for intercalation.[17,23]

2.3. DNA binding using Topo I assay

The relative DNA binding affinity and preferred binding mode of the chalcogenoxanthylium dyes of this study were also investigated using a Topo I assay. Results from the Topo I assay are shown in Figure 3 and minimal concentrations required for complete re-supercoiling are given in Table 1. The Topo I assay gives relative DNA binding abilities for intercalation and not for other binding modes. Under the conditions of our Topo I assay, the lower the concentration required to cause re-supercoiling, the stronger the affinity for DNA.[6,11] The technique can also help tease apart the DNA binding mode adopted by a DNA binder, that is, intercalation vs minor groove binding.

The minimum concentration required for complete re-supercoiling of **2-Se** ($\sim 5 \times 10^{-6}$ M, Table 1) was half that of **2-S** ($\sim 1 \times 10^{-5}$ M, Table 1), suggesting that **2-Se** was a stronger DNA binder than **2-S**. This result was consistent with the trend in relative binding constants for ctDNA (i.e., tighter binding by **2-Se**) and for AT/GC selectivity (0.56/1 and 0.43/1 for **2-S** and **2-Se**, respectively, Table 1) obtained using ITC. The Topo I data suggest that these compounds prefer an intercalative binding mode and that **2-Se** is the tighter binder.

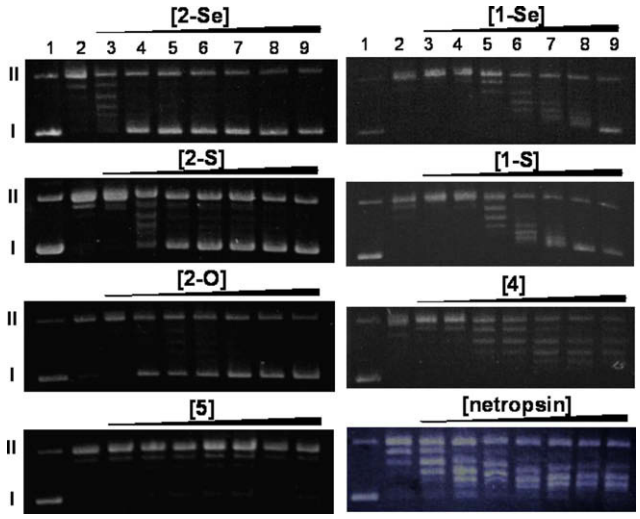

Figure 3. Topo I assay of **2-Se**, **2-S**, **2-O**, **5**, **1-Se**, **1-S**, **4** and netropsin using 5 U of Topo I. Lane 1 contains only DNA and serves as a control. Lane 2 contains DNA and Topo I, but no compound. Remaining lanes (3–9) contain DNA, Topo I, and increasing concentrations of each compound ($2.5–24 \times 10^{-6}$ M). The Topo I assay for netropsin is taken from Ref. [11]. Forms I and II refers to fully supercoiled and relaxed DNA, respectively.

It is expected that compounds that bind to DNA via the minor groove in addition to intercalation will elicit less perturbation of the DNA structure (unwinding) and therefore have less ability to cause re-supercoiling. These mixed-binders will not induce re-supercoiling consistent with their relative DNA binding affinities in our Topo I assays, since re-supercoiling will be dependent on the relative contributions of intercalation vs minor groove binding. The greater the contribution from minor groove binding, the smaller the re-supercoiling effect.

For the 9-phenyl derivatives (compounds **1-S** and **1-Se**), results from Topo I assay show no significant difference between these two compounds; that is, both caused complete re-supercoiling at similar concentrations ($\sim 2 \times 10^{-5}$ M, Table 1). These are higher concentrations than required by **2-S** and **2-Se**. Furthermore, ITC studies showed 2- to 3-fold stronger binding for **1-S** and **1-Se** to ctDNA relative to **2-S** and **2-Se**. These data suggest that another binding mode is involved with **1-S** and **1-Se** in addition to intercalation. Furthermore, based on the moderate AT preference found for **1-Se**, and to a lesser extent **1-S**, in the ITC study, the other mode is likely a minor groove binding mode. The fact that we observed complete re-supercoiling during the Topo I assay of **1-S** and **1-Se** shows that intercalation is involved in the binding of these dyes to DNA. It should also be noted that 2- to 4-fold higher concentrations of **1-S** and **1-Se** were required for complete re-supercoiling (Table 1) relative to **2-O**, **2-S**, and **2-Se** implying lower intercalative contributions for the binding of the **1-E** dyes. Taken together, these results suggest a mixed binding mode for both **1-Se** and **1-S**, with a greater involvement from minor groove binding for **1-Se**.

The Topo I assay of compound **5** showed negligible re-supercoiling even at the highest dye concentration used (2.4×10^{-5} M, $[dye]/[DNA_{bp}] = 0.9$) and the observed re-supercoiling was no greater than the control lane containing topoisomerase and no dye (lane 2, Fig. 3). These data are consistent with the observation from ITC showing negligible DNA binding.

As shown in Figure 3, compound **4** caused apparent partial re-supercoiling of topoisomers, but complete re-supercoiling was never observed even at the highest concentrations employed. Interestingly, the Topo I assay results for compound **4** were very similar to that reported by us (Fig. 3)[11] and others[21,24] for the known minor groove binder, netropsin. Minor groove binding for **4** was also predicted by its strong AT preference in ITC studies (Table 1).

For netropsin, the apparent partial re-supercoiling observed was attributed to non-specific, non-intercalative binding by the compound resulting in increased stiffness and thus changes in hydrodynamic mobility of the DNA. Breslauer and coworkers[21] did viscometric studies on netropsin and observed a slight increase in DNA solution viscosity with no further increase after $r([dye]/[DNA_{bp}]) = 0.34$, which was very similar to what we observed at $r \sim 0.3$ in an earlier Topo I assay study of netropsin.[11] In the present Topo I assay of **4**, the extent of re-supercoiling was essentially unchanged after $r \sim 0.4$. Here, we propose a similar explanation for the binding of **4** to DNA based on the close similarities between our Topo I assay results and the results for the minor groove binder, netropsin.

3. Summary and conclusions

The chalcogenoxanthylium dyes bind tightly to ctDNA with values of K_b on the order of 10^6–10^7 M^{-1}. The 9-aryl or heteroaryl substituent contributes strongly to the binding mode of the chalcogenoxanthylium compounds of Chart 1. None of the **2-E** series of dyes with a 9-(2-thienyl) substituent showed a preference

for [poly(dAdT)]$_2$ relative to [poly(dCdG)]$_2$ by ITC with **2-S** and **2-Se** showing a preference for [poly(dCdG)]$_2$. The **2-E** series of dyes also showed the lowest concentrations for re-supercoiling in the Topo I assay (Table 1). The **1-E** series of dyes showed a slight preference for [poly(dAdT)]$_2$ by ITC and required higher concentrations for re-supercoiling in the Topo I assay. Selenoxanthylium dye **4** showed a strong preference for [poly(dAdT)]$_2$ relative to [poly(dCdG)]$_2$ by ITC with essentially no binding to [poly(dCdG)]$_2$ and did not show complete re-supercoiling in the Topo I assay. These data are consistent with a preference for intercalation by the **2-E** dye series, mixed binding modes for the **1-E** series of dyes (minor groove binding and intercalation), and essentially only minor groove binding by dye **4**. A 9-(3-thienyl-2-diethylcarboxamide) gave a chalcogenoxanthylium dye, which exhibits essentially no binding to ctDNA as measured by ITC.

The chalcogen atom has some impact on the observed binding to ctDNA and appears to be a combination of both resonance and inductive effects. The π-framework of the xanthylium dyes has contributions from both benzenoid-type resonances and cyanine-type resonances as shown in Chart 2. Xanthylium and thioxanthylium dyes have similar contributions from each type. The selenoxanthylium dyes have poorer overlap of the Se 4p orbitals with the carbon 2p orbitals, which increases contributions from the cyanine-type resonances and affinity for DNA if the two N atoms delocalizing the positive charge interact directly with DNA. The cyanine contributions should be more pronounced in the telluroxanthylium dyes, but the Te atom is significantly more electropositive than the Se atom and is actually electropositive relative to C. The net result is an inductive increase in electron density in the carbon framework diminishing the net delocalized positive charge at the two N atoms. Consequently, the selenoxanthylium dyes show tighter binding to ctDNA relative to xanthylium, thioxanthylium, and telluroxanthylium analogues.

The chalcogenoxanthylium dyes represent a versatile scaffold for designing sensitizers for PDT. Small changes in structural parameters, such as the 9-aryl or heteroaryl substituent and the ring chalcogen atom, lead to pronounced changes in binding modes to genomic material and may permit the design of highly selective agents for specific viral or bacterial pathogens.

4. Experimental

4.1. General methods

Solvents and reagents were used as received from Sigma–Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted. Concentration in vacuo was performed on a Büchi rotary evaporator. NMR spectra were recorded on an Inova 500 instrument with residual solvent signal as internal standard: $CDCl_3$ (δ 7.26 for proton, δ 77.0 for carbon), CD_2Cl_2 (δ 53.8 for carbon). Infrared spectra were recorded on a Perkin-Elmer FTIR instrument. UV–vis near-IR spectra were recorded on a Perkin-Elmer Lambda 12 spectrophotometer or on a Shimadzu UV-3600 spectrophotometer in quartz cuvettes with a 1-cm path length. Elemental analyses were conducted by Atlantic Microanalytical, Inc. The series of compounds **1-E** and **2-E** were prepared according to Refs. 14 and 15 Compound **4** was prepared according to Ref. 16. Chalcogenoxanthenones **6** and **7** (Chart 3) were prepared as described in Refs. 25 and 26, respectively and were used in the synthesis of dyes **3** and **5**.

4.2. Preparation of selenoxanthylium dye 3

2-Bromothiophene (0.30 mL, 3.0 mmol) was added to a stirring solution of ground magnesium powder (0.080 g, 3.5 mmol) in 3.0 mL of anhydrous THF and heated at reflux for 1.0 h. The reaction mixture was then transferred via cannula into a solution of selenoxanthone **6**, (0.10 g, 0.25 mmol) dissolved in 3.0 mL of anhydrous THF. The resulting mixture was heated at reflux until homogeneous, cooled to ambient temperature, then glacial acetic acid (0.25 mL) was added and the reaction mixture was poured into a 10% by wt./wt. solution of aqueous hydrobromic acid. The precipitate was collected by filtration after 1.0 h and washed with water and Et_2O. The final dye was obtained following recrystallization from CH_3CN/Et_2O: 0.11 g (77%) as dark green powder, mp 209–210 °C; ^{1}H NMR (CD_2Cl_2) δ 7.69 (dd, 1 H, J = 1.0, 5.3 Hz), 7.54 (d, 1 H, J = 9.5 Hz), 7.36 (d, 1 H, J = 2.5 Hz), 7.30 (s, 1 H), 7.28 (dd, 1 H, J = 3.5, 5.3 Hz), 7.16 (dd, 1 H, J = 1.0, 3.5 Hz), 6.85 (dd, 1 H, J = 2.5, 9.5 Hz), 3.56 (m, 4 H), 3.24 (s, 6 H), 2.78 (t, 2 H, J = 6.5 Hz), 2.71 (t, 2 H, J = 6.5 Hz), 2.19 (quintet, 2 H, J = 6.5 Hz), 1.99 (quintet, 2 H, J = 6.5 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 152.1, 150.4, 148.7, 141.6, 140.5, 136.6, 136.0, 133.7, 130.6, 128.9, 127.5, 125.2, 119.7, 118.8, 116.8, 114.9, 109.1, 51.0, 50.1, 38.7, 27.0, 25.3, 19.8, 19.5; λ_{max} (H_2O) 610 nm ($\varepsilon = 7.0 \times 10^4\ M^{-1}\ cm^{-1}$); HRMS (ESI) m/z 465.0920 (calcd. for $C_{25}H_{25}N_2S^{80}Se^+$: 465.0898). Anal. Calcd for $C_{25}H_{25}N_2SSe{\cdot}PF_6{\cdot}3H_2O$: C, 50.17; H, 5.22; N, 4.68. Found: C, 50.51; H, 4.83; N, 4.71. (The waters of crystallization were visible by ^{1}H NMR spectroscopy.)

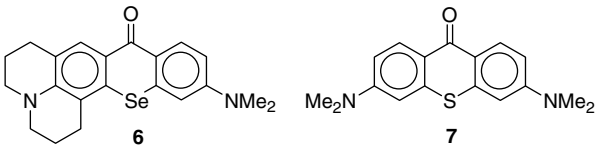

Chart 3. Structures of chalcogenoxanthenones **6** and **7**.

4.3. Preparation of *N*,*N*-diethyl 3,6-bis-(dimethylamino)-9-(3-thienyl-2-carboxamide)thioxanthylium hexafluorophosphate (5)

A solution of *sec*-BuLi (5.6 mL of a 1.0 M solution, 5.6 mmol) was added dropwise to *N*,*N*-diethyl 5-bromothiophene-2-carboxamide (0.70 g, 2.7 mmol) dissolved in freshly distilled anhydrous THF (5.0 mL) cooled to -78 °C. After 1 min, the resulting solution was added via cannula to a solution of 2,7-bis(dimethylamino)-9-thioxanthenone (**7**, 0.20 g, 0.74 mmol) in THF (10 mL) at ambient temperature. The 2-lithio-thiophene-5-carboxamide rearranged to the 3-lithio-thiophene-2-carboxamide above −78 °C. After stirring for 0.5 h, glacial acetic acid (0.33 g, 5.6 mmol) was added to quench any organolithium species and the reaction mixture was poured into a 10% by wt. solution of aqueous hexafluorophosphoric acid. The precipitate was collected and washed with water and ether after 1.0 h of stirring. The crude crystalline product was purified via chromatography on SiO_2 eluted with 1:2:7 MeOH/EtOAc/CH_2Cl_2 followed by recrystallization from CH_3CN/Et_2O (2×) to give 84 mg (19%) of **4** as dark green crystals, mp 229–231 °C: ^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.72 (d, 1 H, J = 5 Hz), 7.54 (d, 2 H, J = 9.6 Hz), 7.10 (d, 1 H, J = 5 Hz), 7.07 (d, 2 H, J = 2.4 Hz), 6.98 (dd, 2 H, J = 2.4, 9.6 Hz), 3.28 (s, 12 H); ^{13}C NMR (125 MHz, CD_2Cl_2) δ 163.0, 156.0, 154.6, 144.5, 136.9, 136.8, 130.5, 128.7, 119.7, 116.3, 106.5, ∼44 (br, 2 C's), 41.0, ∼14 (br, 2 C's); λ_{max} (H_2O) 586 nm (ε $5.6 \times 10^4\ M^{-1}\ cm^{-1}$); HRMS (ESI) m/z 464.1818 (Calcd for $C_{26}H_{30}N_3OS_2^+$: 464.1825). Anal. Calcd for $C_{26}H_{30}N_3OS_2{\cdot}PF_6$: C, 51.22; H, 4.96; N, 6.89. Found: C, 50.98; H, 4.99; N, 7.04.

4.4. Isothermal titration calorimetry (ITC)

Calorimetric titrations were carried out on a MicroCal VP-ITC (MicroCal Inc., Northampton, MA). The data was analyzed using the Origin 7.0 software provided by the manufacturer. All experi-

ments using ctDNA were run at 30 °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). For the experiments using $[poly(dAdT)]_2$ and $[poly(dCdG)]_2$, MES buffer containing 40 mM NaCl was used in order to enhance the stability of the DNA. For each experiment, exactly 12 μL of the drug solution (generally ~0.5–1.0 × 10^{-4} M) was injected into a buffered solution of DNA (generally ~1.0–1.2 × 10^{-5} M in bp, 1.4 mL) over 24 s at 240-s intervals using a 250-μL syringe rotating at 307 rpm. Samples were degassed at 25 °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 solution into the buffer. The heat of dilution for titrating buffer into DNA was found to be negligible. The heat released upon binding was directly proportional to the amount of binding that occurred. A binding isotherm of heat released as a function of the drug/DNA molar ratio was constructed and the data fitted by nonlinear least square fitting analysis.

4.5. Topoisomerase I DNA unwinding assay

Typically, 2.4 × 10^{-7} g of supercoiled pUC19 plasmid DNA was incubated with 5 U of human topoisomerase I (Topo I) enzyme (TopoGen) 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 h at 37 °C. The reaction was terminated using 0.5% SDS and 5 × 10^{-4} g/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).

4.6. Nucleic acids and enzymes

Ultrapure calf thymus DNA (ctDNA) was obtained from Invitrogen (Carlsbad, CA). Supercoiled pUC19 plasmid DNA (1.0 μg/μL) was purchased from Bayou Biolabs (Harahan, LA). Poly(dAdT) and poly(dGdC) DNA sequences were purchased from Midland Certified Reagent Company (Midland, TX) as HPLC-purified and desalted products. To ensure optimal duplex formation, a buffered solution of each DNA sequence [i.e., poly(dAdT) and poly(dGdC)] was heated to 90 °C and slowly cooled to room temperature (22–23 °C) before use. The resulting DNA duplexes ($[poly(dAdT)]_2$ and $[poly(dCdG)]_2$) were equilibrated in MES buffer containing 40 mM NaCl by dialyzing for 48 h using Spectra/Por DispoDialyzer membranes (Spectrum). DNA duplex concentrations (in bp) were determined spectrophotometrically using ε_{260} = 12,824 M^{-1} cm^{-1} for ctDNA, ε_{262} = 13,200 M^{-1} cm^{-1} for $[poly(dAdT)]_2$, and ε_{256} = 16,800 M^{-1} cm^{-1} for $[poly(dGdC)]_2$. Purified human topoisomerase I and proteinase K enzymes were obtained from TopoGEN Inc. (Port Orange, FL) and Fisher Scientific, respectively.

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