

Syntheses and DNA photocleavage by mono- and bis-phenothiazinium—piperazinexylene intercalators

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Abstract

Chromophore systems consisting of one (compound **5**) or two (compound **6**) phenothiazine rings covalently attached to a bis-piperazine-xylene chain were synthesized and evaluated as DNA photocleaving agents. In the presence of DNA, the compounds were shown to monointercalate in their deaggregated forms and to strongly absorb red wavelengths of light. Reactions containing micromolar concentrations of compound produced robust photocleavage of plasmid DNA under near-physiological conditions of temperature and pH (22 °C and pH 7.0). Phenothiazines **5** and **6** increased the T_m of calf thymus DNA by 17 and 19 °C, indicating that significant levels of duplex stabilization were produced.

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1. Introduction

Phenothiazines are redox active, basic dyes that have been utilized as electrophores in supramolecular assemblies¹ and in photogalvanic cells for potential solar energy conversion.² In biological systems, phenothiazines have shown a preference for interacting with GC base pairs within double-helical DNA, where they bind by intercalation.^{3,4} Because light-sensitized phenothiazines are efficient generators of singlet oxygen (1O_2),⁵ they produce extensive photooxidative damage to DNA^{4,6} by forming alkaline labile lesions and direct strand breaks at guanine bases.^{7,8} In this regard, phenothiazines have been attracting considerable attention for use as photosensitizers in photodynamic therapy (PDT), a new therapeutic approach in which red light is used to treat malignant tumors and non-cancerous diseases.^{5,6} The advantages of phenothiazines

include strong absorption⁵ within the therapeutic window for PDT (600–800 nm),⁹ low cellular toxicity in the absence of light,⁵ and selective accumulation of phenothiazines in tumor cells.^{5,6} Accordingly, the phenothiazinium compounds methylene blue ($\lambda_{max}=665$ nm, $\epsilon=8.16 \times 10^4$ M⁻¹ cm⁻¹)⁴ and toluidine blue ($\lambda_{max}=636$ nm, $\epsilon=7.45 \times 10^4$ M⁻¹ cm⁻¹)⁴ have been used in a clinical setting to produce efficient photoactivity against AIDS-related Kaposi's sarcoma.¹⁰ Moreover, phenothiazines have exhibited efficient in vitro and in vivo photoinactivation of bacterial, fungal, and viral pathogens.^{4–6,11–13} A number of these studies have directly linked light-sensitized DNA cleavage to the photodynamic action of methylene blue.^{4,5,11,12}

Herein we describe the syntheses and characterization of two phenothiazinium photonucleases containing either one or two phenothiazine rings covalently attached to a bis-piperazine-xylene linker (7-dimethylamino-3-(1,1'-[1,4-phenylenebis(methylene)bispiperazine])phenothiazin-5-ium iodide (**5**) and *N,N'*-bis[(7-dimethylamino)phenothiazin-5-ium-3-yl]-1,1'-[1,4-phenylenebis(methylene)bispiperazine] diiodide (**6**). In addition to evaluating DNA photocleavage efficiencies, we have

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compared binding affinities, and have determined the DNA binding modes of the two compounds.

2. Results and discussion

2.1. Synthesis

We incorporated two key structural features into the design of **5** and **6**. A phenothiazine based on methylene blue was selected as the chromophore, due to its ability to intercalate into DNA^{3,4} and effect efficient DNA photooxidation within the PDT therapeutic window (600–800 nm).¹⁴ Bis-piperazine-xylene (**4**) was employed as a linker to enhance duplex stability and DNA binding affinity. In intercalating and groove binding compounds, xylene¹⁵ and piperazine^{16,17} rings have been used to stabilize DNA by making extensive van der Waals contacts within the nucleic acid grooves. In the case of piperazine, hydrogen bonding and electrostatic interactions are also possible.^{16,17} Finally, the structural composition of compound **5** (one intercalating phenothiazine ring and a terminal piperazine groove binding element) versus that of compound **6** (two intercalating ring systems) was intentionally included in our design rationale in order to evaluate DNA photocleavage and binding affinity as function of DNA binding mode.

Known compounds phenothiazin-5-ium tetraiodide hydrate (**1**),¹⁸ 3-(dimethylamino)phenothiazin-5-ium triiodide (**2**)¹⁸ (Scheme 1), and 1,1'-[1,4-phenylenebis(methylene)]bis(4-piperazinecarbaldehyde) (**3**)¹⁹ (Scheme 2) were prepared according to published procedures. (In the case of compound **2**, the reaction proceeded in higher yield when chloroform was employed as solvent in lieu of methanol.) Boykin et al. utilized HCl to hydrolyze compound **3**.¹⁹ However, we found that bis-piperazinexylene **4** was more readily obtained by basic hydrolysis. Thus, known compound **4**¹⁹ was prepared in 78% yield by refluxing **3** in aqueous potassium hydroxide

for 24 h (Scheme 2). (Under acidic conditions, we obtained mixtures of starting material with minor amounts of product even after 48 h of reflux.)

In our first attempts to synthesize mono-phenothiazine **5**, the molar equivalents of **2** and **4** were varied and alternative solvents and reaction times were employed (Table 1). Ultimately, we found that treating 1 mol equiv of each reagent in methanol afforded compound **5** in 34% yield (Scheme 3, Table 1). A small quantity of impure compound **6** (10%) was also recovered. (We had anticipated that the latter transformation might require more effort, since two aromatic nucleophilic additions (compound **6**) versus one aromatic nucleophilic addition (compound **5**) would be needed.) After a number of procedural modifications, the synthesis of compound **6** was achieved in 41% yield by reacting 2 mol equiv of phenothiazine **2** with 1 mol equiv of the bis-piperazinexylene linker **4** in the presence of a base (cesium carbonate) and DMF as solvent (Scheme 3). Although several syntheses of 3,7-disubstituted phenothiazin-5-ium salts have appeared in the literature,^{18,20,21} to the best of our knowledge, no synthetic accounts of bis-phenothiazinium salts incorporating our design features have been reported.

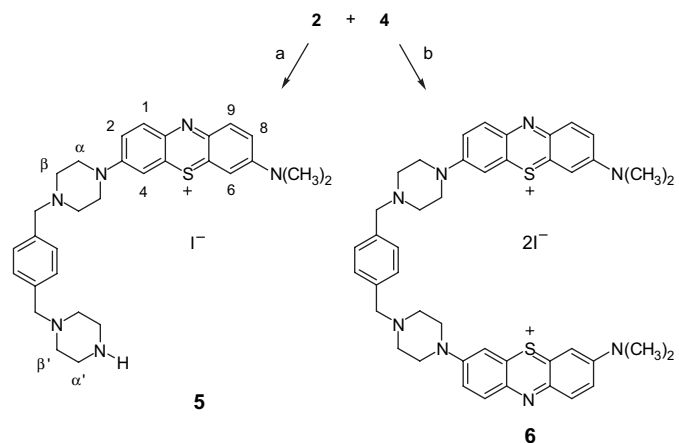
Table 1
Synthesis of compound **5**

Trial	Compound 2 (mmol)	Compound 4 (mmol)	Solvent		Time ^a (h)	Yield (%)
			Type	mL		
1	0.161	0.322	CHCl ₃	12	24	Trace
3	0.076	0.153	CHCl ₃	7	72	16
2	0.164	0.164	CH ₃ OH	10	24	15
4	0.815	0.815	CH ₃ OH	25	72	34

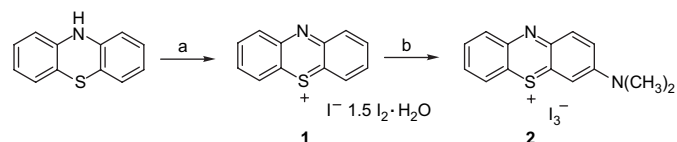
^a All reactions were run at 22 °C over the time intervals indicated.

2.2. UV–visible spectra

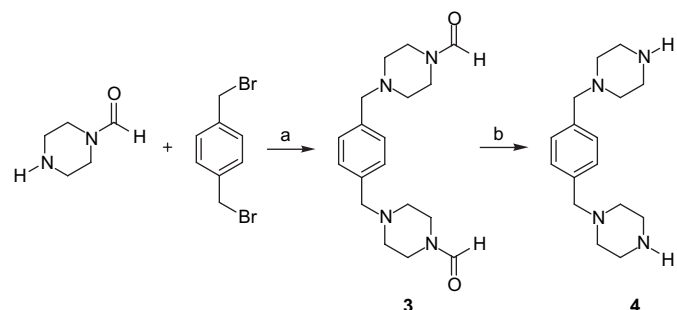
Phenothiazines undergo dimerization in aqueous solutions as a function of increased ionic strength and phenothiazine concentration. In general, the absorption band of the dimer is hypochromic and blue-shifted with respect to the absorption band of corresponding monomer.^{4,6,22,23} Taking this into account,



Scheme 3. Syntheses of compounds **5** and **6**. Reagents and conditions: (a) CH₃OH, rt, 72 h, 34%; (b) Cs₂CO₃, DMF, rt, 48 h, 41%.



Scheme 1. Preparation of **1** and **2**. Reagents and conditions: (a) I₂, CHCl₃, 5 °C, 2 h, 80%; (b) HN(CH₃)₂ in CH₃OH, rt, 4 h, 55%.



Scheme 2. Preparation of **3** and **4**. Reagents and conditions: (a) K₂CO₃, CH₃OH, reflux, 24 h, 75%; (b) KOH, 3:2 CH₃CH₂OH–H₂O (v/v), reflux, 24 h, 78%.

UV–visible spectra of 10 μM concentrations of compounds **5** and **6** were recorded in the absence and presence of 1% (w/v) sodium dodecyl sulfate (SDS), a negatively charged surfactant that disrupts phenothiazine dimerization (Fig. 1, Table 2). Upon the addition of SDS, it is evident that a pronounced red shift and significant hyperchromicity are produced only in the case of bis-phenothiazine **6**, indicating that this compound forms dimers under the experimental conditions employed.

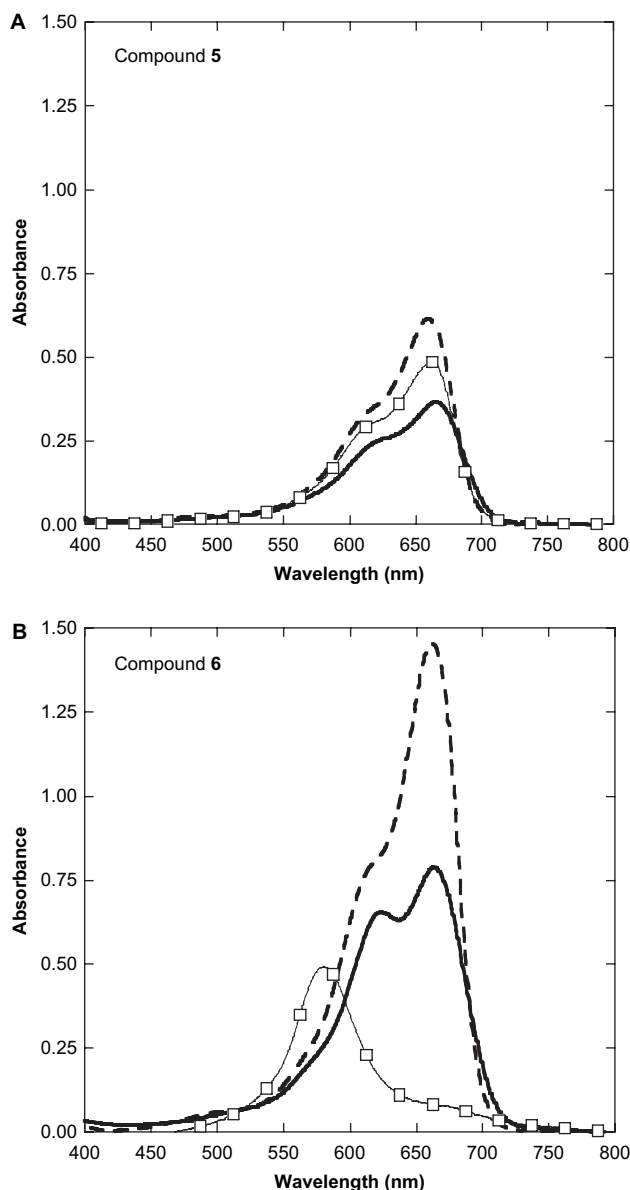


Figure 1. UV–visible spectra recorded at 22 °C in 10 mM sodium phosphate buffer pH 7.0 of: (A) 10 μM of compound **5** (line with squares) in the presence of 380 μM bp CT DNA (solid line) or 1% SDS (w/v) (dashed line); (B) 10 μM of compound **6** (line with squares) in the presence of 380 μM bp CT DNA (solid line) or 1% SDS (w/v) (dashed line). The samples containing DNA were pre-equilibrated for 12 h in the dark at 22 °C.

DNA base-stacking interactions give rise to bathochromic wavelength shifts and hypochromic absorption in the electronic spectra of the majority of DNA intercalators. Thus, as a preliminary test for DNA intercalation, UV–visible spectra of 10 μM

Table 2

Absorbance data^a

Compound	λ_{max} (nm)	$\epsilon \times 10^4$ ($\text{M}^{-1} \text{cm}^{-1}$)	$\epsilon \times 10^4$ ($\text{M}^{-1} (\text{bp}) \text{cm}^{-1}$)
5	662	4.56	na
5 +1% SDS	660	5.81	na
5 +CT DNA	665	na	3.66
6	580	nd	na
6 +1% SDS	662	14.6	na
6 +CT DNA	665	na	7.80

^a Extinction coefficients for compounds **5** and **6** were determined in 10 mM sodium phosphate buffer pH 7.0 using solutions containing 1–10 μM of dye in the absence and presence of 1% SDS (w/v) or 38–380 μM bp CT DNA. The samples containing DNA were pre-equilibrated for 12 h in the dark at 22 °C; na=not applicable; nd=not determined due to aggregation in buffer.

concentrations of compounds **5** and **6** were recorded in the presence of 380 μM bp of calf thymus (CT) DNA (Fig. 1, Table 2). The data indicate that compounds **5** and **6** exhibit bathochromicity and hypochromicity when SDS is replaced by nucleic acid. Upon the addition of 1.0% SDS, the λ_{max} values of 10 μM of compounds **5** and **6** are 660 nm ($\epsilon=5.81 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}$) and 662 nm ($\epsilon=14.6 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}$), respectively. In the presence of CT DNA, the respective λ_{max} values are 665 nm ($\epsilon=3.66 \times 10^4 \text{ M}^{-1} (\text{bp}) \text{cm}^{-1}$) and 665 nm ($\epsilon=7.80 \times 10^4 \text{ M}^{-1} (\text{bp}) \text{cm}^{-1}$). It can be inferred from these data that both compounds are in their monomeric, deaggregated forms and are likely to be intercalated within the DNA. More importantly, DNA-bound phenothiazines **5** and **6** exhibit absorbance maxima well within the therapeutic window used in photodynamic therapy (600–800 nm).

2.3. Viscometric measurements

Our next goal was to obtain direct evidence of DNA intercalation. Viscosity measurements provide a simple and stringent assay for determining the binding modes of DNA interacting ligands. In order for an intercalator to insert itself between adjacent base pairs within duplex DNA, the helix must first unwind to create a gap large enough to accommodate the incoming ligand. The unwinding process increases the contour length of the helix, and as a result, the DNA becomes more viscous. Because groove binding compounds do not require a base pair gap, DNA lengthening does not occur, and viscosity is not significantly changed. Thus, viscometric measurements can be employed to distinguish between different DNA binding modes.²⁴ According to the theory of Cohen and Eisenberg, linear plots of the cubed root of the relative DNA viscosity $(\eta/\eta_0)^{1/3}$ versus the molar ratio of bound ligand to DNA bp (r) should result in a slope close to 0.0 for groove binding compounds and 1.0 for classical monointercalators.²⁵ The slope of a bisintercalator is then expected to be approximately twice that observed in the case of monointercalation.

Viscometric measurements were carried out using individual, pre-equilibrated solutions consisting of 200 μM bp of CT DNA and 10 mM sodium phosphate buffer pH 7.0 in the presence (η) and the absence (η_0) of dye. The viscosity data were plotted as $(\eta/\eta_0)^{1/3}$ versus r , for r values ranging from 0.01 to 0.06 (Fig. 2). The slopes obtained from the mono-

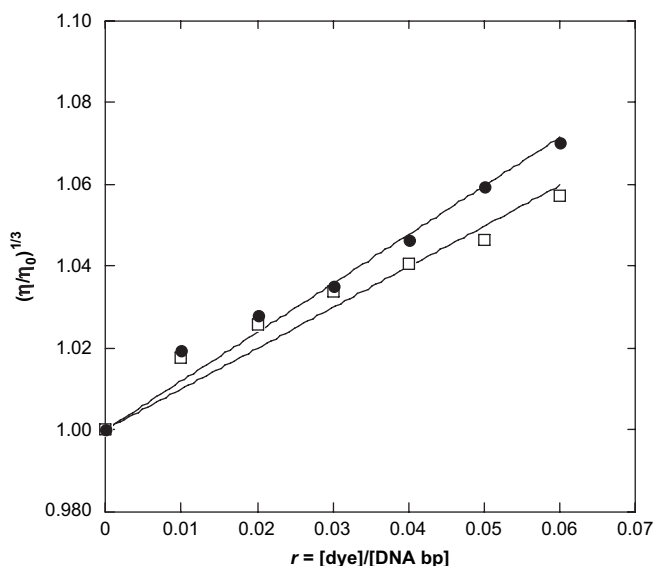


Figure 2. Viscometric measurements of 200 μM bp calf thymus DNA pre-equilibrated with 0, 2, 4, 6, 8, 10, 12 μM of compound **5** or compound **6** for 12 h at 22 $^{\circ}\text{C}$ (10 mM sodium phosphate buffer pH 7.0): compound **5** (□, slope=0.99, $R=0.972$) and compound **6** (●, slope=1.20, $R=0.990$).

and bis-phenothiazine plots were 0.99 and 1.20, respectively. Clearly, compounds **5** and **6** both interact with CT DNA as monofunctional intercalators.

The neighbor-exclusion principle²⁶ states that every second base pair site along the length of the DNA double-helix will remain unoccupied during intercalation. In order to prevent violation of nearest neighbor-exclusion, bisintercalators should possess a linker chain whose length is ≥ 10 Å.²⁷ While the bis-piperazinexylene linker of compound **6** meets this requirement,²⁸ it is conceivable that conformation constraints imposed by DNA lower the free energy for intercalative binding of a second ring, preventing its insertion into the nucleic acid duplex.²⁹ An additional consideration is linker rigidity. Partial double bond character between the C3 atom of phenothiazine and the proximal nitrogen atom of piperazine may result if there is significant delocalization of the nitrogen lone-pair electrons. Such a restriction might preclude intercalation of the second phenothiazine ring of compound **6**.

2.4. DNA photocleavage

In our next experiment, DNA photocleavage was evaluated as a function of decreasing dye concentration. Individual reactions consisted of 10–0.25 μM of compound **5** or **6** pre-equilibrated with 38 μM bp pUC19 plasmid DNA in 10 mM sodium phosphate buffer pH 7.0. The samples were aerobically irradiated for 60 min in a ventilated Rayonet Photochemical Reactor fitted with twelve 575 nm lamps (spectral output 400–650 nm). DNA direct strand breaks were then detected on a 1.0% non-denaturing agarose gel stained with ethidium bromide. As shown in Figure 3, compounds **5** and **6** produced robust levels of DNA photocleavage in a concentration dependent manner. The highest DNA cleavage yields (conversion of supercoiled plasmid to the nicked form) were 96% (Fig. 3, Lane 3) and 93% (Fig. 3,

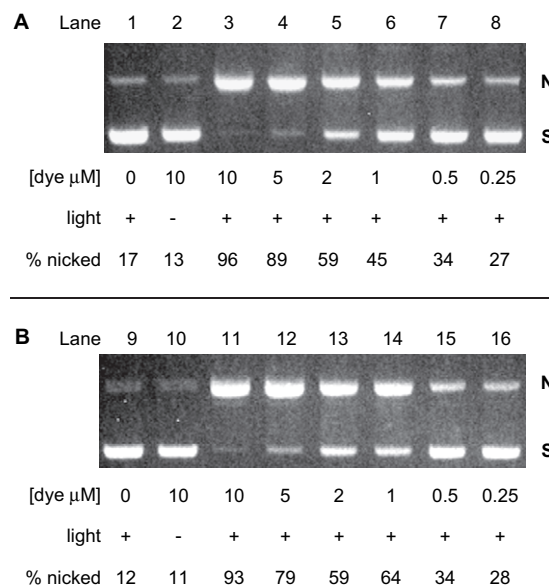


Figure 3. (A) Compound **5** and (B) compound **6**: 1% nondenaturing agarose gels showing photocleavage of pUC19 plasmid DNA. Samples contained 10 mM sodium phosphate buffer pH 7.0 and 38 μM bp DNA in the absence and presence of dye. After being equilibrated for 12 h in the dark at 22 $^{\circ}\text{C}$, the samples were aerobically irradiated for 60 min at 22 $^{\circ}\text{C}$ in a Rayonet Photochemical reactor fitted with twelve 575 nm lamps. Lanes 1 and 9: DNA controls (no dye). Lanes 3–8: 10–0.25 μM compound **5**. Lanes 11–16: 10–0.25 μM compound **6**. Lanes 2 and 10: 10 μM **5** and 10 μM **6** (no $h\nu$). Abbreviations: N=nicked; S=supercoiled.

Lane 11) for samples irradiated in the presence 10 μM of **5** and 10 μM of **6**, respectively. In addition, both phenothiazines exhibited detectable levels of photocleavage at concentrations of compound as low as 0.25 μM (Lanes 8 and 16). Cleavage was minimal in parallel control reactions run in the dark (Lanes 2 and 10) and in samples irradiated for 60 min in the absence of phenothiazine (Lanes 1 and 9).

We then employed 10 μM concentrations of compounds **5** and **6** to monitor reaction kinetics. Individual samples containing 38 μM bp pUC19 plasmid DNA in 10 mM sodium phosphate buffer pH 7.0 were irradiated as described above, at time points ranging from 5 to 60 min. As shown in Figure 4, DNA photocleavage by compounds **5** and **6** was substantial after only 5 min of irradiation and steadily increased as a function of increasing irradiation time. Maximal levels of nicked plasmid (93 and 95%) were generated at the 60 min time point.

2.5. DNA thermal denaturation

During intercalation and/or groove binding, free energy contributions arising from π – π , van der Waals, electrostatic, and hydrogen bonding interactions between ligands and DNA stabilize the DNA double-helix. As a result, the melting temperature (T_m) of the helix is increased. Because the T_m value of ligand-bound DNA is related to the affinity of the ligand for the nucleic acid, melting assays have been used to access the relative binding affinities of compounds that associate with double-helical DNA.³⁰

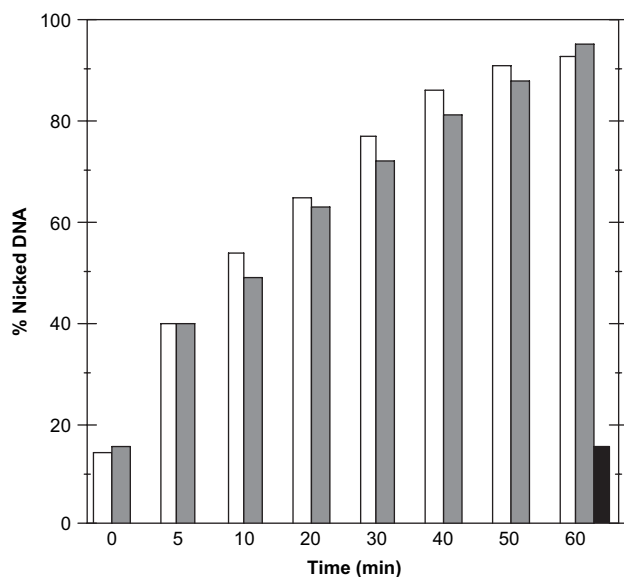


Figure 4. % DNA photocleavage of 38 μM bp pUC19 plasmid DNA in the presence of 10 μM compound **5** (white bars) and 10 μM compound **6** (gray bars) (10 mM sodium phosphate buffer pH 7.0 at 22 °C). After pre-equilibration for 12 h in the dark at 22 °C, individual samples were aerobically irradiated for 0, 5, 10, 20, 30, 40, 50, and 60 min at 22 °C in a Rayonet Photochemical reactor fitted with twelve 575 nm lamps. The black bar represents DNA that was irradiated for 60 min in the absence of dye (10 mM sodium phosphate buffer pH 7.0).

Taking into account the neighbor-exclusion principle,²⁶ we recorded DNA melting isotherms at a dye to DNA bp molar ratio of 0.6 (Fig. 5). Compared to CT DNA ($T_m=69^\circ\text{C}$), compound **5** exhibited a T_m of 86 °C ($\Delta T_m=17^\circ\text{C}$), while compound **6** produced a T_m of 88 °C ($\Delta T_m=19^\circ\text{C}$). Some typical ΔT_m values for positively charged, DNA monointercalators similar in design to **5** and **6** (i.e., positively charged intercalators consisting of three fused aromatic rings attached to a groove binding side chain) are: 7.2 °C for ethidium, 13.1 °C

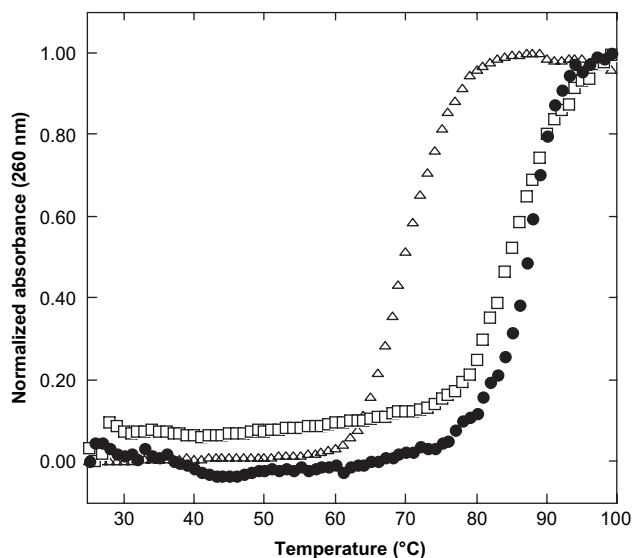


Figure 5. Melting isotherms of: 12.5 μM bp CT DNA in 10 mM sodium phosphate buffer pH 7.0: CT DNA (Δ , $T_m=69^\circ\text{C}$); CT DNA with 7.5 μM of compound **5** ($\square$, $T_m=86^\circ\text{C}$); CT DNA with 7.5 μM of compound **6** ($\bullet$, $T_m=88^\circ\text{C}$).

for adriamycin, and 14.7 °C for quinacrine.³¹ Clearly, the ΔT_m values produced by **5** and **6** are significant, providing evidence for strong binding of both compounds to DNA and considerable levels of duplex stabilization.

Because the ΔT_m values produced by compounds **5** and **6** are nearly equivalent, it can be inferred that the DNA binding affinities of both dyes are similar. This result is consistent with our observation that **5** and **6** interact with CT DNA as monofunctional intercalators. It is also consistent with the dicationic nature of the dyes at pH 7.0, the experimental conditions employed here. Typically, basic phenothiazines have pK_a values greater than 12.³² In the case of compound **5**, the bis-piperazinexylene substituent would be expected to bear one positive charge (the secondary amino group of the terminal piperazine), as the piperazine tertiary amino groups would not be protonated due a reduction in charge density arising from the electron withdrawing effects of the xylene¹⁹ and phenothiazine rings.³² Therefore, the dicationic charge density of compound **6** is imparted by the two phenothiazines, while in compound **5** the bis-piperazinexylene substituent is expected to furnish one positive charge and the phenothiazine ring the other positive charge. The melting temperature and viscosity data can also be used to interpret the results of the absorbance and photocleavage experiments presented in this paper. Although bis-phenothiazine **6** possesses two DNA-photosensitizing phenothiazinium rings and absorbs light more strongly than mono-phenothiazine **5** in the DNA-bound form (Fig. 1, Table 2), **5** and **6** both produce equivalent levels of photocleavage (Figs. 3 and 4). Furthermore, both compounds monointercalate and are likely to possess similar DNA binding affinities. Taken together, these findings suggest that, similar to methylene blue,⁷ intercalative binding promotes efficient photosensitization of direct DNA strand breaks by the phenothiazine chromophores in **5** and **6**. In the case of methylene blue, van der Putten and co-workers observed a significant reduction in O_2 -dependent DNA photocleavage in the presence of 20 mM of Mg^{2+} , a magnesium ion concentration which changed binding of the dye from an intercalative to a Mg^{2+} -insensitive external binding mode.⁷ Levels of photocleavage were further reduced when the singlet oxygen scavenger sodium azide was added to this reaction. The authors concluded that methylene blue must be in close proximity to reactive sites within the DNA in order for efficient O_2 -dependent photocleavage to occur.

3. Conclusions

In summary, we report the syntheses of cationic photodynamic cancer therapy. At micromolar concentrations of compound, robust levels of DNA photocleavage are produced under near-physiological conditions of temperature and pH (22 °C and pH 7.0). Thus, the systems

described in the report may serve as a good starting point for the development of new phototherapeutic agents.

4. Experimental

4.1. General

Melting points were determined using a Stuart Scientific model SMP10 apparatus. ^1H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, on either a Varian Unity One or a Varian Mercury-VX-300 spectrometer. Carbon and proton assignments were based on HSQC and HMBC experiments. Infrared spectra were acquired with an FT-IR Perkin–Elmer Spectrum One spectrophotometer. Elemental analyses (CHNS) were done on a Leco CHNS-932 automatic analyzer. Iodine composition was performed by oxygen flask combustion and by ion chromatography (Atlantic Microlabs, Inc. Norcross, GA). Electrospray ionization (ESI) mass spectra were generated using a Micromass Q-ToF hybrid mass spectrometer. UV–visible spectra were recorded on a UV-1601 Shimadzu spectrophotometer and thermal melting curves on a Cary Bio 100 UV–vis spectrophotometer. Merck silica gel 60 (230–400 ASTM mesh) was employed for flash column chromatography.

Distilled, deionized water was utilized in the preparation of all buffers and all aqueous reactions. Chemicals were of the highest available purity and were used without further purification. Anhydrous magnesium sulfate, cesium carbonate, chloroform, α,α' -dibromo-*p*-xylene, dichloromethane, dimethylamine (2 M solution in methanol), dimethylformamide, ethidium bromide, iodine, methanol, 10*H*-phenothiazine, 1-piperazinecarbaldehyde, potassium hydroxide, potassium carbonate, sodium phosphate dibasic, and sodium phosphate monobasic were obtained from the Aldrich Chemical Company. The transformation of *Escherichia coli* competent cells (Stratagene, XL-blue) with pUC19 plasmid DNA (Sigma) and growth of bacterial cultures in LB broth were performed according to published procedures.³³ A Qiagen Plasmid Mega Kit was used to purify the plasmid DNA from the bacterial cultures. Ultra Pure™ calf thymus (CT) DNA (Invitrogen Lot no. 15633-019, 10 mg/mL, average size ≤ 2000 bp) was used without purification. The concentration of CT DNA solutions was determined by UV–visible spectrophotometry using the extinction coefficient $\epsilon_{260} = 12,824 \text{ M}^{-1}(\text{bp}) \text{ cm}^{-1}$.

4.2. Synthesis

4.2.1. Phenothiazin-5-ium tetraiodide hydrate (**1**)

A solution of 10*H*-phenothiazine (0.566 g, 2.84 mmol) in 20 mL of chloroform was stirred at 5 °C and iodine (2.16 g, 8.51 mmol) dissolved in 50 mL of chloroform was added dropwise over 1 h. The reaction mixture was stirred at 5 °C for an additional hour, while its progress was monitored by silica gel TLC. The resulting precipitate was then filtered, washed with a copious amount of chloroform, and was dried overnight in vacuo to afford 1.63 g (80%) of dark-blue solid **1**. TLC (chloroform): $R_f = 0.09$. Mp = 170 °C (dec). ^1H NMR (300 MHz,

acetone- d_6 , δ (ppm)): 8.01 (m, 2H), 7.92 (m, 2H), 7.64 (m, 4H). ^{13}C NMR (75 MHz, acetone- d_6 , δ (ppm)): 153.6, 130.7, 129.5, 128.6, 125.5, 123.5. IR (film, ν (cm^{-1})): 2967, 1558, 1467, 1440, 1311, 1233, 1131, 1067, 1023, 841, 705. LRMS (ESI): m/z calcd for $\text{C}_{12}\text{H}_8\text{NS} [\text{M}]^+$ 198.04, found 199.0.

4.2.2. 3-(Dimethylamino)phenothiazin-5-ium triiodide (**2**)

To a solution of phenothiazin-5-ium tetraiodide hydrate (0.400 g, 0.553 mmol) in 20 mL of chloroform was added dimethylamine in methanol (0.553 mL, 1.11 mmol) dropwise over 4 h. Reaction progress was monitored by silica gel TLC. The resulting precipitate was filtered, washed with chloroform, and allowed to air dry. Product **2** (189 mg, 55%) was obtained as a dark-blue solid. TLC (3:7 10% aqueous ammonium acetate–methanol (v/v)): $R_f = 0.28$. Mp 144–145 °C. ^1H NMR (300 MHz, DMSO- d_6 , δ (ppm)): 8.22 (dd, $J = 8.0$, 1.6 Hz, 1H, H-9), 8.17 (dd, $J = 8.0$, 1.6 Hz, 1H, H-6), 8.10 (d, $J = 10.0$ Hz, 1H, H-1), 8.04 (dd, $J = 10.0$, 2.4 Hz, 1H, H-2), 8.00 (d, $J = 2.4$ Hz, 1H, H-4), 7.85 (m, 2H, H-7, H-8), 3.64 and 3.60 (2s, 6H, $\text{N}(\text{CH}_3)_2$). ^{13}C NMR (75 MHz, DMSO- d_6 , δ (ppm)): 156.1, 144.1, 139.8, 139.6, 138.0, 134.6, 133.2, 129.8, 126.3, 126.1, 125.8, 109.7, 43.4, 42.9. IR (film, ν (cm^{-1})): 2800, 1617, 1559, 1489, 1429, 1411, 1252, 1118, 1078, 887, 835, 772. LRMS (ESI): m/z calcd for $\text{C}_{14}\text{H}_{13}\text{N}_2\text{S} [\text{M}]^+$ 241.08, found 241.1. Anal. Calcd for $\text{C}_{14}\text{H}_{13}\text{N}_2\text{SI}_3$: C, 27.03; H, 2.11; N, 4.50; S, 5.15; I, 61.20. Found C, 27.12; H, 1.97; N, 4.46; S, 5.23; I, 60.94.

4.2.3. 1,1'-[1,4-Phenylenebis(methylene)]bis(4-piperazine-carbaldehyde) (**3**)

A solution containing α,α' -dibromo-*p*-xylene (2.60 g, 0.010 mol), 1-piperazine carboxaldehyde (2.30 mL, 0.022 mol), and potassium carbonate (1.40 g, 0.010 mol) in 40 mL of methanol was refluxed under an open atmosphere for 24 h. Reaction progress was monitored by silica gel TLC. After the starting materials were consumed, the reaction was concentrated to dryness under reduced pressure. The resulting solid was then dissolved in 25 mL of water and extracted with dichloromethane (50 mL) three times. The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to yield 2.47 g (75%) of white solid **3**. TLC (44:8:1 chloroform–methanol–28% ammonium hydroxide): $R_f = 0.69$. Mp = 140–142 °C. ^1H NMR (300 MHz, DMSO- d_6 , δ (ppm)): 7.97 (s, 2H, NCHO), 7.25 (s, 4H, Ph), 3.47 (s, 4H, CH_2 -Ph), 3.38–3.30 (m, 8H, CH_2 - α), 2.34 and 2.28 (2 t, $J = 4.9$ Hz, 4H, CH_2 - β). ^{13}C NMR (75 MHz, DMSO- d_6 , δ (ppm)): 160.9 (CHO), 136.7 (C_{ipso} Ph), 128.9 (Ph), 61.8 (CH_2 -Ph), 53.3 and 52.1 (CH_2 - α), 44.9 and 39.4 (CH_2 - β). IR (film, ν (cm^{-1})): 2870, 2805, 1668, 1514, 1489, 1431, 1400, 1226, 1125, 1017, 998, 841, 821, 770. LRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{27}\text{N}_4\text{O}_2 [\text{M}+\text{H}]^+$ 331.21, found 331.1.

4.2.4. 1,1'-[1,4-Phenylenebis(methylene)]bis(piperazine) (**4**)

A solution of **3** (0.500 g, 1.50 mmol) in 3 mL of ethanol and 2 mL of water was treated with potassium hydroxide (0.421 g, 7.50 mmol) and refluxed under an open atmosphere for 24 h. Reaction progress was monitored by silica gel

TLC. After the starting material was consumed, the reaction mixture was allowed to cool and was then extracted with dichloromethane (10 mL) three times. The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to yield 0.321 g (78%) of white solid **4**. TLC (44:8:1 chloroform–methanol–28% ammonium hydroxide): R_f =0.45. Mp=146–148 °C. ^1H NMR (300 MHz, DMSO- d_6 , δ (ppm)): 7.19 (s, 4H, Ph), 3.36 (s, 4H, CH₂–Ph), 2.64 (t, J =4.6 Hz, 8H, CH₂– α), 2.23 (t, J =4.6 Hz, 8H, CH₂– β). ^{13}C NMR (75 MHz, DMSO- d_6 , δ (ppm)): 136.7 (C_{ipso} Ph), 128.6 (Ph), 62.6 (CH₂–Ph), 54.1 (CH₂– β), 45.6 (CH₂– α). IR (film, ν (cm^{–1})): 3244, 2880, 2804, 1514, 1453, 1360, 1335, 1249, 1134, 1008, 858, 830, 765. LRMS (ESI): m/z calcd C₁₆H₂₇N₄ [M+H]⁺ 275.22, found 275.2.

4.2.5. 7-Dimethylamino-3-(1,1'-[1,4-phenylenebis(methylene)bis(piperazine)]phenothiazin-5-ium iodide (**5**)

A solution of **2** (0.507 g, 0.815 mmol) and **4** (0.224 g, 0.815 mmol) in 25 mL of methanol was vigorously stirred for 72 h at rt. Progress was monitored by silica gel TLC. Upon completion, the reaction was concentrated under reduced pressure and the resulting solid was purified by flash column chromatography (diameter 4 cm, 70 g silica gel) using 9.5:0.5 dichloromethane–methanol as the eluent to afford 170 mg (34%) of dark-blue solid **5**. TLC (9.5:0.5 dichloromethane–methanol): R_f =0.2. Mp>300 °C. ^1H NMR (300 MHz, DMSO- d_6 , δ (ppm)): 7.90 (m, 2H, H-1, H-9), 7.68 (br s, 1H, H-6), 7.62 (dd, J =9.6, 1.8 Hz, 1H, H-8), 7.53–7.49 (m, 2H, H-2, H-4) 3.85 (br s, 4H, CH₂– α), 3.54 (s, 4H, CH₂–Ph), 3.37 (s, 6H, NCH₃), 3.09 (t, J =5.0 Hz, 4H, CH₂– α'), 2.55 (br, 8H, CH₂– β , β'). ^{13}C NMR (75 MHz, DMSO- d_6 , δ (ppm)): 154.2 and 153.0 (C-3, C-7), 138.1 and 138.0 (C-1, C-9), 136.7, 135.8, 135.0 and 134.1 (C_{ipso} –Ph, C-4a, C-5a, C-9a, and C-10a), 129.0 (Ph), 119.7 (C-2), 118.9 (C-8), 107.2 and 107.1 (C-4, C-6), 61.2 and 61.1 (CH₂–Ph), 52.4 (CH₂– β'), 49.1 (CH₂– β), 47.4 (CH₂– α), 43.2 (CH₂– α'), 41.4 (NCH₃). IR (film, ν (cm^{–1})): 1743, 1593, 1487, 1386, 1352, 1229, 1136, 1042, 990, 882, 825, 779. HRMS (ESI): m/z calcd for C₃₀H₃₈N₆S [M+H]²⁺ 257.1439, found 257.1462.

4.2.6. N,N'-Bis[(7-dimethylamino)phenothiazin-5-ium-3-yl]-1,1'-[1,4-phenylenebis(methylene)bis(piperazine)] diiodide (**6**)

To a solution of **2** (0.200 g, 0.322 mmol) in 20 mL of dimethylformamide were added **4** (0.044 g, 0.160 mmol) and cesium carbonate (0.156 g, 0.480 mmol). The reaction was vigorously stirred at rt for 48 h and then concentrated under reduced pressure. The progress of the reaction was monitored by silica gel TLC. The resultant solid was purified via flash column chromatography (diameter 3 cm, 39 g silica gel) using 9.5:0.5 dichloromethane–methanol as the eluent to afford 66 mg (41%) of dark-blue solid **6**. TLC (9.5:0.5 dichloromethane–methanol): R_f =0.3. Mp>300 °C. ^1H NMR (300 MHz, DMSO- d_6 , δ (ppm)): 7.93 (app d, J =9.7 Hz, 4H, H-1, H-9), 7.70 (d, J =2.7 Hz, 2H, H-6), 7.63 (dd, J =9.7, 2.7 Hz, 2H, H-8), 7.55–7.51 (m, 4H, H-2, H-4), 7.32 (s, 4H, Ph), 3.85 (br s, 8H, CH₂– α), 3.56 (s, 4H, CH₂–Ph), 3.38 (s, 12H, NCH₃), 2.57 (br s, 8H, CH₂– β).

^{13}C NMR (75 MHz, DMSO- d_6 , δ (ppm)): 154.3 and 153.0 (C-3, C-7), 138.2 and 138.1 (C-1, C-9), 135.9, 135.1, 134.2 and 133.9 (C_{ipso} –Ph, C-4a, C-5a, C-9a and C-10a), 129.1 (Ph), 119.9 and 119.0 (C-2, C-8), 107.3 (C-4, C-6), 61.2 (CH₂–Ph), 52.5 (CH₂– β), 47.5 (CH₂– α), 41.5 (NCH₃). IR (film, ν (cm^{–1})): 2954, 2884, 2834, 1590, 1488, 1385, 1347, 1228, 1140, 1035, 1001, 881, 778. HRMS (ESI): m/z calcd for C₄₄H₄₈N₈S₂ [M]²⁺ 376.1722, found 376.1725.

4.3. UV–visible spectrophotometry

The extinction coefficients of compounds **5** and **6** were obtained using 500 μL solutions containing 1–10 μM of dye and 10 mM sodium phosphate buffer pH 7.0 in the absence and presence of 38–380 μM bp CT DNA. The solutions were pre-equilibrated for 12 h (rt, no $h\nu$), after which spectra were recorded at 22 °C in 1 cm quartz cuvettes and absorbance was plotted as a function of concentration. Linear least square fits to the data yielded slopes that were averaged over three trials (KaleidaGraph version 3.6.4 software). Using the procedure described above, extinction coefficients in the absence of calf thymus DNA were also recorded in the presence of a final concentration of aqueous 1% SDS (w/v).

4.4. Viscometric measurements

In a total volume of 1 mL, individual solutions containing 200 μM bp of CT DNA (average length ≤ 2000 bp) and 10 mM sodium phosphate buffer pH 7.0 in the absence and presence of 2–12 μM of compound **5** or **6** were allowed to pre-equilibrate for 12 h (rt, no $h\nu$). A Cannon-Ubbelohde size 75 capillary viscometer immersed in a thermostated water bath set at 25 ± 0.2 °C was then used to measure DNA viscosity. The flow times of the buffer, DNA in buffer, and dye–DNA in buffer were measured with a stopwatch and were averaged over four trials to an accuracy of ± 0.2 s. After subtracting the averaged flow time of the buffer, DNA (η_0) and dye–DNA (η), averaged flow times were plotted as $(\eta/\eta_0)^{1/3}$ versus the molar ratio of dye to DNA bp. Slopes were obtained by conducting linear least square fits to the data (KaleidaGraph version 3.6.4 software). The conventional method for performing viscosity assays involves the gradual titration of ligand into a DNA solution inside a single viscometer. In the procedure reported here, a series of individual DNA solutions containing different concentrations of ligand were prepared and allowed to pre-equilibrate before the viscosity of each solution was measured. This alternative technique may be particularly useful for compound **6** as well as for other phenothiazines (e.g., 1,9-dimethyl methylene blue and methylene blue) for which pre-equilibration with DNA is required to disrupt ligand dimerization.^{4,6,22,23}

4.5. DNA photocleavage

Individual solutions containing 38 μM bp of pUC19 plasmid DNA, 10, 5, 2, 1, 0.5, 0.25, and 0.0 μM concentrations of compound **5** or **6**, and 10 mM sodium phosphate buffer pH 7.0 were

prepared in a total volume 20 μ L. After a 12 h pre-equilibration period (rt, no $h\nu$), the samples were kept in the dark or aerobically irradiated for 60 min in a ventilated Rayonet Photochemical reactor fitted with twelve 575 nm lamps (spectral output 400–650 nm; Southern New England Ultraviolet Co.). In time course experiments, individual, pre-equilibrated 20 μ L solutions consisting of 10 μ M of **5** or of **6** and 38 μ M bp pUC19 plasmid DNA in 10 mM sodium phosphate buffer pH 7.0 were aerobically irradiated as described above for 0, 5, 10, 20, 30, 40, 50, and 60 min. Cleaved DNA products were electrophoresed on 1% nondenaturing agarose gels containing ethidium bromide (0.5 μ g/mL), visualized on a transilluminator set at 302 nm, photographed, and scanned. ImageQuant version 5.2 software (Amersham Biosciences) was then used to quantify supercoiled and nicked plasmid DNA. The density of the supercoiled DNA was multiplied by a correction factor of 1.22 to compensate for the decreased binding affinity of ethidium bromide to supercoiled versus nicked DNA forms. Photocleavage yields were calculated from the data according to the formula [nicked DNA/total DNA] $\times$ 100.

4.6. Thermal melting studies

Individual 3 mL solutions containing 10 mM sodium phosphate buffer pH 7.0 and 12.5 μ M bp calf thymus DNA in the absence and presence of 7.50 μ M of compound **5** or **6** were placed in 3 mL (1 cm) quartz cuvettes obtained from Starna. After a 12 h pre-equilibration period (rt, no $h\nu$), absorbance was monitored at 260 nm while the DNA was melted using a Peltier heat block to increase the temperature from 25 to 100 $^{\circ}$ C at a rate of 0.5 $^{\circ}$ C min $^{-1}$. KaleidaGraph version 3.6.4 software was then used to calculate the first derivative of a plot of $\Delta A_{260}/\Delta T$ versus temperature. The maximum of the first derivative plot was used to identify the T_m value of the DNA.

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