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Novel Cytotoxic DNA Sequence and Minor Groove Targeted Photosensitizers: Conjugates of Pyrene and Netropsin Analogues

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Abstract—The design, syntheses, photochemical and biological properties of conjugates of pyrene with pyrrole- (**1**) and imidazole-containing (**2**) analogues of netropsin are reported. The results of an ethidium displacement assay and circular dichroism (CD) titration studies show both compounds bind with a higher affinity to poly(dA–dT) than to poly(dG–dC). In addition they bind as strongly to T4 coliphage DNA as to calf thymus DNA suggesting the binding occurs in the minor groove. The quenching rate constants of the singlet excited states of agents **1** and **2** by molecular oxygen were found to be 8.5×10^9 $M^{-1}s^{-1}$ and 7.7×10^9 $M^{-1}s^{-1}$, suggesting the involvement of singlet oxygen. Both compounds showed some cytotoxicity to human chronic myeloid leukemia K562 cells in the dark. Upon irradiation the activities were significantly enhanced resulting in photoinduced dose modifications of 8 and 14 for **1** and **2**, respectively under the conditions employed. Both agents were markedly more phototoxic than 1-pyrenebutyric acid **8**. To address the mechanism of action of compounds **1** and **2** their photoactivated abilities to produce DNA strand breaks were measured. Both agents caused increased single strand breakage with increasing UV exposure. The concentrations (EC_{50}) of **1** and **2** needed to cause 50% single-strand cleavage of pBR322 DNA upon UV-A activation were found to be 40 μM and 45 μM, respectively. In contrast, no DNA strand breaks were observed in the dark with either conjugate or with **8** following irradiation. DNA strand breaks were measured in drug treated K562 cells using alkaline elution. Extensive strand breaks were observed following irradiation of the cells which were rapidly repaired.

Introduction

One of the main problems of cancer chemotherapy is the dose limiting toxicities of the drugs, and this has been attributed to their lack of selectivity for killing cancerous tissues.[1] Consequently there is an interest in the design of anticancer agents that not only have potent cytotoxicities but also selectively kill cancer cells without affecting normal tissues. One strategy towards this objective is the use of photoactivatable drugs which can be selectively activated by light at the target tissues.[2] Numerous substances are known to be phototoxic toward microorganisms, and many drugs cause phototoxic or photoallergic reactions in animals.[3] Some of these photoactive compounds, such as psoralens[2b,4] and hematoporphyrins,[5] have been used to treat a number of cancers. Psoralens, which act by the Type I mechanism, are known to exert their activity through the formation of DNA interstrand crosslinks upon UV irradiation.[6] However, photosensitizers such as hematoporphyrins, pyrene and DNA binding anthracenediones work by other mechanisms, in which they exert their biological activities by producing singlet oxygen and/or superoxide and/or hydroxy radicals upon illumination.[2,7] Further studies have also shown that anthracenediones can cause DNA single-strand breaks upon activation by visible light, albeit with poor sequence selectivity.[8] Therefore, the main objective of this study is to develop novel photosensitizers which demonstrate both DNA affinity and enhanced DNA sequence selectivity.

In this paper, the design, syntheses and the biological properties of conjugates of the photoactive agent pyrene tethered to pyrrole- (**1**) and imidazole-containing (**2**) netropsin analogues, or lexitropsins, are described. The rationale for this design is based on our previous studies which showed that conjugates of psoralen with netropsin analogues (**3** and **4**, see Fig. 1) are significantly (> 333 fold) more photocytotoxic than psoralen itself.[9] Netropsin analogues were chosen for this study because of their unique DNA minor groove and sequence selectivities, in which the pyrrole and imidazole analogues generally exhibit AT and GC sequence selectivity, respectively.[10] Furthermore, netropsin analogues have been shown to readily penetrate cellular membranes and concentrate in the nucleus,[10b,11] thus making them attractive for the delivery of photoactive groups, such as pyrene, to DNA. A pyrene moiety was chosen as the photosensitizer in compounds **1** and **2** because a pyrene–phosphatidyl choline conjugate had been shown to exhibit significant photocytotoxicity, and pyrene has a different mechanism of action from the psoralens.[12] It was proposed that upon UV-A activation the pyrene–lipid conjugate caused oxidative damage to the lysosomal membrane presumably by singlet oxygen, which subsequently released the toxic lysosomal hydrolases

into the cytoplasm.[13] In contrast, conjugation of pyrene to a DNA binding ligand should allow oxidative damage to be concentrated on the DNA.

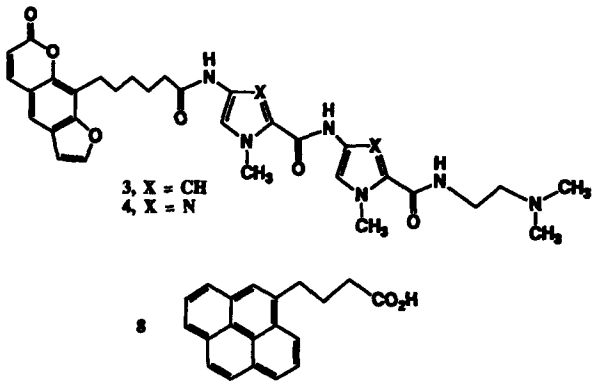

Figure 1. Structures of psoralen conjugates **3** and **4**, and 1-pyrenebutyric acid **8**

Results and Discussion

Synthesis

As depicted in Figure 2, the target compounds **1** and **2** were prepared by coupling 1-pyrenebutanoyl chloride **5** with the respective amines **6**[14] and **7**[15] in the presence of triethylamine in 44 and 43% yield, respectively. The structures of these compounds were confirmed by 300 MHz ^{1}H NMR, IR and high resolution mass spectral analyses.

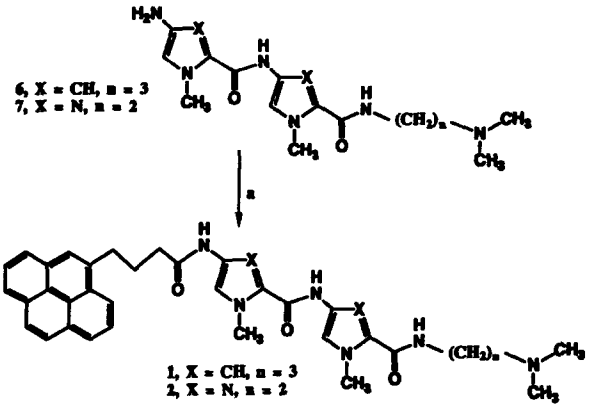

Figure 2. Synthesis of the target pyrene–lexitropsin conjgates **1** and **2**. [a] 1-Pyrenebutanoyl chloride **5**, triethylamine, CH_2Cl_2, 0 °C to room temperature.

DNA sequence and groove selectivity

The apparent binding constants of compounds **1** and **2** to calf thymus, T4 coliphage, poly(dG–dC) and poly(dA–dT) were determined using an ethidium displacement assay.[16] The results given in Table 1 illustrate that these compounds bind strongly to the DNAs studied, and except for poly(dA–dT), they bind with higher affinity to the DNAs than distamycin. The enhanced DNA binding affinities of conjugates **1** and **2** could be attributed to the interaction of both the lexitropsin and the pyrene moieties with DNA. In addition, binding of both compounds to T4 DNA suggests that they interact in the minor groove because the major groove of this DNA is blocked by α-glycosylation of the cytidine residues.[17] It is also worth noting that compounds **1** and **2** bind slightly more strongly to poly(dA–dT) than to poly(dG–dC). However, in both cases, the conjugates lack the marked AT sequence specificity of distamycin and netropsin.[18]

CD titration studies

The reversible interactions of agents **1** and **2** with DNA were also measured by CD titration studies. The CD spectrum of the DNA itself was collected (trace a, Figs 3A and 3B, for poly(dA–dT) and poly(dG–dC), respectively) and drug aliquots were added at specified r' values, moles of agent to moles of base pairs. Titration of compound **1** to poly(dA–dT) at an r' value of 0.3 produced positive DNA induced ligand bands at 290 nm (1.30 mdeg), 330 nm (0.52 mdeg) and 350 nm (0.61 mdeg) (trace b, Fig. 3A). In the complete titration study an isodichroic point was seen at 265 nm. Since compound **1** itself does not produce a CD spectrum, appearance of DNA induced ligand bands was taken as evidence for the complexation of this compound with the DNA. Based on the UV spectrum of compound **1**, the CD band at 290 nm is presumably due to the π to π^* absorption of the lexitropsin moiety, while the longer wavelength bands belong to the π to π^* absorption of the pyrene moiety. Since asymmetry was induced to both chromophores of **1**, these data support the hypothesis that both the lexitropsin and the photosensitizer moieties are interacting with the DNA. Furthermore, titration of this agent to poly(dG–dC) did not produce any DNA induced bands (trace b, Fig. 3B), clearly indicating a preference for poly(dA–dT) Titration of compound **2** to poly(dA–dT) also produced a positive band at 290 nm (1.40 mdeg, r' = 0.3) (trace. c, Fig. 3A), while addition to poly(dG–dC) produced a band at 290 nm (1.1 mdeg, r' = 0.3) (trace c, Fig. 3B). These results, in agreement with the results from the ethidium diplacement assay, suggest that compound **2** binds with a slightly higher affinity to poly(dA–dT) than

Table 1. The apparent DNA binding constants ($K_{app} \times 10^6$ M^{-1}) of compounds **1** and **2**

	K_{app} ($\times 10^6$ M^{-1})			
Agent	Calf thymus	T4	poly(dA–dT)	poly(dG–dC)
1	4.6 ± 0.4	8.7 ± 0.6	12.1 ± 0.2	2.8 ± 0.8
2	4.2 ± 0.5	7.3 ± 0.1	4.2 ± 0.7	2.4 ± 0.5
distamycin[a]	0.77	0.65	34.8	0.20

[a] Taken from Ref. 9a.

to poly(dG–dC) even though it is an imidazole-containing analogue of netropsin. In Figures 3A and 3B the negative and positive Cotton effects at 245 and 260 nm were retained at an r' value of 0.3 indicating that the DNA has retained the B-conformation. Thus, binding of **1** and **2** to DNA only causes minor conformational changes.

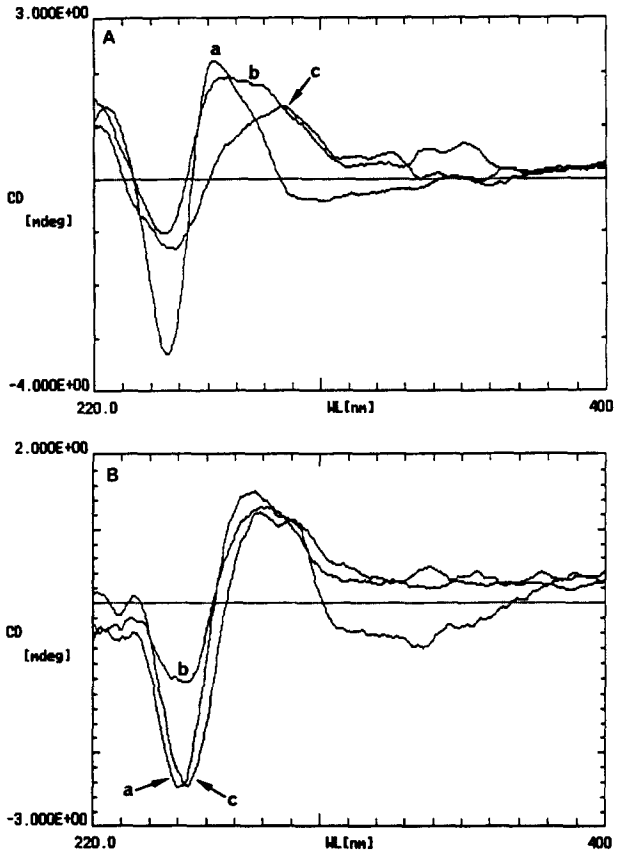

Figure 3. In part A, the CD titration spectra of compounds **1** and **2** to poly(dA–dT) are given. Part B contains the titration spectra of conjugates **1** and **2** to poly(dG–dC). Traces a to c of part A correspond to free poly(dA–dT); **1** and poly(dA–dT); **2** and poly(dA–dT), respectively. Traces a to c of part B correspond to free poly(dG–dC); **1** and poly(dG–dC); **2** and poly(dG–dC), respectively. The r' values of the drug:DNA complexes were 0.3.

Photophysical studies

The electronic spectrum of both conjugates displayed absorption maxima for the lexitropsin and pyrene residues (see the Experimental section). When the π to π^* absorption band at 344–346 nm of the pyrene moiety of **1** or **2** was excited, distinct emission bands were observed at 380, 400 and 420 nm. Because photochemical excitation of pyrene moieties has been proposed to produce singlet oxygen molecules to elicit their biological activities,[2a,12] the quenching rate constants of the triplet excited states of the pyrene moiety of the conjugates by oxygen were detemined. The intensities of the emission bands were measured at three oxygen concentrations; 0, 0.0003 and 0.0013 M for nitrogen, air and oxygen saturated aqueous solutions, respectively. From these data, the Stern–Volmer plots (ϕ_0/ϕ versus concentration of oxygen) were constructed. Using a lifetime of 129 ns for the excited state (determined for 1-pyrenebutyric acid in water at 15 °C)[20] the oxygen quenching rate constants for compounds **1** and **2** were calculated to be 8.5×10^9 $M^{-1}s^{-1}$ and 7.7×10^9 $M^{-1}s^{-1}$, respectively. The results indicate that the excited states of the conjugates are quenched by oxygen with rates approaching the diffusion limit, suggesting that singlet oxygen could be involved in the DNA cleavage process.

Further emission studies of compounds **1** and **2** were conducted with various concentrations of DNA (base pairs) specified by r' values ranging from 0 to 3.3. The intensities of the emission bands at 384–392 nm for both agents were only enhanced by about 5 times at an r' of 3.3 (data not shown). These data suggest that the pyrene moiety might only be pseudointercalated or merely binding in the minor groove because the emissions of classical intercalators such as ethidium bromide are generally increased by > 25 times upon binding to DNA.[16]

In-vitro photoinduced cytotoxicity

The cytotoxicities of compounds **1** and **2** against human chronic myeloid leukemia K562 cells were determined in the dark and following irradiation at 366 nm using the MTT assay.[21] The growth curves for both compounds are given in Figure 4 and the IC_{50} values are listed in

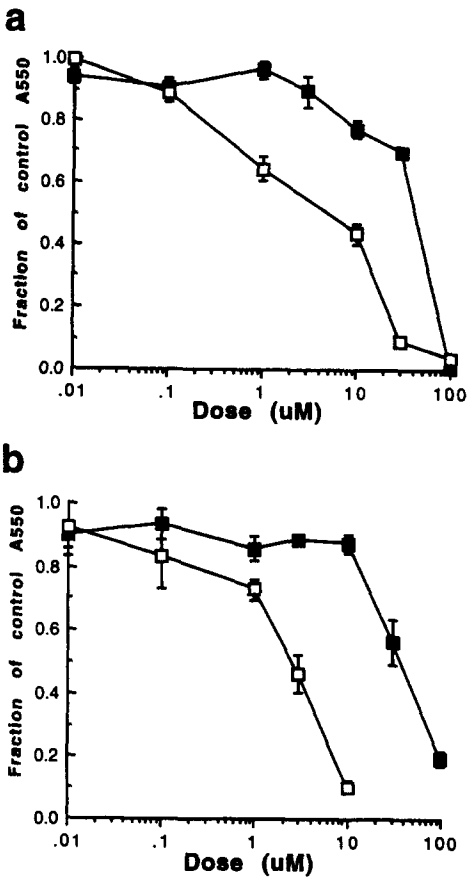

Figure 4. Viability curves for compounds **1** (a) and **2** (b). K562 cells were treated with drug for 1 h at 37 °C in the dark and grown for 72 h either in the dark (filled boxes) or following 2 min exposure to 366 nm UV (open boxes). Values are the mean SD of eight values within a single experiment.

Table 2. In both cases the compounds showed some cytotoxicities under dark conditions. Upon irradiation for 2 min the activities were significantly enhanced resulting in photoinduced dose modifications of 8 and 14 for agents **1** and **2**, respectively, under the conditions used. This level of irradiation is completely non-toxic to the cells in the absence of agent. The data also show that both compounds are more photocytotoxic than the non-targeted 1-pyrenebutyric acid **8** (Table 2).

DNA cleavage studies

Supercoiled plasmid DNA (form I) is converted to the open circular form (II) by the presence of a single-strand break and this form migrates more slowly on an agarose gel. The production of double-strand breaks (or closely opposed single-strand breaks) generates linear DNA molecules which migrate intermediately between forms I and II. Using this method the abilities of compounds **1**, **2** and **8** to produce DNA strand breaks in pBR 322 DNA were measured both in the dark and following irradiation at 366 nm. None of the compounds gave any detectable strand breakage in the dark at doses up to 1 mM. In addition no breaks were detected with **8** following 366 nm irradiation for 2 min (data not shown). In contrast, both **1** and **2** gave extensive strand breakage following irradiation. Figure 5 shows the effect of UV exposure on plasmid DNA reacted with 100 μM **1**. The amount of open circular DNA increased with time of irradiation up to 4 min and then decreased

Table 2. Photoinduced cytotoxicities of compounds **1**, **2** and **8**

	IC_{50}[a] (μM)		Photoinduced
Agent	dark	irrad.[b]	dose modification
1	40 μM	5 μM	8
2	35 μM	2.5 μM	14
8	> 100 μM	100 μM	> 1

[a]Concentration of drug required to inhibit growth of K562 cells by 50% following 1 h exposure.
[b]Irradiation was at 366 nm for 2 min.

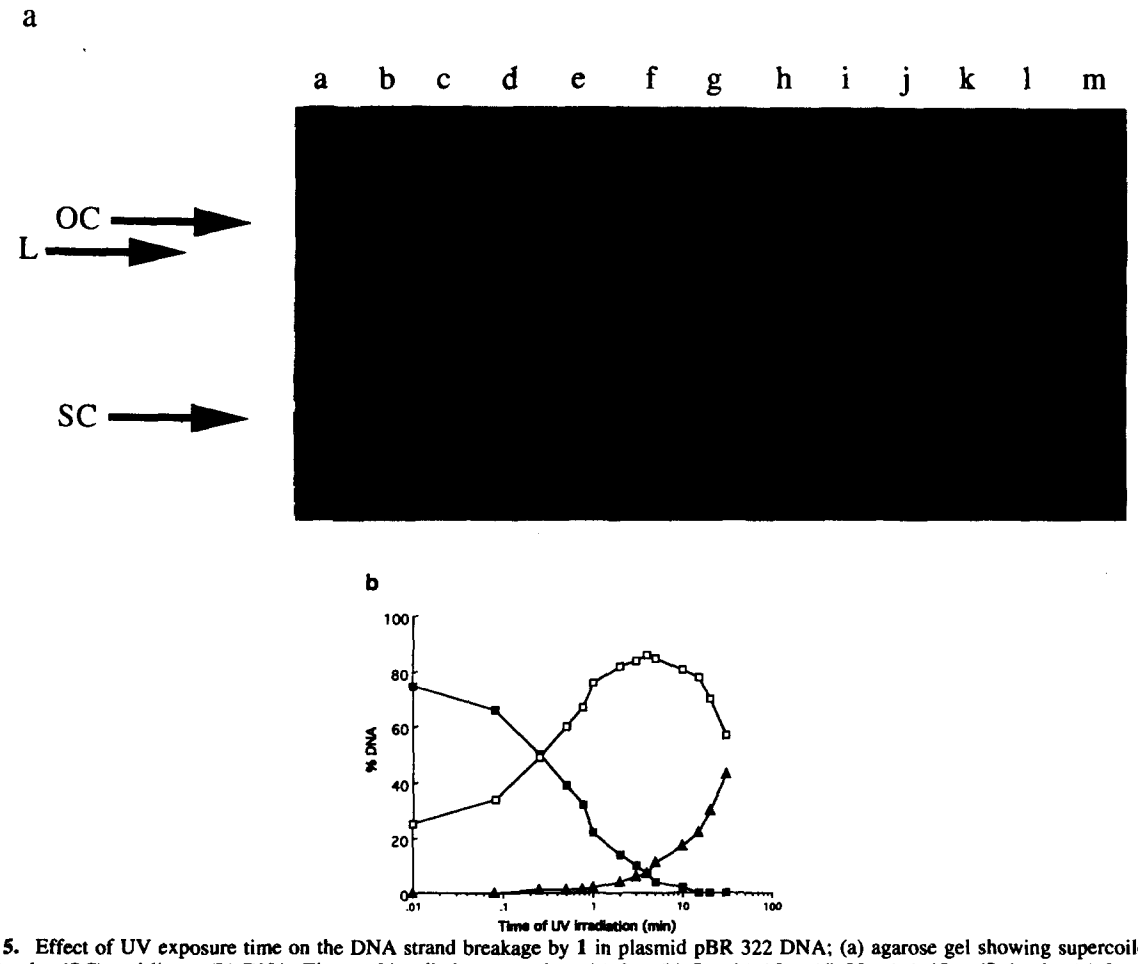

Figure 5. Effect of UV exposure time on the DNA strand breakage by **1** in plasmid pBR 322 DNA; (a) agarose gel showing supercoiled (SC), open circular (OC) and linear (L) DNA. Times of irradiation were: lane (a) 0 s, (b) 5 s, (c) 15 s, (d) 30 s,(e) 45 s, (f) 1 min, (g) 2 min, (h) 3 min, (i) 4 min, (j) 5 min, (k) 10 min, (l) 15 min, (m) 20 min; (b) amount of supercoiled DNA (filled boxes), open circular DNA (open boxes) and linear DNA (filled triangles) from gel in (a) above.

with the corresponding increase in linear DNA. Similar results were obtained with compound **2** (data not shown). No breaks were observed following 30 min UV in the absence of drug. At a constant level of irradiation (2 min) the dose of drug required to produce single-strand breaks in 50% of the DNA samples was 40 μM and 45 μM for **1** and **2**, respectively.

DNA strand breakage was also measured in cells using the technique of alkaline elution. Figure 6a shows that following treatment of cells with 12.5 μM **1** for 1 h, a low level of single-strand breakage was observed when the cells were kept in the dark. Following 2 minutes irradiation extensive strand breakage was observed corresponding to the marked photoinduced cytotoxicity of this compound. Similar results were observed with compound **2** (data not shown). Compounds **1** and **2** clearly demonstrate a significant increase in cytotoxicity following UV exposure which corresponds to the activation of the compounds to produce species which damage DNA in cells by single-strand breakage. Both compounds are more phototoxic and more efficient at producing DNA damage than the non-targeted 1-pyrene butyric acid **8** indicating efficient delivery to the DNA target. Although both agents produce significant photoinduced dose modifications the magnitude of the effect is less than observed previously for the corresponding psoralen-containing analogues **3** and **4**. This may suggest that single-strand breakage of DNA is a less cytotoxic lesion than an interstrand crosslink. An important determinant of cytotoxicity may also be repair of the lesions. Figure 6b clearly indicates that the single strand breaks produced in cells by activation of **1** are rapidly repaired with > 50% removed within 1 h.

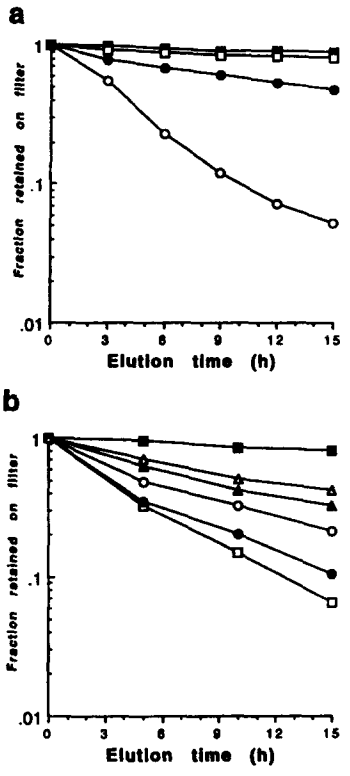

Figure 6. Alkaline elution profiles for **1** in K562 cells. (a) Cells were untreated (squares) or treated with 12.5 μM drug (circles) for 1 h at 37 °C in the dark and then processed immediately (filled symbols) or exposed to UV for 2 min (open symbols); (b) cells were treated with 12.5 μM drug for 1 h at 37 °C and either kept in the dark (filled boxes) or irradiated for 2 min at 366 nm (open boxes, filled circles, open circles, filled triangles, open triangles). Samples were either analyzed immediately (open boxes), or following varying times of post-incubation at 37 °C: (filled circles) 15 min, (open circles) 30 min, (filled triangles) 45 min, (open triangles) 60 min.

Experimental

N-[3-(Dimethylamino)propyl]-1-methyl-4-[1-methyl-4-[1-pyrenebutanamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide (1)

A suspension of *N*-[3-(dimethylamino)propyl]-1-methyl-4-[1-methyl-4-nitropyrrole-2-carboxamido]pyrrole-2-carboxamide[14] (311 mg, 0.74 mmol) in chilled methanol (50 mL) and 0.14 g of 5% Pd/C was hydrogenated for 5 h at atmospheric pressure and room temperature. The catalyst was removed by filtration, and upon concentration of the filtrate the residue, compound **6**,[14] was coevaporated with dry methylene chloride (twice, 20 mL each).

1-Pyrenebutanoyl chloride was made by dissolving 1-pyrenebutyric acid (334 mg, 1.16 mmol) in dry THF (2 mL) and oxalyl chloride (2 mL), and refluxing the mixture for 40 min under a drying tube. The excess oxalyl chloride was removed under pressure and the product collected was coevaporated twice with dry methylene chloride (10 mL). The acid chloride was dissolved in dry methylene chloride (15 mL) and added dropwise to a chilled (0 °C) solution of the amine, triethylamine (0.11 mL, 0.79 mmol), and dry methylene chloride (35 mL). The reaction mixture was warmed to room temperature overnight. The methylene chloride was removed under reduced pressure, and water (40 mL) was added along with sodium hydroxide (2 M) to bring the pH of the aqueous layer to 12. The solution was then extracted with chloroform (50 mL, three times), and the organic extracts were combined, dried (Na_2SO_4) and concentrated. The product was purified by silica gel chromatography beginning with 2.5% MeOH/$CHCl_3$ and ending with 10% MeOH/$CHCl_3$. The desired fractions were collected and concentrated to give compound **1** as a yellow foam (0.20 g, 44%); mp 125–127 °C; TLC (50% MeOH/$CHCl_3$) R_f 0.4; ^{1}H NMR ($CDCl_3$): δ 1.52 (*q*, 5.6, 2H), 2.08 (*s*, 6H), 2.11 (*m*, 2H), 2.25 (*t*, 6.9, 2H), 2.27 (*t*, 5.6, 2H), 3.17 (*q br*, 6.9, 2H), 3.33 (*m*, 2H), 3.68 (*s*, 3H), 3.70 (*s*, 3H), 6.55 (*d*, 1.2, 1H), 6.58 (*d*, 0.9, 1H), 7.00 (*d*, 0.9, 1H), 7.09 (*d*, 0.9, 1H), 7.42 (*t br*, 6.9, 1H), 7.64 (*d*, 7.8, 1H), 7.77–8.02 (*m*, 7H), 8.08 (*d*, 7.8, 1H), 8.39 (*s br*, 1H), 8.50 (*s br*, 1H); IR: ν 3295, 2964, 1643, 1579, 1531, 1452, 1433, 1397, 1259, 1098, 1024, 847, 783, 751 cm^{-1}; UV-vis (water): 242 ($\varepsilon = 4.7 \times 10^4$ M^{-1} cm^{-1}), 268 ($\varepsilon = 2.67 \times 10^4$), 278 ($\varepsilon = 3.3 \times 10^4$), 314 ($\varepsilon = 2.3 \times 10^4$), 328 ($\varepsilon = 2.3 \times 10^4$), 344nm ($\varepsilon = 1.9 \times 10^4$); MS (FAB, NBA) m/z (rel. intensity): 617 (M + H^+ 1.5); HRMS (FAB, NBA) m/z 617.3237 ($C_{37}H_{41}N_6O_3$ requires 617.3240).

N-[2-(Dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-[1-pyrenebutanamido]imidazole-2-carboxamido]imidazole-2-carboxamide (2)

Compound **2** was prepared using a similar procedure to that for **1**, except *N*-[2-(dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-nitroimidazole-2-carboxamido]imidazole-2-carboxamide[15] (424 mg, 1.16 mmol) was used. The product was isolated as a yellow foam (0.32 g, 43.3%); mp 97–100 °C; TLC (10% MeOH/$CHCl_3$) R_f 0.56; ^1H NMR ($CDCl_3$): δ 2.28 (*s*, 6H), 2.32 (*q*, 7.5, 2H), 2.50 (*t*, 7.5, 2H), 2.51 (*t*, 6.5, 2H), 3.45 (*m*, 4H), 4.01 (*s*, 3H), 4.02 (*s*, 3H), 7.38 (*s*, 1H), 7.39 (*s*, 1H), 7.62 (*t br*, 7.5, 1H), 7.71 (*s br*, 1H), 7.89 (*d*, 9.5, 1H), 7.78 (*t*, 8.2, 1H), 8.03 (*s*, 2H), 8.08–8.19 (*m*, 4H), 8.32 (*d*, 9.5, 1H), 9.16 (s br, 1H); IR: ν 3391, 3274, 2953, 1729, 1665, 1536, 1467, 1365, 1189, 1018, 847, 751 cm^{-1}; UV-vis (water): 244 ($\varepsilon = 7.2 \times 10^3$ M^{-1} cm^{-1}), 268 ($\varepsilon = 4.2 \times 10^3$), 278 ($\varepsilon = 5.3 \times 10^3$), 328 ($\varepsilon = 4.6 \times 10^3$), 346 nm ($\varepsilon = 3.7 \times 10^3$); MS (FAB, NBA) m/z (rel. intensity): 605 (M + H^+ 36); HRMS (FAB, NBA) m/z 605.2979 ($C_{34}H_{37}N_8O_3$ requires 605.2989).

Ethidium displacement assay

The fluorescence of the DNA (25 μL of a $2A_{260}$ solution) in 2 mL of an ethidium bromide buffer solution (10 mM Tris, 1mM EDTA, 1.3 μM EtBr, pH 7.4) was measured (excitation wavelength of 525 nm, emission wavelength of 605 nm) for the maximum fluorescence. Aliquots of drug were then added and the fluorescence recorded until a 50% reduction occurred. The binding constants were then determined as described previously.[9]

CD titration studies

An initial DNA spectrum was taken by injecting a $2A_{260}$ DNA solution [154 μM (bp), 130 μL] into a 160 μL cell with a 1 mm path length. Ten additions of a 1.0 mM drug solution were made in increments poly(dA–dT): 0.5, 0.5, 1.0, 1.0, 1.0, 2.0, 2.0, 4.0, 4.0, 4.0 μL which gave *r'* values of 0.025, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.60, 0.80, 1.0, respectively. For poly(dG–dC) DNA, increments of 1.0 mM drug were added in the following manner: 0.8, 0.8, 0.8, 0.8, 1.6, 1.6, 3.2, 3.2, 3.2 μL which gave *r'* values of 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.60, 0.80, 1.0, respectively. Scan parameters and experimental conditions were identical to those described previously.[9]

Plasmid nicking assay

Plasmid pBR 322 DNA (500 ng per sample) and the appropriate concentration of drug were incubated in the dark for 1 h in a triethanolamine buffer (25 mM triethanolamine, 1 mM EDTA, pH 7.2). Irradiation with a 366 nm lamp (UVP Inc., San Gabriel, CA, 50 Hz, 420 μW cm^{-2}) was performed for the times listed in Figure 5. After irradiation, the samples were ethanol precipitated, dried and taken up in a sucrose loading buffer. The samples were electrophoresed on a 0.8% agarose gel at 40 V overnight. The gels were stained with ethidium bromide, illuminated with a transilluminator and photographs were taken with type 57 high speed polaroid film. Quantification of the bands was achieved using a "DeskScan II" interfaced with a MacIIfx and "Image 1.52b2" software.

Oxygen quenching studies

Fluorescence studies were performed on an Aminco SLM 8000 instrument. Fluorescence studies of **1** and **2** were scanned from 360 nm to 600 nm with an excitation wavelength of 344 nm. The studies were recorded under nitrogen-saturation, air-saturation, and oxygen-saturation. Stern–Volmer plots of ϕ_0/ϕ versus quencher concentration [O_2] M for compounds **1** and **2** were constructed, where ϕ_0= relative emission intensity at 384 nm in the absence of quencher, and ϕ = relative emission intensity in the presence of quencher.

Fluorescence titration studies

In these studies, 5 μL of 1 mM drug were combined with 2 mL of a 10 mM sodium phosphate buffer, pH 7.4. The emission spectra (360 nm to 600 nm) were recorded observed with an excitation wavelength of 344 nm. The drug solution was titrated with calf thymus DNA ($2A_{260}$) with specified *r'* values of 3.3, 1.65, 0.83, 0.42, 0.21, 0.16, 0.10, 0.075, and 0.05, respectively.

Cytotoxicity studies

The IC_{50} values were determined using the MTT assay.[21] The K562 human chronic myeloid leukemia cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. Drug incubation was for 1 h at 37 °C in the dark. Drug-containing medium was removed by centrifugation and the cells resuspended in 2 mL fresh medium. Alternatively, the samples to be irradiated were washed once in ~1 mL sterile phosphate buffer saline (PBS) and resuspended in PBS (37 °C). Irradiation of the samples was for 2 min with a 366 nm lamp (UVP Inc., San Gabriel, CA, 50 Hz, 420 μW cm^{-2}) that was clamped 5 cm from the open Petri dish (35 mm diameter). After irradiation the cells were centrifuged and resuspended in fresh medium.

Following the appropriate drug treatment and/or irradiation, the cells were transferred to 96-well microtitre plates, 10^4 cells per well, 8 wells per sample. Plates were then kept in the dark at 37 °C in a humidified atmosphere containing 5% CO_2. After 72 h, the plates were processed as described previously.[9a]

Alkaline elution

DNA interstrand crosslinking was measured by the technique of alkaline elution.[19] Early logarithmic cells were labeled for 24 h with 0.015 μCi [^{14}C]-thymidine mL^{-1} (specific activity 56 μCi $mmol^{-1}$; Amersham,

U.K.), centrifuged and resuspended in fresh medium for 1 h before drug treatment. Cells were treated with drug for 1 h at 37 °C in the dark, and following incubation centrifuged to remove drug-containing medium, washed with sterile PBS and resuspended in 1 mL sterile PBS (37 °C). Irradiation was then performed as described for the cytotoxicity studies. Immediately following irradiation the cells were placed on ice, or alternatively, if a post-incubation period was required, the cells were resuspended in fresh medium and stored in the dark at 37 °C for the appropriate time. Elution was through 2 μm polycarbonate filters at pH 12.1 in the presence of proteinase K.

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