Quaternarized pdppz: synthesis, DNA-binding and biological studies of a novel dppz derivative that causes cellular death upon light irradiation†
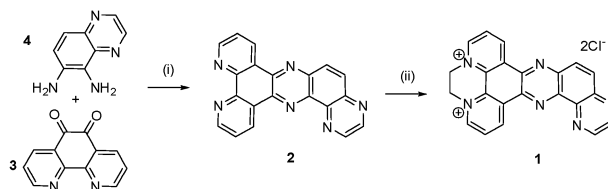
Robert B. P. Elmes,[a] Marialuisa Erby,[b] Suzanne M. Cloonan,[b] Susan J. Quinn,*[c] D. Clive Williams*[b] and Thorfinnur Gunnlaugsson*[a]

The quaternarized pdppz derivative 1 was shown to bind strongly to DNA with concomitant changes in its ground and excited state photophysical properties. Furthermore, the compound also showed rapid cellular uptake, and induced apoptosis upon light irradiation in various cancer cell lines after 24 hours of incubation.

The development of novel low molecular weight cancer therapeutics is a highly topical area of research. Such molecules should ideally possess good water solubility, possess rapid cellular uptake, undergo selective localisation and be able to induce programmed cell death of the cancer cells.[1–4] In recent work, we have demonstrated that pyrrolo-1,5-benzoxazepine,[5] sulfur-substituted α-alkyl phenethyl-amine,[6] naphthalimide,[7] and polyamide[8] structures show potential as anticancer agents in a variety of cancer cell lines, as well as in solid tumours.[9] In other related work, we have developed new polypyridyl ligands possessing various organic fluorophores, either directly attached, or conjugated *via* short spacers to the Ru(II)-polypyridyl unit, for use as DNA targeting ligands and imaging agents.[10] Herein, we present the synthesis of 1, a novel quaternarized polypyridyl molecule formed from 2, pyrazino[2,3-*h*]dipyrido[3,2-*a*:2′,3′-*c*]phenazine, or pdppz. This new species is based on combining, in a single structure, two well known polypyridyl ligands, dipyrido[3,2-*a*:2′,3′-*c*]phenazine (dppz) and 1,4,5,8-tetraazaphenanthrene (TAP), that are commonly used as DNA binding ligands in Ru(II) polypyridyl complexes,[11–13] and has not, to the best of our knowledge, been synthesised before. The quaternarisation of polypyridyl ligands has recently been shown by Thomas *et al.*[14] to be an effective way of increasing the DNA binding affinity of such organic structures. Hence, we embarked on investigating the ability of 1, to bind to DNA, as due to its extended aromatic surface, in concert with its cationic character, we foresaw that 1 would be an ideal candidate as a DNA intercalator. Moreover, its cationic nature also furnishes 1 with good aqueous solubility and should facilitate its cellular uptake in various cancer cell lines, where its ability to initiate apoptosis could potentially be controlled by light irradiation.

[a] *School of Chemistry, Centre for Synthesis and Chemical Biology, Trinity College Dublin, Dublin 2, Ireland. E-mail: gunnlaut@tcd.ie; Fax: +353 1 671 2826; Tel: +353 1 896 3459*
[b] *School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland. E-mail: clive.williams@tcd.ie; Fax: +353 1 8963130; Tel: +353 1 8962596*
[c] *School of Chemistry and Chemical Biology, Centre for Chemical Synthesis and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland*

† Electronic supplementary information (ESI) available: Synthesis, 22 figures and 2 tables. See DOI: 10.1039/c0cc04303f

Scheme 1 Synthesis of 2 (free ligand) and corresponding dicationic organic derivative 1. (i) EtOH, reflux; (ii) dibromoethane, reflux.

The synthetic pathway of 1 and 2 is shown in Scheme 1. The synthesis of 2 was achieved by condensation of 5,6-diaminoquinoxaline[15] 4 with 1,10-phenanthroline-5,6-dione[16] 3 by reflux in EtOH yielding 2 as a grey solid in 95% yield. Subsequent reflux in dibromoethane over 48 h and precipitation from methanol with diethyl ether yielded 1 as a pure beige solid in 78% yield. Synthesised as its chloride salt, 1 was water-soluble, and its photophysical properties were investigated in 10 mM phosphate-buffered aqueous solutions at pH 7.4.

The characteristic absorption spectrum together with the excitation and emission spectra of 1 are shown in Fig. 1. The absorption spectrum of 1 shows absorbance maxima at *ca.* 285 nm, 310 nm, 370 nm, 390 nm along with a broad shoulder centred at 460 nm. The band at 285 nm ($\varepsilon = 31\,400$ cm^{-1} M^{-1}) is characteristic of π–π* transitions of the *phen* moiety while the intense band at 310 nm ($\varepsilon = 44\,100$ cm^{-1} M^{-1}) is attributed to π–π* transitions with less intense bands at 370 nm and 390 nm ($\varepsilon = 6900$ cm^{-1} M^{-1}) attributed to n–π* transitions within the phenazine part of the ligand.[17] These transitions are typical of the related compound dppz. Upon excitation into the intense band at 310 nm 1 gave intense emission with λ_{max} at 515 nm ($\Phi_F = 0.053$). Similarly, the excitation of 1 ($\lambda_{em} = 515$ nm) yielded a spectrum structurally identical to the absorption spectrum.
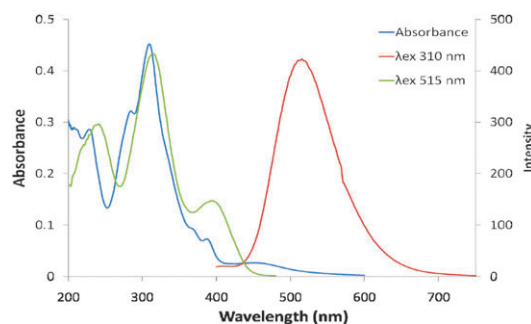

Fig. 1 The UV/Visible absorption spectra, the excitation and fluorescence emission spectra of 1 (10 μM) in aerated 10 mM phosphate buffer, at pH 7.4.

We next investigated the ability of **1** to bind to salmon testes DNA (stDNA) at pH 7.4 (10 mM phosphate buffer), using the absorption and emission spectroscopy of **1**, Fig. 2. As can be seen from these results, the absorption spectrum was significantly affected over the entire wavelength range. From these changes, we were able to determine the affinity binding constant K_b as 6.5×10^5 M^{-1} (± 0.5) (and a binding site size of 2.6 (± 0.03)) using the method of Bard *et al.*[18] This indicates a strong binding affinity for DNA, comparable to that seen for many transition metal based intercalators, and of similar magnitude to that reported by Thomas *et al.*[14] Concomitantly, the changes in the emission spectra were also dramatic, where the emission was almost completely 'switched off', as demonstrated by an inset in Fig. 2. Similar effects were seen in the fluorescence emission spectra upon excitation at other wavelengths such as 390 nm. Again, analysis of these changes (of which 80% occurred over $0 \rightarrow 10$ P/D, see ESI†) by fitting data using the model of McGhee and von Hippel,[19] gave $K_b = 3.55 \times 10^5$ M^{-1} (± 0.02), consistent with that seen in the absorption spectra. We also investigated the effect of ionic strength on these changes by carrying out a back titration in the presence of increasing concentrations of NaCl (see ESI†). This showed that the binding of **1** to DNA was only marginally affected; indicating that electrostatic interactions are not the main form of interaction with DNA. Furthermore, while no induced circular dichroism (CD) was observed, significant changes were evident in the linear dichroism (LD) spectrum (see ESI†), which resulted in the formation of a strong negative band at 310 nm, resulting from the π–π^* transition of the phenazine part of the ligand; strongly supporting an intercalative mode of DNA interaction for **1**.[20]

Similar spectroscopic titrations were also carried out using the homo-polymers poly(dG–dC) and poly(dA–dT) (see binding isotherms in ESI†); the results showed that the emission of **1** was again significantly quenched in both cases. As seen for the titration of stDNA, the major changes occurred with the addition of 5 P/D for poly(dG–dC) (95% quenched); while 25 P/D was needed to achieve 70% quenching for poly(dA–dT), demonstrating a clear preference for the binding of **1** to poly(dG–dC). This was further confirmed by fitting the changes in the absorption and the emission spectrum, which for the former resulted in $K_b = 1.35 \times 10^7$ M^{-1} (± 0.55)

(and a binding site size of 1.7), while for poly(dA–dT) a significantly lower K_b of 4.85×10^5 M^{-1} (± 0.33) was determined. Thermal denaturation studies also showed that these compounds greatly stabilized the stDNA structure ($\Delta T_m > 5$–10 °C, see ESI†).

Having established strong affinity of **1** for DNA, we next wished to evaluate its DNA photocleavage efficiency. Agarose gel electrophoresis of pBR322 plasmid DNA was undertaken, Fig. 3. When incubated in the dark **1** showed no DNA cleavage. However, after 30 minutes of irradiation at a low intensity (2 J cm^{-1}), under aerobic conditions at a P/D ratio of 10, **1** showed extremely efficient photocleavage, resulting in essentially complete conversion of the supercoiled DNA into nicked circular form (Fig. 3, lane 8). Efficient photocleavage was also observed when 320 nm and 400 nm cut off filters were employed (Fig. 3, lanes 8 and 6, respectively). Interestingly, when irradiated in the presence of the known singlet oxygen scavenger, NaN$_3$, **1** showed diminished photocleavage efficiency suggesting that reactive oxygen species could be the primary mechanism aiding in the photocleavage of the DNA after treatment with **1**.

We anticipated that **1** would be taken up by cells due to its cationic nature, possibly through receptor mediated transport, or by diffusion driven by plasma membrane potential. Fig. 4 shows the fluorescence confocal laser scanning microscopy picture of live HeLa (cervical cancer) cells upon excitation at 310 nm after incubation with **1** (10 μM) 24 h prior to imaging (Fig. 4A). The image clearly demonstrates the localisation of **1** in all cells, with apparent localisation in the cytoplasm and to a lesser extent in the nucleus, as demonstrated in Fig. 4B, a bright field image showing co-localisation of **1** (green) with the nucleic acid stain DAPI (blue). To assess the anticancer

Fig. 3 Agarose gel electrophoresis of pBR322 DNA (1 mg ml^{-1}) after 30 min irradiation (2 J cm^{-2}) in 10 mM phosphate buffer, pH 7.4; lane 1: plasmid DNA control; lane 2: Ru(bpy)$_3$$^{2+}$ (P/D 20); lane 3: NaN$_3$ control; lane 4: **1** in the dark (P/D 10); lanes 5 and 6: **1** (P/D 20, 10) ($\lambda > 400$ nm); lanes 7 and 8: **1** (P/D 20, 10) ($\lambda > 320$ nm); lanes 9 and 10: **1** + 10 mM NaN$_3$ (P/D 20, 10). See further information in ESI†.
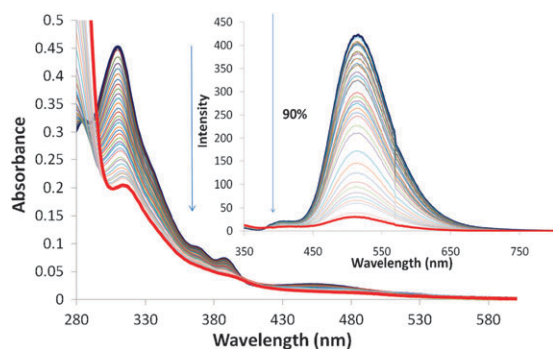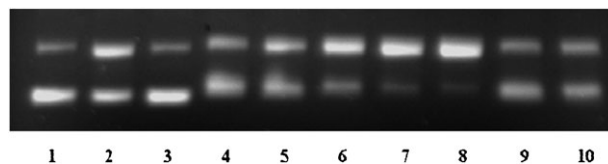

Fig. 2 Changes in the absorption spectrum of **1** (10 μM) with increasing concentration of stDNA (0–172 μM). Inset: the corresponding changes in the fluorescence emission spectrum of **1** (10 μM) (λ_{ex} 310 nm). The blue and the red spectra indicate the beginning and the end point of the titrations.
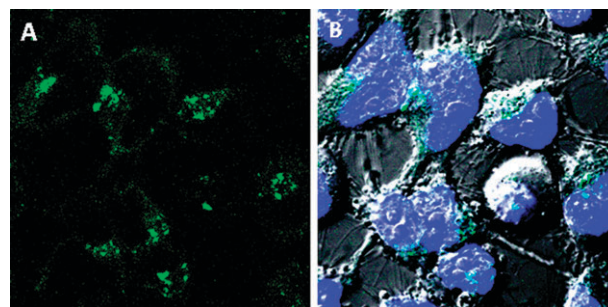

Fig. 4 Confocal laser scanning microscopy live cell images of **1** (10 μM) with HeLa cells. Shown is the image obtained with cells (A) stained with **1** (green) and (B) overlay of **1** (green), nuclear co-stain DAPI (blue) and the bright field view after 24 h of incubation.

potential of **1**, its antiproliferative effects were assessed in a number of malignant cell lines, including HeLa cells, the mesothelioma cell lines ONE58 and CRL5915 and the Burkitt's lymphoma cell lines, DG-75 and MUTU-I. Fig. 5A illustrates that **1** (100 μM) was found to reduce the viability of these malignant cells by approximately 50–60% in the dark and by 80–90% in the light indicating both a dark and a light-dependent cytotoxic death effect (IC$_{50}$ values for **1** were determined to be between 52–200 μM in the dark, and 34–47 μM under light irradiation in these cell lines). This cytotoxicity occurs at a similar potency to the well known platinum-based chemotherapeutic cisplatin (shown as C in Fig. 5A).[7b] Further studies carried out using propidium iodide FACS (fluorescent activated cell sorter) analysis, which detects the proportion of apoptotic bodies in the pre-G1 phase of the cell cycle,[21] also suggest that **1** induces light-dependent apoptosis in HeLa cells (Fig. 5B). This indicates that cytotoxicity may involve a programmed cell death mechanism. These results clearly demonstrate that these small molecular weight agents have the potential to be used as novel analogues to classical photodynamic therapeutic (PDT) drugs, as has also recently been demonstrated by O'Shea et al.[3]

In summary the di-cationic derivative **1**, developed using the newly prepared ligand **2**, has been shown to have high affinity for DNA; binding by intercalation and giving rise to light mediate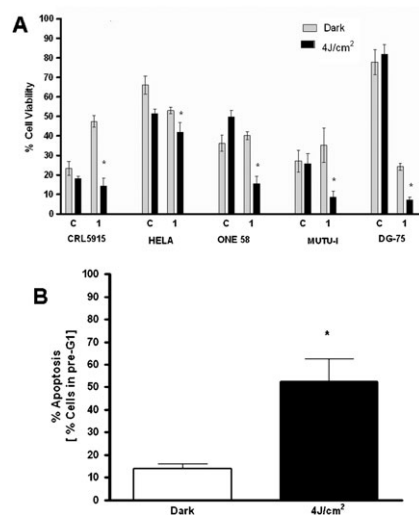d cleavage of plasmid DNA. We have shown that **1** is readily taken up by various cancer cell lines, and that **1** induces cell death, which is enhanced upon photoactivation.

Notes and references

1 P. B. Dervan, A. T. Poulin-Kerstien, E. J. Fechter and B. S. Edelson, *Top. Curr. Chem.*, 2005, **253**, 31.
2 (a) Z. Chen, X. Liang, H. Zhang, H. Xie, J. Liu, Y. Xu, W. Zhu, Y. Wang, X. Wang, S. Tan, D. Kuang and X. Qian, *J. Med. Chem.*, 2010, **53**, 2589; (b) R. Lalor, H. Baillie-Johnson, C. Redshaw, S. E. Matthews and A. Mueller, *J. Am. Chem. Soc.*, 2008, **130**, 2892.
3 (a) S. O. McDonnell, M. J. Hall, L. T. Allen, A. Byrne, W. M. Gallagher and D. F. O'Shea, *J. Am. Chem. Soc.*, 2005, **127**, 16360; (b) W. M. Gallagher, L. T. Allen, C. O'Shea, T. Kenna, M. Hall, A. Gorman, J. Killoran and D. F. O'Shea, *Br. J. Cancer*, 2005, **92**, 1702; (c) A. T. Byrne, A. E. O'Connor, M. Hall, J. Murtagh, K. O'Neill, K. M. Curran, K. Mongrain, J. A. Rousseau, R. Lecomte, S. McGee, J. J. Callanan, D. F. O'Shea and W. M. Gallagher, *Br. J. Cancer*, 2009, **101**, 1565.
4 (a) J. Andersson, S. M. Li, P. Lincoln and J. Andreasson, *J. Am. Chem. Soc.*, 2008, **130**, 11836; (b) J. Fujimoto, T. Bando, M. Minoshima, G. Kashiwazaki, S. Nishijima, K. Shinohara and H. Sugiyama, *Bioorg. Med. Chem.*, 2008, **16**, 9741; (c) I. Ott, Y. F. Xu, J. W. Liu, M. Kokoschka, M. Harlos, W. S. Sheldrick and X. H. Qian, *Bioorg. Med. Chem.*, 2008, **16**, 7107; (d) R. B. P. Elmes and T. Gunnlaugsson, *Tetrahedron Lett.*, 2010, **51**, 4082.
5 S. Maria Nathwani, S. Butler, D. Fayne, N. N. McGovern, B. Sarkadi, M. J. Meegan, D. G. Lloyd, G. Campiani, M. Lawler and D. C. Williams, *Cancer Chemother. Pharmacol.*, 2010, **66**, 585.
6 S. M. Cloonan, J. J. Keating, S. G. Butler, A. J. S. Knox, A. M. Jørgensen, G. H. Peters, D. Rai, D. Corrigan, D. G. Lloyd, D. C. Williams and M. J. Meegan, *Eur. J. Med. Chem.*, 2009, **12**, 4862.
7 (a) E. B. Veale and T. Gunnlaugsson, *J. Org. Chem.*, 2010, **75**, 5513; (b) E. B. Veale, D. O. Frimannsson, M. Lawler and T. Gunnlaugsson, *Org. Lett.*, 2009, **11**, 4040.
8 D. O. Frimannsson, T. McCabe, W. Schmitt, M. Lawler and T. Gunnlaugsson, *Supramol. Chem.*, 2010, **22**, 483.
9 S. A. Bright, A. M. McElligott, J. W. O'Connell, L. O'Connor, P. Carroll, G. Campiani, M. W. Deininger, E. Conneally, M. Lawler, D. C. Williams and D. M. Zisterer, *Br. J. Cancer*, 2010, **102**, 1474.
10 (a) G. J. Ryan, S. J. Quinn and T. Gunnlaugsson, *Inorg. Chem.*, 2008, **47**, 401; (b) A. M. Nonat, S. J. Quinn and T. Gunnlaugsson, *Inorg. Chem.*, 2009, **48**, 4646.
11 A. E. Friedman, J. C. Chambron, J. P. Sauvage, N. J. Turro and J. K. Barton, *J. Am. Chem. Soc.*, 1990, **112**, 4960–4962.
12 I. Ortmans, B. Elias, J. M. Kelly, C. Moucheron and A. Kirsch-DeMesmaeker, *Dalton Trans.*, 2004, 668–676.
13 S. Vasudevan, J. A. Smith, M. Wojdyla, T. McCabe, N. C. Fletcher, S. J. Quinn and J. M. Kelly, *Dalton Trans.*, 2010, **39**, 3990.
14 (a) T. Phillips, I. Haq, A. J. H. M. Meijer, H. Adams, I. Soutar, L. Swanson, M. J. Sykes and J. A. Thomas, *Biochemistry*, 2004, **43**, 13657; (b) T. Phillips, C. Rajput, L. Twyman, I. Haq and J. A. Thomas, *Chem. Commun.*, 2005, 4327; (c) P. Waywell, J. A. Thomas and M. P. Williamson, *Org. Biomol. Chem.*, 2010, **8**, 648.
15 R. Nasielski-Hinkens, J. Kotel, T. Lecloux and J. Nasielski, *Synth. Commun.*, 1989, **19**, 511.
16 L. Calucci, G. Pampaloni, C. Pinzino and A. Prescimone, *Inorg. Chim. Acta*, 2006, **12**, 3911.
17 R. M. Hartshorn and J. K. Barton, *J. Am. Chem. Soc.*, 1992, **114**, 5919.
18 M. T. Carter, M. Rodriguez and A. J. Bard, *J. Am. Chem. Soc.*, 1989, **111**, 8901.
19 J. D. McGhee and P. H. von Hippel, *J. Mol. Biol.*, 1974, **86**, 469.
20 B. Norden and T. Kurucsev, *J. Mol. Recognit.*, 1994, **7**, 141.
21 *Measurements of cell death by flow cytometry*, in *Techniques Apoptosis: A User's Guide*, ed. Z. Darzynkiewicz, X. Li, T. G. Cotter and S. J. Martin, Cytometry, London, U.K., 1996.

Fig. 5 (A) The antiproliferative effects of **1** (100 μM) on the malignant cell lines CRL5915, HeLa, ONE58, MUTU-I and DG-75. $1–5 \times 10^4$ cells 200 μl^{-1} were seeded and treated with cisplatin (C) (10 μM) or **1** for 24 h, irradiated with 4 J cm^{-2} light and incubated for 24 h. Alamar Blue reagent (10 μl) was added to measure cell viability at 590 nm (excitation 544 nm). Values represent the mean ± SEM of six data points and untreated cells represented 100% cell viability. (B) Compound **1** (100 μM) induces light-dependent apoptosis in HeLa cells. 0.25×10^6 cells were treated with **1** (100 μM) for 24 h, irradiated with 4 J cm^{-2} light, incubated for 24 h, harvested by centrifugation and fixed in 100% ethanol. FACS analysis was carried out upon incubation with propidium iodide and RNAse A. 10 000 cells were counted using appropriate gates. Values represent the mean ± SEM of three independent experiments. Cells treated with **1** were statistically compared to control cells by unpaired T-test using GraphPad prism software. * = p < 0.05.