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Interaction between an 8-methoxypyrimido[4′,5′:4,5] thieno (2,3-*b*)quinoline-4(3H)one antitumour drug and deoxyribonucleic acid

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Abstract. The interaction of 8-methoxypyrimido[4′,5′:4,5]thieno(2,3-*b*)quinoline-4(3H)one (MPTQ) with DNA was studied by UV-Vis and fluorescence spectrophotometry as well as by hydrodynamic methods. On binding to DNA, the absorption spectrum underwent bathochromic and hypochromic shifts and the fluorescence was quenched. Binding parameters, determined from spectrophotometric measurements by Scatchard analysis, indicated a binding constant of 3.56×10^6 M^{-1} for calf thymus DNA at ionic strength 0·01 M. Binding to the GC-rich DNA of *Micrococcus lysodeikticus* was stronger than the binding to calf thymus DNA at ionic strength 0·01 M. The MPTQ increased the viscosity of sonicated rod-like DNA fragments, producing a calculated length of 2·4 Å/bound MPTQ molecule. The binding of MPTQ to DNA increased the melting temperature by about 4°C. This research offers a new intercalation functional group to DNA targetted drug design.

Keywords. DNA; intercalative binding; 8-methoxy pyrimido[4′,5′:4,5]thieno(2,3-*b*) quinoline-4(3H)one; fluorescence quenching.

1. Introduction

Ellipticine, 9-methoxy ellipticine and olivacine occur as alkaloids in the plants of *Aspidosperma ochrosia* and *Tabernaemontana* genera of the family Apocyanaceae.[1] Svoboda and collaborators established that these alkaloids possess experimental antitumour activity exhibiting a broader spectrum than most of the available clinically active agents.[2,3] There is evidence that antitumour activity is due to the intercalation between the base pairs of DNA and interference with normal functioning of the enzyme topoisomerase II which is involved in the breaking and releasing of DNA strands.[4] The intercalative binding of these drugs is due to the presence of planar linearly fused tetracyclic heterocyclic system. This finding has stimulated the research in finding new antitumour drugs containing planar fused ring system. Various fused systems such as thiophene,[5] furan and pyridine analogues of ellipticine[6] and benzothiazoloquinolines[7] have been studied for their intercalative property. Recently Cao and He studied DNA affinity properties of Safranine T which features a planar phenazine ring and have shown that electrostatic binding plays an important role in the intercalation of Safranine T.[8] The

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results of these various binding studies have been useful in designing new and promising anticancer agents for clinical use.[9] Synthesis of pyrimidothienoquinolines have been recently reported in the literature.[10] These compounds belong to a new class of linearly fused tetracyclic heterocyclic systems.

In this paper, we studied the DNA binding properties of 8-methoxy pyrimido [4′,5′:4,5]thieno(2,3-*b*)quinoline-4(3H)one (MPTQ) which features planar fused tetracyclic heterocyclic rings (figure 1c). The planar hydrophobic moiety of MPTQ is expected to facilitate intercalation into the relatively nonpolar interior of the DNA helix. The strong absorption, fluorescence and hydrodynamic characteristics of MPTQ drug provide a sensitive spectroscopic handle to study its interaction with DNA. The change in the intensities of these spectra can be used to decipher the nature and strength of the stacking interaction between the chromophore and the DNA base pairs.

2. Materials and methods

2.1 *Chemicals*

Calf thymus DNA (42% GC), *Micrococcus lysodeikticus* DNA (72% GC) and *Clostridium perfringens* (28% GC) were from Sigma. DNA solutions were prepared by dissolving the solid material, normally at 1–2 mg/ml, in 0·05 M *tris*-buffer with HCl (*p*H 7·0). The DNA concentrations per nucleotide [C(p)] were determined spectrophotometrically at 260 nm using a molar extinction coefficient of 6,600 M^{-1} cm^{-1}.[11]

MPTQ was synthesised by Dr S Y Ambekar, Department of Chemistry, Mysore University, Mysore, India.[10] Drug solutions were prepared freshly by dissolving a few milligrams in *tris*-HCl buffer. The concentrations of drug solutions were determined spectrophotometrically at 343 nm using a molar extinction coefficient of 35,880 M^{-1} cm^{-1}.

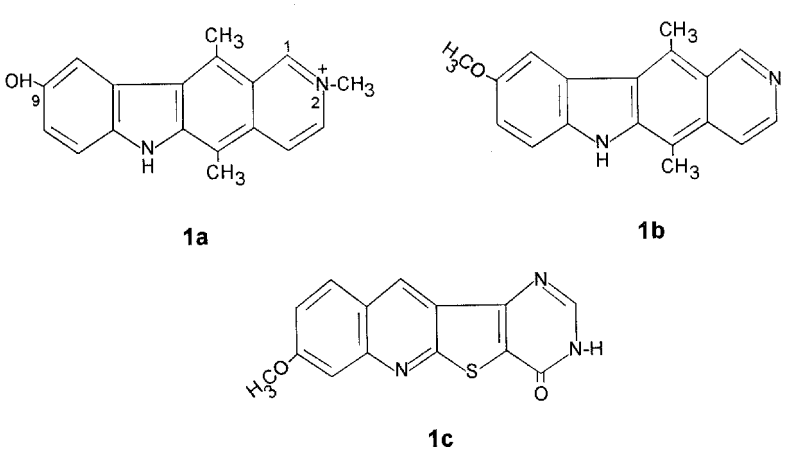

Figure 1. Structures of (a) 2-N-methyl 9-hydroxyellipticinium; (b) 9-methoxy ellipticinium; (c) 8-methoxypyrimido[4′,5′:4,5]thieno(2,3-*b*)quinoline-4(3H)one.

2.2 *Spectral measurements*

2.2a Ultraviolet-visible absorption spectra were determined in a Perkin–Elmer model 554 UV-Vis recording spectrophotometer using quartz cuvettes of 10 mm light-path. The parameters of interaction between MPTQ and calf thymus DNA were determined spectrophotometrically using a Beckman 25 double beam spectrophotometer. Aliquots of a concentrated DNA solution (0·18 mM–1·125 mM) were added to a cuvette filled with MPTQ solution (12–25 mM) and thoroughly mixed. Extreme care was taken to ensure that optical reference solutions were prepared in an identical manner.

The binding data were expressed in the form of a Scatchard plot.[12] The variables of r (moles of ligand bound/mole of nucleotides) and C (the molar concentration of free drug) were calculated from the absorption measurements according to the method of Peacocke and Skerrett.[13] The intrinsic binding constant K_a and maximum number of available binding sites/nucleotide (n) were deduced from Scatchard plot.

2.2b *Fluorescence measurements:* Fluorescence spectra were determined in Shimadzu FL-510 spectrofluorophotometer. The fluorescence titrations with DNA were conducted by keeping the concentration of MPTQ constant, and varying the DNA concentrations. The samples were excited at 343 nm and the fluorescence intensity was monitored at 456 nm. The intrinsic binding constants of MPTQ with calf thymus DNA, *Cl. Perfringens* and *M. lysodeikticus* DNA were determined by fluorescence titrations.

Data from the fluorescence titrations were used to determine the binding constant of MPTQ with DNA, by the modified Scatchard (1) given by McGhee and von Hippel:[14]

$$r/c_f = K_a(1 - nr)[(1 - nr)/[1-(n - 1)r]]^{n-1}, \quad (1)$$

where K_a is the intrinsic binding constant and n represents the number of mononucleotides occluded by the binding of single ligand molecule. Plot of r/c_f vs r was constructed, where r is equal to c_b /DNA. c_b, c_f are concentration of bound and free MPTQ, respectively. The binding data were treated by least square methods and values of K_a and n were obtained.

2.2c *Viscosity measurements:* Viscosity measurements were made according to published procedures[15,16] using a semimicro dilution capillary viscometer (Viscomatic Fica MgW) with a thermostated bath D40S at 20°C. The flow time for water was 71·3 s. For the viscosity experiments samples of calf thymus DNA were sonicated[7] to fragments having an estimated molecular weight of approximately 500,000.[17,18]

2.2d *Thermal denaturing studies:* The DNA melting studies were done by controlling the temperature of the sample cell with a Shimadzu circulating bath while monitoring the absorbance at 260 nm. The temperature of the solution was continuously monitored with a thermo-couple attached to the sample holder.

3. Results and discussion

The UV-Vis absorption spectrum of MPTQ was characterized by two maxima at 328 and 343 nm. The addition of increasingly higher concentrations of DNA led to bathochromic and hypochromic changes, as seen in figure 2, i.e. the interaction of MPTQ with DNA resulted in a strong decrease of the absorption intensity at both peaks, accompanied by a

shift towards higher wavelengths. A 22·6% reduction in absorption was observed at the 343 nm peak maximum in the presence of an excess of DNA at a molar ratio of DNA nucleotide: MPTQ (P/D) equal to 15. Two isosbestic points were observed at 320 and 348 nm, respectively. Hypochromism was suggested to be due to strong interactions between the electronic states of the intercalating chromophore and that of the DNA base pairs.[19] The spectral changes that we observed (hypochromicity, red shift and isosbestic points) were consistent with the intercalation of the chromophore into the stack DNA base pairs.

Scatchard plot[12] prepared for the DNA-MPTQ system to obtain binding parameters for the intercalation process is shown in figure 3. Calculations of r and r/C were performed

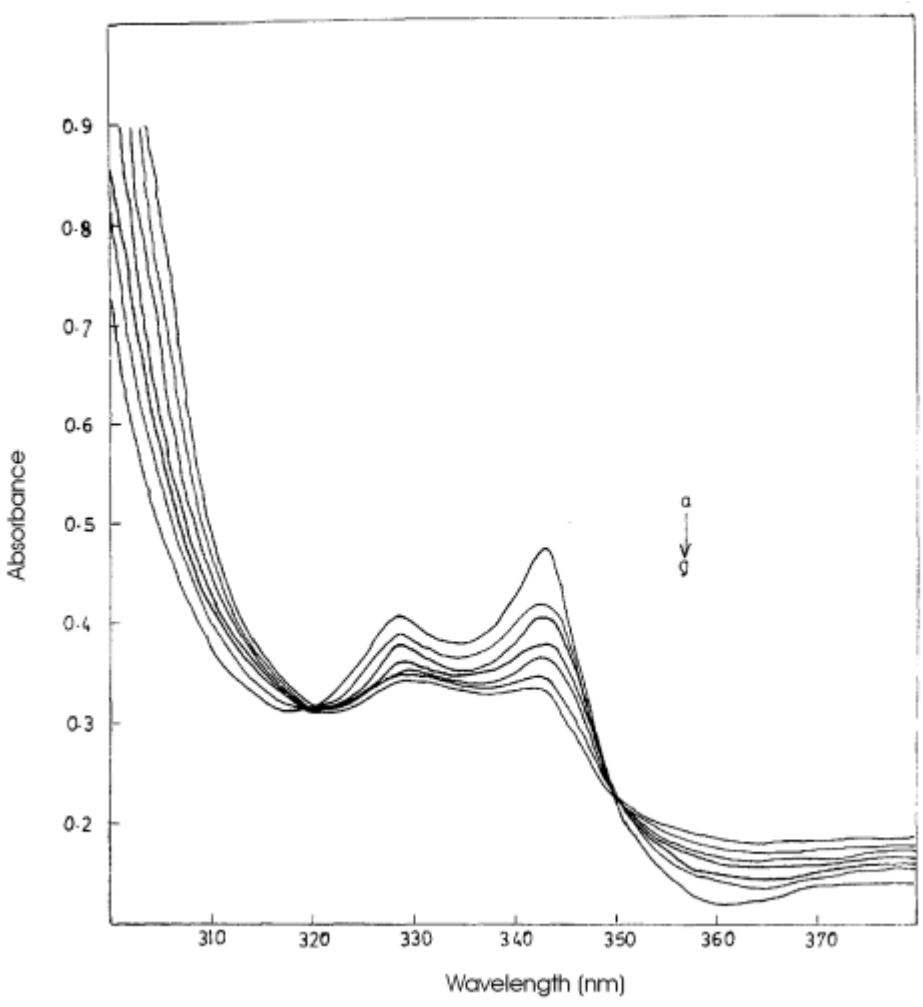

Figure 2. UV-Vis absorption spectra of MPTQ with calf thymus DNA. (a) 12·82 µM MPTQ (b) 0·18 (c) 0·375 (d) 0·562 (e) 0·75 (f) 0·937 (g) 1·125 mM DNA.

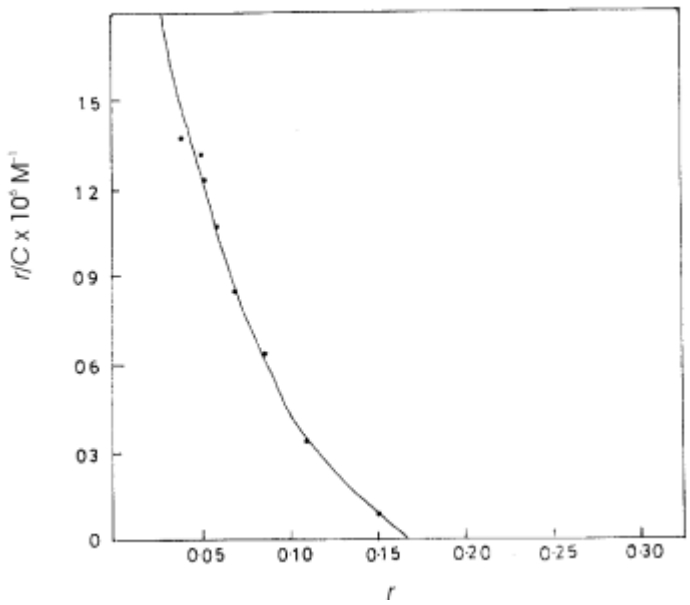

Figure 3. Scatchard plot of binding of MPTQ to calf thymus DNA in phosphate buffer. The total concentration was 25 *m*M.

using the absorbances measured at 343 nm. This wavelength was chosen because of the large absorbance change. The intrinsic binding constant (K_a) $2{\cdot}4 \times 10^6$ M^{-1} and the apparent number of binding sites per nucleotide (n) 1·45 were deduced.

The binding of MPTQ to DNA was further studied by fluorescence spectroscopy. The fluorescence spectrum of MPTQ was observed with a peak of emission centered ~456 nm when exited at 343 nm. In the presence of increasing concentrations of calf thymus DNA, *Cl. Perfringens* DNA and *M. lysodeikticus* DNA, the fluorescence were substantially quenched. The binding did not show saturation of the MPTQ fluorescence spectra even when large amounts of DNA were added, which would have already saturated in the absorption spectra. This indicated that only a fraction of the binding sites changed the electronic state of chromophore of MPTQ. The fluorescence quenching of MPTQ by DNA was fitted to the static quenching model,[20] i.e. a complex was formed between the base pairs and the chromophore of MPTQ. These results indicate that there are two kinds of fluorophores in solution.[20] The changes of fluorescence spectra of MPTQ after adding DNA also indicated that there were strong interactions between the chromophore of MPTQ and the base pairs of DNA. Combined with the changes of absorption spectra, the strong interactions should be considered as the intercalative binding of drug with DNA.

Because it was the static quenching of DNA to MPTQ, the quenching constant was considered as a measure of the formation constant of MPTQ and DNA,[21] i.e. the binding constant of MPTQ with DNA. The quenching constant of MPTQ by DNA was determined from (2):[21]

$$(I_0 - I)^{-1} = I_0^{-1} + K^1_q I_0^{-1} [Q]^{-1}, \tag{2}$$

where I_0, I are the fluorescence intensities in the absence and presence of DNA, [Q] is the DNA concentration and $K_q^{-1} = K_a$ is the binding constant. From the double reciprocal plot of $(I_0 - I)^{-1}$ versus $[Q]^{-1}$ (figure 4a), the binding constant (K_q) of $(3{\cdot}5 \pm 0{\cdot}3) \times 10^6$ M^{-1} was obtained. Since a number of drugs have been reported to exhibit sequence-specificity in binding to DNA,[22] the interaction of MPTQ with *Cl. perfringens* DNA and *M. lysodeikticus* DNA were also studied (figures 4b, c). The binding isotherm yielded K_a of $(1{\cdot}29 \pm 0{\cdot}08) \times 10^5$ M^{-1} and $(4{\cdot}44 \pm 0{\cdot}05) \times 10^6$ M^{-1}, respectively. It can be seen that these para-

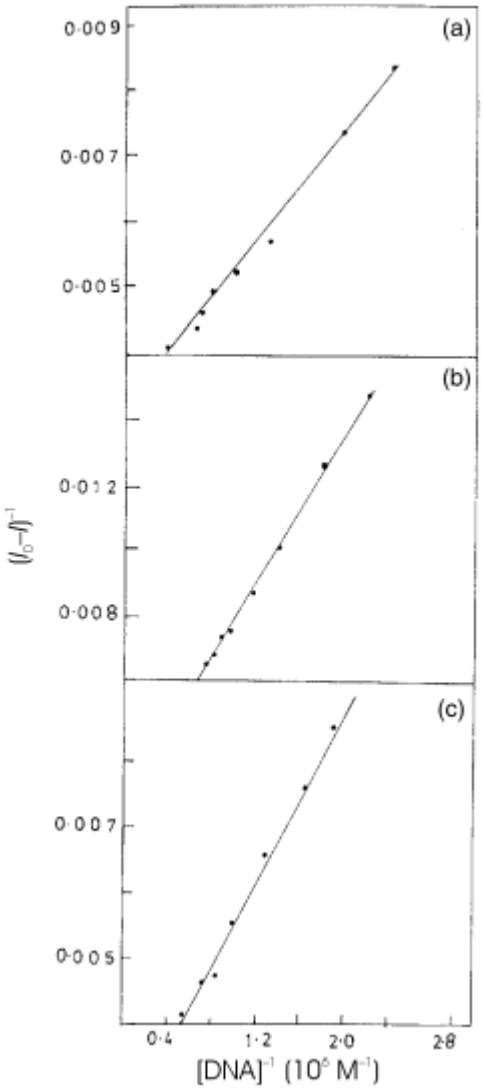

Figure 4. Double-reciprocal plot of fluorescence quenching of MPTQ ($12{\cdot}8 \times 10^{-6}$ M) by (**a**) calf thymus DNA, (**b**) *Cl. Perfringens* DNA and (**c**) *M. lysodeikticus* DNA (l_{ex} = 343 nm, l_{em} = 456 nm).

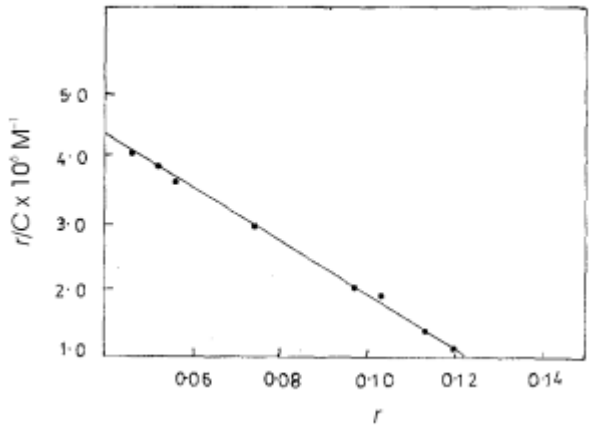

Figure 5. Scatchard plot of the MPTQ fluorescence titration by calf thymus DNA (l_{ex} = 343, l_{em} = 456). The binding data were treated by the least squares method.

meters differ from those measured for calf thymus DNA, revealing that *M. lysodeikticus* DNA, which is characterized by a higher content of $G + C$ (72%), is bound to MPTQ more efficiently, followed by calf thymus DNA (42%) and *Cl. perfringens* DNA (28%).

The fluorescence titration data were also used to estimate the binding constant and the binding size of MPTQ with calf thymus DNA by the modified Scatchard (1). The plot of r/C vs r is shown in figure 5. From the best fit of the data to (1), an intrinsic binding constant (K_a) of $6{\cdot}11 \times 10^6$ M^{-1} and a site size of 6·7 base pairs were obtained.

3.1 *Viscosity measurements*

An increase in viscosity of native DNA is regarded as a diagnostic feature of an intercalation process.[6,23] We have measured the viscosity changes in short, rod like DNA fragments. The relative length increase (L/L_0) of the complex formed between MPTQ and DNA is shown in figure 6. It is evident that binding of MPTQ increased the viscosity of DNA, corresponding to an increase in the contour length of the DNA fragments. The measured slope of the plot $1{\cdot}43 \pm 0{\cdot}05$, falls within 72% of the slope of a theoretical curve for an idealized intercalation process $(1 + 2r)$.[24,25] On this basis we calculate that intercalation of one drug molecule provoked an increase of 2·4 Å in the contour length of DNA. Since the size of these sonicated fragments was significantly greater than the persistence length, the estimated 2·4 Å lengthening is probably best regarded as a lower limit.

3.2 *Melt studies*

Other strong evidence for the intercalative binding of MPTQ into the double helix DNA was obtained from DNA melting studies. The intercalation of small molecules into the double helix is known to increase the DNA melting temperature (T_m), at which the double helix denatures into single stranded DNA, owing to the increased stability of the helix in the presence of an intercalator.[26] The molar extinction coefficient of DNA bases at

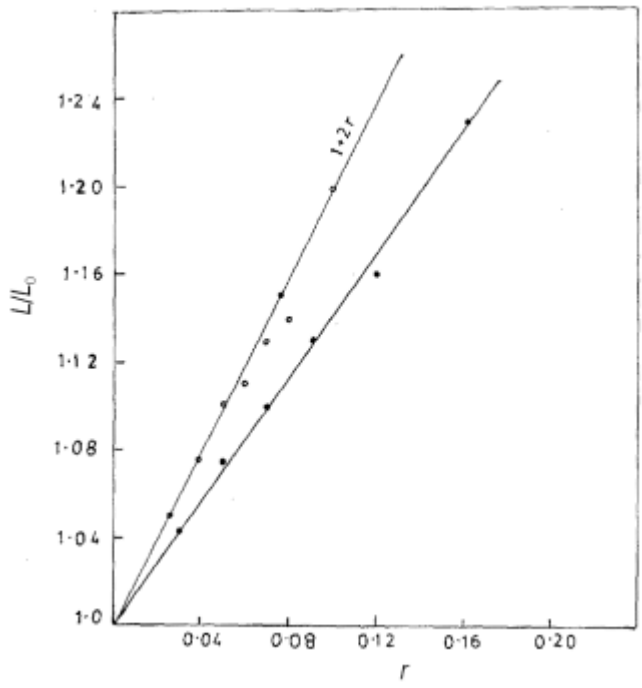

Figure 6. Effect of MPTQ on the relative contour length of sonicated calf thymus DNA fragments. L represents the contour length of fragments with MPTQ bound at the indicated binding ratio r; L_0 is the contour length of control MPTQ free DNA. The line labeled $1 + 2r$ represents the theoretical relation for an idealized intercalation process. The line fitted to the experimental points was computed by the method of least squares and constrained to pass through the point (0, 1). Its slope corresponds to the relation $L/L_0 = 1 + (1{\cdot}43 \pm 0{\cdot}05)r$. Each point represents the mean of the three experiments.

260 nm in the double helical form is much less than the single stranded form; hence, melting of the helix leads to an increase in the absorbance at 260 nm.[27] Thus the helix to coil transition temperature can be determined by monitoring the absorbance of DNA at 260 nm as a function of temperature. The DNA melting studies were carried out with calf thymus DNA in the absence and presence of MPTQ [1 : 5 ratio of MPTQ to DNA-c(P)]. The T_m for calf thymus DNA with no MPTQ present was 80°C and with MPTQ a single transition at 84°C was observed. The advantage of this method is that it is much easier to identify when more than one transition occurs.[28] These various DNA melting experiments strongly supported the intercalation of MPTQ into the double helix DNA.

4. Conclusion

The results presented in this paper provide coherent evidence that association of MPTQ with DNA can be explained by a mechanism of intercalation. In many ways MPTQ behaved as an ideal intercalating agent:[6] on binding to DNA we saw typical batho-

chromic and hypochromic shifts in its absorption spectrum; its fluorescence was quenched; the binding constants determined at ionic strength 0·01 M were of the order of 10^6 M^{-1}, commonly reported for such drugs; and hydrodynamic changes consistent with extension and unwinding of the DNA helix were clearly seen.

Binding to DNA showed some dependence on the nucleotide content and/or sequence, as evidenced by the increase in the association constant for *M. lysodeikticus* DNA which has a higher GC content than calf thymus DNA. These findings would be in accordance with the results previously reported[29,30] for the 2-N-methyl 9-hydroxy ellipticinium and 9-hydroxy ellipticinium, which ostensibly display a preference for GC base pairs. The most solid evidence for intercalation mode of binding comes from viscometric and DNA melting studies. The calculated value for the DNA helix extension of 2·4 Å, although lower than the idealized value expected for the intercalation model of 3·4 Å, lies well within the range of values reported for other intercalating agents, i.e., between 1·8 and 4·5 Å.[22,31] We conclude that the binding of MPTQ to DNA occurs by a mechanism of intercalation, which probably accounts for its reported antitumour activity.

Acknowledgements

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