DNA binding by pixantrone†

Najia Adnan,[a] Damian P. Buck,[a] Benny J. Evison,[b] Suzanne M. Cutts,*[b] Don R. Phillips[b] and J. Grant Collins*[a]

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The binding of the anticancer drug pixantrone (6,9-bis[(2-aminoethyl)amino]benzo[g]-isoquinoline-5,10-dione dimaleate) to the octanucleotide duplexes d(ACGATCGT)$_2$ and the corresponding C-5 methylated cytosine (^{5Me}C) analogue d(A^{5Me}CGAT^{5Me}CGT)$_2$ has been studied by NMR spectroscopy and molecular modelling. The large upfield shifts observed for the resonances from the aromatic protons of pixantrone upon addition to either d(ACGATCGT)$_2$ or the corresponding ^{5Me}C analogue is consistent with the drug binding the octanucleotides by intercalation. The selective reduction in the sequential NOEs between the C_2-G_3 and C_6-G_7 nucleotides in NOESY spectra of either octanucleotide with added pixantrone confirms the intercalative binding mechanism. Strong NOEs from the side-chain ethylene protons of pixantrone to the H5 protons and the 5-CH_3 protons of the C_2 and C_6 residues of d(ACGATCGT)$_2$ and d(A^{5Me}CGAT^{5Me}CGT)$_2$, respectively, indicate that pixantrone predominantly intercalates from the DNA major groove at the 5′-CG and 5′-^{5Me}CG sites. Simple molecular models based on the conclusions from the NMR experiments indicated that the ^{5Me}C groups do not represent a steric barrier to intercalation from the major groove. However, the observation of weak NOEs from the ethylene protons of pixantrone to a variety of minor groove protons from either octanucleotide suggests that the drug can also associate in the minor groove.

Introduction

Anthracycline antibiotics are a major group of effective *anti*-neoplastic drugs, derived from the bacteria *Streptomyces peucetius*, that were originally recognised for their *anti*-bacterial characteristics in 1939.[1,2] Daunorubicin (daunomycin) and Adriamycin (doxorubicin) were isolated in the 1960s and continue to play an important role in the treatment of various forms of cancer.[3,4]

Even though anthracyclines are active against a wide variety of tumours and haematological malignancies, their clinical use is limited by tumour resistance and toxicity towards healthy tissues.[5] Their main side-effect is a life-threatening cardiotoxicity.[6] This dose-limiting toxicity has promoted the search for analogues with comparable therapeutic activity, but with reduced cardiotoxicity. In this regard, many anthracycline-based derivatives have been produced in recent years. One good example is the anthracenedione-compound pixantrone (6,9-bis[(2-aminoethyl)amino]benzo[g]isoquinoline-5,10-dione dimaleate - see Fig. 1), which has emerged as the most promising candidate.[7–10] As distinct from the parent anthracyclines, which are thought to cause heart damage through the production of free-radicals, pixantrone is significantly less toxic.[7] Pre-clinical research has shown that pixantrone is active against a range of cancers, having excellent potential for the treatment of haematological malignancies, particularly lymphomas and leukaemia, with no visible cardiotoxicity.[7,8] Pixantrone has passed phase II clinical trials for the treatment of non-Hodgkin's lymphoma,[9,10] and is currently in phase III studies.[10]

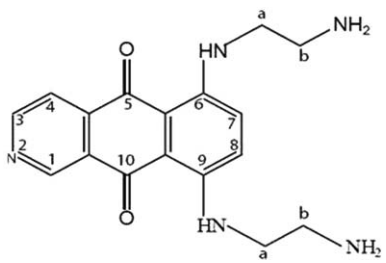

Fig. 1 Structure and atom numbering of pixantrone.

While the precise mechanism of action of pixantrone is yet to be fully understood, its activity is related to its ability to bind DNA.[11–16] Pixantrone is known to inhibit topoisomerase II *via* its ability to intercalate within DNA.[11] Topoisomerase II poisoning stabilises the DNA protein complex and stimulates topoisomerase II mediated DNA cleavage.[12,13] However, this interaction and resultant DNA cleavage does not correlate with drug cytotoxicity.[12,13] This suggests that the observed anticancer activity may be due to another, as yet undefined, mechanism. One such mechanism, involving the formation of drug-DNA covalent adducts at the N-2 amino group of guanine residues, has recently been proposed by Phillips, Cutts and co-workers.[14–16] *In vitro* transcriptional analysis established that formaldehyde-activated pixantrone can form covalent adducts selectively at 5′-CpG and 5′-CpA dinucleotide sites.[15]

More recently, Phillips, Cutts and co-workers have also shown that pixantrone, and the structural analogue mitoxantrone, form 2–5 fold more covalent adducts at 5′-CpG sequences when the cytosine is methylated at the C-5 position.[16,17] 5′-CpG sequences are unevenly distributed across the mammalian genome, with clusters of 5′-CpG sequences being disproportionately found

[a]*School of Physical, Environmental and Mathematical Sciences University College, University of New South Wales, Australian Defence Force Academy, Northcott Drive, Campbell, ACT 2600, Australia. E-mail: g.collins@adfa.edu.au; Fax: +61 (02) 6268 8017*
[b]*Biochemistry Department, La Trobe University, Bundoora, VIC, 3083, Australia. E-mail: s.cutts@latrobe.edu.au*

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in regulatory promoter regions.[18,19] Furthermore, the cytosine residue of CpG sequences is often methylated, with methylation typically occurring after DNA replication.[20] It has also been previously reported that C-5 methylation of cytosine can affect DNA recognition by small molecules, including the anthracycline drug daunomycin.[21]

In order to gain a better understanding of the ability of pixantrone to form covalent bonds with DNA, it is important to develop a detailed picture of the initial reversible intercalative binding at both 5′-CpG and the corresponding methylated (5′-^{5Me}CpG) sites. The DNA site where pixantrone covalently binds will be governed by the initial reversible intercalative binding; the pre-covalent association of the drug with the DNA will affect the rate and site of adduct formation. An increased local concentration of pixantrone will increase the probability of a covalent reaction at these sites. Based upon the structural similarity with mitoxantrone, it has been assumed that pixantrone will also intercalate from the DNA major groove.[16] As the N-2 amino group of guanine is located in the minor groove, it has been proposed that the C-5 methyl group (located in the major groove) sterically hinders intercalation from the major groove, thereby increasing binding from the minor groove.[16] In this study we have examined the intercalative binding of pixantrone with two oligonucleotides, d(ACGATCGT)$_2$ and the corresponding ^{5Me}C analogue d(A^{5Me}CGAT^{5Me}CGT)$_2$, by NMR spectroscopy and molecular modelling.

Experimental

Materials

Pixantrone was kindly supplied by Cell Therapeutics Europe (CTE), Bresso, Italy. The oligonucleotides d(ACGATCGT)$_2$ and d(A^{5Me}CGAT^{5Me}CGT)$_2$ were obtained from GeneWorks, South Australia. D_2O (99.9% and 99.96% D) was obtained from Aldrich and CM-Sephadex was obtained from Sigma-Aldrich.

Sample preparation for NMR analysis

Both oligonucleotides were initially purified using a reverse-phase Waters C18 Sep-Pak cartridge. The cartridge was activated with methanol (10 mL) and water (2 × 10 mL) prior to an aqueous oligonucleotide solution being loaded. The Sep-Pak was then washed with water (2 × 3 mL) and the oligonucleotide subsequently eluted with 50% v/v acetonitrile–water. Fractions of approximately 2 mL each were collected and those fractions containing the oligonucleotide (as determined spectrophotometrically) were freeze-dried.

The oligonucleotides were then converted from a triethylammonium salt to a sodium salt using a CM-Sephadex column that had been equilibrated with 1 M NaCl. After elution from the column, 650 μL of phosphate buffer (pH 7.0) containing 20 mM NaCl and 1 mM Na_2H_2EDTA was added and the solution freeze-dried. The oligonucleotide was freeze-dried once more from D_2O, and finally dissolved in 650 μL of 99.96% D_2O prior to use. The concentration of the oligonucleotide (1–1.25 mM) was estimated from the A_{260} absorbance, using an extinction coefficient of 6,600 $M^{-1}cm^{-1}$ per nucleotide. Stock solutions of pixantrone were prepared in 99.96% D_2O. Additions of the pixantrone stock solutions were made directly to the NMR tube containing the oligonucleotide solutions. The precise pixantrone to oligonucleotide duplex ratio was determined from the relative integrals of the ^{1}H NMR signals.

NMR spectroscopy

NMR experiments were recorded at 400 MHz for the ^{1}H nuclei using a Varian Unityplus-400 spectrometer. One-dimensional spectra recorded in 90% H_2O/10% D_2O solution were collected using the WATERGATE solvent suppression technique of Piotto *et al.*[22] Phase-sensitive NOESY spectra were acquired using the method of States *et al.*,[23] with 2048 data points in t_2 for 256 t_1 values with a pulse repetition delay of 1.7 s for mixing times ranging of 100 and 300 ms. DQFCOSY spectra were similarly recorded using 2048 data points in t_2 for 256 t_1 values and a pulse repetition delay of 1.7 s.

Molecular modelling

Molecular modelling of the oligonucleotide was carried out using HyperChem 7.5 software.[24] The octanucleotides were generated from the nucleic acid database and energy restraints were added to preserve the hydrogen bonds expected of duplex DNA during optimisation procedures, which were conducted *in vacuo* using the Amber 99 force field and a Polak–Ribiere conjugant-gradient algorithm with a 5×10^{-5} kcal/(Å mol) convergence criteria.

The point charges of pixantrone were determined from semi-empirical calculations using the AM1 program. The pixantrone complex point charges resulting from different AM1 calculations appeared to vary only slightly with the conformation. Therefore the point charges resulting from an optimised conformation were used to approximate the charges for the subsequent molecular modelling AMBER optimizations with the DNA octanucleotide. The complex was manually inserted into the free DNA model to reflect the observed intermolecular NOEs formed with the DNA protons. Energy restraints were applied to preserve the observed intermolecular NOEs and base pair hydrogen bonds during the subsequent interactive optimization using AMBER and then removed to confirm the resulting structure was an energy minimum. The added 5-methyl group atoms were assigned appropriate amber atom types and charge approximations.[25]

Results

Assignment of the ^{1}H NMR resonances of pixantrone and the octanucleotides

The aromatic peaks of pixantrone were assigned using standard one-dimensional and COSY NMR experiments. The H3 proton was assigned from the observed long range coupling to the H1 in a COSY experiment. The Ha methylene protons were assigned on the basis of the larger difference in the chemical shift of the non-equivalent Ha geminal protons, compared to the non-equivalent Hb protons. The ^{1}H NMR resonances of the free and bound octanucleotides were assigned by standard techniques.[26–28] In agreement with many other NMR studies of oligonucleotides,[26–29] analysis of short mixing-time NOESY spectra and DQFCOSY spectra indicated that both octanucleotides adopted a B-type duplex conformation in the aqueous buffer. However, it was noted that the sequential NOEs between guanine H8 protons and the H2′/H2″ protons of the 5′-cytosine residues were relatively weaker

Table 1 ^{1}H NMR shifts of pixantrone and pixantrone bound to d(ACGATCGT)$_2$ and the ^{5Me}C analogue d(A^{5Me}CGAT^{5Me}CGT)$_2$, at drug to DNA duplex ratios (R) = 2 at 25 °C, in D_2O (pixantrone) and pH 7 phosphate buffer (octanucleotide bound pixantrone)

Pixantrone	H1	H3	H4	H7,8	Ha	Hb
Free	9.09	7.83	8.74	7.20	3.74	3.26
d(ACGATCGT)$_2$ Bound	8.62 (–0.47)	7.36 (–0.47)	8.35 (–0.39)	6.76 (–0.44)	3.51 (–0.23)	3.26 (0.00)
d(A^{5Me}CGAT^{5Me}CGT)$_2$ Bound	8.61 (–0.48)	7.28 (–0.55)	8.26 (–0.48)	6.63 (–0.57)	3.47 (–0.27)	3.27 (0.01)

for the ^{5Me}C-octanucleotide compared to the control duplex. In addition, the NOEs between the H8 and H3′ protons of G_3, A_4 and G_7 were relatively stronger for the ^{5Me}C-octanucleotide compared to the control duplex. Consequently, while the ^{5Me}C-octanucleotide does adopt a B-type DNA structure, it is clear that the methylation of the cytosine residues does affect the duplex structure to some degree. The assignment of the H3′ and H4′ resonances of the pixantrone-bound octanucleotides could not be determined because of the significant broadening and overlap of the resonances.

Pixantrone binding to d(ACGATCGT)$_2$

Titration of the control duplex d(ACGATCGT)$_2$ with pixantrone induced significant broadening of the resonances from the drug and oligonucleotide as shown in Fig. 2, indicating that pixantrone binds with intermediate exchange kinetics (on the NMR time scale). Intermediate exchange kinetics is consistent with relatively strong binding by the drug. While the base H8/H6/H2 resonances of the control duplex only exhibited small changes in chemical shift upon pixantrone binding, the resonances from the aromatic protons from the drug shifted significantly upfield (0.39 to 0.47 ppm, see Table 1), consistent with pixantrone binding by intercalation. Alternatively, the resonances from the aliphatic ethylene side-chain protons shifted upfield to a lesser extent (0.23 and 0.00 ppm) upon addition to the oligonucleotide, consistent with being positioned within a duplex groove.

A pixantrone to d(ACGATCGT)$_2$ binding constant could not be determined by following the changes in the chemical shift of resonances from the octanucleotide as a function of added pixantrone, due to the relatively small shifts of the generally broad and in some cases overlapping resonances. However, a binding constant can be estimated from the pixantrone resonances. The association constant for the binding of pixantrone to an oligonucleotide can be expressed as:

$$K_{ass} = \frac{[\text{P-DNA}]}{[\text{P}][\text{DNA}]}$$

where [P-DNA] is the concentration of the pixantrone-bound oligonucleotide and [DNA] and [P] are the concentrations of the free oligonucleotide and pixantrone respectively. [P-DNA], [P] and [DNA] in the above equation can be estimated from the following equation and the initial concentrations of the pixantrone and octanucleotide.[30]

$$\delta_{obs} = \chi_f\delta_F + \chi_b\delta_b$$

δ_{obs} is the observed chemical shift of the pixantrone resonances, χ_f and χ_b are the mole fractions of free and bound pixantrone and δ_F and δ_b are the chemical shifts of the resonances of the free and bound pixantrone. The value for δ_b is taken from the shift at the lowest ratio of pixantrone to oligonucleotide that the resonance can be assigned, while the value for δ_F is taken from the spectrum of the free pixantrone. On the basis of the changes in chemical shift of the pixantrone H7/8 resonance in the titration, it was estimated that at least 96% of the drug is bound at a pixantrone to oligonucleotide duplex ratio (R) of 2. Given the millimolar concentration used in the experiment and assuming that binding at each symmetric 5′-CpG site is independent (*i.e.* a 1 : 1 binding model), a binding constant of at least 2×10^5 M^{-1} can be calculated.

The changes in chemical shift for the resonances from the octanucleotide at $R = 0.6$ are given in the ESI.† The determination of a preferred binding site based upon the observation of selective changes in chemical shift of the resonances from the octanucleotide should be more clearly detected at low R. However, no selective shifts were observed.

NOESY spectra were acquired at drug to oligonucleotide duplex ratios of 0.6, 2 and 2.3. The NOESY spectra, as shown in Fig. 3, show that the strongest intermolecular NOEs are from the pixantrone Ha and Hb methylene protons to the octanucleotide H5 protons of C_2 and C_6, – indicating major groove intercalation at the expected 5′-CpG sites. At $R = 0.6$, NOEs are only observed to C_2/G_3 and C_6/G_7 major groove H8/H6/H5 protons (see Table 2). At $R = 2$, very weak NOEs are also observed to the A_4H1′ from the side-chain pixantrone protons. As it would not be possible to have the side-chain protons within 5 Å of the A_4H1′ when pixantrone is intercalated from the major groove, the observation of an NOE to the A_4H1′ suggests some minor groove binding. At R = 2.3, clear intermolecular NOEs are observed from the pixantrone ethylene protons to the A_4H1′, and some other H1′

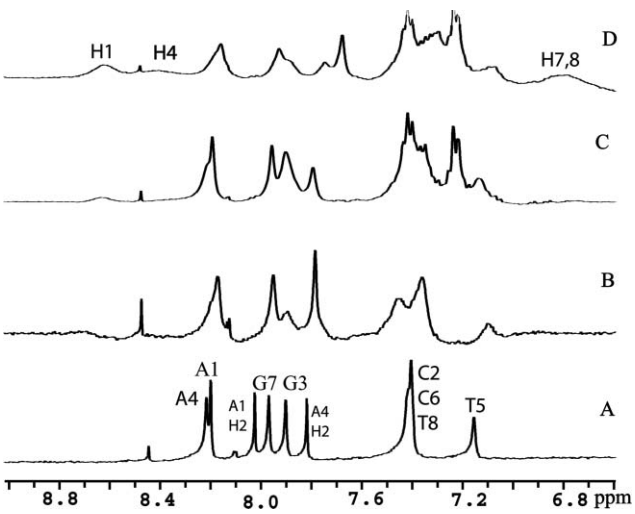

Fig. 2 Titration of d(ACGATCGT)$_2$ with pixantrone. (A) The free octanucleotide, (B) with added pixantrone at a drug to DNA duplex ratio (R) of 0.4, (C) with added drug at R = 0.6 and (D) with added drug at $R = 2$.

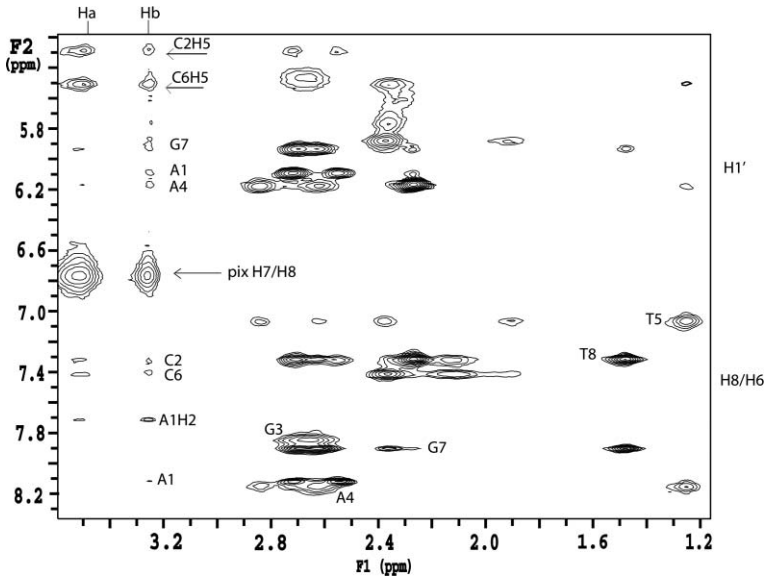

Fig. 3 An expansion of a NOESY spectrum of d(ACGATCGT)$_2$ with added pixantrone, at a drug to DNA duplex ratio of 2.3 at 25 °C. The expansion shows the NOE connectivities from the aromatic base and sugar H1′ protons (5.20 to 8.3 ppm) to the sugar H2′/H2″ and T-methyl protons (1.20 to 3.50 ppm). Intermolecular NOEs between the pixantrone Ha and Hb protons with various octanucleotide protons are indicated.

Table 2 NOE cross-peaks observed between pixantrone and d(ACGATCGT)$_2$, at pixantrone to duplex ratios of 0.6 and 2.3 in phosphate buffer (pH 7) observed at 25 °C and/or 35 °C

Pixantrone	DNA proton at $R = 0.6$	DNA proton at $R = 2.3$
Hb	C_2H5, C_6H5	C_2H5, C_6H5 *Weak NOEs* C_2H6, C_6H6, A_1H2, A_1H8, A_1H1', A_4H1', G_7H1'
Ha	C_2H5, C_6H5 *Weak NOEs* C_2H6, G_7H8	C_2H5, C_6H5 *Weak NOEs* C_2H6, C_6H6, A_1H2, G_7H1', A_4H1'

protons. Also of note, in the base H8/H6 to the sugar H2′/H2″ region, the G_7-C_6 sequential NOEs (and G_3-C_2, although these are weaker) are still observed even at $R = 2.3$. This signifies that even though there is an excess of pixantrone, not all of the 5′-CpG sites are bound. Intercalation increases the separation of the bases at the binding site from 3.4 to 6.8 Å; that is, beyond the 5 Å distance that NOEs would be detected. Consequently, although major groove intercalation at 5′-CpG sites is dominant, some proportion of the drug binds at other sites, and given that NOEs were also observed to minor groove protons, the additional binding sites include minor groove association. It is not possible to accurately determine the proportion of the drug that binds in the minor groove from the NOE cross-peak volumes, as the integral of the cross-peaks is a function of both inter-proton distance and concentration. However, an approximate estimate of 15% (with a corresponding binding constant of 10^4–10^5 M^{-1}) is consistent with the relative cross-peak volumes and the loss of G_7-C_6 sequential NOEs.

Pixantrone binding to d(A^{5Me}CGAT^{5Me}CGT)$_2$. Titration of the ^{5Me}C-duplex with pixantrone also induced significant broadening of the resonances from the drug and oligonucleotide as shown in Fig. 4, indicating that pixantrone binds with intermediate exchange kinetics (on the NMR time scale). Similarly to that observed with the control duplex, the base H8/H6 resonances of the ^{5Me}C-duplex only exhibited small changes in chemical shift upon pixantrone binding (see ESI†), while the resonances from the pixantrone aromatic protons shifted significantly upfield (0.48 to 0.66 ppm, see Table 1), consistent with pixantrone binding by intercalation. The resonances from the pixantrone side-chain ethylene protons only shifted 0.27 and 0.01 ppm upon binding, consistent with the side-chains being positioned within the oligonucleotide groove. On the basis of the changes in chemical shift of the pixantrone H7/8 resonance in the titration, it was estimated that 89% of the drug is bound at $R = 2$. Given

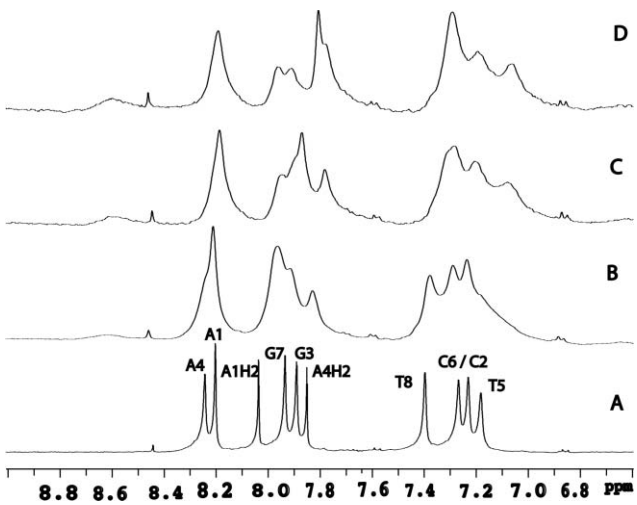

Fig. 4 Titration of d(A^{5Me}CGAT^{5Me}CGT)$_2$ with pixantrone. (A) The free octanucleotide, (B) with added drug at R = 0.5, (C) with added drug at R = 1 and (D) with added drug at R = 2.

the millimolar concentration used in the experiment, a binding constant of about 3×10^4 M^{-1} can be calculated.

NOESY spectra, at a range of pixantrone to ^{5Me}C-duplex ratios (R) and temperatures, were recorded to obtain a more detailed picture of the binding. In the NOESY spectrum at R = 2, strong NOE cross-peaks were observed from each oligonucleotide base H8/H6 to the H2′ and H2″ protons of their own and 5′-sugar residues, except from G_7 to C_6 and G_3 to C_2 as shown in Fig. 5 and 6. The observation of NOEs from each base H8/H6 to the H2′ and H2″ protons of their own and 5′-sugar residues indicates the ^{5Me}C-duplex maintains a B-type duplex upon addition of pixantrone. Also consistent with this conclusion was the observation of an imino resonance from all base pairs, except the terminal A.T, in spectra recorded in 90% H_2O/10% D_2O. The very weak sequential NOEs from the G_7 to C_6 and G_3 to C_2 indicates that pixantrone intercalates between these bases.

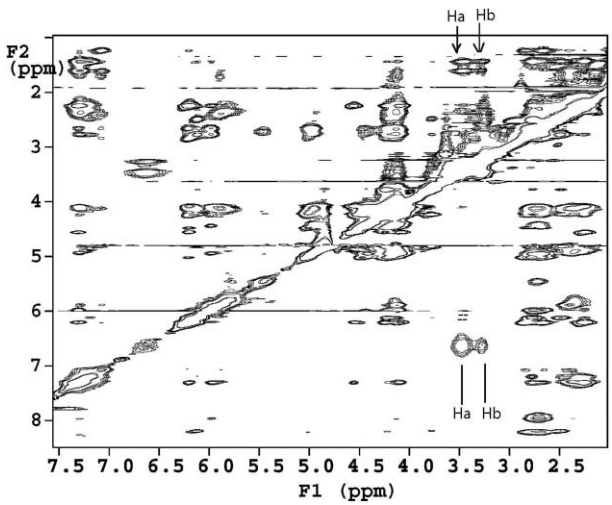

Fig. 5 NOESY spectrum of d(A^{5Me}CGAT^{5Me}CGT)$_2$ with added pixantrone, at a drug to DNA duplex ratio of 2 at 25 °C. The spectrum shows the NOE cross-peaks between the pixantrone Ha/Hb protons (3.47 and 3.27 ppm) and the pixantrone H7/H8 protons (6.63 ppm) and the C_2 and C_6 methyl protons (1.43 and 1.59 ppm).

A range of intermolecular NOEs were observed in NOESY spectra of pixantrone bound to the ^{5Me}C-duplex at $R = 2$, as summarised in Table 3. The strongest intermolecular NOEs are between the drug side-chain ethylene protons and the C_2–CH_3 and C_6–CH_3, with a number of other weaker NOEs being observed to a range of major and minor groove ^{5Me}C-duplex protons. The NOE data indicates that pixantrone predominantly intercalates at the symmetric 5′-^{5Me}CpG sites from the major groove. In general the outer methylene protons formed weaker NOEs than the inner methylene protons. The observation of NOEs between the drug side-chain ethylene protons and a range of minor groove AH2 and H1′ protons (see Fig. 6) suggests that a small proportion (approximately 10%, binding constant = 10^3–10^4 M^{-1}) of the pixantrone binds from the minor groove.

Modelling

As only exchange-averaged resonances were observed upon addition of pixantrone to either of the two octanucleotide duplexes, the NMR data did not allow the determination of a quantitative

Table 3 NOE cross-peaks observed between pixantrone and d(A^{5Me}CGAT^{5Me}CGT)$_2$ at a pixatrone to duplex ratio of 2, in 10 mM phosphate buffer (pH 7) at 25 °C

Pixantrone	DNA Atom
Hb	C_2CH_3, C_6CH_3
	Weak NOEs
	C_6H6, (A_1H2 – very weak)
Ha	C_2CH_3, C_6CH_3
	Weak NOEs
	C_6H6, A_4H1′, G_7H1′, A_1H1′,
	C_2H1′, (A_1H2, C_2H6 – very weak)

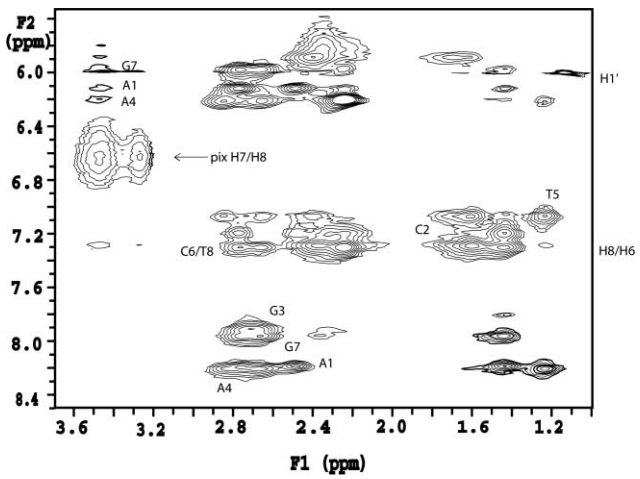

Fig. 6 NOESY spectrum of d(A^{5Me}CGAT^{5Me}CGT)$_2$ with added pixantrone, at a drug to DNA duplex ratio of 2 and 25 °C. The spectrum shows the NOE cross-peaks between the pixantrone Ha/Hb protons (3.47 and 3.27 ppm) and the pixantrone H7/H8 protons (6.63 ppm) and various octanucleotide H1′ minor groove protons (5.7 to 6.2 ppm).

structure. However, simple HyperChem binding models were constructed to examine the proposed pixantrone binding at the 5′-CpG site of d(ACGATCGT)$_2$ and the corresponding ^{5Me}C analogue d(A^{5Me}CGAT^{5Me}CGT)$_2$.

Fig. 7 (and a figure in the ESI†) show representative models of pixantrone binding from the major groove at the 5′-CpG and 5′-^{5Me}CpG sites in d(ACGATCGT)$_2$ and d(A^{5Me}CGAT^{5Me}CGT)$_2$, respectively, that are consistent with the observed intermolecular NOEs. The ethylenediamine side-chains optimized with the primary amines interacting with the two sugar-phosphate chains. Steric hindrance from the additional ^{5Me}C groups is not apparent from the intercalated models. Indeed, van der Waals interactions between the pixantrone ethylene carbons and the additional methyl groups appear possible, which could potentially act to stabilize the intercalated conformation. However, any steric hindrance to intercalation by the ^{5Me}C groups would take place before intercalation, possibly resulting in a slower kinetic intercalative association.

Models of pixantrone intercalating from the minor groove were also examined to account for the observed intermolecular NOEs to minor groove protons. It was not possible for pixantrone to intercalate at the 5′-CpG or 5′-^{5Me}CpG sites in d(ACGATCGT)$_2$ or d(A^{5Me}CGAT^{5Me}CGT)$_2$ with both ethylenediamines in the minor groove without bringing the ethylene protons close to the G_3H1′

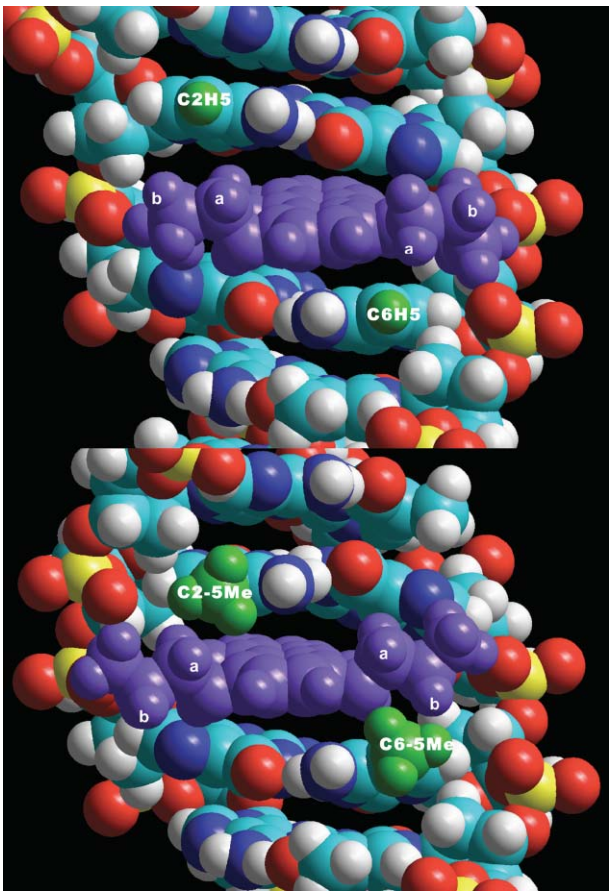

Fig. 7 Energy minimised HyperChem models of pixantrone intercalated into one symmetric 5′-CG or 5′-^{5Me}CG site in d(ACGATCGT)$_2$ (top) and d(A^{5Me}CGAT^{5Me}CGT)$_2$ (bottom).

proton. No intermolecular NOEs were observed to the G_3H1' proton. However, the observation of intermolecular NOEs to both the A_4 and A_1 minor groove protons with both duplexes suggests that several minor groove-binding modes exist.

It was noted that the optimal DNA conformation required to accommodate both ethylenediamine side-chains in the minor groove differed markedly from that observed for duplexes with either ethidium or parent anthracylines (*e.g.* Adriamycin and daunomycin) intercalated from the minor groove (data not shown). These DNA duplexes exhibit some under-winding at the intercalation site, as well as increased base-rise between the base pairs at the intercalation site. The relatively larger side-chains of the parent anthracyclines sit in the minor groove, but the duplex still resembles that where the intercalating drug is much smaller like ethidium.

Unlike the parent anthracyclines, the side-chains of pixantrone are substituted directly onto the intercalating chromophore. If the ethylenediamine side-chains were substituted onto the chromophore at the same positions as the alicyclic substituent of Adriamycin or daunomycin, there would still be enough room for minor groove intercalation without altering the duplex conformation beyond the normal increase in base distance and reduction in helix twist. Instead, the ethylenediamine groups are substituted onto opposite sides of the pixantrone chromophore. This increases the distance between the two substituents and the subsequent side chains. It also confers the rigidity of sp^2 bond angles on the first members of the chains, the amine group bound to the aromatic ring system. Although the remainder of the chains have rotational flexibility, the distance and rigidity between the two anilino groups means that the extra volume of the other side-chain members induces a rearrangement of the minor groove around them. As a result, model duplexes were overwound as the minor groove followed the more planar geometry of the pixantrone side-chains. There was little indication from the NMR spectra of any such change in the duplex conformation upon addition of pixantrone to the ^{5Me}C-duplex.

This duplex rearrangement would not be required for parent anthracyclines intercalating from the minor groove. The substituents equivalent to pixantrone side-chains terminate in hydroxyl groups. Parent anthracycline side chains also project from the chromophore along sp^2 hybrid orbitals, but they project from adjacent instead of opposite positions, and are consequently closer to each other. Also, the ring position of parent side-chain directs them almost directly towards the groove instead of in opposite directions towards the phosphate backbones. In the groove, the bulky alkyl groups are directed away from the chromophore plane by the subsequent sp^3 hybrid orbitals. Thus, parent anthracyclines are better equipped to occupy the minor groove on intercalation than are pixantrone or mitoxantrone.

Models of intercalated pixantrone with one ethylenediamine side-chain in each of the major and minor DNA grooves were also examined. Such an intercalation orientation may arise if, after the chromophore has intercalated, one of the pixantrone side-chains, were to transiently intercalate and emerge at the other groove. However, these models reflected the observed NOEs poorly, as ethylene protons still came within short distances of minor groove protons at the intercalation site. It therefore appears that the ethylenediamine substituents are sufficient to stabilize the intercalation orientation to that of the major groove. Accordingly, a more general low population minor groove binding model without intercalation better accounts for the observed weak NOEs to minor groove protons, such as with A_4H1'.

Discussion

Although the anticancer drug candidate pixantrone is a topoisomerase II poison, this mechanism of action does not appear to fully account for its anticancer activity.[12,13] Since pixantrone-DNA adducts have been demonstrated to have a clear preference for ^{Me}CpG sequences,[16] modulation of methylation status and subsequent epigenetic gene regulation (as reported previously for mitoxantrone)[31] is a possible additional mechanism of action. In this study we investigated whether the non-covalent binding mode of pixantrone was influenced by CpG methylation.

Consistent with previous research,[11] the results of this study demonstrate that pixantrone binds the octanucleotide d(ACGATCGT)$_2$ by intercalation. The large upfield shifts observed for the resonances from the pixantrone aromatic protons, coupled with substantial loss of sequential NOE intensity in NOESY spectra, provide strong evidence for intercalation. Furthermore, the loss of sequential NOE intensity from the G_7 to C_6 and G_3 to C_2 nucleotides and the observation of intermolecular NOEs between pixantrone and the H5 protons of the symmetrically related C_2 and C_6 residues are consistent

with intercalation at the expected 5′-CpG site in the control octanucleotide, d(ACGATCGT)$_2$. However, weak G_7 to C_6 and G_3 to C_2 NOEs were observed even when a slight excess of pixantrone was added, suggesting that other minor binding sites also occur. The observation of weak NOEs to the A_4H1' is consistent with this proposal. Based on the observation of very strong NOEs from the side-chain ethylene protons of pixantrone to the H5 protons C_2 and C_6 residues, it is concluded that pixantrone, like its parent compound mitoxantrone, predominantly intercalates from the DNA major groove.

The NMR results indicate that methylation of the cytosine residues of d(ACGATCGT)$_2$ does not change, to a first approximation, the binding mode of pixantrone. The observed changes in chemical shift of pixantrone aromatic resonances upon binding the methylated octanucleotide, coupled with strong intermolecular NOEs to the C_2 and C_6 methyl protons, again indicate binding through intercalation from the major groove at the 5′-^{5Me}CpG sites. The slightly weaker sequential NOEs between G_7 and C_6 and G_3 to C_2 nucleotides, as compared with the control duplex, suggest that the preference for the 5′-CpG sites may be slightly stronger when the cytosines are methylated. However, the estimated binding affinity of pixantrone was slightly lower for the methylated octanucleotide, tentatively suggesting, weaker but more selective binding.

The structure and electronic properties of cytosine and its base paired guanine are affected by C-5 methylation.[32,33] Yet little or no alteration in the site preference of pixantrone for 5′-CpG sites was observed. The sequence-specific determinants for 5′-CpG preference therefore probably do not include C-5 pyrimidine methylation *per se* to any detectable degree. If it did, the intermolecular NOEs to C_2 and/or C_6 H5/CH_3 would become weaker while NOEs to other octanucleotide protons concomitantly stronger. Site selection must rely on other factors, like the effect on the guanine N-2 amino group, or on the extra thermodynamic stability conferred upon C-G base pairs by the third hydrogen bond.

Mitoxantrone, a structural analogue of pixantrone where the ethylenediamine side chains contain an additional 2-hydroxyethyl group, should present similar steric determinants for intercalation orientation. However, formaldehyde-activated mitoxantrone adducts form with the guanine N-2 and the adduct formation is increased when cytosine is C-5 methylated.[17,34] These observations, and subsequent molecular modelling, suggested that minor groove intercalation became more favourable upon C-5 methylation. Although mitoxantrone preferentially intercalates from the major groove,[34–36] the presence of 5-methyl groups on the cytosines was thought to place an impediment to major groove intercalation and hence, make minor groove intercalation relatively more favourable, resulting in a 2–3 fold increase in adduct formation.[34] A similar increase of adducts at methylated CpG sequences was observed using pixantrone, and this was also thought to be due to favourable minor groove intercalation at methylated sites.[16] However, the electronic effect of the methylation of cytosine can also make the complementary guanine more reactive at the N-2 position,[32] and other guanine positions.[37] The results of our present study suggest some non-intercalative minor groove binding could be a more likely explanation for the guanine N-2 adducts, with the electron-donating effect of the cytosine 5-methylation causing the increase in guanine N-2 adduct formation.

The NMR interpretation that a small population of non-intercalative minor groove binding conformations account for adduct formation is consistent with the most effective predictor of adduct formation - the energy calculations for the formation of hydrogen bonds with groups in the minor groove.[34] It is possible that intercalation and minor groove binding are cooperative processes that combine to form adducts at the 5′-CpG site, with selective intercalation from the major groove causing a localized increase in base pair rise and reduced helix wind, resulting in better minor groove access and guanine N-2 adduct formation.

In conclusion, the results of this study demonstrate that pixantrone predominantly intercalates from the DNA major groove. However, evidence of minor groove association was also observed. The minor groove association provides a basis for the formation of covalent adducts at the guanine N-2 sites that are proposed to contribute to the anticancer activity of pixantrone. Furthermore, it is possible that by decreasing the ability of a pixantrone derivative to intercalate, thereby increasing minor groove association, could increase the amount of covalent adducts formed, and thus, the anticancer activity.

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