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Abstract

The ability of a bis-amino mitoxantrone anticancer drug (named WEHI-150) to form covalent adducts with DNA, after activation by formaldehyde, has been studied by electrospray ionisation mass spectrometry and HPLC. Mass spectrometry results showed that WEHI-150 could form covalent adducts with d(ACGCGCGT)₂ that contained one, two or three covalent links to the octanucleotide, whereas the control drugs (daunorubicin and the anthracenediones mitoxantrone and pixantrone) only formed adducts with one covalent link to the octanucleotide. HPLC was used to examine the extent of covalent bond formation of WEHI-150 with d(CGCGCG)₂ and d(CG5MeCGCG)₂. Incubation of WEHI-150 with d(CG5MeCGCG)₂ in the presence of formaldehyde resulted in the formation of significantly greater amounts of covalent adducts than was observed with d(CGCGCG)₂. In order to understand the observed increase of covalent adducts with d(CG5MeCGCG)₂, an NMR study of the reversible interaction of WEHI-150 at both CpG and 5MeCpG sites was undertaken. Intermolecular NOEs were observed in the NOESY spectra of d(ACGGCCGT)₂ with added WEHI-150 that indicated that the drug selectively intercalated at the CpG sites and from the major groove. In particular, NOEs were observed from the WEHI-150 H_{2,3} protons to the H1' protons of G3 and G7 and from the H_{6,7} protons to the H5 protons of C2 and C6. By contrast, intermolecular NOEs were observed between the WEHI-150 H_{2,3} protons to the H2'' proton of the 5MeC3 in d(CG5MeCGCG)₂, and between the drug aliphatic protons and the H1' proton of G4. This demonstrated that WEHI-150 preferentially intercalates at 5MeCpG sites, compared to CpG sequences, and predominantly via the minor groove at the 5MeCpG site. The results of this study demonstrate that WEHI-150 is likely to form interstrand DNA cross-links, upon activation by formaldehyde, and consequently exhibit greater cytotoxicity than other current anthracenedione drugs.

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Reversible and formaldehyde-mediated covalent binding of a bis-amino mitoxantrone analogue to DNA

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Graphical Abstract

The ability of the anthracenedione anticancer drug WEHI-150 to form covalent adducts with DNA, after activation by formaldehyde, has been studied by mass spectrometry, HPLC and NMR spectroscopy.

Abstract

The ability of a bis-amino mitoxantrone anticancer drug (named WEHI-150) to form covalent adducts with DNA, after activation by formaldehyde, has been studied by electrospray ionisation mass spectrometry and HPLC. Mass spectrometry results showed that WEHI-150 could form covalent adducts with d(ACGCGCGT)₂ that contained one, two or three covalent links to the octanucleotide, whereas the control drugs (daunorubicin and the anthracenediones mitoxantrone and pixantrone) only formed adducts with one covalent link to the octanucleotide. HPLC was used to examine the extent of covalent bond formation of WEHI-150 with d(CGCGCG)₂ and d(CG^{5Me}CGCG)₂. Incubation of WEHI-150 with d(CG^{5Me}CGCG)₂ in the presence of formaldehyde resulted in the formation of significantly greater amounts of covalent adducts than was observed with d(CGCGCG)₂. In order to understand the observed increase of covalent adducts with d(CG^{5Me}CGCG)₂, an NMR study of the reversible interaction of WEHI-150 at both CpG and ^{5Me}CpG sites was undertaken. Intermolecular NOEs were observed in the NOESY spectra of d(ACGGCCGT)₂ with added WEHI-150 that indicated that the drug selectively intercalated at the CpG sites and from the major groove. In particular, NOEs were observed from the WEHI-150 H_{2,3} protons to the H1' protons of G₃ and G₇ and from the H_{6,7} protons to the H5 protons of C₂ and C₆. By contrast, intermolecular NOEs were observed between the WEHI-150 H_{2,3} protons to the H2'' proton of the ^{5Me}C₃ in d(CG^{5Me}CGCG)₂, and between the drug aliphatic protons and the H1' proton of G₄. This demonstrated that WEHI-150 preferentially intercalates at ^{5Me}CpG sites, compared to CpG sequences, and predominantly via the minor groove at the ^{5Me}CpG site. The results of this study demonstrate that WEHI-150 is likely to form interstrand DNA cross-links, upon activation by formaldehyde, and consequently exhibit greater cytotoxicity than other current anthracenedione drugs.

Introduction

Anthracyclines (e.g. daunorubicin, see Figure 1) are an important class of anticancer agents that continue to play a significant role in the treatment of cancer.¹⁻³ However, their clinical use is limited by their cardiotoxicity.⁴ This dose-limiting toxicity has promoted the search for new anthracenedione, and aza-anthracenedione derivatives, with comparable cytotoxicity but lower cardiotoxicity. Mitoxantrone and pixantrone (see Figure 1) are two examples of synthetic second generation anthracycline derivatives that exhibit significantly lower cardiotoxicity but maintain potent anticancer activity.⁵⁻⁸ Similar to the parent anthracyclines, the anthracenedione analogues can bind DNA by intercalation between the base pairs and thereby induce their cytotoxic effect through the impairment of topoisomerase II.⁹⁻¹¹ In addition, in an attempt to develop an alternative pathway to anticancer activity, anthracenediones have also been used to target G-quadruplex DNA structures.^{12,13} Recently, it has been demonstrated that daunorubicin, mitoxantrone and pixantrone can form drug-DNA covalent adducts at the N-2 amino groups of guanine nucleotides after activation by formaldehyde.¹⁴⁻¹⁶ As the anthracenedione analogues are more cytotoxic to cells and solid tumours that produce higher levels of formaldehyde, it has been proposed that covalent bond formation is an important contributing mechanism to the anticancer properties of these compounds. Additionally, it has been established that the combination of an anthracycline drug and a formaldehyde-releasing pro-drug is synergistic in cancer cell lines, and leads to apoptosis independently of topoisomerase II-mediated damage.¹⁷⁻¹⁹

Daunorubicin can only form one formaldehyde-mediated covalent bond with DNA (a N-CH₂-N link), whereas, mitoxantrone and pixantrone can potentially form two covalent bonds, as they each contain two potentially reactive amine groups. Mitoxantrone-DNA adducts have a half-life of 50 minutes and appear to be formed between the secondary amine of one side chain and the N-2 of guanine at a CpG sequence.²⁰ The more recently synthesised anthracenedione pixantrone forms DNA adducts 10- to 100-fold more efficiently at CpG sites

than mitoxantrone and while the pixantrone-DNA adducts are still labile, they are more stable than those formed by mitoxantrone.²¹ Previously, Phillips, Cutts and co-workers have also shown that pixantrone and mitoxantrone form 2-5 fold more covalent adducts at 5'-CpG sequences when the cytosine is methylated at the C-5 position.²² The cytosine residue in CpG sequences (found disproportionately in regulatory promoter regions) is often methylated, with methylation typically occurring after DNA replication.²³

Figure 1. Structure of daunorubicin, mitoxantrone, pixantrone and WEHI-150. The atom numbering of WEHI-150 is also given.

A bis-amino mitoxantrone analogue, named herein WEHI-150 (see Figure 1), was synthesised in an attempt to develop anthracenedione analogues that are more reactive with DNA than mitoxantrone and pixantrone, but are also more likely to form covalent cross-links between the two DNA strands, through the formation of an adduct with a covalent bond to a guanine residue on each strand of the duplex.^{24,25} By the replacement of the terminal hydroxyl group on each mitoxantrone side chain with an amine group, it was hypothesised

that WEHI-150 would be able to rapidly form covalent linkages with DNA and more importantly, it would be able to form multiple covalent linkages with DNA. DNA sequence specific *in vitro* transcription studies revealed probable interstrand cross-linking sites for WEHI-150.²⁵

In this study we have examined by mass spectrometry and HPLC the ability of WEHI-150 to form covalent adducts with oligonucleotides that contain either a CpG or a 5-methylated cytosine in the CpG (⁵MeCpG) site. As the results indicated that WEHI-150 readily forms multiple covalent adducts with DNA, and to a significantly greater extent when the oligonucleotide contains a ⁵MeCpG site, we have also examined the reversible intercalative binding of WEHI-150 at both CpG and ⁵MeCpG sites by NMR spectroscopy and molecular modelling. The mode of reversible binding of WEHI-150 at the CpG sequence (i.e. from the major or minor groove), will affect the rate of adduct formation with the guanine N-2 amino group that is located in the minor groove. It has been established by x-ray studies that daunorubicin intercalates at CpG sites from the DNA minor groove,²⁶ whereas, mitoxantrone and pixantrone bind from the major groove.^{27,28} However, in the NMR study of the binding of pixantrone to the octanucleotide d(ACGATCGT)₂ some non-intercalative minor groove binding was also observed. It was proposed that this small population of minor groove bound pixantrone could be responsible for the guanine N-2 adducts.²⁸

Experimental

Materials

Oligonucleotides were obtained from GeneWorks, South Australia. D₂O (99.9% D) was obtained from Cambridge Isotope Laboratories, and CM-Sephadex from Amersham-Pharmacia-Biotech. Formaldehyde (37% solution) was purchased from Sigma-Aldrich (St Louis, USA). Ammonium acetate (NH₄OAc), acetonitrile (ACN, HPLC grade) and ammonia were obtained from Ajax Finechem (Seven Hills, Australia), while methanol was obtained

from Aldrich chemical company. De-ionised Milli-Q water was used for all the experiments. Pixantrone (BBR 2778) was kindly supplied by CTI BioPharma, Seattle, while mitoxantrone and daunorubicin were purchased from Sigma-Aldrich. WEHI-150 was synthesised as previously described.²⁹

Sample preparation for mass spectrometry

A d(ACGCGCGT)₂ solution (1 mM) was obtained by annealing the appropriate amounts of the self-complementary oligonucleotide in 100 mM ammonium acetate, pH 7.5, by heating to 90 °C for 15 min and then allowing the sample to cool slowly to room temperature. Drug stock solutions were prepared by dissolving the appropriate amount in 100 mM ammonium acetate to obtain a final concentration of 1 mM. All stock solutions were stored at -20 °C until required for experiments. DNA-drug cross-linking samples were prepared by adding the appropriate amount of double-stranded DNA (1 mM stock) and drug (1 mM stock) to obtain a 1:5 molar ratio with a final DNA concentration of 40 µM in 100 mM ammonium acetate. Solutions were left to equilibrate for one hour, then formaldehyde was added to the DNA-drug mixture to obtain a formaldehyde concentration of 2 mM. Samples were incubated at room temperature (21 °C) for six hours and then 10 µL of the mixture was taken and diluted just prior to ESI-MS analysis with 70 µL of 100 mM ammonium acetate and immediately analysed.

Mass spectrometry

All drug-DNA complexes were analysed under the same instrumental conditions using a Waters Q-ToF Ultima™ ESI mass spectrometer equipped with a Z-spray ionisation source and a quadrupole mass analyser with a *m/z* range of 32,000. Samples were injected into the mass spectrometer at a flow rate of 20 µL/min using a Harvard Model 22 syringe pump (Natick,

MA, USA). All spectra were acquired using a MCP potential of 1850V, Rf lens energy of 70V, capillary voltage of 2.50 kV and sampling and extraction cone voltages were 70V and 4 V, respectively. The source temperature was 80 °C and the desolvation gas flow rate was 300 L/h at a temperature of 120 °C. All spectra were acquired in negative ion mode over the range m/z 500-4000 and typically 30 acquisitions were combined to obtain a representative spectrum. Each spectrum was then background subtracted using a polynomial order of 2 with 20% below curve, then smoothed using the MassLynx software, where 10 channels were smoothed twice. Prior to analysis the instrument was externally calibrated with a solution of 1 mg/mL caesium iodide (CsI) over the m/z acquisition range.

Sample preparation for HPLC

Oligonucleotides solutions (20 μ M) were incubated with WEHI-150 (40 μ M) and formaldehyde (2 mM) in 100 mM ammonium acetate pH 6.8 at 37 °C for 10 hours, then the un-reacted (including the reversibly bound) WEHI-150 was removed by phenol/chloroform extraction and the DNA precipitated by the addition of absolute ethanol. The WEHI-150-DNA adducts, a blue precipitate, were dissolved in 100 mM ammonium acetate pH 6.8 prior to injection into the HPLC.

HPLC

HPLC analysis was performed on a Shimadzu prominence (UFLC) instrument, consisting of an auto sampler (SIL-20A_{HT}), pump (LC-20AD) and PDA detector (SPD-M20A). UV/Vis detection, 260 nm for DNA and 660 nm for the WEHI-150, was used. The reaction between WEHI-150 and the oligonucleotide was monitored by HPLC where mobile phase A was 0.1% TEA in water pH 7 adjusted with acetic acid and mobile phase B was HPLC grade acetonitrile with 10% water. A C18 column, 4.6 x 150 mm, 5 micron, reverse phase (Alltech Apollo) was

used at room temperature at a flow rate of 1.0 mL/min. The chromatography gradient elution was: 0 min, 0% B; 30 min, 30% B; 40 min, 50% B; 50 min, 50% B.

Sample preparation for NMR

Oligonucleotides were purified from minor impurities by passing them through a C18 Sep-Pak cartridge and then converted to the sodium salt using a CM-Sephadex C-25 ion exchange resin column, as previously described.³⁰ The oligonucleotide was dissolved in 650 μ L of phosphate buffer (pH 7.0), containing 20 mM NaCl and 1 mM Na₂H₂EDTA, and the solution was freeze-dried. Finally the oligonucleotide was dissolved in 650 μ L of 99.9% D₂O and the solution heated to 90 °C and then cooled slowly to achieve the most stable duplex conformation prior to use. The concentration of the oligonucleotide was estimated by the UV absorbance (at A₂₆₀), using an extinction coefficient of 6,600 M⁻¹cm⁻¹ per nucleotide. Stock solutions of WEHI-150 were prepared in 99.9% D₂O, and directly added to the NMR tube containing the oligonucleotide and the ¹H NMR spectrum recorded.

NMR Spectroscopy

NMR experiments were recorded at 400 MHz for the ¹H nuclei using a Varian Unityplus-400 spectrometer at 25 °C unless otherwise stated and the data processed using the Agilent software Vnmrj 2.2C. Chemical shift values were referenced to HDO at 4.78 ppm relative to 2,2-dimethylsilapentane-5-sulfonic acid. One- and two-dimensional spectra were recorded in 99.9% D₂O for the non-exchangeable protons whereas for the exchangeable protons, spectra were recorded in 90% H₂O/10% D₂O using the WET solvent suppression technique. Phase-sensitive NOESY spectra were acquired using the method of States *et al.*,³¹ with 2048 data points in t_2 and 256 in t_1 values with pulse repetition delay of 1.7 s for mixing time ranging of 100 and 300 ms. DQFCOSY spectra were recorded using the same t_2 , t_1 and pulse repetition delay as stated above.

Molecular modelling

Molecular modelling was performed using HyperChem 7.5 software. Double helix B type oligonucleotide was generated from the nucleic acid database and energy restraints were added to preserve the hydrogen bonding between base pairs expected of duplex DNA during the optimisation procedure, which were conducted *in vacuo* using the Amber 99 force field and a Polak-Ribiere conjugant-gradient algorithm with 5×10^{-5} kcal/(Å mol) convergence criteria.

The point charges of WEHI-150 were determined from semi-empirical calculations using the AM1 program. The WEHI-150 complex point charges resulting from different AM1 calculations appeared to vary only slightly with the conformation. Therefore, the point charges resulting from an optimised confirmation were used to approximate the charges for the subsequent molecular modelling AMBER optimisations with the DNA oligonucleotide. The compound was manually inserted into the free DNA model to reflect the intermolecular NOEs observed with 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 the restraints were removed to confirm the resulting structure was an energy minimised structure.

Results

Mass spectrometry analysis of covalent binding

Mass spectrometry was used to identify drug-oligonucleotide complexes, both reversibly bound and covalent adducts, for WEHI-150 and the control anthracenedione drugs daunorubicin, mitoxantrone and pixantrone. Preliminary experiments indicated that under the conditions used the hexanucleotide sequence d(CGCGCG)₂ was not stable in the gas phase and mostly separated into its single strands, with only small amounts of the free and drug-bound duplex being observed. Consequently, to increase duplex stability and the intensity of

the observed drug-DNA adducts the octanucleotide d(ACGCGCGT)₂ was used for the mass spectrometry experiments. For the covalent adducts, it was possible to determine the number of covalent CH₂ links each drug formed with the oligonucleotide. Ionisation by electrospray produces a range of charge states for oligonucleotides as a function of the sequence and length, and for d(ACGCGCGT)₂ the charge states of 4⁻ and 3⁻ were observed as the most abundant species in the spectrum under the conditions used. While it was noted that some drug-DNA adducts contained one and two bound drugs, and a very small proportion contained three bound drugs (spectra not shown), the nature of the DNA used (a self-complementary sequence) created peak overlap for some of the charge states (single- versus double-stranded). Although the resolution obtained in each spectrum allows determination of the exact number of covalent CH₂ links each adduct formed with the oligonucleotide, it was not possible to unambiguously attribute the proportion of CH₂ cross-links made to the oligonucleotide for each drug when more than one drug was bound. In order to avoid any of these issues, the 3⁻ charge state was examined in detail, with a focus on the duplex with only one drug, either reversibly or covalently bound. Table 1 shows that the obtained *m/z* values for the drug-oligonucleotide complexes containing one bound drug were in agreement with the calculated *m/z* values.

Table 1. Calculated and experimental m/z values for reversible and covalent drug-DNA complexes for d(ACGCGCGT)₂ with one drug bound for the 3⁻ charge state. Numbers in bracket indicate the obtained m/z values. (ND = Not Detected).

[DNA] ³⁻ Alone	Drug	0 CH ₂ (Reversible binding)	1 CH ₂	2 CH ₂	3 CH ₂	4 CH ₂
1606.06 (1606.26)	Daunorubicin Mwt 527.53	1781.90 (1781.85)	1785.91 (1785.90)	--	--	--
1606.06 (1606.02)	Mitoxantrone Mwt 444.49	1754.22 (1754.22)	1758.22 (1758.12)	1762.22 (ND)	--	--
1606.06 (1606.10)	Pixantrone Mwt 325.37	1714.52 (1714.52)	1718.52 (1718.54)	1722.52 (ND)	--	--
1606.06 (1606.35)	WEHI-150 Mwt 442.52	1753.57 (1753.58)	1757.57 (1757.54)	1761.57 (1761.52)	1765.57 (1765.50)	1769.57 (ND)

Figure 2 shows the 1700 to 1820 m/z region of the mass spectra of the drug-oligonucleotide complexes after a 6-hour incubation with formaldehyde. Control experiments were conducted to determine the effect of the addition of 2 mM formaldehyde to the oligonucleotide in the absence of drug. No significant changes were observed in the mass spectrum, indicating that formaldehyde did not react with the oligonucleotide (data not shown). As observed in Figure 2, the bulk of the single bound daunorubicin existed as a covalent adduct containing one covalent link to the oligonucleotide. This is the expected result, as daunorubicin contains only one amine group; however, of note is the almost absence of any reversibly bound daunorubicin. For mitoxantrone, only a relatively small proportion of the bound drug is covalently linked to the oligonucleotide, with the reversibly bound species dominating. While mitoxantrone is theoretically capable of forming adducts with two covalent links to the oligonucleotide, only adducts with one covalent linkage were observed. Similar results were observed for pixantrone, although the proportion of the covalent adduct

was significantly greater. Interestingly, the profile for WEHI-150 was different to that of the other drugs. Although not to the same degree as observed with daunorubicin, the proportion of covalently bound oligonucleotide adducts was greater than that of the reversibly bound WEHI-150. Furthermore, adducts with one, two and even three covalent links to the oligonucleotide were observed.

Figure 2. Mass spectra showing the adducts formed after a 6-hour incubation at room temperature of d(ACGCGCGT)₂ with daunorubicin (**A**), mitoxantrone (**B**), pixantrone (**C**) and WEHI-150 (**D**) in the presence of formaldehyde at 1:5 ratio of DNA duplex to drug with [DNA= 5 μ M]. The spectra show the 1770-1820 m/z region where the drug-DNA adducts charge state is 3⁻ with one bound drug. Numbered empty shapes (○) indicate the number of CH₂ cross-links formed between the double stranded DNA and the drug. Zero indicates reversible binding by the drug. Note: peaks around m/z 1740 in (**A**) correspond at 2 drugs for single stranded 2⁻ charge state with m/z 1737 = 1 CH₂ and m/z 1743 = 2 CH₂. The peaks around m/z 1800 in (**B**) are of unknown origin.

HPLC analysis of covalent binding

While the results from the mass spectrometry experiments demonstrated that WEHI-150 could form one, two or three covalent bonds with d(ACGCGCGT)₂, it was not possible to determine the extent of the covalent adducts that were formed relative to unreacted and reversibly-bound oligonucleotide. Consequently, HPLC was used to examine the extent of covalent bond formation of WEHI-150 with d(CGCGCG)₂ and d(CG^{5Me}CGCG)₂. Figure 3 shows the HPLC elution profiles of the oligonucleotide mixtures obtained after WEHI-150 was incubated with either d(CGCGCG)₂ or d(CG^{5Me}CGCG)₂ in the presence of formaldehyde and then repeatedly extracted with a phenol/chloroform solution before being precipitated by the addition of ethanol. With d(CGCGCG)₂, three approximately equal small product peaks (1', 2' and 3') were observed in the HPLC profile, along with a large peak for the free oligonucleotide. Virtually all non-covalently bound WEHI-150 was removed by the phenol/chloroform extraction after the incubation of the drug with the oligonucleotide. In addition, any remaining small amount of reversibly bound drug dissociates from the oligonucleotide on the HPLC column and is separately eluted after the three small peaks seen in Figure 3, at approximately 27 minutes. Consequently, the observed small peaks can be identified as covalent adducts due to their characteristic WEHI-150 UV/Vis absorption at 660 nm. By contrast, incubation of WEHI-150 with d(CG^{5Me}CGCG)₂ in the presence of formaldehyde resulted in the formation of a significantly greater amount of covalent adducts. Of further note, only one major covalent adduct was observed in the HPLC elution profile, possibly suggesting preferential binding at the ^{5Me}CpG site.

Figure 3. HPLC elution profiles of the oligonucleotide mixture obtained after incubation with WEHI-150 in the presence of formaldehyde. A: $d(CGCGCG)_2$, where the free DNA peak is denoted as 1 and the WEHI-150-DNA covalent adducts 1', 2' and 3'. B: $d(CG^{5Me}CGCG)_2$, where the free oligonucleotide peak is denoted as 2 and the dominant covalent adduct 2*.

NMR analysis of reversible binding at CpG and $^{5\text{Me}}$ CpG sites

As the HPLC experiments indicated that WEHI-150 formed significantly more covalent adducts with $\text{d}(\text{CG}^{5\text{Me}}\text{CGCG})_2$ than with the corresponding non-methylated oligonucleotide in the presence of formaldehyde, an NMR study of the reversible interaction of the drug at both CpG and $^{5\text{Me}}$ CpG sites was undertaken. In order to simplify the analysis of the binding of WEHI-150 at a CpG site, the octanucleotide $\text{d}(\text{ACGGCCGT})_2$ was used for the NMR experiments. The octanucleotide contains two symmetric CpG sites (indicated in bold), rather than the three potential CpG binding sites in $\text{d}(\text{CGCGCG})_2$. However, for the analysis of WEHI-150 binding at a $^{5\text{Me}}$ CpG site, the hexanucleotide $\text{d}(\text{CG}^{5\text{Me}}\text{CGCG})_2$ was used, as this oligonucleotide also allowed a comparison of the relative preference for a $^{5\text{Me}}$ CpG compared to the non-methylated dinucleotide.

Assignment of free WEHI-150

The aromatic protons in the ^1H NMR spectrum of WEHI-150 were assigned using standard one-dimensional COSY and NOESY NMR experiments and by comparison with the previously reported assignments for mitoxantrone and pixantrone.^{28,32} The H6,7 protons were assigned by the observation of a strong NOE to the Ha protons and weak NOEs to the Hb, Hc and Hd protons.

WEHI-150 binding to $\text{d}(\text{ACGGCCGT})_2$

The resonances in the ^1H NMR spectra of the free and WEHI-150 bound oligonucleotide were assigned by standard techniques.³³⁻³⁵ Consistent with many NMR studies of oligonucleotides,³³⁻³⁶ the analysis of short mixing-time NOESY and DQFCOSY spectra indicated that the octanucleotide adopted a B-type conformation in the aqueous buffer.

Figure 4 shows the spectra of the imino protons for the free and WEHI-150-bound d(ACGGCCGT)₂ at 10 °C. Two new sets of peaks, corresponding to 1:1 and 2:1 drug to oligonucleotide duplex binding, were observed upon addition of WEHI-150 to the oligonucleotide. This indicates that WEHI-150 binds the oligonucleotide with slow exchange kinetics (on the NMR time scale). The observation of only three imino resonances at a drug to oligonucleotide ratio of 2 indicates that the two WEHI-150 molecules were bound to d(ACGGCCGT)₂ in a symmetrical fashion, suggesting selective binding at the two CpG sites. Significant upfield shifts were observed for the imino resonances from the G₃ and G₇, again consistent with intercalation at both the C₂pG₃ and C₆pG₇ sites. Two new sets of peaks from the aromatic protons of d(ACGGCCGT)₂ were also observed upon addition of WEHI-150 to the oligonucleotide (see Figure 5). Table 2 summarises the chemical shifts of the resonances from the WEHI-150-bound oligonucleotide and the change in chemical shift upon addition of WEHI-150 to a drug to duplex ratio of 2. The resonances from the WEHI-150 protons shifted upfield (0.24 to 0.35 ppm, see Table 3), consistent with WEHI-150 binding by intercalation, while the resonances from the aliphatic side chain protons shifted upfield but to a lesser extent.

Figure 4. ^1H NMR spectrum of the imino resonances of $\text{d}(\text{A}_1\text{C}_2\text{G}_3\text{G}_4\text{C}_5\text{C}_6\text{G}_7\text{T}_8)_2$ (A) and with added WEHI-150, at a drug to duplex ratio $R = 0.8$ (B), $R = 1.4$ (C) and $R = 1.9$ (D) at 10°C , in 90%/10% $\text{H}_2\text{O}/\text{D}_2\text{O}$ in pH 7 phosphate buffer. In the spectra, * represents 1:1 drug to oligonucleotide binding, while # indicates 2:1 binding.

Figure 5. ^1H NMR spectra of the titration of $\text{d}(\text{A}_1\text{C}_2\text{G}_3\text{G}_4\text{C}_5\text{C}_6\text{G}_7\text{T}_8)_2$ with WEHI-150. (A) free oligonucleotide, (B) with added WEHI-150 to DNA duplex ratio (R) of 0.8 (C) with added WEHI-150 at $R = 1.4$ (aromatic region). In the spectra, * represents 1:1 drug to oligonucleotide binding, while # indicates 2:1 binding.

Table 2. ^1H NMR assignment of the resonances of $\text{d}(\text{A}_1\text{C}_2\text{G}_3\text{G}_4\text{C}_5\text{C}_6\text{G}_7\text{T}_8)_2$ with added WEHI-150 at $R = 2$, at $25\text{ }^\circ\text{C}$ in pH 7 phosphate buffer. * Imino proton shifts were recorded at $10\text{ }^\circ\text{C}$. Changes in chemical shift upon WEHI-150 binding are given in brackets, with a negative sign indicating an upfield shift.

Base	H8/H6	AH2/CH5	H1'	H2'	H2''	Methyl	Imino*
A ₁	8.25 (+0.05)	7.95 (-0.09)	6.21 (-0.01)	2.62 (0.00)	2.80 (+0.06)		
C ₂	7.44 (+0.01)	5.36 (-0.14)	5.42 (+0.01)	2.09 (-0.01)	2.36 (-0.01)		
G ₃	8.09 (+0.22)		5.86 (+0.19)	2.73 (+0.03)	2.73 (-0.04)		12.63 (-0.51)
G ₄	7.71 (-0.09)		5.81 (-0.14)	2.60 (-0.01)	2.60 (-0.14)		13.04 (+0.02)
C ₅	7.39 (+0.01)	5.17 (-0.13)	5.88 (-0.09)	2.08 (0.00)	2.47 (-0.01)		
C ₆	7.52 (+0.10)	5.57 (-0.03)	5.63 (0.00)	1.93 (+0.01)	2.27 (-0.07)		
G ₇	8.27 (+0.27)		6.09 (-0.03)	2.70 (-0.04)	2.36 (-0.38)		12.41 (-0.53)
T ₈	7.49 (+0.04)		6.28 (+0.02)	2.26 (-0.01)	2.65 (+0.38)	1.37 (-0.32)	

Table 3. ^1H NMR shifts of free WEHI-150 and WEHI-150 bound to $\text{d}(\text{ACGGCCGT})_2$ and $\text{d}(\text{CG}^{5\text{Me}}\text{CGCG})_2$ at drug to DNA ratios (R) = 2 and 1, respectively, in pH 7 phosphate buffer. Changes in chemical shift are given in brackets, with a negative sign indicating an upfield shift.

WEHI-150 protons	H2,3	H6,7	Ha	Hb	Hc	Hd
Free	7.24	7.46	3.91	3.49	3.47	3.42
d(ACGGCCGT)₂ Bound	7.00 (-0.24)	7.11 (-0.35)	3.81 (-0.10)	3.38 (-0.11)	3.49 (+0.02)	3.53 (+0.11)
d(CG^{5Me}CGCG)₂ Bound	6.65 (-0.59)	6.76 (-0.70)	3.35 (-0.56)	2.97 (-0.52)	3.30 (-0.17)	3.27 (-0.15)

NOESY spectra were recorded to obtain a detailed picture of the WEHI-150-oligonucleotide binding. Strong intermolecular NOEs were observed from the WEHI-150 H2,3 to the H1' protons of G₃ and G₇ and from the H6,7 protons to the H5 protons of C₂ and C₆ (see Figure 6). In addition, weak NOEs were also observed from the H6,7 protons of the WEHI-150 to the H6 protons of C₂ and C₆. These NOEs indicate that WEHI-150 preferentially binds at the CpG sites of the oligonucleotide as expected. Furthermore, NOEs between the ethylene side chain protons of WEHI-150 and the major groove protons of the C₂ and C₆ were also observed. Taken together, the observed pattern of intermolecular NOEs indicates that WEHI-150 intercalates at CpG sites from the major groove.

Figure 6. An expansion of a NOESY spectrum of d(A₁C₂G₃G₄C₅C₆G₇T₈)₂ with added WEHI-150, at a ratio of R = 0.8 to DNA at 20 °C. It shows the NOE connectivities from the aromatic protons C₂H5, C₆H5 and G₃, G₇ sugar H1' protons of the oligonucleotide to the WEHI-150 H6,7 and H2,3 respectively.

WEHI-150 binding to $d(CG^{5Me}CGCG)_2$

The addition of WEHI-150 to $d(CG^{5Me}CGCG)_2$ induced an upfield shift for the G₄ imino resonance (see Figure 7), suggesting that WEHI-150 bound at the ^{5Me}CpG site. Significant upfield shifts (0.59 to 0.70 ppm, see Table 3) were observed from the WEHI-150 aromatic protons upon addition to $d(CG^{5Me}CGCG)_2$, consistent with WEHI-150 binding by intercalation. Interestingly, the resonances from the Ha and Hb aliphatic side chain protons also shifted considerably upfield. While the aromatic resonances from the oligonucleotide generally broadened upon addition of WEHI-150, multiple peaks for the H6 of the $^{5Me}C_3$ were observed (see Table 4 and Figure 8), suggesting there were at least two binding modes in slow exchange.

Figure 7. 1H NMR spectra of the imino resonances of the $d(C_1G_2^{5Me}C_3G_4C_5G_6)_2$ A, with added WEHI-150 at R = 1 B, at 10 °C in 90%/10% H_2O/D_2O in pH 7 phosphate buffer. G₂ and G₄ imino protons of the free and bound oligonucleotide are indicated.

Table 4 ^1H NMR assignment of the resonances of $\text{d}(\text{C}_1\text{G}_2^{5\text{Me}}\text{C}_3\text{G}_4\text{C}_5\text{G}_6)_2$ with added WEHI-150 at $R = 1$ in pH 7 phosphate buffer at 30 °C. * Imino proton shifts were recorded at 10 °C. Changes in chemical shift upon WEHI-150 binding are given in brackets, with a negative sign indicating an upfield shift.

Bases	H8/H6	H5	H1'	H2'	H2''	Methyl	Imino*
C₁	7.63 (-0.04) 7.59 (-0.08)	5.82 (-0.12)	5.73 (-0.07)	2.00 (-0.03)	2.35 (-0.09)		
G₂	7.97 (-0.04) 7.90 (-0.11)		5.97 (-0.08)	2.66 (-0.02)	2.82 (-0.04)		13.17 (-0.01)
⁵MeC₃	7.15 (-0.03) 7.21 (+0.03) 7.07 (-0.11)		5.65 (-0.04) 5.71 (+0.02)	 1.98 (-0.05)	 2.39 (0.00)	1.61 (-0.06) 1.54 (-0.13)	
G₄	7.87 (-0.07)		5.86 (-0.03)	2.69 (-0.02)	2.69 (-0.02)		13.12 (-0.09)
C₅	7.31 (-0.05)	5.36 (-0.11)	5.80 (-0.06)	1.95 (+0.01)	2.32 (-0.05)		
G₆	7.89 (-0.07)		6.08 (-0.10)	2.61 (-0.03)	2.39 (-0.01)

Figure 8. Aromatic region of the ^1H NMR spectrum of free $\text{d}(\text{CG}^{5\text{Me}}\text{CGCG})_2$ (A) and with added WEHI-150 at 30 °C in 10 mM phosphate buffer pH 7.0, at a drug to duplex ratio (R) of 0.25 (B), (C) R = 0.37, (D) R = 0.5, (E) R = 1.0, free WEHI-150 (F). The H8 and H6 protons of the hexanucleotide and the H2,3 and H6,7 protons of the free and bound WEHI-150 are indicated.

Figure 9 shows an expansion of a NOESY spectrum of $\text{d}(\text{CG}^{5\text{Me}}\text{CGCG})_2$ with added WEHI-150. An intermolecular NOE was observed between the WEHI-150 H2,3 protons to the H2'' proton of the $^{5\text{Me}}\text{C}_3$. In addition, NOEs were also observed from the WEHI-150 aliphatic protons to the H1' proton of G₄. These unambiguous NOEs indicate that WEHI-150 predominantly intercalates via the minor groove at the $^{5\text{Me}}\text{CpG}$ site. However, additional weak NOEs were also observed from WEHI-150 aliphatic protons to the H8 and H6 protons

of the G₄ and ⁵MeC₃ protons, indicating that some proportion of WEHI-150 also intercalates via the major groove.

Figure 9. An expansion of a NOESY spectrum of d(CG⁵MeCGCG)₂ with added WEHI-150, at a drug to DNA duplex ratio of 1, at 35 °C in D₂O and pH 7 phosphate buffer. The expansion shows the NOE connectivities from the WEHI-150 H2,3 and Ha, Hc and Hd protons (3.1-3.45 ppm) to the octanucleotide H2'' of the ⁵MeC₃ and H1' of the G₄ protons respectively.

Molecular modelling

Mass spectrometry indicated that WEHI-150 could form multiple covalent bonds with a 5'-GCGC duplex sequence, and NMR studies indicated that covalent bond formation occurred when the drug predominantly intercalated from the minor groove. Due to the broad and overlapping resonances from the two binding forms, the NMR data did not allow the determination of a quantitative structure of WEHI-150 intercalated at the 5'-⁵MeCpG site of d(CG⁵MeCGCG)₂. However, simple HyperChem binding models were constructed to examine the feasibility of WEHI-150 forming two to four covalent bonds with the four guanine residues in a 5'-G⁵MeCGC duplex sequence. Molecular models were constructed (see Figure

10) with the WEHI-150 intercalated (non-covalently bound) from the minor groove at the 5'-^{5Me}CpG site of the hexanucleotide d(CG^{5Me}CGCG)₂. The WEHI-150-oligonucleotide structure is consistent with the intermolecular NOEs observed in the NMR experiments. Models were examined where the WEHI-150 was intercalated from the major groove at the 5'-^{5Me}CpG site; however, the distances between the WEHI-150 and oligonucleotide protons that gave NOE cross-peaks in NOESY spectra were significantly greater than the maximum distance (5 Å) for which an NOE can be observed. For example, a strong intermolecular NOE was observed between the WEHI-150 aliphatic Ha proton and the G₄H1' from d(CG^{5Me}CGCG)₂: the minimum distance between these protons for the drug intercalated from the major groove was 9.25 Å; whereas the distance with WEHI-150 intercalated from the minor groove was 3.3 Å. The model clearly demonstrates that, in principle, WEHI-150 can readily form between one and four covalent bonds with the four guanine residues in the 5'-G^{5Me}CGC duplex sequence. Unless the DNA is significantly distorted, the model suggests that when WEHI-150 forms three covalent bonds, it must form an interstrand cross-link where each strand of the duplex is covalently bound to the drug.

Figure 10. Energy minimized HyperChem model of WEHI-150 intercalated (non-covalently bound) at the ⁵MeCpG site via the minor groove. The guanine residues are shown in yellow, the cytosine residues in grey, guanine NH₂ groups are red, and the DNA backbone in cyan. The WEHI-150 is shown in green and the primary and secondary amines of WEHI-150 are in blue.

Discussion

Anthracenedione compounds can bind DNA by intercalation and thereby exert anticancer activity through the impairment of topoisomerase II.^{9,10} However, it has also been proposed that anthracenedione drugs containing side chain amine groups can induce apoptosis in cancer cells through formation of a covalent adduct with the N-2 amino group of a guanine residue at CpG sites after activation by formaldehyde.¹⁴⁻¹⁶ The results of this study confirm that daunorubicin, mitoxantrone and pixantrone can form covalent adducts with duplex DNA. WEHI-150 was synthesised in an attempt to develop anthracenedione analogues that can form covalent cross-links between the two DNA strands, through the formation of covalent bonds to a guanine residue on each strand of the duplex. The results from this study indicate that

WEHI-150 can form at least three covalent bonds with duplex DNA at GpCpGpC sequences after activation by formaldehyde.

The HPLC results presented in this study demonstrated that WEHI-150 formed considerably more covalent adducts with the hexanucleotide d(CG^{5Me}CGCG)₂, than with the non-methylated hexanucleotide d(CGCGCG)₂. Previous studies have demonstrated a 2-5 fold increase in covalent adducts with mitoxantrone and pixantrone when the DNA contained ^{5Me}CpG sequences.²² As the initial reversible binding of WEHI-150 at a CpG or ^{5Me}CpG site must to some degree influence the formation of the subsequent covalent bond formation, an NMR study of WEHI-150 binding with d(CGCGCG)₂ and d(CG^{5Me}CGCG)₂ was carried out. The results confirmed that WEHI-150 preferentially intercalated at CpG sites, and demonstrated that the drug intercalated from the DNA major groove. This is consistent with the results of previous NMR studies with the closely related analogues mitoxantrone and pixantrone.^{27,28} Furthermore, the results demonstrated WEHI-150 preferentially intercalates at ^{5Me}CpG sites, compared to CpG sites, and of greater consequence, the drug predominantly intercalated from the minor groove at ^{5Me}CpG sequences.

Although pixantrone forms more covalent adducts at ^{5Me}CpG sites than at CpG sites, previous NMR studies have established that pixantrone predominantly intercalates from the major groove at both ^{5Me}CpG sites and CpG sites.²⁸ However, some minor groove binding was observed.²⁸ Consequently, it is possible that the additional DNA adducts formed by pixantrone at ^{5Me}CpG sites could be due to the increased nucleophilicity of the guanine N-2 due to the electronic effect of the methylation of the complementary cytosine. For WEHI-150, the electronic effects of C-5 methylation may contribute to the increased adducts formed at ^{5Me}CpG sites; however, the switching from intercalation from the major groove to the minor groove is likely to be more important. It is not clear why WEHI-150, unlike pixantrone, intercalates from the minor groove at ^{5Me}CpG sites. Examination of molecular models

suggests that there are no steric barriers to major groove intercalation at ⁵MeCpG sites for either pixantrone or WEHI-150. Base stacking is the main driving force for the formation of duplex DNA,³⁷ and the stacking between an intercalated drug and the base pairs at the binding site also provides favourable thermodynamic driving forces for the formation of the drug-DNA complex.³⁸ However, DNA groove-binding interactions are predominantly entropy driven, and it has been established that intercalation is enthalpically driven.³⁸ Consequently, it is likely that the stacking interactions of WEHI-150 are maximised for minor groove intercalation at ⁵MeCpG sites. In particular, the electrostatic dipole-dipole interactions between WEHI-150 and the DNA base pairs are a significant stabilising force for the intercalative binding, and play a role in determining the geometry of the binding. The anthracenedione moiety of WEHI-150 produces a different pattern of dipole moments, compared to the aza-anthracenedione pixantrone, which consequently results in a different binding geometry – minor groove intercalation for WEHI-150, and major groove intercalation for pixantrone.

Taken together, the results of this study demonstrate that WEHI-150 is likely to form interstrand DNA cross-links. Although it is yet to be established, it is possible that the interstrand cross-links formed by WEHI-150 would disrupt DNA-mediated processes to a greater degree than anthracenedione drugs that are capable of forming only one covalent bond with DNA. Furthermore, the observed reversible binding preference for ⁵MeCpG sites, compared to CpG sites, could also potentiate the anticancer properties of WEHI-150. Gene expression is largely affected by promoters, which often contain CpG “islands”.³⁹ Although cancer cells generally contain hypomethylated genomes, CpG islands are hypermethylated.³⁹ Consequently, preferential binding in the DNA minor groove at the methylated CpG islands in promoter regions could allow for the optimal activity of WEHI-150 through the formation of covalent bonds with guanine residues after activation by formaldehyde.

In conclusion, the results of this study indicate that WEHI-150 can form interstrand cross-links with DNA after activation by formaldehyde. In addition, WEHI-150 predominantly intercalates from the DNA major groove at CpG sites, but from the minor groove at ⁵MeCpG sequences. As intercalation at ⁵MeCpG sites is preferred compared to non-methylated sites, WEHI-150 forms considerably more covalent adducts at sequences containing ⁵MeCpG sites with adjacent guanine residues.

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