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Disciplines

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Isolation and structural analysis of the covalent adduct formed between a bis-amino mitoxantrone analogue and DNA: a pathway to major–minor groove cross-linked adducts †

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The major covalent adduct formed between a ^{13}C-labelled formaldehyde activated bis-amino mitoxantrone analogue (WEHI-150) and the hexanucleotide d(CG^{5Me}CGCG)$_2$ has been isolated by HPLC chromatography and the structure determined by NMR spectroscopy. The results indicate that WEHI-150 forms one covalent bond through a primary amine to the N-2 of the G$_2$ residue, with the polycyclic ring structure intercalated at the ^{5Me}C$_3$pG$_4$/G$_{10}$p^{5Me}C$_9$ site. Furthermore, the WEHI-150 aromatic ring system is oriented approximately parallel to the long axis of the base pairs, with one aliphatic side-chain in the major groove and the other side-chain in the minor groove. This study indicates that mitoxantrone derivatives like WEHI-150 should be capable of forming major–minor groove cross-linked adducts that will likely produce considerably different intracellular biological properties compared to known anthracycline and anthracenedione anticancer drugs.

Introduction

The dose-limiting toxicities of the anthracycline class of anticancer drugs (*e.g.* doxorubicin) have promoted the search for new anthracenedione derivatives with comparable cytotoxicity but lower cardiotoxicity. Mitoxantrone (see Fig. 1) is a synthetic second-generation anthracycline derivative that is clinically used for the treatment of a variety of cancers.[1–3] It exhibits significantly lower cardiotoxicity than the anthracyclines but maintains potent anticancer activity.[4,5] Mitoxantrone and related derivatives can bind DNA by intercalation and induce a cytotoxic effect through the impairment of topoisomerase II.[6,7] More recently, it has been demonstrated that mitoxantrone can also form a covalent adduct at the N-2 amino group of a guanine nucleotide in DNA, after activation of a side-chain amine by formaldehyde, that potentially leads to apoptosis independently of topoisomerase II-mediated damage.[8,9] However, the mitoxantrone–DNA covalent adduct is yet to be structurally characterised.

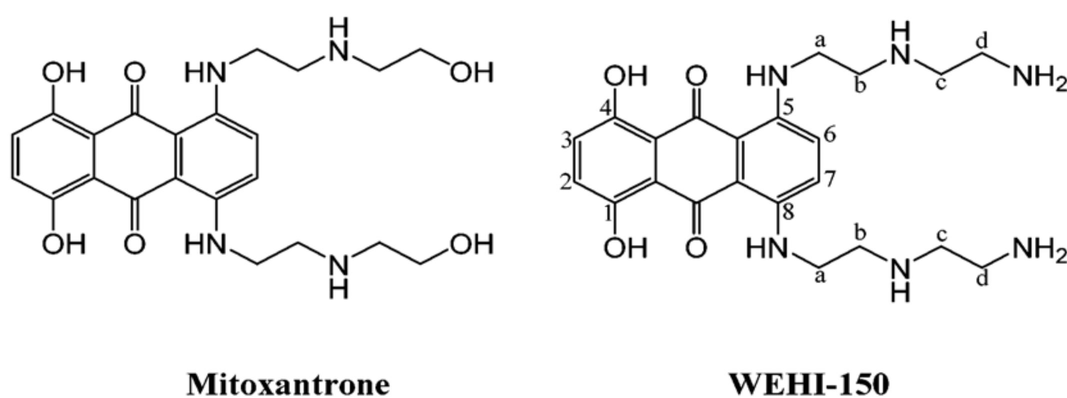

Fig. 1 Structure of mitoxantrone and WEHI-150. The atom numbering of WEHI-150 is given.

WEHI-150 is a bis-amino mitoxantrone analogue (see Fig. 1) that contains two potentially reactive amine groups in each side-chain. As a consequence WEHI-150 can more rapidly and more extensively form covalent adducts with DNA than mitoxantrone.[10] In order to further develop the anthracenedione class of anticancer drugs it is important to determine the structure of a mitoxantrone– or mitoxantrone derivative–DNA adduct. Previous studies have demonstrated that WEHI-150 preferentially intercalates and then forms covalent adducts with DNA at CpG sites.[10] In addition, WEHI-150 forms significantly more covalent adducts when the cytosine at the CpG site is methylated at the C-5 position (^{5Me}CpG).[10] Consequently, we sought to isolate by HPLC the major covalent adduct formed between WEHI-150 and the hexanucleotide d(CG^{5Me}CGCG)$_2$ and then examine its structure by NMR spectroscopy.

Materials and methods

The oligonucleotide d(CG^{5Me}CGCG)$_2$ was obtained from GeneWorks, South Australia, and WEHI-150 was synthesised as previously described.[11] The covalent adduct was produced by incubating the oligonucleotide d(CG^{5Me}CGCG)$_2$ (20 μM) with WEHI-150 (40 μM) and formaldehyde (^{13}C, 2 mM) in 100 mM ammonium acetate pH 6.8 at 37 °C for 10 hours. 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 separation was repeatedly performed on a Shimadzu prominence (UFLC) with Waters Delta pak C4, 3.9 × 150 mm, 5 micron, analytical column using a 100 mM ammonium acetate (pH 6.8) and acetonitrile gradient as the mobile phase. The major covalent adduct (collected at 90% ammonium acetate and 10% acetonitrile) from each reaction mixture was collected manually into a vessel containing chilled phosphate buffer (10 mM, pH 7.0 containing 20 mM NaCl and 1 mM EDTA) to avoid adduct degradation. The isolated covalent adduct contained a small amount (≈15%) of the free hexanucleotide. The free hexanucleotide did not interact with the WEHI-150–hexanucleotide adduct in the subsequent NMR experiments.

The molecular modelling investigations were carried out using HyperChem Release 7.5 software as previously described.[10] Duplex B-DNA was generated from the nucleic acid database. Energy restraints were added to maintain the H-bonds as expected in duplex DNA during the optimisation procedures. The duplex DNA was optimised *in vacuo* by applying the Amber 99 force field and a Polak-Ribiere conjugant algorithm with a 5×10^{-5} kcal (Å mol)$^{-1}$ convergence criterion. WEHI-150 atom charges were defined using the AM1 semi-empirical method. The models were constructed based on the intermolecular NOEs observed between the drug and oligonucleotide. Energy restraints (force constant 7 kcal Å^{-2}) were then applied to maintain a maximum distance of 3.5 Å between any drug and oligonucleotide protons that gave strong NOEs in the NOESY spectra. The drug-oligonucleotide complex was then energy minimised using the AMBER 99 molecular mechanics optimisation process. The distance restraints were then removed and the structure re-optimised to obtain a local energy minimum.

Results and discussion

In preliminary experiments, the reactions of WEHI-150 with guanosine, guanosine monophosphate (GMP) and the oligonucleotide d(CG^{5Me}CGCG)$_2$, in the presence of formaldehyde, were monitored by HPLC. The results demonstrated that significant amounts of the covalent adduct were formed with d(CG^{5Me}CGCG)$_2$ in 4 hours, whereas with the single nucleotides no covalent adduct formation was observed in 24 hours. These results demonstrate the need for the initial reversible association with DNA in order to form the WEHI-150–DNA covalent adduct.

Experiments were carried out to determine the stability of the major covalent adduct as a function of pH (after a 12-hour incubation) and incubation time at pH 7.0. The percentage of remaining intact covalent adduct was determined by HPLC with UV detection. The results showed that the adduct was more stable at basic pH compared to acidic conditions. No adduct was observed after a 12-hour incubation at pH 3.1 at 37 °C, whereas approximately 75% of the adduct remained intact at pH 7, indicating the WEHI-150–d(CG^{5Me}CGCG)$_2$ adduct was moderately stable at neutral pH. The covalent adduct was incubated at 37 °C in 10 mM phosphate buffer at pH 7.0 and the amount of remaining intact covalent adduct monitored by HPLC at different time intervals. Approximately 50% of the covalent adduct was observed after 33 hours incubation at 37 °C, indicating that the covalent adduct was highly stable compared to the parent drug mitoxantrone.[12] It was also noted that WEHI-150–d(CG^{5Me}CGCG)$_2$ adduct appeared to be degraded to free DNA.

Mass spectrometry was used to identify the number of WEHI-150 molecules and the number of CH_2 covalent links formed in the major WEHI-150–d(CG^{5Me}CGCG)$_2$ covalent adduct that was isolated by HPLC. Ionisation by electrospray produces a range of charge states for oligonucleotides as a function of the sequence and length. Under the conditions used the 3^- charge state was observed as the most abundant species in the spectrum for the WEHI-150–d(CG^{5Me}CGCG)$_2$ covalent adduct. The obtained *m*/*z* values for the covalent adduct are in agreement with the calculated *m*/*z* values for the presence of one WEHI-150 molecule that contained one CH_2 covalent link (see Fig. 2).

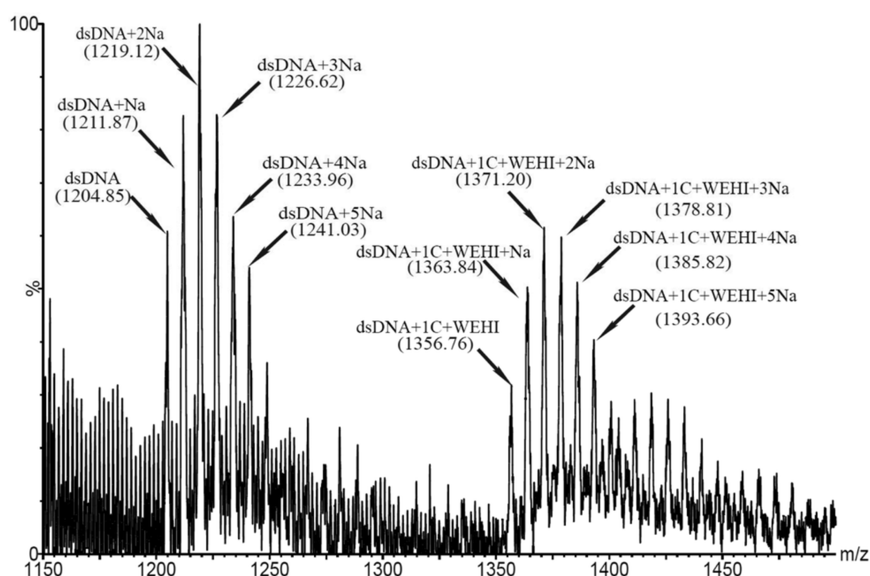

Fig. 2 Negative ion electrospray mass spectrum of the major WEHI-150–d(CG^{5Me}CGCG)$_2$ covalent adduct focussed on the 3^- state mass range, showing one CH_2 link between WEHI-150 and the oligonucleotide duplex d(CG^{5Me}CGCG)$_2$. The obtained *m*/*z* values are given in brackets which are in agreement with calculated *m*/*z* values.

The major WEHI-150–d(CG^{5Me}CGCG)$_2$ adduct was then examined by ^{1}H NMR spectroscopy. A HSQC experiment was performed to confirm the results observed by mass spectrometry that indicated the covalent adduct contained only one CH_2 link between the drug and the oligonucleotide. The results showed one ^{13}C signal at 58.8 ppm with two ^{1}H signals for the non-equivalent methylene protons at 3.44 and 4.04 ppm (see ESI Fig. S1†), consistent with formation of a single CH_2 link in the covalent adduct. The aromatic region of the ^{1}H NMR spectrum of the covalent adduct is shown in Fig. 3, with the full width spectra given in Fig. S2 in the ESI.† The observation of multiple sharp signals in the spectrum indicates the loss of the oligonucleotide duplex symmetry upon covalent adduct formation with WEHI-150. The oligonucleotide base residue numbers are given from 1 to 12 for the two DNA strands, S-1 and S-2 (see Fig. 3). Upon covalent adduct formation, the resonances from the aromatic protons of WEHI-150 shifted significantly upfield (see ESI Table S1†), compared to the free drug, consistent with intercalation in addition to covalent binding.

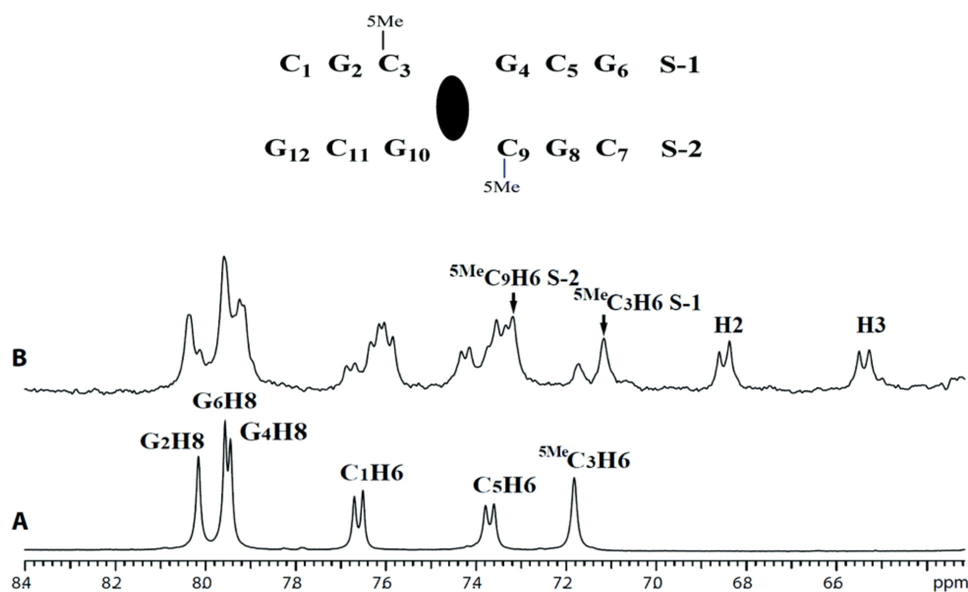

Fig. 3 Oligonucleotide residue numbering is shown (top). The aromatic region of the ^{1}H NMR spectrum of the free oligonucleotide d($C_1G_2{}^{5Me}C_3G_4C_5G_6$)$_2$ (A) and the WEHI-150 covalent adduct with d($C_1G_2{}^{5Me}C_3G_4C_5G_6$)$_2$ (B) in pH 7.0 phosphate buffer in D_2O at 10 °C. H6 and H8 protons of the free hexanucleotide are shown in A. The WEHI-150 H2, H3 protons and the H6 protons of the 5-methylated cytosines are indicated in B for the covalent adduct.

OESY and DQFCOSY spectra were recorded to obtain a detailed picture of the WEHI-150 covalent adduct with d(CG^{5Me}CGCG)$_2$. The resonances at 6.82 and 6.50 ppm were assigned to the H2 and H3 protons of WEHI-150, respectively, as these protons showed coupling in DQFCOSY spectra with no connectivity to the aliphatic protons of WEHI-150 in NOESY spectra. The resonance at 6.09 ppm was assigned to the WEHI-150 H6/H7 protons as these protons showed strong NOEs with the aliphatic resonances of WEHI-150. A range of intermolecular NOEs were observed between the WEHI-150 and the d(CG^{5Me}CGCG)$_2$ protons. In particular, NOEs were observed from the WEHI-150 H2 and H3 protons to the methyl and the H2″ protons of the ^{5Me}C$_3$ of S-1 (see Fig. 4). In addition weak NOEs were observed from the WEHI-150 H3 proton to the H6 and H8 protons of the ^{5Me}C$_3$ and G$_4$ residues of S-1. These intermolecular NOEs strongly suggest that WEHI-150 binds preferentially at the ^{5Me}C$_3$pG$_4$/G$_{10}$p^{5Me}C$_9$ site. In addition, the loss of the sequential NOEs from ^{5Me}C$_3$ to G$_4$ confirms WEHI-150 binds at the ^{5Me}CpG site.

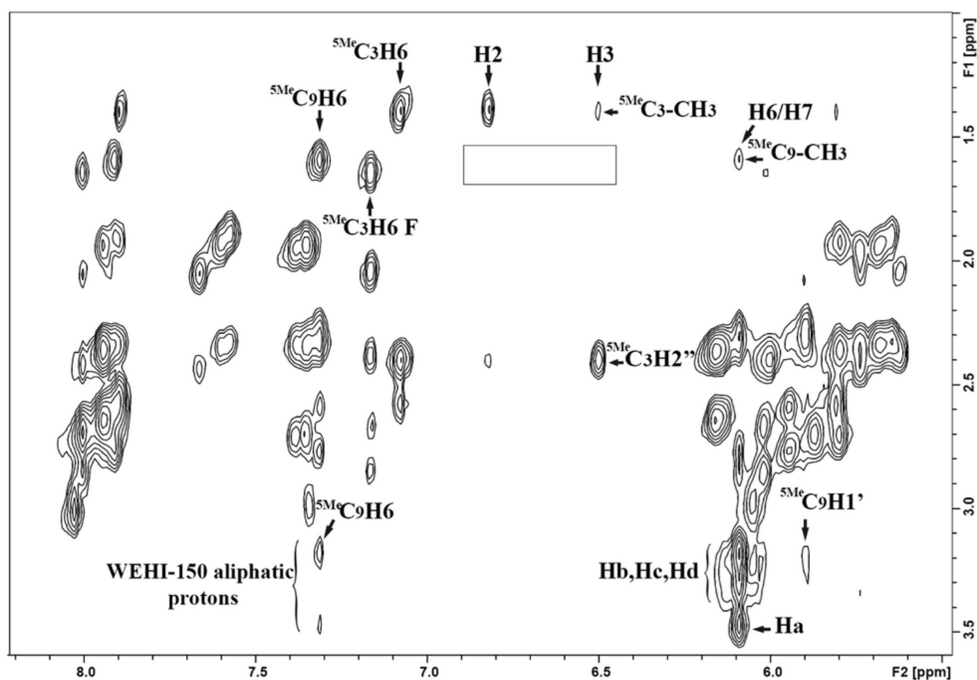

Fig. 4 Expansion of a NOESY spectrum of the WEHI-150 covalent adduct with d(C$_1$G$_2$^{5Me}C$_3$G$_4$C$_5$G$_6$)$_2$ in pH 7.0 phosphate buffer in D$_2$O at 10 °C. The expansion shows NOE connectivities from the WEHI-150 H2, H3 and H6/7 protons to the oligonucleotide methyl and sugar H2″ protons of the 5-methyl cytosines, and from the WEHI-150 aliphatic protons to the ^{5Me}C$_9$H1′ proton. In the empty box region cross peaks would be expected if the WEHI-150 H2 and H3 protons are close to the methyl protons of the 5-methyl cytosine (^{5Me}C$_9$ S-2). Absence of these cross peaks suggests that the WEHI-150 chromophore is oriented parallel to the DNA base pair.

A relatively strong NOE was observed between the H2 of WEHI-150 and the oligonucleotide $^{5Me}C_3$ methyl protons, whereas, only a weak NOE was observed between the H3 and the $^{5Me}C_3$ methyl protons. In contrast, a strong NOE was noted between the H3 and the $^{5Me}C_3H2''$ protons and a weak NOE between the H2 and $^{5Me}C_3H2''$. These observations suggest the H3 proton is located under the $^{5Me}C_3$ base whereas the H2 proton is positioned more towards the major groove of the oligonucleotide. Of further note, NOEs of medium intensity were observed from the WEHI-150 H6/H7 protons to the oligonucleotide $^{5Me}C_9H6$ and $^{5Me}C_9CH_3$ protons. Observation of NOEs from the WEHI-150 H2, H3 and H6/H7 protons to only major groove oligonucleotide protons suggests that the long axis of the polycyclic ring system of WEHI-150 is positioned more parallel, than perpendicular, to the base pairs at the intercalation site. Consistent with this proposition was the observation of NOEs from the aliphatic side-chain protons to both major ($^{5Me}C_9H6$ and $^{5Me}C_9CH_3$) and minor groove (G_2H1' and C_9H1') protons (see Fig. 4 and ESI Fig. S3†).

NOESY experiments were carried out in 90% H_2O/10% D_2O to assign the imino resonances of the free and WEHI-150-bound covalent adduct (see ESI Table S2†). While significant upfield shifts were observed for the G_2, G_4, G_8 and G_{10} imino resonances, the G_2 imino exhibited the greatest shift. An NOE was observed between the G_2 imino proton and a NH proton at 10.00 ppm. The large shifts noted for the G_2 imino and amino protons suggests that WEHI-150 forms a covalent adduct with the N-2 of G_2. Consistent with this conclusion is the assignment of the NH proton of a N-2 alkylated guanine at 9.57 ppm by Moore *et al.* in their study of the covalent reaction of ecteinascidin 743 with an oligonucleotide.[13] As it would not be possible for WEHI-150 to reach the N-2 of G_2 through the formation of a covalent link with one of the secondary amines while intercalating at the ^{5Me}CpG site, it can be further concluded that the covalent adduct was formed through the reaction of a primary side-chain amine group.

Taken together the MS and NMR results indicate that WEHI-150 forms one covalent bond through a primary amine to the N-2 of the G_2 residue, with the polycyclic ring structure intercalated at the $^{5Me}C_3pG_4/G_{10}p^{5Me}C_9$ site. Furthermore, the WEHI-150 aromatic ring system is oriented approximately parallel to the long axis of the base pairs, with one aliphatic side-chain in the major groove and the other side-chain in the minor groove. Although the NMR data was not sufficient to allow the determination of the structure of the covalent adduct by restrained molecular dynamics, simple energy minimised (to remove steric clashes) models could be constructed that were consistent with the NMR data. The model shown in Fig. 5 indicates that the proposed "threaded" structure (aliphatic side-chains in major and minor grooves) is feasible. Models were constructed keeping both aliphatic side chains in the minor groove as observed with the reversible binding;[10] however, the resultant models were not consistent with the observed NOEs and thus the models were discounted (*e.g.* see ESI Fig. S4†). A number of compounds, both organic (*e.g.* nogalamycin) and transition metal complexes (*e.g.* dinuclear ruthenium complexes), have been reported to bind DNA in a threaded intercalative manner.[14–17]

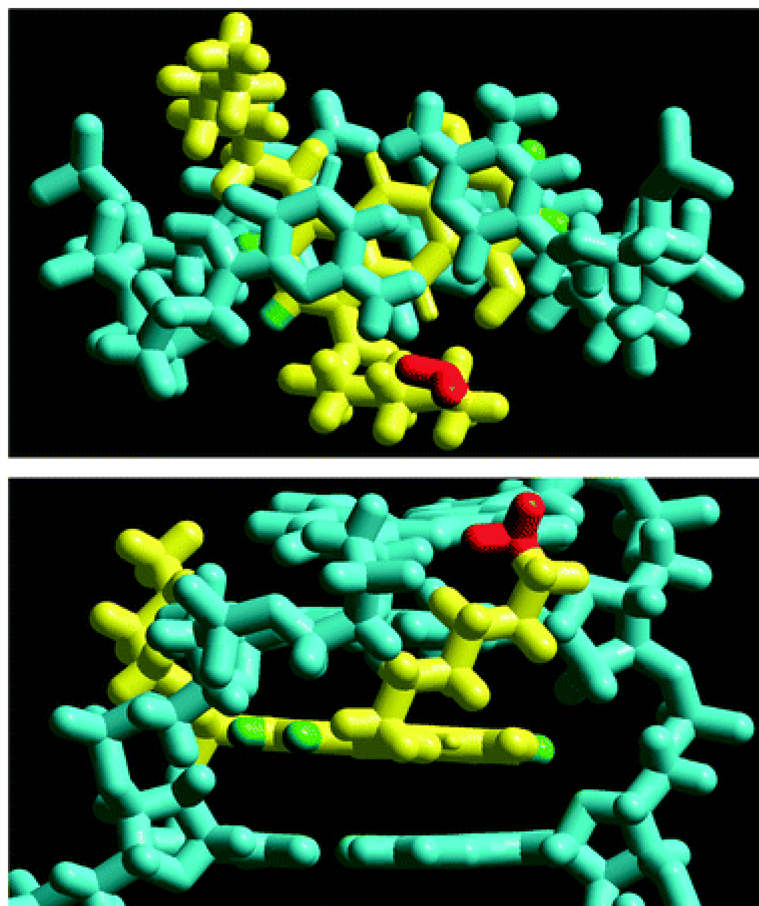

Fig. 5 Energy minimized HyperChem model of the WEHI-150 covalent adduct with d(CG^{5Me}CGCG)$_2$. The DNA is shown in cyan, the WEHI-150 in yellow, the WEHI-150 H2, H3 and H6/H7 protons in green and the CH_2 derived from formaldehyde in red. The model shows one side-chain located in the major groove and other side-chain in the minor groove.

The structures of formaldehyde-cross-linked adducts of the anthracyclines daunorubicin and doxorubicin with oligonucleotides have been previously reported;[18,19] however, this study reports for the first time (to our knowledge) the structure of a mitoxantrone analogue–DNA covalent adduct. Although the anthracycline–DNA adducts contain a CH_2 link to the N-2 of a guanine residue, the polycyclic ring system is positioned perpendicular to the base pairs at the intercalation site in a similar manner to that found for the reversibly bound drugs.[20] Furthermore, the entire covalently bound anthracycline drug resides in the DNA minor groove.

The results of this study indicate that WEHI-150 remains intercalated at the ^{5Me}CpG site and forms a covalent link through a methylene group to the N2-amino group of a guanine residue adjacent to the

intercalation site. However, and interestingly, the NMR results were only consistent with a threaded structure where the WEHI-150 has one side-chain in the major groove and the other in the minor groove. Furthermore, the results clearly demonstrated that the primary amine of WEHI-150 was significantly more reactive than the secondary amine. This suggests a mitoxantrone derivative with an amine in each side-chain, with extended methylene chains, might be the optimal anthracenedione. Furthermore, the addition of asymmetric side-chains may yield an anthracenedione capable of facilitating dual covalent bond formation, and thus higher stability as well as posing a greater challenge for cellular DNA repair responses. In particular, our work raises the likelihood that the threaded structure is capable of covalent linkages in both the DNA minor and major grooves. In a previous study,[10] it was found that one WEHI-150 could form adducts with one, two and even three CH_2 covalent links to an oligonucleotide. Consequently, it is likely that one of the amine groups located in the major groove by the threaded structure can form a covalent link, after activation by formaldehyde, with a nucleophilic nitrogen (*e.g.* the N7 of a guanine residue) in the major groove. Additionally, in order to potentially increase the proportion of such a major–minor groove covalent cross-linking drug, novel mitoxantrone analogues can now be envisioned that contain an electrophilic carbon on one side-chain (through replacement of the terminal amine with a CH_2-Cl) that would more readily react with nucleophilic groups in the DNA major groove.

In conclusion, the threaded adduct structure we have observed for the mitoxantrone analogue WEHI-150, and the proposed major–minor groove cross-linked adduct (a new type of drug-DNA binding motif) will likely produce considerably different intracellular biological properties compared to anthracycline–DNA covalent adducts. For example, the stabilised threaded structure is more likely to inhibit transcription due to interfering with the binding of transcription factors in the DNA major groove.

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Footnote

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6ob02100j

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