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Formaldehyde-Activated Pixantrone Is a Monofunctional DNA Alkylator That Binds Selectively to CpG and CpA Doublets

Benny J. Evison, Francis Chiu, Gabriella Pezzoni, Don R. Phillips, and Suzanne M. Cutts

Department of Biochemistry, La Trobe University, Victoria, Australia (B.J.E, D.R.P., S.M.C);
Centre for Drug Candidate Optimisation, Victorian College of Pharmacy, Monash University, Parkville, Victoria, Australia (F.C); and Cell Therapeutics Europe, Bresso, Italy (G.P.)

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ABSTRACT

The topoisomerase II poison mitoxantrone is important in the clinical management of human malignancies. Pixantrone, a novel aza-anthracenedione developed to improve the therapeutic profile of mitoxantrone, can efficiently alkylate DNA after formaldehyde activation. In vitro transcriptional analysis has now established that formaldehyde-activated pixantrone generates covalent adducts selectively at discrete CpG or CpA dinucleotides, suggesting that the activated complex binds to guanine or cytosine (or both) bases. The stability of pixantrone adduct-induced transcriptional blockages varied considerably, reflecting a mixture of distinct pixantrone adduct types that may include relatively labile monoadducts and more stable interstrand cross-links. 6,9-Bis-[[2-(dimethylamino)ethyl]amino]benzo[*g*]isoquinoline-5,10-dione (BBR 2378), the dimethyl *N*-substituted analog of pixantrone, could not form adducts, suggesting that pixantrone alkylates DNA through the primary amino functions located in each side chain of the drug. Pixantrone generated DNA adducts only when guanine was present in substrates and exhibited a lack of adduct formation with inosine-containing polynucleotides, confirming that the N2 amino group of guanine is the site for covalent attachment of the drug. Mass spectrometric analysis of oligonucleotide-drug complexes confirmed that formation of covalent pixantrone-DNA adducts is mediated by a single methylene linkage provided by formaldehyde and that this occurs only with guanine-containing double stranded oligonucleotide substrates. CpG methylation, an epigenetic modification of the mammalian genome, significantly enhanced the generation of pixantrone-DNA adducts within a methylated DNA substrate, indicating that the methylated dinucleotide may be a favored target in a cellular environment.

Agents that covalently modify DNA occupy an important niche in the treatment of human malignancies. The DNA alkylators induce a range of lesion types, including monofunctional adducts, intra- and interstrand cross-links, and DNA protein cross-links. It is noteworthy that the cellular responses elicited by each of these lesion types is clearly distinct; consequently, a knowledge of adduct structure is crucial to fully exploit the DNA-damaging properties of a given alkylator.

DNA-damaging agents characteristically impair normal cellular processes, including DNA replication, transcription, and repair, and provide significant clinical effectiveness in the treatment of human cancer. Although some DNA-interactive agents currently used in the clinic exhibit modest sequence selectivity (e.g., highly reactive alkylating agents), others, such as anthracyclines, display a clearly defined DNA sequence preference that may affect cancer cell selectivity (DeVita et al., 2001; Hurley, 2002). Doxorubicin and its structural analog mitoxantrone are among the most clinically important representatives of this group, and function as potent topoisomerase II poisons via their ability to intercalate tightly within DNA (Capranico and Binaschi, 1998; Palumbo et al., 2002). Initial studies that probed the sequence preference of each drug yielded conflicting results; however, a general consensus for nucleic acids containing alternating sequences, particularly GC base pairs, was achieved for both drugs (DuVernay et al., 1979; Lown et al., 1985). Footprinting analyses demonstrated that both drugs preferentially intercalate at discrete pyrimidine (3′–5′) purine sequences. A clear specificity for 5′-CpA dinucleotide sequences was detected for both intercalators, with mitoxantrone also favoring 5′-CpG doublets (Fox et al., 1986; Trist and Phillips, 1989; Panousis and Phillips, 1994).

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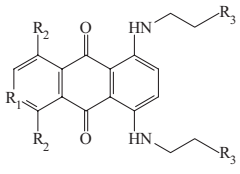
ABBREVIATIONS: BBR 2778, pixantrone (6,9-bis[(2-aminoethyl)amino]benzo[g]isoquinoline-5,10-dione); BBR 2378, 6,9-bis[[2-(dimethylamino)ethyl]amino]benzo[*g*]isoquinoline-5,10-dione; doxorubicin, (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxohexopyranosyl)oxy]-8-glycolyl-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione; mitoxantrone, 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione; bp, base pair(s); PBS, phosphate-buffered saline; ds, double stranded; FLT, full-length transcript.

A novel form of doxorubicin-DNA interaction was discovered after the identification of binding sites additional to the CpA doublet recognized by intercalated doxorubicin. This form of DNA interaction was distinctive because the DNA lesion exhibited vastly greater stability and sequence selectivity for GpC (van Rosmalen et al., 1995). Subsequent studies revealed that the unique lesion was a covalently bound doxorubicin-DNA monoadduct mediated through the primary amino group of the drug. The adduct was stabilized by partial intercalation within the DNA duplex and strong hydrogen bonding to the opposite, noncovalently bound strand (Wang et al., 1991; Taatjes et al., 1997; Zeman et al., 1998). A central theme of this work was that the covalent linkage was mediated by formaldehyde and constituted an absolute requirement for the generation of doxorubicin-DNA adducts. Mitoxantrone undergoes reaction with formaldehyde to generate an analogous covalent DNA adduct. In vitro transcription analysis revealed that these mitoxantrone-DNA adducts formed primarily at CpG and CpA dinucleotides (Parker et al., 2000), consistent with the specificity of intercalated mitoxantrone (Panousis and Phillips, 1994).

The considerable clinical utility of doxorubicin and mitoxantrone has been tempered chiefly by the development of cardiomyopathies and drug resistance in patients (Faulds et al., 1991; DeVita et al., 2001). These deleterious consequences have prompted a search for novel anthracycline and anthracenediones that possess improved therapeutic efficacy, together with reduced side effects. Among the most promising anthracenedione analogs to emerge from these studies is pixantrone (also known as BBR 2778) (Fig. 1), a 2-aza-anthracenendione that is devoid of cardiotoxicity (Cavalletti et al., 2007) and is progressing through phase III clinical trials for the treatment of aggressive non-Hodgkin's lymphoma (Engert et al., 2006). Like its parent compound mitoxantrone, pixantrone intercalates within DNA and impairs topoisomerase II activity by stimulating enzyme-mediated DNA cleavage (De Isabella et al., 1995). Formaldehyde-activated pixantrone can covalently bind to DNA more efficiently than the analogous mitoxantrone reaction (Evison et al., 2007). Pixantrone possesses primary amino functionalities in both side arms, and this seems to provide an improvement over the secondary amino functionalities inherent to mitoxantrone.



Compound	R_1	R_2	R_3
Mitoxantrone	CH	OH	$NH(CH_2)_2OH$
Pixantrone	N	H	NH_2
BBR 2378	N	H	$N(CH_3)_2$

Fig. 1. The chemical structures of the anthracenediones studied.

In an effort to characterize this novel drug-DNA interaction, the sequence specific binding of formaldehyde-activated pixantrone to DNA was the subject of this investigation. Modified DNA substrates and a pixantrone derivative devoid of a primary amino functionality were also used to probe the nature of the interaction. In theory, pixantrone has the potential to generate two distinct types of formaldehyde-mediated lesion, given that it possesses two primary amino groups, one at the distal end of each side chain. Covalent binding through either or both side chains of the drug would permit the generation of a monofunctional adduct or interstrand cross-link, respectively. Given the potential for two distinct structures, the focus of the present study was to characterize the genuine form of the pixantrone-DNA adduct and its structural requirements. Taken together, the results have provided insight into the structure, sequence specificity, and stability of these unique pixantrone-DNA adducts.

Materials and Methods

Drugs and Reagents. Pixantrone, [^{14}C]pixantrone (21.5 mCi/mmol), and BBR 2378 were kindly supplied by Cell Therapeutics Europe (Bresso, Italy), whereas doxorubicin was a gift from Pfizer (Milan, Italy), and mitoxantrone was purchased from Sigma. Formaldehyde solution [40% (v/v)] was obtained from British Drug House (Poole, Dorset, UK). A Maxi Plasmid Purification Kit was purchased from QIAGEN (Valencia, CA). Ultrapure NTPs and the synthetic polynucleotides poly(dI-dC) and poly(dG-dC) were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). *Escherichia coli* RNA polymerase and glycogen were obtained from Roche, whereas SssI methylase and *S*-adenosylmethionine were from New England Biolabs (Ipswich, MA).

Pixantrone, mitoxantrone, and doxorubicin stock solutions were prepared by dissolving each in Milli-Q water (Millipore, Billerica, MA) to an approximate concentration of 2 mM. BBR 2378 was prepared by dissolving solid crystals in dimethyl sulfoxide and subsequently storing the solution at 4°C. Accurate concentrations of each drug were determined spectrophotometrically using $\varepsilon^{1\%}_{1\ cm}$ = 296 at 641 nm, ε = 19,200 $M^{-1}\ cm^{-1}$ at 608 nm, and ε = 11,500 $M^{-1}\ cm^{-1}$ at 480 nm for pixantrone, mitoxantrone, and doxorubicin, respectively. Formaldehyde solutions were freshly prepared on each day of use.

In Vitro Transcription Assay. An ultrapure 512-bp fragment containing the *lac* UV5 promoter from the plasmid pCC1 was prepared by electroelution and phenol/chloroform extraction. Covalent drug-DNA adducts were generated in a reaction mixture consisting typically of the 512-bp DNA fragment (25 μM_{bp}), formaldehyde, and either pixantrone, BBR 2378, mitoxantrone, or doxorubicin in PBS, pH 7.0, at 37°C. Drug-reacted DNA was subsequently ethanol-precipitated to remove unreacted formaldehyde and then resuspended in 1× transcription buffer consisting of 40 mM Tris, pH 8.0, 100 mM KCl, 3 mM $MgCl_2$, and 0.1 mM EDTA. The in vitro transcription assay employing RNA polymerase transcription from the *lac* UV5 promoter was performed as described previously (Phillips et al., 2001). Each reaction was terminated by the addition of an equal volume of loading/termination buffer (9 M urea, 10% sucrose, 40 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue in 2× Tris-borate/EDTA). All transcription samples were denatured at 90°C for 5 min and immediately quenched on ice. Transcripts were subsequently resolved by electrophoresis in 12% denaturing polyacrylamide gels and detected by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analysis. Band quantitation was performed using ImageQuant software (Molecular Dynamics).

Sequencing of RNA Transcripts. The sequences of RNA transcripts were determined by incubating nondrug treated initiated transcription complexes with chain-terminating 3′-*O*-methyl nucle-

otides in the elongation phase of transcription as described previously (Phillips et al., 2001). Transcriptional elongation was subsequently terminated by the addition of an equal volume of loading/termination buffer and the samples denatured and subjected to PAGE as described above.

In Vitro Cross-Linking Assay. The pCC1 plasmid was linearized with HindIII and 3′-end-labeled as described previously (Evison et al., 2007). Multiple reactions were run in parallel, each containing end-labeled DNA (25 μM_{bp}), 2 mM formaldehyde and either pixantrone (0–20 μM) or BBR 2378 (0–100 μM) in PBS, pH 7.0. Reactions were run at 37°C for 4 h, phenol/chloroform-extracted, and subsequently prepared for electrophoresis as described previously (Evison et al., 2007). Samples were subjected to electrophoresis through 0.8% agarose gels overnight at 30 to 40 V in 1× Tris-acetate/EDTA buffer. Agarose gels were then dried and analyzed by PhosphorImager as described above.

[^{14}C]Pixantrone Studies. The synthetic polynucleotides poly(dI-dC) and poly(dG-dC) were initially dissolved in 1× Tris/EDTA buffer. The concentration of DNA was determined spectrophotometrically using ε = 6900 M^{-1} cm^{-1} at 251 nm and ε = 8400 M^{-1} cm^{-1} at 254 nm for poly(dI-dC) and poly(dG-dC), respectively. [^{14}C]Pixantrone-polynucleotide adducts were generated by incubating each alternating copolymer (25 μM_{bp}) with 2 mM formaldehyde and 0–20 μM pixantrone overnight at 37°C. The reaction was terminated and the DNA purified using a phenol/chloroform extraction followed by an ethanol precipitation. Once resuspended in Tris/EDTA buffer, samples were mixed with Ready Safe liquid scintillation cocktail (Beckman Coulter Australia, Sydney, Australia) and the ^{14}C counts determined using a Wallac 1410 liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Preparation of Drug-Reacted Oligonucleotides. The sequences of oligonucleotides (purchased from Geneworks, Thebarton, South Australia) were as follows: BE1, 5′-TTATTTCCGGCGCCTTATTT; BE2, 5′-AAATAAGGCGCCGGAA-
ATAA; BE4, 5′-TTATTTCCIGCICCTTATTT; and BE5, 5′-AAATAAG-GCICCIGAATAA. Oligonucleotides were purified through denaturing polyacrylamide gels, overnight elution, and subsequent concentration using a Centricon Centrifugal Filter Unit (molecular mass cut-off, 3 kDa) from Millipore. T4 polynucleotide kinase was employed to label the 5′ terminus of oligonucleotides BE1 or BE4 using standard procedures. Unincorporated label was subsequently removed by centrifugation of the sample through a Micro Bio-Spin 6 chromatography column (Bio-Rad Laboratories, Hercules, CA). Labeled oligonucleotide (BE1 and BE4) was added to its nonradiaoctive complement (e.g., labeled BE1 and unlabeled BE2) at a ratio of 1:1 in PBS, pH 7.0. The final mixture was heated to 80°C for 5 min and then cooled slowly to room temperature to facilitate hybridization of duplex DNA. Covalent drug-DNA adducts were generated in a reaction mixture typically consisting of DNA, pixantrone, and formaldehyde in PBS, pH 7.0, at 37°C.

Oligonucleotide Band Shift Assay. After reaction with pixantrone and formaldehyde, ^{32}P end-labeled oligonucleotide samples were denatured in 2 volumes of loading dye (90% formamide, 10 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol) and loaded onto a pre-electrophoresed 19% denaturing polyacrylamide gel. Electrophoresis was performed overnight at 600 V at room temperature. Gels were subsequently fixed in 10% glacial acetic acid/10% methanol, exposed to a PhosphorImaging plate for 2 h, and then quantitated as described previously.

Mass Spectrometry Sample Preparation. Drug-reacted oligonucleotide samples consisted of 50 μM unlabeled ds oligonucleotide, 100 μM pixantrone, and 10 mM formaldehyde in a 500-μl reaction volume. After drug treatment, samples were directly loaded into the trap chamber of an Elutrap Electroelution apparatus (Whatman, Maidstone, UK) immersed in 1× Tris-borate/EDTA. Samples were subsequently electrodialysed to maximize the removal of noncovalently bound drug by applying 100 V for 2 h at 4°C. The purified covalent drug-DNA complex was subsequently filtered via centrifugation and desalted four times into 10 mM ammonium acetate by gel filtration (Micro Bio-Spin 6 chromatography column).

Electrospray Ionization Mass Spectrometry. Mass spectrometry was performed on a micro quadrupole time-of-flight mass spectrometer (Micromass; Waters Corp., Milford, MA) operating in positive mode electrospray ionization at a capillary voltage of 3.2 kV and a cone voltage of 30 V. A desolvation temperature of 250°C, a source block temperature of 90°C, a desolvation gas flow of 250 l/h, and a cone gas flow of 50 l/h were maintained during analysis. Mass calibration was performed over the range of 100–4000 mass units using a sodium iodide-cesium iodide mixture. Samples were introduced by direct infusion at 5 μl/min using a syringe pump. Mass spectra were acquired from 1000 to 3000 mass units at a repetition rate of 1 s for 2 to 3 min per sample. The spectra with multiple charged ions were deconvoluted using MaxEnt to yield the molecular weight of the duplex DNA and adducts.

Preparation of Methylated DNA. The DNA modifying enzyme SssI methylase was used to methylate all cytosine residues within its recognition sequence CpG. Linearized pCC1 plasmid was treated with SssI methylase and 640 μM *S*-adenosyl-methionine overnight at 37°C and then purified using a standard phenol/chloroform extraction and ethanol precipitation. The DNA was subsequently challenged with HpaII, a methylation-sensitive restriction endonuclease. High levels of methylation were confirmed by the inability of the enzyme to cleave the linearized plasmid. The methylated DNA, which was 3′-end labeled, was subsequently used for in vitro cross-linking assays.

Results

Transcriptional Detection of Pixantrone-DNA Adducts. The in vitro transcription assay resulted in an accumulation of truncated transcripts that arose from the impairment of RNA polymerase movement along double-stranded DNA by drug-DNA lesions. The length of each drug-induced truncated transcript provides a direct measure of the location of the lesion on the DNA template. In the absence of drug-induced blockages, RNA polymerase can progress through the DNA template to yield a full-length transcript (FLT) that is 379 nucleotides in length. At first, it was necessary to establish that the in vitro transcription assay was appropriate for the detection of formaldehyde-activated pixantrone-DNA lesions and, for this purpose, a pixantrone concentration-dependence assay was used. Before transcription, the 512-bp fragment containing the *lac* UV5 promoter was reacted with 2 mM formaldehyde and increasing concentrations of pixantrone. In the absence of pixantrone, RNA polymerase transcribed through the DNA template efficiently to yield FLT, indicating that formaldehyde alone was insufficient to induce any transcriptional blockages (Fig. 2A, lane 0). The progression of RNA polymerase through each pixantrone-reacted DNA template was increasingly impaired at specific sites with increasing concentrations of pixantrone. The drug-induced blockage at truncated transcript 52 (denoted by an asterisk at the left of Fig. 2A) was quantitated and is represented as a function of pixantrone concentration in Fig. 2B. This graph shows a linear increase in blockage frequency that was followed by saturation beyond 10 μM pixantrone together with a concomitant loss of FLT (Fig. 2C). The involvement of formaldehyde in the generation of drug-induced transcriptional blockages was subsequently investigated. As increasing concentrations of formaldehyde were introduced to the reaction, a clear concentration-dependent increase in discrete pixantrone-induced transcriptional block-

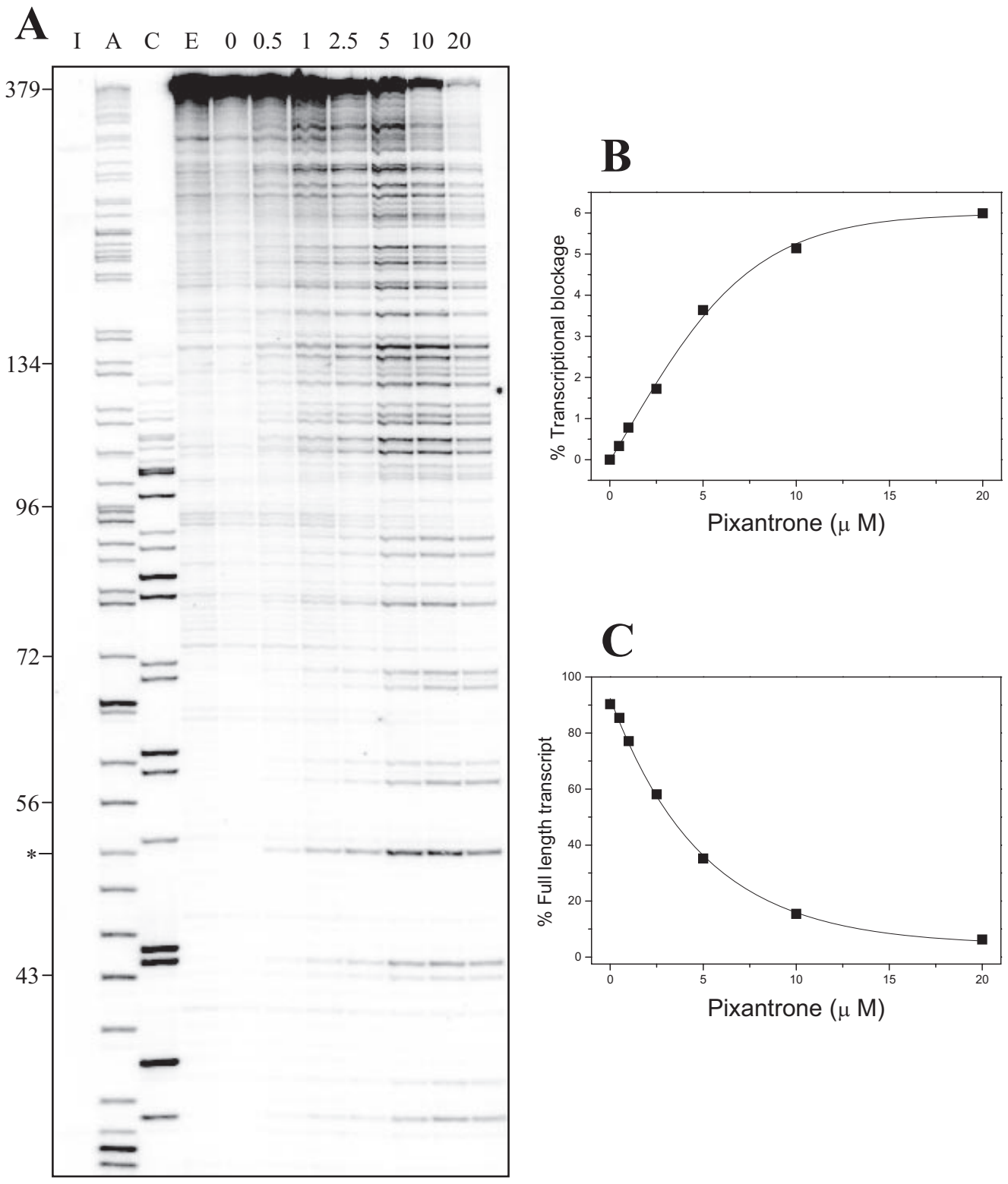


Fig. 2. Drug-induced transcriptional blockages are dependent upon the concentration of pixantrone. A, a 512-bp DNA fragment containing the *lac* UV5 promoter was incubated with 2 mM formaldehyde and 0 to 20 μM pixantrone for 4 h. After ethanol precipitation, the drug-reacted 512-bp fragment was subjected to transcription. Transcripts were initiated from the *lac* UV5 promoter, allowed to elongate for 5 min, and then terminated. Lane I is an initiation control that represents initiated transcripts that have not been elongated; lane E denotes the elongation of this complex to full-length transcript. Lanes A and C are sequencing lanes using the chain terminators 3′-*O*-methoxy-ATP or 3′-*O*-methoxy-CTP, respectively. The length of particular transcripts is shown on the left side of the PhosphorImage. B, the percentage transcriptional blockage of 52-mer (indicated by an asterisk at the left-hand side of A) was quantitated and is expressed as a function of pixantrone concentration. C, the drug-induced decay of full-length transcript expressed as a function of pixantrone concentration.

ages was evident, and when formaldehyde was entirely absent, transcriptional blockages were not obtained (data not shown). This trend supports the idea that an absolute requirement exists for formaldehyde in the production of pixantrone-DNA adducts. The time-dependence of pixantrone-DNA adduct formation was also investigated for defined time periods up to 8 h. Increased times of drug reaction resulted in increasing levels of transcriptional blockages with maximal blockages produced after approximately 4 h (data not shown).

Sequence Specificity of Transcriptional Blockages. The presence of discrete transcriptional blockages induced by the combination of pixantrone and formaldehyde suggests that the pixantrone-DNA adduct may demonstrate a degree of sequence specificity. In parallel with transcriptional blockages induced by pixantrone-DNA adducts, in vitro transcription was also performed on templates reacted with formaldehyde and either mitoxantrone or doxorubicin, two drugs that form sequence selective adducts with DNA after activation by formaldehyde (van Rosmalen et al., 1995; Parker et al., 2000). The mole fraction of each blocked transcript was quantitated and the sequence selectivity of these drugs is summarized in Fig. 3, A–C. The histogram (Fig. 3B) reveals that of the 12 most intense transcriptional blockages induced by formaldehyde-activated pixantrone, 58% (7 of 12) involve transcriptional inhibition at 5′-CpG dinucleotide sequences, whereas all of the remaining intense blockage sites (42%; 5 of 12) occurred at CpA doublets. Transcriptional blockages induced by formaldehyde-activated pixantrone are strikingly similar to those created by mitoxantrone (Fig. 3A). For mitoxantrone, each of the 12 sites with the highest intensity block occur at either CpG (50%; 6 of 12) or CpA (50%; 6 of 12) dinucleotides. The sequence selectivity of formaldehyde-activated doxorubicin (Fig. 3C) contrasts sharply with the CpG and CpA consensus sequence demonstrated by pixantrone and mitoxantrone. Doxorubicin exhibited a strong selectivity for GpC doublets as documented previously (van Rosmalen et al., 1995), the five most intense transcriptional blockages occurring exclusively at this sequence (Fig. 3C).

A Lack of Drug-DNA Adduct Formation by the Pixantrone Analog BBR 2378. The ability of the pixantrone analog BBR 2378 (Fig. 1) to stabilize duplex DNA in the presence of formaldehyde was explored using an in vitro cross-linking assay (Fig. 4). The characteristic stabilization of dsDNA by pixantrone-DNA adducts was clearly evident, yet its close structural analog BBR 2378 was unable to stabilize duplex DNA even at a concentration up to 100 μM. Consequently, it can be concluded that BBR 2378 was unable to exhibit adduct formation in this assay. Despite the inability of BBR 2378 to stabilize dsDNA in the in vitro cross-linking assay, the potential of BBR 2378 to generate monoadducts remained a possibility because the assay is not ideal for the rigorous measurement of drug-DNA adducts that do not stabilize dsDNA. Given its greater sensitivity in the detection of monoadducts, the in vitro transcription assay was used to investigate the potential of BBR 2378 to form such DNA lesions. DNA templates were reacted with 2 mM formaldehyde and BBR 2378 up to 100 μM. No transcriptional blockages were induced by BBR 2378 at any drug concentration examined (data not shown). These results provide convincing evidence that BBR 2378 is incapable of forming formaldehyde-mediated DNA adducts.

Temporal Stability of Pixantrone-DNA Adducts at 37°C. Previous characterization of pixantrone-DNA adducts using an in vitro cross-linking assay established that the lesion is intrinsically unstable (Evison et al., 2007). The in vitro transcription assay provides an ideal alternative for the assessment of adduct lability at clearly defined DNA sites. Loss of drug-DNA adducts during transcription enables the progression of RNA polymerase past the lesion, yielding a clear decay of discrete transcriptional blockages with time. After reaction with pixantrone and formaldehyde, the drug-reacted 512-bp DNA fragment was subjected to transcriptional elongation for up to 4 h (Fig. 5). Isolated transcriptional blockages, such as the 52-mer and 59-mer, decayed with increasing elongation times. In contrast, some sites (e.g., the 108-mer) seemed resistant to decay, and the blockage of RNA polymerase progression persisted for up to 4 h. The intensities of five distinct transcriptional blockages were subjected to first-order kinetic analysis (Fig. 5B). The gradient of each plot yielded the dissociation rate constant for the loss of drug from each site. These dissociation rates (summarized in Table 1) vary considerably from 30 min to greater than 3 h and are a reflection of the wide-ranging stabilities of pixantrone-DNA adducts at distinct binding sites.

Analysis of Pixantrone-DNA Adduct Formation with Modified DNA Substrates. The role of the N2 of guanine in pixantrone-DNA adduct formation was examined by comparing the binding of formaldehyde-activated [^{14}C]pixantrone to poly(dG-dC) and poly(dI-dC) substrates. The level of incorporation of radiolabeled drug into each polynucleotide is shown in Fig. 6A, where there is a large and dose-dependent incorporation of pixantrone into poly(dG-dC) but not poly(dI-dC). Two duplex oligonucleotides were prepared to further characterize formaldehyde-activated pixantrone binding to DNA. Oligonucleotide duplex dsBE12 was designed to include two CpG doublets that could potentially accommodate a single pixantrone adduct at each CpG site. An identical duplex, dsBE45, was also constructed in which the guanine residue of each CpG step was substituted for inosine. As shown in Fig. 6B, drug-induced stabilization of duplex dsBE12 was clearly evident, whereas no detectable effect was obvious with ds BE45 at any of the drug concentrations examined. The doxorubicin-reacted controls (lanes X and Y, Fig. 6B) confirmed that both duplexes remained double-stranded throughout reaction with these drugs.

Mass Spectrometric Analysis of the Covalent Pixantrone-DNA Complex. The molecular composition of the covalent pixantrone-DNA complex was investigated using electrospray ionization mass spectrometry. It was necessary to establish that the technique was suitable for the detection of dsBE12 alone. Upon subjection to mass spectrometry, dsBE12 was detected predominantly as a single peak (Fig. 7A) associated with an observed mass of 12,233 Da, and this is consistent with the putative mass of the protonated form of the duplex (expected mass, 12,232 Da). A minor peak was evident in the mass spectrum with a mass marginally greater (21 Da) than duplex alone. It is most likely that this peak corresponds with dsBE12 complexed with a single sodium cation. Cations were exceedingly difficult to completely eliminate from each reaction sample and therefore remain a consistent yet minor feature of the mass spectra. Given its reactive nature, formaldehyde (10 mM) was included in a second control reaction with duplex dsBE12 to establish

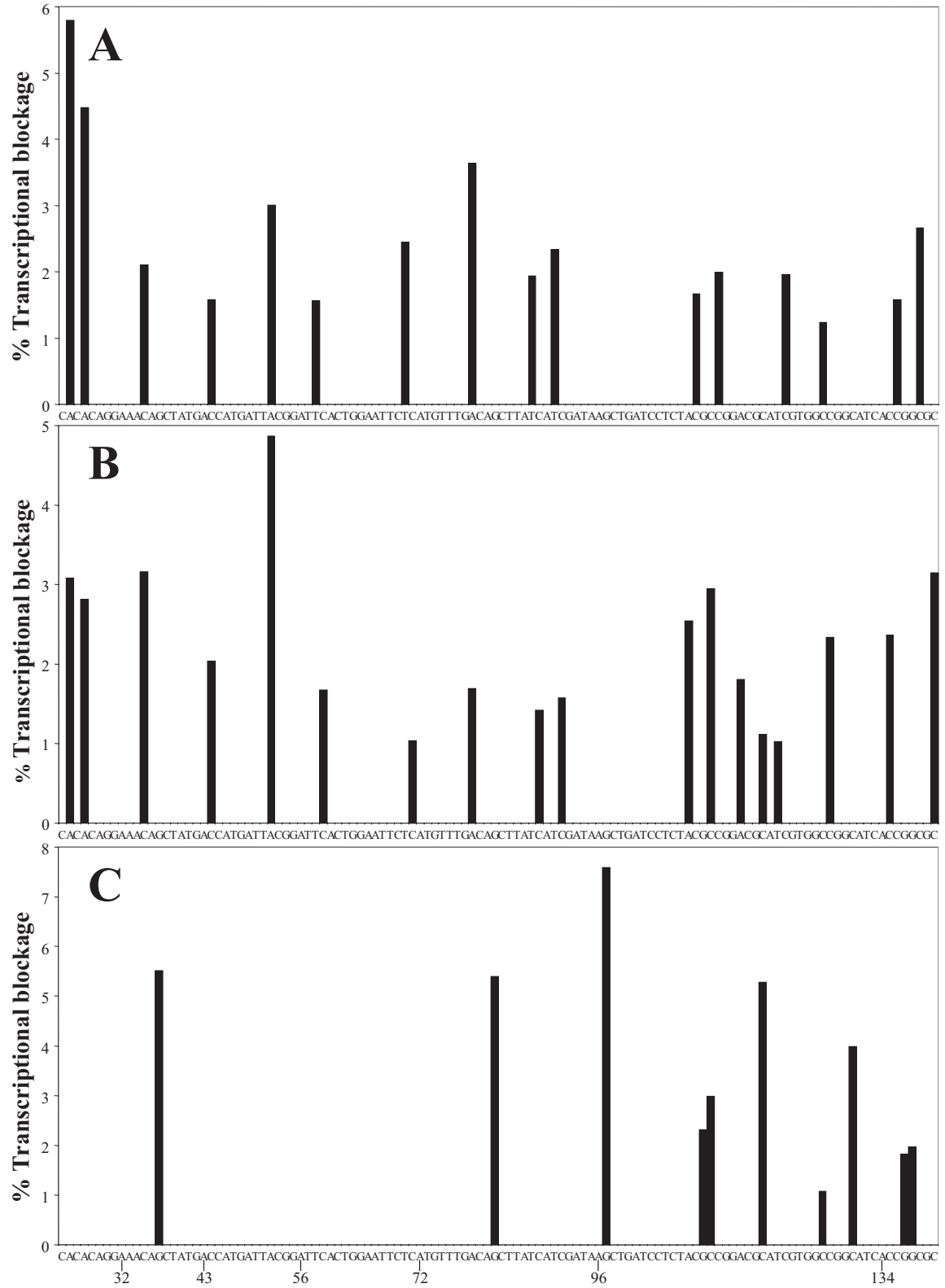


Fig. 3. A direct comparison of drug-induced transcriptional blockages generated by formaldehyde-activated mitoxantrone, pixantrone, and doxorubicin. Multiple reactions were run in parallel, each containing the 512-bp DNA fragment and either of the following: 0 to 20 μM mitoxantrone and 5 mM formaldehyde, 0 to 5 μM pixantrone and 2 mM formaldehyde, or 0 to 1 μM doxorubicin and 1 mM formaldehyde. Each reaction was incubated for 4 h, ethanol precipitated, resuspended, and subjected to transcription. The sequence selectivity of formaldehyde-activated mitoxantrone (A), pixantrone (B), and doxorubicin (C) is depicted in the histograms. The percentage transcriptional blockage of each transcript in either the 20 μM mitoxantrone, 5 μM pixantrone, or 0.25 μM doxorubicin reactions was quantitated and is expressed as a function of the sequence of a portion of the 512-bp DNA fragment. The sequence of the nontemplate DNA strand is shown. Transcriptional blockages less than an arbitrary value of 1% have been omitted. The numbers at the bottom of histogram (C) indicate the length of transcripts.

whether the agent alone was sufficient to covalently alkylate duplex DNA (Fig. 7B). A major peak was again observed at 12,233 Da, suggesting that duplex dsBE12 remained largely intact in the presence of formaldehyde. Several peaks displaying a slightly greater mass than duplex alone are visible, indicating the presence of formaldehyde-DNA adducts, but these peaks are minor relative to the larger peak representing duplex DNA. A third control reaction containing dsBE12 and 100 μM pixantrone was performed next to establish the mass spectral pattern induced by intercalated pixantrone. Mass spectrometric analysis of this complex yielded a spectrum displaying the original oligonucleotide duplex at 12,233 Da and three novel peaks with masses increasing in multiples of 325 Da. This mass corresponds to the molecular mass of pixantrone (expected mass, 325 Da) and reflects the intercalative binding by one, two, or three pixantrone molecules to dsBE12 (data not shown).

To characterize the molecular nature of the covalent bond(s) that stabilize the pixantrone-DNA adducts, dsBE12 was reacted with pixantrone and formaldehyde. The mass spectrum displayed three prominent peaks (Fig. 7C). The first and smallest in mass at 12,232 Da is consistent with duplex dsBE12. A second peak at 12,557 Da corresponds well with a single pixantrone molecule intercalated within dsBE12 (expected mass, 12,557 Da). A third and equally conspicuous peak was evident at 12,569 Da, just 12 Da greater than its neighbor. This mass increase is consistent with the incorporation of a single methylene unit that is derived from formaldehyde and covalently tethers dsBE12 to a single pixantrone molecule (expected mass, 12,569 Da). The molecular composition of the covalent pixantrone-DNA adduct was scrutinized further by substituting oligonucleotide duplex dsBE12 for dsBE45 in the drug reaction mix. Duplex dsBE45, which incorporates an inosine residue in place of guanine at both CpG doublets of the oligonucleotide duplex, was reacted with formaldehyde and pixantrone. The mass spectrum of the drug-oligonucleotide complex (Fig. 7D) shows multiple peaks corresponding to either duplex dsBE45 alone (observed mass, 12,170 Da; expected mass, 12,172 Da) or two distinct pixantrone-dsBE45 intercalation complexes, one containing a single pixantrone molecule (observed mass, 12,496 Da; expected mass, 12,497 Da) and the other accommodating two drug molecules (observed mass, 12,821 Da; expected mass, 12,822 Da). The most important feature of this spectrum is the absence of a prominent peak that could reasonably represent a covalent pixantrone-dsBE45 adduct (expected mass, 12,509).

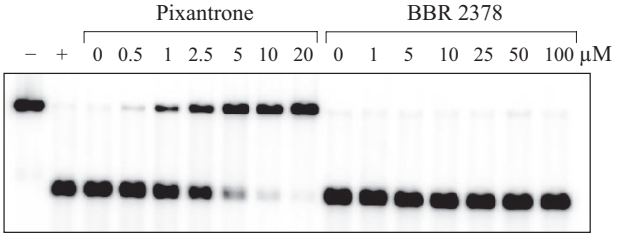


Fig. 4. Formaldehyde-activated pixantrone stabilizes dsDNA in denaturing conditions, yet BBR 2378 does not. Multiple reactions containing end-labeled DNA, 2 mM formaldehyde, and either pixantrone (0–20 μM) or BBR 2378 (0–100 μM) were incubated for 4 h at 37°C. After a routine phenol/chloroform extraction, samples were ethanol-precipitated, resuspended, and denatured in preparation for electrophoresis as described under *Materials and Methods*. Control single-stranded DNA (+) was generated by subjecting unreacted DNA to thermal denaturation, whereas the corresponding dsDNA control (−) was not thermally denatured.

Although a peak possessing this mass is present (observed mass, 12,508 Da), it most likely represents a nonspecific formaldehyde adduct because the peak representing duplex dsBE45 alone (which has no prospect of forming a pixantrone adduct) is associated with a similar cocontaminant.

Global CpG Methylation by SssI Methylase Enhances Pixantrone-DNA Adduct Formation. The in vitro cross-linking assay was employed to directly assess the influence of CpG methylation on the stabilization of dsDNA by formaldehyde-activated pixantrone in vitro. Incubation of unmethylated and SssI methylase-treated DNA samples with formaldehyde and increasing concentrations of pixantrone facilitated a dose-dependent resistance to DNA duplex separation (Fig. 8A), consistent with the generation of covalent DNA adducts by formaldehyde-activated pixantrone. Quantitation of these bands (Fig. 8B) demonstrated that 50% stabilization of CpG-methylated DNA was achieved at approximately 0.6 μM pixantrone. By comparison, unmethylated DNA required a pixantrone concentration far in excess of 2.5 μM to generate a similar level of duplex stabilization (Fig. 8B), indicating that DNA methylation significantly enhances the capacity of formaldehyde-activated pixantrone to generate DNA adducts.

The reaction of formaldehyde-activated pixantrone with CpG-methylated DNA was analyzed in greater detail by following the course of adduct-induced stabilization of duplex DNA with time. Both native and methylated DNA samples were incubated with pixantrone (1 μM) in the presence of formaldehyde for defined time periods up to 8 h. Drug-induced alkylation of methylated DNA was far more rapid compared with native DNA, and this yielded a considerable increase in adduct levels (Fig. 8C). Stabilization of duplex DNA was quantitated and revealed that 50% stabilization of CpG-methylated DNA was attained within 30 min, yet only 15% of native DNA was stabilized after 8 h of reaction time (Fig. 8D). Clearly, then, DNA methylation at CpG sequences greatly enhanced the alkylation of DNA by formaldehyde-activated pixantrone.

Discussion

A novel pixantrone-DNA interaction was discovered when it was established that the drug can covalently bind to DNA after extrinsic activation by formaldehyde (Evison et al., 2007). The characterization of novel DNA-ligand interactions represents an important initial step in understanding the biochemical and biological responses induced by the ligand (Hurley, 1989). Moreover, this knowledge provides guidance in the rational design of analogs with enhanced activities (David-Cordonnier et al., 2002). Given the invaluable nature of this knowledge, the structural characterization of formaldehyde-mediated pixantrone-DNA adducts was the primary focus of the present study.

The involvement of formaldehyde in the pixantrone-DNA interaction was confirmed by an absolute dependence of transcriptional inhibition on formaldehyde. The disruption of a crucial DNA-based process such as transcription will have a significant effect on cell viability. Thus, DNA damage caused by formaldehyde-activated pixantrone-DNA adducts may ultimately contribute toward the cytotoxicity of the drug. A distinctive feature of each transcriptional blockage induced by pixantrone-DNA adducts is that they occurred in a sequence-dependent manner. The sequence-selective blockage

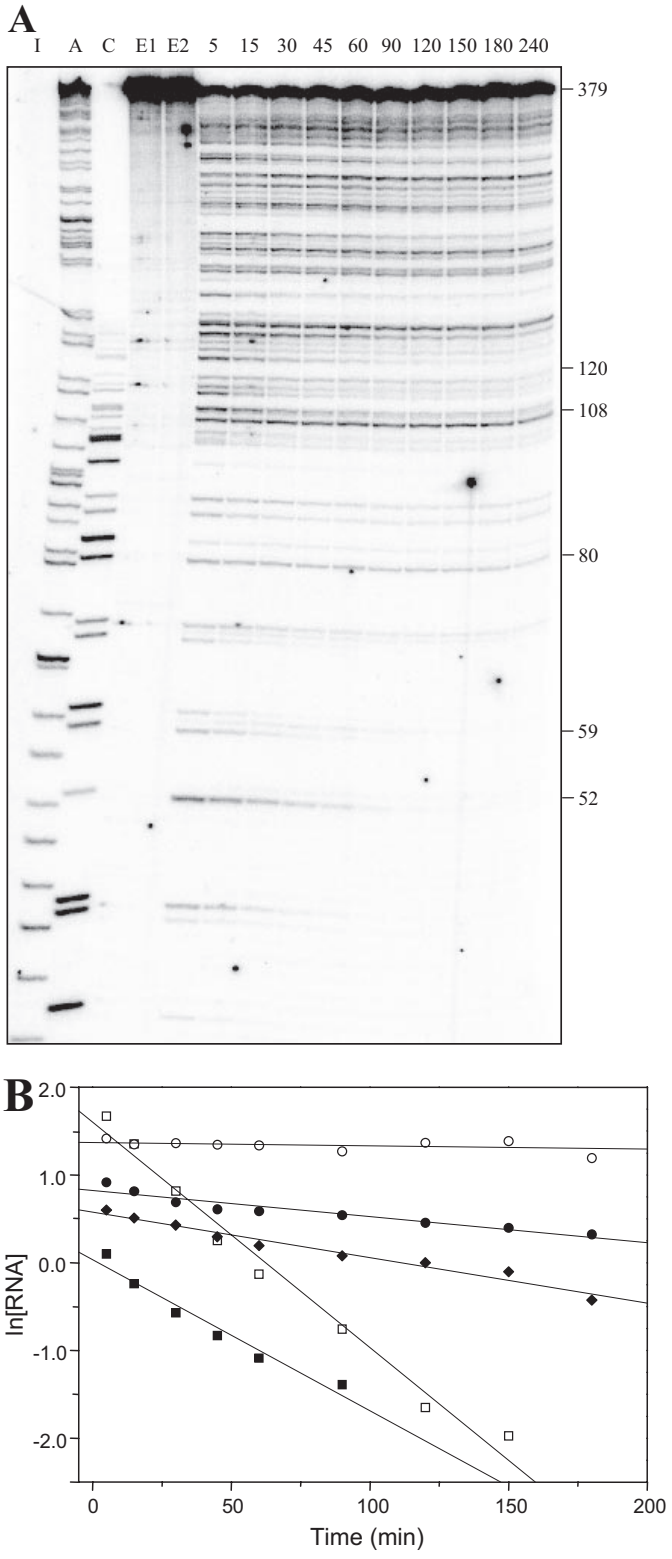


Fig. 5. Elongation of the transcription complex past drug-induced blockage sites. A, the 512-bp fragment was initially reacted with 5 μM pixantrone and 2 mM formaldehyde for 4 h. After ethanol precipitation, drug-reacted DNA was resuspended, and transcription was initiated from the *lac* UV5 promoter. Subsequent elongation of the initiated complex was allowed to proceed at 37°C for time periods ranging from 5 to 240 min. Lanes E1 and E2 are control lanes representing the initiated transcription complex that had been allowed to elongate in the absence of drug for 5 and 240 min, respectively. The remaining controls are as described in Fig. 2A. B, several drug-induced blockage sites, including 52-mer (□), 59-mer (■), 80-mer (●), 108-mer (○), and 120-mer (♦) were selected for quantitation and subjected to first-order kinetic analysis. The half-life of each drug-induced blockage is summarized in Table 1.

of RNA polymerase indicated that pixantrone-DNA adduct formation was associated with CpG and CpA dinucleotide sequences. The DNA sequence selectivity of alkylating agents is primarily governed by steric accessibility and the availability of chemically reactive nucleophilic groups within DNA (Murray, 1999), suggesting that formaldehyde-activated pixantrone binds to either guanine or cytosine (or both bases). Guanine was most likely the base of DNA alkylation by formaldehyde-activated pixantrone because it has been established as the most reactive base within DNA, and numerous compounds alkylate DNA at this base (Hemminki and Ludlum, 1984). As expected, these compounds include formaldehyde-activated mitoxantrone, which exhibits a conspicuous similarity to pixantrone in sequence selectivity (Fig. 3A), and formaldehyde-activated doxorubicin, each of which bind to guanine via the exocyclic N2 amino group (Zeman et al., 1998; Parker et al., 2004). Given the close structural homology shared by pixantrone and these drugs (Fig. 1), it was likely that pixantrone was similarly covalently attached to DNA through this amino group of guanine via a methylene group mediated by formaldehyde. The potential reaction of formaldehyde-activated pixantrone with inosine-substituted DNA substrates was investigated in three independent ways: binding to polynucleotide sequences, stabilization of ds oligonucleotides and mass spectrometric analysis of drug-DNA complexes. Pixantrone-DNA adduct formation was not observed using any of these inosine-substituted substrates. Because inosine lacks the N2 exocyclic amino group at position 2 of the guanine nucleobase, these results collectively implicate N2 as the site of DNA alkylation by formaldehyde-activated pixantrone.

The CpG doublet targeted by formaldehyde-activated pixantrone is particularly pertinent given the unique role this dinucleotide assumes in the mammalian genome. Globally, the human genome is severely depleted of the CpG motif; however, it is repeated frequently in relatively small stretches of DNA called CpG islands, which are often associated with the promoter region of many genes. The existence of CpG islands may represent selective "hotspots" for pixantrone-DNA adduct formation in a cellular environment. A further characteristic feature of CpG doublets within the mammalian genome is that the vast majority are methylated at position 5 of the cytosine base by a family of DNA methyltransferases. The methylation status of each doublet in the context of a specific CpG island has an important bearing on the expression of the associated gene (Momparler and Bovenzi, 2000; Laird, 2003). Because the natural target of formaldehyde-activated pixantrone is typically methylated in vivo, the effect of CpG methylation on pixantrone adduct formation was investigated. Adducts exhibited a dramatic preference for methylated substrate (Fig. 8), and in a cellular environment, this may endow these adducts with the capacity to alter the expression of the genes in which they occur; this is presently under examination. Indeed, other CpG-specific alkylators, including formaldehyde-activated mitoxantrone, mitomycin C, and the carcinogen benzo(*a*)pyrene diolepoxide, are also enhanced by CpG methylation (Li et al., 2000; Parker et al., 2001).

A deeper insight into a potential structure of the pixantrone-

DNA adduct can be gleaned from analysis of its close structural analog BBR 2378 (Fig. 1). BBR 2378, a dimethyl *N*-substituted analog of pixantrone, can neither stabilize dsDNA in vitro nor form monoadducts in the presence of formaldehyde. That formaldehyde-activated pixantrone exhibits both of these properties suggests a critical role for either or both of the primary amino functions located in each side chain of pixantrone. It would seem that the formaldehyde-mediated methylene group extends from a guanine residue and forms an aminal bridge (N–C–N) with either or both pendant primary amino groups of pixantrone.

TABLE 1

The stability of pixantrone-DNA adducts at discrete binding sites

The underlined nucleotide (nontemplate sequence) represents the dominant site of each transcriptional blockage selected for quantitation in Fig. 5.

Site	Symbol in Fig. 5.	Sequence	Half-Life
			min
52	□	A<u>C</u>GG	30
59	■	T<u>C</u>AC	40
80	●	A<u>C</u>AG	>180
108	○	<u>A</u>CGC	>180
120	◆	<u>T</u>CGT	134

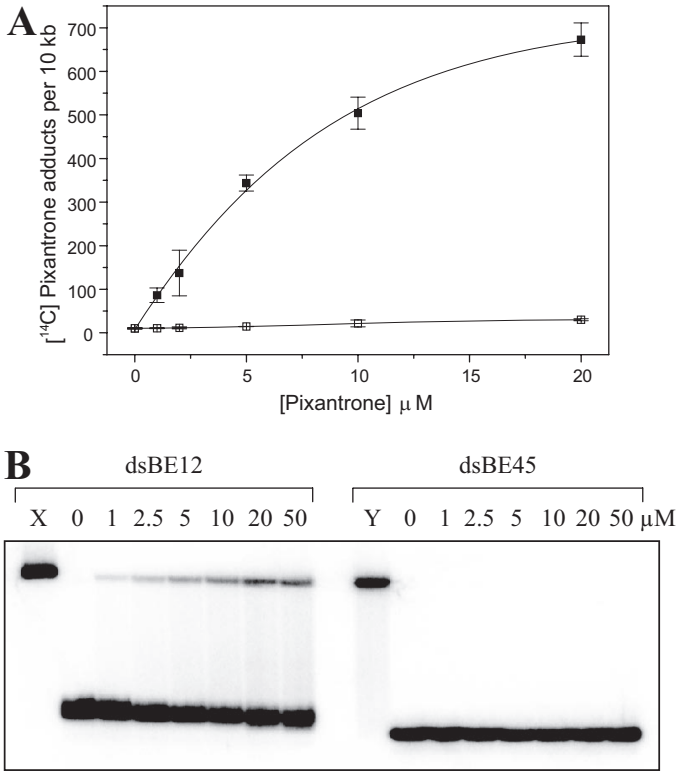


Fig. 6. Formaldehyde-activated pixantrone is efficiently incorporated into CpG- but not CpI-containing substrates. A, [^{14}C]pixantrone (0–20 μM) was reacted with either 25 μM_{bp} poly(dG-dC) (■) or poly(dI-dC) (□) and 2 mM formaldehyde overnight at 37°C. Noncovalently bound drug was removed by phenol/chloroform extraction. Incorporation of [^{14}C]pixantrone into each synthetic polynucleotide was determined by scintillation counting and is expressed as the number of pixantrone adducts per 10 kilobases of DNA. Error bars represent 95% confidence intervals of duplicate experiments. B, pixantrone (0–50 μM as indicated) was incubated with either 25 μM_{bp} ^{32}P-labeled duplex dsBE12 (CpG-containing) or duplex dsBE45 (CpI-containing) and 2 mM formaldehyde overnight at 37°C. Samples were subsequently resolved in denaturing conditions by electrophoresis and visualized by PhosphorImager.

In vitro transcriptional analysis of numerous transcriptional blockage sites established that different pixantrone-DNA adducts exhibit a wide range of temporal stabilities, varying from 30 min to greater than 3 h (Table 1), and this provides a substantial enhancement over the established stability of mitoxantrone-DNA adducts (Parker et al., 2000). One interpretation of variation in drug stability may be the existence of two distinct adduct types. Pixantrone-binding sites that exhibit temporal lability, such as the 52-mer blockage, are likely to represent a monoadduct in which formaldehyde-activated pixantrone alkylates just a single DNA strand. In contrast it was possible that extremely stable sites, including the 108-mer blockage, may be a functional

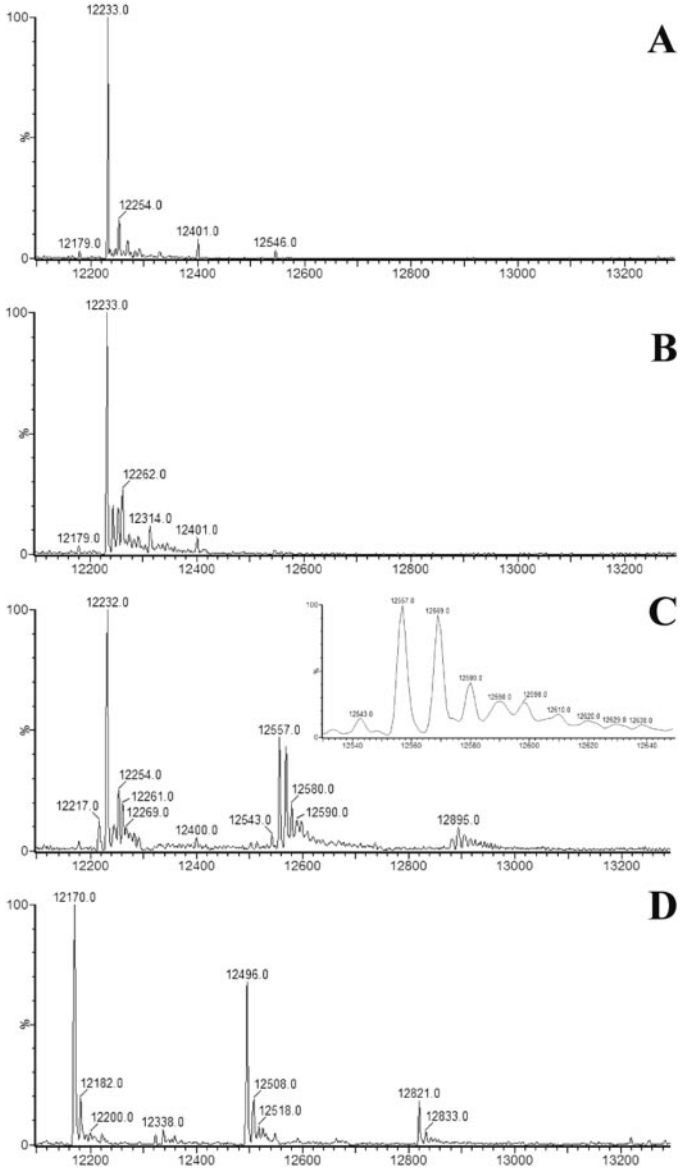


Fig. 7. Positive ion electrospray mass spectrometric analysis of dsBE12. The oligonucleotide duplex dsBE12 (50 μM) was incubated in either isolation (A), the presence of 10 mM formaldehyde (B), or the presence of both 10 mM formaldehyde and 100 μM pixantrone in PBS at 37°C overnight (C). D, duplex dsBE45 was reacted as described for C. All samples were processed using electrodialysis and subjected to mass spectrometry as described under *Materials and Methods*. The inset of C is an expansion of the mass range 12530 to 12650 Da of the corresponding mass spectrum.

consequence of a classical covalent interstrand cross-link, because coupling via both side chains of pixantrone would generate a highly stable lesion. The potential for pixantrone-DNA interstrand cross-links was investigated by mass spectrometry. Despite the exceptional stability of adducts occurring within the octamer 5′-CCGGCGCC-3′ as established by transcriptional inhibition, mass spectral studies indicate that formaldehyde-activated pixantrone is covalently attached to only a single DNA strand. The monofunctional adduct is mediated by a moiety of 12 Da, a mass that is fully consistent with the formation of a single methylene bridge (accompanied by the loss of two protons during formation of the aminal linkage). The prospect of the adduct existing as an interstrand cross-link can be eliminated given that there is no evidence of a mass increase of 24 Da as would be anticipated with the generation of two methylene bridges, one covalently tethering the drug to each DNA strand.

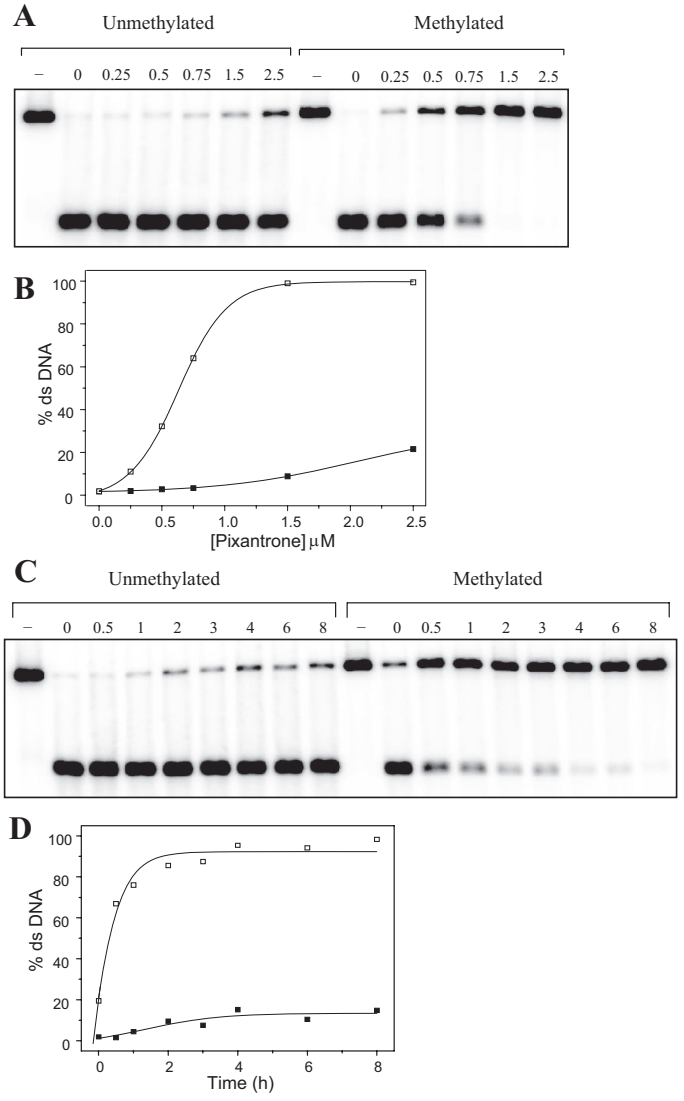


Fig. 8. Global CpG methylation by SssI methylase enhances pixantrone-DNA adduct formation. A, both native (unmethylated) and SssI methylase-treated DNA samples (25 μM_{bp}) were reacted with 2 mM formaldehyde and pixantrone (0–2.5 μM as indicated) for 4 h. Each sample was subsequently extracted twice with phenol, once with chloroform, and then ethanol-precipitated. After resuspension, samples were denatured at 52°C for 5 min in 60% formamide and then subjected to agarose electrophoresis overnight. Lane (−) is a control lacking pixantrone that had not been thermally denatured and represents the migration of dsDNA. B, the fraction of dsDNA represented in A was quantitated and is expressed as a function of pixantrone concentration for both native (■) and methylated (□) DNA. C, pixantrone (1 μM) was incubated with either native or CpG-methylated end-labeled DNA (25 μM_{bp}) for 0 to 8 h in the presence of 2 mM formaldehyde. Each sample was routinely processed and analyzed as described previously. D, the percentage of DNA remaining as double-stranded in C was quantitated and is plotted as a function of reaction time for both native (■) and methylated (□) DNA.

Each of the conclusions made above can be used to assemble a putative structure of the covalent pixantrone-DNA adduct. In this model, the chromophore portion of the drug molecule is intercalated within a CpG or CpA doublet, and the side chains of the drug protrude out into the minor groove, which accommodates the N2 exocyclic amino group of the guanine nucleobase, a center now established as the site of covalent attachment. The drug is alkylated to a single DNA strand via an aminal linkage that extends out from the N2 reactive center of DNA. The methylene component of the aminal linkage is derived from formaldehyde, where the linkage is completed and bridged by either of the pendant primary amino groups of pixantrone. Most probably, the monofunctional adduct is stabilized by secondary hydrogen bonding to the opposite DNA strand in a situation analogous to doxorubicin-DNA adducts (Zeman et al., 1998).

Much evidence has recently accumulated to suggest that the generation of a covalent linkage with DNA is an important and pertinent cytotoxic event of the anthracyclines (Skladanowski and Konopa, 1994a,b; Cutts et al., 2001, 2007; Swift et al., 2006). Mitoxantrone can also induce adducts in cancer cells; however, drug doses far in excess of growth inhibitory concentrations were required to generate these lesions, suggesting that this mechanism of action is biologically irrelevant (Parker et al., 2004). Pixantrone provides a vast improvement over mitoxantrone in terms of potential adduct formation because adducts form much more readily and are more stable (Evison et al., 2007). The formaldehyde required for pixantrone-induced DNA alkylation exists at significantly higher levels in cancer cells in vitro and in vivo (Ebeler et al., 1997; Kato et al., 2001), an attribute that may naturally predispose these cancers to pixantrone cytotoxicity. Cellular formaldehyde levels may also be bolstered by applying formaldehyde-releasing prodrugs, an approach that has been successfully applied in combination with the anthracyclines. Combining both agents potentiates the generation of cellular anthracycline-DNA adducts with an accompanying synergistic cytotoxic response (Cutts et al., 2001, 2007; Swift et al., 2006). There is now a clear rationale to investigate potential pixantrone and formaldehyde-releasing drug combinations for cancer therapy.

Pixantrone-DNA adducts are monofunctional in nature, yet the most potent and effective alkylating agents used in cancer therapy are typically those that covalently cross-link the complementary strands of DNA, rather than alkylation of just one. Interstrand cross-links are generally considered to be a more lethal class of DNA lesion given their difficulty to repair and their capacity to impair replication fork progression (Lawley, 1995; Rajski and Williams, 1998; Hurley, 2002; Beljanski et al., 2004). The CpG doublet favored by formaldehyde-activated anthracenediones provides an ideal locale for an interstrand cross-link, given the availability of the two proximal N2 exocyclic amino groups of guanine on opposing DNA strands. The clinical utility of both mitoxantrone and pixantrone suggests that these drugs provide a strong basis

for the design of novel compounds that can potentially harness this clinical usefulness and can act as more potent DNA-damaging agents.

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Address correspondence to: Dr. Suzanne M. Cutts, Department of Biochemistry, La Trobe University, Victoria 3086, Australia. E-mail: s.cutts@latrobe.edu.au