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DNA sequence specificity of mitoxantrone

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ABSTRACT

An *in vitro* transcription assay was used to determine the sequence specificity of binding of mitoxantrone to a 497 bp fragment of DNA containing the lac UV5 promoter. Transcriptional blockages of the *E.coli* RNA polymerase were observed dominantly prior to 5′-CpA sequences (64% occurrence), and to a lesser extent 5′-CpG sequences (29%). Overall, 93% of all blockage sites were prior to pyrimidine (3′–5′) purine sequences. An effect of flanking sequences was evident since the blockage sites contained an A/T base pair 5′ prior to the consensus CpA and CpG intercalation sites. The consensus sequences for the preferred mitoxantrone intercalation sites are therefore 5′-(A/T)CA and 5′-(A/T)CG. The location of transcriptional blockages one base pair prior to the intercalation site is consistent with the fact that the mitoxantrone side chains lie in the major groove.

INTRODUCTION

The anthracenedione-based anticancer drug mitoxantrone (1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)-amino]-ethyl]amino]-9,10-anthracenedione dihydrochloride), a synthetic analogue of the anthracycline antibiotics (Fig. 1), has shown significant clinical effectiveness in the treatment of a range of human malignancies [1,2]. In contrast to the anthracyclines, mitoxantrone produces less side-effects such as nausea, vomiting, alopecia and cardiac toxicity [3,4].

Mitoxantrone exhibits a range of intracellular effects, the most dominant of which appears to be the induction of long-term DNA damage. This may relate to its ability to intercalate into DNA with high affinity [5,6], causing condensation and compaction of chromatin [7], but may also relate to disruption of protein–DNA interactions [8] or to impairment of topoisomerase activity [9–11].

Since DNA appears to play an important role in many of the intracellular effects caused by mitoxantrone, many studies of the DNA binding specificity of mitoxantrone have been conducted. However, these have for the most part been indirect and inconclusive. Spectrophotometric studies of the association of mitoxantrone with DNA revealed some evidence of a 5′-GC intercalation sequence specificity [12], while other investigators have failed to demonstrate any distinct sequence preference [13,14].

Theoretical calculations on the binding of mitoxantrone to double-stranded tetranucleotides have revealed that mitoxantrone has a preference for oligomers with a pyrimidine (3′–5′) purine sequence at the intercalation site over those with the isomeric purine (3′–5′) pyrimidine sequence [15], and this has subsequently been supported by DNA footprinting studies [16].

We present here the results of an *in vitro* assay of transcriptional blockages induced by mitoxantrone on a 497 bp DNA fragment, and show that the consensus sequence of drug binding sites is at 5′-(A/T)CA and 5′(A/T)CG sequences.

MATERIALS AND METHODS

Materials

Mitoxantrone was supplied by Lederle Laboratories, (Pearl River, NY) and was prepared fresh daily by dissolving in distilled water to a final concentration of 2 mM.

Methods

In vitro transcription. A 497 bp P*vu* II/S*al* I restriction fragment of a modified pBR322 vector contained the *lac* UV5 promoter and was utilised for *in vitro* transcription with *E.coli* RNA polymerase [17]. Mitoxantrone was added to defined concentrations and the reaction mixtures equilibrated at 10°C for 20 min. Elongation of the transcripts was then initiated and transcription terminated at various time points up to 60 min, as described previously [18]. In order to detect transcriptional blockages, the elongation phase was conducted at 10°C in a high salt (400 mM KCl) transcription buffer, in the presence of 50 or 100 μM mitoxantrone. The use of these conditions has been shown to be necessary for the detection of intercalative binding specificity of drugs with short half lives [18]. These conditions decrease the dissociation rate of mitoxantrone from the DNA template, minimise background pausing and enable transcription inhibition to be detected at convenient time intervals.

Electrophoresis and autoradiography. Electrophoresis and autoradiography were performed as described previously [19]. Quantitation of the drug induced transcription blockages was carried out using Image-Quant software on a Molecular Dynamics Model 400B PhosphorImager (Molecular Dynamics, CA).

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RESULTS

Mitoxantrone induced transcriptional inhibition on the 497 bp fragment containing the UV5 promoter is shown in Fig. 2. In the absence of drug the RNA polymerase progressed through the DNA template to produce a full length transcript. Incubation of the initiated transcription complex with 50 or 100 μM mitoxantrone resulted in the sequence specific blockage of the RNA polymerase. The optimum drug concentration required in order to detect blockages at 10°C in this system was 100 μM. At 50 μM the same blockages were present, however the lower intensity of these blocked transcripts precluded accurate quantitation. Short lived transcription blockages were also only detected at 100 μM drug concentration. Since some losses of mitoxantrone are inevitable (binding occurs to the reaction vessel and to proteins in the buffer), the effective free mitoxantrone concentration available for binding to DNA would be somewhat less than the stated 50 and 100 μM. The fact that transcriptional blockages are detected at 50 μM of drug, together with the results of a recent study which showed that the *in vivo* concentration of mitoxantrone within human leukemic cells is approximately 10 μM [20], shows that the concentrations of mitoxantrone utilised in this study are in the region of physiologically relevant levels. No transcriptional blockages were detected when the elongation phase was carried out at 37°C (data not shown).

Table 1. Site and sequence of blocked transcripts induced by mitoxantrone on the 497 bp UV5 DNA template

Site of Blocked Transcript	Sequence
23	TCA
26	ACACA
41	TGA
	ACT
47	TGA
	ACT
52	ACG
58	TCA
70	TCA
73	TGT
	ACA
80	ACA
86	ATCA
90	TCG
101	GATC
109	TACG
111	CCG

The underlined nucleotide represents the major location of the transcriptional blockage, detected after 2 min of elongation in the presence of 100 μM mitoxantrone. All sequences shown are for the non-templated DNA strand, except for sequences where both strands are shown to illustrate the blockage site in relation to the 5'-CA sequence. Low intensity blockages of less than 0.1% of the total intensity have not been shown.

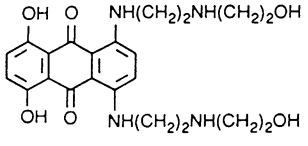

Figure 1. Structure of mitoxantrone.

Mitoxantrone-induced blockages observed in the two minute elongation lane (100 μM mitoxantrone) were calculated as the mole-fraction of blocked transcript at each site and this measure of relative drug occupancy is shown in Fig. 3. Of the fourteen unambiguous blockage sites induced by mitoxantrone, 64% (9/14) involve transcriptional inhibition prior to 5'-CA (or 5'-TG of the complementary strand) sites, whilst all of the remaining blockages except one were prior to 5'-CG sequences (4/14) of the non-template strand (Table 1). Analysis of the flanking sequences associated with the transcriptional blockages revealed that six out of nine 5'-CA blockages contained a thymine residue immediately 5' to the binding site, with the other three having adenine at that position (Table 1). Since all nine 5'-CA blockages contained either thymine or adenine prior to the intercalation site, the consensus flanking sequence preference is clearly 5'-(A/T)CA.

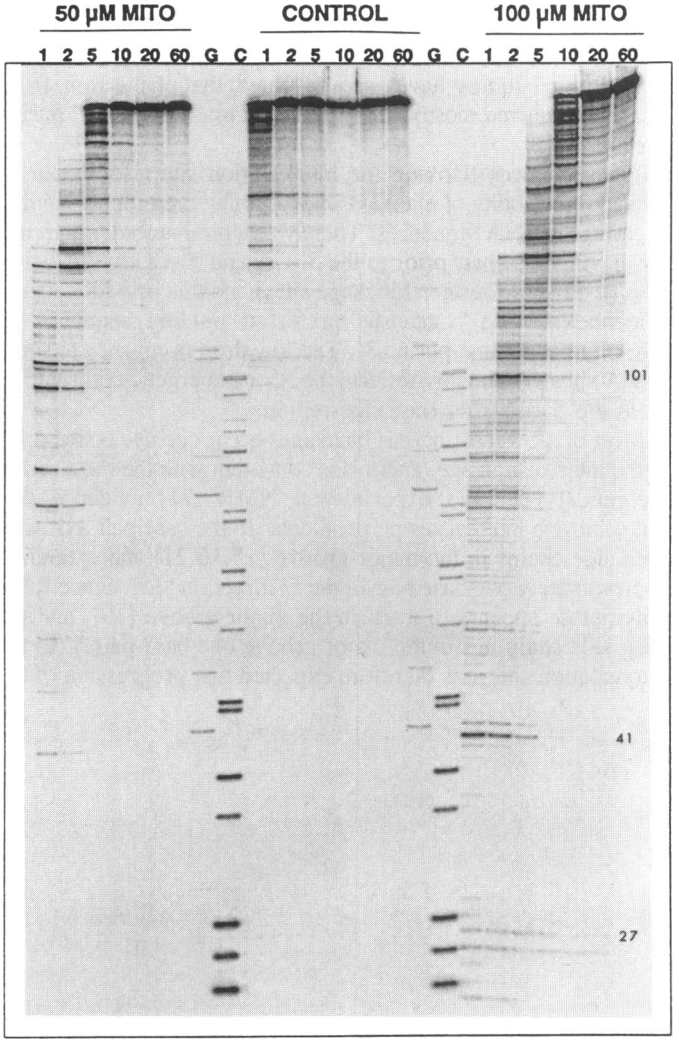

Figure 2. Mitoxantrone-induced transcriptional blockages. Autoradiogram of transcriptional blockages at 10°C following incubation of the initiated complex in the absence of drug ('control') or in the presence of either 50 or 100μM mitoxantrone. Elongation of the initiated complex was for 1, 2, 5, 10, 20 or 60 min. The C and G sequencing lanes were obtained by elongation of the initiated transcription complex in the presence of 3'-methoxy CTP and 3'-methoxy GTP respectively [17]. The sequence numbering begins from guanine of the initiating GpA nucleotide, and corresponds to the −1 position of the lac UV5 transcript [17].

A similiar consensus flanking sequence of 5′-(A/T)CG is also evident for 5′-CG blockage sites, since three out of four of these sites were preceeded by thymine or adenine. There was no such consensus in the 3′ flanking region.

DISCUSSION

Mitoxantrone was investigated for sequence specific reversible binding to the 497 bp UV5 DNA template, using an *in vitro* transcription assay. From these studies the preferred intercalation sites were found to be 5′-CA (64% occurrence) and 5′-CG (29%), representing an overall 93% preference for pyrimidine (3′−5′) purine sites. Theoretical minimum energy calculations performed on the binding of mitoxantrone to double stranded tetranucleotides also revealed a pronounced energy preference for pyrimidine (3′−5′) purine sequences at the intercalation site [15]. These theoretical studies also revealed that the tricyclic chromophore of the drug is not exclusively responsible for imparting binding specificity, rather there is a large energetic contribution from the basic side chains of the drug. DNA footprinting studies have also revealed that protection from cleavage occurred mostly at or around pyrimidine (3′−5′) purine sites [16].

The sequences flanking the intercalation site also appear to influence the ability of mitoxantrone to exhibit preferential binding at particular DNA sequences. There was a pronounced preference for an A/T base pair prior to the 5′-CA and 5′-CG intercalation sites (twelve of fourteen blockage sites) and this provides strong evidence for the concensus preferred binding sequence as 5′-(A/T)-pyrimidine-purine-3′. The low drug occupancy at some 5′-CA sites presumably reflects the strong energetic contribution from the 5′ flanking sequence region.

Most of the transcriptional blockages occur one nucleotide prior to the intercalation site, and this is consistent with the most recent theoretical [15] and experimental NMR [21] evidence that mitoxantrone intercalates perpendicular to the base pair axis with both side chains in the major groove [15,16,21] and extending approximately one base pair in the 5′ direction [15]. Since RNA polymerase appears to track in the major groove [19], and the drug side chain lies in the major groove one base-pair 5′ to the intercalation site, it is therefore expected that progression of the polymerase will be halted one base pair prior to the intercalation site. In contrast, Adriamycin (which also displays a 5′-CA sequence preference as indicated by the same transcription assay [18]) binds with the sugar moiety in the minor groove, and in this case most transcriptional blockages are at the cytosine of the 5′-CA intercalation site. Hence, the RNA polymerase is able to progress right up to the Adriamycin intercalation site and is not blocked earlier by any drug appendage protruding into the major groove, as occurs with mitoxantrone.

The reason for the large blockage at the 101 site is unknown since this site is not associated with either 5′-CA or 5′-CG sequences. It possibly reflects either a high affinity site associated with the unique palindromic GATC sequence at that site, or a complex phenomena involving the potential hairpin helix in that region (80−87 and 93−100). In this context it must also be noted that if the preferred binding site is a three base pair unit, all such possible combinations cannot be probed in the limited sequence distribution afforded by the present assay. Clearly, a much more extensive analysis will be required to confirm the validity of the concensus sequences detected here, and to confirm the significance of the large transcriptional blockage at the 101 site.

Whilst it is not clear how the 5′-(A/T)CA and 5′-(A/T)CG sequence specificities relate to drug action, it is possible that the drug is localised preferentially in specific control regions of the genome, such as in transcriptional promoter or enhancer regions, where it may result in a critical effect on the regulation of transcriptional processes.

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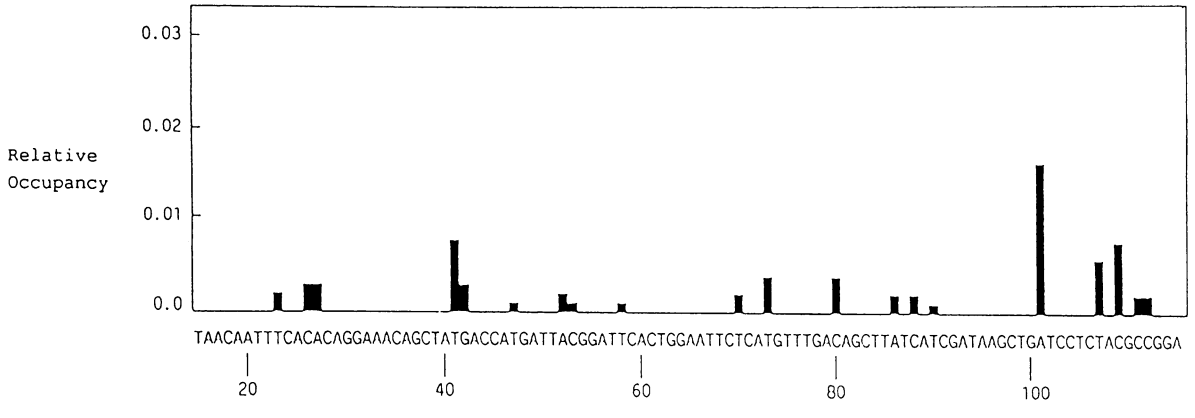

Figure 3. Sequence specificity of mitoxantrone-induced transcriptional blockages. The relative intensity of blocked RNA transcripts is shown for the 2 min elongation lane exposed to 100μM mitoxantrone (Fig. 1). The mole-fraction of blocked transcript was calculated for each of these blockages and represents the relative drug occupancy at each drug-binding site [17]. The sequence shown is that of the non-template strand, numbered from guanine of the the initiating GpA nucleotide [17].

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