DNA sequence preferences for the anti-cancer drug mitoxanthrone and related anthraquinones revealed by DNase I footprinting

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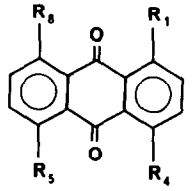
The interaction has been studied of several anthraquinone-based intercalating drugs, including the anti-cancer agent mitoxantrone, with defined sites of DNA. A 160 base pair DNA sequence from *tyrT* was employed for footprinting with DNase I. The anthraquinones had aminoalkylamino substituents in various positions of the ring system. Inhibition of enzymatic cutting of the DNA was observed at various positions on the sequence, mostly around some of the pyrimidine-3′,5′-purine sites. Enhancements to cutting were observed clustered around AT-rich regions. The compounds showed differences in detailed footprinting behaviour, which have been related to differences in their mode of interaction with DNA as found in earlier computer modelling studies.

Anthraquinone *Mitoxanthrone* *Intercalator* *Footprinting*

1. INTRODUCTION

The aminoalkylamino-disubstituted anthraquinone compound I, mitoxanthrone (fig.1), has established experimental anti-tumour activity [1,2] and is now coming into clinical use for the treatment of human cancers [3,4]. A number of studies have shown that it binds to DNA in vitro, and that intercalative processes are involved [5–9]. Thus, mitoxanthrone unwinds closed circular duplex DNA by 14–17° per bound drug molecule, displaces bound ethidium from its DNA complex, and itself produces spectroscopic changes on binding characteristic of an intercalator. The drug is an inhibitor of nucleic acid synthesis [10] and causes DNA strand breaks in vivo [11]. It is generally presumed that these effects are causally related to its anti-tumour activity.

A recent study has been made on a series of substituted anthraquinones (II–V, fig.1) that differ in the pattern of substitution, using a combination of solution methods for investigating DNA



I $R_1 = R_4 = NH(CH_2)_2\overset{+}{N}H(CH_2)_2OH$, $R_5 = R_8 = OH$

II $R_1 = NH(CH_2)_2\overset{+}{N}H(C_2H_5)_2$, $R_4 = R_5 = R_8 = H$

III $R_1 = R_4 = NH(CH_2)_2\overset{+}{N}H(C_2H_5)_2$, $R_5 = R_8 = H$

IV $R_1 = R_5 = NH(CH_2)_2\overset{+}{N}H(C_2H_5)_2$, $R_4 = R_8 = H$

V $R_1 = R_8 = NH(CH_2)_2\overset{+}{N}H(C_2H_5)_2$, $R_4 = R_5 = H$

Fig.1. Structural formulae of anthraquinones used in this study.

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binding, and computerised molecular modelling [12]. The 1,4-disubstituted compound is analogous to mitoxanthrone itself, with the side chain in this series differing only slightly from that of the established drug. All four compounds were shown to bind intercalatively to DNA. There were significant differences in the strength of binding amongst the members of the series, which correlated with the ranking order of calculated interaction energies. The modelling also suggested that there are differences in the orientation and positioning of these anthraquinones when bound in their respective DNA intercalation sites.

This investigation extends these studies to questions of DNA site selectivity, preference and dynamics. Footprinting, which involves examining drug-induced inhibition of cleavage of defined-sequence DNA fragments by either enzymatic [13] or chemical [14] agents has been shown to be an effective technique for addressing these questions. Structurally 'complex' intercalators such as actinomycin [15,16], the quinoxaline antibiotics [13,14], and the anthracycline nogalamycin [17] all induce large changes in the cleavage pattern, consistent with the existence of definite nucleotide sequence preferences. 'Simple' intercalators do not generally produce clear footprinting patterns, presumably because they lack selectivity. The technique is also dependent on the relative persistence of drugs at different sites on DNA, and simple intercalators generally dissociate rapidly from DNA rendering their positions more difficult to detect by standard footprinting methods.

2. EXPERIMENTAL

Mitoxanthrone (I) and the anthraquinones II–V were synthesised according to published procedures. Nogalamycin was a gift from Dr P.F. Wiley of the Upjohn Co., Kalamazoo, MI. Stock solutions of each antibiotic were prepared by direct weighing and dissolved in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl. DNase I was obtained from Sigma and prepared as a 7200 U/ml stock solution in 0.15 M NaCl containing 1 mM $MgCl_2$. This was stored at −20°C and diluted to working concentrations immediately before use.

2.1. *DNA substrates*

The 160 base-pair *tyrT* fragment was isolated and labelled as described [13,18,19]. Incubation with reverse transcriptase, dGTP and [α-^{32}P]dCTP led to selective labelling of the 3′-end of the top strand (fig.3) whereas incubation with dTTP and [α-^{32}P]dATP led to selective labelling at the 3′-end of the bottom strand. Bands in the *tyrT* digestion pattern were assigned by running dimethylsulphate-piperidine markers specific for guanine, as previously described.

2.2. *DNase I footprinting*

Digestion with DNase I was performed as previously described. Samples of the labelled DNA fragment (3 pmol in base-pairs per gel lane) were incubated with antibiotic solution at 37°C for 30 min (sufficient to ensure an equilibrium of the ligand), then cooled to 4°C and digested with DNase I dissolved in 20 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$ at a final enzyme concentration of 0.01 U/ml. Experiments were performed at this low temperature to increase the persistence time of the ligands on the DNA and sharpen the protection pattern (Fox, unpublished). Aliquots (3 μl) were removed from the mixture after various times and the reaction was stopped by adding 2.5 l of 80% bromophenol blue, 10 mM EDTA and 1 mM NaOH. Samples were heated at 100°C for at least 3 min prior to electrophoresis.

2.3. *Gel electrophoresis*

The products of *tyrT* digestion were analysed on 0.3 mm 8% (w/v) polyacrylamide gels containing 8 M urea and Tris-borate-EDTA buffer (pH 8.3). After 2 h electrophoresis at 1500 V the gel was soaked in 10% acetic acid for 10 min, transferred to Whatman 3MM paper, dried under vacuum at 80°C and subjected to autoradiography at −70°C with an intensifying screen.

3. RESULTS

Fig.2 shows an autoradiograph of DNase I digestion patterns for the *tyrT* fragment labelled on the lower strand in the presence and absence of mitoxanthrone (I), the four synthetic anthraquinones (II–V) and nogalamycin.

The changes in the cleavage pattern induced by nogalamycin (extreme right lanes) reveal a number of positions that are protected from cleavage by the enzyme. These are centred around positions 21,

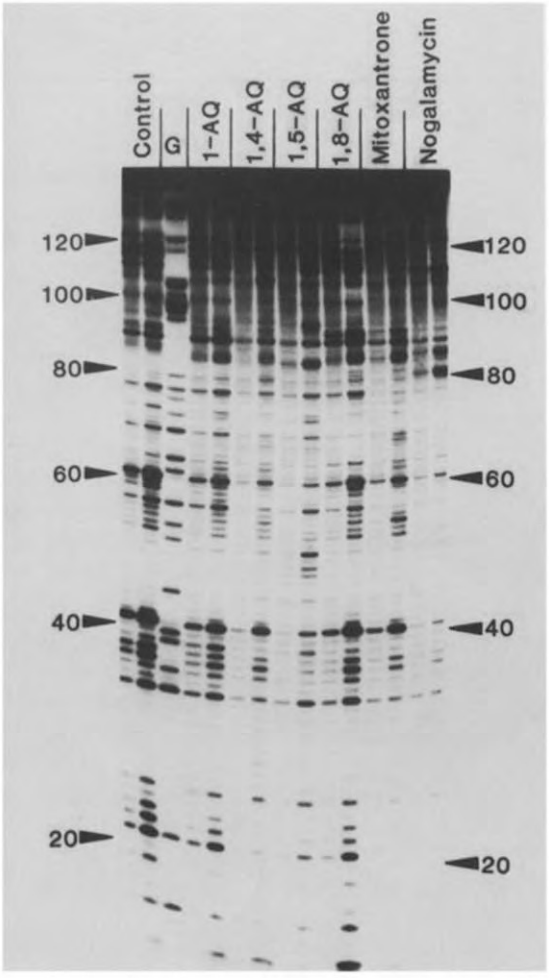

Fig.2. DNase I digestion patterns for the *tyrT* DNA, labelled at the 3′-end of the lower strand, in the presence of mitoxanthrone and related anthraquinones (final concentration 20 M), and nogalamycin (1 M). Each pair of lanes represents digestion by the enzyme for 2 and 10 min. Track G is a dimethylsulphate-piperidine marker specific for guanine.

37, 41, 60, 92 and 117 and are in accord with a recent study on this drug. As previously noted a number of bands appear strongly enhanced in the presence of this antibiotic, due to increased sensitivity to the enzyme. Enhanced cleavage is most noticeable at positions 81, 86, 110 and 127 (fig.2).

The five anthraquinones all provoke changes in the DNase I cleavage pattern. These changes are different for the various ligands and are generally less pronounced than those produced by nogalamycin, even at 10-fold higher concentration. The bands which show a decreased rate of cleavage are summarised in table 1.

Various regions where DNase I cleavage is enhanced have also been identified (table 2). These enhancements are not identical for each of the different anthraquinones and are distinct even on visual inspection. For example, the intense enhancement produced by nogalamycin around position 81 (visible on the lower strand) is paralleled only very weakly by the 1,8-anthraquinone, although this anthraquinone is the only one to pro-

Table 1

Major positions of protection against DNase I cutting for the *tyrT* DNA fragment

1-AQ	1,4-AQ	1,5-AQ	1,8-AQ	Mito-xanthrone	Nogala-mycin
	21–23	21–23 (weak)		21–23	21–23
	37–38	37–41	37–38 (weak)	37–38	37–38
					41
		55 (weak)		57–61	55–57
59–60	59–61	59–61			59–61
86–87	86–87	86–87			69
	91 (weak)	91–92			91
	100				117
	111				120

The first four columns refer to the aminoethyl (aminodiethyl)-substituted anthraquinones as indicated

Table 2

Major sites of enhancement towards DNase I cutting on the *tyrT* DNA fragment

1-AQ	1,4-AQ	1,5-AQ	1,8-AQ	Mito-xanthrone	Nogala-mycin
28–30	28–30	28–30	28–30	28–30	28–30
39		48–51			
				53–55	50
84–85	84–85 (weak)	84	85	85	
	107	94	91		86
				121	
	127	125		127	127

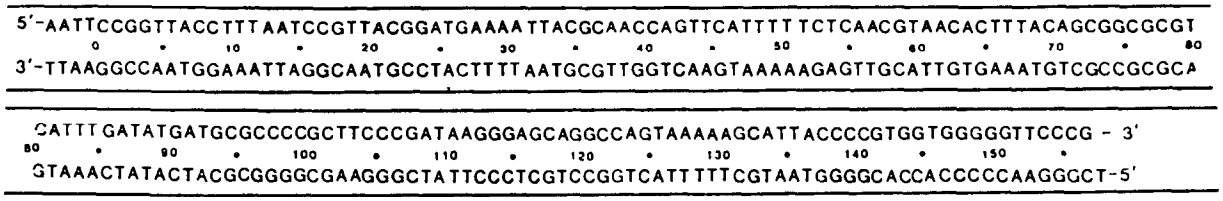

Fig.3. Sequence and numbering scheme for the *tyrT* DNA fragment.

duce a marked enhancement of cutting around position 91.

Fig.3 shows the sequence of the *tyrT* fragment used. There is no general correlation between the two types of site enhancement or reduced cleavage; some protected sites are clustered together with enhanced ones, whereas a number of sites showing enhanced cleavage appear remote from protected ones. The protected sites are mostly at, or close to the sequences CG, CA and TA (reading in the 5′–3′ direction). As previously noted the majority of the enhanced regions lie in, or close to, short non-alternating AT runs.

4. DISCUSSION

It is now well established that certain structurally complex intercalators such as echinomycin, actinomycin or nogalamycin interact non-randomly in a sequence-specific manner with DNA. For all of these drugs, as well as for the AT-specific groove binders netropsin [18] and distamycin [15], plausible molecular mechanisms have been advanced to explain their sequence-specificity that take account of their structural complexity. It is then at first sight surprising that in this study even the simplest anthraquinones have been shown to exhibit some ability (albeit limited) to discriminate between various sequences, not only as regards protection from nuclease attack but also in producing non-random enhancement of the enzyme cutting rate. The sites inhibited by the drugs are mostly of the pyrimidine-3′,5′-purine type. This is in accord with a number of theoretical predictions which have indicated that such sites require less energy to open up from 3.4 to 6.8 Å compared with the other possible dinucleotide combinations [20–22]. NMR studies on simple intercalators [23], as well as mitoxanthrone itself bound to short-length oligonucleotides [9], have led to the same conclusion.

It is not surprising that the least changes in the cleavage pattern are induced by the simplest anthraquinone, substituted with a single side chain. The other three compounds, possessing two identical side chains at different positions of substitutions, produce varying patterns of cutting. We have previously shown by computerised molecular modelling that these three anthraquinones (the 1,4; 1,5; 1,8 derivatives) take up distinct geometrical positions as regards intercalation, associated with characteristic stabilisation energies. The ranking order of energy correlates with the measured kinetics of dissociation from DNA [24], and is (in order of decreasing stability for an intercalated complex) 1,5; 1,4; 1.8; 1. Since the ability of any ligand to induce a footprinting pattern depends on the strength of interaction and the slowness of drug-DNA dissociation, the number of altered regions and the level of protection afforded would be expected to follow the same order. Inspection of table 1 shows that this is indeed the case. We note that the number of sites displaying enhanced cleavage also follows this same ranking order.

Of the six DNA-binding compounds examined here, four may be grouped into two pairs. Firstly, the 1,4-anthraquinone clearly bears a strong structural resemblance to the anti-cancer drug mitoxanthrone. Both have alkylamino side chains, each bearing a single protonated nitrogen atom separated from its point of attachment to the chromophore by a two-carbon link, although they differ slightly in the terminal atoms of the side chain. Their behaviour in the footprinting experiments parallels this structural similarity with relatively little difference between them in terms of protected sites. The slightly greater number of sites protected by mitoxanthrone may be due to involvement of its side-chain hydroxyl groups in secondary interactions imparting extra stabilisation at certain binding sites. Secondly, molecular modelling studies on the 1,5-anthraquinone [12] and

nogalamycin [25] have indicated that they share a common non-classical mode of intercalation, with their chromophores 'spearing' base-pairs at the intercalation site and their sterically bulky substituents (aminoalkyl side chains and sugar groups, respectively), situated in both major and minor grooves of the DNA. Thus, for these compounds intercalation has to be preceded by binding to a transiently opened-up site on DNA. Many features of this model have been confirmed by kinetic [26,27] and footprinting studies on nogalamycin [17]. The comparative footprinting studies reported here suggest a marked correspondence between these two drugs as regards their preferred binding sites, together with differences from the others in the series, the most notable occurring at site 41 (which is only protected by these two compounds).

It is remarkable that all of the protected and enhanced regions for the DNA-binding ligands examined in this study cluster around similar stretches of sequence. By no means all of the pyrimidine-3′,5′-purine sites are protected from cutting by bound drug: those in the GC-rich stretches 71–79 and 93–102 are especially prominent in this regard. These stretches of sequence are not major sites of enhancement towards DNase I cutting either. Regions of enhancement lie mostly (though not exclusively) in the AT-rich sections such as 28–30 and around 127. It is known that DNase I cleavage is especially sensitive to perturbations in the double-helical geometry of DNA [19], and thus the enzyme can sense both local and long-range changes induced by the drugs. It is of interest that in the absence of drug the enzyme cuts least well at long AT and GC runs, presumably because in these regions the enzyme cannot readily accommodate the DNA groove widths pertaining there. This question of groove widths is also of importance in determining differential accessibility as regards drug binding. Molecular modelling has indicated that the drugs examined here do not necessarily bind via the same DNA groove. For example, the 1-substituted anthraquinone can adopt a number of equi-energetic bound states with the side chain projecting into either major or minor grooves. By contrast, the 1,8-anthraquinone requires a wide groove for its side chains, which are too large to reside in the minor groove. Sequence-dependent variations in groove dimensions would thus be expected to exert significant influence on the binding of this drug. No such effect is actually observed since the 1,8-anthraquinone affords the least protection in the series, but this way may not be too surprising since DNase I cuts from the opposing minor groove. The enhancements of cutting produced by this drug are perhaps the most marked in the series, suggesting that the location of its side chain in the major groove induces some long-range perturbations.

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