DNA recognition by two mitoxantrone analogues: influence of the hydroxyl groups

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Abstract The clinically useful anticancer drug mitoxantrone intercalates preferentially into 5′-(A/T)CG and 5′-(A/T)CA sites on DNA. The 5,8 hydroxyl substituents on its anthracenedione chromophore are available to interact with the double helix. Footprinting experiments with two anthraquinone derivatives structurally related to mitoxantrone and ametantrone have been undertaken to assess the influence of the hydroxyl groups on the DNA recognition process. The results confirm that they do play a role in the recognition of preferred nucleotide sequences and suggest that the binding of anthraquinones to a 5′-(A/T)CG site is dependent on the presence of the 5,8 hydroxyl substituents whereas binding to 5′-(A/T)CA sites appears to proceed just as well without them.

Key words: Mitoxantrone; DNA recognition; Anticancer drug; Footprinting

1. Introduction

The synthetic antitumour drug mitoxantrone (Fig. 1) has major clinical value in the treatment of several leukemias as well as solid tumours such as breast cancer because it is less cardiotoxic than the classical anthracycline antibiotics [1,2]. This 1,4-[bis-(aminoalkyl)amino]-anthracene-9,10-dione derivative binds to cellular nucleic acids, causes chromatin compaction, produces damaging radicals and interferes with topoisomerase II activity so as to induce DNA strand breaks [3–7]. The interaction with DNA and consequent damage are thought to be essential for its therapeutic effect [8,9].

Spectroscopic studies originally showed that mitoxantrone binds preferentially to GC-rich DNA and synthetic polynucleotides [10–15]. A preference for alternating purine-(3′,5′)-pyrimidine sequences was subsequently inferred from footprinting and theoretical studies [16,17]. More recently, selective recognition of 5′-(A/T)CG and 5′-(A/T)CA trinucleotides was deduced from in vitro transcription assays [18]. Although the molecular determinants implicated in DNA recognition by mitoxantrone have yet to be identified, it is believed that the two hydroxyethylaminoalkyl side chains are, at least in part, responsible for its sequence selectivity since the length, position and nature of these positively charged side chains strongly influence the stability of drug–DNA complexes [13,15,19,20]. In addition, it has been postulated that the hydroxyl groups at positions 5 and 8 on the anthraquinone chromophore might also contribute to the interaction because mitoxantrone dissociates more slowly from DNA than does its non-hydroxylated homologue ametantrone [15]. Various studies comparing the DNA binding properties of mitoxantrone and ametantrone concur that the 5,8-$(OH)_2$ groups stabilize the drug–DNA complex [3,13,21]. To elucidate the role of these phenolic hydroxyl substituents we have investigated the sequence-specific recognition of DNA by two mitoxantrone analogues which both lack the hydroxyethyl groups on the aminoalkyl side chains and differ by the presence or the absence of hydroxyl substituents at positions 5 and 8 (Fig. 1). DNase I footprinting experiments suggest that the hydroxyl groups are involved in the recognition of 5′-(A/T)CG trinucleotides but not of 5′-(A/T)CA.

2. Materials and methods

2.1. Drugs

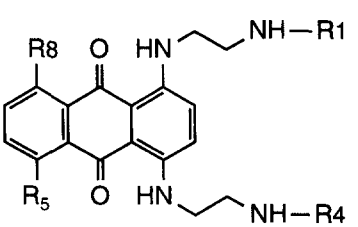
Compounds A and B were prepared by condensation of leucoquinizarine or 5,8-dihydroxyleucoquinizarine with t-Boc-ethylenediamine followed by air oxidation of the dihydro intermediates as previously described [22,23]. Stock solutions were prepared in 10 mM Tris-HCl buffer containing 10 mM NaCl (pH 7.0) and stored at 4°C.

2.2. Viscometry

Viscosity measurements were carried out using a specially constructed capillary viscometer as previously described [24]. Unwinding angles were estimated with reference to that of ethidium bromide (26°) used as a control [25].

2.3. DNA fragments

The 117 bp fragment was cut out of plasmid pBS with *Eco*RI and *Pvu*II and 3′-end labelled at the *Eco*RI site with [α-^{32}P]dATP in the



mitoxantrone	R1=R4=CH_2CH_2OH	R5=R8=OH
ametantrone	R1=R4=CH_2CH_2OH	R5=R8=H
compound A	R1=R4=H	R5=R8=OH
compound B	R1=R4=H	R5=R8=H

Fig. 1. Structures of anthracenedione drugs.

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Abbreviations: bp, base pairs; DAP, 2,6-diaminopurine.

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presence of AMV reverse transcriptase. *tyrT*(A93) DNA was cut out of plasmid pKMp27 with *Eco*RI and *Ava*I and 5′-end labelled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase [26,27]. Molecules substituted with inosine and/or DAP were prepared by pcr [27]. Labelled DNA was purified by 6% polyacrylamide gel electrophoresis prior to being resuspended in 10 mM Tris-HCl, pH 7.0 buffer containing 10 mM NaCl.

2.4. DNase I footprinting

DNase I experiments were performed essentially according to the original protocol [28]. Briefly, the digestion of 6 μl samples of the labelled DNA fragment was initiated by adding 2 μl of a DNase I solution so as to yield a final enzyme concentration of about 0.01 unit/ml. After 3 min, the digestion was stopped by freeze drying, samples were lyophilized, washed once with 50 μl of water, lyophilized again and then resuspended in 4 μl of an 80% formamide solution containing tracking dyes. Samples were heated at 90°C for 4 min and chilled in ice for 4 min prior to electrophoresis.

2.5. Electrophoresis and quantitation by storage phosphor imaging

DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea). After electrophoresis (about 2.5 hours at 60 Watts, 1600 V in TBE buffer, BRL sequencer model S2), gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80°C, and examined by autoradiography using either a phosphorimager or X-ray films (Fuji R-X) exposed at −70°C with an intensifying screen. For quantitative analysis, a Molecular Dynamics 425E PhosphorImager was used to collect data from storage screens exposed to the dried gels overnight at room temperature. Base line-corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software.

3. Results and discussion

Prior to investigating sequence specific binding to DNA, viscometric measurements using closed circular duplex DNA were performed in order to compare the effects of the two anthraquinone derivatives on DNA supercoiling. Both drugs unwind the double helix to a similar extent. The unwinding angle, measured by titrating drug into supercoiled DNA at various concentrations, was estimated to be 16° for compound A and 15° for compound B respectively. Similar unwinding angles have been reported for mitoxantrone and structurally related anthraquinones [11,19,29] confirming that both derivatives A and B can also intercalate into DNA.

In the first series of footprinting experiments, performed with a 117 bp restriction fragment from plasmid pBS in the presence of increasing concentrations of compounds A and B, the cleavage pattern differs substantially from that in the control samples (Fig. 2) suggesting that both ligands are capable of interacting with DNA in a sequence-selective fashion. At 5 μM the involvement of a specific binding process can easily be seen by the blockage of nuclease activity around nucleotide positions 31, 55 and 74. The ratio of fractional cleavage in the presence of drug to that in the drug-free control was calculated for each internucleotide linkage and used to construct differential cleavage plots (Fig. 3). They reveal that a feature common to both drugs is the enhancement of cutting by DNase I at phosphodiester bonds between bases 62–69 and 80–90 indicating that binding to AT-rich sequences is disfavoured, just as reported with mitoxantrone [16]. Similarly, GC-containing homopurine·homopyrimidine tracts do not afford binding sites for compounds A and B since in neither case are the trinucleotides GGG and CCC at positions 61, 68 and 79 protected from

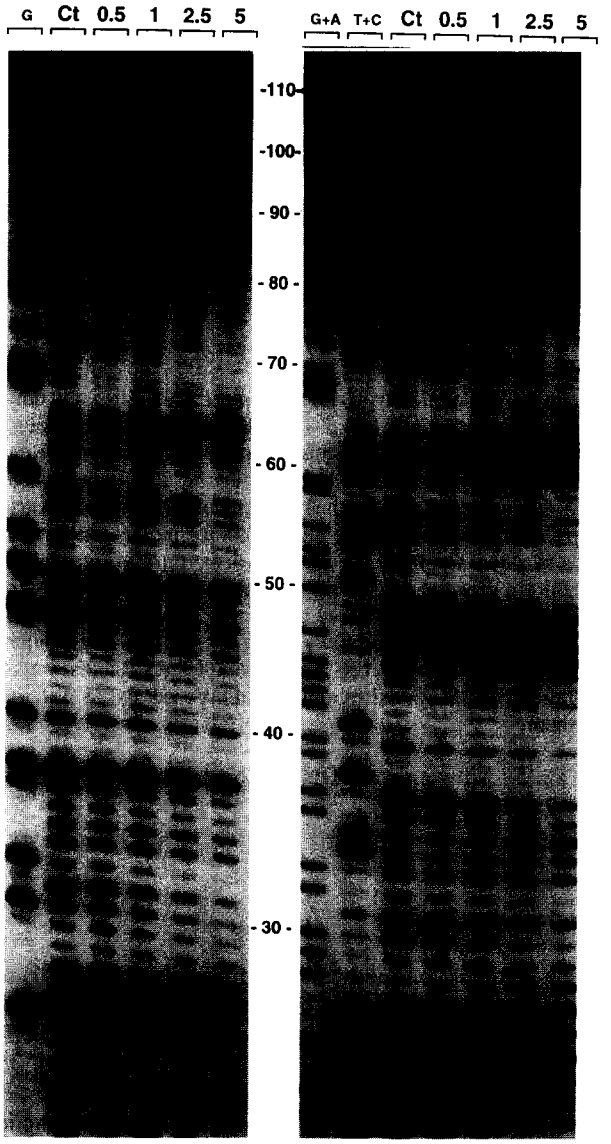

Fig. 2. DNase I footprinting with the 117-mer fragment in the presence of compound A (left panel) and B (right panel) at concentrations (μM) shown at the top of each gel lane. The control tracks labelled 'Ct' contained no drug. The tracks labelled T+C, G+A and G represent Maxam-Gilbert sequencing markers specific for pyrimidine, purine, and guanine residues respectively. Numbers refer to the nucleotide sequence shown in Fig. 3.

attack. The two drugs bind preferentially to alternating purine–pyrimidine sequences containing both A·T and G·C base pairs. With the dihydroxy-anthraquinone derivative A every region protected from DNase I cleavage (a presumptive drug binding site) lies squarely around the trinucleotide ACG or 5′-(A/T)CA (which is equivalent to 5′-TG(A/T)), marked respectively by black and hatched rectangles in Fig. 3A. 5′-(A/T)CG or 5′-(A/ T)CA triplets are the consensus sequences reported for mitoxantrone [18]. This observation immediately establishes that the hydroxyethyl terminal groups which distinguish mitoxantrone from compound A are not responsible for the sequence- selective recognition process.

The sequences which are protected from DNase I cleavage by compound B are comparable to but distinct from those

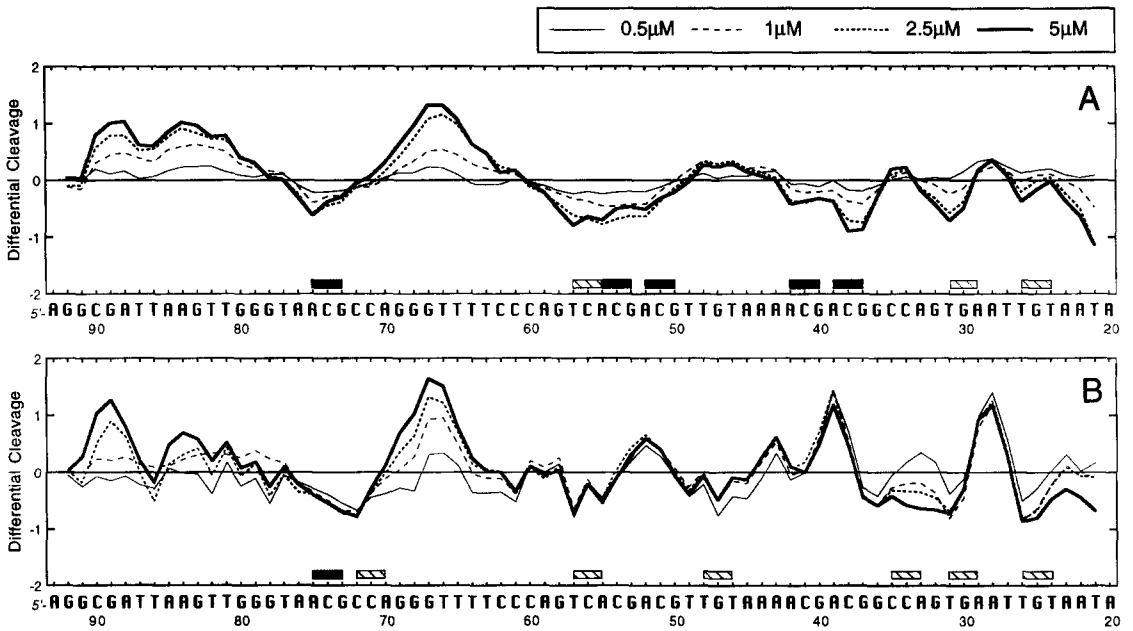

Fig. 3. Differential cleavage plots showing differences in susceptibility of the 117-mer fragment to DNase I cleavage in the presence of drugs A (top panel) and B (bottom panel). Vertical scales are in units of $\ln(f_a) - \ln(f_c)$, where f_a is the fractional cleavage at any bond in the presence of the drug and f_c is the fractional cleavage of the same bond in the control. Negative values correspond to a ligand-protected site and positive values represent enhanced cleavage. Black boxes indicate the triplet sequences 5′-ACG. Hatched boxes mark the triplets 5′-AGT or 5′-TCA.

protected by compound A. There is rather poor correspondence between the drug B-protected sites and the ACG triplets. On the other hand, there is a relatively good correspondence with the sites containing a CA or TG step such as the trinucleotides CCA, TCA and TGT marked as hatched rectangles in Fig. 3B. Therefore, the results suggest that binding to a 5′-(A/T)CG site is dependent on the presence of the 5,8 hydroxyl groups on the anthracenedione chromophore whereas binding to 5′-(A/T)CA sites proceed to a large extent irrespective of the presence or absence of hydroxyl groups.

The generality of these observations has been confirmed with the 160 bp *tyr*T(A93) DNA fragment which was previously employed to study the sequence specificity of mitoxantrone [16] as well as a variety of other DNA-binding drugs [30]. This DNA fragment offers weaker binding sites for the anthraquinones than the 117-mer. However, the differential cleavage plots shown in Fig. 4 corroborate the conclusions drawn with the pBS fragment. The regions of reduced nuclease cleavage in the presence of compounds A and B match quite well, though not exclusively, with CG- or CA (TG)-containing sites. In most

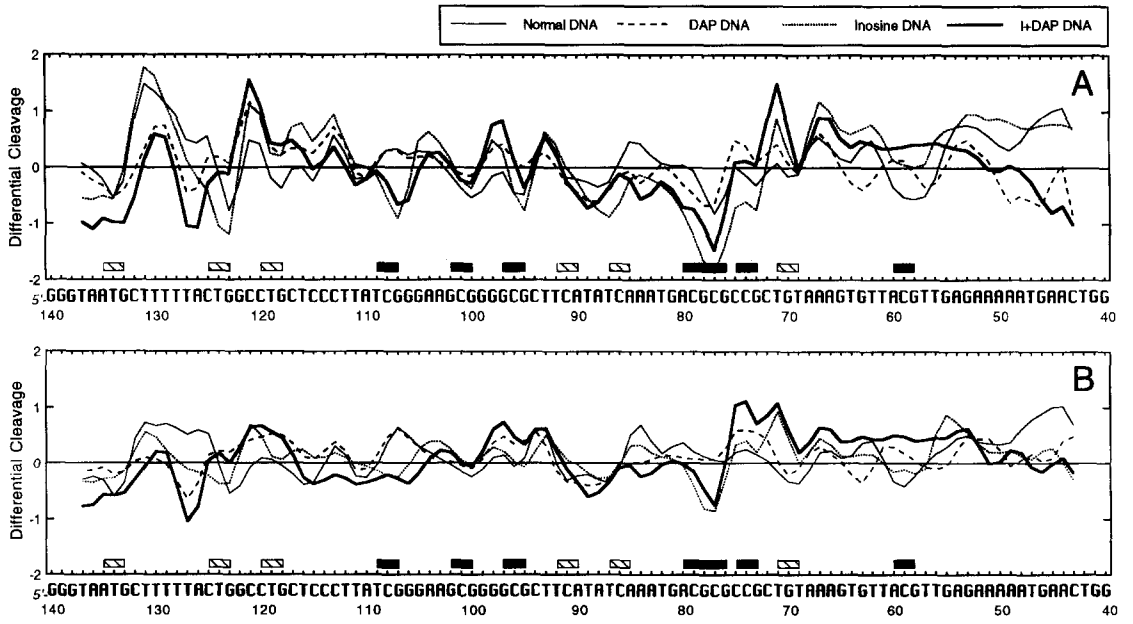

Fig. 4. Nuclease cleavage of *tyr*T(A93) DNA containing natural nucleotides, inosine in place of guanosine, DAP in place of adenine, or both inosine and DAP, in the presence of 5 μM drugs A and B. Other details as for Fig. 3.

cases, footprints at XCG sites (black rectangles) are observed with the bis-hydroxylated compound A whereas they are less pronounced or absent with the non-hydroxylated analogue B. This is particularly noticeable around the sequence ACGCGCCG extending from positions 80 to 73 which denotes a strong binding site for drug A but not for drug B. Conversely, footprints at CA or TG steps are detected almost identically with compounds A and B.

Finally, the influence of a structural modification which affects the minor groove of the DNA helix was examined, namely the effect of transposing the purine 2-amino group from guanine to adenine nucleotides [27]. Both compounds A and B were found to footprint on the modified DNA molecules in much the same way as they do on natural *tyr*T DNA (Fig. 4), showing that sequence recognition by these molecules cannot depend upon interaction with the 2-amino group of guanine located in the minor groove. This is consistent with an intercalation process which would leave the alkylamine side chains sitting in the major groove, as is believed to be the case for mitoxantrone [18] and bis-naphthalimide drugs [25].

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References

[1] van de Graaf, W.T.A. and de Vries, E.G.E. (1990) Anti-Cancer Drugs 1, 109–125.
[2] Faulds, D., Balfour, J.A., Chrisp, P. and Langtry, H.D. (1991) Drugs 41, 400–449.
[3] Kapuscinski, J. and Darzynkiewicz, Z. (1986) Proc. Natl. Acad. Sci. USA 83, 6302–6306.
[4] Fox, M.E. and Smith, P.J. (1990) Cancer Res. 50, 5813–5818.
[5] Fisher, G.R., Brown, J.R. and Patterson, L.H. (1990) Free. Rad. Res. Commun. 11, 117–125.
[6] Fisher, G.R., Gutierrez, P.L., Oldcorne, M.A. and Patterson, L.H. (1992) Biochem. Pharmacol. 43, 575–585.
[7] Capranico, G., De Isabella, P., Tinelli, S., Bigioni, M. and Zunino, F. (1994) Biochemistry 32, 3038–3046.
[8] Lown, J. W., Reszka, K., Kolodziejczyk, P. and Wilson, W. D. (1987) in: Molecular Mechanisms of Carcinogenic and Antitumor Activity (Chagas, C. and Pullman, B., Eds.) pp. 243–274, Adenine Press, New York.
[9] Reszka, K., Kolodziejczyk, P., Hartley, J.A., Wilson, W.D. and Lown, J.W. (1988) in: Bioactive Molecules (Lown, J.W., Ed.) Vol. 6, Elsevier, Amsterdam, pp. 401–445.
[10] Kapuscinski, J., Darzynkiewicz, Z., Traganos, F. and Melamed, M. R. (1981) Biochem. Pharmacol. 30, 231–240.
[11] Lown, J.W., Morgan, A.R., Yen, S.-F., Wang, Y.H. and Wilson, W.D. (1985) Biochemistry 24, 4028–4035.
[12] Gandecha, B.M., Brown, J.R. and Crampton, M.R. (1985) Biochem. Pharmacol. 34, 337–346.
[13] Krishnamoorthy, C.R., Yen, S.-F., Smith, J.C., Lown, J.W. and Wilson, W.D. (1986) Biochemistry 25, 5933–5940.
[14] Kotovych, G., Lown, J.W. and Tong, J.P.K. (1986) J. Biomol. Struct. Dyn. 4, 111–125.
[15] Denny, W.A. and Wakelin, L.P.G. (1990) Anti-Cancer Drug Des. 5, 189–20.
[16] Fox, K.R., Waring, M.J., Brown, J.R. and Neidle, S. (1986) FEBS Lett. 202, 289–294.
[17] Chen, K.-X., Gresh, N. and Pullman, B. (1986) Nucleic Acids Res. 14, 3779–3812.
[18] Panousis, C. and Phillips, D.R. (1994) Nucleic Acids Res. 22, 1342–1345.
[19] Collier, D.A. and Neidle, S. (1988) J. Med. Chem. 31, 847–857.
[20] Tanious, F.A., Jenkins, T.C.., Neidle, S. and Wilson, W.D. (1992) Biochemistry 31, 11632–11640.
[21] Palumbo, M., Palù, G., Gia, O., Ferrazzi, E., Gastaldi, S., Antonello, C. and Meloni, G.A. (1987) Anti-Cancer Drug Des. 1, 337–346.
[22] Greenhalgh, C.W. and Hughes, N. (1968) J. Chem. Soc. 1284.
[23] Morier-Teissier, E., Boitte, N., Helbecque, N., Bernier, J.L., Pommery, N., Duvalet, J.L., Fournier, C., Hecquet, B., Catteau, J.P. and Hénichart, J.P. (1993) J. Med. Chem. 36, 2084–2090.
[24] Waring, M.J. and Henley, S.M. (1975) Nucleic Acids Res. 2, 567–586.
[25] Bailly, C., Braña, M. and Waring, M.J. (1996) Biochemistry, submitted.
[26] Bailly, C. and Waring, M.J. (1993) Biochemistry 32, 5985–5993.
[27] Bailly, C. and Waring, M.J. (1995) Nucleic Acids Res. 23, 885–892.
[28] Low, C.M.L., Drew, H.R. and Waring, M.J. (1984) Nucleic Acids Res. 12, 4865–4877.
[29] Islam, S.A., Neidle, S., Gandecha, B.M., Partridge, M., Patterson, L.H. and Brown, J.R. (1985) J. Med. Chem. 28, 857–864.
[30] Waring, M.J. and C. Bailly (1994) J. Mol. Recognition 7, 109–122.