A simple ligation assay to detect effects of drugs on the curvature/flexibility of DNA

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Received 30 August 1996

Abstract Circular DNA molecules can readily be formed from the 169 bp *tyr*T fragment in the presence of T4 DNA ligase. We have analyzed the formation of DNA circles in the presence of the clinically important antitumour drugs amsacrine, mitoxantrone and daunomycin. All three are intercalating agents but they affect the closure reaction differently: daunomycin and mitoxantrone progressively inhibit the formation of circles whereas at low concentrations amsacrine strongly enhances the yield of circles suggesting that this drug can increase the flexibility and/or curvature of DNA. The ligation assay described here may prove useful and widely applicable for investigating the effects of small molecules on the secondary structure of DNA.

Key words: Anticancer drug; DNA binding; DNA structure

1. Introduction

Upon binding to DNA certain proteins such as the catabolite gene activator protein (CAP), the integration host factor (IHF) and the factor for inversion stimulation (FIS) induce a bending of the double helix [1,2]. The DNA bends induced by such proteins are often specifically required for the initiation of transcription or site-specific recombination as well as other enzymatic processes. The phenomenon of DNA curvature/flexibility and its relation to DNA supercoiling and packaging is a crucial determinant of both the function and architecture of protein-DNA complexes [3].

DNA bending can also be induced by peptides and small molecules. Moreover, it is an intrinsic characteristic of DNA molecules containing certain sequences such as adenine-thymine tracts [4,5]. The intrinsic flexibility of particular DNA sequences contributes importantly to the target-site selection by DNA-bending ligands [6,7]. Bending and other structural distortions of DNA induced by small molecules are likely to represent an essential part of their mechanism of action. The antibiotics actinomycin and mithramycin, which are frequently used in chemotherapy of cancer, bend the DNA into the major groove upon binding to the minor groove of GC-rich sequences [8,9]. On the other hand, the antibiotic CC-1065 bends the helix axis towards the minor groove upon covalent attachment into that groove [10,11]. Other antitumour drugs such as ditercalinium and bizelesin can recognize and straighten out bent DNA [12,13]. Evidently, drugs can interfere with the biological functions of DNA by inhibiting protein-induced bending or by mimicking an intrinsic or protein-induced curvature. These considerations have prompted us to develop a simple assay to evaluate the effects of DNA-binding ligands on secondary structure and DNA curvature/flexibility in particular. The assay described in this paper is based on the circularisation of the 169 base pair *tyr*T DNA fragment in the presence of DNA ligase and depends upon measuring the extent to which small molecules interfere with the formation of circular DNA molecules. Results obtained with the antitumour drugs daunomycin, mitoxantrone and amsacrine are reported.

2. Materials and methods

2.1. Drugs and enzymes

Mitoxantrone (Novantrone) was obtained from Laboratoires Léderlé (Oullins, France). Amsacrine and daunomycin were purchased from Sigma Chemical Co. (La Verpillière, France). Enzymes *Eco*RI, alkaline phosphatase, T4 polynucleotide kinase, T4 DNA ligase and exonuclease III were purchased from Boehringer (Mannheim, Germany) and used according to the supplier's recommended protocol in the activity buffer provided.

2.2. Purification and labelling of the tyrT fragment

The plasmid pKMp27 [14,15] containing the *Eco*RI-*Eco*RI 169 bp *tyr*T fragment was obtained by courtesy of Dr. A.A. Travers (MRC Laboratory of Molecular Biology, Cambridge, UK). The plasmid was isolated from *E. coli* by a standard SDS-NaOH lysis procedure and purified by banding in CsCl-ethidium bromide gradients. It was cut with *Eco*RI, treated with alkaline phosphatase and then labelled at the two protruding 5′-termini using T4 polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP (6000 Ci/mmol, Amersham). The labelled *tyr*T fragment was purified by electrophoresis.

2.3. Formation of DNA circles

In a typical experiment, each sample contained 1 μl of ^{32}P-labelled DNA (approx. 0.3 μg/ml, 50 counts per s), 6 μl of water, 10 μl of drug at the desired concentration (or water in the controls) and 2 μl of 10×ligase buffer. After 30 min incubation to ensure equilibration, 1 μl (5 units) of ligase was added to each tube and the reaction was continued at room temperature for 1–60 min (generally 20 min when testing a drug). The ligase was then denatured by heating at 65°C for 5 min and samples were immediately electrophoresed. To verify that the ligation products corresponded to circular DNA molecules, 1 μl of exonuclease III (100 units) was added to the ligase-treated sample (after heat denaturation of the ligase) and incubated for 30 min at 37°C prior to electrophoresis.

2.4. Electrophoresis and data analysis

Ligation products were separated by polyacrylamide gel electrophoresis under native conditions (6% acrylamide gels, 1.5 mm thick). Electrophoresis was carried out for about 1.5 h at 150 V in TBE buffer. Gels were transferred to Whatman 3MM paper, dried under vacuum and scanned with a Molecular Dynamics 445SI PhosphorImager using ImageQuant version 4.1 software. Data in Figs. 2 and 4 were compiled from quantitative analysis of three independent gels such as those shown in Figs. 1 and 3, respectively, and must be considered as a set of averaged values.

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Abbreviations: bp, base pairs; exo III, exonuclease III; SDS, sodium dodecyl sulphate

PII S0014-5793(96)01079-4

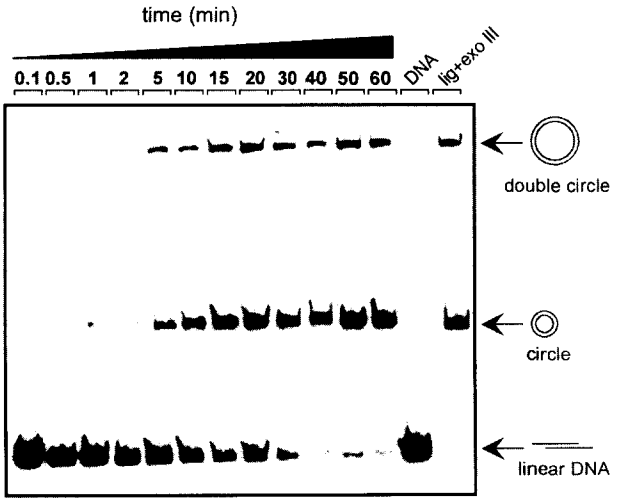

Fig. 1. Time dependence of ligation of the 169 bp *tyr*T DNA into circular molecules. The 5′-end labelled restriction fragment was incubated with the ligase for various lengths of time (0.1–60 min) and the samples were subjected to electrophoresis on a 6% non-denaturing gel. The starting material, incubated without ligase, is shown in the track labelled 'DNA'. The monomeric and dimeric circular products are resistant to digestion by exonuclease III (lane lig+exo III).

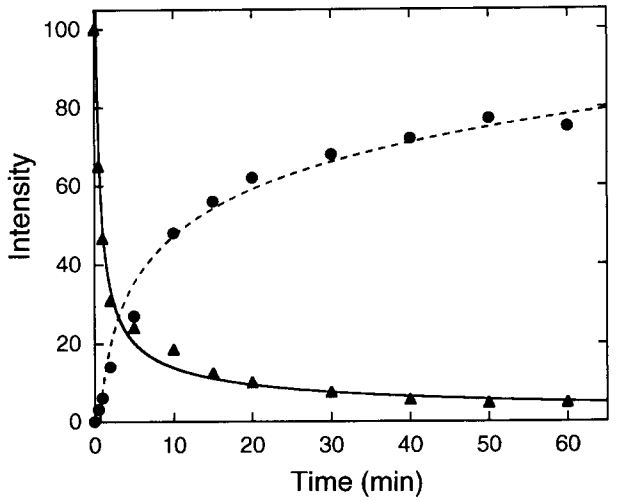

Fig. 2. Time course of formation of monomeric circular DNA (●) upon treatment of linear DNA (▲) in the presence of T4 DNA ligase. Band intensities (%) were determined by densitometric analysis of three autoradiograms like the one shown in Fig. 1.

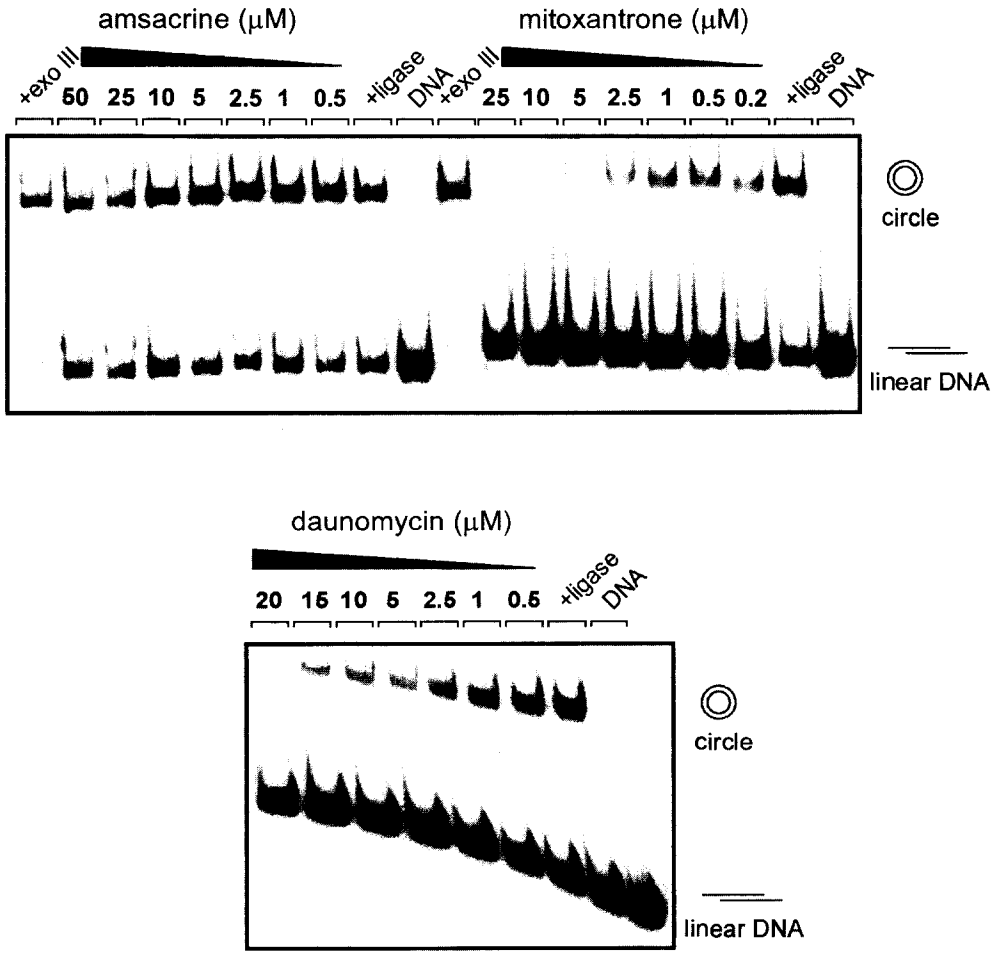

Fig. 3. Effects of intercalating drugs on the formation of circular DNA. The drug concentration (μM) is indicated at the top of each gel lane. The linear DNA was exposed to drug for 30 min prior to reaction with the ligase for 20 min. The ligation products were separated by electrophoresis on a 6% non-denaturing gel.

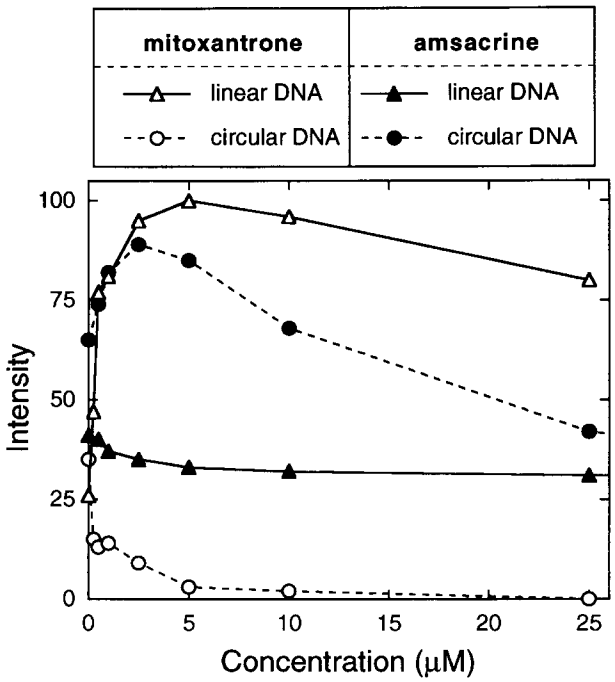

Fig. 4. Comparison of the production of circular molecules (○,●) from linear *tyr*T DNA (△,▲) in the presence of increasing concentrations of mitoxantrone and amsacrine. Band intensities were determined by densitometric analysis of three autoradiograms like that shown in Fig. 3.

3. Results and discussion

Initial experiments were performed in the absence of drug in order to establish optimal conditions for the formation of DNA circles. The 169 bp *tyr*T fragment was incubated with various amounts of ligase for different periods of time at room temperature and the ligation products were separated by electrophoresis (Fig. 1). Under the experimental conditions adopted, three DNA species were obtained: the unreacted 169 bp linear DNA and two types of circular DNA molecules which migrate much more slowly than the linear DNA in the polyacrylamide gel. These two products can be identified as circles by their complete resistance to digestion by exonuclease III. They correspond to the monomeric 169 bp circle and the dimeric 338 bp circle, which both give rise to a single product that comigrates with the linear starting material upon redigesting the ligation products with *Eco*RI (not shown). Large quantities of circular DNA can thus be obtained when using a very low concentration of nucleic acid (< 0.02 μg/ml) so as to avoid the formation of linear dimers. The formation of the circular DNA molecules is time-dependent. As shown in Fig. 2, more than 80% of the starting material is converted into circular products after 20 min reaction. Monomeric DNA circles are formed rapidly, within 15–30 min. Accordingly for subsequent experiments in the presence of drugs the same DNA samples were incubated with the ligase for 20 min so as to obtain 40–60% circular DNA in the controls. Under these conditions, both an increase and a decrease in the formation of circles in the presence of the test drug can be detected.

Fig. 3 shows typical gels which illustrate the results of reacting the linear *tyr*T fragment with DNA ligase in the presence of increasing concentrations of the antitumour drugs amsacrine, mitoxantrone and daunomycin. One can immediately see that these three drugs exert different effects on the circularization of the fragment. Both mitoxantrone and daunomycin strongly inhibit the formation of circles. In contrast, at low concentrations amsacrine increases the yield of circles but inhibits the ligation at concentrations ≥ 10 μM. The differences between amsacrine and mitoxantrone are plainly evident from the quantitative analysis shown in Fig. 4. The observation that these two intercalating agents interfere differently with the closure reaction suggests that they exert different effects on DNA structure. Their inhibitory effects on the circularisation are unlikely to result from direct interference with the ligase itself because the level of inhibition was not particularly affected by varying the enzyme concentration, through we cannot formally exclude the possibility from these experiments. Moreover, the inhibition is not likely to result from drug binding to the true substrates of reaction, i.e. the single-stranded ends of the *tyr*T fragment, given the known preference of intercalators for binding to double helical DNA [16]. Far more likely is the proposition that the observed effects arise from multiple interactions with the DNA molecule so as to alter the ease with which its ends can become apposed.

All three antitumour drugs tested here are classical intercalating agents. Daunomycin, mitoxantrone and amsacrine unwind the DNA double helix by 12–18° [16,17] and provoke an elongation of the DNA helix at the intercalation site in order to insert their planar chromophore between two adjacent base pairs. The inhibition of synthesis of DNA circles observed with the drugs is thus easily attributable to the lengthening and straightening effects which are associated with the intercalation process. More interesting is the observation that at concentrations ≤ 5 μM amsacrine favours the formation of DNA circles. This suggests that the intercalation of its acridine chromophore may be accompanied by a slight increase in DNA flexibility and/or curvature of the double helix. This peculiar effect of amsacrine on DNA structure could well be a prelude to its effect on enzyme-mediated DNA metabolism and hence to its antitumour activity. While the bending of DNA by amsacrine remains speculative at present, it is entirely possible that some such subtle effect on DNA structure contributes to the mechanism by which the drug inhibits replicative events. Then the different effects observed with amsacrine compared to mitoxantrone and daunomycin could explain why, despite their generally assumed common mode of action (i.e. stabilization of topoisomerase II-DNA complexes), there are significant differences in the mechanism by which these drugs interfere with DNA metabolism [18,19]. At all events, the present results serve to indicate the general utility of the ligation assay described here as a means of detecting possible effects of small molecules on DNA secondary structure, including its curvature and flexibility. This simple and rapid test may prove useful in screening experiments to compare novel series of drugs.

Acknowledgements: C.B. thanks Cédric Corvoisier for technical assistance. This work was supported by research grants (to C.B.) from the INSERM, the Ligue Nationale Contre le Cancer (Comité du Nord) and the Association pour la Recherche sur le Cancer; (to M.J.W.) from the Cancer Research Campaign, the Wellcome Trust, Association for International Cancer Research and the Sir Halley Stewart Trust.

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