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Detection of elsamicin–DNA binding specificity by restriction enzyme cleavage

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The sequence specificity of elsamicin A, an anti-tumour antibiotic, binding to DNA was elucidated considering the inhibition of the rate of digestion of linearised pBR322 DNA by *Aat*II, *Cla*I, *Eco*RI, *Hin*dIII and *Nru*I restriction enzymes. Elsamicin A inhibits the rate of digestion by *Nru*I (recognition sequence TCG/CGA) to a greater extent than it does for the other enzymes, thus evidencing the sequence-selective binding of elsamicin to CGC regions in DNA. Our results also show the important role of the neighbouring sequences in the elsamicin A–DNA interactions and their effects on the cleavage by restriction enzymes.

Elsamicin A; Antibiotic–DNA binding; Restriction enzyme

1. INTRODUCTION

Anti-tumour antibiotics presently in use, and many of those in clinical trials are believed to exert their activity by binding to DNA [1, 2]. In a few cases even the determinants of the antibiotic–DNA interactions are quite well understood.

Among the antibiotics that, because of their preclinical activities against various tumours, are nowadays selected for phase I trials, there is a new and novel fermentation product: elsamicin A [3–5], the binding of which to DNA has recently been characterised [6–8]. This antibiotic appears to bind preferentially to sequences containing a CpG step, possibly as a triplet 5'NCG3' [6]. Moreover, elsamicin produces extensive DNA breakage in intact cells [4] that can be also observed in vitro in the presence of a reducing agent and Fe(II) [8]. In fact, the chromophore moiety of elsamicin (chartarin) acts as a true catalyst promoting the production of hydroxyl radicals that act as the ultimate reactive species that cleaves DNA [7,8]. Whereas the mechanisms of DNA breakage induced by elsamicin is well established by experimental [7,8] and theoretical [8] evidence, the exact composition of its sequence-selective binding site remains partially unknown. As mentioned above, it is perfectly established that the thicker binding site for elsamicin A contains the CpG step [6], but as for the antibiotic daunomycin (which contains a different chromophore bound to a sugar moiety [9]) we have tentatively suggested that the sequence specificity of elsamicin requires us to consider, at least, a triplet site [6]. In order to clarify the sequence specificity of elsamicin A, we have undertaken a complementary approach to DNAse footprinting based on the use of different restriction enzymes containing, or not, the CpG step within or near their cleaving sequence. Observation of differential sensitivity at restriction enzyme sites is a corroborated probe for the specific binding of antibiotics to DNA [9–13].

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2. MATERIALS AND METHODS

2.1. *Antibiotics, DNA and restriction enzymes*

Elsamicin A (BMY-28090) was a generous gift from Bristol-Myers, Wallingford, USA. 100 μM solutions of elsamicin were prepared by direct weighing and dissolving in distilled water. Fresh diluted solutions were prepared using the appropriate restriction enzyme buffer. Plasmid PBR322 was purchased from Boehringer-Mannheim, linearised using *Bam*HI and the linearisation was verified by agarose gel electrophoresis. The following enzymes: *Aat*II (Sigma), *Cla*I, *Eco*RI, *Hin*dIII and *Nru*I (Boehringer Mannheim) which contain a unique site in pBR322 were used for the restriction nuclease assays.

2.2. *Determination of binding stoichiometry*

This determination was performed using different concentrations of linearised pBR322 DNA and elsamicin A to obtain different input ratios of drug to DNA, between 0 and 1. Absorbance measurements at 421 nm, the maximum of absorbance of the antibiotic [3], were recorded using a Cobas-Bio autoanalyzer (Roche). Absorbance at 421 nm was plotted versus the input ratio of antibiotic (μM) to DNA (μM, in base pairs). The ratio corresponding to the break point, calculated by a least-squares fit, renders the stoichiometric value.

2.3. *Effect of elsamicin A on the rate of digestion of PBR322 DNA by restriction enzymes*

We followed the method described by Malcom and Moffatt [10] to analyze the effect of elsamicin A on the first-order rate constant at the individual restriction enzymes sites. All the digests were carried out at 37°C using the buffer suggested by the suppliers. In a typical

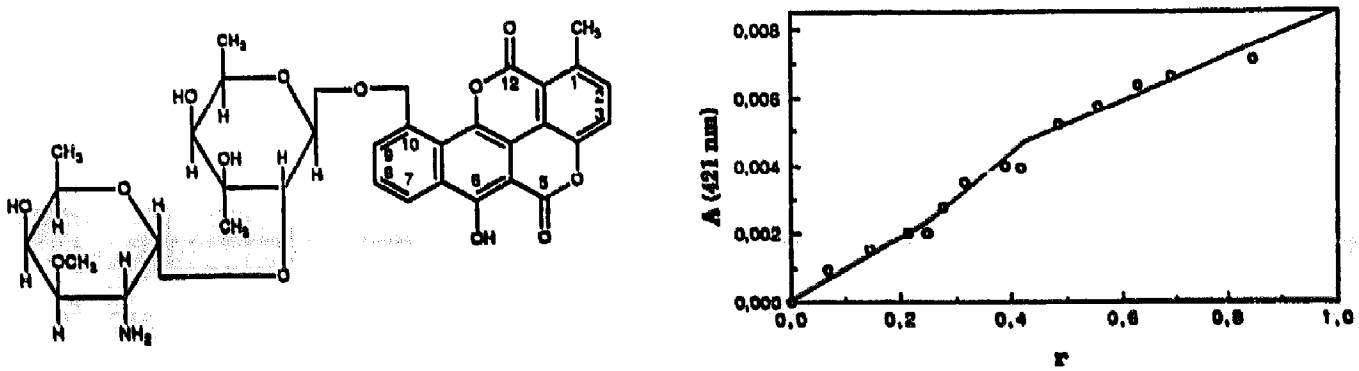

Fig. 1. Structural formula of elsamicin (left panel) and plot of the absorbances at 421 nm against the concentration of the antibiotic added to linearised pBR322 DNA (right panel). The binding stoichiometry was calculated from this plot as described in the main text.

experiment DNA solutions containing between 15 and 30 μM DNA (in base pairs) were incubated at 37°C in the presence of different antibiotic concentrations to render a drug/DNA ratio below the stoichiometric value. The digestions of linearised pBR322 in the presence or absence of the antibiotic were initiated by the addition of the appropriate enzyme. At known time intervals, aliquots were removed from the reaction tube and the reactions were stopped as described in [3]. The digestion products were resolved by electrophoresis on 1% agarose gels in TBE buffer. Gels were loaded with about 0.1 μg DNA/track, stained with ethidium bromide and photographed after running. Negatives were scanned, and the relative area of each band was determined using a PhastImage (Pharmacia) densitometer device. Linear least-squares fit of the logarithm of the relative amount of uncut DNA versus time renders the first order rate constant for cleavage at each restriction site.

3. RESULTS

The binding stoichiometry (r) was determined from the break in the straight lines resulting from the plot displayed in Fig. 1. For the linearised pBR322 we detected one binding site, which saturates at about one elsamicin A molecule per four base pairs (r = 0.25), and another weaker binding site that saturates at a higher ratio of elsamicin to DNA (r = 0.43). These values were used to design a study on the inhibition of the cleavage of linearised pBR322 by five different restriction enzymes (see section 2) in the presence/absence of elsamicin. We analyzed the differential reactivity at the restriction sites using input ratios of antibiotic to DNA around or below the stoichiometric value, thus analyzing differences in cleavage that could be compared straightforward, in order to define differences in the antibiotic binding sites. The enzymes used in our experiments, whose target sites are displayed in Fig. 2, have a unique cutting site in pBR322. The recognition sites for *Aat*II, *Cla*I and *Nru*I contain the CpG step that has been previously described by DNase Footprinting as a component of the sequence-selective site for elsamicin [6]. Digestion of *Bam*HI linearised pBR322 DNA by these enzymes results in the initial appearance of three bands after electrophoresis on an agarose gel. They are the complete linearised pBR322 and two fragments, one smaller than the other, which are the products of the enzyme cleavage. Fig. 3 shows an example of such results (note that the smaller fragment has migrated too far to be seen). The four panels displayed in Fig. 3 depict the time course of digestion of linearised pBR322 by *Nru*I in the absence of elsamicin and in the presence of three different antibiotic/DNA input ratios. All the digestions were performed with each restriction enzyme under conditions in which the reactions follow first-order kinetics. Fig. 4 presents a plot of the logarithm of the relative amount of uncut pBR322 versus digestion time in the presence/absence of different input ratios (r) of drug to DNA. As described in section 2, the slopes of these plots were used to calculate the first-order rate constants. However, we obtained poor reproducibility in the calculations of the first-order rate constant for *Cla*I, possibly reflecting some unknown peculiarities in

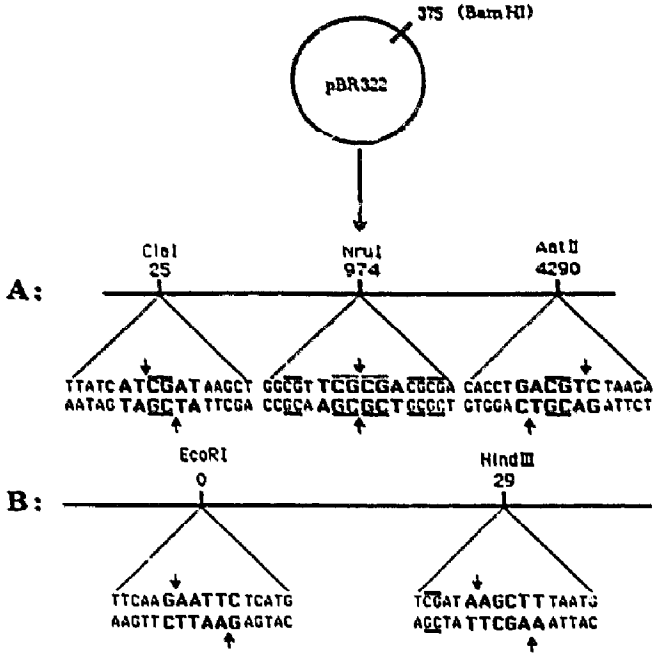

Fig. 2. Map of *Bam*HI linearised pBR322, showing the sequences surrounding the *Cla*I, *Nru*I, *Aat*II, *Eco*RI and *Hind*III sites. *Cla*I, *Nru*I and *Aat*II cleavage sites contain the sequence CpG (A), while *Eco*RI and *Hind*III cleavage sites do not (B).

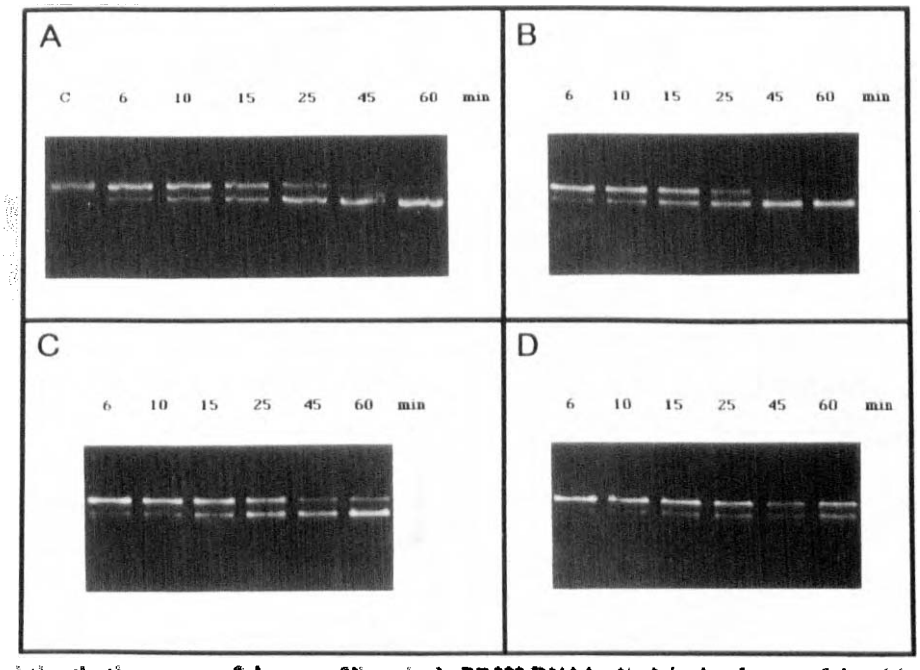

Fig. 3. Agarose gels depicting the time course of cleavage of linearised pBR322 DNA by *Nru*I, in the absence of elsamicin, and its presence at input ratios of antibiotic/DNA of 0.1, 0.15 and 0.25.

its kinetic behaviour. The presence of elsamicin inhibits the cleavage of their putative sequences by *Aat*II, *Nru*I (Fig. 4A and B) and *Cla*I, but enhances the rate of cleavage by *Hin*dIII and *Eco*R1 that do not recognise sequences containing the CpG step needed for sequence-selective binding of elsamicin [6,7]. The meaning of these enhancements in restriction enzyme cutting will be considered in the following discussion.

Fig. 5 shows a comparison of the relative rate of digestion of linearised pBR322 DNA as a function of the antibiotic added. These plots show that *Nru*I (recognition sequence: TCG/CGA) cleavage is 50% inhibited at an input ratio of about 0.18, while *Aat*II (GACG/TC) is not inhibited by more than 15% at the same ratio. Moreover, even though data for *Cla*I (AT/CGAT) should be used with caution, for the reasons given above, the analysis of its agarose gels leads us to consider that the degree of inhibition is even smaller than at the *Aat*II sites. These results are consistent with the sequence-selective binding of elsamicin within, or in the vicinity of, the *Nru*I recognition site.

4. DISCUSSION

In this study, we have used restriction enzymes to disclose which is the preferential nucleotide (N) located at the 5′ site of the elsamicin binding site: 5′NCG3′ deduced from footprinting studies [6]. The rationale of our approach is that a triplet appears to be a more 'realistic' binding site for antibiotics consisting of a chromophore plus a sugar moiety [2,9,13] than the CpG step, where the chromophore would be intercalated. Restriction enzymes seem to be a good probe to determine such triplet sequences [9,13]. At first glance, the relatively stronger inhibition of the *Nru*I reaction by elsamicin A compared to the *Aat*I, *Cla*I, *Eco*R1 and *Hin*dIII reaction observed in Fig. 5 is consistent with the preferential binding of elsamicin to the triplet 5′GCG3′, so elsamicin appears to follow an order of binding preference: TCG < ACG < GCG. Hence, a purine seems to be favoured at the 5′ site, in contrast to what happen with daunomycin, in which a sequence containing an AT pair is favoured [9,14]. The disaccharide moiety of elsamicin could favour different kinds of contact with the nucleotides at the 5′ site, not to mention more subtle dissimilarities that would arise from the different aglycone moiety. Nevertheless, a more rigorous analysis of the possible structural basis of the elsamicin sequence specificity will require us to wait until crystallographic and NMR data are available. It is noteworthy that the related antibiotic chartreusin also recognises CGC triplet sequences [6,15].

The presence of the sequence TCGCGA at the *Nru*I restriction (see Fig. 2) raises a question as to whether we could be observing a mixture of bindings to TCG and GCG sites, although because TCG sites (digested by *Cla*I) are poorly protected we consider that the stronger protection arises mainly from elsamicin binding to 5′GCG3′. The binding to alternating C+G sequences is especially interesting since one could conjecture that, because of that preference, elsamicin might behave as an antitumour drug by interfering with DNA

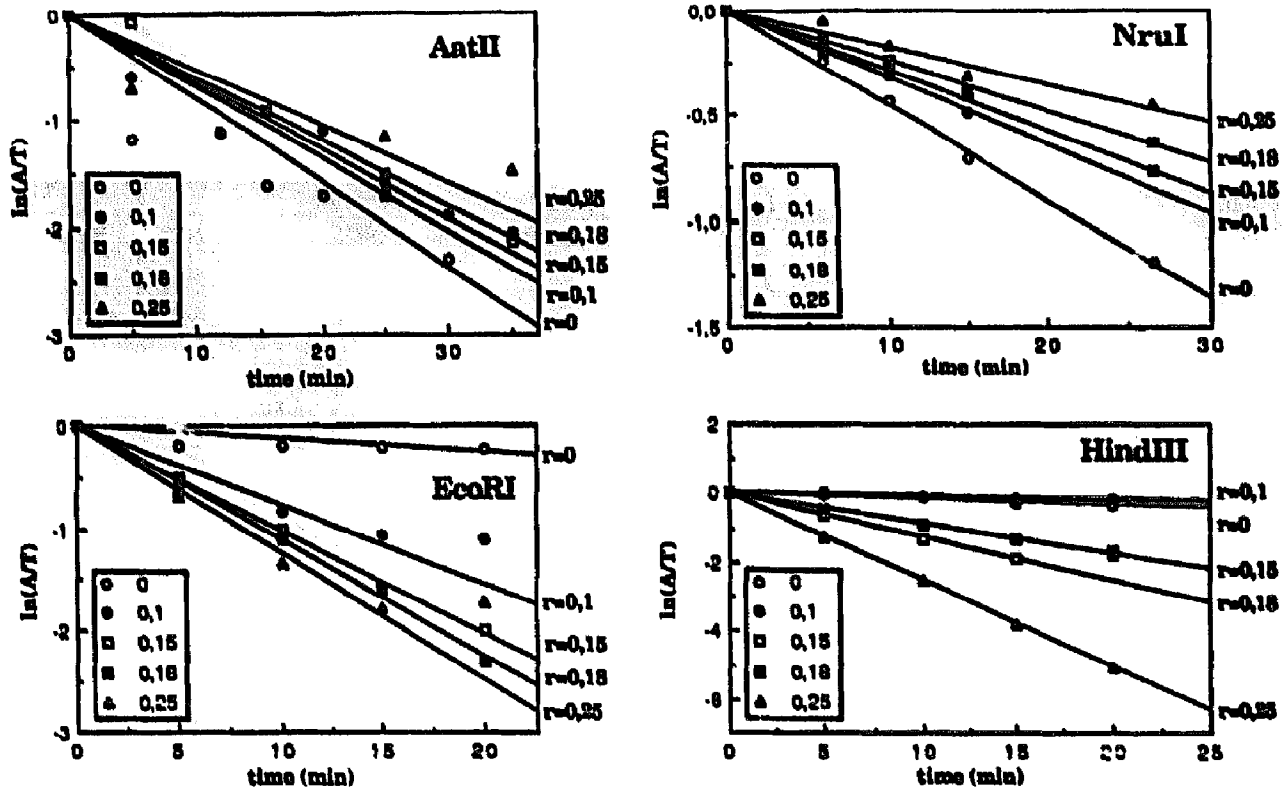

Fig. 4. Comparison of the rates of cleavage of the *Aat*II, *Nru*I, *Eco*RI and *Hin*dIII sites in the presence of different input ratios of elsamicin to DNA. The plot displays the uncleaved fraction (T is the total DNA and A the uncleaved band observed on an agarose gel) of pBR322 DNA as a function of time. Rate constants were determined from the slopes of the least squares fit of the experimental data.

regions that may adopt the Z-DNA conformation in vivo [16,17]. Direct evidence for such an effect is, at present, under investigation in our laboratory.

*Hin*dIII and *Eco*RI reaction sites do not contain binding sites for elsamicin [6], see Fig. 2, and, therefore, no protection of its cleaving activity is detected, but

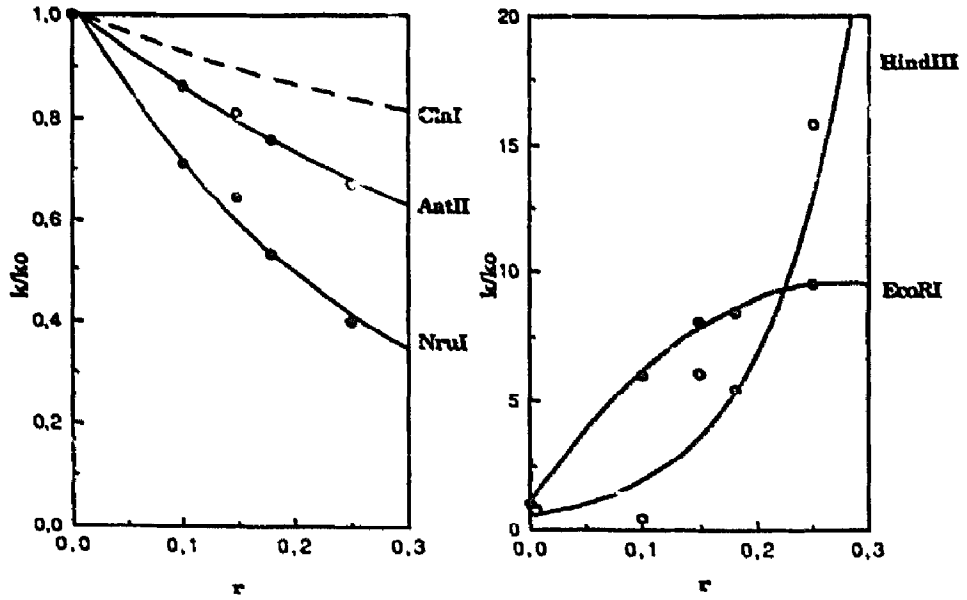

Fig. 5. Comparison of the relative rate of digestion of linearised pBR322 DNA by *Cla*I, *Aat*II, *Nru*I (left panel) and *Hin*dIII and *Eco*RI (right panel) as a function of different input ratios of elsamicin to DNA. The data show that inhibition of the cleavage reaction occurs at lower antibiotic concentration for *Nru*I relative than for the other restriction enzymes. *Hin*dIII and *Eco*RI cleaving reactions are enhanced by the presence of elsamicin A.

cleavage enhancements are observed in Figs. 4 and 5. They can be attributed to changes in the DNA structure induced by elsamicin binding in the vicinity of the restriction sites. Indeed, as can be seen in Fig. 2, putative elsamicin binding sites are found near the *Eco*R1 and *Hin*dIII cleavage sites, and the interpretation of the enhanced cleavage as a result of DNA distortions is not at variance with the explanations given to the differences observed in *Eco*R1 cutting rates [18,19]. Since all the experiments have been performed at smaller antibiotic concentrations than those required to obtain a stoichiometric ratio of elsamicin to DNA ($r < 0.25$), our results cannot be related to 'weak' antibiotic-binding sites.

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