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Visualising the dissociation of sequence selective ligands from individual binding sites on DNA

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Abstract **We have used a modification of the footprinting technique to measure the dissociation of mithramycin, echinomycin and nogalamycin from their binding sites in a natural DNA fragment. Complexes with radiolabelled DNA were dissociated by addition of unlabelled DNA. Samples were removed at various times and subjected to DNase I digestion, and the rate of dissociation from each site was estimated from the time-dependent disappearance of the footprints. For echinomycin the slowest rate of dissociation is from ACGT, while the slowest site for mithramycin contains four contiguous guanines. The dissociation of nogalamycin is extremely slow, even from its weaker sites; the slowest rate was from ACGTA, which took longer than 4 h, even at 37°C.**

Key words: Echinomycin; Mithramycin; Nogalamycin; Dissociation

1. Introduction

Echinomycin, mithramycin and nogalamycin are antitumour agents which are thought to act by virtue of their ability to bind to DNA [1]. Echinomycin is a bifunctional intercalator, which is selective for the dinucleotide CpG [2–5]. Mithramycin binds as a dimer within the DNA minor groove [5,6] and is selective for GC-rich regions of DNA [7–9]. Nogalamycin is an unusual anthracycline antibiotic, possessing bulky groups at both ends of its chromophore, which binds by intercalation, spearing between the DNA base pairs [10–12]. Since it requires local disruption of DNA duplex before it can bind, it has very slow kinetic parameters [13,14], and binds fastest to regions which are easiest to disrupt, such as poly(dA-dT) [13]. It binds especially well to regions containing alternating purines and pyrimidines, especially TGC [15,16].

It has been suggested that, for a series of related compounds, there is a correlation between their biological activity and their persistence time on the DNA lattice. Each of these ligands is known to dissociate slowly from DNA with half-lives of several minutes. The dissociation rates have typically been measured by SDS sequestration techniques, in which a complex between the ligand and DNA is dissociated by adding SDS to sequester the free ligand as it dissociates from the DNA. For echinomycin [17,18], nogalamycin [13,14] and mithramycin [19] the dissociation from mixed sequence DNAs is a complex function requiring several exponentials for its complete description. In contrast the dissociation of echinomycin and nogalamycin from synthetic DNAs is described by a single exponential, suggesting that the complexity observed with natural DNA is due to the parallel dissociation from different binding sites, each of which has different microscopic kinetic constants.

We have recently developed a modification of the footprinting technique [20] for visualising the dissociation of ligands from individual binding sites in a mixed sequence DNA. In this technique a complex between the ligand and radiolabelled DNA is dissociated by addition of excess unlabelled DNA. Samples are removed from the reaction mixture at various times and subjected to DNase I footprinting. Dissociation from each binding site is visualised as the time-dependent disappearance of the footprint. In this paper we use this technique to examine the dissociation of echinomycin, mithramycin and nogalamycin from *tyr*T DNA, and obtain the first estimates for the dissociation of these ligands from individual binding sites.

2. Materials and methods

2.1. Drugs and enzymes

Echinomycin was obtained from the National Cancer Institute USA and stored at 4°C as a stock solution of 1 mM in dimethylsulphoxide. Mithramycin was a gift from Pfizer Inc. USA and was stored at −20°C as a stock solution of 1 mM in 10 mM Tris-HCl pH 8.0, containing 10 mM NaCl. Nogalamycin was a gift from Dr. P.F. Wiley, Upjohn Co., Kalamazoo. DNase I was purchased from Sigma and stored at −20°C at a concentration of 7200 units/ml. All other enzymes were purchased from Promega. [α-^{32}P]dATP (3000 Ci/mmol) was purchased from Amersham.

2.2. DNA fragments

The *tyr*T DNA fragment (Fig. 1) was prepared as previously described [2,8,15], by cutting with *Eco*RI and *Ava*I, and was labelled at the 3′-end of the *Eco*RI site with [α-^{32}P]dATP using reverse transcriptase. The radiolabelled fragment was separated from the remainder of the plasmid on a 6% (w/v) polyacrylamide gel.

2.3. DNase I footprinting

Samples for footprinting were prepared by mixing 9 μl of radiolabelled DNA (about 50 pmol base pairs) with 9 μl of ligand, dissolved in 10 mM Tris-HCl pH 8.0, containing 10 mM EDTA, at the concentration described in the text. This mixture was left to equilibrate for at least 30 min. Dissociation of the antibiotic was initiated by adding 10 μl calf thymus DNA (3 mM base pairs). 4 μl aliquots were removed from this mixture at various times and digested with 2 μl DNase I (150 units/ml dissolved in 2 mM $MgCl_2$, 2 mM $MnCl_2$, 20 mM NaCl). The reaction

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  AATTCCGGTTACCTTTAATCCGTTACGGATGAAAATTACGCAACCAGTTCATTTTTCTCAACGTAACAC
    0         10        20        30        40        50        60
3'-AAGGCCAATGGAAATTAGGCAATGCCTACTTTTAATGCGTTGGTCAAGTAAAAAGAGTTGCATTGTG

TTTACAGCGGCGCGTCATTTGATATGATGCGCCCCGCTTCCCGATAAGGGAGCAGGCCAGTAAAAAGCATT
   70        80        90        100       110       120       130
AAATGTCGCCGCGCAGTAAACTATACTACGCGGGGCGAAGGGCTATTCCCTCGTCCGGTCATTTTTCGTAA

ACCCCGTGGTGGGGGTTCCC
  140       150
TGGGGCACCACCCCCAAGGGCT-5'
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Fig. 1. Sequence of the *tyrT* DNA fragment.

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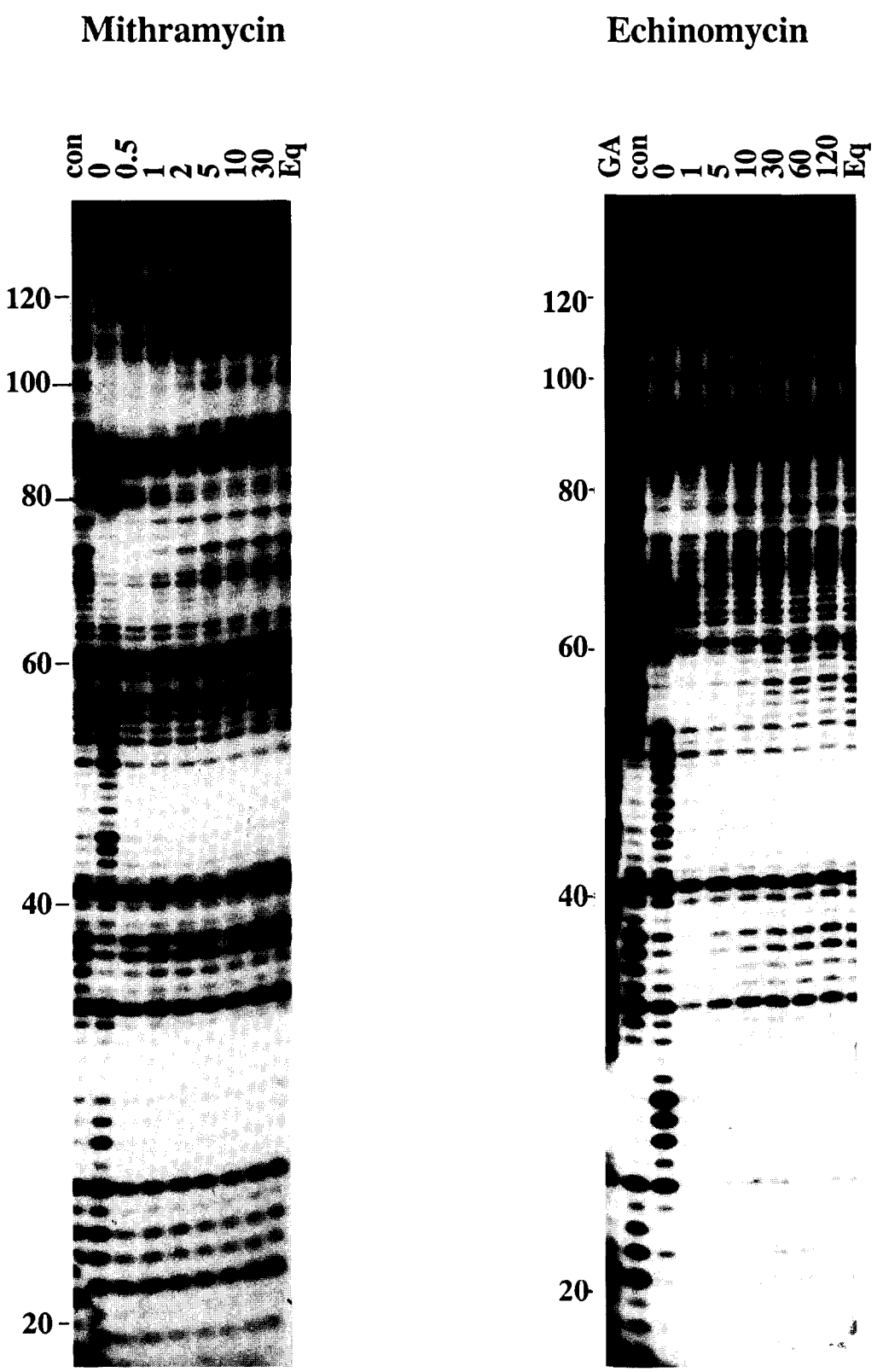

Fig. 2. Dissociation of mithramycin and echinomycin from *tyr*T DNA at 20°C. Aliquots were removed from mixtures of radiolabelled *tyr*T DNA and mithramycin (50 μM) or echinomycin (20 μM) at various times after adding unlabelled calf thymus DNA and subjected to short (12 s) digestion by DNase I. The time after adding the competitor DNA (minutes) is indicated at the top of each lane. 'con' indicates digestion of the DNA alone in the absence of any ligand; '0' corresponds to digestion of a complex with the ligands before adding the unlabelled DNA. In the track labelled 'Eq' the labelled and unlabelled DNA were mixed before adding the ligand, and represents the true equilibrium distribution of the ligand in the dissociating mixture. The track labelled 'GA' is a Maxam-Gilbert marker specific for purines. The numbers correspond to the sequence shown in Fig. 1.

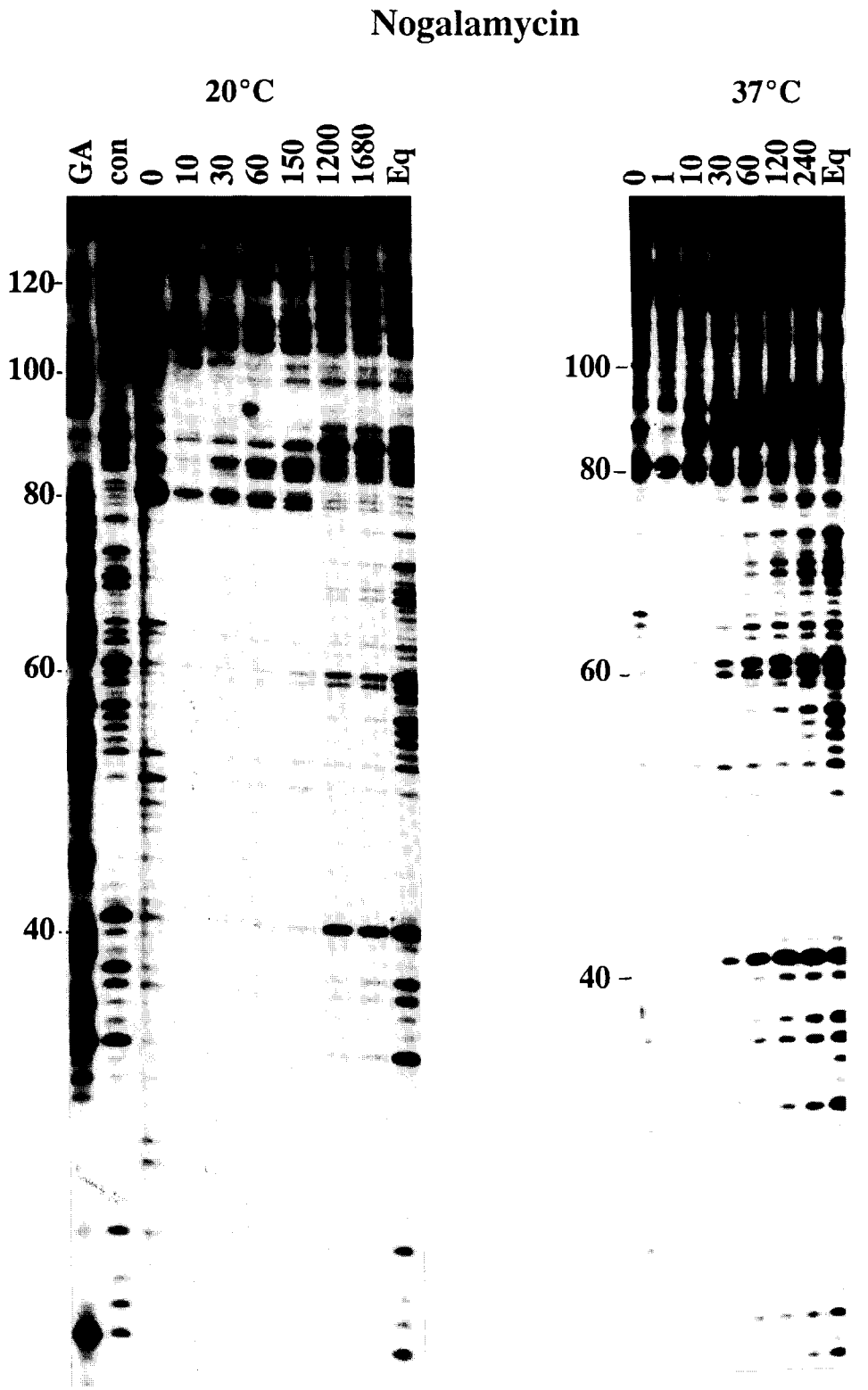

Fig. 3. Dissociation of nogalamycin from *tyr*T DNA measured at 20°C and 37°C. Aliquots were removed from complexes of radiolabelled *tyr*T DNA with nogalamycin (3 μM) at various times after adding unlabelled calf thymus DNA and subjected to short (12 s) digestion by DNase I. The time after adding the competitor DNA (minutes) is indicated at the top of each lane. 'con' indicates digestion of the DNA alone in the absence of any ligand; '0' corresponds to digestion of a complex with the ligands, before adding the unlabelled DNA. In the track labelled 'Eq' the labelled and unlabelled DNA were mixed before adding the drug, and represents the true equilibrium distribution of the ligand in the dissociating mixture. The track labelled 'GA' is a Maxam-Gilbert marker specific for purines. The numbers correspond to the sequence shown in Fig. 1.

was stopped after 12 s by addition of 4 μl formamide containing 10 mM EDTA and 0.1% (w/v) bromophenol blue. The footprinting pattern at equilibrium was determined by mixing the radiolabelled and unlabelled DNA before adding the antibiotics and digesting in the same manner. Digestion patterns of the native DNA and the complex before dissociation were obtained in the usual manner by mixing 1.5 μl radiolabelled DNA with 2 μl drug and digesting for 1 min with 2 μl DNase I (0.03 units/ml).

2.4. Gel electrophoresis

All samples were heated to 95°C for 3 min prior to electrophoresis. The products of digestion were resolved on 8% (w/v) polyacrylamide gels containing 8M urea. Gels were run at 1500 V for about 2 h. These were fixed in 10% acetic acid, transferred to Whatmann 3MM paper, dried under vacuum at 80°C and subjected to autoradiography at −70°C with an intensifying screen. Bands in the digests were assigned by comparison with Maxam-Gilbert markers specific for guanine and adenine.

3. Results and discussion

3.1. Mithramycin

Fig. 2 shows DNase I footprinting profiles for the dissociation of mithramycin from *tyr*T DNA. Three clear DNase I footprints are evident in the 'time 0' lane, as previously reported [8,9], between positions 35–40, 65–78 and 91–122. Each of these is located in a GC-rich region and the latter two contain several overlapping ligand binding sites. It can be seen that, on adding the competitor DNA, each of these footprints disappears in a time-dependent fashion, which is not the same for each site. Looking first at the top site (91–122) it can be seen that the uppermost bands around position 115–120 return to the control pattern by the first time point suggesting that dissociation from GGCC (121) and CCC (113) is relatively fast; this was also too fast to measure at 4°C (not shown). Dissociation from 106–112 (presumably corresponding to binding at the CGGG located at position 106) is a little slower; bands in this region still show attenuated cleavage after 0.5 min but not 1 min. In contrast dissociation from around position 100 (within the sequence GCGGGGCGC) is much slower, and bands only begin to reappear 5 min after adding the competitor DNA. Dissociation from the middle footprint (65–78, which contains three overlapping mithramycin binding sites [9]) also shows an intermediate rate and is complete by 1 min. Similar experiments performed at 4°C (not shown) suggested that dissociation from the top of this footprint was slower than from the bottom. Dissociation from the lowest site (35–40) is also relatively fast and is largely complete by the first time point. This region contains only two GC residues (GpG) and is a much poorer binding site.

These data confirm that the dissociation of mithramycin from its GC binding sites is relatively slow and that the dissociation time constants from different sites vary between 0.5–5 min. The longest rates are observed from the uninterrupted stretch of GC residues around position 100, and probably result from the slow dissociation from GGGG. Several regions of enhanced DNase I cleavage are apparent in the 'time 0' lane (especially around positions 80–90 and 123–125). These are no longer apparent by the first time point, suggesting that they arise from changes in the ratio of free DNA to enzyme in the presence of the drug [21], and are not caused by drug-induced changes in DNA structure, since the latter should disappear at a similar rate to the footprints.

3.2. Echinomycin

Fig. 2 also shows the time course for the dissociation of echinomycin from *tyr*T DNA. The fragment contains 8 echinomycin binding sites (CpG) between positions 25–110, each of which has been shown to yield clear DNase I footprints [2]. The top three CpG sites (CCGA, CCGC and GCGC) overlap to produce a single DNase I footprint. Dissociation from this region is complete by 5 min. The next footprint down (68–82) also contains three overlapping echinomycin binding sites (GCGT, GCGC and GCGG). Bands in the lower portion of this footprint reappear sooner than those in the upper region, suggesting that GCGG may be a poorer echinomycin binding site than GCGT or GCGC. The footprint around position 60 contains an isolated echinomycin binding site ACGT; dissociation from this site takes about 30 min, slower than from any of the other sites on this fragment. Another isolated CpG site is responsible for the footprint seen around position 37 (ACGC). Dissociation from this site is relatively fast (about 5 min) even though it represents only a single base change from the next site up. Once again several regions of enhanced DNase I cleavage are apparent in the 'time 0' lane, which have disappeared by the first time point. Their rapid removal suggests that they are not caused by drug-induced changes in DNA structure.

Based on these results it appears that echinomycin dissociates most slowly from ACGT, requiring about 30 min for the footprint to disappear, compared with dissociation from GCGG which is complete within 1 min.

3.3. Nogalamycin

Fig. 3 shows the dissociation of 3 μM nogalamycin from *tyr*T DNA. This ligand does not have an absolute sequence binding preference, binding best to regions of alternating purines and pyrimidines [15,16]; it therefore causes a general decrease in DNase I cleavage throughout a large part of the fragment, which makes the dissociation profiles difficult to interpret. Since the dissociation is extremely slow at 20°C we have repeated the experiments at 37°C to obtain a clearer estimate of the different relative dissociation times. The region between 80–105 contains a good nogalamycin binding site (ATGCG) around position 94 [15]. The ligand dissociates from the upper part of this region with a time constant of about 20 h at 20°C, compared with bands in the lower part of this footprint which reappear after only about 60 min. Most of the remainder of the fragment shows a single large protection which persists for most of the 24 h time course at 20°C. Exceptions to this are the bands at positions 41, 60 and 61 which have returned by 20 h. Clearer information about the dissociation from this region can be obtained by examining the footprinting patterns obtained at 37°C. For most of the region below position 80 the cleavage pattern has returned to that in the control by 120 min after adding the competitor DNA. As noted at 20°C the bands at 41, 60 and 61 return the fastest. Bands around position 57 (corresponding to ligand binding to the sequence ACGTA [15]), only begin to reappear after 120 min and cleavage in this region has not returned to that in the control even at the longest time point (4 h).

These results demonstrate that the dissociation of nogalamycin is extremely slow. This takes up to 30 min at 37°C from its weaker binding sites, and requires longer than 4 h at the optimum binding sites.

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