The DNA sequence specificity of cyanomorpholinoadriamycin

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Cyanomorpholinoadriamycin (1 μM) was reacted in a transcription buffer with DNA of an initiated transcription complex. Subsequent elongation of the initiated complex revealed permanent transcriptional blockages at 16 sites after only 5 min of drug-DNA reaction time. The most dominant sites were immediately prior to 5'-CC (six) and 5'-GG (six) sequences of the non-template strand, consistent with the presence of intrastrand crosslinking between adjacent guanine residues. Minor levels of blockage were at 5'-GC and 5'-CG sequences and may reflect low levels of interstrand crosslinking.

Cyanomorpholinoadriamycin; DNA; Intrastrand crosslink; Transcription; Sequence specificity

1. INTRODUCTION

Adriamycin, an anthracycline antibiotic with an extremely rich biochemistry has been one of the most successful antitumour agents in clinical use throughout the last two decades [1–3]. Its use, however, has been restricted by several factors including cardiotoxicity (which limits the absolute level of drug administered to 550 mg/m^2), acquired drug resistance and inactivity of the drug against some tumours [2,3]. As a consequence of these limitations much research has been directed at the design of Adriamycin derivatives with improved antitumour activity and fewer limiting properties [2–4].

The most promising derivative developed so far is the 3'-(3-cyano-4-morpholinyl)-3'-deaminoadriamycin (CMA) which has been shown to be up to 1400-fold more potent than Adriamycin in vitro [5,6] while retaining similar activity in experimental tumours [7]. The chemistry, biochemistry and biological responses of CMA have recently been extensively reviewed by Acton et al. [8]. Two major factors appear to contribute to the enhanced potency of CMA over Adriamycin. Firstly, the increased lipophylicity of CMA may facilitate the rapid passage of the drug into the cell and ultimately the nucleus. Once in the nucleus the second factor, the cyanide moiety, has been suggested to act as a leaving group allowing covalent binding of the drug to DNA [8,9].

CMA has been shown to crosslink DNA in vitro [10–14] with a preference for GC-rich DNA [15] and there is now good evidence that the cytotoxicity of CMA in vivo is directly related to the extent of inter-

strand crosslinks [8]. However, the sequence specificity of the interaction has yet to be established.

In order to determine the nature of CMA crosslinking of DNA, we have utilised an in vitro transcription assay [16,17] and present here the sequence specificity of CMA complexes with DNA established by the length of specific transcriptional blockages in this assay. The blockage sites are consistent with the formation of both intrastrand and interstrand crosslinks.

2. EXPERIMENTAL

2.1. Reagents
CMA was a gift from Dr. E.M. Acton (NCI, Washington). The drug was dissolved in DMF as a 2 mM stock solution and stored in the dark at –20°C. *E. coli* RNA polymerase was from Pharmacia and all other reagents were as described previously [16].

2.2. Source of DNA
The 497 bp *PvuII/SalI* restriction fragment containing the *lac* UV5 promoter was isolated from pRW1 as described previously [17].

2.3. Formation of transcription complex
An initiated transcription complex was formed (126 μl) as described previously [16,17] using *E. coli* RNA polymerase (32 units), 2 μg of 497 bp DNA, 200 μM GpA, 5 μM GTP, CTP and ATP and [α-^{32}P]UTP in transcription buffer comprising 40 mM Tris (pH 8.0), 100 mM KCl, 3 mM MgCl$_2$, 0.1 mM EDTA, 10 mM DTT, 0.125 mg/ml acetylated BSA and 1 unit/μl RNasin.

2.4. Reaction of CMA with initiated transcription complex
The initiated transcription complex was divided into two aliquots of 50 μl; 5 μl of transcription buffer was added to one and 5 μl of CMA (1 μM final concentration) in transcription buffer was added to the other. The reactions were incubated at 37°C and aliquots removed at specified times.

2.5. Elongation of transcription complex
The CMA-treated initiated transcription complexes were elongated for 4 min at 37°C by the addition of GTP, ATP, CTP and UTP at a final concentration of 2 mM in the presence of 400 mM KCl. The

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reaction was terminated by the addition of an equal volume of loading buffer as described previously [16,17].

2.6. Electrophoresis and autoradiography

Electrophoresis was performed as described previously [16,17]. Transcriptional blockages were detected and quantitated using a 400 B PhosphorImager (Molecular Dynamics, CA).

3. RESULTS

3.1. Reaction time

Fig. 1 shows the effect of reaction time on the formation of CMA-induced transcriptional blockages. Increa-
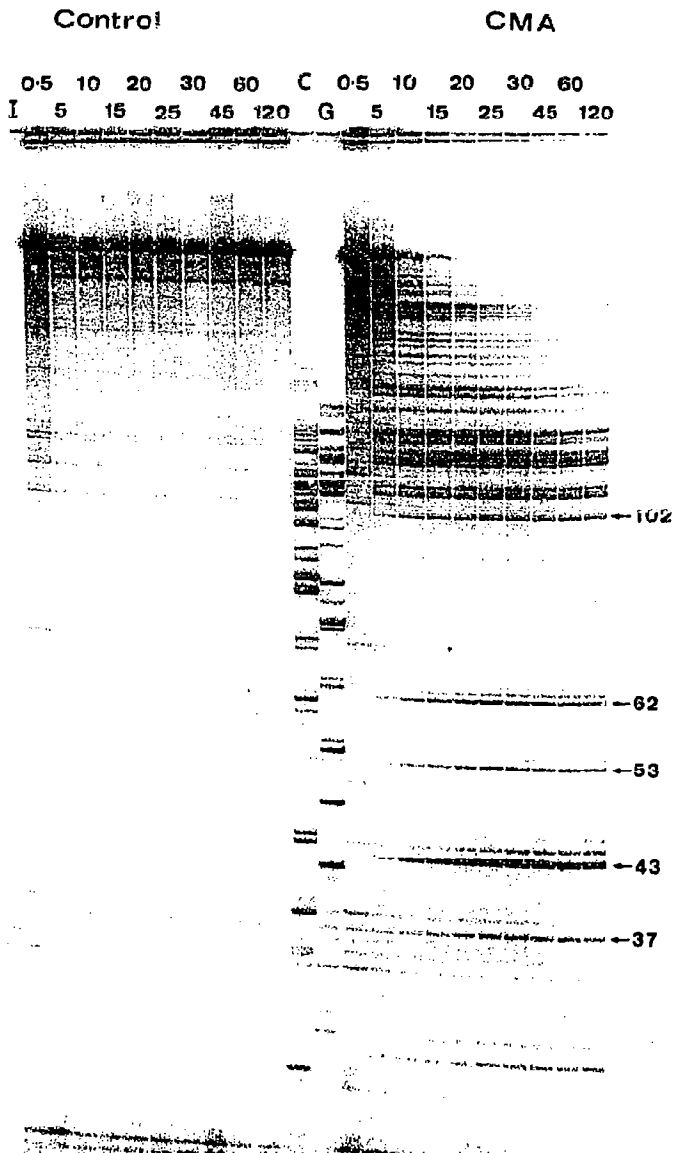
Fig. 1. Transcriptional blockages induced by CMA. The drug (1 μM) was reacted with initiated transcription complexes in transcription buffer (pH 8.0) at 37°C for times ranging from 0.5 to 120 min. The initiated complex is shown in lane I, while C and G denote sequencing lanes where transcription was terminated by methoxy-CTP and methoxy-GTP, respectively [16–18]. The control lanes represent elongation of the initiated complex in the absence of added drug.
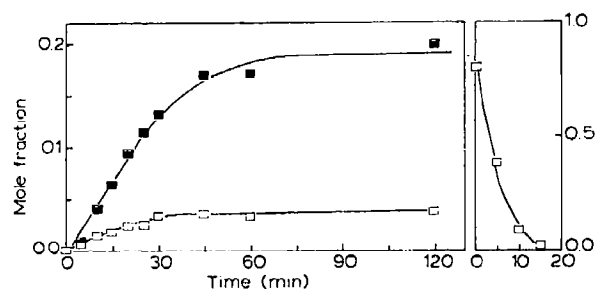
Fig. 2. Rate of reaction of CMA with DNA. The mole fraction of blocked transcripts (□, 37-mer; ■, 43-mer) was calculated from the data shown in Fig. 1, and is shown at drug–DNA reaction times from 0 to 120 min. The small panel shows the decay of full-length transcript from 0 to 20 min.

sing reaction time of the initiated transcription complex with 1 μM CMA revealed transcription blockages not evident in the control lanes. The relative amount of RNA in each band was quantitated using a PhosphorImager, yielding the mole fraction of RNA corresponding to each blockage site.

Blockages are evident after 5 min of incubation of the initiated transcription complex with CMA. The strongest early blockages, yielding RNA 37 and 43 nucleotides long, increased linearly as a function of reaction time for 30 min and reached a maximal level after approximately 1 h (Fig. 2). The cumulative effect of blockages at different sites reduced the full-length transcript to zero after approximately 20 min, with a half-life of approximately 2 min under these conditions.

3.2. Sequence specificity

The sequence specificity of the binding of CMA to DNA under in vitro transcription conditions is presented in Fig. 3. Assignment of bands in the region of 100–140 nucleotides was performed on a separate gel subjected to double the electrophoresis time (data not shown). Analysis of the drug blockage sites reveals 5 major features. In the 380 bp probed by this procedure, 16 blockage sites are well resolved in the 120 bp region from 20–140, with 6 of these occurring at 5′-CC sequences (non-template strand), 6 at 5′-GG sequences, 3 at 5′-GC and one at 5′-CG. The strongest blockages (mole fraction of RNA >0.05) were at 5′-CC (sites 3, 9, 10 and 16) and 5′-GG (site 12). The single most dominant blockage site is at 5′-CC (site 3) and is clearly more lethal than neighbouring 5′-GG sites at 1, 4 and 5. Almost all blockages are one nucleotide prior to the consensus blockage sequences shown in Fig. 3, and this is consistent with transcriptional blockage patterns observed previously for other DNA-acting drugs [18,19]. Only weak blockages are apparent at 5′-GC and 5′-CG sites (2, 6–8).

All of the major blockages shown in Fig. 3 were permanent in that no progression of RNA polymerase was evident at any site for elongation times up to one hour (data not shown).
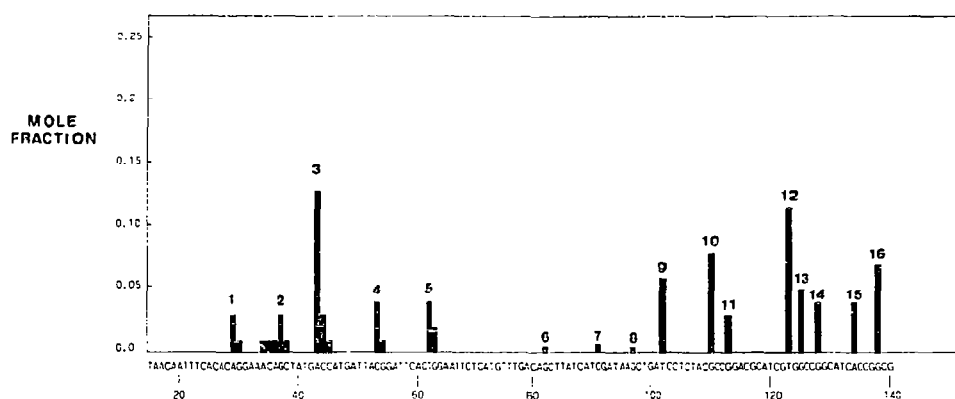
Fig. 3. Sequence specificity of blocked transcripts. The mole fraction of blocked transcripts (30 min reaction time of Fig. 1) is shown at each nucleotide in the transcribed sequence up to transcripts of 140 nucleotides in length.

4. DISCUSSION

4.1. Sequence specificity

Since the dominant blockage sites were all at (or immediately prior to) 5'-CC or 5'-GG (non-template) sites, it appears that these are the major sites of interaction of CMA with DNA in vitro. From the present results it is not possible to conclude whether adducts are on the template or non-template strand. However, because of the known accessibility and reactivity of guanine N-2 and N-7 positions [20], the known reaction of guanine with alkylators and drugs [21,22], and the limited reactivity of cytosine [20,21], it is highly likely that the adducts are at 5'-GG of both the template and non-template strands (i.e. blockages at 5'-CC of the non-template strand arise from adducts at 5'-GG of the template strand, whereas blockages at 5'-GG of the non-template reflect adducts on that strand). For these reasons we conclude that all of the major transcriptional blockages arise from CMA adducts at adjacent guanine residues, and this is indicative of intrastrand crosslinking. The absolute requirement for adjacent guanine residues is also highlighted by the lack of significant transcriptional blockages at any isolated G residues, irrespective of whether on the non-template or template strand.

4.2. Interstrand crosslinking

If the major blockages at 5'-CC and 5'-GG (non-template) sequences represent intrastrand crosslinking at adjacent G-residues, as discussed above, then the minor blockages at 5'-GC probably reflect interstrand crosslinking sites. It has been well documented recently that high levels of intrastrand crosslinks form with DNA in vitro in the absence of metabolic activation [12,14,15], at CMA concentrations in the micromolar range and for reaction times up to 100 min. By comparison, interstrand crosslinking involving metabolic activation occurs more rapidly even at concentrations as low as 10^{-11} M [13]. It therefore appears likely that interstrand crosslinking is metabolically activated in vivo and that only low levels are detected in vitro unless

substantially higher concentrations of CMA are employed. Apparently intrastrand crosslinking in vitro is substantially more favoured than interstrand crosslinking but this may not be the case in vivo.

The observation that all transcriptional blockage sites were at 5'-CC. GG, GC or CG sequences indicates that the extent of intrastrand crosslinking should be highly dependent on the (G+C) content of any DNA, irrespective of the exact nature of the sequences involved with interstrand crosslinking. Such a dependence has recently been reported by Jesson et al. [15] using the ethidium bromide fluorescence assay, and is consistent with the present in vitro transcriptional results.

4.3. Structure of crosslinks

Although the role of the cyano group has been well established as important for interstrand crosslinking, both in vitro and in vivo [8,9,12], and the sequence specificity of the sites has now been defined, it is still not possible to suggest with confidence the nature of the second attachment site. The reduced extent of intrastrand crosslinking by the 5-imino derivative of CMA has demonstrated that the quinone moiety serves some role in interstrand crosslinking [15]. Other mechanisms and structures have been suggested to account for crosslinking [8,9] and it will now be possible to test the validity of these structures based on the apparent assignment of 5'-GG intrastrand crosslinks and tentative 5'-GC interstrand crosslinks.

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