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Distamycins inhibit the binding of OTF-1 and NFE-1 transfactors to their conserved DNA elements

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ABSTRACT

We investigated the effects of the antiviral agent distamycin A and of a distamycin derivative (FCE 24517) which possesses antineoplastic activity on the binding of some regulatory proteins to DNA. Both compounds inhibited the binding to DNA of the ubiquitous octamer binding factor OTF 1 and of the erythroid specific GATAAG protein (NFE 1). This was shown using the electrophoretic mobility shift assay on a DNA fragment of human γ-globin gene promoter (-156 to -201), on the same fragment with a point mutation (T to C mutation) known to have an increased affinity of binding for NFE 1, on a DNA fragment of human histone H2B promoter and on a DNA fragment of mouse α1 globin promoter. The ability of distamycin or of FCE 24517 to inhibit the binding was specific for AT-rich sequences since neither drug inhibited the binding of nuclear protein factors to the sequence CCACACCC of the human ß globin gene. Binding to DNA was investigated by evaluating the drugs' ability to protect selected sequences from DNase I digestion (DNase footprinting). Distamycins binding was highly preferential for DNA sequences containing stretches of AT. These studies indicate that chemicals which have a high degree of DNA sequence-specific binding can selectively inhibit the binding of regulatory proteins to DNA. These effects might be responsible for modification of the transcripion of specific genes and might to some extent account for these drugs' antiviral and antineoplastic activities. This approach offers potential for the investigation of new such drugs.

INTRODUCTION

Distamycin A is an antibiotic originally isolated from Streptomyces distallicus (1) and subsequently obtained by total synthesis (2). It has strong antiviral activity (3). Recently some distamycin derivatives proved to be potent antiproliferative agents with activity against some murine tumors (4,5) , but their mechanism of action is still unknown. They form a strong reversible complex with double helical B-DNA with high selectivity for AT rich sequences.

Distamycin A binds deeply into the minor groove of B-DNA ; NH groups of each of the three pyrrolecarboxamide rings form hydrogen bonds with N-3 of adenine or O-2 of thymine and the CHs of the aromatic pyrroles are bound to the C-2 hydrogens of adenines by Van der Waals interaction (6) . Distamycin A binding to DNA widens the minor groove by several Angstroms by bending the DNA helix and by inducing conformational changes in neighboring DNA (6). These changes can alter the chromatin structure (7,8) and consequently affect normal DNA-protein interactions.

It is widely accepted that transcriptional initiation is mediated by formation of stable

complexes between DNA binding proteins (trans factors) and sequences (cis elements) in promoter and enhancer regions. These elements contain short regulatory boxes that mediate DNA-protein interaction, leading to transcriptional activation (9). By exploiting the gel shift assay and DNase I footprinting techniques, several sequence specific DNA binding proteins have been identified (10). The octanucleotide ATTTGCAT sequence has been shown to be important in trancriptional activation of several genes including human histone H2B, immunoglobin, U2 and U1 small nuclear RNA, and binds a ubiquitous (OTF 1) and a lymphoid specific (OTF 2) protein (11-13); mutations of every single nucleotide (with the possible exception of the first A) greatly impair binding of both proteins. Another conserved sequence (GATAAG) in promoters and enhancers of several globin genes binds a protein (NFE 1) present only in erythroid cells (13,14).

Since distamycin A and its derivatives bind to DNA containing AT sequences, these drugs might alter the binding of protein factors to boxes in which AT sequences are essential for recognition and stable binding, thus possibly interfering with the activation of several genes. This study was undertaken to verify this hypothesis.

MATERIALS AND METHODS

Drugs

Distamycin A and its derivative N-deformyl-N- (4-(N,N-bis-(2-chloroethyl) amino)benzoyl) - distamycin A (FCE 24517) were synthetized by Farmitalia-Carlo Erba, Milan, Italy and dissolved in water or 0.1% DMSO respectively for DNA or cell treatment.

DNA fragments

Sequences of DNA fragments used in these experiments were as follow (only top strand is shown): human γ-globin: from -156 to -201 (CCCTGGCAAAGTCTGTCTATAAACGTAACT CTATCACACCCCTTCC) (13,14) -175 mutant γ-globin: same as above except T to C mutation at -175;

H2B promoter: from -68 to -23 (CTGAAGTTGAGAAGTGGAATAAACGTATTCG CTAAGATATATTTTC)

mouse α1 globin promoter : (CGGGCAACTGATAAGGATCCC) (14).

All fragments were ^{32}P labeled at 5' ends with T4 polynucleotide kinase and [$\gamma\,^{32}P$] ATP according to standard procedures.

Nuclear extracts

Nuclear extracts from human erythroleukemia cells (K562) were prepared according to Dignam et al. (15).

Electrophoretic mobility shift assay

DNA fragments labeled at the 5'-end (0.1-0.5 ng) were preincubated for 30 min at room temperature with different concentrations of Distamycin A or FCE 24517 in the presence of poly (dI-dC) (2 μg) and then incubated with nuclear extracts (5 μg of proteins) for other 30 min, electrophoresed in 50 mM Tris borate pH 8.2 and autoradiographed (16).

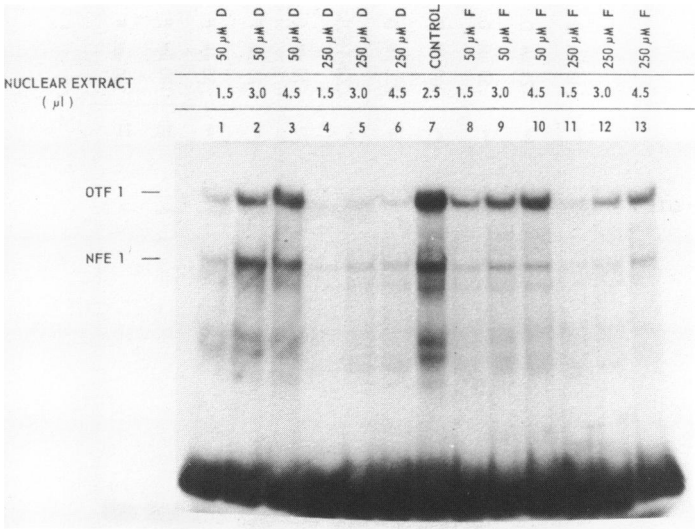

Figure 1
Binding of nuclear factors to human γ-globin fragment. Labeled DNA was preincubated with 50 (lanes 1,2,3) or 250 μM (lanes 4,5,6) distamycin A (D) or with 50 (lanes 8,9,10) or 250 μM (lanes 11,12,13) FCE 24517 (F) for 30 min and then incubated for 30 min with K562 nuclear extracts as indicated in the figure. Lane 7 is untreated DNA.

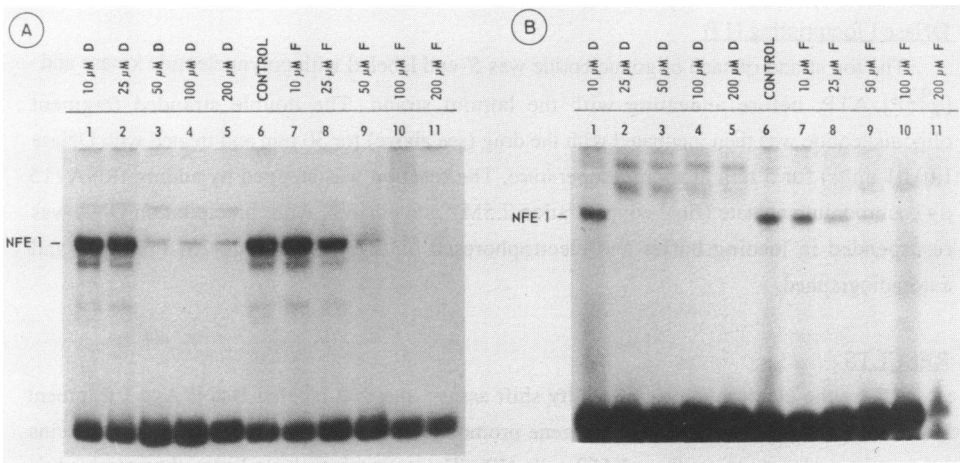

Figure 2
A) Effect of distamycins on NFE 1 binding to -175 fragment; the -175 fragment was preincubated with 10 (lane 1) 25 (lane 2) 50 (lane 3) 100 (lane 4) and 200 μM (lane 5) Distamycin A (D) or with 10 (lane 7) 25 (lane8) 50 (lane 9) 100 (lane 10) and 200 μM (lane 11) FCE 24517 (F) for 30 min before addition of K562 nuclear extract (5μg). Lane 6 is untreated fragment plus nuclear extract.
B) Effect of distamycins on NFE 1 binding to mouse α1 globin fragment.

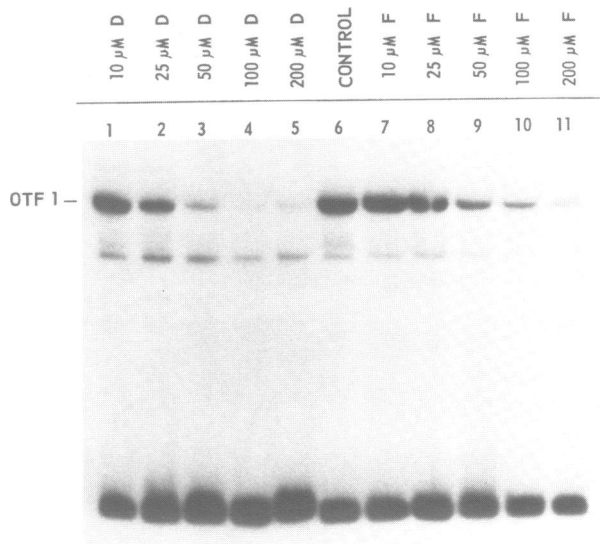

Figure 3
Effect of distamycins on OTF 1 binding to an oligonucleotide derived from the sequence of H2B promoter (from -68 to -23); pretreatment of DNA fragments with distamycins and subsequent treatment with K562 nuclear extract were as described in figure 2.

DNase I footprinting (17)

The top strand of each oligonucleotide was 5'-end labeled with polynucleotide kinase and [γ^{32}P] ATP, before annealing with the bottom strand. The double stranded fragment oligonucleotide was then incubated with the drug (see above) for 30 min and treated with DNase I (0.01 units) for 5 min at room temperature. The reaction was stopped by adding tRNA (15 μg), ammonium acetate (final concentration 2.5M) and ethanol. After precipitation DNA was resuspended in loading buffer and electrophoresed on 20% acrylamide-7M urea gels and autoradiographed.

RESULTS

Using the electrophoretic mobility shift assay the ^{32}P labeled BstNI-Apa I fragment (from -201 to -156) of the γ-globin gene promoter had been shown to bind two proteins present in nuclear extracts from K562 cells (12). These proteins have been characterized in terms of their DNA binding specificity and correspond to the ubiquitous octamer binding protein (OTF 1) (11,12,16) and to the erythroid-specific GATAAG protein (NFE 1) (14,18 and references within). Preincubation of the labeled fragment with distamycin A and FCE 24517 clearly lowered the intensity of both these bands (Fig.1). This effect was already observed at 50 μM distamycin A or FCE 24517 and increased at higher drug concentrations (compare

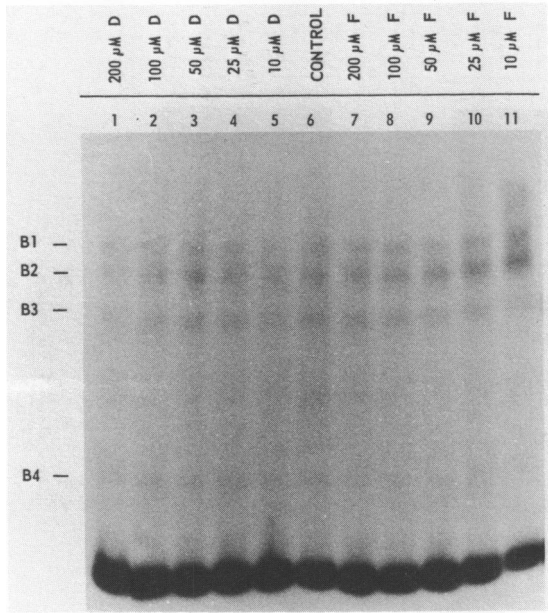

Figure 4
Effect of distamycins on nuclear factors binding to a human ß-globin fragment; the BstNI-Hind III ß-globin fragment was incubated with 200 (lane 1) 100 (lane2) 50 (lane 3) 25 (lane 4) or 10 µM (lane 5) Distamycin A (D) or with 200 (lane 7) 100 (lane 8) 50 (lane 9) 25 (lane 10) and µM (lane11) FCE 25417 (F) for 30 min followed by incubation with K562 nuclear extract for 30 min. Lane 6 is DNA incubated with nuclear extract only.

for example lane 7 with lanes 2,5,9 and 12). Preincubation of the drug with DNA is essential for this effect; when the drug was added together with DNA and protein or preincubated with protein alone, little or no effect was observed (data not shown).

In order to investigate the effect of these drugs on the binding of the two proteins separately, we used a DNA fragment containing a point mutation in the octamer sequence (-175 T to C) ; this mutation, abolishes the binding of OTF 1 and enhances that of NFE 1 (12). As shown in Fig. 2 A the binding of NFE 1 was severely affected by both compounds at 50 µM but a significant reduction was already seen at lower concentrations, particularly in the case of FCE 24517. These results were confirmed by employing a different oligonucleotide (14) derived from mouse α1 globin promoter and showing a high affinity binding site for NFE 1 (GATAAG), as the only homology with the previous one (Fig.2B) .

Using an octa box containing synthetic oligonucleotide derived from human histone H2B promoter (11) and shown to bind OTF 1, a single specific retarded band was generated. Treatment with distamycin A or FCE 24517 prevented protein binding in a concentration dependent manner from 25 to 200 µM (Fig. 3).

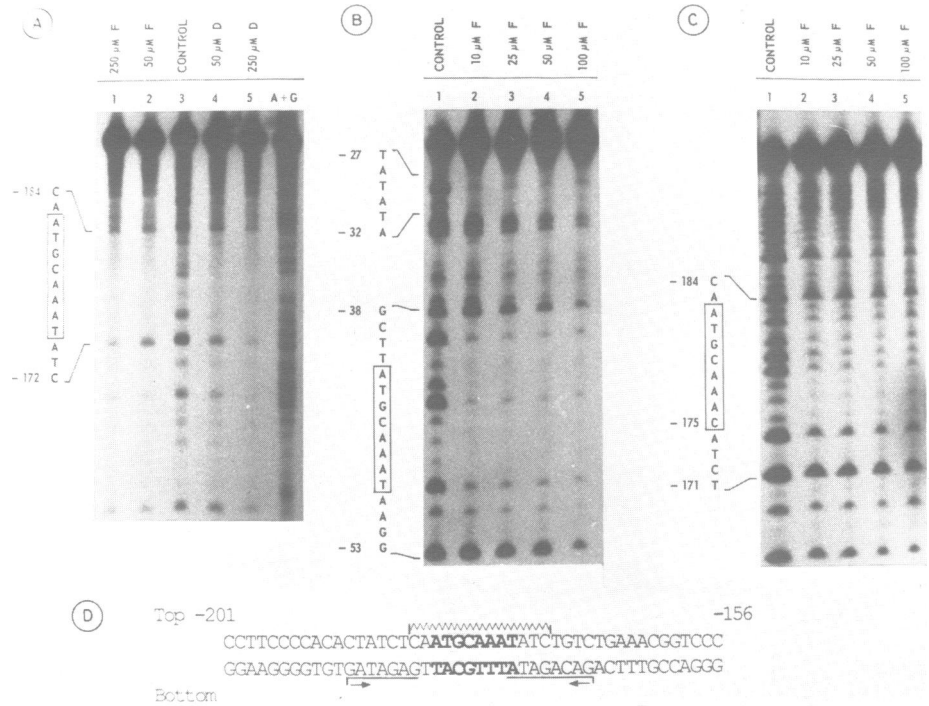

Figure 5
DNase I footprinting of DNA fragments treated with distamycins:
(A) Human γ-globin fragment (5'-end labeled on the top strand) was treated with 50 (lane 2) or 250 µM (lane 1) FCE 24517 (F) or with 50 (lane 4) or 250 µM (lane 5) distamycin A (D) for 30 min and then subjected to cleavage by DNase I. Lane 3 is the untreated control; A+G is the Maxam-Gilbert sequencing reactions. The protected sequence is indicated; the octamer is boxed. (B) Oligonucleotide derived from sequence of human H2B promoter treated with 10 (lane 2) 25 (lane 3) 50 (lane 4) 100 µM (lane 5) FCE24517 (F) for 30 min before addition of DNase I. Lane 1 is the untreated control. The sequence of the protected regions is indicated and the octamer is boxed. (C) -175 mutant γ-globin fragment. Treatments and lanes are the same as in panel B.
(D) The sequence (from -201 to -156) of human γ-globin gene is illustrated. The octamer (binding site of OTF 1) is indicated in bold type ; on the bottom strand the GA(T,C)AGA sequences (binding site of NFE 1) are underlined; the region protected as in panel A is marked with a zigzag above it.

In order to verify whether the ability of distamycin A and FCE 24517 to inhibit protein binding was specific for AT-containing DNA boxes, we investigated the effect of the two drugs on the binding of nuclear proteins to a previously characterized human ß- globin CACCC box containing fragment Hind III -BstNI (see ref. 19). Incubation of this fragment with nuclear extracts from K562 cells generated several retarded bands (B1-B4) shown by DNase I footprint and methylation interference experiments to interact with the CCACACCC sequence (19); they therefore do not require AT stretches for specific recognition. Fig. 4 shows that neither

distamycin A nor FCE 24517 had any detectable effect on binding to this DNA box even at very high concentrations (200 μM).

To establish the precise site of binding of distamycin A and FCE 24517 on the oligonucleotides we investigated these drugs' ability to protect selected sequences from DNase I digestion (DNase I footprinting) (17). When the normal globin fragment was employed, both compounds protected a sequence spanning the octa box from DNase I digestion (Fig. 5A). Similar results were also obtained employing the γ-globin mutated fragment and the H2B oligonucleotide (Fig. 5C and 5B). In both cases GC rich sequences were not protected (see -190/-200 region in γ-globin fragment fig. 5A and 5C) and the corresponding bands were even enhanced. Significant protection was already evident at low drug concentrations (10-25 μM). In the case of the H2B oligonucleotide a second protection was observed in another stretch of AT present in this fragment (-30/–20), corresponding to the histone H2B TATA box (11).

DISCUSSION

The antibiotic distamycin A has potent antiviral activity (3) and some of its derivatives inhibit proliferation of certain murine tumors (4,5). The mechanisms of action of these compounds have still to be elucidated.

The specific binding of distamycin to AT-rich sequences has been already described (2,6) and the chemical basis for this interaction has been well characterized. However, to our knowledge the influence of distamycins interactions with DNA on the binding of regulatory proteins to DNA has not yet been investigated . To this aim we used as a model system two different sequences known to be important in gene regulation. The ATGCAAAT octamer is essential for transcriptional regulation of several ubiquitously or tissue-specifically expressed genes (for a review see refs 9,11,12,16) transcribed by either pol II or pol III (20); additionally, it has been implicated in the stimulation of DNA replication by NF III (21,22) . The GATAAG sequence is present in promoter and enhancer elements of several globin genes, and is affected by mutations up-regulating γ-globin expression in adult erythroid cells (14,18).

We found that distamycin A or its derivative FCE 24517 inhibited the binding of specific nuclear proteins to the ATGCAAAT and GATAAG sequences. The effect of the drugs appears to be selective for these sequences, as indicated by the following observations :

- the drugs have no effect on protein binding to the C-rich CCACACCC sequence (Fig 4);
-footprinting experiments show that the drugs interact preferentially with the AT rich region including the ATGCAAAT motif (Fig 5), an effect that is particularly clear at low drug concentrations; that the octamer is a preferred target is also shown by the fact that the drugs inhibit the binding of nuclear proteins to this motif irrespective of the flanking sequences (see for example the octa motif in the H2B histone and globin genes respectively). As regards the NFE 1 binding site, the footprint shown in fig 5 indicates that the sequence AGAT(G/A) (-171 to

-175) reacted with the drug; this sequence is known, from methylation interference experiments and from the effects of the -175 mutation on binding (14), to be an important part of the NFE 1 recognition motif;

-the drug's effect is not due to interaction with nuclear protein itself, as indicated by preincubation experiments and by its inability to inhibit the binding of nuclear proteins interacting with other motifs (CCACACCC box, fig 4).

Considering that some distamycin derivatives, including FCE 24517, have recently shown potent antineoplastic activity (4,5) our results invite the speculation that these compounds might act by preventing the DNA-binding of specific regulatory proteins, thus inhibiting the transcription of genes which are important for neoplastic cell growth . This would be a novel mode of action for an anticancer agent. Indeed, recent results (unpublished) by this laboratory show that in L1210 mouse leukemia cells these compounds lower (by more than 90%) the level of histone H2B mRNA (whose transcription is dependent on the octa motif) but not that of histone H4 and actin mRNAs.

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