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DISTAMYCIN ANALOGUES WITH IMPROVED SEQUENCE-SPECIFIC DNA BINDING ACTIVITIES

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Abstract—In the present study we have investigated the effect of unprecedented chemical modifications introduced in the distamycin molecule, with the aim of assessing their ability to interfere with sequence-specific DNA–protein interactions *in vitro*. By using an electrophoretic mobility shift assay, we have been able to identify novel distamycin analogues with improved displacing abilities on the binding of octamer nuclear factors to their target DNA sequence. While variations in the number of pyrrole rings and/or reversion of an internal amide bond result in distamycin-like compounds with identical or very similar properties, the reversion of the formamido into a carboxyamido group or its replacement with the charged formimidoyl moiety significantly improves the ability of the resulting novel distamycin derivatives to compete with OCT-1 (octamer 1 nuclear factor) for its target DNA sequence. Tissue-specific octamer-dependent *in vitro* transcription is similarly affected by these chemical modifications, suggesting that the ability of distamycins to bind octamer sequences has a direct influence on the functional state of octamer-containing promoters. These data represent an initial, successful attempt to rationalize the design of DNA binding drugs, using distamycins as a model.

Key words: distamycin; octamer; HLA-DRA; nuclear factors; electrophoretic mobility shift; *in vitro* transcription

Distamycin and its derivatives are compounds endowed with strong antiviral and antitumour activities [1, 2]. Although their mechanism of action is still unknown [3], recent data suggest that they form a molecular complex with a high degree of selectivity for AT-rich DNA sequences [4–8]. Because this interaction induces a conformational change in the DNA structure [9], it is likely to interfere with DNA–protein interactions. In addition, should this occur in genomic regions involved in crucial biological activities, such as DNA replication and/or transcription of cellular genes, DNA binding of distamycin is expected to contribute greatly to its overall cytotoxic effects. Recently, evidence has been provided that distamycin binds to AT-rich regions in the promoter of a variety of eukaryotic genes, including the erythroid-specific γ-globin gene and the human HLA-DRA gene [10–12].

In this report we analyse the effects of distamycin and novel distamycin analogues on the binding of nuclear factors from tumour cell lines to the octamer (ATGCAAAT) sequence which is present in the promoters of many eukaryotic genes and represents the binding site of the OCT¶ family of transcriptional factors [13–17]. Data to be presented suggest that distamycin also binds to the AT sequences in the octamer motif, as assessed by the inhibition in OCT binding activities, and affects cell-free *in vitro* transcription of an OCT-dependent promoter. In addition, we exploit this model to identify novel distamycin analogues with improved DNA binding abilities.

MATERIALS AND METHODS

Distamycins. Distamycin (synonyms distamycin A, stallimycin) hydrochloride (MEN 10399), its four-ring homologue (MEN 10567), its novel *N*-formimidoyl (MEN 10397 and MEN 10559) and retroamide (MEN 10398, MEN 10400 and MEN 10557) analogues were obtained by total synthesis (to be described elsewhere) at the laboratories of Menarini Ricerche Sud, Pomezia, Rome (Italy). These distamycins are depicted, with the relevant code numbers, in Fig. 1.

Synthetic oligodeoxynucleotides. The 5′-3′ strands and the relative complementary 3′-5′ strands were synthesized on a Pharmacia Gene Assembler Plus DNA Synthesizer using the phosphoramidite method. The sequence of the double-strand oligonucleotide used to test OCT binding is as

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¶ Abbreviations: OCT-1, octamer 1 nuclear factor; NP-40, Nonidet P40; TBE, Tris borate EDTA; EBNA-1, Epstein Barr nuclear antigen 1.

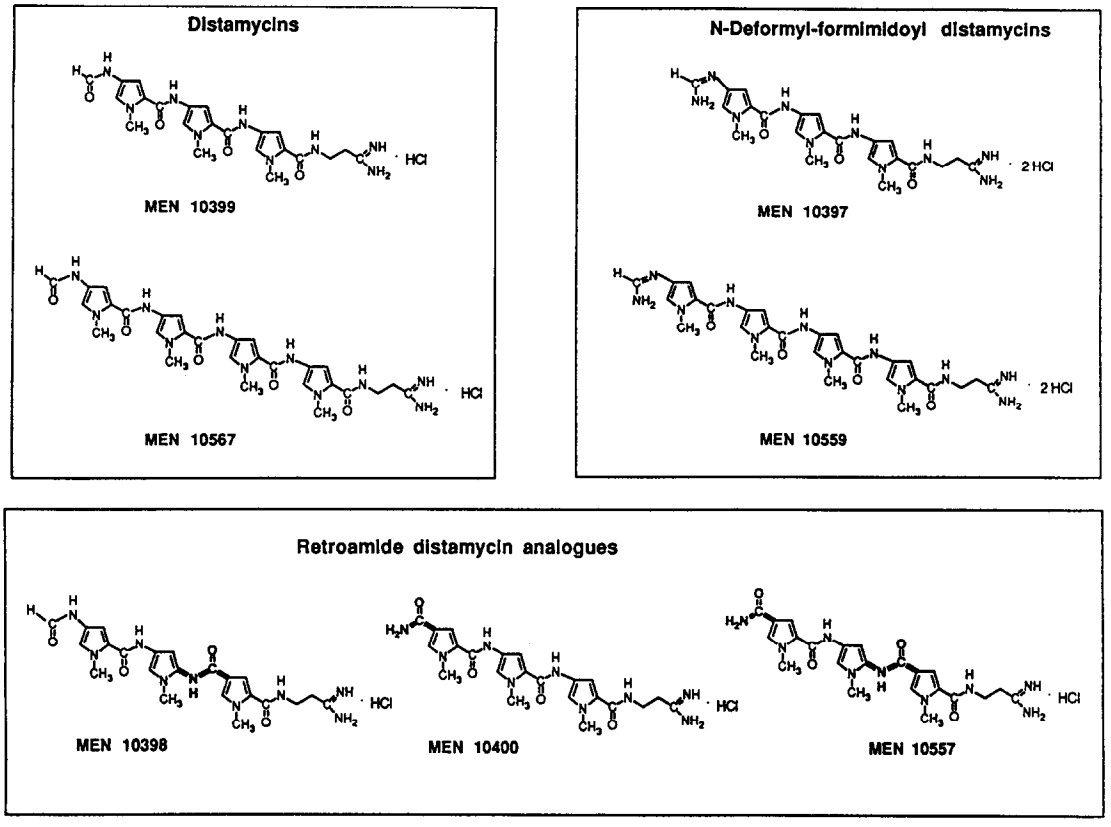

Fig. 1. Chemical structures of distamycin HCl and its analogues. Distamycins are grouped according to their most significant chemical modification. The distamycin HCl and *N*-deformyl-formimidoyl groups are comprised of three- and four-pyrrole ring analogues.

follows (the sense strand in the binding site is underlined):

5′-AATTGCATGCCTGCAGGTCGACTCTA
GAGGATCCATGCAAATGGATCCCCGGGTAC
CGAGCTC-3′

3′-CGTACGGACGTCCAGCTGAGATCTCCTA
GGTACGTTTACCTAGGGGCCCATGGCTCGA
GTTAA-5′

The 64-mer OCT-containing oligo is also available as part of a commercial kit (Pharmacia, Uppsala, Sweden). The annealed, double strand oligo was filled in with [α-^{35}S]dATP by the Klenow DNA polymerase fragment. Double-strand oligos (sense strand indicated below) mimicking the DRA (12) X box (5′-ACCCTTCCCCTAGCAACAGATGC GTCATCT-3′) and the SP1 binding site (5′-ATTCGATCGGGGCGGGGCGAGC-3′) were used as controls.

Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared at concentrations of 1–5 mg/mL according to Dignam *et al.* [18] from the following human continuous cell lines: WIL-2 (B lymphoid); Molt-4 (T lymphoid); HL-60 (myelomonocytic leukaemia); Colo 38 and MRN-1 (malignant melanoma). Nuclear extracts for *in vitro* transcription were also prepared from HeLa cells. The electrophoretic mobility shift assay (shortly, gel retardation) was performed as originally described [19] with minor modifications [20]. Binding reactions were, unless otherwise specified, set up in binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 5% glycerol and 0.1% NP-40), in the presence of 50 ng poly *d*(I-C)·poly *d*(I-C) (Pharmacia, Uppsala, Sweden), 3 μg of nuclear extract proteins and 0.25 ng of end-labelled double-stranded oligonucleotides (approximately 50,000 cpm), in a total volume of 20 μl. After 30 min at room temperature, samples were electrophoresed at constant voltage (300 V for 2 hr) through a low ionic strength (0.35 × TBE) buffer (1 × TBE = 0.089 M Tris borate; 0.089 M boric acid; 0.008 M EDTA) on 6% polyacrylamide gels until tracking dye (Bromophenol blue) reached the end of a 16 cm slab. Gels were dried and exposed at −80°. The order of addition of the reagents was as follows: (a) poly *d*(I-C)·poly *d*(I-C); (b) competitor DNA (if required); (c) distamycin; (d) labelled oligonucleotides; and (e) nuclear extracts. For supershift experiments, antibodies to OCT-1 and OCT-2 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.) and added last to the mixture.

In vitro *cell-free transcription.* Transcription was performed as described [20] using 60 mg of nuclear

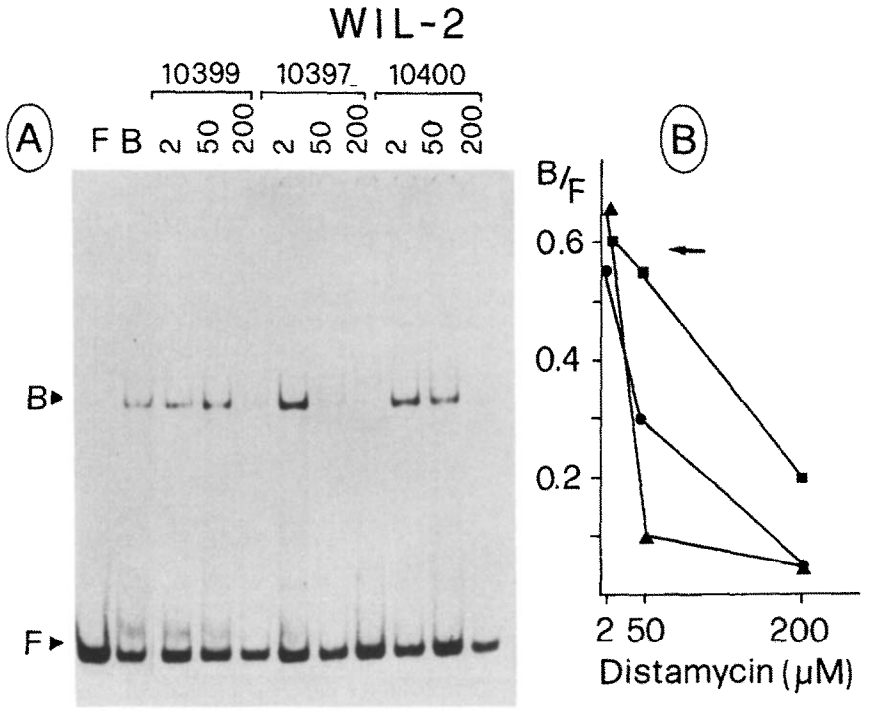

Fig. 3. Effect of different distamycins on OCT-1 binding in WIL-2 nuclear extracts. Panel A: Different concentrations (μM), as indicated, of distamycin HCl (MEN 10399), *N*-formimidoyl distamycin (MEN 10397) and a retroamide analogue (MEN 10400) were tested in a band shift assay for their ability to compete with OCT binding activities in WIL-2 cells. Free (F▶) and OCT-bound (B▶) double-stranded oligonucleotides are indicated. As a control, the binding was performed in the absence of distamycin, and in the absence (F, first lane to the left) and presence (B, second lane to the left), respectively, of nuclear extracts. Panel B: In order to compare minor variations in the amounts of input radioactivity from lane to lane, data are expressed quantitatively following densitometric scanning in the form of the ratio of bound vs free oligos against distamycin concentration. The arrow marks baseline B/F ratios in absence of distamycins. The symbols are as follows: MEN 10399 = ■; MEN 10397 = ▲; Men 10400 = ●.

(MEN 10400) analogues on OCT binding is shown. Displacement of OCT binding can be appreciated by the decrease in the bound/free OCT ratio obtained in the gel retardation experiments run in the presence of distamycins, as compared to those run in its absence. This decrease is often not appreciable at low distamycin concentrations, where a slight paradoxical increase in apparently bound oligo is often observed (Figs 3–5). Displacement of OCT binding is, however, clearly dose-dependent, and both analogues exert more efficient displacing abilities than the original distamycin molecule. A quantitative, densitometric evaluation of this displacement is provided in panel B (Fig. 3). It is clearly apparent that MEN 10397 already exhibits suboptimal activity at 50 μM concentration and is therefore the best OCT competitor under these assay conditions. MEN 10397 did not generate, in this experiment, large amounts of slowly migrating bands.

We next verified whether the same competing efficiencies of MEN 10397 and MEN 10400 are reproducibly obtained in nuclear extracts from different cell lines. In spite of the variable OCT binding activities detected, MEN 10397 and MEN 10400 are also more efficient than distamycin HCl in competing OCT binding in nuclear extracts from other cell lines. A synopsis of these results is provided in Table 1. These data altogether demonstrate that the efficacy of OCT displacement by distamycin and its analogues is not dependent on the cell line used. Thus, it can be concluded that MEN 10397 and MEN 10400 are both more effective than distamycin HCl in competing OCT binding.

Effect of amide bond reversion and number of pyrrole rings on the OCT displacing activities of distamycin analogues

To verify whether the above observations could be generalized to other distamycin analogues carrying different chemical modifications (see Fig. 1), we compared the OCT displacing abilities of the four-ring distamycin homologue MEN 10567, two *N*-formimidoyl derivatives (three ring MEN 10397 and four-ring MEN 10559) and different retroamide (MEN 10398, MEN 10400 and double retroamide MEN 10557) analogues. Results describing testing of these analogues are shown in Figs 4 and 5 and Table 1. The additional, slower migrating band seen in Fig. 2 and at least one other slow component at

Table 1. Effect of distamycin HCl and its analogues on OCT-1 binding in nuclear extracts from different transformed cell lines

Drug	μM	WIL-2*	COLO 38	MRN-1	MOLT-4	HL-60
	(—)	0.5†	0.17	0.7	0.6	0.12
	2	0.6‡	0.16	0.7	0.8	0.2
10399	50	0.55	0.15	0.4	0.6	0.1
	200	0.15	0.13	0.35	0.5	0.16
	2	0.65	0.11	0.65	0.8	0.2
10397	50	0.1	0.4/0.1§	0.1/0.5	0.1	0/0.2
	200	0.05	0.2/0.06	0.15	0.1	0/0.2
	2	0.55	0.16	0.4	0.95	0.15
10400	50	0.3	0.09/0.09	0.35	0.5	0.12
	200	0.05	0.05/0.05	0.3	0.45	0.1
	2	1.2/1.2				
10567	50	0.2/0.1	NT	NT	NT	NT
	200	0.5/0.5				
	2	1.2/1.2				
10559	50	0.2/0.5	NT	NT	NT	NT
	200	0.1/0.2				
	2	0.65				
10398	50	0.8	NT	NT	NT	NT
	200	0.7				
	2	0.8				
10557	50	0.15	NT	NT	NT	NT
	200	0.05				

* Results from WIL-2 cells are reported in panel B of Figs 3–5.

† Ratios of densitometric scan values of bound over free (B/F) retarded oligos.

‡ At 2 μM concentrations, an apparent facilitation in OCT binding in certain nuclear extracts is occasionally observed.

§ Densitometric B/F values of the additional, slowly migrating band (see Results for further details) seen with formimidoyl derivatives.

the top of the gel are also visible in these experiments. They are more abundant with formimidoyl derivatives, but, as shown in the displacement experiment in Fig. 2, nonspecific. Therefore, only the OCT-1 band was taken into account. It is clearly evident from Fig. 4 that the introduction of the *N*-formimidoyl group improves, by itself, and irrespective of the number of pyrrole rings, the OCT competing ability of these molecules (compare MEN 10567 and MEN 10559). Along the same lines, displacement is virtually complete at 50 μM concentration, with both MEN 10397 (three rings) and MEN 10559 (four rings). In addition, experiments shown in Fig. 5, while confirming that the reversion of the formamido group (MEN 10400) greatly enhances the OCT competing abilities of distamycin, demonstrate that the reversion of the internal amide bond, as in MEN 10398, abolishes the displacing effect of the parent distamycin. Double reversion (MEN 10557), however, does not significantly modify the performance of the single reversion carried by MEN 10400. Thus, it can be concluded that the position of the amide bond reversion is critical for the achievement of OCT displacing abilities by distamycins.

Effect of distamycins on the in vitro *transcription of an octamer-containing promoter*

To determine whether the DNA binding activity of distamycin and its derivatives might influence the transcription ability of an octamer-dependent promoter, two plasmids were used as templates in an *in vitro* cell-free transcription system, in the presence of the parental distamycin and two members of the families of *N*-formimidoyl and retroamide analogues. The pWT (wild-type) and pMUT (five base mutant) plasmids contain identical sequences except for a mutation which affects the octamer activity. It can be seen in Fig. 6 that an abundant transcript of more than 2 kb (possibly resulting from inefficient *in vitro* termination of CAT transcription) is detectable with the wild-type pWT-oct template and barely visible with the pMUT-oct template using WIL-2 extracts. At variance, both pWT-oct and pMUT-oct plasmids generate comparable amounts of this transcript when HeLa extracts are used. Therefore, as previously reported [20], the octamer sequence is necessary for transcription from the DRA promoter in cell-free systems supplemented with lymphoid (WIL-2) but not epithelial (HeLa)

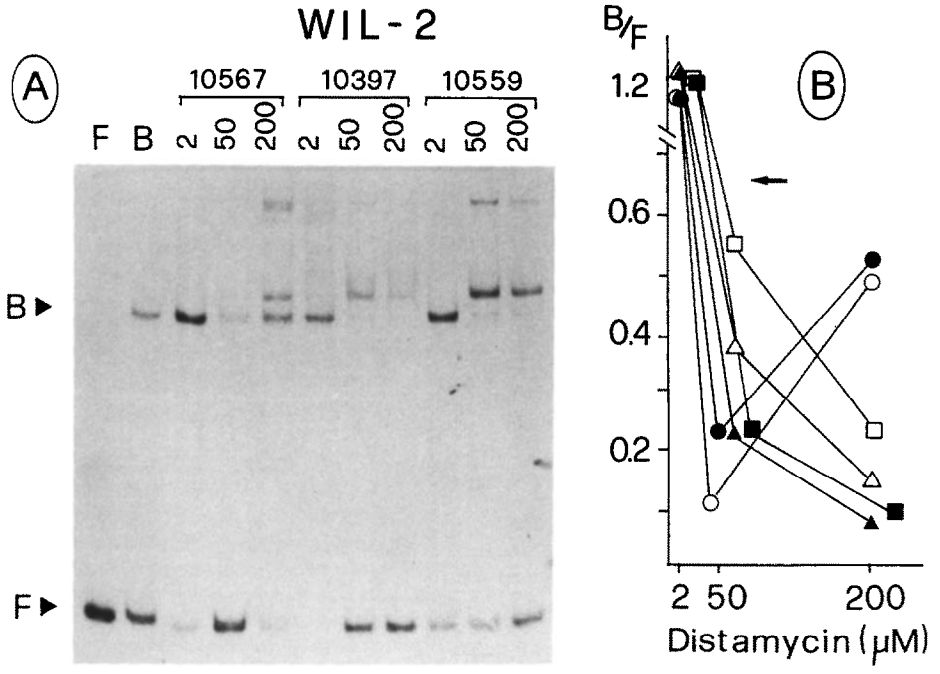

Fig. 4. Effect of the addition of one extra pyrrole ring in distamycin HCl and its formimidoyl derivatives. Panel A: Different concentrations (μM), as indicated, of a distamycin homologue (MEN 10567) and *N*-formimidoyl analogues (MEN 10397 and MEN 10559) were tested in a band shift assay for their ability to compete with OCT binding activities in WI-L2 cells. (For further details see legend to Fig. 3.) Panel B: See legend to Fig. 3. Symbols are as follows: MEN 10567 = ●; MEN10397 = ▲; MEN 10559 = ■. Open symbols correspond to the additional, slower migrating bands of unknown origin. (See text for details.)

nuclear factors. Because transcription is strictly dependent on the OCT sequence when WIL-2 cell extracts are used, this experimental system was selected to test experimentally the ability of parent (MEN 10399), *N*-formimidoyl (MEN 10397) and retroamide (MEN 10400) distamycins to inhibit *in vitro* transcription. Tissue-specific transcription is affected by the three distamycins to approximately the same extent as in the band shift assays, with the sole exception that parent distamycin appears to be more potent in the *in vitro* transcription system than in the gel retardation assay. The two remaining distamycins maintain their relative potencies and MEN 10400 exhibits, at the low distamycin concentration, a paradoxical enhancing effect on transcription similar to that observed in gel retardation experiments. These data further indicate that the additional, slowly migrating bands seen in certain experiments (i.e. Figs 4 and 5) with *N*-formimidoyl and other derivatives reflect the generation of non-physiological complexes of labelled oligonucleotide in presence of distamycins. They also demonstrate that distamycins are capable of affecting transcription of an octamer-dependent reporter gene by specifically altering its functional properties.

DISCUSSION

In this study, we have assessed the comparative ability of six novel distamycin analogues to interfere with sequence-specific DNA–protein interactions *in vitro*. Our data demonstrate that either increasing the number of pyrrole rings to four or inverting the direction of an internal amide bond results in compounds with similar or lower OCT displacing properties, respectively, as compared to the parent distamycin. However, the reversion of the formamido into a carboxyamido group or its replacement with the charged formimidoyl moiety result in novel distamycin analogues with improved ability to compete successfully with OCT factors for their target sequences.

The extra positive charge of the formimidoyl group most likely provides MEN 10397 and MEN 10559 with a higher affinity for strongly electronegative AT-rich regions in the minor groove of double-strand DNA. The explanation for the behaviour of retroamide distamycins, in contrast, is not obvious. Amide reversion, in fact, does not appear to alter significantly the charge or dimensions of the drug molecule. The fact that these modifications are most effective on the N-terminal residue suggests that this region is strongly implicated in the DNA recognition process.

It is of interest to note that our approach measures the ability of distamycins to compete, *in vitro*, with DNA–protein interactions taking place in a complex mixture of nuclear proteins. This experimental approach, at variance from physico-chemical esti-

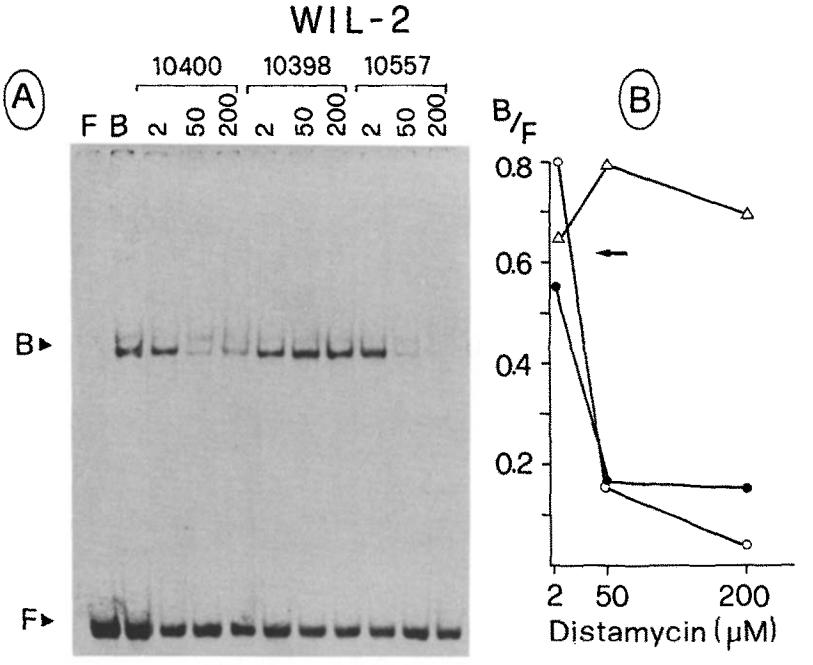

Fig. 5. Effect of different amide bond reversions on OCT-1 binding in WIL-2 nuclear extracts. Panel A: Different concentrations (μM), as indicated, of retroamide analogues (MEN 10400, MEN 10398, MEN 10557) were tested in a band shift assay for their ability to compete with OCT binding activities in WIL-2 cells. (For further details see legend to Fig. 3.) Panel B: See legend to Fig. 3. The symbols are as follows: MEN 10400 = ●; MEN 10398 = △; MEN 10557 = ○.

mates of drug binding to DNA [22, 23], is likely to provide information related, although indirectly, to the physiological regulation of the genes controlling cell cycle and differentiation state in both normal and transformed cells.

Recently, we have found [24] that distamycins compete for DNA binding with a purified, recombinant factor, EBNA-1, with efficiency similar, although not identical (MEN 10400 is scarcely active on EBNA-1), to that observed in the present study. Experimental systems involving just a few interacting molecular species, such as the EBNA-1 recombinant factor, target DNA and distamycins, are certainly more quantitative and less prone to the influence of non-physiological DNA–drug interactions than approaches using unfractionated nuclear extracts, such as the OCT-1 system herein described. The remarkable similarities detected in the two experimental models, however, do suggest that the improved DNA binding ability of certain distamycins has also been correctly evaluated in the case of the octamer sequence. The main advantage of the octamer model, therefore, is that a contextual evaluation has been possible, at biochemical and functional levels, of the effect of DNA binding drugs in the presence of the entire machinery necessary for tissue-specific transcription. Thus, in spite of intrinsic limitations, we show here that distamycins interfere with physiological interactions of OCT-1 with its target DNA sequence.

If this effect on octamer-dependent transcription also occurs *in vivo*, it might be exploited to alter important phenotypic traits of normal and neoplastic cells. For instance, the expression of the DRA gene triggers a number of immunological phenomena, such as allograft rejection and autoimmune responses [19]. Increases in the intracellular levels of the OCT family of transcriptional factors during cell proliferation have been shown, and lead to a cell-cycle regulation in the transcription of a variety of genes, including histone H2B [25]. In agreement with a role of OCT-1 in cell cycle progression, monoclonal antibodies against OCT-1 display antiproliferative activity toward tumour cell lines [26]. Obviously, it remains to be demonstrated to what extent transcription factors, such as proteins of the OCT family, are one of the *in vivo* targets of distamycins and other DNA binding drugs [27]. In spite of this lack of information, however, the present report demonstrates that it is possible to manipulate and improve DNA binding drugs by the most conventional and widely applied pharmacological strategy, i.e. the introduction of a series of selected and simple side-groups or changes in an otherwise identical chemical backbone.

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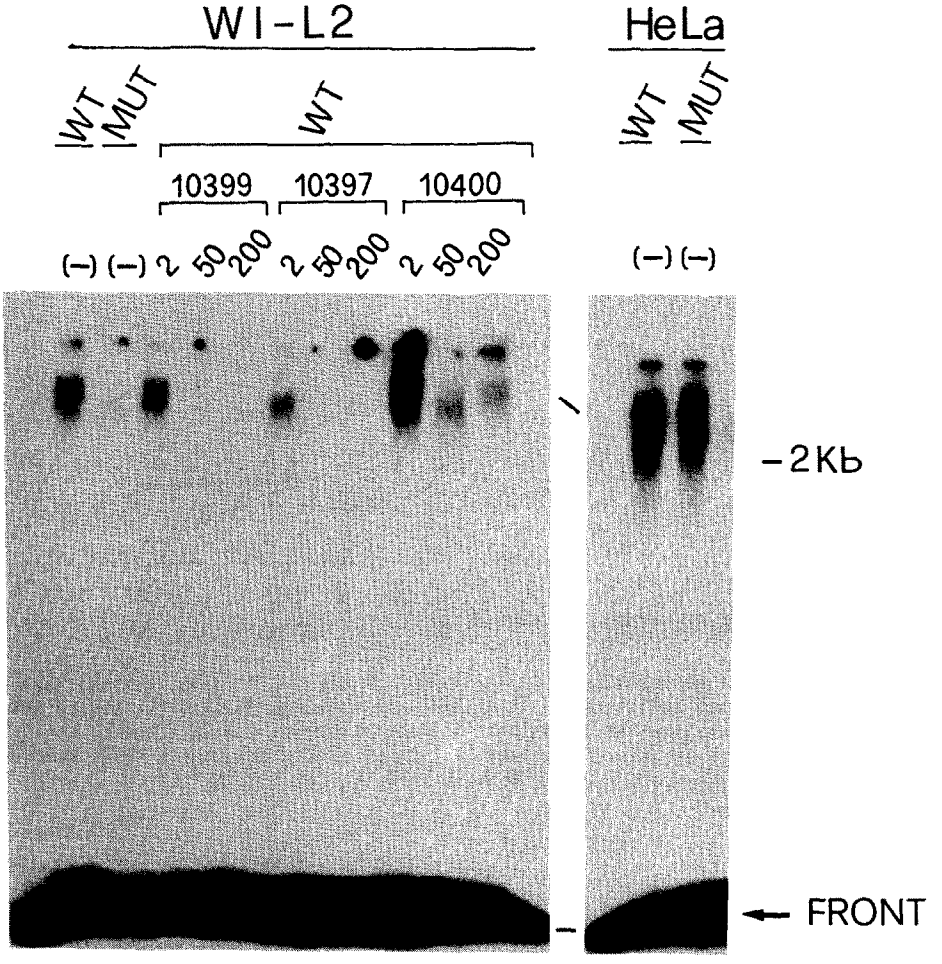

Fig. 6. *In vitro* cell-free transcription of an OCT-dependent promoter in the presence of distamycins. The ability of WIL-2 and HeLa nuclear extracts to support octamer-dependent transcription from a wild-type promoter (WT) containing the promoter region of the HLA-DRA gene, and a mutant promoter containing an altered octamer sequence (MUT) was assessed in the presence of different concentrations of distamycin analogues, as indicated. Transcripts were analysed in a denaturing acrylamide gel with sizing markers in a contiguous lane.

pMUT-oct plasmids, and useful suggestions for the *in vitro* transcription assay. A.C. is grateful to Dr Antonello Punturieri for helpful discussions.

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