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Interactions of nucleic acids with distamycins. Binding of Dst-3 to $d(CGTTTAAACG)_2$ and $d(CGTACGTACG)_2$

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ABSTRACT

The binding between Distamycin 3 and the palindromic duplexes $d(CGTTTAAACG)_2$ and $d(CGTACGTACG)_2$ was investigated by two indipendent techniques: UV-Vis absorption in the Job's plot approach and Induced Circular Dichroism. Both decamers bind two molecules of peptide per duplex, with close overall affinities. This result indicates that a row of six A:T base pairs can accommodate two molecules of drug and that the minimal binding site of Distamycin 3 can consist of just two A:T base pairs.

INTRODUCTION

Netropsin (Nt) and Distamycins (Dst) are oligopeptides with high antiviral activity[1], whose primary target is DNA.

Studies of the structure, energetics and dynamics of their binding to synthetic and natural DNAs are of great significance both for drug design purposes and for molecular recognition studies. In fact the binding specificity of these ligands to the DNA minor groove is an important topic for our understanding of the molecular basis of DNA sequence recognition processes by proteins and small molecules[2].

Significant progress in this area has been achieved by the interplay of footprinting methods, studies by X-ray diffraction, optical and magnetic resonance spectroscopies and thermodynamical measurements. The X-ray and two-dimensional NMR analyses of the complexes between Nt or Dst and oligonucleotides[3–8] provided evidence that binding occurs within the minor groove of the DNA. This preferred location was recently confirmed by the flow Linear Dichroism techniques also for the full chains of calf thymus DNA[9].

Extensive investigations were carried out on the site selectivity of these drugs employing natural or synthetic DNA polymers, as well as short oligonucleotides. While Nt exhibits a very large preference for A:T pairs in clusters[10–12], Dst-3 (see chart 1) shows some additional, although lower, affinity to GC pairs[5–7,13,]. However, no systematic study has been done on the dependence of the binding affinity upon the sequence and length of the A:T clusters and the structure of the flanking sequences. Obviously this information is needed to understand whether specific genes in bacterial and eucaryotic cells can be selectively affected.

One way of addressing the problem is to evaluate the relative binding affinity between the peptide and many short oligomers (typically decamers and dodecamers) containing A:T-rich sites of different structures. Moreover, these systems allow the determination of the stoichiometry of the complexes, which is particularly significant when multiple occupation of a binding site can be envisaged. In fact, while this work was in progress, ^{1}H-NMR results by Pelton and Wemmer[14,15] gave, for the first time, unequivocal evidence that the sequences d(CGCAAATTGGC) and $d(CGCAAATTTGCG)_2$ bind two molecules of Dst-3. The formation of complexes containing two molecules of Dst 3 or Dst 5 had also been suggested[16] for the dodecamer $d(CACAAGCTTGTC)_2$, which has two separate and close binding sites.

The binding affinity of different sequences for the drug may be evaluated from the Induced Circular Dichroism (ICD) signals of Distamycins[10,13]. This approach has also been extensively applied to study the difference in the binding specificity of synthetic analogues of Distamycins[17].

The binding stoichiometry can be determined by the Job's method of continuous variations[18], which has been used to calculate the binding site size of Netropsins to different polynucleotides[19] and to study the formation of two- and three-stranded helices between poly A and poly U[20].

We applied these methods to investigate the binding of Distamycin 3 to the self-complementary decamers **$d(CGTTTAAACG)_2$ [T_3A_3]** and **$d(CGTACGTACG)_2$ [TACGTA]**. [T_3A_3] has a strong binding site of six A:T pairs in a row, while [TACGTA] has two separate sites well below the minimum size considered necessary for significant binding to occur[8]. Thus, we expected the two oligomers to exhibit considerable differences in binding to Dst-3. Instead, under the experimental conditions of our experiments, both decamers bind two molecules of peptide per duplex and have close overall affinities.

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EXPERIMENTAL

Absorption spectra were recorded with a Varian Cary 2300 UV-Visible Spectrophotomer; CD spectra with a Jobin Yvon Mark IV Dichrograph. Dst-3 (ϵ_{303} = 34000 M^{-1} cm^{-1})[10] was a gift from Dr. Mongelli (Farmitalia C.E., Milano).

The protected decanucleotides were synthetized in solution by the hydroxybenzotriazole phosphotriester method[21]. The crude material obtained after removal of the protecting groups was purified on a DEAE-Sephacell column by elution with a linear salt gradient from 0.05 to 2 M aqueous triethylammonium bicarbonate (TEAB), pH 7.4. The collected fractions were analyzed by HPLC (Spherisorb C_{18} column, eluant: gradient from 0 to 30% of CH_3CN in 0.05 M KH_2PO_4, pH 4.5, T 40°C). Fractions with HPLC purity ≥ 94% were pooled, coevaporated several times with water to remove TEAB, converted to the ammonium salt by repeated coevaporation with concentrated NH_4OH, and finally lyophilized. The extinction coefficients , ϵ_{260} = 8970 and 8330 M^{-1} cm^{-1} for [T_3A_3] and [TACGTA] respectively, were determined by alkaline hydrolysis[22], and further confirmed by measuring the absorbance of a solution of each decamer at 80°C and comparing the experimental data with that calculated for the single stranded mono- and di-nucleotide contributions (see CRC Handbook of Biochemistry and Molecular Biology, 1975).

Solution concentrations of Dst-3 and of the oligonucleotides were determined spectroscopically from the extintion coefficients reported above.

All UV and CD measurements for the binding experiments were done in 0.1 M Tris HCl (pH 7) and 0.2 M NaCl, at 20°C.

The optical melting curves[23] (fig.1) were registered at 260 nm in stoppered thermostatted quartz cells, with a temperature increase of 0.5°C/min starting at 10°C. In both cases, the melting transition starts above 20°C. Thus, under the conditions of the binding experiments, the duplex → to single-strand equilibrium is largely shifted towards the duplex state.

The solutions used for the Job's plot were prepared by mixing equimolar solutions (4×10^{-5} M) of Dst-3 and the oligomer duplex in different proportions[18,24] and their absorption measured at 20°C. The Circular Dichroism (CD) signals (reported in figures as ΔOD) were obtained at constant molar concentration of the duplexes (3.6×10^{-5} M), with a 1 cm pathlength, at 20°C.

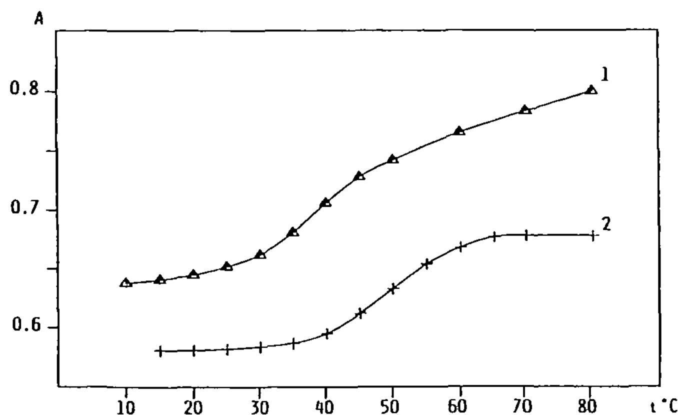


Figure 1: Melting curves of d(CGTTTAAACG) (1) and d(CGTACGTACG) (2). A is the optical absorbance at 260 nm.

RESULTS AND DISCUSSION

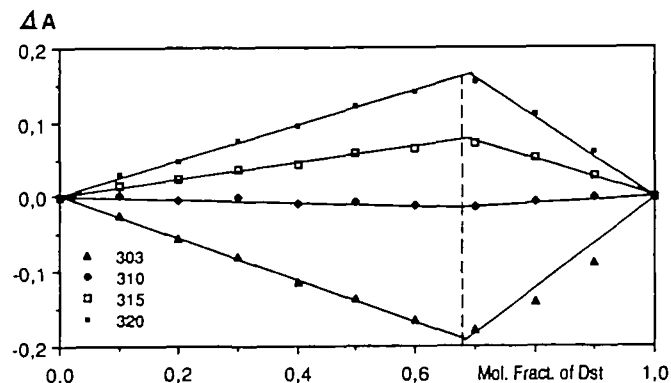
Job's plots or continuous variations of UV-Vis absorption

According to this procedure[18], equimolecular solutions of ligand and duplex are mixed in various proportions, so that the sum of the imput concentrations is kept constant, and the UV absorption of the resulting mixture is measured at a suitable wavelength. The difference (ΔA) between each experimental value and that calculated as the sum of the contributions of the two separate components is plotted against the molar fractions. The resulting curves display a break point at the molar fraction corresponding to the stoichiometry of the complex. It is worth stressing that the position of the break point is independant of the equilibrium constant. As pointed out by Vosburgh et al[24], when more than one complex may be formed, the plots should be done at several wavelengths. If the break points occur at the same composition, the **probability** is that only one compound is formed.

The Job's plots of the two systems, at different wavelengths, are reported in figure 2.

For all wavelengths the break points are centered, for the two substrates, at x = 0.68, corresponding to two Dst-3 molecules per duplex.

This result indicates that both $d(CGTTTAAACG)_2$ and $d(CGTACGTACG)_2$ provide two binding sites for Dst-3.



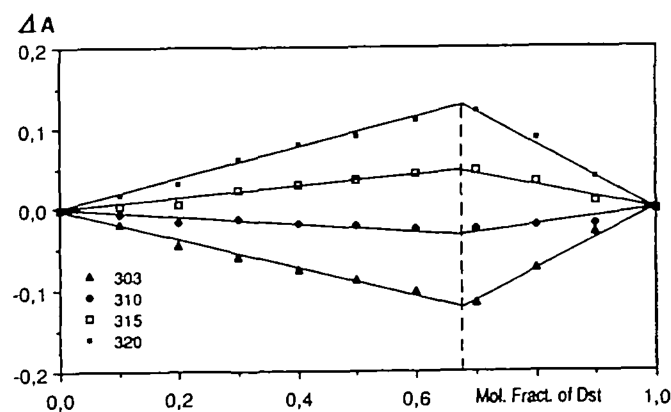


Figure 2: Job's plots of Dst-3 binding to $d(CGTACGTACG)_2$ (top) and $d(CGTTTAAACG)_2$ (bottom) at 20°C, at wavelengths: 303, 310, 315, 320 nm. Total concentration (drug + duplex) = 4×10^{-5} M. ΔA is the calculated absorption difference (see text).

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In favourable cases, the shape of the curves can help in deciding whether more than one complex is formed [20, 24]. The plots of Fig. 2, within the limited number of data points available, do not present any observable deviation from linearity at mole fraction x = 0.5, corresponding to the formation of 1 : 1 complexes. In the present cases, however, we feel that this cannot be considered as a clear cut evidence that 2 : 1 complexes are formed exclusively, for the following reasons. The UV absorption spectrum of a bound Distamycin molecule is expected to be practically the same in the 2 : 1 and 1 : 1 complexes, whereas the molar extinction coefficient of the latter should be about half that of the former. The equilibrium constants for the reactions: Dst + duplex → Dst.duplex and Dst + (Dst.duplex) → 2Dst.duplex are likely to be of the same order of magnitude. Pelton and Wemmer[15] followed by ^{1}H-NMR the titration of $(CGCAAATTTGCG)_2$ with Distamycin and, while detecting the presence of both types of complexes, found that the 2 : 1 form is already present in larger proportion at Dst mole fraction x = 0.5. Thus we suggest that, in the present experiment, the lack of evidence of the formation of the 1 : 1 complexes is due to an unfavorable combination of their equilibrium constants and molar extinction coefficients in respect to those of the 2 : 1 adducts.

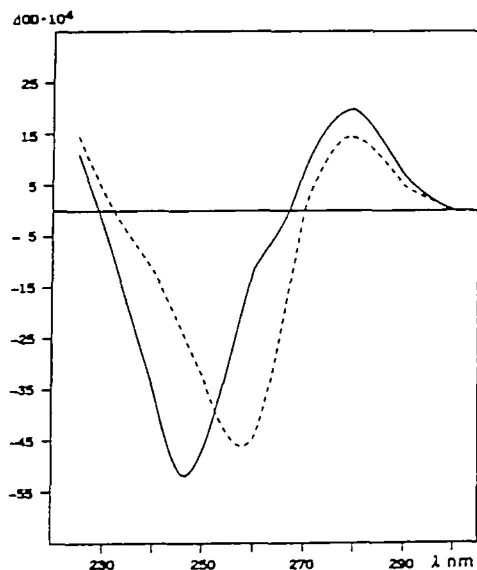

Figure 3: Circular Dichroism spectra at 20°C of $d(CGTTTAAACG)_2$ (full line) and $d(CGTACGTACG)_2$ (dashed line).

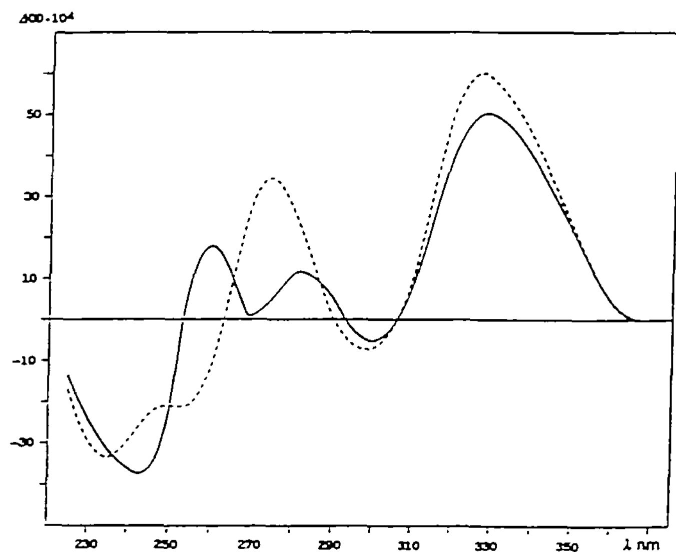

Figure 4: Circular Dichroism spectra at 20°C of the complexes between Dst-3 and $d(CGTTTAAACG)_2$ (full line) and $d(CGTACGTACG)_2$ (dashed line) at the Dst/duplex ratio R = 3 .

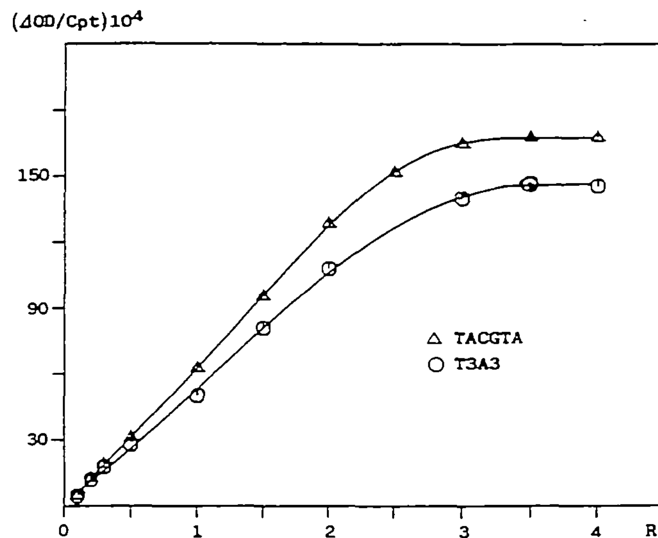

Figure 5: CD titration curves at 20°C. R is the ratio Dst/duplex, ΔOD the Circular Dichroism signal at 328 nm, C_{pt} the total duplex concentration (3.6×10^{-5}M).

Figure 6: Schematic of a sliding model of Dst-3 location within the minor groove of the two oligonucleotides. Circles represent drug pyrrole rings

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Induced Circular Dichroism of Dst-3 bound to DNA fragments

The CD spectra at 20°C of the oligonucleotide solutions, before and after addition of three molar equivalents of drug per duplex, are reported in figs. 3 and 4, respectively.

The CD spectra of the complexes between Dst-3 and the two decamers (fig. 4) result from the composition of three exciton doublets: one from the DNA fragments (centered at 260 nm) and two from Dst-3 (centered at 320 and 240 nm).

Dst-3 is a non chiral molecule; therefore its solutions do not exhibit any CD signals, unless a chiral interacting agent is added. The shape of the CD spectrum induced in Dst-3 upon its binding to DNA fragments indicates that the drug assumes a non-planar chiral conformation, with the pyrrole chromophoric units helically twisted in a propeller-like structure. A twist of about 10° between the planes of the pyrrole rings was found by X-ray diffraction of the crystalline complex formed between Dst-3 and $d(CGCAAATTTGCG)_2$[3].

The CD spectra of the two complexes are different in the spectral region where the higher energy doublet of Dst (260−230 nm) overlaps that of the oligonucleotides, which has the same sign and is slightly red shifted. In the [TACGTA] case these two couplets are so overlapped that only one broad couplet can be detected. In the $[T_3A_3]$ case, instead, the positive components of the doublets of DNA and Dst-3 are well resolved at 280 and 260 nm, respectively. The lowest energy ICD couplet of Dst-3 is strongly non-conservative and its shape is practically the same for both complexes. Therefore the intensity of this last band was used as a probe of Dst-3 binding.

Titration curves were obtained, at the same oligonucleotide concentration, by recording ICD spectra at constant temperature (20°C) and different drug/duplex ratios (R) (fig. 5).

The curves in fig. 5 show three main features:

a) a sizeable difference of the ICD value at saturation for the two oligomers.

b) no discontinuity in the slopes is detectable up to $R = 2.5$ and saturation occurs at $3 \leq R \leq 3.5$ in both cases.

c) the ratio ICD/ICD(saturation) for the two decamers is very close at all R values investigated.

The difference in the ICD values at saturation is most likely due to different chiral conformations of the bound Dst-3 molecules in the two complexes. Dst-3 ICD signals can be analyzed in terms of the coupled oscillator or exciton mechanism[25]. The exciton CD signals change sign and vanish at the Dst conformations in which the pyrrole chromophores are all coplanar or perpendicular to each other. The sensitivity of the CD technique to conformational changes is the highest near this turning point. As the dihedral angles between the pyrroles of bound Dst-3 were found to be around 10°[3], ICD measurements are in this case very sensitive to drug conformational changes.

Points b) and c) indicate that the two decamers have very similar overall affinity for Dst-3 despite the different structures of their individual binding sites.

Binding models

The results of Job's treatment of UV-Vis absorption measures and the ICD titration curves show that each duplex provides Dst-3 with two binding sites which cannot be discriminated by our data.

As far as [TACGTA] is concerned, the most likely structures of the complex should be (a1) and (a2), whereas for $[T_3A_3]$ three limiting situations (b1, b2 and b3) can be envisaged, following the binding scheme proposed by Pelton and Wemmer[15] for the complex between Dst-3 and $d(CGCAAATTTGCG)_2$. The real structures could also result from an equilibrium among the above limit models.

As already mentioned by other authors[15], the presence of complexes with more than one molecule of drug at the 'same' site (i.e. $[T_3A_3]$ in the present case) must be considered when working with comparable concentrations of ligand and substrate. This aspect is particularly important in calorimetric and quantitative footprinting experiments such as those reported[26] with $d(GGTATACC)_2$.

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