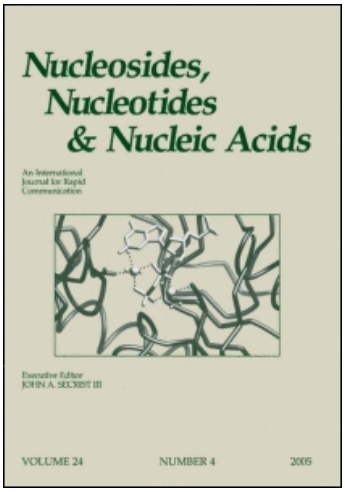

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BINDING OF ACTINOMYCIN D TO SINGLE-STRANDED DNA

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Abbreviations used: AMD, actinomycin D; 7AAMD, 7-aminoactinomycin D; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; NMR, nuclear magnetic resonance. Sequences are of DNA and are shown with 5'-3' polarity.

ABSTRACT: The sequence specificity and structural aspects of the mode of interaction of the antitumor drug actinomycin D (AMD) with single-stranded DNA were studied by fluorescence, absorption and NMR spectroscopy, calorimetry, ultrasonic velocity and density measurements, and molecular modeling. The binding is length and sequence dependent, with the tetranucleotide motif TAGT showing the highest affinity. A "hemi-intercalation" model for the interaction is proposed.

Actinomycin D (AMD) is a potent antibiotic used commonly in the treatment of rhabdomyosarcoma and Wilms' tumor in children as a component of the clinically used VAC therapy (Vincristine, Actinomycin D, Cyclophosphamide). The pharmacological action of AMD is generally attributed to its tight interactions with DNA, which cause inhibition of transcription elongation by a blockage of RNA polymerase. Actinomycin D is also known to produce inhibition of DNA-DNA and DNA-RNA hybridization.[1]

The binding of AMD to dsDNA has been extensively characterized by crystallographic and NMR analysis.[2-10] The drug is regarded as a prototypic intercalator. However, we have demonstrated that AMD also binds tightly and specifically to single-stranded (ss) DNA.[11,12] The presence of an unpaired guanine residue without need of a base-paired cytosine or an ordered structure is a necessary though not sufficient

requirement. That is, the single-strand binding exhibits a somewhat complex sequence dependence.[12] Complexes of AMD with ssDNA have also been reported in a study by Hsieh *et al.* based on ion spray and tandem mass spectrometric analysis.[13]

We have extended the characterization of AMD binding to ssDNA using a variety of biophysical techniques, including fluorescence, absorption, and NMR spectroscopy, calorimetry, and ultrasound velocity and density measurements. The fluorescent derivative of AMD, 7-aminoactinomycin D (7AAMD), a compound with the biological activity and dsDNA-recognition properties of AMD,[14] was used in the fluorescence studies. [11,12] We report here a summary of the newest findings.

RESULTS AND DISCUSSION

Absorption and fluorescence spectroscopy. From quantitative measurements of the changes in absorption and fluorescence of 7AAMD upon interaction with a series of non self-complementary oligonucleotides (TABLE I) we determined that those containing the TAGT motif exhibit the highest affinity, one that increases with the length of the DNA. The residues flanking the ssDNA binding site are critical in determining the binding properties of the drug (see S3, S1, and R5 in TABLE I).

NMR and molecular modeling: A model proposed[11] for the interaction between AMD and the TAGT target based on NMR experiments, is featured in Figures 1 and 2. There are 5 hydrogen bonds between the AMD ligand and the ssDNA: cyclopeptide chain I (benzenoid side, Figure 1), O and NH of threonine to N1H/N2H (bifurcated bond) and O6 of G, respectively; cyclopeptide chain II (quinoid side, Figure 1), O and NH of threonine to N6H and N1 of A, respectively. The distances between the phenoxazone system and donor-acceptor groups of the DNA exclude the possibility of hydrogen bond formation between these substructures.

The orientation of the AMD chromophore with respect to the sugar–phosphate backbone in the hemi-intercalation model is different from the canonical dsDNA intercalation (Figure 2). In the latter, AMD intercalates between two base pairs (C-G)-(C-G) from the minor groove side, and the chromophore methyl groups point toward the major groove, whereas in the AMD-ssDNA complex these methyl groups face the sugar phosphate backbone. As a result of this orientation there are differences in the interaction of the phenoxazone and the cyclopeptide chains with respect to the flanking bases of the DNA strand. The model suggests that for most effective binding, the aligment of AMD phenoxazone system is perpendicular to that in complexes with dsDNA. As a consequence, the N6 of adenine plays for ssDNA the role of the guanine N2 in dsDNA,

TABLE I: ssDNA Binding to 7-aminoactinomycin D. Data from [11]. $\Delta F/F_0$ is the maximum relative fluorescence increase observed over the concentration range covered in the experiment. K_a is the equilibrium association constant.

	DNA	λ_{abs} (nm)	λ_{em} (nm)	$\Delta F/F_0$	K_a (M^{-1})
D1:	5'AAAAAAAAAATAATTTTAAATATTT 3'	505	665	0	-
D5:	5'AAAAAGAAAGTAGTTTTAAGTATTT 3'	540	630	5.6	$8.3 \cdot 10^5$
R5:	5'AAAAAAAAAATAGTTTTAAATATTT 3'	530	630	11.6	$6.3 \cdot 10^5$
R7:	5'AAATATTTAAAAGTATTTTTTTTTT 3'	525	640	1.8	$8.8 \cdot 10^4$
R8:	5'AAAAAAAAAAGAATTTTAAATATTT 3'	510	645	0.7	$3.5 \cdot 10^4$
R9:	5'TTTTTTTTTTGTTAAAATTTATAAA 3'	510	640	1.3	$2.5 \cdot 10^4$
R10:	5'AAATATTTAAAATTGTTTTTTTTTT 3'	510	645	1.7	$3.3 \cdot 10^4$
R6:	5'TTTTTTTTTTATGAAAATTTATAAA 3'	505	650	0.2	-
S4:	5' AAAAAAATAGTTTTAAATA 3'	512	630	11.6	$8.1 \cdot 10^4$
S2:	5' AAAAATAGTTTTAA 3'	512	640	8.6	$1.5 \cdot 10^4$
S5:	5' AAAAAAAAAATAGTTTT 3'	505	640	17.0	$3.3 \cdot 10^4$
Sc:	5' TAGTTAGT 3'	505	640	2.1	$1.8 \cdot 10^4$
S1:	5' TTTTTTTTTTAGTAAAATTTATAAA 3'	507	630	9.1	$3.6 \cdot 10^4$
S3:	5' TTTATAAATTTTAGTAAAAAAAAAA 3'	507	630	9.4	$3.3 \cdot 10^4$
poly d(AGT):	($5'...TAGT...3'$)	---	640	2.5	$9.1 \cdot 10^5$

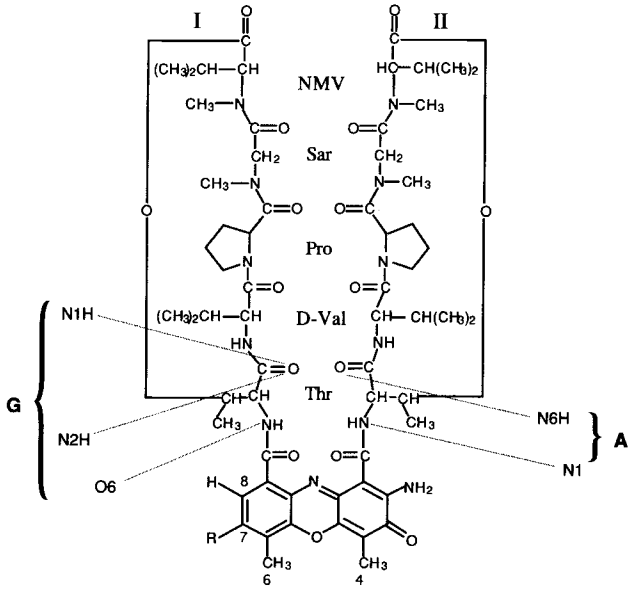

FIGURE 1: Structure of AMD and H-bonding pattern of AMD-ssDNA complex from NMR study.[11] R = H, AMD; R = NH_2, 7AAMD. Hydrogen-bonds to guanine and adenine in the TTTAGTTT:AMD ssDNA model are shown.

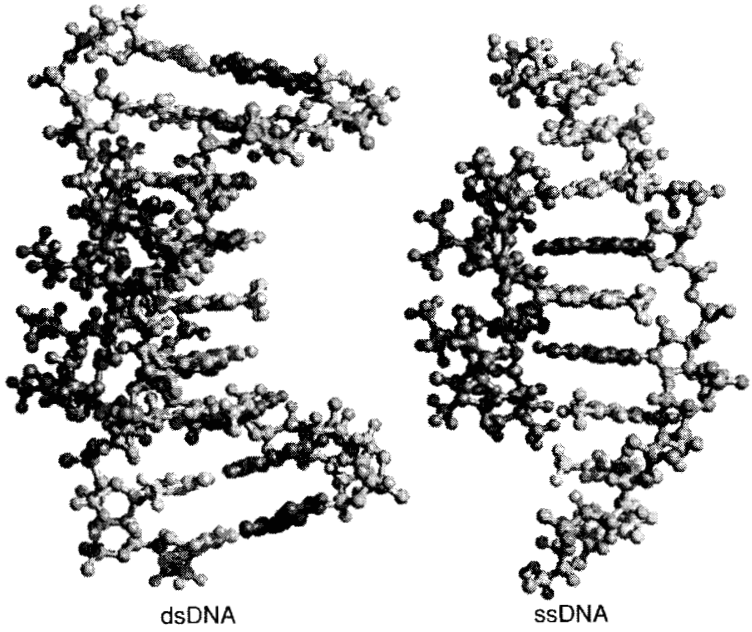

FIGURE 2: Comparison between dsDNA and ssDNA interaction with AMD. Left, model from crystallographic analysis.[15] Right, model from NMR study.[11]

i.e. it provides a hydrogen bond donor for AMD recognition. The purine residues make a total of five hydrogen bonding interactions with the two threonines of the cyclopeptide side chains (Figure 1). Replacement in the model of either the G by an A or the A by a G led to structures in which the exchanged purine was no longer hydrogen bonded to the AMD. The particular pattern of hydrogen donor and acceptor groups identified in the AMD-TAGT complex may account, at least in part, for the high sequence and compositional specificity of AMD interactions with ssDNA.

A more recent study[16] involving 2-dimensional NMR was performed in order to refine the model for hemi-intercalation. A number of correlations between MeVal C$\underline{H}_3$, Val C$\underline{H}_3$ and THR C$\underline{H}_3$ with Pro $\underline{H}\,\delta$ andVal $\underline{H}\,\alpha$ appeared in the 1:1 complex NOESY spectra but were absent in the AMD spectra acquired under the same conditions. These data are indicative of a change in the conformation of the drug upon interaction with ssDNA (Figure 3).[16]

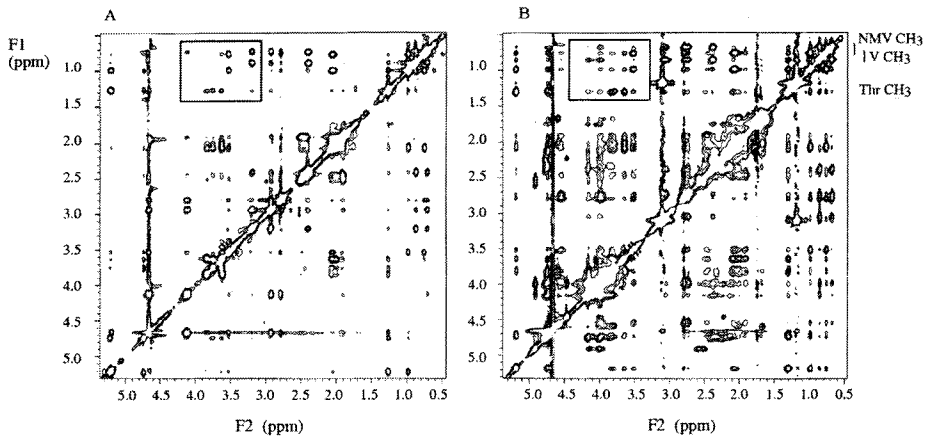

FIGURE 3: Section of the phase-sensitive NOESY spectra of AMD (A) and TTTAGTTT:AMD (B, both components at 1.4 mM, pD 7.0, D_2O). The rectangle in B contains diagnostic NOE crosspeaks for NMV, Val and Thr methyl groups with actinomycin resonances such as Pro protons δ, Val H α. These NOEs are absent from spectrum A.

Calorimetry. Direct calorimetric titrations of d(TAGT) with AMD, using a titration calorimeter from Microcal (Northampton, MA) yielded an exothermic binding heat of ~1 kcal/mol after correcting for the heat of dilution, and a K_a of $5 \cdot 10^4$ M^{-1}.[17]

Ultrasonic velocity and density measurements. The changes in volume and compressibility accompanying stoichiometric binding of AMD to TAGT are 19 cm^3 mol^{-1} and $3.4 \cdot 10^{-3}$ cm^3 mol^{-1} bar^{-1}, respectively.[18] As in the case of AMD dimerization, the change in apparent adiabatic compressibility is positive, indicative of dehydration changes accompanying binding. Additionally, 4 to 6 H-bonds are created during the interaction according to the volume and compressibility data.[18] These results are compatible with the hemi-intercalation model. We note that the bathochromic shift of 7AAMD upon binding to the TAGT motif is also consistent with such a buried drug-DNA complex. Furthermore, a reduced accessibility of water to the 7AAMD chromophore would be expected to inhibit solvent-mediated fluorescence quenching, thereby accounting for the increased emission quantum yield of the complex.[11,12]

Biological consequences of ssDNA binding to AMD. Binding to the single-stranded sites can be directly correlated with the extent of transcriptional inhibition reported in the literature.[19] It is tempting to speculate that one mode of action of AMD in inhibiting RNA polymerase may involve binding to the transcription bubble rather than upstream of

the enzyme, as is often assumed. This idea is consistent with a model for the mechanism of AMD inhibition presented by Sobell and colleagues[20,21] in which the drug binds to melted regions of DNA. A mechanism of this type is also supported by the observed tendency of the drug to inhibit DNA-DNA and DNA-RNA hybridization.[1,22] In addition, a recent study[23] reported the inhibition by AMD of HIV reverse transcriptase and other replication polymerases, presumably mediated by sequence-specific binding to ssDNA. The loci corresponding to the strongest inhibition featured a TAG motif in both strands. We conclude that the interaction of AMD with ssDNA may be of relevance for the understanding of the biological action of this important drug.

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