NMR AND FLUOROMETRIC CHARACTERIZATION OF MITHRAMYCIN IN AQUEOUS SOLUTION

N. RAMA KRISHNA[†], DONALD M. MILLER[†,††] and TED T. SAKAI*[,†]

[†]Comprehensive Cancer Center and
the Departments of Biochemistry and [††]Medicine,
University of Alabama at Birmingham,
Birmingham, AL 35294, U.S.A.

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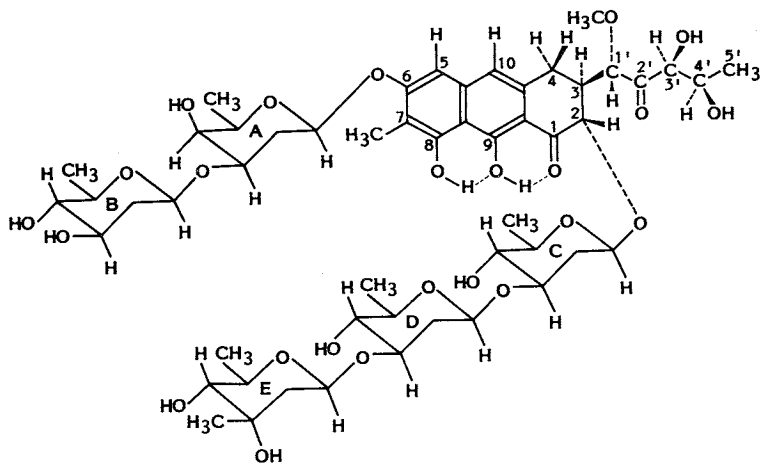
The assignments for the resonances in the ^{1}H and ^{13}C NMR spectra of mithramycin in aqueous solution have been determined by a combination of 2D NMR methods. Specific NOESY contacts observed between different moieties indicate that the drug assumes a compact conformation in aqueous solution. Fluorescence measurements are consistent with a compact structure for mithramycin in water and confirm the preference of mithramycin for binding to dG-containing nucleic acids. These studies provide a basis for the further physico-chemical characterization of mithramycin-DNA complexes.

Mithramycin, produced by several *Streptomyces* strains, is an antitumor antibiotic which is a member of the aureolic acid family (for a general review of aureolic acid drugs, see ref 1). It is characterized by a polyaromatic aglycone to which are attached a hydrophilic side chain and two oligosaccharide side chains, one containing two sugars and the other three sugars (Fig. 1). Mithramycin differs from other members of the aureolic acid group, *e.g.*, chromomycins and olivomycins, primarily in the identity of the sugar residues as well as of substituents on the sugars[1]. An additional difference in some derivatives in the absence of the 7-methyl group on the aglycone (olivomycins). These antibiotics form complexes with DNA and, in the presence of Mg^{2+}, serve as potent inhibitors of DNA-directed RNA synthesis[1~8]. We have shown that mithramycin prevents protein binding to GC-rich regulatory regions of the SV40[9] and *c*-myc[10] promoters. These drugs have been shown to exhibit antitumor activity against a number of experimental tumors and are used clinically in the treatment of testicular tumors and of hypercalcemia and hypercalciuria

Fig. 1. Structure of mithramycin A, showing numbering system used in this study.



associated with malignancies[1,11,12].

Mithramycin has been characterized by ^{1}H and ^{13}C NMR spectroscopy by THIEM and MEYER[13] using acetone as the solvent. No NMR data have been reported for this drug in the physiologically relevant solvent, water. By utilizing high resolution 2D NMR spectroscopic techniques at 600 MHz, we have made the ^{1}H and ^{13}C resonance assignments of mithramycin in aqueous solution and characterized its free solution conformation. In addition, we have characterized some of the fluorescence properties of mithramycin which lend themselves for use in the study of mithramycin binding to DNA.

Materials and Methods

Mithramycin A (99.8% pure), calf thymus DNA and poly(dG-dC) were purchased from Sigma Chemical Company (St. Louis, MO); poly(dA-dT) was obtained from Pharmacia P-L Biochemicals (Piscataway, NJ). D_2O was from MSD Isotopes (Québec, Canada) or Stohler Isotope Chemicals (Waltham, MA).

NMR samples in D_2O were prepared by dissolving 1 mg of mithramycin in 0.5 ml of solvent. The sample also contained 0.10 M sodium chloride and 0.01 M sodium phosphate buffer (mixed mono- and dibasic phosphates, total phosphate concentration = 0.01 M, pH 7.0).

All NMR experiments were performed at an ambient probe temperature of 23°C on a Bruker AM-600 spectrometer operating at 600 MHz for protons. Quadrature detection was employed for all NMR experiments. 2D COSY experiments were performed with a standard two-pulse sequence[14]. A spectral width of 6,600 Hz was used with a 256 × 1,024 time domain data matrix and 1.5 seconds relaxation delay. For each of the t1 values, 32 scans were accumulated. Phase-sensitive 2D NOESY spectra[15] were obtained with mixing times of 200 and 500 mseconds using the same spectral width as in the 2D COSY experiments, giving a 512 × 2,048 hypercomplex data matrix. All 2D data processing was done on a micro VAX II computer utilizing the FT NMR software written by Dr. DENNIS HARE (Hare Research, Inc., Woodinville, WA). The COSY data were zero-filled to 1,024 × 2,048 and processed with a non-shifted sine bell squared window function in both t1 and t2 dimensions. For 2D NOESY experiments, data were zero-filled to 1,024 × 2,048 data points and multiplied by a 30-degree shifted sine bell squared window function in each dimension before Fourier transformation. The [^{1}H-^{13}C] correlation experiments were performed in the inverse detection mode[16].

Fluorescence measurements were made at ambient temperature (22 ~ 24°C) on a Perkin-Elmer MPF-3 spectrofluorometer. All solutions contained 0.01 M sodium phosphate buffer (pH 7.0) and 0.01 M sodium chloride. Concentrations of mithramycin were measured spectrophotometrically at 400 nm using a calculated molar absorptivity of 10,000 M^{-1}/cm.

Samples of calf thymus DNA, poly(dG-dC) and poly(dA-dT) were dialyzed successively against 0.01 M sodium phosphate buffer (pH 7.0) - 1 M sodium chloride - 0.05 M EDTA, several changes of distilled water and then the buffered saline using in these studies. Concentrations of calf thymus DNA and poly(dA-dT) were measured spectrophotometrically at 260 nm using molar absorptivities of 6,700 and 5,600 M^{-1}/cm, respectively[16]; those of poly(dG-dC) were measured at 254 nm using a molar absorptivity of 8,400 M^{-1}/cm[17].

Results and Discussion

NMR Measurements

Chemical Shift Assignments

Mithramycin A in Aqueous Solution: Mithramycin A has a fairly low solubility in pure D_2O, but it will dissolve readily in aqueous buffered saline. Experiments were routinely performed in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.10 M sodium chloride.

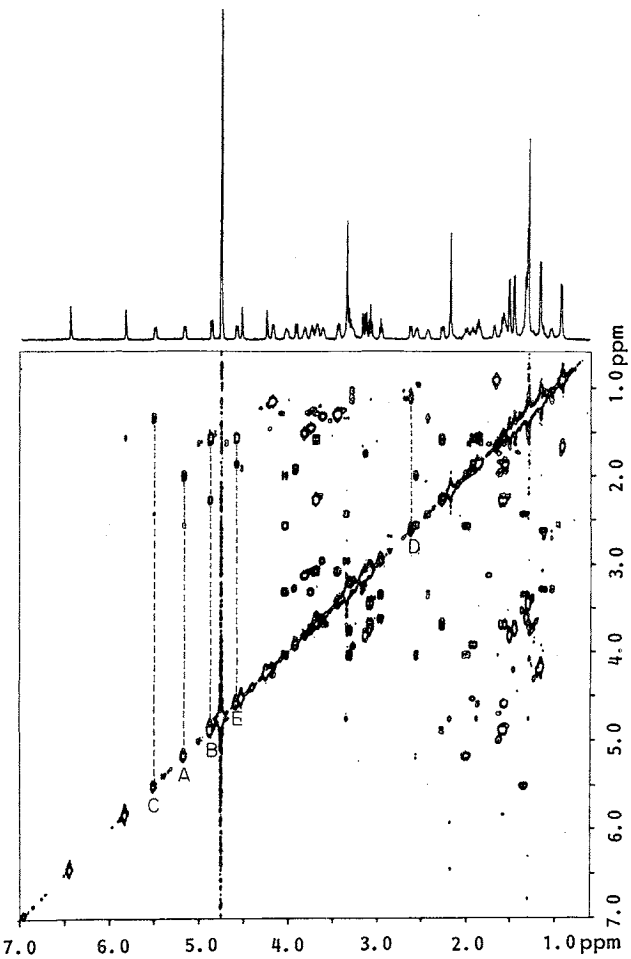
Figs. 2 and 3 show the 2D COSY and NOESY spectra, respectively, obtained at 600 MHz of

Table 1. ^{1}H NMR chemical shifts[a] of mithramycin in D_2O[b].

Carbohydrate	1-H	2-H_e	2-H_a	3-H	4-H	5-H	6-H	3-CH_3
A	5.16	2.56	2.00	4.02	3.31	3.75	1.46	
B	4.86	2.25	1.58	3.68	3.08	3.44	1.30	
C	5.49	2.43	1.34	3.35	2.96	3.62	1.32	
D	2.63	1.13	1.03	3.29	3.16	1.68	0.92	
E	4.58	1.86	1.56	—	3.13	3.81	1.51	1.29
Aglycone	2-H	3-H	4-H_e	4-H_a	5-H	10-H	7-CH_3	
	3.91	1.93	1.84	1.58	6.44	5.83	2.17	
Side chain	1′-H	3′-H	4′-H	5′-CH_3	OCH_3			
	4.52	4.24	4.17	1.17	3.33			

[a] All chemical shifts are referenced to the DOH resonance at 4.76 ppm.
[b] Solution contains 0.01 M sodium phosphate buffer - 0.1 M NaCl (pH 7.0).

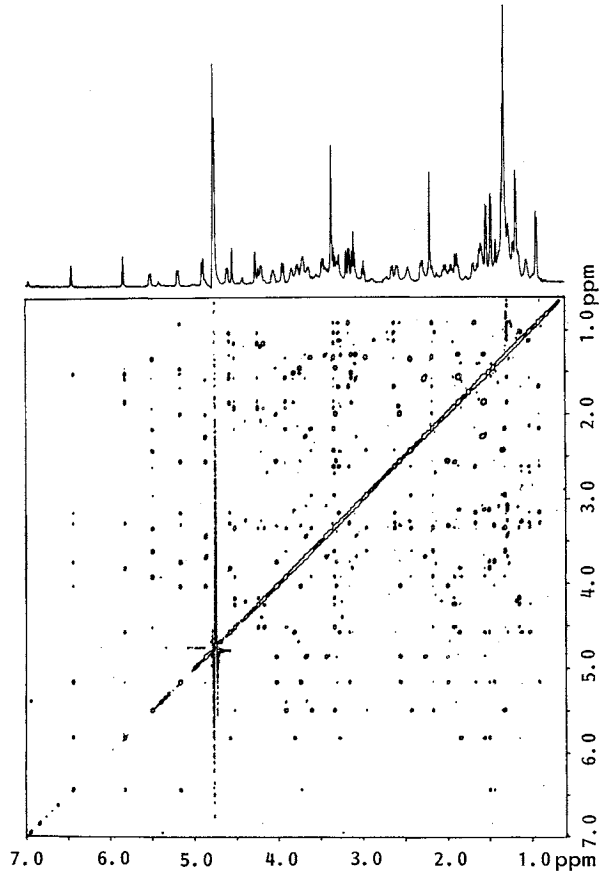
Fig. 2. 600 MHz 2D COSY NMR spectrum of mithramycin in D_2O containing 0.01 M sodium phosphate buffer (pH 7.0, meter reading) and 0.10 M sodium chloride.



The COSY cross peak assignments for the anomeric protons are shown. The corresponding 1D NMR spectrum is shown along the top.

Fig. 3. 600 MHz 2D NOESY NMR spectrum of mithramycin in D_2O containing 0.01 M sodium phosphate buffer (pH 7.0, meter reading) and 0.10 M sodium chloride.

A mixing time of 500 mseconds was used.



mithramycin A in aqueous solution. The corresponding 1D spectra are shown along the top of each plot.

The complete assignments for the protons of mithramycin in D_2O were obtained from the 2D COSY and 2D NOESY data. Specifically, 2D COSY was employed to identify networks of *J*-coupled protons within each sugar and within the hydrophilic side chain. The 2D NOESY spectrum was then used to obtain sequence specific assignments for the different sugars and for the different protons in the aglycone.

The anomeric protons for four of the five sugars were easily identified in the 2D COSY spectrum from their characteristic low field shift positions (δ 5.49, 5.16, 4.86 and 4.58), as well as from the pair of cross peaks exhibited by these protons because of connectivities to the 2-H_{eq} and 2-H_{ax} protons within each sugar. Identification of the remaining proton resonances for each sugar by following the *J*-connectivity networks was straightforward. In the case of sugar D, it was necessary to complement the COSY data with the NOESY data to complete the proton assignments. The anomeric proton of this sugar exhibited an unusually large upfield shift and resonates at 2.63 ppm. The origin of this upfield shift is discussed later.

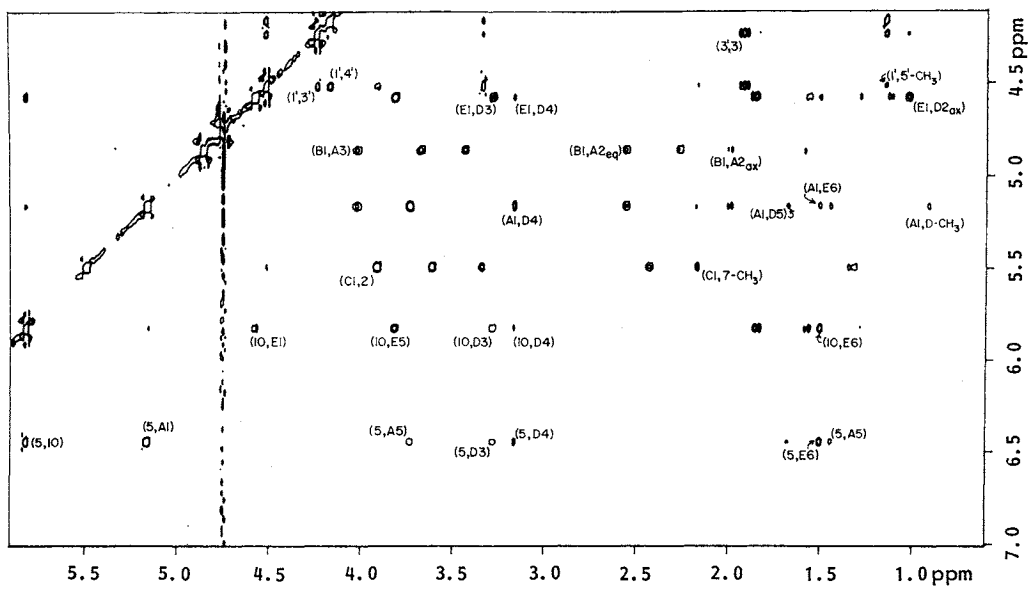
In the 2D NOESY spectrum, an aromatic proton (a singlet at 6.44 ppm) showed NOESY contacts to another singlet aromatic proton at 5.83 ppm and to the anomeric proton at 5.16 ppm from one of the sugars. Based on the NOESY contacts predicted from the primary structure of the drug (Fig. 1), the

Table 2. ^{13}C NMR chemical shifts[a] of mithramycin in D_2O[b].

Carbohydrate	C-1	C-2	C-3	C-4	C-5	C-6	3-CH_3
A	102.47	38.54	80.50	77.34	75.11	18.12	
B	102.10	41.18	73.06	79.39	75.21	27.31	
C	108.06	38.76	84.41	78.09	74.37	17.73	
D	102.64	31.68	75.27	71.76	72.51	16.17	
E	100.61	45.75	—	79.39	73.62	18.94	17.73
Aglycon	C-2	C-3	C-4	C-5	C-10	7-CH_3	
	83.48	44.27	29.36	103.59	114.83	7.78	
Side chain	C-1′	C-3′	C-4′	5′-CH_3	OCH_3		
	85.17	80.32	70.46	19.07	60.83		

[a] All chemical shifts are referenced to external dioxane at 66.50 ppm.
[b] Solution contains 0.01 M sodium phosphate buffer - 0.1 M NaCl (pH 7.0).

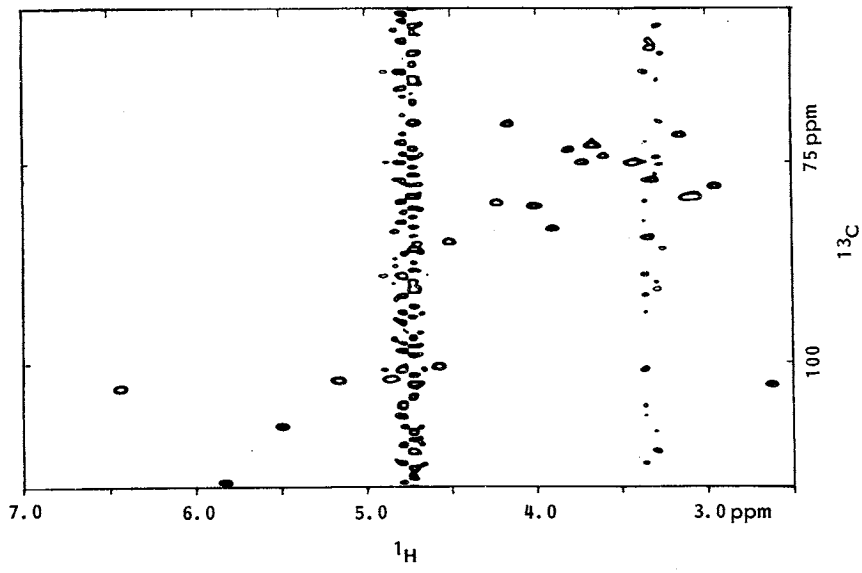
Fig. 4. 600 MHz phase-sensitive 2D NOESY spectrum of mithramycin in D_2O obtained with a mixing time of 200 mseconds.



aromatic resonances at 6.44 and 5.83 ppm were assigned, respectively, to the 5-H and 10-H protons of the aglycone, while the anomeric proton was assigned to sugar A. Other interesting NOESY connectivities across interresidue linkages that were helpful in establishing specific assignments were: B-1-H to A-3-H; B-1-H to A-2-H_{eq}; C-1-H to aglycone-2-H; D-1-H to C-3-H; E-1-H to D-3-H; and E-1-H to D-2-H_{ax}. Some of these are shown in the 200 mseconds NOESY spectrum (Fig. 4).

After completing the proton resonance assignments, the ^{13}C resonances were assigned by ^{1}H-^{13}C COSY using the inverse detection technique on the AM-600. This correlation spectrum (Fig. 5) clearly showed that a proton resonance resonating at 2.63 ppm was attached to a ^{13}C resonating at 102.6 ppm, a value typical of an anomeric carbon involved in a glycosidic linkage.

Fig. 5. Part of the ^{1}H-^{13}C correlation spectrum of mithramycin in D_2O obtained using the inverse mode.



Conformational Features of Mithramycin in Water

The 2D NOESY spectra exhibited a wealth of information that can be used to define the solution conformation of the drug in water. The anomeric proton resonance of sugar D is shifted to an unusually high field position at 2.63 ppm. The origin of this shift is the strong ring current interaction experienced by sugar D from the aromatic aglycone. Evidence for this suggestion is obtained from the NOESY contacts experienced by the 3-H, 4-H and 5-H protons of sugar D and by the 1-H, 5-H and 6-H protons of sugar E with the 5-H and 10-H protons of the aglycone (Figs. 3 and 4). This observation, together with the large upfield shift experienced by the 1-H of sugar D, suggests that the C–D–E trisaccharide moiety is folded over the aglycone in such a way that the 1-H proton of sugar D is exposed to a large ring current field from the aromatic group. Sugar E, on the other hand, is positioned in such a way that it does not experience a significant ring current shift, but is close enough to the 5-H and 10-H protons of the aglycone to experience NOESY contacts. We have also observed weak NOESY contacts between the 1-H of sugar A and the 4-H of sugar D. Thus, the A–B disaccharide unit is oriented in such a way as to bring sugar A closer to sugar D.

A weak NOESY contact between 1′-H and 5′-CH_3 of the hydrophilic side chain suggests that it folds back slightly, bringing the methyl group closer to the aglycone.

These data suggest that the conformational manifold of mithramycin in aqueous solution contains some well-defined folded conformations. The hydrophobic nature of the molecule appears to result in a stacking interaction between the aglycone and the "aliphatic" side of sugar D, minimizing water access to the aromatic ring and enhancing solvation of the hydroxyl groups of sugar D.

These results pose an interesting contrast to chromomycin A_3 which exists in an extended conformation in methanol, a relatively polar solvent, and assumes a folded structure in dichloromethane, a fairly non-polar solvent[18]. Mithramycin in organic solvent (acetone) is found to assume an extended structure[13], whereas our present studies show the drug is folded in aqueous solution. The differences presumably reside in the fact that chromomycin A_3 has three of its sugar hydroxyls blocked (one with a methyl group and

two with acetyl groups).

Fluorescence Measurements

Fluorescence excitation and emission spectra of mithramycin were obtained in the same buffer used in the NMR experiments. Typical excitation and emission spectra are shown in Fig. 6. The free drug exhibited several excitation maxima, including major peaks at 285 and 400 nm, with a shoulder at about 245 nm. The emission spectrum shows a very broad peak with the maximum at about 525 nm. These maxima are to slightly lower wavelength than those reported in the literature[19,20] and probably reflect the lower ionic strength (approximately 0.01 M) and higher pH (pH 8) used in those studies. As reported by others[20], the fluorescence emission of mithramycin is quenched by the presence of Mg^{2+}, indicating interaction of the metal ion with the fluorophore. The Mg^{2+}-induced quenching of mithramycin is markedly slower in the buffered saline than in a buffer comprised of 0.01 M Tris-HCl (pH 7.2) or 0.01 M Tris-HCl - 0.10 M sodium chloride, indicating a competition between phosphate and mithramycin for the metal ion.

In the presence of excess calf thymus DNA and Mg^{2+}, the excitation peaks below 300 nm disappear as expected due to absorption of light by the DNA itself and the excitation peak at 400 nm is reduced in intensity.

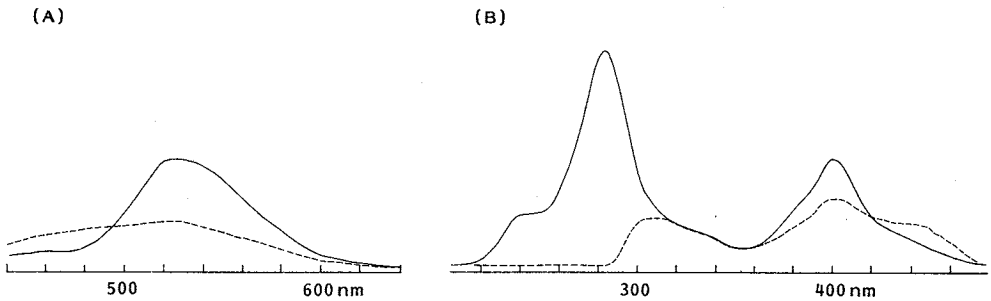
Addition of calf thymus DNA to mithramycin solutions containing Mg^{2+} results in a slow increase of mithramycin fluorescence, however, the final fluorescence intensity is less than that of the corresponding mithramycin solution in the absence of Mg^{2+}. This suggests that in the presence of DNA there may be a redistribution of metal ion from a position in which the chromophore is affected (*e.g.*, the aromatic hydroxyls) to another site (either on the mithramycin molecule or on the DNA). Similar results have been reported by others[20]. Metal ions are reported to bind to a unique site in complexes of the related chromomycin A_3 to DNA[21].

HILL[19] reported that mithramycin fluorescence increased linearly with DNA concentrations and suggested this as a basis for quantitating DNA in cells; however, it is likely, on the basis of our results and those of CONS and FOX[20], that that observed DNA-mediated increase in the fluorescence of mithramycin was the result of the "unquenching" by DNA of Mg^{2+}-quenched mithramycin fluorescence.

Quenching of mithramycin fluorescence was observed with both calf thymus DNA and poly(dG-dC);

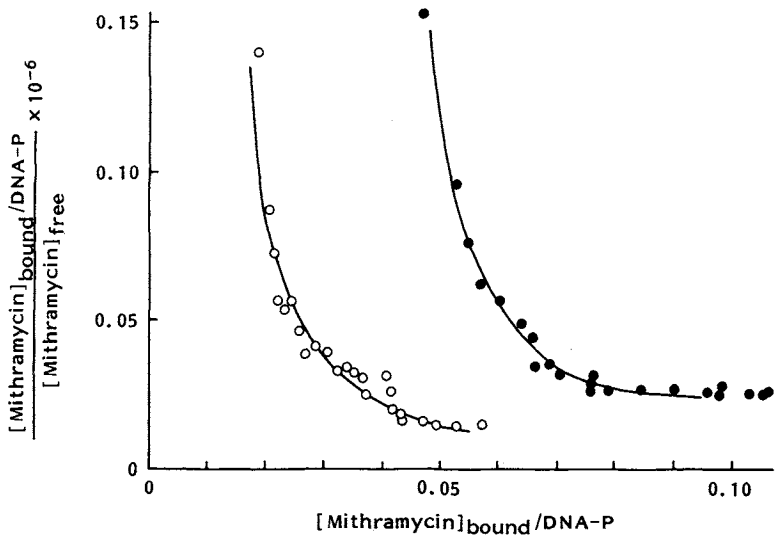
Fig. 6. Fluorescence excitation and emission spectra of mithramycin alone and in the presence of calf thymus DNA.

(A) Emission, (B) excitation. —— Mithramycin, ------ DNA.



Spectra were obtained at ambient temperature (24 °C). For mithramycin (0.01 mM), the solution contains 0.01 M sodium phosphate buffer (pH 7.0) and 0.10 M sodium chloride. Solutions with DNA (0.002 M in DNA-phosphate) also contained 0.05 M $MgCl_2$.

Fig. 7. Scatchard plots for the binding of mithramycin to calf thymus DNA and to poly(dG-dC).
○ DNA, ● poly(dG-dC).



Data obtained at 24°C in 0.01 M sodium phosphate buffer (pH 7.0) - 0.10 M sodium chloride - 0.05 M $MgCl_2$. Mithramycin concentration is 0.05 mM.

however, little change was seen with poly(dA-dT). These data are consistent with the known specificity of mithramycin for G-C sites in DNA[1,22]. Scatchard plots[23] indicated the presence of two classes of binding sites for both calf thymus DNA and poly(dG-dC) (Fig. 7). For calf thymus DNA, the higher affinity site showed an apparent *Ka* of about $2 \times 10^5\ M^{-1}$ with an average stoichiometry of 1 site/40 ~ 50 base pairs; the lower affinity site has a *Ka* of about $6 \times 10^3\ M^{-1}$ with a stoichiometry of about 1 site/9 base pairs. The binding constants are estimates based on straight-line extrapolations of the linear portions of the Scatchard plots. This treatment may be used when the dissociation constants differ by about two orders of magnitude or more[24,25]. Multiple sites might only be observable by fluorescence since no mention is made of multiple classes in previous UV-visible spectral measurements.

The binding constants obtained for mithramycin binding to poly(dG-dC) are similar to those found with DNA, with *Ka* values of about $2 \times 10^5\ M^{-1}$ and $5 \times 10^3\ M^{-1}$ for the two classes of sites; however, the Scatchard plot is displaced to the right and shows roughly double the binding sites per base pair, as would be expected in going from a DNA which is about half G-C to one which is completely G-C. The smaller number of mithramycin binding sites observed in our studies compared to those found by CONS and FOX[20] may reflect the higher ionic strength used in our studies.

The use of changes in the UV-visible spectrum of mithramycin and related compounds to monitor its interactions with DNA has long been known[22,26,27]. Although mithramycin fluorescence has been suggested as a means of quantitating DNA isolated from cells[19], its utility in quantitating the interaction of the drug with DNA is fairly new and was reported[20] as these studies were in progress. These data show that it will be possible to study the interaction of mithramycin with various DNA's using fluorescence techniques. The findings that the spectral changes take time to attain equilibrium indicate that it may also be possible to monitor the kinetics of complexation between drug and DNA (and possibly metal ion).

The irreversible loss of fluorescence peaks (both excitation and emission) upon prolonged exposure

of mithramycin solutions in the fluorometer shows the great sensitivity of mithramycin to light and the need to take care in the measurement (and interpretation) of fluorescence data.

The net decrease in mithramycin fluorescence upon binding to DNA indicates that the fluorophore is going from a comparatively hydrophobic environment to one which is more accessible to solvent since solvent would be expected to quench fluorescence in these molecules. The NMR experiments indicate that mithramycin exists in a folded structure in water so that it is likely this folding contributes to the fluorescent character of the molecule. This suggests that the interaction of mithramycin with DNA affects this intramolecular interaction in such a way that a decrease in fluorescence is observed. Studies by others[21,28~30] on the interaction of the related chromomycin A_3 with DNA oligomers show that the side chains of the molecule are indeed extended away from the chromophore in such complexes.

The fluorescence data are in agreement with a mode of mithramycin binding to DNA in which the fluorophore is more exposed to solvent in the complex than it is in free solution. This suggests that intercalation is an unlikely mode of binding since intercalation might be expected to lead to an increase in drug fluorescence. Once again, studies with the related chromomycin A_3 indicate that the drug is a groove-binder[21,28~30].

Conclusions

NMR measurements have been made at 600 MHz on mithramycin in aqueous solution. The conformational manifold of mithramycin in water exhibits some well-defined conformations in which the trisaccharide moiety is folded over the aglycone. Further, sugar A experiences spatial proximity to sugar D. The hydrophilic side chain appears to loop back on itself so that the end of the chain is near the chromophore. The molecule assumes something of a micellar structure, minimizing exposure of its hydrophobic areas to solvent and increasing exposure of its hydrophilic group.

Fluorometric measurements are consistent with the existence of the mithramycin fluorophore in a fairly hydrophobic environment such as that which might result from the intramolecular stacking observed by NMR. Measurements in the presence of DNA show that mithramycin has a preference for G-C containing DNA's and are consistent with the assumption by mithramycin of an extended conformation upon binding to DNA.

Acknowledgments

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