Volume 289, number 2, 210–212 FEBS 10140 September 1991
© 1991 Federation of European Biochemical Societies 00145793/91/$3.50
ADONIS 001457939100852L

NMR investigation of the interaction of mithramycin A with d(ACCCGGGT)$_2$

Max A. Keniry[1], Debra L. Banville[2], Corey Levenson[3] and Richard H. Shafer[2]

[1]*Research School of Chemistry, The Australian National University, GPO Box 4, Canberra, ACT 2601, Australia,* [2]*Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143, USA* and [3]*Cetus Corporation, 1400 53rd Street, Emeryville, CA 94608, USA*

Received 22 April 1991; revised version received 10 July 1991

The binding of mithramcyin A to d(ACCCGGGT)$_2$ has been investigated by one- and two-dimensional ^{1}H NMR spectroscopy. Titration of the drug into the octamer solution results in loss of the oligonucleotide C_2 symmetry at stoichiometric ratios less than 4 drug molecules per duplex. However, at a ratio of 4:1 (drug/duplex), the C_2 symmetry of the oligonucleotide is restored. From these data it is evident that more than one complex forms at ratios less than 4:1 while only one complex predominates at the ratio 4:1. This is the first report of a DNA octamer which binds 4 large drug molecules. These results are compared to those we have recently reported for mithramycin binding to d(ATGCAT)$_2$, where only a single, bound complex is observed, with a stoichiometry of 2:1.

Mithramycin; Drug–DNA interaction, ^{1}H NMR

1. INTRODUCTION

Drugs based on the aureolic acid backbone have, over the years, shown many atypical binding properties with DNA [1]. This family of relatively large drugs (molecular weight ca. 1200 g/mol), that includes chromomycin [2,3], mithramycin [4] and olivomycin [5], binds to DNA in the presence of divalent cations and shows a preference for GC base pairs. Recent NMR studies with short oligonucleotides have demonstrated that mithramycin and chromomycin bind to DNA as dimers in the minor groove [2–4].

Recently we have shown that chromomycin prefers 5′-GC-3′ over 5′-GC-3′ sites [3]. In addition, it is known that one of the most favored sites is a 5′-CCC-3′ sequence [6]. Here we present data on the association of mithramycin A in the presence of Mg^{2+} with d(ACCCGGGT)$_2$ [7], a sequence that contains the less favored 5′-CG-3′ sequence at the center of the oligonucleotide and the highly favored 5′-CCC-3′ sequence off-center. We are now able to demonstrate that an oligonucleotide with just eight base pairs binds four molecules of mithramycin and does so in a manner that retains the original symmetry of the oligonucleotide when all the binding sites are occupied. This represents the first example of such a short DNA oligonucleotide binding four large drug molecules.

The ^{1}H NMR spectra of a drug–oligonucleotide complex can be very complicated, particularly if the symmetry of a self-complementary sequence is removed by drug binding and/or there is a mixture of free and drug-associated oligonucleotide. There are, however, two regions of the NMR spectrum that are excellent indicators of the nature and extent of the drug association. The imino proton region is ideal for investigating drug binding because it is well separated from all other nucleotide signals and because each base pair contributes only one resonance to this region. The non-exchangeable cytosine H5 and H6 base protons are less frequently used for this purpose but they can be separated from other base resonances if the scalar coupling between these two protons is exploited. We have used the 2D HOHAHA experiment [8] to show how the number of distinct cytosine resonances changes as the mithramycin/d(ACCCGGGT)$_2$ ratio is altered.

2. MATERIALS AND METHODS

Mithramycin A, produced by *Streptomyces plicatus*, was purchased from Sigma (St. Louis, MO) and used without further purification. The octamer d(ACCCGGGT)$_2$ was synthesized and purified as described previously [7]. All NMR samples were 1 mM in duplex d(ACCCGGGT)$_2$ in 20 mM phosphate, 40 mM NaCl, 0.05 mM EDTA, pH 7.2. Duplex concentration was determined spectrophotometrically, assuming a duplex molar extinction coefficient of 103 000/M-cm which was determined by phosphate analysis. The mithramycin-d(ACCCGGGT)$_2$ complex was made by solubilizing lyophilized aliquots of mithramycin in the d(ACCCGGGT)$_2$ solution that had Mg^{2+} in slight excess of a 4:1 molar ratio of Mg^{2+}/d(ACCCGGGT)$_2$. The concentration of the stock solution of mithramycin was calculated with a molar extinction coefficient of 13 000/M-cm (in a 1 M NaCl, 100 mM borate buffer, pH 8.0, at room temperature) which we determined spectrophotometrically from the dry weight. NMR experiments

---

Correspondence address: R.H. Shafer, Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143 USA. Fax: (1) (415) 476-0688.

Published by Elsevier Science Publishers B.V.

were carried out on a Varian VXR500S spectrometer. Experimental details are given in the figure legends.

3. RESULTS

Taking into consideration the symmetry of d(ACCCGGGT)$_2$, we expected only three guanine imino proton resonances in the lowfield region of the spectrum of H_2O. These three resonances are shown in Fig. 1A. The thymine imino proton resonance is not observed at 15°C because of rapid exchange with the solvent at the terminal base pairs. For similar symmetry considerations, only three H5/H6 crosspeaks are observed in the HOHAHA spectrum of the octamer obtained in D_2O, illustrated in Fig. 2A (the splitting of each peak due to the 7 Hz coupling constant is evident in the F2 dimension).

On addition of mithramycin to d(ACCCGGGT)$_2$ in the ratio 1:1 (drug molecules per duplex), at least eight additional imino resonances are observed in the region 11–14 ppm (Fig. 1B). It has been shown recently that both chromomycin and mithramycin bind to self-complementary DNA duplexes with retention of C_2 symmetry [2–4]. If the binding site is at the center of the d(ACCCGGGT)$_2$ duplex, as in all previous studies, then only three additional imino resonances are expected. The two resonances between 16.5 and 15.5 ppm arise from one phenolic proton of each of the bound mithramycin molecules. The other phenolic proton is in fast exchange with water and has not been observed in any previous NMR spectra of chromomycin or mithramycin complexes with DNA [2–4]. When the binding site is at the center of a symmetrical duplex, only one phenolic proton resonance is observed. The presence of two phenolic resonances in unequal ratios in combination with the appearance of at least eight additonal guanine imino protons suggests that at a drug/duplex ratio of 1:1 there are at least two binding sites, for example at 5′-CCC-3′ and 5′-CCG-3′. similar effects can also be seen in the HOHAHA data at a drug/duplex ratio of 1.5 (Fig. 2B). Here a small amount of free DNA can be discerned, along with many new crosspeaks.

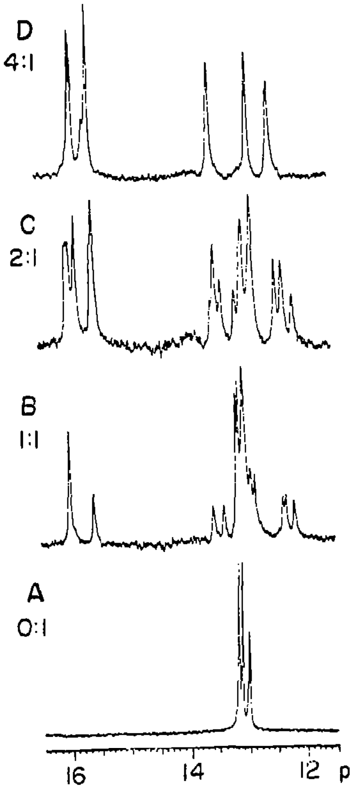

Fig. 1. 500 MHz ^1H NMR spectra of d(ACCCGGGT)$_2$ and the mithramycin–d(ACCCGGGT)$_2$–Mg^{2+} ternary complex 1 mM in DNA duplex, in 20 mM sodium phosphate, 40 mM NaCl, 0.05 mM EDTA, pH 7.2 in 10% D_2O/90% H_2O. All spectra were taken at 15°C. (A) 0:1, (B) 1:1, (C) 2:1, (D) 4:1, mithramycin/d(ACCCGGGT)$_2$. The water resonance was suppressed by a $1\,\tau\,\bar{3}\,\tau\,3\,\tau\,\bar{1}$ sequence. Each spectrum required 128 scans over a sweepwidth of 12.5 kHz and τ was set at 96 μs.

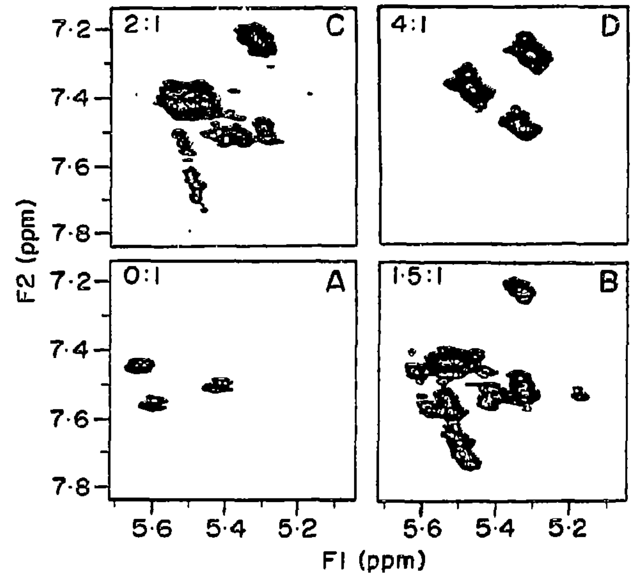

Fig. 2. 500 MHz ^1H 2D HOHAHA spectra of d(ACCCGGGT)$_2$ and the mithramycin–d(ACCCGGGT)$_2$–Mg^{2+} ternary complex, 1 mM in DNA duplex in 20 mM sodium phosphate, 40 mM NaCl, 0.05 mM EDTA, pH 7.2 in 99.996% D_2O. All spectra were taken at 20°C. (A) 0:1, (B) 1.5:1, (C) 2:1, (D) 4:1, mithramycin/d(ACCCGGGT)$_2$. Each data set consisted of 256 complex increments, 2K points per FID over a sweep width of 4.5 kHz. The data set was zero filled to a size of 1K × 2K real points and each dimension was apodized with a 60 degree shifted sinebell.

On further addition of mithramycin to d(ACCCGGGT)$_2$ to a ratio of 2:1 the original guanine imino resonances have disappeared and there are at least nine peaks in the region 11–14 ppm (Fig. 1C), each distinct from the three original resonances of free DNA. In addition, a third resonance appears in the 16.5–15.5 ppm spectral region. The absence of free DNA at this ratio can be seen more clearly in the HOHAHA spectrum for the 2:1 ratio (Fig. 2C). Furthermore, the relative intensities of the new crosspeaks have changed substantially from those observed at the 1.5:1 ratio.

At a 4:1 drug/duplex ratio here are only three distinct guanine imino proton resonances, which suggests that the original symmetry of the duplex has been restored. Also, there are only two drug phenolic proton resonances, one of which appeared at the 2:1 drug/duplex ratio. Taken together, these two observations are consistent with binding of two mithramycin dimers at two equivalent off-center sites on d(ACCCGGGT)$_2$. A small shoulder on the 15.2 ppm peak suggests the possibility of some heterogeneity in the binding sites and requires further investigation. We note that this shoulder cannot arise from free drug since its phenolic proton resonates at 18 ppm (data not shown). The restoration of the original duplex symmetry is confirmed by the presence of only three cytosine H5/H6 crosspeaks in the HOHAHA spectrum at 4:1 (Fig. 2D).

The large number of imino proton resonances and cytosine crosspeaks is inconsistent with a single binding site at drug/duplex ratio less than 2:1. One might account for the extra peaks by a mixture of free duplex, duplex bound by one drug dimer and duplex bound by two drug dimers. However, the imino proton and HOHAHA spectra clearly show that there is no 4:1 complex present at drug/duplex ratios less than 1.5:1 and that there is no free duplex at ratios of 2:1 or greater.

4. CONCLUSIONS

We have sown that the octamer duplex d(ACCCGGGT)$_2$ can bind as many as four molecules of mithramycin and effectively double its molecular weight. Results from the titrations establish that there is a variety of binding sites at ratios of drugs per duplex less than 4:1. The occupancy of these binding sites is dependent on the amount of added drug. In addition to the anticipated drug–drug interactions within each dimer, these results imply that dimer–dimer interactions must also play a role in determining the exact binding sites of the four mithramycin molecules on the octamer. Hence, the binding sites observed at the intermediate ratios, but not at the 4:1 ratio, must reflect differences in the preferred binding site prior to saturation. Presumably, at saturation, binding sites closer to the center are less favorable because of Van der Waal's interactions between dimers. Preliminary 2D NOESY spectra indicate that the binding site at the 4:1 ratio is in the minor groove and spans all four base pairs making up half of the duplex, suggesting that dimer–dimer contact is quite close. A more detailed 2D NMR study is underway to accurately determine the binding site and to ascertain whether binding of a second dimer entials relocation of the binding sites present at the 2:1 drug/ duplex ratio. A thorough analysis of the mithramycin– octamer complex structure should assist in determining the physical basis of the preference for the -CCC- binding site.

Acknowledgements: This work was supported by Grant CA 27343 from the National Cancer Institute, USPHS, and benefitted from the use of NMR equipment at the Australian National University NMR Center.

REFERENCES

[1] Zimmer, Ch. and Wahnert, U. (1986) Prog. Biophys. Molec. Biol. 47, 31–122.

[2] Gao, X. and Patel, D.J. (1989) Biochemistry 28, 751–762.

[3] Banville, D.L., Keniry, M.A., Kam, M. and Shafer, R.H. (1990) Biochemistry 29, 6521–6534.

[4] Banville, D.L., Keniry, M.A. and Shafer, R.H. (1990) Biochemistry 29, 9294–9304.

[5] Brikenshtein, V.Kh., Pitrina, L.R., Barenboim, G.M. and Gurskii, G.V. (1983) Molek. Biol. 18, 1606.

[6] Van Dyke, M.W. and Dervan, P.B. (1983) Biochemistry 22, 2373–2377.

[7] Keniry, M.A., Levenson, C. and Shafer, R.H. (1987) J. Biomol. Struct. Dyn. 4, 745–756.

[8] Bax, A. and Davis, D.G. (1985) J. Magn. Reson. 63, 207–213.