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A ^{19}F NMR study of the interaction of 3-fluoro-4-demethoxydaunomycin with the hexanucleotide d(TCCGGA)$_2$

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^{19}F NMR spectroscopy has been used to study the binding of the fluorinated anthracycline 3-fluoro-4-demethoxydaunomycin with the hexanucleotide d(TCCGGA)$_2$. In the spectrum of the 1 : 1 anthracycline–d(TCCGGA)$_2$ complex four resonances of approximately equal intensity were observed. This indicated that 4-demethoxydaunomycin intercalated at all possible sites with similar affinity. This suggests that the specific high affinity binding sites that are observed in anthracycline–DNA footprinting experiments are strongly regulated by the local DNA conformation.

^{19}F NMR; Anthracycline; Sequence specificity; Oligonucleotide; DNA

1. INTRODUCTION

While other modes of action have been suggested [1–3], it is generally believed that anthracyclines exert their anti-tumour activity mainly by interaction with DNA. Consequently, there has been considerable interest in establishing the preferred DNA binding site(s) of anthracycline drugs [4,5]. Recent studies aimed at establishing the preferred DNA binding site(s) have provided conflicting results. Chaires et al. [4], using DNA footprinting titration experiments, have reported that daunomycin exhibits a preference for 5′-CG (or GC) sites which are flanked on the 5′ side by an A·T base pair. Alternatively, Phillips and co-workers [5] have used DNA footprinting and transcription assays to provide evidence that daunomycin prefers 5′-CA sites.

We have previously demonstrated that by incorporating a ^{19}F label into the anthracycline molecule, the relative binding affinity at specific two base sequences in an oligonucleotide can be determined by ^{19}F NMR spectroscopy [6]. In this initial study it was shown that 4-demethoxydaunomycin shows no significant preference for any particular binding site in the hexanucleotide d(CTGCAG)$_2$, which contains both 5′-GC and 5′-CA sites. This suggested that, contrary to the results of DNA footprinting and transcription experiments with the parent anthracyclines, 4-demethoxydaunomycin shows no over-riding binding preference for any particular sequence of two bases in small segments of DNA. In order to evaluate the general validity of our initial findings we have examined the binding of 3-fluoro-4-demethoxydaunomycin (3FD), shown in Fig. 1, with another synthetic hexanucleotide, d(TCCGGA)$_2$, that has a different base sequence to that used in the previous study. This hexanucleotide not only contains different two and three base pair combinations, but also contains a run of pyrimidine bases and a run of purine bases, with only the central dinucleotide having the alternating pyrimidine–purine sequence that is generally considered to be favourable for anthracycline binding [4,5].

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2. EXPERIMENTAL

2.1. *Materials*

3-Fluoro-4-demethoxydaunomycin hydrochloride was prepared as previously described by Irvine et al. [7]. The hexanucleotide, d(TCCGGA), and Sephadex G-15 were purchased from Pharmacia P-L Biochemicals. D_2O (99.96% D) was obtained from Sigma Chemical Co. All reagents used were of analytical grade.

2.2. *Preparation of the 1 : 1 3FD-d(TCCGGA)$_2$ complex*

To a 10 ml solution of d(TCCGGA)$_2$ (1.73 μmol) in 10 mM phosphate (pH 7) containing 100 mM NaCl and 1 mM EDTA was added a 3-fold excess of solid 3FD (which is insoluble in water) and the suspension was stirred overnight with occasional heating to 40°C. The suspension was then filtered and the filtrate freeze-dried to a red powder. A solution of this powder was made up in water and passed down a Sephadex G-15 column with water as the eluant. The 3FD–d(TCCGGA)$_2$ complex eluted at the void volume. No other coloured fractions were eluted, although a very thin red band remained at the head of the Sephadex column.

The residual 3FD was dissolved in methanol and quantified from its absorption at 485 nm. The 1 : 1 binding stoichiometry of the complex was also confirmed by comparison of the signal area of the 3FD

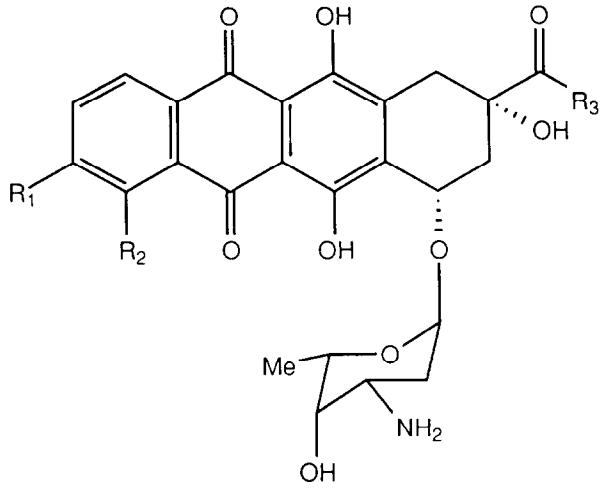

Fig. 1. Structures of relevant anthracyclines: $R_1 = F$, $R_2 = H$, $R_3 = CH_3$, 3-fluoro-4-demethoxydaunomycin (3FD); $R_1 = H$, $R_2 = OCH_3$, $R_3 = CH_2OH$, adriamycin.

methyl resonance with the hexanucleotide's thymidine methyl signal in the ^1H NMR spectrum.

2.3. *NMR spectroscopy*

All NMR spectra were recorded on a Varian XL-300 spectrometer operating at 282.2 MHz for the ^{19}F nucleus. The spectra were accumulated with a 1.6 s recycle time and a 60° pulse. No proton decoupling could be carried out.

3. RESULTS

Fig. 2 shows the ^{19}F NMR spectrum of the 1 : 1 3FD–d(TCCGGA)$_2$ mixture. Since free 3FD is insoluble in water, these ^{19}F signals must have arisen from fluorinated anthracycline that is bound to the hexanucleotide, furthermore the presence of four peaks indicates that 3FD exists in at least four unique environments. As only a 1 : 1 ratio of 3FD to d(TCCGGA)$_2$ was obtained, although excess 3FD was initially added to the nucleotide solution (see section 2), and that 4-demethoxydaunomycin has a high DNA binding constant ($K = 10^6$) [8], it is concluded that the binding stoichiometry is 1 : 1, with little or no 2 : 1 complex present in the mixture. Addition of excess 3FD directly to the 1 : 1 complex in the NMR tube resulted only in a small increase in the area of peak A. This indicates that the hexanucleotide is capable of binding a second 3FD molecule, but in agreement with our previous study [6] no co-operativity in binding was observed.

Studies on the interaction of anthracyclines with DNA have shown that these drugs bind to DNA by intercalation rather than associate in other ways [9,10]. Non-intercalative association with DNA has been proposed as a prelude to intercalation [11], however, these forms are thermodynamically and kinetically unfavourable and would not be detected by NMR spectroscopy. In addition the results of Yen et al. [12] discount the possibility of the anthracycline sugar lying in the DNA major groove. Given these restraints it is concluded that the four resonances observed in Fig. 2 are due to the four unique intercalation isomers shown in Fig. 3. These conclusion are strongly supported by the results of our earlier study [6] of the binding of 3FD with the hexanucleotide d(CTGCAG)$_2$. In an analogous fashion, four ^{19}F resonances were also observed and assigned to the four possible intercalation isomers; that is where the 3FD bound between the 5′-CT-3′, 5′-TG-3′, 5′-GC-3′ and 5′-CA-3′ with the drug sugar moiety lying in the minor groove and pointed in the 3′ direction in each case. This proposal was further supported by a ^1H NMR study in which additional nucleotide proton resonances were observed upon addition of 3FD to the d(CTGCAG)$_2$ solution that allowed identification of individual binding sites [13]. It was not possible to obtain similar ^1H NMR results for the 3FD–d(TCCGGA)$_2$ complex, presumably due to the faster nucleotide dissociation rate of 3FD which results in only one exchange averaged resonance being observed for each nucleotide proton.

While it was not possible to unambiguously assign each resonance in Fig. 2 to one of the intercalation isomers shown in Fig. 3, tentative assignments of peaks A and B were made. Previous work suggests that at low NaCl concentrations the broadest signal is due to the intercalation isomer where the sugar moiety is located between the penultimate and terminal base pairs [6], i.e. structure 4 in Fig. 3. At low NaCl concentrations the hexanucleotide is subject to maximum 'fraying' of the terminal base pairs, particularly the terminal base pair of the oligonucleotide that is not additionally stabilized by the stacking interaction of the anthracycline aromatic ring system. This results in a relatively faster dis-

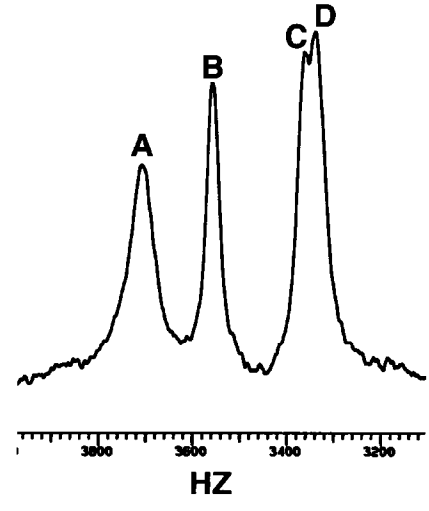

Fig. 2. ^{19}F NMR spectrum of the 1 : 1 3FD–d(TCCGGA)$_2$ complex in D_2O, 3 mM phosphate, pH 7, and 0 mM NaCl at 5°C. No fluorine–proton coupling is observed as the line-width of each resonance is significantly broadened due to 3FD exchange between binding sites. Above 35°C only one exchange averaged resonance is observed.

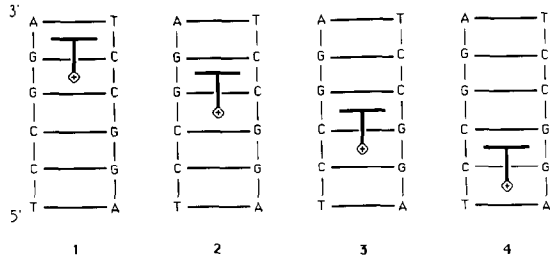

Fig. 3. A scheme showing the four intercalation isomers observed for the 1 : 1 3FD–d(TCCGGA)$_2$ complex. The DNA duplex, idealized as a ladder, is shown with the minor groove facing out of the page. The intercalating, aromatic moiety of the 3FD molecule is shown as a heavy line parallel to the base pair 'rungs' of the ladder, while the aminoglycoside moiety is shown as a heavy line leading down the centre of the minor groove, terminated by a positive charge due to the amine cation. (n.b. the intercalation site occupied in form 2 is not the same as that occupied in form 4. Relative to the DNA host, the entire drug molecule is rotated 180° going from 2 to 4, and hence can be considered to 'perceive' a different base sequence in each form.)

sociation rate of the bound drug from this site, and hence the corresponding resonance shows the largest degree of exchange broadening. At higher NaCl concentrations, where the duplex is significantly more stable, the results of the ^{1}H NMR study of 3FD–d(CTGCAG)$_2$ complex showed that the structure corresponding to isomer 1 in Fig. 3 exhibited the fastest 3FD dissociation rate [13]. This is consistent with other studies which have shown that the individual base pair melting temperature progressively increases in going from the terminal base pairs to the central residues of an oligonucleotide [14,15]. Although intercalation of an anthracycline will increase the individual base pairs' melting temperatures, the order of increasing thermal stability is preserved [14].

In Fig. 2 it is observed that at 5°C and low salt concentration peak A is significantly wider than peaks B, C, and D, and becomes even broader at 18°C as shown in Fig. 4. It is also observed in Fig. 4 that as the NaCl concentration is increased peak A becomes relatively sharper. This indicates that the dissociation rate of the 3FD becomes relatively slower as the intercalation site becomes more stable. Alternatively, as the NaCl concentration increases peak B broadens (Fig. 4), suggesting that the intercalation site corresponding to peak B is progressively less stable with increasing NaCl concentration. In addition the resonances due to the intercalation isomers where the 3FD is bound towards the centre of the hexanucleotide (isomers 2 and 3) are the most kinetically stable with respect to temperature and salt concentration. From these considerations the four resonances observed in Fig. 2 are tentatively assigned as follows; peak A to isomer 4, peak B to isomer 1, while peaks C and D are assigned non-specifically to isomers 2 and 3.

4. DISCUSSION

The results from this study show that the 4-demethoxydaunomycin binds at all possible sites present in the hexanucleotide d(TCCGGA)$_2$, including purine–purine and pyrimidine–pyrimidine sequences which have always been considered as lower affinity binding sites [4,5]. The ^{19}F NMR results are a direct measure of the thermodynamic preference at each of the competing binding sites in this hexanucleotide. These results confirm the proposal of our previous study [6], and hence it is concluded that 4-demethyoxydaunomycin does not show a significant intrinsic preference for any particular two base sequence in its binding to an oligonucleotide.

Although less well studied, recent evidence has been presented that 4-demethyoxydaunomycin, like the parent anthracyclines, does bind to DNA at a limited number of high affinity sites [8]. This, coupled with the results presented here, indicates that factors other than the sequence at the specific intercalation site can significantly regulate the relative anthracycline binding affinity to DNA. It has been known for some time that DNA is not homogeneous in structure but that along the polymer strand there exists a heterogeneity of conformation. This can significantly alter the DNA groove dimensions thereby affecting the electrostatic and hydrogen bonding interactions between the anthracycline and DNA. Furthermore, the degree to which the anthracycline planar aromatic system can overlap with the DNA bases would also be significantly affected. Therefore, it is suggested that the DNA binding specificity of anthra-

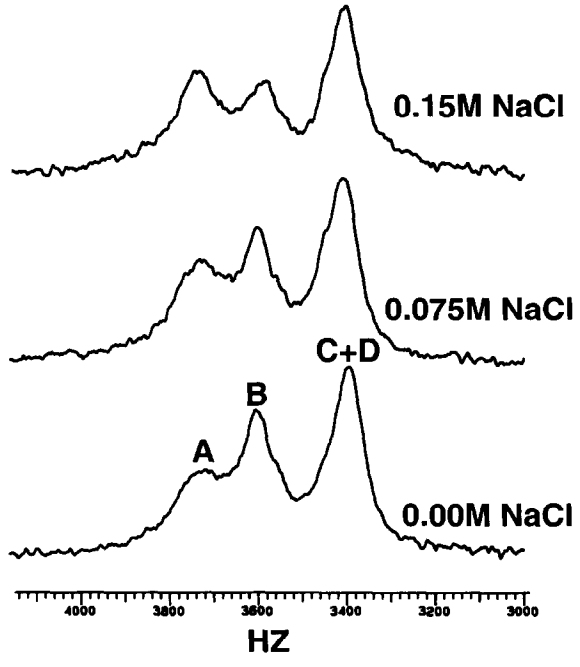

Fig. 4. ^{19}F NMR spectrum of the 1 : 1 3FD-d(TCCGGA)$_2$ complex in D_2O, 10 mM phosphate, pH 7, over a range of NaCl concentrations (given to the right of each spectrum) at 18°C.

cyclines is significantly controlled by long-range structural effects, in addition to specific hydrogen bonds at the intercalation site.

While 4-demethoxydaunomycin has a slightly higher anti-tumour activity than daunomycin [8], it has been shown that the long axis of the bound anthracycline is oriented differently in the intercalation pocket compared to the parent anthracyclines [8,16]. This may result in different binding preferences being observed for 4-demethyoxydaunomycin; indeed, based on the tentative assignments, the 3FD showed a marginally higher preference for the 5′-GG-3′ sequence. Consequently, the conclusions drawn from this study may only reflect the case for 4-demethoxydaunomycin, however, the results presented here support the recent study of Fox and Alam [17] who demonstrated that the DNA binding preference of the anthracycline nogalamycin is affected by local DNA dynamic and structural effects, i.e. by relatively long-range effects.

DNA footprinting experiments have shown that anthracyclines are capable of binding at a variety of different DNA sites, with a range of binding affinities [4,5,17]. It is the determination of the highest affinity sites that has produced conflicting results. In addition, in these footprinting studies it is generally observed that the anthracycline does not bind significantly at all proposed high affinity sequences. It would therefore appear reasonable to expect that local conformational differences in DNA would strongly influence the anthracycline–DNA binding affinity at any particular sequence.

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