

## Nuclear Magnetic Resonance Probing of Binding Interactions used by Minor Groove Binding, DNA-directed Ligands: Assignment of the Binding Site of Hoechst 33258 on the Self-complementary Oligonucleotide, d(CGCGAATTCGCG)

John A. Parkinson,<sup>a</sup> Jill Barber,<sup>a</sup> Kenneth T. Douglas,<sup>a</sup> John Rosamund,<sup>b</sup> and Derek Sharples<sup>a</sup>

<sup>a</sup> Department of Pharmacy, and <sup>b</sup> Department of Biochemistry and Molecular Biology, University of Manchester, Manchester M13 9PL, U.K.

The binding of Hoechst 33258 to the minor groove of the duplex formed by the self-complementary oligonucleotide d(CGCGAATTCGCG) has been shown by means of high-field <sup>1</sup>H nuclear magnetic resonance spectroscopy to be across the central AATT region of the duplex.

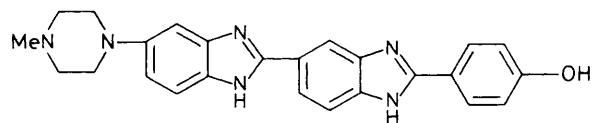
At a fundamental level our understanding of DNA–ligand interactions on a submolecular scale is much less advanced than for protein–ligand interactions, partly because of the smaller library of *X*-ray diffraction data for DNA–ligand complexes. However, at a more practical level, recent molecular biological advances have made the development of drugs based on DNA-sequence recognition an apparently accessible (if long term) goal.

Hoechst 33258 (**1**) binds preferentially in the minor groove of A-T rich regions of right-handed double-stranded DNA<sup>1</sup> and is widely-used as a fluorescent DNA strain. It binds to the minor groove of the duplex of the self-complementary oligonucleotide d(CGCGAATTCGCG) and two groups have crystallised the ligand–duplex complex and reported structural details of ligand binding.<sup>2,3</sup> The earlier crystal structure<sup>2</sup> proposed that (**1**) lies near one end of the AATT region with its protonated piperazine ring protruding into, and binding in, the adjacent CG region, *i.e.* that binding occurs at the ATTC sequence (see Figure 1). The other model<sup>3</sup> has (**1**) bound essentially centrally to the minor groove of the duplex occluding the AATT region only.

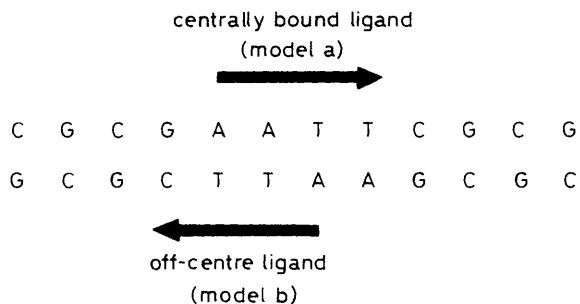
In view of this discrepancy, we have investigated the interaction of Hoechst 33258 with d(CGCGAATTCGCG)<sub>2</sub> by n.m.r. spectroscopy; n.m.r. is an attractive complement to *X*-ray crystallography, providing dynamic as well as static information. We now report some preliminary results of a solution n.m.r. study.

Hoechst 33258 (Aldrich) was used without further purification. Before use its purity was assessed by h.p.l.c. [reversed phase Rainin Dynamax C<sub>18</sub> Microsorb analytical column, 0.1 M potassium dihydrogen phosphate (pH 7.0)/methanol, 88/200]. The oligonucleotide, d(CGCGAATTCGCG), was synthesised on an Applied Biosystems Solid Phase Oligonucleotide Synthesiser using phosphoramidite chemistry, and this crude product (24 mg) was purified by reversed-phase h.p.l.c. using a C<sub>18</sub> column (Rainin Dynamax semi-preparative, 250 × 10 mm) with 0.1 M ammonium acetate (pH 7.0) and 0.1 M ammonium acetate/20% acetonitrile (pH 7.0) as eluant. N.m.r. data were obtained at 500 MHz (<sup>1</sup>H).

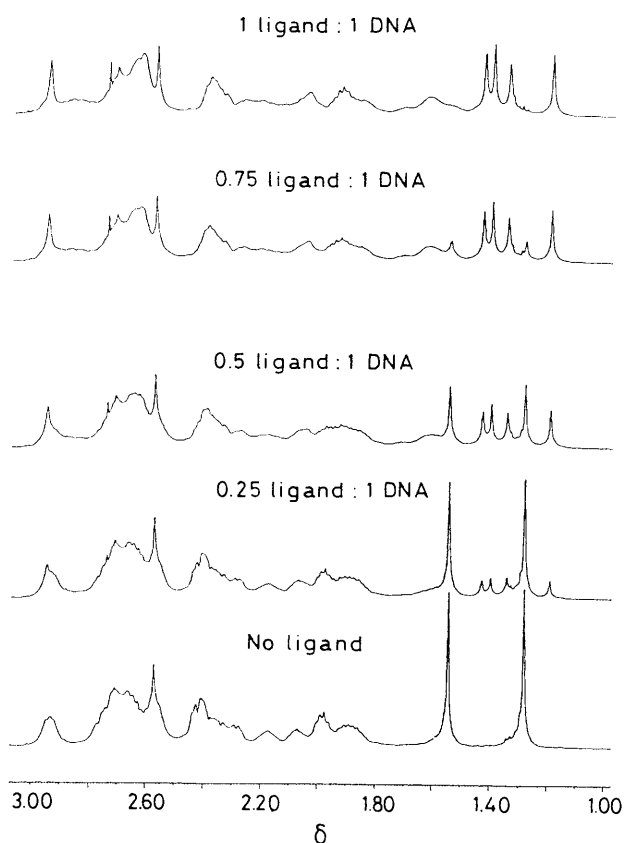
Using spectrofluorometric Scatchard analysis we found that the pure oligonucleotide binds Hoechst 33258 in a 1 : 1 molar ratio with a dissociation constant of  $3.47 \pm 0.01 \times 10^{-6}$  M. This value suggested that the complex would be in slow exchange with its free components on the n.m.r. time-scale. The <sup>1</sup>H n.m.r. spectrum of the free ligand was assigned by a



(**1**) Hoechst 33258

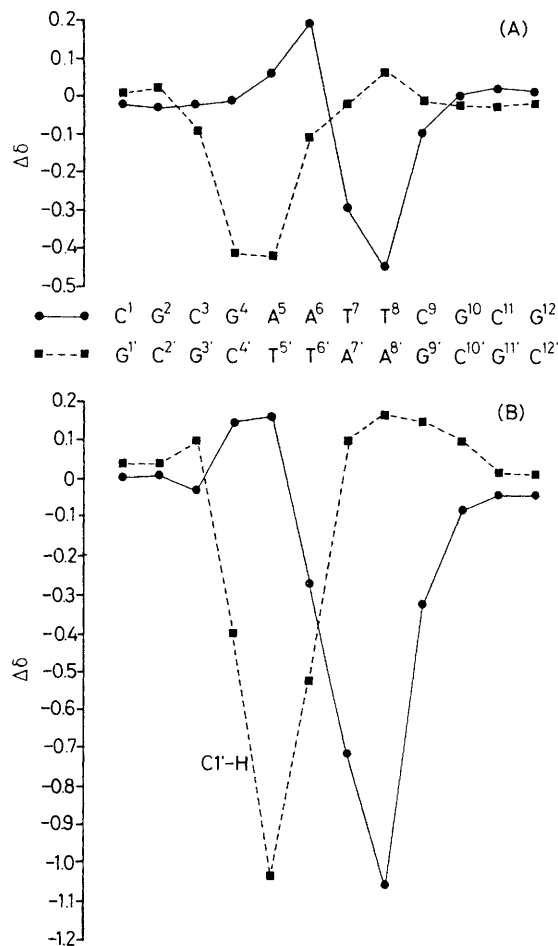


**Figure 1.** Two possible modes of binding of a non-symmetrical ligand [such as Hoechst 33258, (1)] to the B-DNA duplex of d(CGCGAATTCGCG). The heavy arrows indicate the approximate positions of the bound ligand in the minor groove with the head of the arrow indicating that the ligand has directionality. Model (a) is essentially that proposed in ref. 3; model (b) is essentially that of ref. 2.



**Figure 2.** N.m.r. spectra (one-dimensional) of the thymine methyl region of d(CGCGAATTCGCG)<sub>2</sub> in 10 mM phosphate, 100 mM NaCl, 0.1 mM NaN<sub>3</sub> at pH<sub>meas</sub> 7.0, 99.96% D<sub>2</sub>O referenced to internal 3-trimethylsilyl[2,2,3,3-<sup>2</sup>H<sub>4</sub>]propionate. Spectra were recorded at 500 MHz (<sup>1</sup>H) in the presence of the indicated molar ratios of added Hoechst 33258. The resonance at  $\delta$  2.95 in the 1:1 complex is the N-methyl resonance of Hoechst 33258.

spin-diffusion technique.<sup>4</sup> A published assignment<sup>5</sup> of the <sup>1</sup>H n.m.r. spectrum of the free oligonucleotide was confirmed by analysis of the phase-sensitive NOESY spectrum. Free oligonucleotide (in 10 mM phosphate buffer, 100 mM NaCl, 0.1 mM NaN<sub>3</sub>, pH<sub>meas</sub> = 7.00, 99.96% D<sub>2</sub>O) was titrated with aliquots of a concentrated solution of ligand and a series of one-dimensional <sup>1</sup>H spectra acquired, portions of which are



**Figure 3.** Plots of difference in chemical shift for corresponding, assigned protons in bound and free forms of the oligonucleotide d(CGCGAATTCGCG)<sub>2</sub> with Hoechst 33258 (1:1) as ligand: conditions as described in the legend to Figure 2. In (A), the solid line connects experimental data for aromatic protons (for C-6H, T-6H, A-8H, G-8H) of one of the oligonucleotide chains of the duplex, the dotted line shows the data for the other, antiparallel chain. In (B), the solid line shows the experimental data for the sugar rings (C1'-H) for one of the chains and the dotted line shows the corresponding data for the other chain. For both (A) and (B), the solid lines and solid circles for data refer to oligonucleotide C<sup>1</sup>G<sup>2</sup>C<sup>3</sup>G<sup>4</sup>A<sup>5</sup>A<sup>6</sup>T<sup>7</sup>T<sup>8</sup>C<sup>9</sup>G<sup>10</sup>C<sup>11</sup>G<sup>12</sup> and the dotted lines and solid squares for data to G<sup>12</sup>C<sup>11</sup>G<sup>10</sup>C<sup>9</sup>T<sup>8</sup>T<sup>7</sup>A<sup>6</sup>A<sup>5</sup>G<sup>4</sup>C<sup>3</sup>G<sup>2</sup>C<sup>1</sup>.

shown in Figure 2. The absence of signal averaging confirmed the complex and its components are in slow exchange. Accordingly, 2D-NOESY and -COSY data sets of the 1:1 complex were acquired to allow assignments of its <sup>1</sup>H n.m.r. spectrum.

Of immediate interest are the thymine methyl resonances which appear in the uncrowded region of the spectrum ( $\delta$  1.1–1.6). In the free oligonucleotide duplex these appear as two singlets because the oligonucleotide is self-complementary and thus symmetrical about the centre of the AA|TT region. In the presence of Hoechst 33258 the oligonucleotide symmetry is lifted as a result of lack of symmetry of the bound ligand, see Figure 1, and there are four thymine methyl resonances (singlets at  $\delta$  1.43, 1.40, 1.35, and 1.19).

To determine the position of the drug with respect to the four T sites it is necessary to consider the entire length of the oligonucleotide chain. As examples, in Figure 3 we show the chemical shifts of some of the assigned protons in the complex compared with those of the corresponding protons in the free

oligonucleotide, the differences in these pairs of chemical shifts being plotted against the position in the oligonucleotide chain. The differences in free and bound chemical shifts for some resonances are quite small, notably for some of the aromatic ring protons and some of the sugar protons, especially those from base sites distal to the ligand binding site. Both graphs of Figure 3 are very close to being symmetrical about the centre of the duplex; in both graphs the lines representing the two strands of the oligonucleotide cross each other between the two central bases. This is clear evidence for gross symmetry in the complex about the AATT centre. The drug molecule is not symmetrical, however, and graphs 3A and B both show that it perturbs the GC region of one oligonucleotide strand more than it does the other. This is most probably due to the protonated piperazine ring exerting a greater electronic effect on the GC region than does the phenol ring. Another aspect of dissymmetry is that the drug does not affect both oligonucleotide strands equally along their chains. This is probably caused as follows.

At the end of the minor groove corresponding to the AATT stretch, one end of the ligand, say the phenolic, is more closely associated with one oligonucleotide chain than with the other. Because of the helical duplex the other end of the ligand (the piperazine) is then more tightly associated with the opposite oligonucleotide strand at the other end of the AATT minor groove region.

Comparing the two structures<sup>2,3</sup> for this oligonucleotide-ligand complex proposed from X-ray crystallographic data, the major difference between them is essentially that in model

AATT

(a) the ligand lies centrally bound across the TTAA stretch, in

the minor groove<sup>3</sup> and in the other model,<sup>2</sup> (b), it is bound  
AATC

across the minor groove of the off-central TAAG stretch, see Figure 1. Clearly, symmetrical effects of ligand binding on oligonucleotide resonances as one moves towards the chain termini from the AATT midpoint would not be expected for model (b) and the n.m.r. data at this stage of refinement clearly support (a) as originally proposed by Teng *et al.*<sup>3</sup> We see no evidence in the n.m.r. data to indicate multiple binding modes in solution. Detailed analyses of nuclear Overhauser enhancement data and of dynamics will be reported in due course.

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