Enhanced binding of a hexacyclic amidine analogue of Hoechst 33258 to the minor groove of DNA: ^{1}H NMR and UV melting studies with the decamer duplex d(GGTAATTACC)$_2$

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NMR and UV melting studies with the decamer duplex d(GGTAATTACC)$_2$ show that replacing the *N*-methylpiperazine ring of the DNA minor groove binding agent Hoechst 33258 with a hexacyclic amidine leads to both an increase in duplex stability ($\Delta T_m \approx 7$ °C) and enhanced A–T specific recognition; the amidine NH exchanges slowly with the solvent and has a small temperature coefficient (< 3 ppb/°C) supporting its involvement in intermolecular hydrogen bonding, however, the data suggest that the ring is flipping rapidly even at 278 K even though the interaction appears to contribute significantly to complex stability.

The bis-benzimidazole Hoechst 33258 [Fig. 1(*a*), **1**] binds to the minor groove of A–T rich DNA sequences.[1] Specific hydrogen bonding interactions between the benzimidazole NHs and adenine N3/thymine O2 of the DNA bases on the floor of the groove provide key components for binding and recognition; the complex has been studied extensively by NMR[2–6] and X-ray analysis.[7–12] In contrast, the role of the *N*-methylpiperazine ring in the recognition process is less well defined. Its cationic charge clearly contributes to complex stability, while van der Waals contacts are also observed with the floor of the minor groove.[10] However, both the conformation and orientation of the piperazine ring appear to be variable including, in some cases, the observation of preferential binding to G–C regions at the end of A–T tracts of DNA where the wider groove has been proposed to more readily accommodate this bulky substituent.[7,9,11]

One rational approach to the design of new Hoechst analogues with enhanced functional properties is to replace the *N*-methylpiperazine component with one capable of specific hydrogen bond-mediated recognition of the minor groove. Here we describe such an analogue containing a hexacyclic amidine group, Hoechst 43254 **2**. A recent X-ray structure[13] has shown that a Hoechst analogue with a five-membered amidine ring does not have the correct geometry to allow the amidine NH to hydrogen bond to the floor of the minor groove.

Energy minimisation of Hoechst 43254 using MACROMODEL[14] suggests that a twist of *ca*. 20° away from coplanarity is necessary to relieve steric repulsions between amidine NHs and phenyl hydrogens on the adjacent benzimidazole ring. Energy minimisation and molecular dynamics calculations on the DNA complex of **2** (modelled from the X-ray coordinates of the DNA complex of **1** described by Quintana *et al.*[10]) suggest that the ligand should bind with enhanced specificity to the 5′-AATT sequence through the interaction of an amidine NH with the N3 of the second adenine base [see Fig. 1(*b*) *vs*. 1(*c*)]. The twisted conformation of the six-membered amidine ring (*cf*. the five-membered ring) appears to optimise the geometry for such an interaction.

In Fig. 2, UV 'melting' curves for d(GGTAATTACC)$_2$ are shown plotted as absorbance at 260 nm *vs*. temperature at a duplex concentration of 3.2 μm (buffered with 0.01 m sodium phosphate and 0.11 m sodium chloride, pH 7.0). The duplex alone gives a broad melting curve with a T_m of 27 °C. Hoechst 33258 **1** increases the T_m significantly to 45 °C with the melting transition becoming appreciably more cooperative. However, with Hoechst 43254 **2** bound the stabilisation effect is even more pronounced with the T_m rising further to 52 °C ($\Delta T_m \approx$ 7 °C), in good agreement with previous studies with related ligands and other A–T tract duplexes.[15]

In Fig. 3 we show by ^{1}H NMR that **2** binds tightly to d(GGTAATTACC)$_2$ and is in slow exchange on the chemical shift time-scale. Binding of the asymmetric ligand lifts the dyad symmetry of the duplex such that the two strands of DNA are non-equivalent in the complex. The high field methyl region (δ 1–1.8) and low field imino proton region of the spectrum (δ 9.0–14.5) of the ligand-free and ligand-bound duplex are illustrated. The number of thymine methyl [(*a*) *vs.* (*b*)] and imino proton resonances from the A–T base pairs [(*c*) *vs.* (*d*)] doubles on ligand binding indicating that the drug is located at a single high affinity site. Generally, the pattern of ligand-induced perturbations to base H6/H8 (major groove) and deoxyribose H1′ resonances (minor groove) are suggestive of a very similar binding location for both **1** and **2** that spans the central AATT site of the decamer duplex (data not shown). Many intermolecular NOEs have so far been identified that support this model.

The ^{1}H NMR spectrum of the complex shown in Fig. 3(*d*) also provides evidence for hydrogen bonding interactions between the ligand and minor groove. Downfield shifted resonances are observed for the benzimidazole NHs, one of which is resolved at δ 11.65, the other overlapped at δ 12.74.

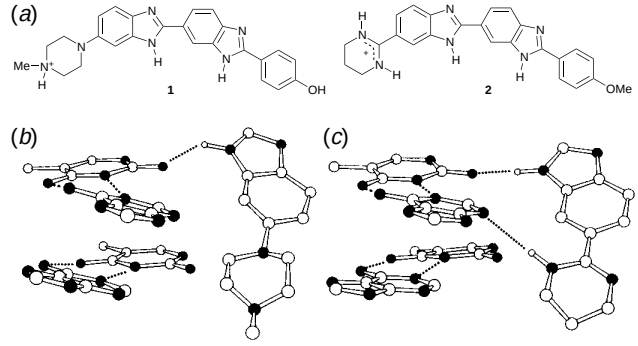

Fig. 1 (*a*) Structure of Hoechst 33258 **1** and Hoechst 43254 **2**. (*b*) Portion of the X-ray structure of Hoechst 33258 (described by Quintana *et al.*[10]) illustrating the position and orientation of the *N*-methylpiperazine ring in the minor groove. (*c*) Hoechst 43254 complex modelled using the same X-ray coordinates and MACROMODEL software.[14] The ligand structure was energy minimised separately to determine the preferred conformation and orientation of the amidine ring. This conformation was adopted in initial modelling studies of the complex of **2**, keeping all other dihedral angles and ligand–DNA interactions the same as in the X-ray structure. The complex was subsequently energy minimised and subjected to 100 ps of unrestrained room temperature molecular dynamics. The resultant amidine NH → adenine N3 hydrogen bonding distance, which was not added as a constraint, was found to be 2.3 Å.

Both are shifted appreciably further downfield than the corresponding resonances in the complex of Hoechst 33258 with the same DNA sequence (δ 11.02 and 12.03, respectively).[5] Given that both ligands appear to bind similarly to the central AATT binding sequence, we interpret these larger binding shifts for **2** as consistent with stronger electrostatic interactions[16,17] and with overall tighter binding within the minor groove. Thus, introducing a more favourable interaction at the amidine end of the ligand appears to cooperatively enhance other intermolecular interactions at the ligand–DNA interface. Further, the well-resolved resonance at δ 9.39 is assigned to the amidine NH of the bound ligand on the basis of cross-peaks in 2D NOESY spectra to adjacent methylene protons (δ 3.82) on the ring. Identification of this resonance has enabled us to probe the role of the hexacyclic amidine ring in intermolecular interactions. The amidine NH has a small temperature coefficient (< 3 ppb/°C), a very slow rate of exchange (as judged by variable temperature and saturation transfer experiments), and gives a number of intermolecular NOEs that support both its close proximity to the floor of the minor groove, and possible involvement in specific hydrogen bonding with it. It is notable that the amidine NH signal has an integrated intensity equivalent to two protons, indicating that the solvent-exposed and solvent-buried ring NH are equivalent even at 278 K. Averaging of the signals from the C3 and C5 methylene protons adds further weight to the conclusion that in the bound state the ring is undergoing 180° flips (or free rotation) such as to rapidly interchange the environments on either side of the ring. Thus, a proposed hydrogen bond that appears to contribute significantly to complex stability also appears to be a dynamic interaction.

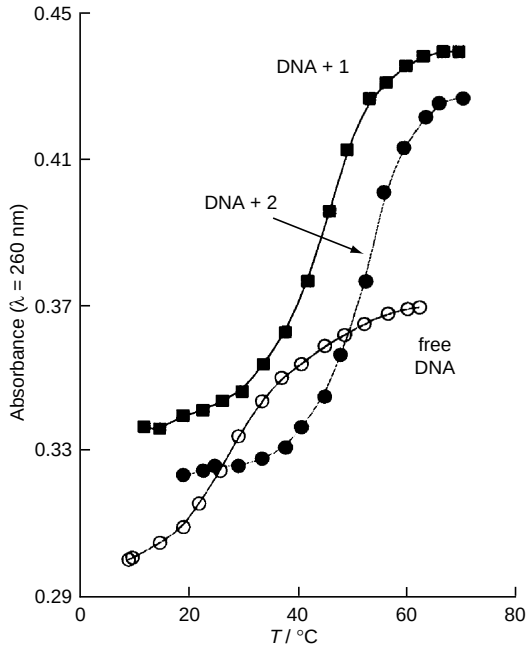

Fig. 2 UV melting curves for the decamer duplex d(GGTAATTACC)$_2$ with and without bound ligands (**1** and **2**). Errors in T_ms were estimated to be less than ±2 °C. Sample concentrations and buffers are indicated in the text. The sample of Hoechst 33258 was purchased from Aldrich, while Hoechst 43254 was kindly provided by Hoechst, Frankfurt, Germany. Both samples were checked by ^{1}H NMR and used without further purification. The oligonucleotide duplex was synthesised trityl-on using solid-phase phosphoramidite chemistry and purified by reverse-phase HPLC using 0.1 m triethylammonium acetate (TEAA) buffer and an acetonitrile gradient. The trityl group was removed by treatment with 50% aqueous acetic acid for 1 h at 35 °C and the oligonucleotide finally dialysed to remove TEAA and introduce Na^+ as the counter-ion.

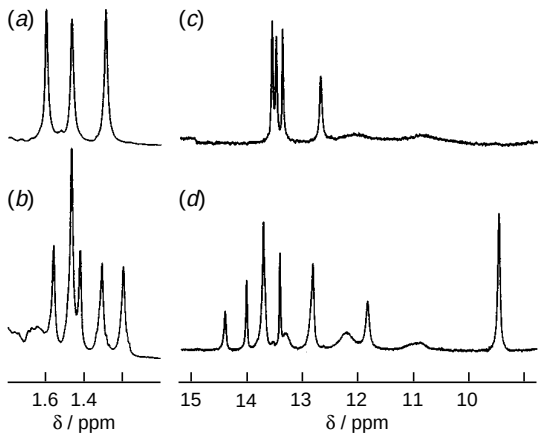

Fig. 3 ^{1}H NMR spectra (500 MHz) of ligand-free DNA (*a*) and (*c*), and of the complex with Hoechst 43254 (*b*) and (*d*), recorded in 90% H_2O solution at 298 K using a 1-1 jump-return pulse sequence to suppress the solvent peak. A spectral width of 24 ppm was employed with a delay of 70 μs to optimise excitation at the position of the imino proton resonances (~ 13 ppm).

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