Experimental Precedent for the Need To Involve the Primary Hydration Layer of DNA in Lead Drug Design

Sophia Y. Breusegem,[‡,†] Seyed E. Sadat-Ebrahimi,[||,⊥] Kenneth T. Douglas,*[,||] Elena V. Bichenkova,[||] Robert M. Clegg,[§] and Frank G. Loontiens[‡]

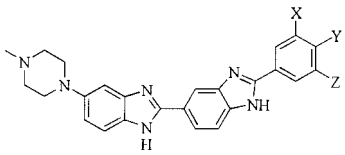
Department of Biochemistry, Physiology and Microbiology, Faculty of Sciences, Ghent University, Ledeganckstraat, 9000 Gent, Belgium, Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois, West Green Street, Urbana, Illinois 61801, and School of Pharmacy and Pharmaceutical Sciences, The University of Manchester, Manchester M13 9PL, U.K.

Received March 2, 2001

Abstract: The increase in fluorescence on binding of *m*-phenyl substituted hydroxy derivatives of Hoechst 33258 with poly-[d(A−T)], d(CGCGAATTCGCG)$_2$, and with the corresponding T_4-looped 28-mer AATT hairpin was used to monitor binding by equilibrium titrations and stopped-flow kinetics. Replacing the *p*-OH substituent of Hoechst 33258 (association constant $K_a = 5.2 \times 10^8$ M^{-1} for 28-mer hairpin) by *m*-OH increases the AATT site binding energy by 1.1 kcal mol^{-1}, $K_a = 3.8 \times 10^9$ M^{-1}. Addition of a second *m*-hydroxy group (bis-*m*-OH Hoechst) further strengthens binding, giving $K_a = 1.9 \times 10^{10}$ M^{-1}, and the binding energy increases by about 2.1 kcal mol^{-1} compared to *p*-OH Hoechst. The value of K_a determined at equilibrium equaled that determined from the ratio of association and dissociation rate constants from stopped-flow studies. The increase in affinity of the monohydroxy Hoechst analogue (*m*-OH) may originate from water-mediated hydrogen bonding with the minor groove. The further increase in affinity of the bis-*m*-OH derivative (whose second *m*-OH group must be directed away from the DNA minor groove floor) may arise from a hydrogen-bonded network of water molecules. The potential to increase binding strength through relayed water molecules is proposed as an additional possible input for lead drug design at DNA targets.

Introduction. There are many examples of water networks in the active sites of enzymes, and several ligands bind to enzymes through bridging water molecules (examples from proteases are in Figures 2, 6, and 8 of the review by Leung et al.[1]). In such circumstances the water network can contribute to binding, partly through the enthalpy contribution of hydrogen bonds and partly through entropic effects associated with desolvation of the reactants and solvation of the resulting complex. Target-bound water molecules have rather seldom been explicitly incorporated into the ligand design process, a noteworthy exception being the design of HIV protease inhibitors, which invoked a tightly bound water bridging active site Asp residues.[2] Rational ligand design is less advanced for nucleic acids as targets,[3] although there are groups which have demonstrated notable success in this field both for intercalating[4,5] and minor groove directed ligands.[3,6,7] Our work in this area led us to obtain accurate binding data for minor groove DNA ligands. To our surprise we have found increased binding strength for a ligand through a bulk solvent (i.e., externally) directed OH group. It appears that this can be explained through the participation of a water network that runs across the surface of the DNA.

Scheme 1. Structures of Ligands Used



X = Z = H; Y = OH; *p*-OH Hoechst
X = Y = H; Z = OH; *m*-OH Hoechst
X = Z = OH; Y = H; *bis-m*-OH Hoechst

Hoechst 33258 (*p*-OH Hoechst) binds in the minor groove of B-DNA at $(A/T)_4$ sites (reviewed by Frau et al.). We synthesized a *m*-OH Hoechst derivative in which the *p*-OH substituent of *p*-OH Hoechst was replaced by a *m*-OH substituent. NMR studies of the *m*-OH Hoechst-d(CGCGAATTCGCG)$_2$ complex indicated that the *m*-OH of the ligand is directed into the minor groove and that the *m*-OH phenyl ring does not flip in the minor groove, contrary to the *p*-OH phenyl group of Hoechst 33258, which rotates rapidly on the NMR time scale.[8] These observations supported the existence of direct hydrogen-bonding interactions between the *m*-OH substituent and the minor groove[9] in the 12-mer duplex. X-ray diffraction studies by Clark et al.,[10] using a pyridyl derivative of *m*-OH Hoechst, showed structured water molecules to be involved in indirect hydrogen bonding of the *m*-OH substituent, and that this derivative can bind in two orientations, one with the *m*-OH substituent pointing into the minor groove and one with the *m*-OH substituent pointing outward toward solvent.

We now report accurate association constants, K_a, confirmed by stopped-flow kinetic data, for *p*-OH, *m*-OH, and 3,5-dihydroxy (bis-*m*-OH) Hoechst dye analogues (Scheme 1) binding to d(CGCGAATTCGCG)$_2$, a T_4-looped hairpin 28-mer analogue of this duplex (Figure 1) and poly[d(A−T)]. Use of a DNA hairpin structure avoids melting of a DNA oligomer at low concentrations: this allowed reproducible and accurate determinations of K_a values. The presence in bis-*m*-OH Hoechst of the second *m*-OH group, which is directed *away* from the minor groove toward the solvent, was found to have an unprecedented strengthening effect on binding.

Results. Binding of *p*-OH Hoechst to DNA can be monitored sensitively through the large increase in dye fluorescence.[11,12] The sensitivity of fluorescence, together with precautions against surface adsorption of the Hoechst molecules, enabled accurate work with very low concentrations (1 nM dye). The low concentrations allow sufficient dissociation of the complex to permit determination of the very high association constants that are otherwise impossible to determine. The stoichiometry

* To whom correspondence should be addressed. Tel: 0161 275 2371. Fax: 0161 275 2481. E-mail: Ken.Douglas@man.ac.uk.
‡ Ghent University.
|| University of Manchester.
§ University of Illinois.
† Present address: University of Illinois.
⊥ Present address: Department of Pharmacy, University of Tehran, Iran.

10.1021/jm0100943 CCC: $20.00
Published on Web 07/11/2001

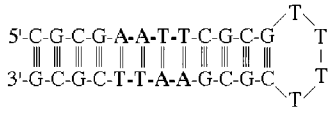


Figure 1. Structure of the 28-mer AATT hairpin.

was shown to be 1:1 for *p*-OH Hoechst binding to the isolated AATT site in the 12-mer duplex or in the 28-mer hairpin by independently measuring dye fluorescence (i) in a classical titration of 0.1 μM dye with excess 12-mer duplex or 28-mer hairpin and (ii) in a continuous variation titration (Job plot) using the 12-mer duplex and a maximum complex concentration of 0.1 μM.

The K_a values for the ligands with 12-mer duplex and 28-mer hairpin (Table 1) were determined in fluorescence titrations (Figure 2). With the 28-mer hairpin, K_a increases from 5.2×10^8 M^{-1} ($-\Delta G°_{20} = 11.7$ kcal mol^{-1}) for *p*-OH Hoechst to 1.9×10^{10} M^{-1} ($-\Delta G°_{20} = 13.8$ kcal mol^{-1}) for bis-*m*-OH Hoechst, or by a factor of 37 (2.1 kcal mol^{-1}). For comparison, we also determined the K_a values with poly[d(A−T)]. In all titrations, a simple binding mechanism was used to simulate the data (see legend to Table 1). We also used stopped-flow kinetics with 2 nM dye and the 28-mer hairpin or poly[d(A−T)] in excess, under pseudo-first-order conditions (Breusegem et al., unpublished). The inverse time constants (for the monoexponential traces) increased linearly with concentration, consistent with simple one-step binding. The dissociation rate parameters k_{off} were determined independently by competitive binding kinetics with poly[d(A-5BrU)]. The kinetic association constants $K_{kin} = k_{on}/k_{off}$ agree perfectly with the K_a values determined at equilibrium.

Discussion. The simple 1:1 stoichiometry found with the 12-mer duplex $d(CGCGAATTCGCG)_2$ and its corresponding 28-mer hairpin structure contrasts with the complicated binding of *p*-OH Hoechst, at concentrations in the micromolar range, to polymeric DNAs such as poly[d(A−T)].[13−15] As shown in Table 1, the accuracy of the K_a values determined at equilibrium is confirmed by their perfect agreement with the kinetically determined association constants K_{kin} ($= k_{on}/k_{off}$), and it allows us to confidently relate binding energies, $\Delta G°_{20}$ ($= -RT \ln K_a$ at 20 °C), with available structural data. The k_{on} values are nearly constant and approach the encounter-controlled limit. Importantly, it is the dissociation rate, k_{off}, that determines the affinity of a modified Hoechst-like dye. Compared to *p*-OH Hoechst, k_{off} for bis-*m*-OH Hoechst is slower by a factor of 34, probably due to breaking the extra, water-relayed hydrogen bonds (see below).

The affinity of the Hoechst dyes for the AATT site in the duplex and the 28-mer hairpin is systematically larger than for poly[d(A−T)]. This might involve a more favorable entropic effect through release of extra water molecules from the spine of hydration at the AATT site,[16−20] which is unlikely to exist in poly[d(A−T)],[21] or by release of Na^+ ions[22] from the groove (the existence of these Na^+ ions has been disputed).[17,18] However, the AATT binding sites in the 12-mer duplex, in the 28-mer hairpin, and also in the alternating polymer poly[d(A−T)] show the same order of affinities, namely *p*-OH < *m*-OH < bis-*m*-OH. With the 28-mer hairpin, K_a increases from 5.2×10^8 M^{-1} for *p*-OH Hoechst to 3.8×10^9 M^{-1} for *m*-OH Hoechst, corresponding to an additional 1.1 kcal mol^{-1} in binding free energy. At first, the observation that the affinity order also applied to poly[d(A−T)] was surprising in view of the suggestion that the *m*-OH group could donate a H-bond to 2-O of C21 and receive one from 2-NH_2 of G4 in the G−C base pair at the 5′ end of the site,[8,9] which is absent in poly[d(A−T)]. If these additional hydrogen bonds were indeed present in the *m*-OH Hoechst:28-mer hairpin complex, the decrease in free energy (1.1 kcal mol^{-1}) appears small, perhaps due to an unfavorable decrease in entropy since, in contrast to the *p*-OH phenyl ring, the *m*-OH Hoechst phenyl ring is not free to rotate in the minor groove.[9]

The X-ray diffraction data of Clark et al.,[10] on the complex formed between a pyridylimidazole *m*-OH Hoechst derivative [*m*-OH Hoechst(N)] and the AATTC site in $d(CGCGAATTCGCG)_2$, offer a possible rationale for the increased affinity, not only of *m*-OH Hoechst but also for bis-*m*-OH Hoechst. The position of *m*-OH Hoechst(N) at the AATT site[10] is very similar to that described for *p*-OH Hoechst,[23] but with two possible orientations of the phenyl ring, one with the *m*-OH group pointing into the minor groove (*m*-OH "in") and another with the *m*-OH group pointing outward (*m*-OH "out"). In the solution-NMR structure of *m*-OH Hoechst bound to the 12-mer duplex[9] there was no evidence from NOESY cross-peaks of any significant contribution from a bound conformation with the *m*-OH "out". Compared to *p*-OH Hoechst, the *m*-OH phenyl group of *m*-OH Hoechst(N) in the "in" orientation is pushed away from the bottom of the minor groove (Figure 5 in Clark et al.,[10]), which makes the distance between *m*-OH and 2-NH_2 of G4 (4.2 Å) in the 12-mer duplex too large to allow the direct hydrogen bonding predicted from NMR.[8,9] However, the *m*-OH "in" hydroxyl group binds two water molecules which bridge bases and deoxyribosidic ring oxygens from opposite strands (N2 of G4 and O4′ of A5; O2 of C21 and O4′ of T20; see Figure 4 in Clark et al.[10]). Water networks have also been described in the interactions of other minor groove directed ligands, in particular bis-amidinophenyl)furan analogues of berenil.[24,25]

A remarkable finding in our work is the very strong binding of bis-*m*-OH Hoechst to the 28-mer hairpin ($K_a = 1.9 \times 10^{10}$ M^{-1}), with ΔG more favorable by 2.1 kcal mol^{-1} when compared to *p*-OH Hoechst (by 1.0 kcal mol^{-1} relative to *m*-OH Hoechst). The ΔG for binding of bis-*m*-OH to the 28-mer hairpin is only about 1.7 kcal mol^{-1} less than ΔG for binding of Eco RI endonuclease to its GAATTC site.[26] This very high affinity is unexpected as the additional *m*-OH group in bis-*m*-OH Hoechst points out toward the solvent.

A tentative correlation of the very high affinity of bis-*m*-OH Hoechst for the AATT site with molecular interactions can be understood using the *m*-OH "out" coordinates of Clark et al.[10] (DDBJ/EMBO/GenBank ×96025) with the calculated coordinates added for the second ("in"-directed) *m*-OH group in bis-*m*-OH Hoechst. From the X-ray model[10] for the *m*-OH Hoechst(N) analogue the *m*-OH "out" is surrounded by three H_2O molecules in close H-bonding distance, two of which are also at H-bonding distance from deoxyribosidic and phosphate oxygens as possible H-bond acceptors. The calculated "in" OH group could form direct and strong hydrogen

Table 1. Association Constants, K_a, for the Binding of Hoechst Derivatives to Poly[d(A−T)], 12-mer d(CGCGAATTCGCG)$_2$ Duplex, and 28-mer Hairpin, with Reaction Rate Parameters, k_{on} and k_{off}, and the Kinetically Defined Association Constant, K_{kin}, for the 28-mer Hairpin at pH 7.5 (50 mM Tris, 0.1 M NaCl) and 20 °C

	poly[d(A−T)]	AATT duplex	28-mer AATT hairpin			
Hoechst phenyl substitution	$10^{-8} \times K_a$ $(M^{-1})^a$	$10^{-8} \times K_a$ $(M^{-1})^b$	$10^{-8} \times K_a$ $(M^{-1})^c$	$10^{-8} \times k_{on}$ $(M^{-1}\ s^{-1})^d$	k_{off} $(s^{-1})^e$	$K_{kin} = k_{on}/k_{off}$ (M^{-1})
bis-*m*-OH	16.8 ± 0.2	120 ± 10	190 ± 50	2.16 ± 0.04	0.0123 ± 0.0016	180 ± 30
m-OH	4.07 ± 0.05	30 ± 2	38 ± 3	2.00 ± 0.07	0.0510 ± 0.0002	39.2 ± 1.5
p-OH	1.68 ± 0.02	4.1 ± 0.1	5.2 ± 0.2	2.46 ± 0.05	0.419 ± 0.005	5.9 ± 0.2

The errors given are for a single titration or for a single stopped-flow concentration series. [a] With poly[d(A−T)], the extent of a repetitive binding site is defined as $N = (2n - 1)$ with n the number of base pair edges covered or excluded from binding at saturation of the minor groove.[30] For p-OH Hoechst, $N \approx 10$ base pairs with $n = 5$ or 6 A−T base pairs in poly[d(A−T)].[15] [b] Equilibrium titrations with the 12-mer duplex as in Figure 2 with 15 to 25 data points per titration, obtained by adding a total of 2−10 μL of 2.2 or 6.6 μM sites to 2.218 mL of 1 nM dye derivative. To minimize melting, the syringe with the freshly diluted 12-mer duplex titrant was kept on ice. [c] Equilibrium titration data from Figure 2. [d] Stopped-flow kinetics with 2 nM dye and 28-mer AATT hairpin in excess (Breusegem et al., unpublished). [e] Determined by dissociation kinetics using poly[d(A-5BrU) in large excess: the value of $k_{off} = 0.419\ s^{-1}$ for *p*-OH Hoechst was obtained by stopped-flow mixing (Breusegem et al., unpublished).

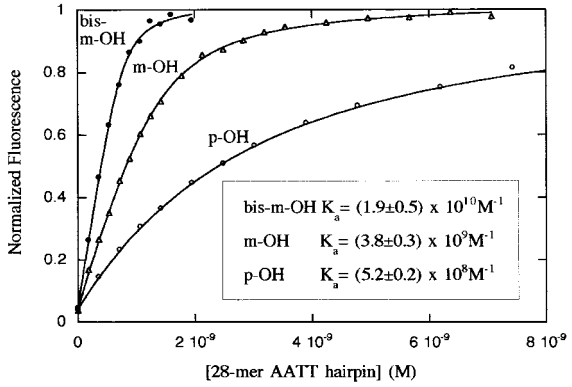


Figure 2. Fluorescence titrations of a fixed concentration (0.7−1.6 nM; 2.218 mL) of bis-*m*-OH, *m*-OH, and *p*-OH Hoechst with 28-mer AATT hairpin (3.54 μM; between 1.1 and 9.0 μL added in minimum portions of 0.11 μL). The data are fitted for a simple binding mechanism, the complex concentration corresponding to the solution of the quadratic binding equation. For the two meta derivatives, the dye concentration was included as a fitting parameter. The plots have been normalized to a common maximal fluorescence.

bonds with O2 of C21 (at 2.7 Å) and with N3 of A5 (at 2.9 Å). Also, the release of previously bound water molecules from the AATT site by the inward *m*-OH group in bis-*m*-OH Hoechst could make a favorable entropic contribution to the binding energy.

Alternatively, one could combine the *m*-OH "in" and "out" conformations of Clark et al.[10] because, except for a 0.5 Å difference in the distance of the phenyl groups from the bottom of the minor groove, both dye conformations can be nearly completely overlaid and have the same dihedral angle of the phenyl group. It requires very little reorganization relative to the experimentally determined X-ray data[10] to accommodate a "hybrid" molecule (of *m*-OH "in" and *m*-OH "out") into the duplex structure. In this case, only very weak direct hydrogen bonds are present between the OH "in" group and the bases. However, both the *m*-OH "in" and the *m*-OH "out" substituents of the dye bind to water molecules that in turn bind to DNA atoms.

Thus, also the "externally" directed OH group of bis-*m*-OH Hoechst is likely to participate in an extended water network such as that described[10] for the HO-out conformation of *m*-OH Hoechst(N) and which links the phenolic OH-out group of the ligand to the major groove. Presumably, this provision of a hydrogen-bonding network on the surface of the DNA molecule allows the HO-out site of bis-*m*-OH Hoechst to increase the binding strength so markedly. Referees have indicated that mention should be made of other possible contributors to this unusual increase in binding energy, such as other types of solvation effects, and the burial of the inwardly directed OH groups perhaps introducing specific hydrophilic interactions with the phosphate backbone, and detailed calculations are planned to investigate this molecular system.

The hypothesis that it is possible to increase the binding affinity by recruiting the primary hydration network of DNA has not been taken into account in any rational ligand design attempts to date for nucleic acids. There is a body of X-ray data, emanating from the berenil family of ligands describing minor groove associated water layers and their part in interactions with DNA. Our results provide the first experimental evidence to our knowledge of the contribution to binding through hydrogen bonding of water molecules that do not act to bridge the ligand directly to the target site. In rational drug design, the use of internal water molecules within the bounds of the active or binding site acting to bridge the substrate to its binding residues is often used. However, it would not normally be considered that one could attain binding strength increases of 5-fold, relative to the single-hydroxy *m*-OH Hoechst, by using water networks stretching across its surface.

Experimental Section. The working conditions and precautions against unspecific adsorption of the dyes to surfaces (Milli-Q water, fresh dust-free solutions, dye solutions in 5 mM HCl, cleaning of glassware with silanization of quartz cuvettes and glass stirrers; use of polystyrene cuvettes) and experimental details have been described.[15,12] These procedures were strictly adhered to. To maintain a low buffer blank, the use of PVC and polyethylene material was avoided. NaCl ("Fractopur") and Tris (GR buffer substance) were from Merck. Poly[d(A−T)], with[27] $\epsilon_{258\ nm} = 13.2 \times 10^3\ M^{-1}\ cm^{-1}$), was from Boehringer; poly[d(A-5BrU)] (with[28] $\epsilon_{260\ nm} = 1.19 \times 10^3\ M^{-1}\ cm^{-1}$), was from Pharmacia. These DNA concentrations in terms of base pairs were expressed as binding sites with $N = 10$ in the titrations (see legend to Table 1). The $\epsilon_{260\ nm}$ values were determined experimentally for d(CGCGAATTCGCG)$_2$ (synthesized by the phosphoramidite method) and for the T_4-looped 28-mer hairpin (Midland, Texas; Figure 1). All measurements were done at 20 °C in 50 mM Tris, 100 mM NaCl (pH 7.5). The concentrations of *p*- and *m*-phenyl substituted analogues of the Hoechst dye were determined using $\epsilon_{338\ nm} = 42 \times 10^3\ M^{-1}\ cm^{-1}$. Hoechst dye analogues were synthesized by literature procedures for similar compounds[29] (details are given in Supporting Information).

Equilibrium Conditions for Measuring Association Constants. Association constants K_a were determined by fluorometrically titrating small concentrations of a dye (2.218 mL of a buffered 1 nM solution in a 1 × 1 × 4.5 mL silanized quartz cuvette that was not removed from the instrument holder during a titration) with an excess of DNA as described[12] (see Supporting Information for details). The experimental increase in fluorescence was fitted (Kaleidagraph, Synergy software) using the sum of fluorescence of free and bound dye as expressed by the solution of the quadratic binding equation, taking even minute dilutions into account. With K_a values larger than about 4×10^9 M^{-1}, the nanomolar concentration of the dye being titrated was included as an additional fitting parameter.

Stopped-Flow Kinetics. The binding kinetics of the Hoechst derivatives and DNA were determined by stopped-flow kinetics following the increase in dye fluorescence on binding. In the stopped-flow instrument (SLM-Aminco Milli-flow mixing unit with a 2 × 10 mm observation cell) fluorescence was excited at 366 nm using a 150-W Hg/Xe lamp, and emitted light was measured through a Schott KV418 cutoff filter (see Supporting Information for full details). All kinetic traces correspond to a monoexponential signal change (Labview or Kaleidagraph). Concentrations given are after mixing.

Molecular Modeling. Molecular modeling was performed using SYBYL 6.4.2 (Tripos Inc.) on a Silicon Graphics Indy R10000 workstation.

Acknowledgment. This work was made possible in part with the help of BOF and FWO (to F.G.L.).

Supporting Information Available: Additional experimental details. This information is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Leung, D.; Abbenante, G.; Fairlie, D. P. Protease Inhibitors: Current Status and Future Prospects. *J. Med. Chem.* **2000**, *43*, 305−341.

(2) Lam, P. Y. S.; Jadhav, P. K.; Eyermann, C. J.; Hodge, C. N.; Ru, Y.; Bacheler, L. T.; Meek, J. L.; Otto, M. J.; Rayner, M. M.; Wong, Y. N.; Chang, C.-H.; Weber, P. C.; Jackson, D. A.; Sharpe, T. R.; Erickson-Viitanen, S. Rational Design of Potent, Bioavailable, Nonpeptide Cyclic Ureas As HIV Protease Inhibitors. *Science* **1944**, *263*, 380−383.

(3) Douglas, K. T. Ligands for DNA and RNA In *Structure, Motion, Interaction and Expression of Biological Macromolecules, Proc. 10th Conversation, SUNY, Albany, NY, 1997;* Sarma, R. H., Sarma, M. H., Eds.; Adenine Press: Schenectady, 1998; pp 279−293.

(4) Chaires, J. B.; Leng, F.; Przewloka, T.; Fokt, I.; Ling, Y. H.; Perez-Soler, R.; Priebe, W. Structure-Based Design of a New Bisintercalating Anthracycline Antibiotic. *J. Med. Chem* **1997**, *40*, 261−266.

(5) Hu, G. G.; Shui, X.; Leng, F.; Priebe, W.; Chaires, J. B. Structure of a DNA-Bisdaunomycin Complex. *Biochemistry* **1997**, *36*, 5940−5946.

(6) Wemmer, D. E.; Dervan, P. B. Targeting the Minor Groove of DNA. *Curr. Opin. Struct. Biol.* **1997**, *7*, 355−361.

(7) Lown, J. W. Lexitropsins: Rational Design of DNA Sequence Reading Agents As Novel Anti-Cancer Agents and Potential Cellular Probes. *Anti-Cancer Drug Des.* **1988**, *3*, 25−40.

(8) Ebrahimi, S. E. S.; Parkinson, J. A.; Fox, K. T.; McKie, J. H.; Barber, J.; Douglas, K. T. Studies of the Interaction of a Meta-Hydroxy Analogue of Hoechst 33258 With DNA by Melting Temperature, Footprinting and High- Resolution ^{1}H NMR Spectroscopy. *J. Chem. Soc., Chem. Commun.* **1992**, 1398−1400.

(9) Parkinson, J. A.; Ebrahimi, S. E.; McKie, J. H.; Douglas, K. T. Molecular Design of DNA-Directed Ligands With Specific Interactions: Solution NMR Studies of the Interaction of a Meta-Hydroxy Analogue of Hoechst 33258 With D(CGCGAATTCGCG)$_2$. *Biochemistry* **1994**, *33*, 8442−8452.

(10) Clark, G. R.; Squire, C. J.; Gray, E. J.; Leupin, W.; Neidle, S. Designer DNA-Binding Drugs: the Crystal Structure of a *Meta*-Hydroxy Analogue of Hoechst 33258 Bound to D(CGCGAATTCGCG)$_2$. *Nucl. Acids Res.* **1996**, *24*, 4882−4889.

(11) Jin, R.; Breslauer, K. J. Characterization of the Minor Groove Environment in a Drug−DNA Complex: Bisbenzimide Bound to the Poly[d(AT).Poly[d(AT)Duplex. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8939−8942.

(12) Loontiens, F. G.; McLaughlin, L. W.; Diekmann, S.; Clegg, R. M. Binding of Hoechst 33258 and 4′6-Diamidino-2-Phenylindole to Self-Complementary Decadeoxynucleotides With Modified Exocyclic Base Substituents. *Biochemistry* **1991**, *30*, 182−189.

(13) Haq, I.; Ladbury, J. E.; Chowdhry, B. Z.; Jenkins, T. C.; Chaires, J. B. Specific Binding of Hoechst 33258 to the D(CGCGAATTCGCG)$_2$ Duplex: Calorimetric and Spectroscopic Studies. *J. Mol. Biol.* **1997**, *271*, 244−257.

(14) Bostock-Smith, C. E.; Searle, M. S. DNA Minor Groove Recognition by Bis-Benzimidazole Analogues of Hoechst 33258: Insights into Structure-DNA Affinity Relationships Assessed by Fluorescence Titration Measurements. *Nucl. Acids Res.* **1999**, *27*, 1619−1624.

(15) Loontiens, F. G.; Regenfuss, P.; Zechal, A.; Dumortier, L.; Clegg, R. M. Binding Characteristics of Hoechst 33258 With Calf Thymus DNA, Poly[d(A−T)], and D(CCGGAATTCCGG)$_2$: Multiple Stoichiometries and Determination of Tight Binding With a Wide Spectrum of Site Affinities. *Biochemistry* **1990**, *29*, 9029−9039.

(16) Drew, H. R.; Dickerson, R. E. Structure of a B-DNA Dodecamer. III. Geometry of Hydration. *J. Mol. Biol.* **1981**, *151*, 535−556.

(17) Johansson, E.; Parkinson, G.; Neidle, S. A New Crystal Form for the Dodecamer C-G-C-G-A-A-T-T-C-G-C-G: Symmetry Effects on Sequence-Dependent DNA Structure. *J. Mol. Biol.* **2000**, *300*, 551−561.

(18) Chiu, T. K.; Kaczor-Greskowiak, M.; Dickerson, R. E. Absence of Minor-Groove Monovalent Cations in the Cross-Linked Dodecamer C-G-C-G-A-A-T-T-C-G-C-G. *J. Mol. Biol.* **1999**, *292*, 589−608.

(19) Soler-Lopez, M.; Malilina, L.; Liu, J.; Huyn-Dinh, T.; Subirana, J. A. Water and Ions in a High-Resolution Structure of B-DNA. *J. Biol. Chem.* **1999**, *274*, 23683−23686.

(20) Soler-Lopez, M.; Malilina, L.; Subirana, J. A. Solvent Organization in an Oligonucleotide Crystal. *J. Biol. Chem.* **2001**, *275*, 23034−23044.

(21) Chuprina, V. P. Anomalous Structure and Properties of Poly-(DA).Poly(DT). Computer Simulation of the Polynucleotide Structure With the Spine of Hydration in the Minor Groove. *Nucl. Acids Res.* **1987**, *15*, 293−311.

(22) Shui, X.; McFail-Isom, L.; Hu, G. G.; Williams, L. D. The B-DNA Dodecamer at High Resolution Reveals a Spine of Water on Sodium. *Biochemistry* **1998**, *37*, 8341−8355.

(23) Teng, M.-K.; Usman, N.; Frederick, C. A.; Wang, A. H.-J. The Molecular Structure of the Complex of Hoechst 33258 and the DNA Dodecamer d(CGCGAATTCGCG)$_2$. *Nucl. Acids Res.* **1988**, *16*, 2671−2690.

(24) Guerri, A.; Simpson, I. J.; Neidle, S. Visualization of Extensive Water Ribbons and Networks in a DNA Minor-Groove Drug Complex. *Nucl. Acids Res.* **1998**, *26*, 2873−2878.

(25) Laughton, C. A.; Tanious, F.; Nunn, C. M.; Boykin, D. W.; Wilson, W. D.; Neidle, S. A Crystallographic and Spectroscopic Study of the Complex Between D(CGCGAATTCGCG)$_2$ and 2,5-Bis(4-Guanylphenyl)Furan, an Analogue of Berenil. Structural Origins of Enhancd DNA-Binding. *Biochemistry* **1996**, *35*, 5655−5661.

(26) Lesser, D. R.; Kurpiewski, M. R.; Jen-Jacobson, L. The Energetic Basis of Specificity in the Eco RI Endonuclease−DNA Interaction. *Science* **1990**, *250*, 776−786.

(27) Schmechel, D. E. V.; Crothers, D. M. Kinetic and Hydrodynamic Studies of the Complex of Proflavine With Poly A-Poly U. *Biopolymers* **1971**, *10*, 465−480.

(28) Latt, S. A.; Wohlleb, J. J. Studies of the Interaction of Hoechst 33258 With DNA, Chromatin and Metaphase Chromosomes. *Chromosoma* **1975**, *52*, 297−316.

(29) Ebrahimi, S. E. S.; Bibby, M. C.; Fox, K. R.; Douglas, K. T. Synthesis, DNA-Binding, Footprinting and in Vitro Antitumour Studies of a Meta-Hydroxy Analogue of Hoechst 33258. *Anti-Cancer Drug Des.* **1995**, *10*, 463−479.

(30) McGhee, J. D.; Von Hippel, P. H. Theoretical Aspects of DNA: Protein Interactions: Co-Operative and Non-Co-Operative Binding of Large Ligands to a One-Dimensional Homogeneous Lattice. *J. Mol. Biol.* **1974**, *86*, 469−489.

JM0100943