Nucleic Acids Research, 1993, Vol. 21, No. 16 3705–3709

The different binding modes of Hoechst 33258 to DNA studied by electric linear dichroism

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Received May 7, 1993; Revised and Accepted June 28, 1993

ABSTRACT

The binding mode of the bisbenzimidazole derivative Hoechst 33258 to a series of DNAs and polynucleotides has been investigated by electric linear dichroism. Positive reduced dichroisms were measured for the poly(dA-dT).poly(dA-dT)- and poly(dA).poly(dT)-Hoechst complexes in agreement with a deep penetration of the drug into the minor groove. Similarly, the drug displays positive reduced dichroism in the presence of the DNAs from calf thymus, *Clostridium perfringens* and Coliphage T4. Conversely, negative reduced dichroisms were obtained when Hoechst 33258 was bound to poly(dG-dC).poly(dG-dC), poly(dA-dC).poly(dG-dT) and poly(dG). poly(dC) as well as with the GC-rich DNA from *Micrococcus lysodeikticus* indicating that in this case minor groove binding cannot occur. Substitution of guanosines for inosines induces a reversal of the reduced dichroism from negative to positive. Therefore, as anticipated it is the 2-amino group of guanines protruding in this groove which prevents Hoechst 33258 from getting access to the minor groove of GC sequences. The ELD data obtained with the GC-rich biopolymers are consistent with an intercalative binding. Competition experiments performed with the intercalating drug proflavine lend credence to the involvement of an intercalative binding rather than to an external or major groove binding of Hoechst 33258 at GC sequences.

INTRODUCTION

These last years increasing interest has appeared in attempts to develop DNA sequence-specific agents, ideally to target any predetermined sequence in DNA, with potential applications in molecular biology, diagnosis and hopefully in antiviral and anticancer chemotherapy [1]. Rational structure modifications of the antibiotics netropsin and distamycin have led to the development of the lexitropsins [2]. Recently, based on the same strategy, the development of analogues of the synthetic dye Hoechst 33258 has been undertaken [3–5].

The interaction of Hoechst 33258 (Fig. 1) with DNA has been studied extensively [6–10]. The primary mode of binding consists of an insertion of the crescent shape molecule into the minor groove of DNA with a strong preference for clusters of AT base pairs [11]. In binding to the dodecamer d(CGCGATATCGCG)$_2$, Hoechst 33258 covers three of the four central AT base pairs and extends its piperazine ring to the neighbouring GC pair [12] while in the presence of the related dodecamer d(CGCGAATTCGCG)$_2$, the drug is located in the central AATT site [13–15]. Hoechst 33258 selectively recognizes AT rather than GC sequences in DNA [16,17]. However, it is beyond doubt that the drug is also able to interact with contiguous GC pairs. The affinity constant of the drug for poly(dG-dC).poly(dG-dC) is high (Ka = 1.1×10^6) though 50-fold lower than the affinity constant for poly(dA-dT).poly(dA-dT) (Ka = 6.3×10^7) [4]. The mode of interaction of Hoechst 33258 with GC sequences is not known precisely. The binding stoichiometries for Hoechst 33258-poly(dG-dC).poly(dG-dC) is 1–2 dyes per 5 GC pairs and was tentatively assigned to the presence of a stacked dimer in the major groove [18,19]. In contrast, CD studies are consistent with dimerization or stacking of the Hoechst 33258 molecules along the alternating copolymer poly(dG-dC).poly(dG-dC) [20]. It is important to better characterize the mode of interaction between Hoechst 33258 and GC base pairs all the more if, as stated above, this drug is to be used as a model compound for molecular recognition of DNA. We have applied linear dichroism which reflects the orientation of the drug relative to the DNA helix axis; the method has previously been used with marked success to elucidate the molecular mechanisms involved in the interaction between DNA and drugs [reviewed in 21–23] among which is Hoechst 33258 [7]. We have analyzed the orientation of Hoechst 33258 bound to a range of natural DNAs with various AT/GC content and to a series of synthetic polynucleotides containing defined base pair arrangements. Particular attention was concentrated on the interaction of Hoechst

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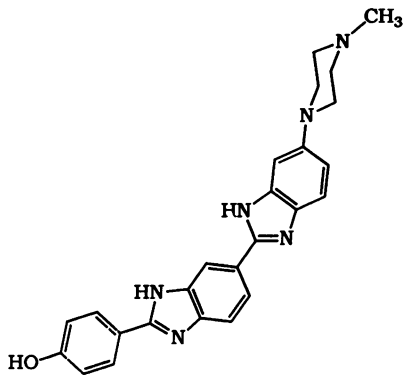

Figure 1. Hoechst 33258. 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2, 5'-bis-1H-bisbenzimide.

33258 with GC-containing polynucleotides by studying the competitive binding with proflavine.

Table I. Extinction coefficients of DNAs and Dichroism Ratio values for the Hoechst–DNA complexes

DNA	ϵ^a	DR[b]
Natural DNAs from		
Micrococcus lysodeikticus (72% GC)	6950	+0.90
Calf Thymus (42% GC)	6600	−0.56
Clostridium perfringens (26% GC)	6600	−0.55
Coliphage T4 (50% GC)	6600	−0.69
Alternating Copolymers		
poly(dA-dT).poly(dA-dT)	6600	−0.74
poly(dG-dC).poly(dG-dC)	8400	+0.83
poly(dI-dC).poly(dI-dC)	6900	−0.55
poly(dA-dC).poly(dG-dT)	6500	+0.45
Homopolymers		
poly(dA).poly(dT)	6000	−0.46
poly(dG).poly(dC)	7400	+0.67
poly(dI).poly(dC)	5300	−0.80

[a]Extinction coefficients (in $M^{-1} \times cm^{-1}$) were taken fom Wells, R.D., Larsen, J.E., Grant, R.C., Shortle, B.E., and Cantor, C.R. (1970) *J. Mol. Biol.* 54, 465–497. [b]$DR = [(\Delta A/A)^{Hoechst-DNA}]/[(\Delta A/A)^{DNA}]$, drug/DNA ratio = 0.1. E = 13kV/cm, in 1mM sodium cacodylate buffer, pH 6.5. The reduced dichroism were measured at 260nm for DNAs and polynucleotides and at 360nm for Hoechst 33258–DNA complexes.

MATERIALS AND METHODS

Drugs and DNA

Hoechst 33258 and proflavine were purchased from Sigma and BDH, respectively. Concentrations of the drug solutions were determined spectroscopically applying molar extinction coefficients of 42,000 $M^{-1} \times cm^{-1}$ at 338 nm for Hoechst 33258 [24] and 41,000 $M^{-1} \times cm^{-1}$ at 444 nm for proflavine [25]. The DNAs from calf thymus, *Clostridium perfringens*, *Micrococcus lysodeikticus* and Coliphage T4 together with the double-stranded polymers poly(dA-dT).poly(dA-dT), poly(dG-dC).poly(dG-dC), poly(dA).poly(dT) and poly(dG).poly(dC) were from Sigma. Poly(dI).poly(dC) and poly(dA-dC).poly(dG-dT) were from Pharmacia and poly(dI-dC).poly(dI-dC) was from Boehringer. Their concentrations were determined applying molar extinction coefficients given in Table I. Calf thymus DNA was deproteinized with sodium dodecyl sulphate (protein content <0.2%) and all DNAs were dialyzed against 1 mM sodium cacodylate buffer pH 6.5.

Electric Linear Dichroism (ELD)

This electrooptical method exploits the fact that, under the influence of a short electric field pulse, the DNA molecule becomes oriented, rendering the solution optically anisotropic. ELD measurements were performed with a computerized optical measurement system built by C.Houssier [26]. The procedures previously outlined were followed [22,23]. The optical set-up of a high sensitivity T-jump instrumentation equipped with a Glan polarizer was used under the following conditions: bandwidth 3nm, sensitivity limit 0.001 in $\Delta A/A$, response time 3μs. The rectangular electric field pulses in the range 0–13 kV/cm were applied to the samples in a 10 mm optical pathlength Kerr cell with a distance between the platinum electrodes of 1.5 mm. The pulse duration was carefully adjusted to reach the steady-state orientation of the molecule (50–100 μs, depending on the electric field strength). Linear dichroism ΔA is defined as the difference between the absorbance for light polarized parallel ($A_{//}$) and perpendicular ($A_\perp$) to the applied field at a given wavelength. The reduced dichroism is $\Delta A/A = (A_{//} - A_\perp)/A$, where A is the isotropic absorbance of the sample measured in the absence of field at the same wavelength and under the same pathlength. Because of axial symmetry around the electric field direction, the changes in absorbance $\Delta A_{//} = A_{//} - A$ and $\Delta A_\perp = A_\perp - A$ are related by $\Delta A_{//} = -2\Delta A_\perp$; thus, measurement of $\Delta A_{//}$ alone is sufficient for the calculation of the reduced dichroism $\Delta A/A$ [22,26]. In these experiments the DNA molecules are oriented by an electric pulse and the dichroism in the region of the absorption bands of DNA or of the ligand bound to DNA is probed using linearly polarized light. When DNA solutions are exposed to the electric field pulses, at 260 nm the absorbance of light polarized parallel to the electric field vector is lower than the absorbance of light polarized perpendicularly ($A_{//} < A_\perp$), indicative of a negative dichroism. Similar negative dichroism signals are observed with intercalator–DNA complexes in the absorption band of the ligand. In contrast, when rectangular electric pulses are applied to a solution of a minor groove binder bound to DNA, the change of the absorption of light in the ligand absorption band is different ($A_{//} > A_\perp$) indicative of a positive dichroism of the complex. Therefore, based on the sign and the amplitude of the observed signals, this technique can reveal the binding mode of the ligand via an estimation of its orientation with respect to the DNA helix. All experiments were conducted in 1 mM sodium cacodylate buffer adjusted to pH 6.5, at room temperature (20°C) and the conductivity of the solutions, measured with a Metrohm conductimeter Model E527, ranged from 0.8 to 1.2 milliSiemens. The dichroism ratio is defined by $DR = [(\Delta A/A)^{ligand-DNA}]/[(\Delta A/A)^{DNA}]$ where the numerator refers to the reduced dichroism of the drug–DNA complex measured at the maximum of the ligand absorption band and the denominator refers to the reduced dichroism of the same DNA sample at 260 nm in the absence of drug. This latter is always negative under the experimental conditions used. The dichroism ratios DR for any given drug–DNA and drug–polynucleotide complexes can be compared with good relative accuracy, independently on the polymer size.

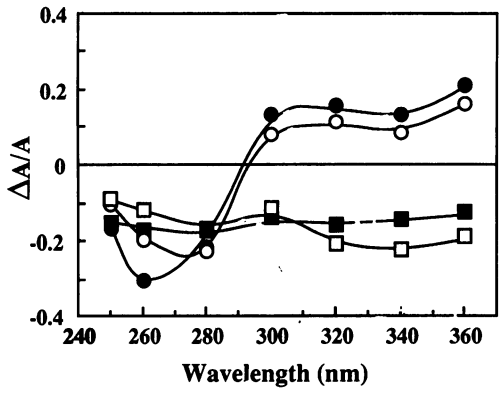

Figure 2. Reduced electric linear dichroism spectra. ELD spectra were obtained in the presence of 10μM Hoechst 33258 bound to 100μM alternating copolymers at 13kV/cm in 1 mM sodium cacodylate pH 6.5. (●) poly(dA-dT).poly(dA-dT), (○) poly(dI-dC).poly(dI-dC), (□) poly(dG-dC).poly(dG-dC), (■) poly(dA-dC).poly(dG-dT).

RESULTS AND DISCUSSION

ELD spectra

ELD spectra were recorded at a constant drug/DNA ratio of 0.1 (100μM DNA−10μM drug) so that we can consider that almost all drug molecules are bound to DNA [7]. Figure 2 shows the ELD spectra of the complexes between Hoechst 33258 and four synthetic polynucleotides with alternating structure: poly(dA-dT).poly(dA-dT), poly(dG-dC).poly(dG-dC), poly(dA-dC).poly(dG-dT) and poly(dI-dC). poly(dI-dC). In each case negative reduced dichroisms are measured in the 240−280 nm band and reflect the orientation of the DNA base pairs perpendicular to the helix axis. As expected, in the presence of poly(dA-dT).poly(dA-dT) positive reduced dichroisms are measured in the 300−380 nm absorption band of Hoechst 33258 and give evidences for a location of the drug in the minor groove of this polynucleotide. Conversely, in the presence of poly(dG-dC).poly(dG-dC) or poly(dA-dC).poly(dG-dT) the reduced dichroism remains negative all along the spectra. Thus, the binding configuration adopted by Hoechst 33258 has markedly changed; in other words, the drug no longer binds to the minor groove of these two GC-containing duplexes. This effect can be directly assigned to the 2-amino group of guanines which does not allow a proper positioning of the two consecutive benzimidazole rings. Indeed, substitution of guanosines for inosines restores minor groove binding of Hoechst 33258 as indicated by the positive reduced dichroism measured in the 300−380 nm band with the Hoechst 33258-poly(dI-dC).poly(dI-dC) complex. The ELD spectra of the complexes between the drug and poly(dA-dT).poly(dA-dT) or poly(dI-dC).poly(dI-dC) are very similar and likely refer to an identical type of drug-minor groove DNA structure while those for the complexes with poly(dG-dC).poly(dG-dC) or poly(dA-dC).poly(dG-dT) obviously correspond to a distinct conformational category. From these ELD spectra, it is clear that Hoechst 33258 adopts two distinct and well defined orientations depending on the DNA sequence to which it binds. To elucidate further this sequence-dependent DNA binding process, ELD measurements were performed using several other synthetic polynculeotides as well as with a series of natural DNAs with various AT/GC content. This effectively provides a means of monitoring the peculiar behaviour of Hoechst 33258 and allows a direct comparison of reduced dichroism values between the Hoechst 33258−DNA complexes. To take into account the intrinsic behaviour of the DNA and polynucleotides under the electric pulses, we refer to the dichroism ratio parameter DR, i.e. the reduced dichroism of the drug−DNA complex measured at 360 nm in the dye absorption band (without contribution from DNA) divided by the dichroism of the DNA measured at 260 nm in the absence of drug (Table I). A positive dichroism ratio indicates that free DNA and the ligand−DNA complex both exhibit negative ELD signals, i.e. that the drug is more or less perpendicular to the electric field direction. Conversely, a negative dichroism ratio indicates that the ligand−DNA complex exhibits a positive reduced dichroism in the ligand absorption band. This immediately implicates an angle lower than 55° between the helix orientation axis and the transition moment of Hoechst 33258. The reduced dichroism of the free DNA being always negative at 260 nm, the DR becomes positive when the dichroism of the ligand is also negative. Measurement of the DR values also represents a convenient means to estimate the angle β between the dye transition moment and the orientation axis of the DNA [22]. A DR value of −0.5 corresponds to an angle β of about 45°. Such DR values have been obtained with four of the DNAs listed in Table I such as the DNA from *Clostridium perfringens* wich contains 26% GC base pairs. It is remarkable that this angle of 45° is coincident with the angle determined from X-Ray analyses of Hoechst 33258−oligonucleotide complexes [12,13]. It is interesting to observe that a highly negative DR value is obtained for the complex of Hoechst 33258 and the DNA from Coliphage T4. This indicates clearly that the blockage of the major groove by glycosyl residues has no effect on the binding of the drug to DNA. Hoechst 33258 binds probably to the minor groove of this DNA which contains more than 50% GC base pairs. With poly(dG-dC).poly(dG-dC) and the DNA from *Micrococcus lysodeikticus* (72% GC) the DR values reach +0.8/+0.9. According to the same method for estimating the angle β and assuming a theoretical angle α of 90° for the DNA bases, Hoechst 33258 would be inclined at about 75° upon binding to GC sequences. Such an angle may be interpreted by (i) an intercalation between propeller-twisted bases as in the case of ethidium bromide (β = 70−75° for α = 90°) [22] or (ii) by an external binding for which the drugs would be stacked along the DNA outside the groove but parallel to the base planes. We think that the data are not compatible with a binding of the drug in the major groove of GC sequences. However, due to the uncertainty associated with the knowledge of the exact orientation of the drug transition moment, we cannot totally exclude this possibility. The data collected with the homopolymers just correlate those obtained with the alternating copolymers. The drug displays positive and negative reduced dichroisms with poly(dA).poly(dT) and poly(dG).poly(dC), respectively. Here again, replacement of inosines for guanines restores minor groove binding of the drug to poly(dI).poly(dC). Therefore, the rigid and sometimes bent structures adopted by homopolymers [27] do not modify the sequence-dependent DNA-binding mode of Hoechst 33258.

Competitive binding

The results presented above may be consistent with an intercalative binding of Hoechst 33258 into GC sequences. To investigate this possibility we tested the mutual interference

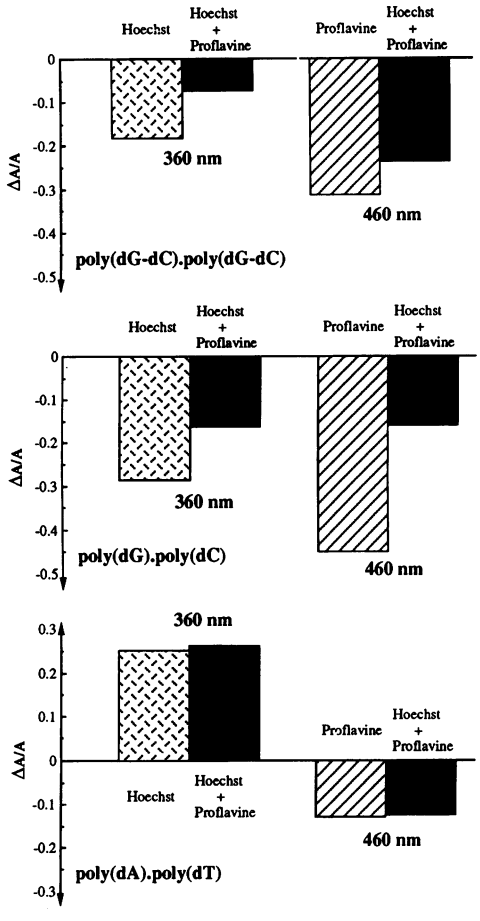

Figure 3. Competitive binding between Hoechst 33258 and proflavine. Variation of the reduced dichroism (ΔA/A) of the complexes between poly(dG-dC).poly(dG-dC) (top), poly(dG).poly(dC) (middle) or poly(dA).poly(dT) (bottom) and Hoechst 33258 (dotted bars), proflavine (hatched bars) or Hoechst 33258 plus proflavine (filled bars). Measurements were made at 13 kV/cm in 1mM sodium cacodylate pH 6.5, at 360nm and 460nm. The DNA concentration is 100μM, the drug concentrations are 10μM.

between Hoechst 33258 and a well characterized intercalating agent upon binding (i) to poly(dG-dC).poly(dG-dC), poly(dG).poly(dC) to which the Hoechst 33258 binding process is not clearly defined and (ii) poly(dA).poly(dT) to which Hoechst 33258 is known to bind in the minor groove. Proflavine was chosen as a typical intercalating drug for several reasons. Firstly, proflavine has been known for a long time to intercalate into DNA positioning its acridine chromophore parallel to the plane of the DNA base pairs in a nearly sequence-independent fashion [25,28,29]. Secondly, the drug was chosen for its spectral properties; it absorbs in the visible range with a maximum at 460 nm in the presence of DNA, i.e. a wavelength at which no interference with Hoechst 33258 can occur. Moreover, the absorbance of proflavine in the 320–380 nm region is very weak, so that measurement of the dichroism at 360 nm for the Hoechst 33258–DNA–proflavine ternary complex mainly reflects the behaviour of Hoechst 33258 with only minimal contribution from proflavine molecules. Thirdly, the binding affinities of Hoechst and proflavine for GC polynucleotides are in the same order of magnitude.

Equal amounts of Hoechst 33258 and proflavine were added to either one of the two GC-containing polymers, poly(dG).poly(dC) and poly(dG-dC).poly(dG-dC), or to the homopolymer poly(dA).poly(dT) to get drugs/DNA ratios of 0.1. The reduced dichroism was then measured at 360 nm and 460 nm for each Hoechst/DNA/proflavine ternary complex and compared to that measured for the complexes of a single drug bound to DNA. The results are summarized in Figure 3 and show that Hoechst 33258 and proflavine interfere significantly with each other in their binding interaction with GC sequences while, upon binding to poly(dA).poly(dT), the interference, if any, is very weak. The negative reduced dichroism measured at 360 nm for the Hoechst 33258/GC polynucleotide complexes becomes less negative in the presence of proflavine. This effect may be due to the displacement of the Hoechst 33258 molecules from their GC binding sites. Conversely, the reduced dichroism measured in the proflavine band at 460 nm is significantly lowered by the addition of Hoechst 33258. The effect is particularly obvious for the competitive binding to the homopolymer poly(dG).poly(dC); in this case the reduced dichroism at 460 nm of proflavine is reduced by 60% in the presence of Hoechst 33258. This implies that either proflavine is displaced from DNA by Hoechst 33258 or that proflavine remains bound to DNA but the parallelism between GC base pairs and the acridine chromophore is disrupted. We suspect the former explanation to be more likely than the latter. It is interesting to observe that the positive and negative reduced dichroisms measured at 360 nm and 460 nm, respectively, for the Hoechst 33258/poly(dA).poly(dT)/proflavine ternary complex are identical to those measured for the Hoechst 33258/-poly(dA).poly(dT) and proflavine/poly(dA).poly(dT) complexes, separately. Therefore, the binding of Hoechst 33258 into the minor groove of poly(dA).poly(dT) is not affected despite the presence of proflavine. It also seems that the binding of Hoechst 33258 into the minor groove of the homopolymer poly(dA).poly(dT) has little effect on the intercalative binding of proflavine while its binding to GC sequences, via a non-minor groove binding process, significantly affects the intercalation of proflavine. At first sight, these results would support the hypothesis that Hoechst 33258 binds to GC sequences through a process similar to that of proflavine, i.e. by intercalation. However, the fact that Hoechst can displace proflavine from GC sequences does not necessarily mean that both drugs get access to the two GC polynucleotides through a similar mechanism. We cannot exclude the possibility that Hoechst 33258 binds to GC sequences through a highly ordered external binding with the long axis of the Hoechst 33258 molecules orientated more or less perpendicular to the double helix axis. This binding configuration though unexpectable cannot be totally discarded. On the basis of the results, intercalative binding of Hoechst 33258 to GC sequences may be envisaged although this mechanism is in some way difficult to envision because of the bulky substituents attached at both ends of the extended bis-benzimidazole chromophore.

CONCLUSION

This study illustrates the usefulness of the electric linear dichroism technique to study drug–DNA sequence-dependent interaction. Indeed, the results clearly indicate that at least two distinct mechanisms are involved in the binding of Hoechst 33258 to AT and GC sequences. The ELD data are fully consistent with the well-characterized minor groove binding of the drug at AT sequences. As anticipated [13,19,30], minor groove binding cannot take place at GC sequences because of the 2-amino group

of guanines protruding in this groove. The negative reduced dichroisms obtained when Hoechst 33258 is bound to GC-rich DNA and polynucleotides reflect an orientation of the drug in a manner more or less parallel to the plane of DNA base pairs. Such an orientation may be consistent with an ordered outside stacking of the dyes (as monomers or dimers) perpendicular to the DNA helix axis as suggested by previous studies [20]. However, the results and in particular the competitive binding between Hoechst 33258 and proflavine to GC sequences can also be interpreted by an intercalation binding mode of Hoechst 33258 at GC sequences. The conventional wisdom argues that intercalators possess a condensed polyaromatic ring but this view is rapidly changing with the discovery of powerful intercalating agents formed by unfused aromatic molecules [31,32] to which Hoechst 33258 may be compared. Moreover, the results found here with Hoechst 33258 are directly reminiscent of those reported with DAPI (4′,6-diamidino-2-phenylindole) which has the property to bind into the minor groove of AT sequences and to intercalate into GC sequences [33,34]. Using flow linear dichroism, Nordén *et al.* [35] showed that DAPI exhibits positive and negative linear dichroism signals at AT and GC sequences, respectively, i.e. exactly as found here with Hoechst 33258. Therefore, by analogy we are inclined to believe that Hoechst 33258 can intercalate into GC sequences. An alternative explanation may be advanced in order to satisfy both the present ELD data and the spectroscopic results of Clegg and his colleagues [18]. Indeed, it can be envisaged that Hoechst 33258 partially intercalates one of its benzimidazole ring and the attached phenolic group between two GC base pairs thus placing the second benzimidazole into the major groove with the positively charged N-methyl-piperazine terminal group protruding outside the DNA helix. This explanation, as yet purely conjectural, seems plausible since a more or less similar situation was recently reported for the drug DAPI bound to poly(dG-dm^{5}C).poly(dG-dm^{5}C) [36]. The peculiar and unexpected mode of binding of Hoechst 33258 to GC sequences undoubtedly merits further investigation. These results should have important implications for our understanding of the molecular mechanisms that drive drug–DNA interactions: when designing Hoechst 33258 derivatives, one should be aware of the fact that these drugs may bind to AT and GC sequences by distinct mechanisms. The binding behaviour of DAPI and Hoechst 33258 may well be a general principle valid for many drugs commonly registered as 'minor groove binders' such as berenil and lexitropsins.

ACKNOWLEDGMENTS

This work was done under the support of research grant FRFC convention 2.4501.91 from the Fonds National de la Recherche Scientifique and from the Institut National de la Santé et de la Recherche Médicale. The INSERM-CFB agreement is acknowledged.

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