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Electric linear dichroism as a new tool to study sequence preference in drug binding to DNA

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Abstract

An original approach using electric linear dichroism (ELD) and natural DNAs and polynucleotides of differing base composition has been developed with the aim to investigate the sequence-dependent recognition of DNA by drugs. Both intercalators and minor groove binders have been studied as well as certain hybrid molecules. The results indicate that the orientation of drugs upon binding to nucleic acids can change markedly according to the target sequence. Among the intercalators tested, only actinomycin D and hycanthone show a clear preference for GC- and AT-rich sequences, respectively. For minor groove binders, the linear dichroism showing a strong dependence on base composition of the DNA and polynucleotides is most pronounced. Netropsin and distamycin bind to DNA with a marked AT specificity. Hoechst 33258, berenil and DAPI exhibit positive and negative dichroism signals at AT and GC sites respectively, suggesting that at least two types of drug–DNA interaction are involved depending on the AT/GC content of the DNA. Further investigations using polynucleotides with inosine substituted for guanosine, and competition experiments with intercalative drugs suggest that Hoechst 33258, berenil and DAPI interact with GC sequences via a non-classical intercalation process.

Keywords: Review; Electric linear dichroism; Intercalation; Groove binding; DNA; Sequence selectivity

1. Introduction

Compounds which bind to DNA with high affinity can influence gene expression and, subsequently, affect cell proliferation and differentiation. Furthermore, such compounds may exhibit antiviral and/or antitumoral activities. Using natural antibiotics as 'lead compounds', it is possible to design and synthesize novel antitumour agents which possess enhanced affinity toward the DNA molecule [1–3]. At this stage, two questions need to be addressed: (1) What are the different binding modes of the drugs to DNA? (2) Have the drugs any sequence preference? In this paper, Electric Linear Dichroism (ELD) has been used to answer these questions.

Although linear dichroism has long been used to determine the orientation of drugs upon binding to nucleic acids (for recent review, see Ref. [4]), application to evaluate the sequence selectivity of the drug binding to DNA was evidenced only recently [5,6]. In this paper, we present a panel of recent results concerning the interactions between synthetic or natural DNAs and several types of drugs. This

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overview mainly concerns the ELD study of these interactions. However, such studies are very often associated with a more complete biophysical investigation including several other spectroscopic techniques (see for example Ref. [7–9]), as we know that the use of only one technique cannot provide a definitive proof of a given binding mode.

2. The drugs

Small ligands can be subdivided into four categories with respect to their mode of binding to DNA: (i) intercalators (mono- and bis-); (ii) groove binders; (iii) covalent binders and (iv) DNA-cleaving agents (Fig. 1). In the following review, we are only concerned with the first two categories.

Classical intercalators generally contain a planar chromophore consisting of two to four fused aromatic rings which may be inserted between stacked nucleic acid base pairs. They bind mainly to double-stranded DNA and their insertion between base pairs causes the helix to unwind and so to lengthen. They generally exhibit poor base specificity and are positively charged at physiological pH (Fig. 2). However, amiloride, which contains only one aromatic ring, was shown to intercalate with a preference for AT-alternating bases [10]. Recently, Wilson and coworkers [11,12] showed that unfused aromatic systems can intercalate.

Non-covalent groove-binding interactions involve direct contact of the molecule with the edges of base pairs in either the major or in the minor groove of DNA. Most of these ligands, such as netropsin and distamycin, are minor groove binders (Fig. 2). Both ligands are positively charged, isohelical with B-DNA, exhibit a marked AT specificity and are held in the minor groove by a combination of hydrogen bonds, van der Waals forces and electrostatic interactions. In the minor groove of AT-rich sequences, the electrostatic potential is highly negative [13].

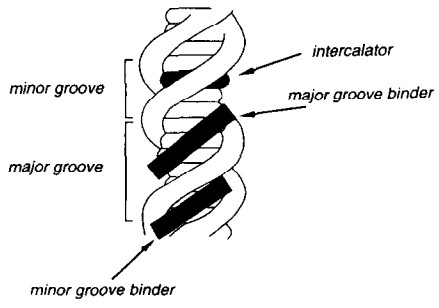

Fig. 1. Modes of DNA–ligand interaction.

Recently, it was shown that the binding mode of compounds which were primarily identified as minor groove binders in calf thymus DNA is dependent upon base sequence and/or composition; these drugs are minor groove binders in AT-rich regions and behave as intercalators in GC-rich regions. Hoechst 33258 and DAPI (4′,6′-diamidino-2-phenylindole) are exhibitors of this behaviour [14].

The design of new drugs can be based on different approaches:

1. de novo synthesis of new compounds similar to natural ones or chemical modifications of natural products in order to modify their sequence specificity. For example, the replacement of the pyrrole units of netropsin by imidazole units produces the so-called 'lexitropsin' in which AT binding selectivity of netropsin is deviated towards GC binding preference. The binding of a prototype lexitropsin molecule to DNA is depicted in Fig. 3. In this case, the heterocyclic nitrogens of the imidazole and thiazole rings are engaged in a hydrogen bond with the amino group of guanines in the minor groove. The fact that the 5'-flanking region of certain oncogenes are particularly GC-rich regions provides the impetus for the design of GC specific ligands [15].
2. the second possibility consists in designing hybrid molecules with multiple binding modes to DNA, called 'combilexins'. Depending on the synthesized drug, the hybrid molecule includes two or three components:

chelating
cleaving
alkylating] – agent

→ sequence specific (pseudo)peptide

→ intercalator

The minor groove peptide moiety (netropsin, for example) serves as a DNA reading element providing sequence selectivity while the intercalating moiety may confer higher DNA binding affinity, or interaction with topoisomerases or facilitate penetration of the compound into the cell nuclei.

The hybrid compounds we shall talk about below are constructed of two linked binding functionalities.

3. Experimental approach

3.1. The ELD technique [16]

Isotropic DNA solutions become anisotropic when they are subjected to external forces (flow, electric field). Induced and/or permanent dipole moments in the molecule are responsible for their orientation in an electric field. Linear dichroism ΔA is defined as the difference between the absorbance for light polarized parallel ($A_{\parallel}$) and perpendicular ($A_{\perp}$) to the field, at a given wavelength. The latter is chosen in the absorption band of DNA or of the ligand bound to DNA. The reduced dichroism is defined as $(A_{\parallel} - A_{\perp})/A$ where A is the isotropic absorbance of the sample measured in the absence of the field at the same wavelength and under the same path length. Due to axial symmetry around the electric field direction, the changes in absorbance $\Delta A_{\parallel} = A_{\parallel} - A$ and $\Delta A_{\perp} = A_{\perp} - A$ are related by

$$\Delta A_{\parallel} = -2\Delta A_{\perp} \quad (1)$$

Therefore, owing to its higher sensitivity, only $\Delta A_{\parallel}$ will be measured to estimate the reduced dichroism $\Delta A/A$.

Fig. 4 shows the typical shape of a negative electro-optical signal (part a) and the model of DNA orientation (part b) assuming an orientation of the DNA helix axis parallel to the electric field direction. Three important technical points have to be mentioned: (i) for a given electric field strength (0 to 13 kV/cm), the pulse length is adequately chosen to get the steady-state orientation of the molecules in the sample solution without degrading or denaturing it; (ii) to avoid Joule effect at high fields, experiments were conducted at low ionic strength (1 mM sodium cacodylate buffer pH 6.5) using a Kerr cell of 1 cm path length with the electrodes separated by 1.5 mm; (iii) measurements were made at low binding ratios (drug over mononucleotide concentration, $D/P = 0.1$) to favour intercalation with respect to external binding when two binding modes are possible. In all experiments, polynucleotide (or DNA) and ligand concentrations were 100 μM and 10 μM, respectively.

At a given electric field strength, the reduced dichroism $\Delta A/A$ is related to the steady-state orientation function Φ by:

$$\Delta A/A = 3/2(3\cos^2\alpha - 1) \cdot \Phi \quad (2)$$

where α is the angle between the orientation axis of the macromolecule and the direction of the optical transition moment in the considered absorption region. At complete orientation of DNA (i.e. at infinite field strength, $\Phi = 1$), the main orientation axis of the macromolecule is parallel to the electric field and the in-plane transition moments of the DNA bases are nearly perpendicular to the field. Consequently, the α value is equal to 90° and the maximum negative value of $\Delta A/A$ amounts to -1.5 (Fig. 4c). In the following, we shall analyze neither the orientation function nor the transient states of the electro-optical signals, but only the steady-state.

In practice, $(\Delta A/A)_{DNA}$ at infinite field strength never reaches the limiting value of -1.5 for several reasons:

1. DNA flexibility increases with its molecular weight so that the mean α-value for DNA is about 72° [17];
2. sequence dependent intrinsic bending of DNA restriction fragments may appear.

3.2. Usefulness of the ELD technique to study DNA–drug interactions

The sequence selectivity of numerous DNA ligands – intercalators and groove binders – has been investigated by the so-called footprinting techniques [18,19]. Although very informative, the footprinting methods using enzymic or chemical nucleases are rather delicate to handle, require the use of high drug/DNA binding ratios and furthermore necessitate the manipulation of radioactive compounds. The sequence preference of drugs binding to DNA can also be examined using spectroscopic techniques such as circular dichroism, stopped-flow, NMR and linear dichroism [20–25]. ELD proves to be a particularly rapid and convenient tool with which one can obtain reliable information on both the binding mode and the sequence preference.

To this aim, we have conducted ELD experiments on complexes of drugs with natural DNAs of different base compositions as well as with polynucleotides containing defined alternating and non-alternating sequences. Inosine, unlike guanosine, has no amino group in its C2 position; hence only two hydrogen bonds are formed in the I–C base pair association, as for an A–T base pair, and there is no steric hindrance of an amino group in the minor groove (Fig. 5).

If α and β, respectively, represent the angles between the transition moment directions of the bases and the ligand chromophores, and the orientation axis of the macromolecule in the electric field, we

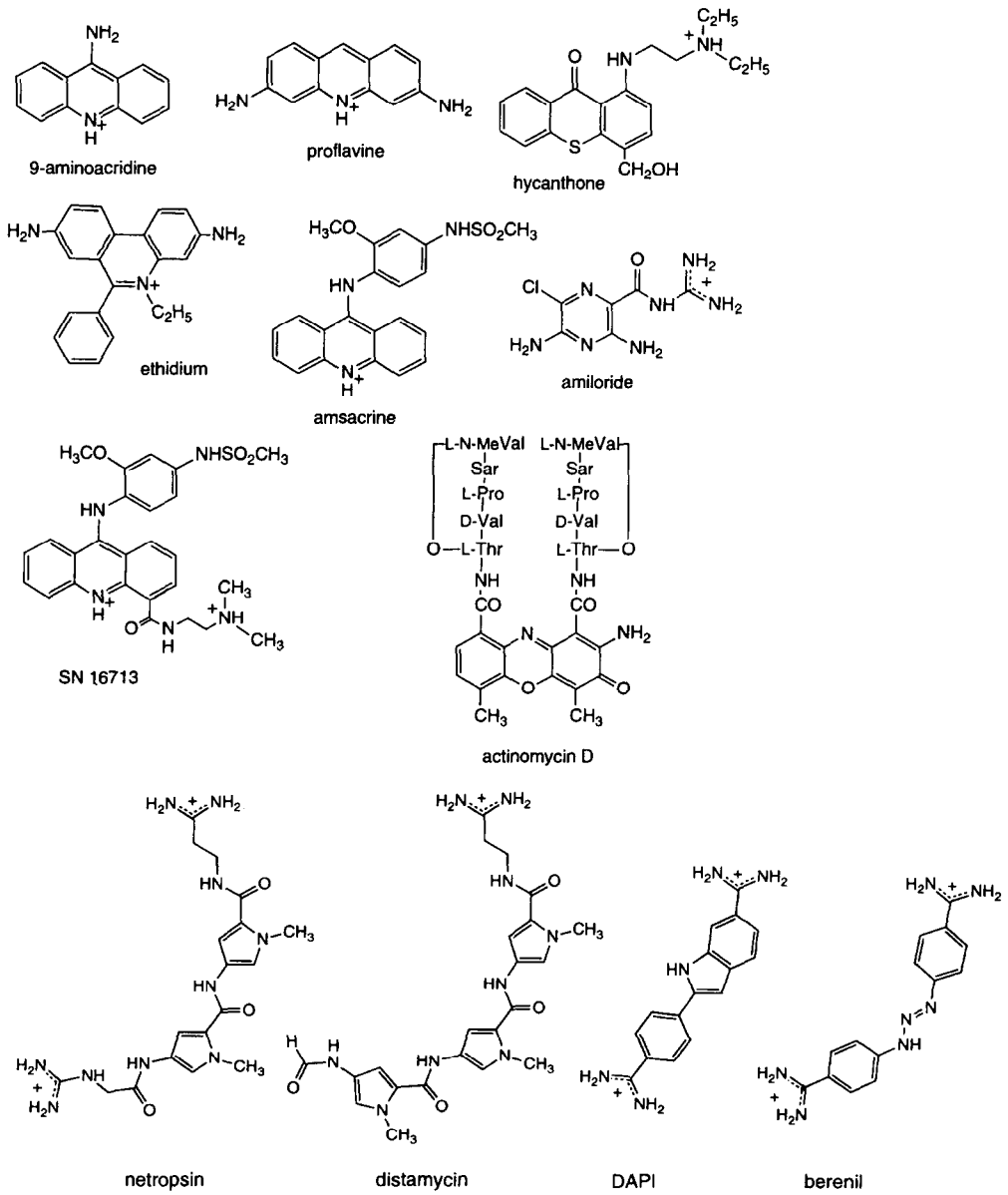

Fig. 2. Chemical structures of intercalating and groove binding drugs.

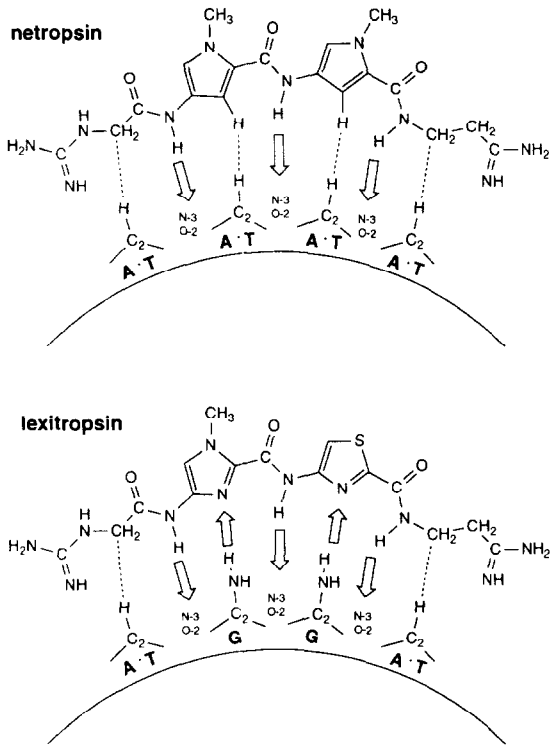

Fig. 3. Representation of the binding to DNA of netropsin and a lexitropsin. Arrows represent hydrogen bonds from donor to acceptor; dashed lines indicate van der Waals interactions. Replacement of the pyrrole rings of netropsin with imidazole or thiazole rings allows hydrogen bonding between the 2-amino group of guanines and the newly introduced heterocyclic nitrogen.

can define at a given field strength the reduced dichroism ratio

$$DR = \frac{(\Delta A/A)_{\text{ligand}}}{(\Delta A/A)_{\text{DNA}}} = \frac{3\cos^2\beta - 1}{3\cos^2\alpha - 1}. \qquad (3)$$

This implies that the degree of orientation Φ in the electric field and the macromolecular structure are not greatly affected by the binding, a reasonable assumption at low amounts of binding. β can be determined using either $\alpha = 90°$ (canonical Watson–Crick structure) or $\alpha = 72°$ (experimental value).

The amplitude and sign of the reduced dichroism $\Delta A/A$ depend on the relative orientation of the drug with respect to the base planes. Therefore, the measurement of $\Delta A/A$ enables to distinguish between intercalating and non-intercalating drugs. For comparative purposes, the reduced dichroism of the ligand–DNA complex measured in the ligand absorption band must be analyzed with respect to the

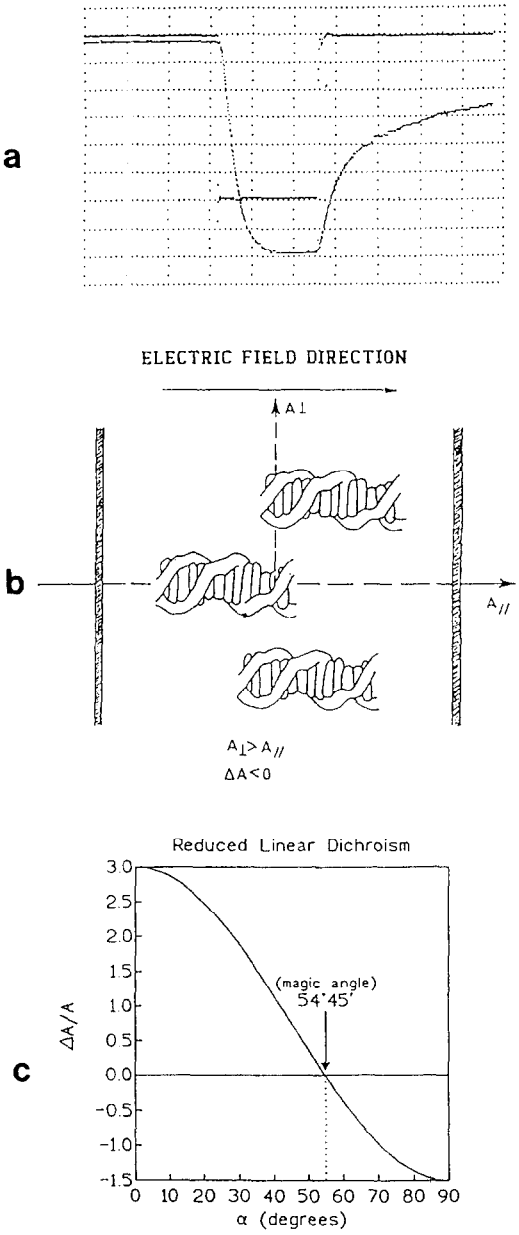

Fig. 4. (a) General shape of electro-optical signal. (b) Model of DNA orientation in the electric field. (c) Variation of the reduced linear dichroism as a function of the angle α between the orientation axis of the macromolecule and the direction of the optical transition moment in the considered absorption region.

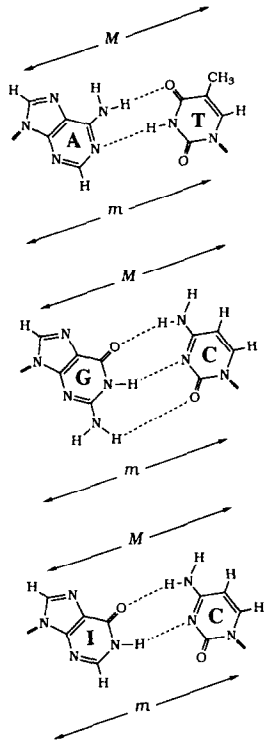

Fig. 5. Structure of hydrogen-bonded purine–pyrimidine base pairs. Broken lines represent hydrogen bonds. I, inosine. The major groove of the helix is indicated by M and the minor groove by m.

reduced dichroism measured for the same DNA or polynucleotide at 260 nm in the absence of drug. Since the reduced dichroism in the base pair absorption region, in the absence of drug, is always negative (due to the stacking of the bases perpendicularly to the helix axis), *DR* is expected to be +1 if the transition moment of the drug chromophore is parallel to the purine–pyrimidine bases, i.e. in the case of an intercalative binding. For groove binders, the angle of the ligand chromophore long axis is inferior to 54.7° with respect to the double helical axis, yielding a positive dichroism for the ligand, so that *DR* should become negative. In these conditions, *DR* values for any given drug–DNA and drug–polynucleotide complex can be mutually compared with good relative accuracy, independently of the size of the polymer. The *DR* values for a given drug–DNA complex is representative of the geometry of the complex but cannot be considered as an indication of the relative affinity of a drug for the DNA used.

The relative preference of a drug for AT versus GC sequences may be characterized by the copolymer *DR* index (*CPI*) and homopolymer *DR* index (*HPI*):

$$CPI = \frac{DR^{\text{poly(dA-dT)}\cdot\text{poly(dA-dT)}}}{DR^{\text{poly(dG-dC)}\cdot\text{poly(dG-dC)}}}$$

$$HPI = \frac{DR^{\text{poly(dA)}\cdot\text{poly(dT)}}}{DR^{\text{poly(dG)}\cdot\text{poly(dC)}}} \qquad (4)$$

The higher the *CPI* value, the more pronounced is the preference of the drug for alternating copolymer dA–dT sequences as compared to dG–dC sequences. Similarly, the higher the *HPI* value, the stronger the preference of the drug for non-alternating homopolymer dA · dT sequences as compared to dG · dC sequences.

4. Results and discussion

4.1. Orientation of DNA and polynucleotides

The three natural DNAs from *Clostridium perfringens* (26% GC), calf thymus (42% GC) and *Micrococcus lysodeikticus* (72% GC) give similar $\Delta A/A$ values of −0.4 at 12.5 kV/cm. The situation is quite different for the synthetic polynucleotides for which a significant dispersion of $\Delta A/A$ values is observed at the same electric field strength (Fig. 6). This behaviour arises from differences in size and

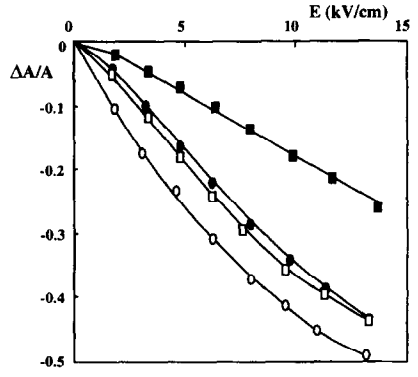

Fig. 6. Electric field dependence of the ELD of synthetic polynucleotides. (●) poly(dA–dT)·poly(dA–dT); (○) poly(dA)·poly(dT); (■) poly(dG–dC)·poly(dG–dC); (□) poly(dG)·poly(dC). ELD data were recorded in the presence of 100 μM polynucleotide in 1 mM sodium cacodylate buffer pH 6.5.

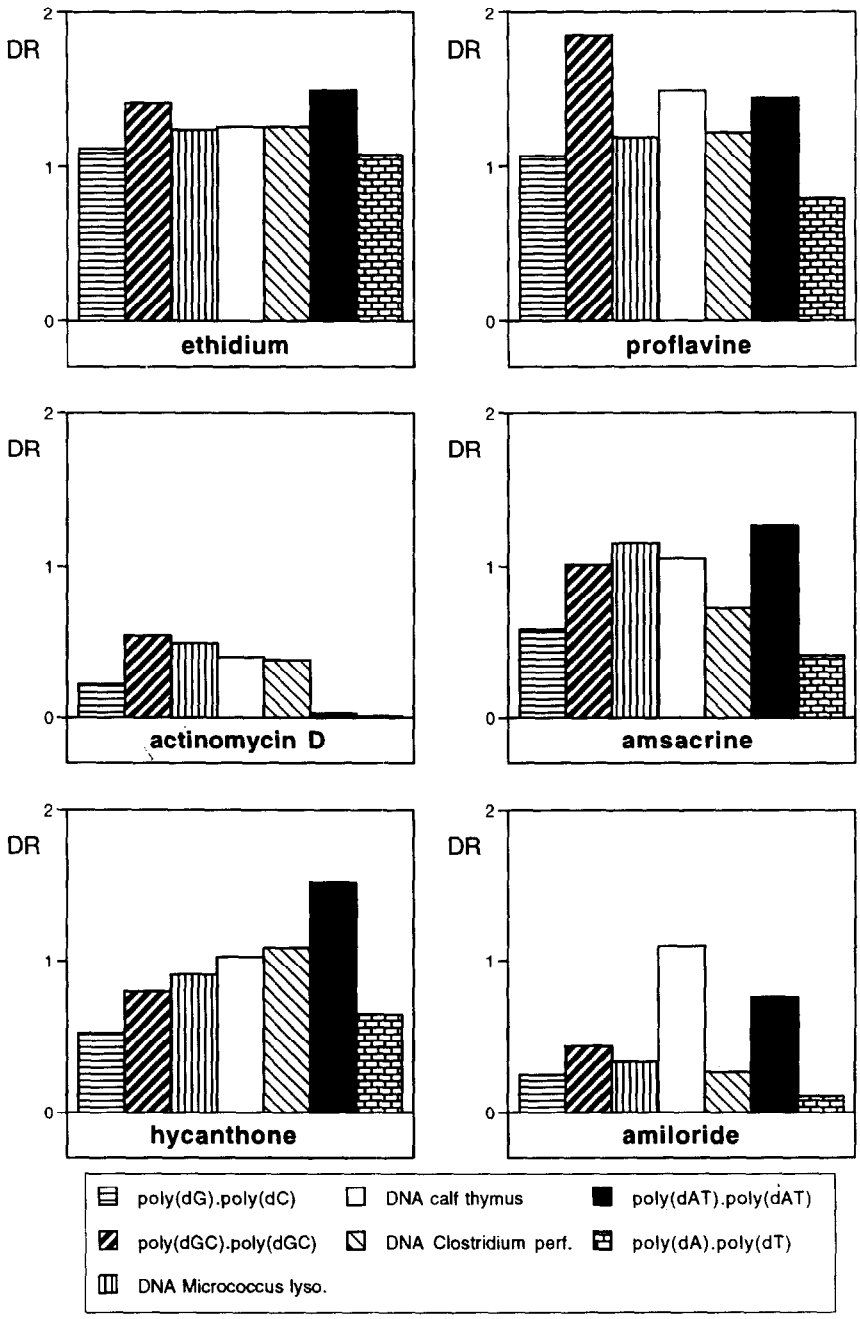

Fig. 7. Variations of the *DR* of ethidium, proflavine, actinomycin D, amsacrine, hycanthone and amiloride bound to DNAs of different base pair compositions. ELD data were recorded in 1 mM sodium cacodylate buffer pH 6.5, at a drug/DNA ratio of 0.1 at 13 kV/cm. ELD measurements were performed at 520 nm for ethidium, 460 nm for proflavine and actinomycin D, 440 nm for amsacrine, 360 nm for amiloride and 340 nm for hycanthone. Polynucleotide (DNA) and ligand concentrations are 100 μM and 10 μM, respectively.

flexibility of the polynucleotides and in variable polydispersity from one batch to another for the same polynucleotide. Therefore, it is always necessary to use the above defined *DR* parameter to get reliable information from ELD measurements.

4.2. Intercalation

Several techniques have to be used concomitantly to assess with some confidence whether or not a drug intercalates into DNA. Let us mention: viscosity of sonicated DNA–drug complex, drug-induced unwinding of supercoiled DNA, ELD and CD (circular dichroism) measurements on DNA–drug complexes.

So far, we have tested about twenty intercalators [6]. In each case, *DR* values are positive indicating that their reduced dichroism in their absorption band has the same sign as that of DNA at 260 nm, i.e. in both cases $\Delta A/A$ is negative. However, the amplitude of the *DR* values varies from drug to drug for a given DNA sample and, for a given drug, from one DNA (or polynucleotide) to another. A *DR* value close to 1 indicates that the chromophore plane of the drug is parallel to the base planes. *DR* values higher than 1 arise from the fact that the intercalation results in a drug-induced stiffening of the DNA molecule. Finally, *DR* values lower than 1 are indicative of a partial intercalation or a tilting of the drug chromophore with respect to the base planes.

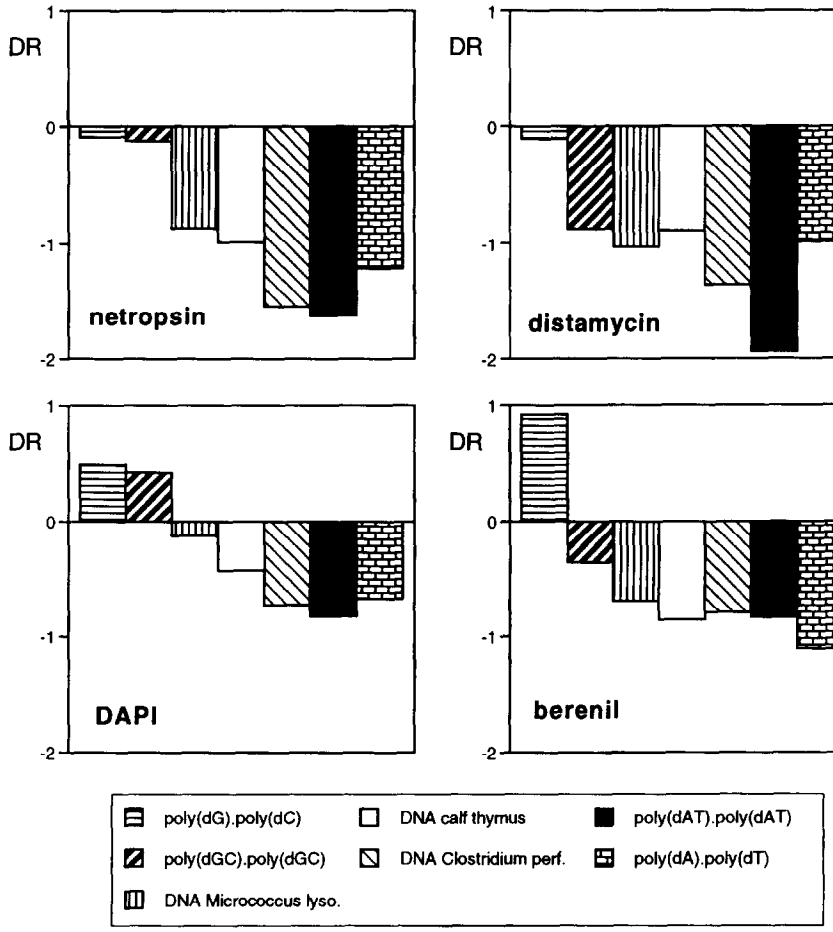

Fig. 8. Variations of the *DR* of netropsin, distamycin, DAPI and berenil bound to DNAs of different base pair compositions. ELD measurements were performed at 310 nm for netropsin, 330 nm for distamycin and 360 nm for DAPI and berenil. Other details as in Fig. 7.

Fig. 7 displays the *DR* values observed in the case of six typical intercalating agents. Ethidium bromide does not show any particular sequence selectivity since the *DR* values do not change appreciably with base composition and the *CPI* and *HPI* values are both close to 1. At most, a slight prefer-

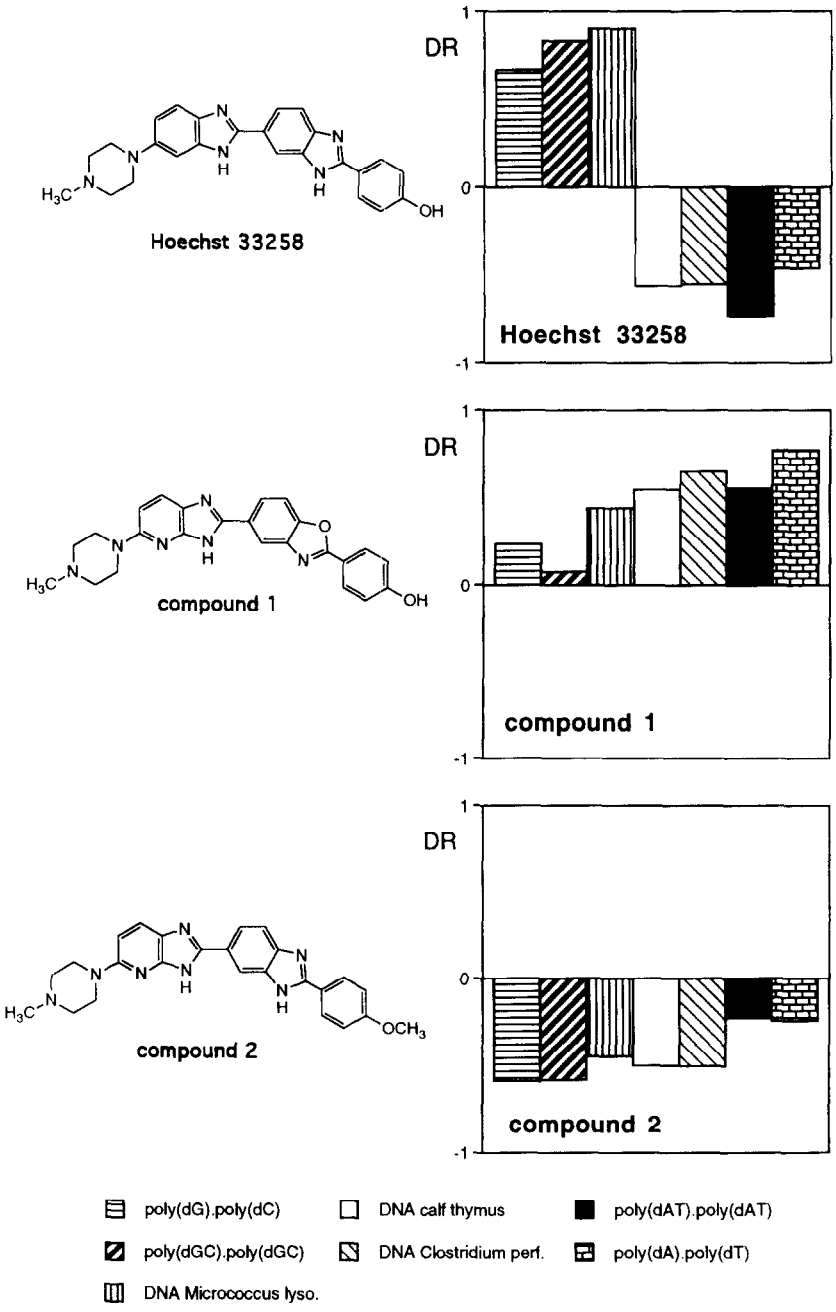

Fig. 9. Chemical structure and variations of the *DR* of Hoechst 33258 and two analogues bound to DNAs of different base pair compositions. ELD measurements were performed at 360 nm for the three compounds. Other details as in Fig. 7.

ence for alternating purine–pyrimidine sequences is observed in agreement with footprinting studies [26]. Proflavine exhibits a certain preference for binding to GC sequences as indicated by the low *CPI* and *HPI* values (= 0.75). But experiments with the three natural DNAs do not confirm this preference. This apparent discrepancy may be understood as follows: there are ten distinct dinucleoside intercalation sites presenting different strengths of interaction with drug chromophores. Depending on the sequence surrounding the intercalation site, the strength of binding can vary significantly and the drug chromophore may adopt slightly different orientations with respect to the base pair planes.

The ability of actinomycin D to intercalate into DNA has long been established and the binding to DNA is characterized by a general requirement for GpC steps [21,27]. The ELD analysis offers corroborating evidence for the GC selectivity. Indeed, the *DR* values increase regularly with the GC content of natural DNAs and the higher value is obtained with the alternating polymer poly(dG–dC).poly(dG–dC). The *DR* values measured with the AT polymers are practically insignificant. It should also be noticed that the *DR* values of any actinomycin D–DNA complex never exceed 0.5 whatever the base composition. This suggests that either full intercalation of the phenoxazinone ring of actinomycin D is pre-

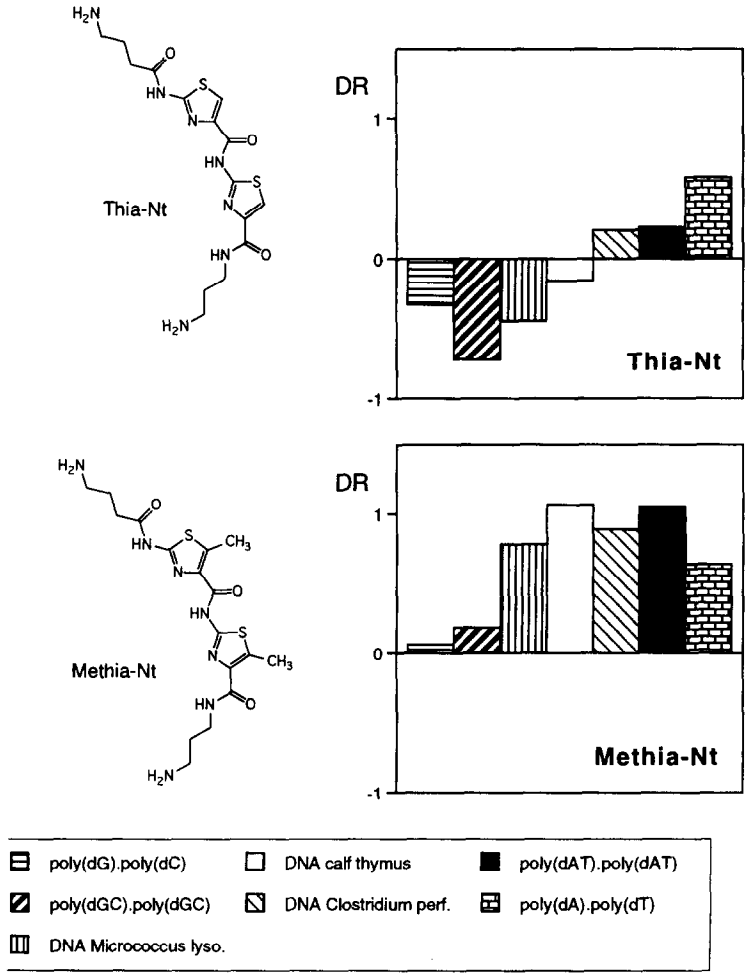

Fig. 10. Chemical structure and variations of the *DR* of two thiazole derivatives of netropsin, Thia-Nt and Methia-Nt, bound to DNAs of different base pair composition. ELD measurements were performed at 310 nm for both compounds. Other details as in Fig. 7.

cluded by the steric hindrance caused by the attached bulky cyclic decapeptide chains or that the DNA is distorted upon drug binding so that the bases are markedly tilted with respect to the helical orientation axis [28].

The ELD analysis for the amsacrine–DNA complexes does not indicate any clear sequence preference, although the *CPI* (= 1.25) values suggest a slight preference for AT over GC sequences, in agreement with theoretical calculations [29], thermodynamic and equilibrium binding studies [30,31]. The amsacrine 4-carboxamide derivative SN 16713 (data not shown) together with actinomycin D is the most discriminative drug among the intercalating agents studied so far. The carboxamide side chain of SN 16713, which is located in the minor groove upon binding to DNA, seems to play a determinant role in the GC selectivity as evidenced by a *CPI* value equal to 0.66; here again, the ELD measurements confirm the selectivity previously evidenced from stopped-flow kinetics and footprinting studies [32,33].

Classical intercalators typically bear two or three fused aromatic rings with protonated heterocyclic nitrogens or protonated side chains attached to these rings. However, two among the intercalators studied here are not classical in a sense that either they possess no charge on the heterocyclic ring (hycanthone) or they contain only one aromatic ring (amiloride). These two compounds give negative reduced dichroism values, whatever the base composition (i.e. *DR* is always positive) and exhibit a marked AT selectivity (*CPI* values between 1.7 and 2) (Fig. 7).

The weaker binding of most intercalators to homopolymers than to alternating ones probably arises from their non-classical B-form (either A-form like or intermediate between A- and B-forms or intrinsically bent DNA). This results in a lower ability of these polymers to accommodate an intercalating chromophore than B-DNA.

4.3. Minor groove binding versus intercalation

Netropsin (Net) and distamycin (Dst) contain two and three N-methylpyrrole rings, respectively. They both hold positive charges (+2 for Net and +1 for Dst). Fig. 8 shows the *DR* values obtained for the DNAs and polynucleotides complexed with these two ligands. *DR* values are negative indicating that the ligand–DNA complex exhibits a positive reduced dichroism in the ligand absorption band. For both ligands, *DR* becomes more negative with increasing AT content of natural DNAs and the most negative value is obtained with the alternating poly(dA–dT).poly(dA–dT). Both the *CPI* and *HPI* values are very large (about 12) reflecting a pronounced selectivity for binding in the minor groove of AT sequences, as several techniques (NMR, crystallography, equilibrium binding) have shown previously [34].

Several other groove binders such as DAPI, Hoechst 33258, berenil, and certain lexitropsins, were considered, until about five years ago, as pure minor groove binders like netropsin and distamycin. Most binding experiments with these ligands used calf thymus DNA (42% GC) or oligonucleotides [35]. However, in 1990, Wilson et al. [24,36] and Norden et al. [37], using GC polynucleotides, proposed that DAPI has the property to intercalate at GC sites. This sequence-dependent binding mode was established on the basis of results from several complementary biophysical techniques including NMR, flow linear dichroism, fluorescence, absorption, circular dichroism and kinetic measurements.

The application of the ELD technology confirmed the sequence-related binding mode of DAPI (Fig. 8), and allowed us to show that similar trends occur for Hoechst 33258 and to a lesser extent for berenil (Figs. 8 and 9). With these three drugs, we obtained negative *DR* values with AT-rich DNA and positive *DR* values with GC-rich DNA and/or GC polynucleotides. The negative *DR* values (positive $\Delta A/A$) reflect the binding of the drugs in the minor groove of AT sequences. On the opposite, the positive *DR* values clearly indicate that these drugs do not bind to the minor groove of GC sequences. For berenil, positive *DR* values (negative $\Delta A/A$) are measured only with the homopolymer poly(dG)·poly(dC) whereas Hoechst 33258 gives rise to positive *DR* upon binding to both the GC polynucleotides and the GC-rich DNA from *Micrococcus lysodeikticus* (Figs. 8 and 9). We know that only one criterion cannot be used to demonstrate an intercalative binding process [38]. However, especially in the case of Hoechst 33258, where high positive *DR*

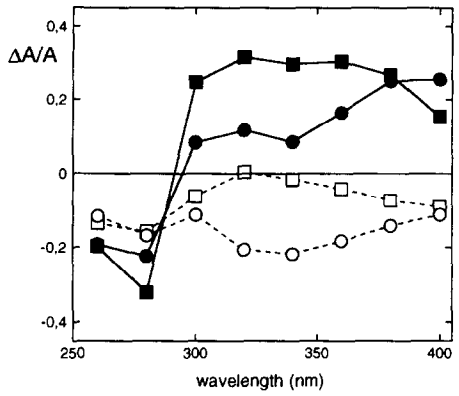

Fig. 11. Reduced linear dichroism ($\Delta A/A$) spectra of the complexes between poly(dI–dC)·poly(dI–dC), poly(dG–dC)·poly(dG–dC) and the drugs Hoechst 33258 and DAPI, at a drug/DNA ratio of 0.1 and at 13 kV/CM in 1 mM sodium cacodylate buffer pH 6.5. poly(dI–dC)·poly(dI–dC) with DAPI (■) and Hoechst 33258 (●) poly(dG–dC)·poly(dG–dC) with DAPI (□) and Hoechst 33258 (○) Other details as in Fig. 7.

values are observed with GC-rich DNAs, neither a non-selective external binding nor a stacking of the dimer (unlikely to occur in our experimental conditions) can be invoked to justify such *DR* values.

Rao and Lown [39] have designed a series of Hoechst 33258 analogues which exhibit altered base and sequence recognition. They studied their DNA binding characteristics to calf thymus DNA and polynucleotides with different base compositions using UV absorption, fluorescence and circular dichroism techniques. Depending on the substitutions in the benzimidazole rings, AT preference or acceptance of GC base pairs adjacent to AT base pairs were observed. In this case again, the ELD technique provided a sensitive tool to show that the mode of binding of these Hoechst analogues varies significantly according to the DNA sequence. Two bisbenzimidazole derivatives differing only by the presence of oxygen or nitrogen in position 2 show a completely different binding mode to DNA (Fig. 9). The first one behaves as an intercalator and the second one as a minor groove binder, whatever the sequence of the receptor site [40].

The compound Thia-Nt is a synthetic lexitropsin for which the pyrrole rings of netropsin have been replaced with thiazole heterocycles with the nitrogen atoms facing the floor of the minor groove. The substitution of the thiazoles for the pyrroles results in

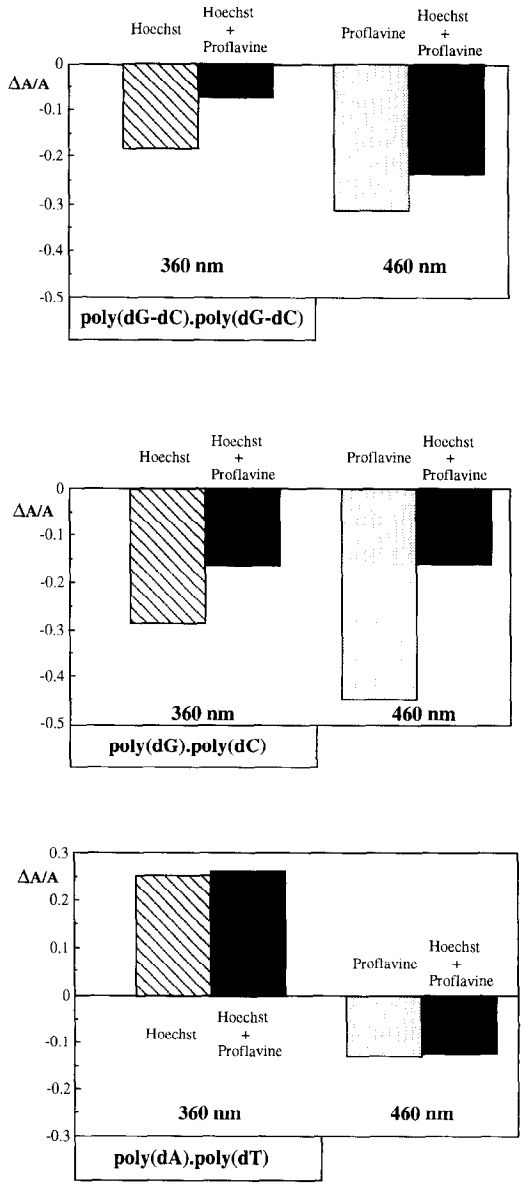

Fig. 12. Competitive binding between Hoechst 33258 and proflavine. Variation of the reduced dichroism $\Delta A/A$ of the complexes between poly(dG–dC)·poly(dG–dC) (top), poly(dG)·poly(dC) (middle) or poly(dA)·poly(dT) (bottom) and Hoechst 33258 (dashed bars), proflavine (dotted bars) or Hoechst 33258 plus proflavine (filled bars). Measurements were made at 13 kV/cm in 1 mM sodium cacodylate buffer pH 6.5, at 360 and 460 nm. The DNA concentration is 100 μM, the drug concentrations are 10 μM.

an altered sequence preference with a shift from AT to GC base pairs. If we compare the variations of *DR*s for netropsin (Fig. 8) and Thia-Nt (Fig. 10), we can conclude that this latter one has an intercalator-like behaviour when the GC content of DNA increases, contrary to netropsin which always behaves as a minor groove binder, whatever the base composition. The introduction of methyl groups on the thiazole rings of Thia-Nt contributes to a better binding to DNA and this new biscationic lexitropsin (called Methia-Nt) is believed to intercalate into DNA [41], independently of the base composition of the DNA whereas the non-methylated analogue Thia-Nt intercalates only in GC-rich DNA and polymers (Fig. 10). Especially with poly(dG–dC) · poly(dG–dC), both lexitropsins show superimposable spectra of reduced dichroism as a function of wavelength in the drug absorption region (data not shown). Complementary CD and viscometric measurements, together with the ELD data, reinforce the view that compound Methia-Net is a DNA-intercalating lexitropsin [41].

4.4. Do 'minor groove binders' intercalate into GC sites?

The particular behaviour of several 'minor groove binders' in the presence of GC-rich DNA and GC polymers prompted us to investigate further this phenomenon.

First, we studied the ability of Hoechst 33258, berenil and DAPI to bind to the alternating polymer

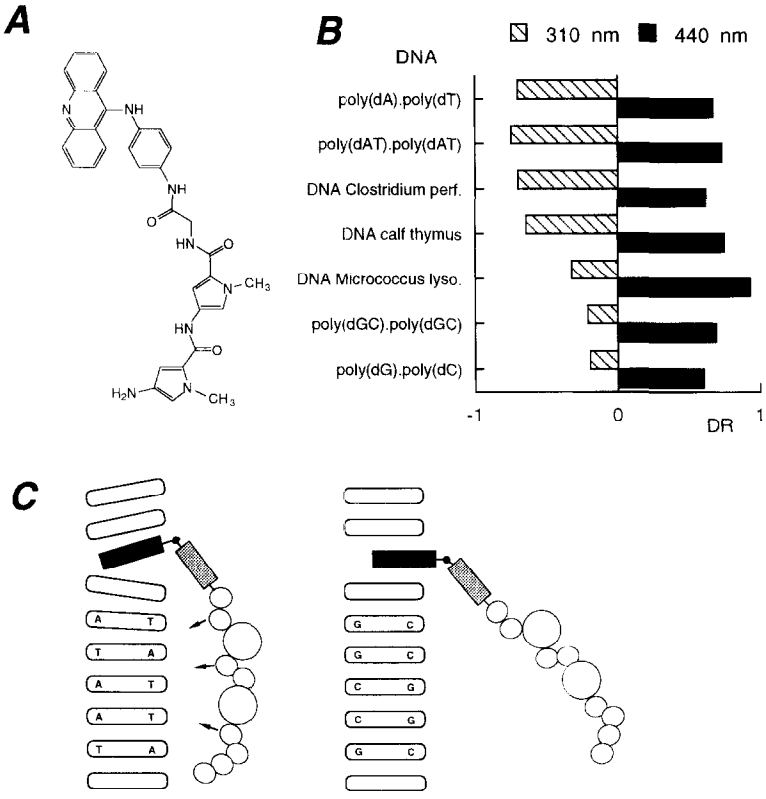

Fig. 13. (A) Chemical structure of the netropsin–anilinoacridine combilexin. (B) Variation of the *DR* of this combilexin bound to DNAs of different base pair compositions. ELD data were recorded at 310 and 440 nm at 13 kV/cm. Other details as in Fig. 7. (C) Schematic illustration showing the proposed orientation of the drug with respect to the DNA base pairs. The acridine ring is represented by a black rectangle, the anilino group by a grey rectangle and the netropsin by the open rings (the two largest ones represent the N-methylpyrrole rings). The DNA is symbolized by a series of superimposed white rectangles representing base pairs. Small arrows indicate the potential formation of hydrogen bonds between the pseudopeptide and DNA.

poly(dI–dC) · poly(dI–dC). Substitution of inosine for guanine completely changes the binding process because *DR* values become negative ($\Delta A/A > 0$) (Fig. 11). The only difference between inosine and guanosine is the absence of exocyclic amino group in inosine. This bulky substituent is located in the minor groove of DNA and participates in the hydrogen bonding of a GC base pair (Fig. 5). A higher negative electrostatic potential is present in the minor groove of AT sequences. The presence of the amino group hinders the three compounds studied to fit snugly into the minor groove as observed with netropsin and distamycin. These two latter ligands (Net, Dst) have four NH sites whereas berenil, Hoechst 33258 and DAPI have only one or two NH groups which they can use to form hydrogen bonds with the base pairs in the minor groove, resulting in a less stringent sequence specificity [42,43].

Second, competition experiments between two ligands for DNA, using the ELD technique, have been pursued with the aim to further characterize the binding mode at GC sites. Proflavine was used to further investigate the mechanism of binding of Hoechst 33258 to GC sequences. Proflavine was selected for three main reasons: (i) the non-specific intercalative binding of proflavine to DNA is well-established; (ii) the absorption maxima of proflavine and Hoechst 33258 are well separated so that we can easily distinguish the test drug from the competing ligand by ELD; (iii) both ligands exhibit comparable affinities for DNA. Proflavine is a classical intercalator and 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. The absorption band of Hoechst 33258 complexed with DNA is centered at 360 nm, i.e. a wavelength at which the absorbance of proflavine is negligible. Finally, both ligands have binding affinities for GC polynucleotides of the same order of magnitude. ELD study of the drugs–DNA complexes (Hoechst 33258-DNA-proflavine) supports the involvement of an intercalative binding rather than an external or major groove binding for Hoechst 33258 at GC sequences [44] as evidenced in Fig. 12. Recently, these competition experiments were extended to berenil and DAPI using a classical intercalator (proflavine) and a DNA-threading intercalating drug (the amsacrine 4-carboxamide derivative SN 16713). The two acridines were selected among intercalating drugs for two main reasons: first, for their spectral properties (see above) and, second, for having identical chromophores but presenting distinct DNA binding properties. Unlike proflavine, SN 16713 exhibits a sharp selectivity for intercalation into GC sequences [33]. However, both ligands display comparable affinities for DNA. Preliminary ELD results (data not shown) support the hypothesis that Hoechst 33258, berenil and DAPI interact with GC sequences via a non-classical intercalation process, which, however, does not seem to be strictly the same for the three ligands.

4.5. Hybrid ligands

Before designing hybrid molecules, we studied the compatibility of binding to DNA of several intercalators and groove binders. In most cases, we found that binding of a drug in the minor groove has little, if any, effect on the intercalation process and vice versa. Recent studies [45] clearly showed that DNA can fully accommodate the minor groove binding of netropsin and the intercalation of amsacrine in close proximity. Such studies using ligands with different mechanisms of binding to DNA led us to envisage the development of combilexin molecules associating an intercalating chromophore (e.g. acridine, ellipticine) and a minor groove binder (e.g. netropsin, distamycin). A typical example of such a conjugate molecule is shown in Fig. 13. Here again, ELD was particularly useful to investigate the binding of the two functionalities of the hybrid. The measurements of the reduced dichroism in the netropsin and the acridine bands (at 310 and 440 nm, respectively) allow the determination of the orientation of both moieties of the hybrid upon interaction with DNA. The positive reduced dichroism measured at 310 nm and the negative dichroism at 440 nm strongly suggest that the two connected moieties interact with DNA in a well defined geometry. The use of several DNAs and polynucleotides showed that the geometry of the hybrid drug–DNA complex can vary according to the target sequence. The ELD data fully support the footprinting data in showing that the acridine moiety of the hybrid can intercalate into DNA whatever the DNA sequence. On the opposite, the netropsin moiety fits into the minor

groove of AT-rich sequences but not of GC-rich sequences. In other words, as depicted in Fig. 13, the netropsin–anilinoacridine combilexin can engage in a bidentate binding at AT sites whereas only the acridine chromophore intercalates at GC sites. Electric linear dichroism proves to be most appropriate to study the DNA binding properties of such hybrid ligands.

5. Conclusion

Linear dichroism represents one of the most valuable techniques for structural studies of drug–DNA complexes in solution. Although several complementary techniques need to be used to elucidate a given mode of binding to DNA, linear dichroism is a suitable method for accurate determination of the orientation of drugs upon binding to nucleic acids [4,38]. This technique, compared with other spectroscopic methods, has the advantage that the unbound ligand molecules do not contribute to the signal. However, one of the limitations is the prerequisite to work at low ionic strength with our apparatus. Our study did not address the field strength dependence of the ELD signal as the aim of the present work was to compare binding modes and DNA sequence selectivity of several ligands. We worked in such experimental conditions that the potential changes in the orientation function are believed to be minimized (though not completely excluded). In a previous study [46], we showed that the electric field dependences of the ELD for calf thymus DNA alone at 260 nm and for DNA complexed with the combilexin Gly–His–Lys–netropsin anilino aminoacridine (GHK–NetGA) at 440 nm were perfectly superimposable.

So far, most linear dichroism studies have been carried out with DNA of random sequences (e.g. calf thymus DNA). Here we show that the use of both natural DNAs of differing base composition and polynucleotides (containing defined alternating and non-alternating sequences) provides insights into the sequence selectivity of drug binding to DNA. Comparative ELD analyses of the binding of drugs to natural DNAs and polynucleotides yield, within a few hours, fruitful information on both the mode of binding and the relative sequence selectivity. In particular, the method appears invaluable in showing the dependence of the orientation of the bound drug on the sequence of DNA target. Whereas there exist numerous techniques to evidence intercalation of a chromophore into DNA, there are only a few to show that a drug binds in the minor groove. NMR techniques are rather time- and material-consuming. By contrast, ELD can provide reliable information on a drug–DNA complex using small quantities of materials. An intercalated drug gives rise to a negative dichroism whereas a drug located in the minor groove usually exhibits a positive dichroism. Because of this unique characteristic, linear dichroism is probably the best technique to distinguish between these two DNA binding modes and therefore it represents the technique of choice to investigate the mechanism of interaction between DNA and intercalator minor groove binder hybrid molecule. The sequence preference deduced from the ELD measurements reported here for a variety of intercalating and minor groove binding drugs greatly improves our knowledge of the DNA recognition properties inferred from other spectroscopic and biochemical studies. In particular, the conclusions drawn for sequence selective drugs such as actinomycin, hycanthone, netropsin or distamycin are totally consistent with the literature data.

In short, the ELD technique is extremely useful to rapidly evaluate intercalating versus non-intercalating drugs, AT versus GC preference and homopolymer versus heteropolymer polynucleotides binding capabilities. ELD should now be considered as a valuable tool to investigate drug–DNA sequence recognition.

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