# Binding Properties and DNA Sequence-Specific Recognition of Two Bithiazole-Linked Netropsin Hybrid Molecules†

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ABSTRACT: We report the DNA binding properties of two hybrid molecules which result from the combination of the DNA sequence-specific minor groove ligand netropsin with the bithiazole moiety of the antitumor drug bleomycin. The drug–DNA interaction has been investigated by means of electric linear dichroism (ELD) spectroscopy and DNase I footprinting. In compound **1** the two moieties are linked by a flexible aliphatic tether while in compound **2** the two aromatic ring systems are directly coupled by a rigid peptide bond. The results are consistent with a model in which the netropsin moiety of compound **1** resides in the minor groove of DNA and where the appended bithiazole moiety is projected away from the DNA groove. This monocationic hybrid compound has a weak affinity for DNA and shows a strict preference for A and T stretches. ELD measurements indicate that in the presence of DNA compound **2** has an orientation typical of a minor groove binder. Similar orientation angles were measured for netropsin and compound **2**. This ligand which has a biscationic nature tightly binds to DNA ($K_a = 6.3 \times 10^5$ $M^{-1}$) and is mainly an AT-specific groove binder. But, depending on the nature of the sequence flanking the AT site first targeted by its netropsin moiety, the bithiazole moiety of **2** can accommodate various types of nucleotide motifs with the exception of homooligomeric sequences. As evidenced by footprinting data, the bithiazole group of bleomycin acts as a DNA recognition element, offering opportunities to recognize GC bp-containing DNA sequences with apparently a preference (although not absolute) for a pyrimidine-G-pyrimidine motif. Thus, the bithiazole unit of bleomycin provides an additional anchor for DNA binding and is also capable of specifically recognizing particular DNA sequences when it is appended to a strongly sequence selective groove binding entity. Finally, a model which schematizes the binding of compound **2** to the sequence 5′-TATGC is proposed. This model readily explains the experimentally observed specificity of this netropsin–bithiazole conjugate.

The design of sequence-selective DNA-binding ligands has attracted much interest due to its significance in understanding the elements of recognition between proteins and DNA and, as a consequence, the specific processes involved in gene expression and control. Moreover, from a pharmacological point of view, sequence specificity of drug binding is also of utmost importance since this may affect the activity of particular genes, leading to a potentially more selective treatment of certain diseases such as cancer.

The widely investigated antibiotic netropsin (Figure 1), which represents the prototype of nonintercalative DNA minor groove binder (Zimmer & Wähnert, 1986), has been subjected to extensive chemical modifications in order to alter its strong AT selectivity toward GC recognition. For that purpose, a strategy has been elaborated and consists in the replacement of the *N*-methylpyrrole rings of netropsin by other appropriate heterocycles such as imidazole, thiophene, thiazole, triazole, furan, or pyrazole. These *lexitropsins*—or information reading oligopeptides—obtained so far, in general, no longer exhibit the pronounced AT specificity typical of most groove binding agents (e.g., netropsin, distamycin, berenil, Hoechst 33258) but bind to AT–GC mixed sequences [for comprehensive review see Lown (1988) and Nielsen (1991)].

In parallel, a few research groups interested in medicinal application of DNA-binding ligands have focused their attention on the development of hybrid ligands combining minor groove binding entities to intercalating agents. The rationale behind the design of such *combilexins*—so-called by analogy with the *lexitropsins* (Bailly & Hénichart, 1991)—is that intercalation combined with groove binding can afford improved affinity for DNA as well as nucleotide sequence selectivity. Moreover, as previously shown with a netropsin–acridine hybrid molecule, the intercalating chromophore (i) can favor the rapid penetration of the hybrid into the cell and its accumulation into the nuclear compartment

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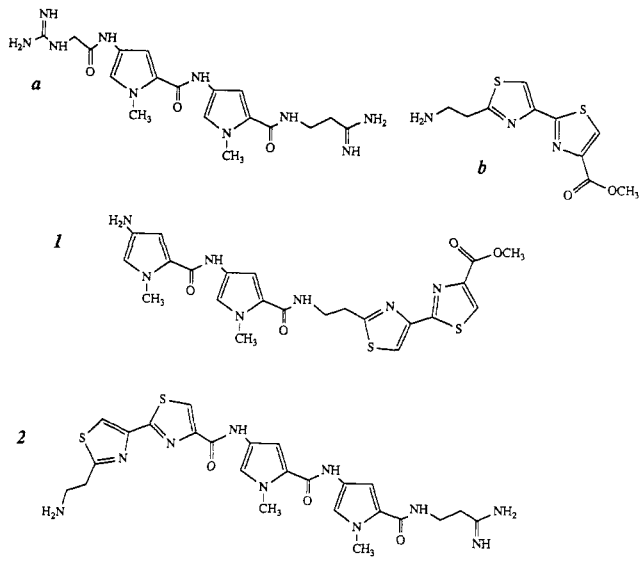


FIGURE 1: Structure of netropsin (a), the bithiazole compound (b), and the netropsin–bithiazole hybrid molecules **1** and **2**.

(Bailly & Hénichart, 1990) and (ii) allows the interaction with topoisomerase II and the modulation of its catalytic activity (Bailly et al., 1992a). This latter point is particularly important since topoisomerases are considered as one of the principal cellular targets for numerous anticancer drugs used in the clinic (Lock & Ross, 1987; Zunino & Capranico, 1990). Thus both *lexitropsin* and *combilexin* approaches show promise and warrant further development.

In the present study, two new hybrid molecules were synthesized; their structural formulae are given in Figure 1. Both include a netropsin skeleton linked to a bithiazole ring either at the carboxyl terminus (compound **1**) or at the amino terminus (compound **2**) of the bis-pyrrole unit. The two compounds mainly differ in two respects. First, they are differently charged: **1** is monocationic while **2** has a biscationic nature. Second, in compound **1** the two moieties are linked by a flexible aliphatic tether while in compound **2** the two aromatic ring systems are directly coupled by a rigid peptide bond. Therefore, because the two drugs are structurally rather different, it is not surprising that these two synthetic DNA ligands exhibit different affinities and specificities for DNA.

The mode of binding to DNA of the bithiazole moiety of bleomycin is still a matter of debate. Classical intercalation, partial stacking between base pairs, binding in the minor groove, and insertion at a bending point of the DNA helix have been proposed, but to date, there is no definitive explanation (see Discussion). Therefore, the first objective of the present work was to examine the mode of binding to DNA of these two new compounds. Furthermore, an attempt was made to see whether the characteristics of their interaction with DNA might correlate with their sequence-specific recognition features. To date, efforts to elucidate the sequence selectivity of the bithiazole moiety of bleomycin have met with some success (Morii et al., 1986, 1987) but do not yet permit a definitive explanation of its sequence selectivity. The pronounced sequence specificity of netropsin makes these hybrid ligands useful probes to study the intrinsic binding selectivity of the bithiazole moiety. Elucidation of the role played by the bithiazole moiety of bleomycin in its complex with DNA represents a crucial element to understand the ability of this clinically useful antitumor agent to act as a sequence-specific DNA cleaving agent and to design new drugs.

The principal motivation behind the design of these two new DNA ligands was to determine whether the bithiazole unit of bleomycin merely provides an additional anchor for DNA or is also capable of specifically recognizing particular DNA sequences when it is appended to a strongly sequence selective groove binding entity. The experimental approach was to apply (i) electric linear dichroism (ELD) spectroscopy and (ii) DNase I footprinting methodology, to reveal the nature and location of the drug binding site.

## MATERIALS AND METHODS

*Chemicals.* Netropsin was purchased from Serva; stock solutions were prepared in water. The synthesis and complete spectral characterization of the netropsin–bithiazole ligands **1** and **2** have been reported recently (Mrani et al., 1992). Ligand concentrations were determined spectroscopically through the molar extinction coefficient or by direct weighing. Electrophoretic reagents [Tris, acrylamide, bis(acrylamide), urea, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and ammonium persulfate] were from BDH. X-ray films and developing chemicals were from Kodak. Bromophenol blue and xylene cyanol were from Serva. All other chemicals were analytical grade reagents, and all solutions were prepared by using distilled, deionized, and filtered water from a "Milli-Q" water purification system (Millipore).

*Biochemicals.* Calf thymus (CT) DNA (highly polymerized sodium salt) was purchased from Sigma Chemical Co. and deproteinized twice with sodium dodecyl sulfate and then extensively dialyzed against the appropriate buffer. DNase I (Sigma Chemical Co.) was stored as a 7200 units/mL solution in 20 mM NaCl, 2 mM $MgCl_2$, and 2 mM $MnCl_2$, pH 8.0 at −20 °C, and was freshly diluted to the desired concentration immediately prior to use. Restriction enzymes *Ava*I, *Bst*EII, *Eco*RI, and *Pvu*II were from New England Biolabs, and reverse transcriptase was from Pharmacia. [$\alpha$-$^{32}$P]dATP and [$\alpha$-$^{32}$P]dCTP (6000 Ci/mmol) were purchased from New England Nuclear. The plasmid pBS was from Stratagene (La Jolla, CA). The plasmids pKMΔ-98 and pMLB1048 (Lamond & Travers, 1983) were isolated from *Escherichia coli* by an alkaline lysis procedure and were purified by banding twice in CsCl–ethidium bromide gradients as previously described (Drew & Travers, 1984; Low et al., 1984; Fox & Waring, 1984). Ethidium was removed by several 2-propanol extractions followed by exhaustive dialysis against Tris–EDTA buffer to remove the CsCl. The purified plasmid was then precipitated and resuspended in appropriate buffer prior to digestion by the restriction enzymes.

*Buffers.* Footprinting experiments were performed in 10 mM Tris-HCl buffer, pH 7.0, containing 10 mM NaCl. Electrophoresis was carried out in TBE buffer (8.9 mM Tris base, 8.9 mM boric acid, 2.5 mM $Na_2EDTA$, pH 8.3). Electric dichroism experiments were conducted in 1 mM sodium cacodylate, pH 6.5, and the conductivity of the solutions ranged from 1.0 to 1.2 mS.

*DNA Restriction Fragments Employed for Enzymic Footprinting.* Four DNAs of different base composition, (i) a 117 bp and (ii) a 253 bp DNA fragment from the plasmid pBS, (iii) the 160 bp *tyrT* DNA fragment containing the tyrosine tRNA promoter, and (iv) the 166 bp *pTyr2* DNA fragment, were used in the nuclease cleavage study. The *tyrT* DNA was obtained by digestion of the plasmid pKMΔ-98 with *Eco*RI and *Ava*I in order to generate sticky ends of unique sequence. It was labeled (i) at the *Eco*RI site on the lower strand (the Crick strand) with [$\alpha$-$^{32}$P]dATP and (ii) at the *Ava*I site on the upper strand (the Watson strand) with [$\alpha$-$^{32}$P]dCTP and

reverse transcriptase, in order to give specific 3′-end labeling. The *pTyr2* DNA fragment was obtained by cleavage of pMLB1048 with *Eco*RI and *Bst*EII endonucleases and was radiolabeled at the *Eco*RI site with [$\alpha$-$^{32}$P]dATP. The 117-mer and 253-mer were obtained from the plasmid pBS digested with *Pvu*II/*Eco*RI and *Pvu*II/*Ava*I, respectively. These digestions also yielded fragments suitable for 3′-end labeling by the reverse transcriptase. The detailed procedures for isolation, purification, and labeling of these duplex DNA fragments have been described recently (Bailly et al., 1990b, 1992b; Plouvier et al., 1991).

*DNase I footprinting, gel electrophoresis, and data processing* were performed essentially according to the published protocols (Low et al., 1984; Fox & Waring, 1984). DNase I footprinting experiments were performed at 37 °C with aliquots removed from the digestion mixture 1 and 5 min after the addition of the nuclease. DNase I experiments included 0.02 unit/mL enzyme, 20 mM NaCl, 2 mM $MgCl_2$, and 2 mM $MnCl_2$. The reactions were stopped by adding 3 $\mu$L of 80% formamide containing 10 mM EDTA, 1 mM NaOH, 0.1% bromophenol blue, and 0.1% xylene cyanol. Samples were heated to 90 °C for 4 min prior to electrophoresis. The cleavage products of the nuclease reactions were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea) capable of resolving DNA fragments differing in length by one nucleotide. After 2-h electrophoresis at 1500 V (BRL sequencer, Model S2), the gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80 °C, and subjected to autoradiography at −70 °C with an intensifying screen. Autoradiography were scanned using a multichannel computer-operated gel scanner at the Medical Research Council Laboratory of Molecular Biology, Cambridge, by kind permission of Sir Aaron Klug and Dr. J. M. Smith. Gel profiles were plotted and displayed on a raster graphics screen. Quantitative analysis of the gel electrophoresis profiles was performed by integration of the area under each peak, using a computer program developed specially for the purpose (Smith & Thomas, 1990). The area under each peak was integrated by simple addition of the pixels under the curve. Data are presented in the form ln ($f_a$) − ln ($f_c$), representing the differential cleavage at each bond relative to that in the control ($f_a$ is the fractional cleavage at any bond in the presence of the drug and $f_c$ is the fractional cleavage of the same bond in the control). The results are displayed on a logarithmic scale for the sake of convenience; positive values indicate enhanced cleavage whereas negative values indicate cleavage inhibition.

*Electric Linear Dichroism (ELD).* The pulse generator and the computerized optical measurement system used for linear dichroism have been previously described (Bailly et al., 1990a). The electric field pulses in the range 0–13 kV/cm were applied to the samples in a quartz cell with 10-mm optical path length and 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 $\mu$s, depending on the electric field strength) without degrading or denaturing it. Experiments were conducted in 1 mM sodium cacodylate buffer, pH 6.5, at room temperature (18–20 °C). The heating of the sample under electric pulses was negligible. Linear dichroism $\Delta A$ is defined as the difference between the absorbance for light polarized parallel ($A_\parallel$) and perpendicular ($A_\perp$) to the applied field at a given wavelength. The reduced dichroism is $\Delta A/A = (A_\parallel - A_\perp)/A$, 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 $\Delta A_\parallel = A_\parallel - A$ and $\Delta A_\perp = A_\perp - A$ are related by $\Delta A_\parallel = -2\Delta A_\perp$; thus, measurement of $\Delta A_\parallel$ or $\Delta A_p$-rp alone suffices for the calculation of the reduced dichroism $\Delta A/A$. $\Delta A_\parallel$ was chosen for its higher measurement sensitivity.

In these experiments the DNA molecules are oriented by an electric pulse, and the dichroism in the region of the absorption bands of the ligand bound to DNA is probed using linearly polarized light. When DNA solutions are exposed to the electric field pulses, the absorbance of light at 260 nm polarized parallel to the electric field vector is lower than the absorbance of light polarized perpendicularly ($A_\parallel < A_\perp$), 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 such as netropsin bound to DNA, the change of the absorption of light at 320 nm is different ($A_\parallel > A_\perp$), indicative of a positive dichroism of the complex. Therefore, on the basis of the sign and the amplitude of the observed signals, this technique can reveal the binding mode of the ligand and allows an estimation of its orientation with respect to the DNA helix.

The angles $\alpha$ and $\beta$ between the transition moments (of the bases and the dye chromophore, respectively) and the orientation axis of the DNA molecules were determined from the ratios of the reduced dichroism at a given field, for the bases and for the ligand, assuming the same orientation degree $\Phi$ in the two cases:

$$[(\Delta A/A)_{\text{ligand}}]/[(\Delta A/A)_{\text{DNA}}] = (3\cos^2\beta - 1)/(3\cos^2\alpha - 1) \quad (1)$$

The orientation angles $\beta$ were calculated using $\alpha = 73°$, the experimental angle measured after extrapolation of the reduced dichroism of DNA to infinite field (Hogan et al., 1978), and with $\alpha = 90°$, corresponding to the theoretical angle between the bases and the helix axis when DNA is in the classical Watson–Crick structure in the B conformation. The theory and complete details for measurements of the linear dichroism are fully described elsewhere (Fredericq & Houssier, 1973; Houssier, 1981; Houssier & O'Konski, 1981).

## RESULTS

*General Characteristics.* In a recent study (Mrani et al., 1991), focused on the synthesis and biological activities of **1** and **2**, we reported that the compounds bind effectively to DNA as judged from the red shifts of, respectively, 3.5 and 12 nm in their absorption maxima observed in the presence of CT-DNA. Their affinity constants, determined by absorption spectroscopy, are reported in Table I. Attachment of the bithiazole part of bleomycin to the amino or carboxyl terminus of the netropsin skeleton results in very different affinities of the hybrids for DNA. The greater affinity of **2** compared with that of netropsin may reflect the influence of its bithiazole moiety which can provide additional contacts with DNA. In contrast, the weak affinity for DNA of compound **1**, together with the observation that the viscosity of low molecular weight CT-DNA molecules is significantly decreased in the presence of **1**, led us to conclude that its bithiazole moiety does not intercalate DNA.

However, these preliminary results did not permit a full analysis of the DNA-binding processes of these two ligands. Therefore, we decided to apply electric linear dichroism and footprinting techniques in order to elucidate further their

Table I: Electric Dichroism and Binding Parameters for Ligand/Calf Thymus DNA Interactions

| | λ (nm)[a] | $K_a$ ($M^{-1}$)[b] | D/P[c] | $\Delta A/A$[d] | $[(\Delta A/A)_{ligand}]/[(\Delta A/A)_{DNA}]$ | β (deg)[f] α = 90° | β (deg)[f] α = 73° | references |
|---|---|---|---|---|---|---|---|---|
| bithiazole | 310 | $<5.0 \times 10^3$ | 0.01 | −0.13 | +0.28 | 61 | 59 | Povirk et al., 1979 |
| netropsin | 320 | $2.5 \times 10^5$ | 0.1 | +0.38 | −0.84 | 38 | 42.5 | Dattagupta et al., 1980 |
| compound **1** | 320 | $1.0 \times 10^4$ | 0.01 | +0.12 | −0.25 | 50 | 51 | |
| compound **2** | 330 | $6.3 \times 10^5$ | 0.1 | +0.37 | −0.83 | 39 | 43 | |
| proflavin | 450 | | | | +1.09 | 90 | 75 | Hogan et al., 1979 |
| dibutylproflavin | 435 | | | | +0.18 | 59 | 57.5 | Bontemps et al., 1974 |

[a] Monitoring wavelength. [b] Affinity constant for CT-DNA. [c] Drug to phosphate–DNA ratio. [d] ELD values at the indicated D/P ratio and under an electric field strength of 12.5 kV/cm. [e] Ratio of the ELD values at 12.5 kV/cm. [f] Orientation angle between the transition moment of the ligand and the DNA helix axis. β is determined from eq 1.

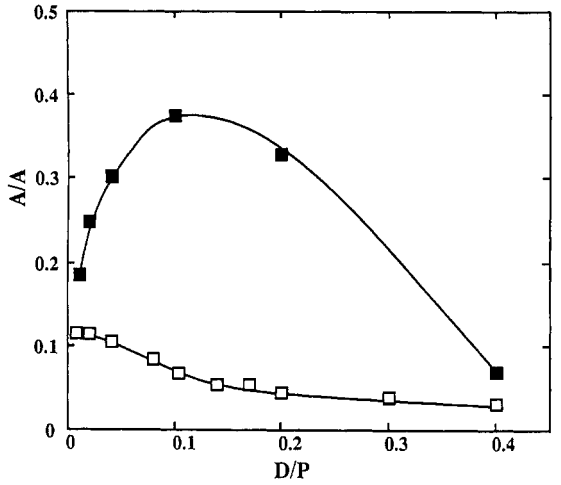



FIGURE 2: Dependence of the reduced dichroism $\Delta A/A$ on the drug to phosphate–DNA ratio (D/P), in the presence of (□) compound **1** and (■) compound **2**, respectively, at 320 and 330 nm, under a field strength of 12.5 kV/cm.

propensity to bind DNA in terms of both mode of binding and sequence specificity.

*Electric Linear Dichroism.* Figures 2 and 3 show a typical experimental set of data for the dependence of the reduced dichroism $\Delta A/A$ on the drug to DNA–phosphate ratio (Figure 2) and the wavelength and field strength dependence (Figure 3), for calf thymus DNA at 1 mM sodium cacodylate, pH 6.5, in the presence of ligands **1** and **2**, by comparison with netropsin or the bithiazole moiety alone. The variations of the reduced dichroism with the binding ratio (D/P) for **1** and **2** clearly show that the two ligands behave differently. The positive ELD values are the highest for D/P ratios of 0.01 and 0.1, respectively, for compounds **1** and **2**. These observations suggest that **1** has a weaker affinity for CT-DNA than **2** and thus corroborate the quantitative spectroscopic measurements reported in Table I. Beyond these ratios of maximal binding, the lower dichroism values are due to the presence of a large excess of DNA, namely, 3–15 times higher than the DNA concentration used for the measurements in the 260-nm band. The decrease of the ELD signals above the maxima is due to the appearance of free ligand in the solution with increasing D/P ratio.

The modes of binding of the two ligands were analyzed on the basis of the highest ELD values obtained when they are fully bound to DNA, i.e., at D/P ratios of 0.01 and 0.1 for **1** and **2**, respectively. The ELD spectra of the hybrid ligand–DNA complexes are compared with those of the netropsin–DNA and bithiazole–DNA complexes and DNA alone in Figure 3a. Both mixed ligands give positive dichroism values in the 300–350-nm region, though considerably less positive for **1** than for **2** or netropsin. It is very likely that **2** and netropsin, which display identical reduced dichroism spectra and a similar dependence upon field strength (Figure 3b), adopt the same binding geometry in the minor groove of DNA. Similar orientation angles are measured for compound **2** and netropsin (Table I). This is in excellent agreement with the orientation angle previously reported for netropsin (Dattagupta et al., 1980).

Three synthetic precursors of compound **2** were studied in order to determine the influence of its cationic amidine side chain on the binding to DNA. These three derivatives (**3**–**5**), which bear either an amide, a nitrile, or an ester group, all exhibited positive dichroism in the presence of CT-DNA. However, the magnitude of the dichroism signals varied significantly depending on the nature on the side chain (Table II). Identical dichroisms were measured with the amidine **2** and the amide **3**, thus showing that the presence of a second positively charged group is not an absolute prerequisite for minor groove binding. Moreover, compound **3** gave similar footprints as **2** on the 117-mer pBS fragment (not shown). At a fixed drug/DNA ratio of 0.02, the measured dichroism values are lower with the nitrile **4** than with **2** and **3** and are even more reduced with the ester **5**. This directly reflects their affinities for DNA (Table II).

The situations is completely different for the bithiazole moiety studied alone, the dichroism of which is weakly negative (Figure 3). At 310 nm, for a bithiazole/DNA ratio of 0.01, a negative dichroism of −0.126 is measured. It corresponds to an orientation angle of about 60° (Table I).

The two binding units of compound **1** are tethered by a short aliphatic linker, and one may plausibly imagine that minor groove binding of the netropsin half of the hybrid is accompanied by an intercalation (at least partially) of the bithiazole. As the two heterocycles absorb in the same region of the spectrum, we cannot, by this technique, distinguish one from the other. Theoretically if such a bimodal binding process were to take place, the positive dichroism conferred by the netropsin moiety ($\Delta A/A$ = +0.38 at 320 nm), added to the negative signal of the bithiazole ($\Delta A/A$ = −0.13 at 310 nm), would give a total dichroism positive in sign and comparable with that measured for compound **1** at 320 nm (Table I). Therefore, we cannot definitely exclude that this bimodal process effectively takes place. However, this seems very unlikely (vide infra).

*DNase I Footprinting Experiments.* To investigate the sequence-selective binding of compounds **1** and **2** by comparison with netropsin, four different DNA substrates were used. Typical autoradiograms of the footprinting gels performed both with the 253-mer and with the 117-mer fragments are shown in Figure 4. It can be observed that the extent of nuclease cleavage varies along the DNA sequence and that the cutting is strongly reduced in several places even in the absence of drug (control lanes). The internucleotide phos-

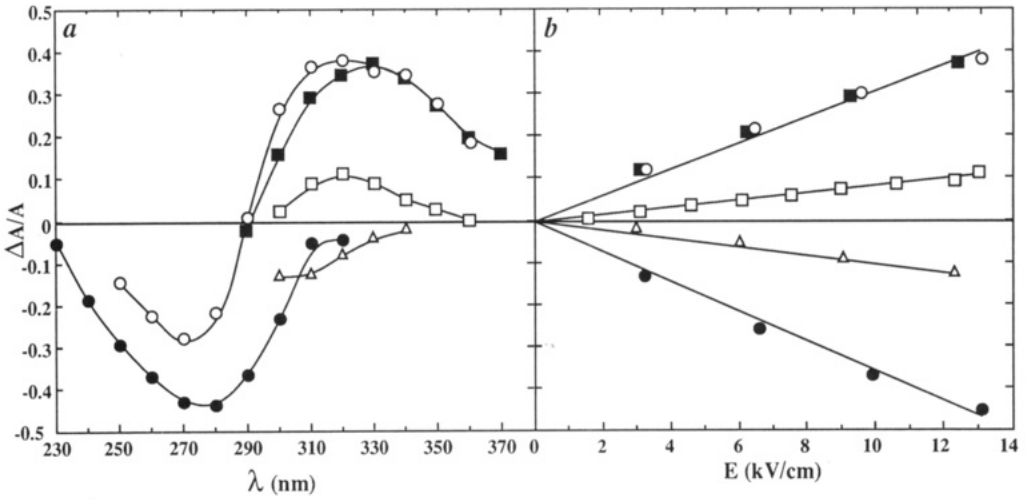
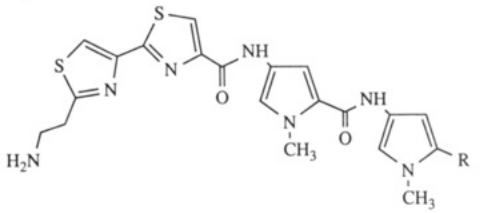
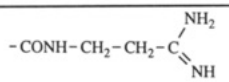
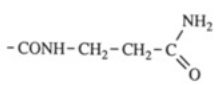
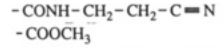
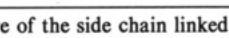



FIGURE 3: Dependence of the reduced dichroism $\Delta A/A$ on (a) wavelength and (b) electric field strength, in the presence of (●) DNA alone, and of complexes of DNA with (○) netropsin, (△) the bithiazole compound, (□) compound **1**, and (■) compound **2**. Conditions: D/P = 0.1 for netropsin and compound **2**; D/P = 0.01 for the bithiazole and compound **1**. (a) $E$ = 12.5 kV/cm; (b) λ = 310 nm for the bithiazole; λ = 320 nm for netropsin and compound **I**; λ = 330 nm for compound **2**; λ = 260 nm for DNA alone.

Table II: Electric Dichroism and DNA Binding Parameters for Ligand **2**–**5**/CT-DNA Interaction



| compd | side chain R[a] | $\Delta A/A^b$ | $K_a$ ($M^{-1}$)[c] |
|---|---|---|---|
| **2** |  | +0.25 | $6.3 \times 10^5$ |
| **3** |  | +0.25 | $1.2 \times 10^5$ |
| **4** |  | +0.20 | $4.3 \times 10^4$ |
| **5** |  | +0.12 | $1.9 \times 10^4$ |

[a] Structure of the side chain linked to the carboxyl terminus of the netropsin skeleton. [b] ELD values at a drug/DNA ratio of 0.02, at 330 nm and under an electric field strength of 12.5 kV/cm. [c] Affinity constant for CT-DNA.

phodiester bonds are cleaved differentially by DNase I throughout the entire DNA fragment, thus generating a non-random pattern of electrophoresis. This reflects the susceptibility of the enzyme to local DNA structural changes. Indeed, it is known that DNase I cleaves DNA with lower efficiency both when the minor groove is wider than average B-DNA (e.g., at GC-rich regions) and when it is narrower (e.g., in runs of A and T) (Drew & Travers, 1984).

In the presence of the drugs several "gaps" in the gels can be seen, showing that both ligands are able to recognize specific DNA sequences. But before discussing in detail the nature of the protected sites, it should be noted that very different concentrations of the ligands are required to observe footprints. Only a few representative drug levels are shown in order to accommodate all drug effects on one sequencing gel, but the effects of the two drugs have been examined over a large range of concentrations. With compound **1**, clear footprints were generally obtained with concentrations equal or superior to 50 μM. Compound **2** binds DNA much more tightly, and concentrations as low as 0.5 μM allow the detection of footprints. Even with 0.1 μM compound **2**, the nuclease

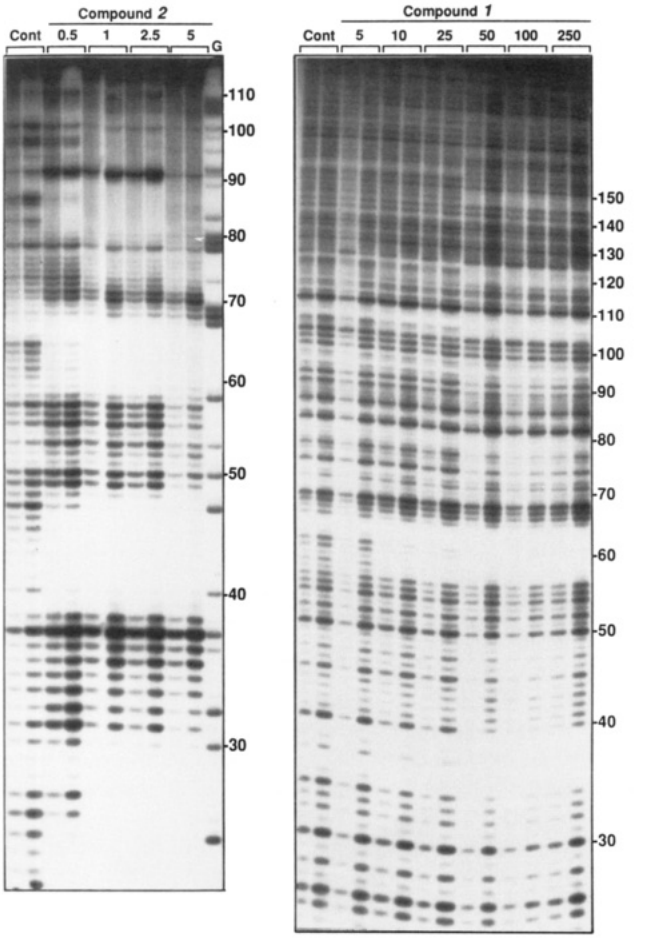



FIGURE 4: DNase I footprinting with the 117-mer *Pvu*II/*Eco*RI fragment (left-hand panel) and the 253-mer *Pvu*II/*Ava*I fragment (right-hand panel) of the plasmid pBS in the presence of different concentrations of compounds **1** and **2**. The concentration (μM) of the drug tested is shown at the top of the appropriate gel lanes. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The tracks labeled "cont" contained no drug. The track labeled "G" represents a dimethyl sulfate–piperidine marker specific for guanine. Numbers at the right side of the gel refer to the sequence shown in the corresponding differential cleavage plots in Figure 6.

activity remains inhibited at some high-affinity sites. These observations further reveal the greater affinity for DNA of the biscationic compound **2** than the monocationic hybrid **1**,

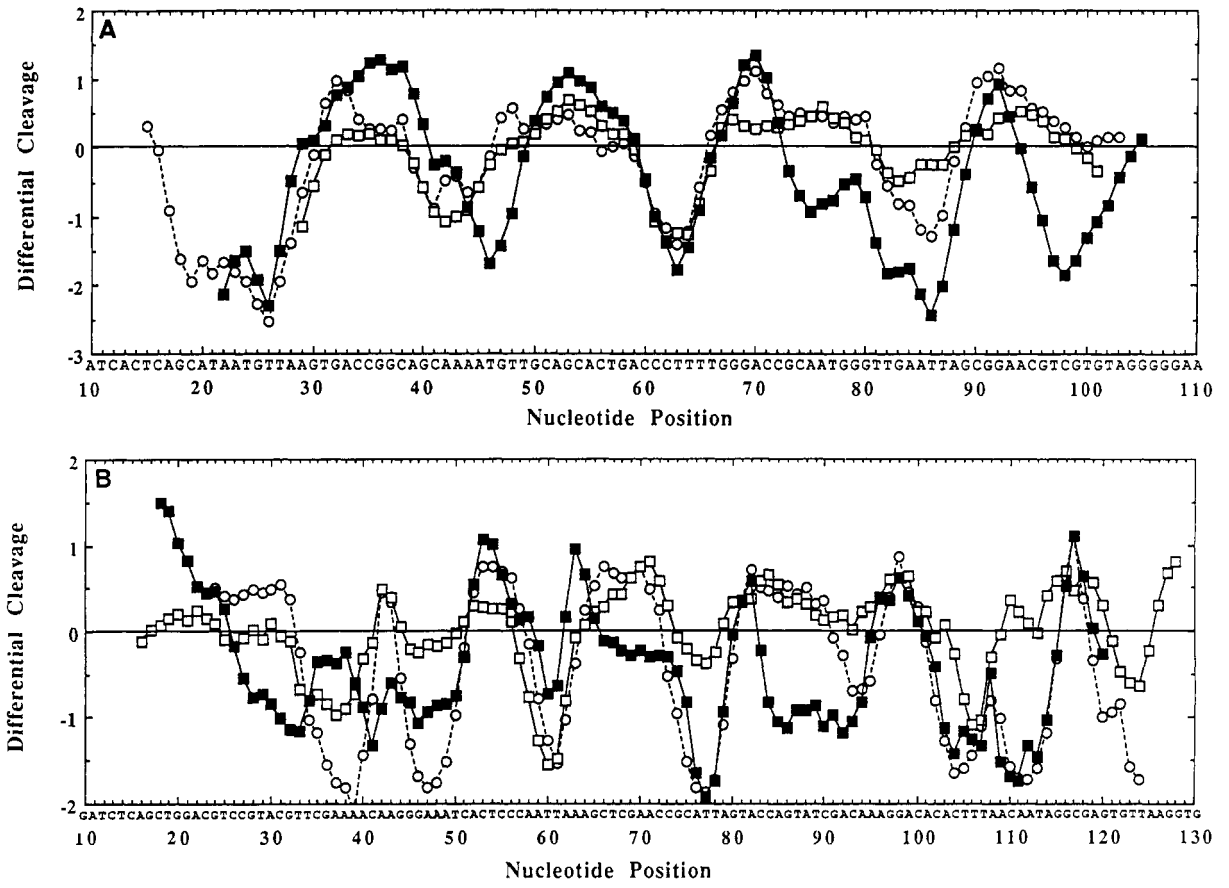



FIGURE 5: Differential cleavage plots showing differences in susceptibility of (A) the 117-mer *Pvu*II/*Eco*RI fragment and (B) the 253-mer *Pvu*II/*Ava*I fragment of the plasmid pBS to DNase I cleavage in the presence of (○) netropsin (10 μM), (□) compound **1** (100 μM), and (■) compound **2** (5 μM). Vertical scales are in units of ln ($f_a$) − ln ($f_c$), where $f_a$ is the fractional cleavage at any bond in the presence of the drug and $f_c$ is the fractional cleavage of the same bond in the control, given closely similar extents of overall digestion. Positive values indicate enhancement and negative values blockage.

and thus support our previous conclusion drawn from the spectrophotometric binding studies (vide supra).

In order to afford a better comparison of the binding specificity of the two compounds with that of netropsin, intensities from selected gel lanes (in Figure 4 and from many other gels not shown) were measured by densitometry and converted to numerical probabilities of cleavage. The differential cleavage plots in Figure 5 show negative values which indicate sites of drug protection from DNase I cleavage and positive values which indicate regions of drug-induced enhancement of cleavage. Stretches of DNA of about 115 bp within the 254-mer and 90 bp within the 117-mer were sufficiently well resolved among the different gels scanned to provide quantitative data. With both fragments, the pattern of protection and enhancement produced in the presence of the netropsin is as expected, with protection occurring at AT-rich sites and enhancement at GC-containing sequences.

In general, compound **1** behaves more or less like netropsin and recognizes AT-rich sequences too but with lower affinity (the extent of protection is weaker). With this drug, the clearest footprints on the 253-mer correspond to the sequences 3′-CGAAA (35–39), 3′-CAATTAAA (57–64), 3′-CATT (75–78), 3′-CTTTA (104–108), and 3′-GTTA (122–125). To identify the sites of drug binding from these sites of inhibited DNase I cleavage, a 3′-shift of about 2–3 bp must be considered because of the bias introduced by the nuclease upon DNA cleavage (Dabrowiak & Goodisman, 1989). The enzyme possesses a loop which extends into the minor groove and which spans about 3–4 bp of DNA (Suck & Oefner, 1986; Suck et al., 1988). Therefore, from the catalog of sequences recognized by the two compounds (Table III), it appears that compound **1**-binding sites are confined predominantly to short runs of A's and T's and to a lesser extent to a few alternating AT sequences. The two clearest footprints on the 253-mer, 3′-CGAAA (35–39) and 3′-CAATTAAA (57–64), remain of identical length as the drug **1** concentration is raised from 10 to 250 μM. This suggests that drug **1** presents a very sharp selectivity for homooligomeric sequences of A's and T's. No additional lower affinity sites can be detected with increasing concentrations of compound **1**. Around positions 94 and 112 of the 253-mer, there exist two netropsin binding sites which a AT sequence is flanked by a cytosine. These sites, 3′-CAAA (93–96) and 3′-CAATA (110–114), are not protected by compound **1**. This hybrid ligand is thus a pure A/T reader; in other words, it binds exclusively to those portions of DNA having a narrow minor groove. A similar case is that of thiazole–lexitropsins that invoke the concept of base-site avoidance and show 100% AT specific binding as a result (Rao et al., 1990). The shortest footprint observed with compound **1** clearly encompasses 5 nucleotides (3′-CGAAA) which, because of the large size of the enzyme relative to that of the drug, probably correspond to an effective binding site of 4 or 3 base pairs. Such a small site size is compatible with a binding of only one moiety of the hybrid, most likely the bis-pyrrole one which bears the cationic group. In contrast, the largest footprint of 8 bp in length (3′-CAATTAAA) can correspond either to a binding of the two parts of the molecule or more likely to an overlapping of two binding sites, with two netropsin moieties inserted into the narrow minor groove of this AT-

Table III: Sequences of the pBS DNA Fragments Showing Protection in the Presence of Netropsin and Compounds 1 and 2[a]

| netropsin | compound 1 | compound 2 |
|---|---|---|
| | 117-mer, from pBS | |
| 3′-CATAATGTTAA (19–29) | | 3′-AATGTTA (22–28) |
| 3′-AAAAT (42–46) | 3′-AAAAT (42–46) | 3′-AATGTTG (44–50) |
| 3′-TTTT (63–66) | 3′-TTTT (63–66) | 3′-TTTT (63–66) |
| 3′-AATTA (84–88) | 3′-AATTA (84–88) | 3′-AATG (75–78) |
| | | 3′-TGAATTAGC (82–90) |
| | | 3′-TCGTGTA (97–103) |
| | 253-mer, from pBS | |
| 3′-GAAAAC (36–41) | 3′-GAAAAC (36–41) | 3′-TACGTTC (29–35) |
| 3′-GAAAT (46–50) | 3′-AAAT (47–50) | 3′-ACAAGGGAAAT (40–50) |
| 3′-TTAA (60–63) | 3′-ATTAA (59–63) | 3′-TAA (61–63) |
| 3′-CATTAG (75–80) | 3′-ATTA (76–79) | 3′-CATTAG (75–80) |
| 3′-CAAA (93–96) | 3′-TTAA (105–109) | 3′-AGTATCGACAA (85–95) |
| 3′-CTTTA (104–108) | 3′-TTAA (123–126) | 3′-CTTTA (104–108) |
| 3′-AATAG (111–115) | | 3′-AATAG (111–115) |
| 3′-TGTTAA (121–126) | | |

[a] The position of the sequence, with reference to the numbering schemes in Figure 6, is indicated in parentheses. The sites of protection take into account a cross-strand stagger of about 2 bonds in the 3′ direction.

rich sequence and the two bithiazole units protruding outside the helix.

To further probe the influence of compound **1** on DNA, we have conducted other analyses showing that (i) DNase I produces only weak enhanced cleavage sites in the presence of **1**, (ii) a total absence of hyperreactivity is observed when the same piece of DNA preincubated with compound **1** is treated with diethyl pyrocarbonate, and (iii) no unwinding of closed circular DNA is detected in topoisomerization assays (not shown). These different observations suggest that the hybrid **1** does not induce any dramatic changes in DNA structure around its binding sites and once again strongly argue against the involvement of a full or partial intercalative binding process of its bithiazole moiety. It is also worth noting that neither the bithiazole compound presented in Figure 1 nor four other bithiazole derivatives having different side chains give any footprint with DNase I under similar experimental conditions.

In the footprinting experiments performed with compound **2** this ligand appears mainly as a AT-specific reader since most of the sites protected by the reference drug netropsin are also identified with **2**. This is illustrated by a similar protection of the sequences 3′-TTTT (63–66) on the 117-mer and 3′-ATTA (76–79), 3′-TTTA (105–108), and 3′-AATA (111–114) on the 253-mer by netropsin and **2**. On the other hand, this minor groove binder differs from netropsin in that it proves able to bind to certain AT/GC mixed sequences. For example, the sequences 3′-ATGTT (45–49), 3′-AATGG (75–79), and 3′-GTCGTGT (96–102) on the 117-mer and 3′-GTACGTT (28–34) and 3′-AGTATCGACA (85–94) on the 253-mer are identified by DNase I as binding sites for compound **2** but not for netropsin (Figure 5 and Table III). Searching for a common characteristic to these sequences specifically recognized by compound **2**, one can observe immediately that they all contain the 3′-CG and/or 3′-TG dinucleotides. This observation is significant considering that cleavage of DNA by the bleomycin–iron complex has been shown to occur best at dinucleotide sequences 3′-CpG and 3′-TpG, with less frequent cutting at 3′-ApG and negligible activity at 3′-GpG sequences (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Sugiura & Suzuki, 1982; Murray & Martin, 1985; Fox et al., 1987; Murray et al., 1988) while the bleomycin–cobalt complex shows a preference for cutting at the dinucleotide 3′-TpG and 3′-ApG (McLean et al., 1989). In this context, we may logically suggest that protection of these sequences necessarily reflects the binding specificity of the bithiazole moiety of the ligand. In contrast to compound **1**, the two moieties of the lexitropsin **2** both participate in the DNA-binding process and confer to this ligand distinct DNA-recognition properties.

In order to further clarify and extend these observations, additional DNase I footprinting experiments were carried out with two other DNA fragments, the 160 bp *tyrT* fragment and the 166 bp *ptyr2* fragment, which have been used previously to map the binding sites of bleomycin and its analog phleomycin (Fox et al., 1987; Fox & Grigg, 1988; McLean et al., 1989) and those of netropsin (Portugal & Waring, 1987a,b). Thus direct comparison between bleomycin, netropsin, and compound **2** would be possible. DNase I digestion patterns observed in the absence and presence of netropsin and compound **2** with the *tyrT* fragment are illustrated in Figure 6, and the corresponding differential plots are displayed in Figure 7. As mentioned above, very low concentrations of **2** are sufficient to detect footprints. The footprints are readily apparent for concentrations below 1 μM. The higher drug **2** loading lanes in the gel corresponding to the Watson strand (Figure 6) are shown to emphasize the protection and also to illustrate that the size of the footprints does not vary with increasing concentrations. The most significant observation is that, consistently on both strands of the *tyrT* DNA fragment, the sequence protected by compound **2** around position 85–95 is clearly 3–4 bp longer than that protected by netropsin (Figures 6 and 7) and extends in the 3′ direction of the top strand toward the sequence 5′-GATGC (91–95). Moreover, it is also interesting to note that the sequences 3′-TGT (69–71) on the bottom strand and 5′-AGTA (124–127) on the top strand are specifically recognized by **2** and not by netropsin. In contrast, the sequence 3′-GTCAA (41–45) located on the top strand does not appear as a possible binding site for **2** and phleomycin while netropsin and bleomycin do recognize this sequence (Table IV). Comparison of our data with those previously reported for bleomycin (McLean et al., 1989) leads to two significant conclusions. First, the netropsin-protected sites markedly differ from those reported for bleomycin or phleomycin. Indeed, major protection with bleomycin occurs predominantly at GC-rich sequences while netropsin avidly binds AT-rich sites. Second, bleomycin-protected sites frequently contain a pyrimidine-G-pyrimidine trinucleotide (Table IV), i.e., a motif which often distinguishes the binding site of compound **2** from that of netropsin. To illustrate the latter observation, we can mention the trinucleotides 3′-TGT (69–71) and 5′-TGT (93–

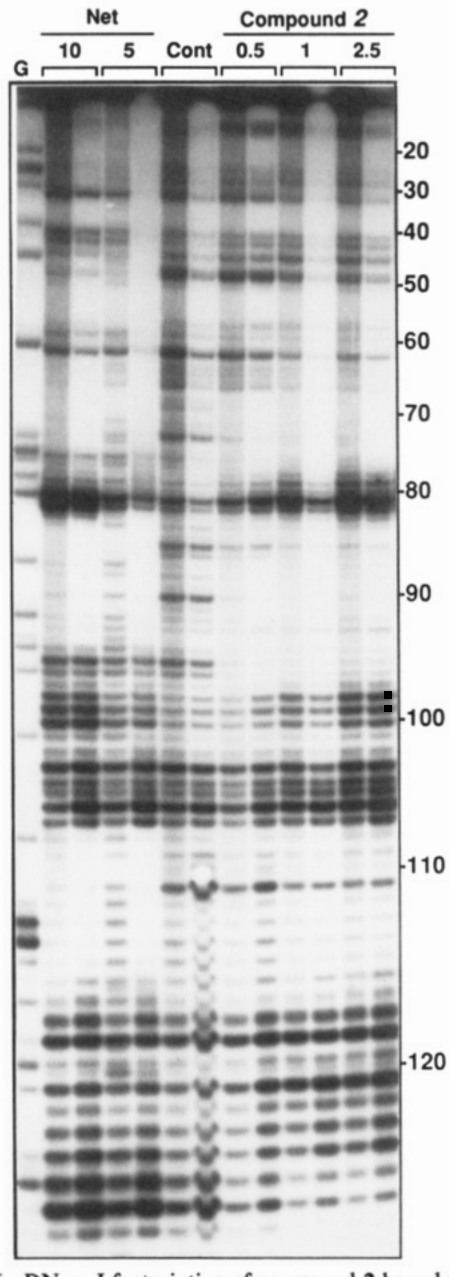



FIGURE 6: DNase I footprinting of compound **2** bound to the 160 bp *tyrT* DNA fragment containing the tyrosine tRNA promoter region. The DNA was labeled with [$\alpha$-$^{32}$P]dCTP for the Watson strand in the presence of AMV reverse transcriptase. Numbers refer to the numbering scheme of Drew and Travers (1984) used in Figure 8. Other details as for Figure 5.

95), respectively, on the bottom strand and the top strand of the *tyrT* fragment, and the sequences 3′-TGT (46–48) and 3′-CGTGT (98–102) on the 117-mer and 3′-CGT (31–33) on the 253-mer of the plasmid pBS, which all constitute or are adajcent to potential binding sites for compound **2** but not for netropsin.

Turning next to the footprinting experiments carried out with the *ptyr2* DNA, Figure 8 shows that the differential cleavage plot produced in the presence of compound **2** closely matches the one obtained in the presence of netropsin. The only difference is observed at positions 37–38 where the dinucleotide step 3′-AC (versus 5′-TG) is protected by **2** and not by netropsin. Despite these differences it is important to emphasize that, within the *tyrT* fragment and again more evidently within the *ptyr2* fragment (Table IV), there are a large number of DNase I-protected sites which are identically revealed in the presence of either netropsin or compound **2**. Therefore, this second set of footprinting experiments supports the previous conclusion that compound **2** is mainly an AT-specific groove binder. But, depending on the nature of the sequence flanking the AT site first targeted by its netropsin moiety, the bithiazole moiety of **2** can accommodate various types of nucleotide motifs with the exception of homooligomeric sequences.

To sum up these DNase I footprinting experiments, it is evident that no unique consensus sequence typifying the binding selectivity of the bithiazole unit can be proposed. However, from the variety of sequences recognized by the lexitropsin molecule **2** we can unambiguously assert that the bithiazole moiety of bleomycin provides opportunities for recognizing GC-containing sequences with apparently a preference (although not absolute) for a pyrimidine-G-pyrimidine motif. The way in which the bithiazole moiety of bleomycin can engage contacts with AT as well as with GC residues is discussed below.

## DISCUSSION

The bithiazole unit (Figure 1b) represents the DNA binding moiety of the antitumor drug bleomycin (Stubbe & Kozarich, 1987; Hecht, 1986), and the interaction between the bithiazole of bleomycin and DNA appears to be primarily responsible for the guanine-pyrimidine (5′→3′) specificity of cleavage of the iron–bleomycin system (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Sugiura & Suzuki, 1982; Murray & Martin, 1985; Fox et al., 1987; Murray et al., 1988). The importance of this bithiazole moiety was confirmed by the study of simplified synthetic bleomycin models in the structure of which the bithiazole ring is lacking (Sugiura et al., 1983; Hénichart et al., 1985a; Otsuka et al., 1986a,b, 1990; Brown et al., 1989; Hudson & Mascharak, 1989; Suga et al., 1989) or is linked to the simplified complexing part of bleomycin by a connective peptide chain (Kenani et al., 1987, 1989). However, the exact nature of the bleomycin–DNA interaction is still controversial. The interpretation of the binding initially proposed, in which the bithiazole binds to DNA by a classical intercalation process with the two thiazole rings in complete overlap with base pairs (Murakami et al., 1976; Takeshita et al., 1978; Lin & Grollman, 1981; Fisher et al., 1985; Miller et al., 1985), appears now very unlikely but cannot be definitively excluded. For simple bithiazole compounds an intercalative binding model is demonstrated (Kuroda et al., 1982; Sakai et al., 1982; Riordan & Sakai, 1983, Houssin et al., 1986), but because of steric hindrance it is difficult, if not impossible, for the bleomycin–metal complex to intercalate its bithiazole moiety between DNA base pairs. Several other investigations have converged to propose a model in which only one thiazole ring would be partially intercalated (Glickson et al., 1981; Booth et al., 1983; Gamcsik et al., 1990) or inserted at a bending point of the DNA helix (Hénichart et al., 1985b). This model has been reconsidered, and minor groove binding has been also postulated (Kross et al., 1982; Kuwahara & Sugiura, 1988). Despite all these studies, and without crystallographic data of the bleomycin–DNA complex, the position of the bithiazole system cannot be unequivocally determined and seems to depend upon the nature of the bulky peptide chain to which it is linked. Nevertheless, if DNA sequences have been delineated to be specific sites for deoxyribose cleavage (Murray et al., 1985, 1988; McLean et al., 1989), no preferential binding directly induced by the bithiazole ring has been reported. So, it seemed of interest to introduce on the bithiazole motif a peptide chain known to bind specific sequences in the minor groove of double-stranded DNA.

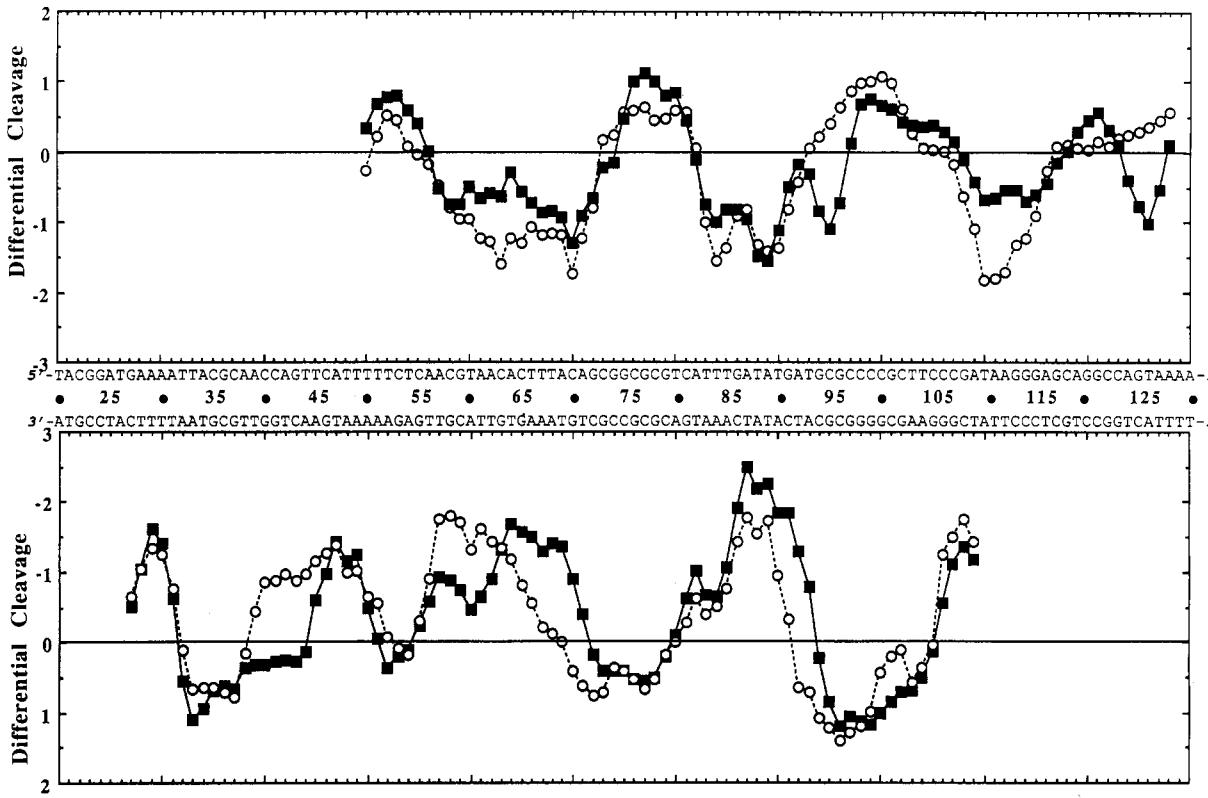



FIGURE 7: Differential cleavage plots for the susceptibility of the *tyrT* DNA fragment to DNase I in the presence of (■) compound **2** (1 μM) and (○) netropsin (10 μM). The upper panel shows differential cleavage of the upper "Watson" strand, and the lower of the complementary "Crick" strand. The ordinate scales for the two strands are inverted, so that the deviation of the points *toward* the lettered sequence corresponds to a ligand-protected site and deviation *away* represents enhanced cleavage. Other details as for Figure 6.

ELD spectroscopy is a very sensitive and suitable electrooptical technique for rapidly determining the orientation of ligands binding to DNA (Norden et al., 1992). This method only requires low amounts of drugs and directly provides quantitative information about the binding geometry of the DNA-bound molecules since the free ligand does not contribute to the dichroism. The dichroism can be scanned as a function of wavelength; thus, the orientation of the ligand–DNA complexes in the electric field can be followed by monitoring the ELD signal within the wavelength range of the absorption spectrum of the drug. There exist several criteria and techniques to provide evidence of intercalative binding (Long & Barton, 1990), but in contrast, there are a few, among which is linear dichroism, which can give evidence for a groove binding process.

The identical shape of the ELD spectra of the netropsin- and compound **2**–DNA complexes leaves no room for doubt that compound **2** is a pure minor groove binding agent. The results also reveal that replacement of the chemically unstable terminal amidine group of **2** by a stable amide group slightly decreases the affinity for DNA (Table II) but changes neither the mode of binding to DNA nor the sequence specificity of the ligand. The monocationic drug **3** may be compared with the natural antibiotic distamycin, and one may rise the question if two molecules, associated in a head-to-tail manner, can simultaneously bind to the minor groove of DNA as reported under certain conditions for distamycin (Pelton & Wemmer, 1989). Each of the three amide NH groups of netropsin forms hydrogen bonds, and the $CH_2$ methylene groups of the amidine side chain are involved in close van der Waals contacts with adenine and thymine bases of DNA (Kopka et al., 1985a,b). Therefore, it is not surprising that the affinity for DNA of the ester **5**, which lacks both the terminal amide NH group and the methylene groups of its side chain, is considerably lower than that of the amide **3**. Compound **4** contains these crucial amide bonds together with the methylene groups appended to the nitrile function, but its affinity for DNA is about 3-fold lower than that of the amide **3**. This difference could be explained by the presence in the amide **3** of an extra terminal NH group which may be engaged in a hydrogen bond with a heteroatom of a DNA base. Therefore, it appears that the deletion of the (alkylamino)carbonyl side chain of compound **2** has marked effects on its binding properties. These results indirectly confirm that the amidine side chain of netropsin is a determining contributor of its affinity for DNA (Kopka et al., 1985a,b).

From the weakly negative dichroism signal observed with the bithiazole–DNA complex, it is not conceivable that this heterocyclic ligand is a classical intercalator. The corresponding orientation angle is about 60° and is close to that reported for the tripeptide S, a degradation fragment of bleomycin containing the bithiazole ring. However, the tripeptide S is structurally different from the bithiazole compound we used in that it contains both a threonine residue linked to the amino terminus and a sulfonium group attached to the carboxyl end. On the basis of spectrophotometric, viscometric, and electric dichroism measurements, Povirk et al. (1979) concluded that the tripeptide S fully intercalates into DNA. However, they observed that the reduced linear dichroism extrapolated to 100% orientation was for DNA alone (at 260 nm) almost twice that of the bithiazole moiety of the tripeptide S measured in its absorption band (320 nm). Although we have calculated a closely similar angle to Povirk et al. (1979) for the bithiazole moiety orientation, we rather believe that such an angle is more representative of a partial intercalation process. This point of view is supported by the observation that the orientation angle measured for the bithiazole compound is closer to that reported for dibutylproflavin which does not

Table IV: Sequences of the *tyrT* and *ptyr2* DNA Fragments Showing Protection in the Presence of Compound **2**, Netropsin, Bleomycin, and Phleomycin[a]

| compound **2** | netropsin | bleomycin | phleomycin |
|---|---|---|---|
| | *tyrT* Fragment, from pKMΔ98 | | |
| 3′-TTTTA (28-32) | 3′-TTTTA (28-32) | 3′-ATGCC (20-24) | 3′-GCAATGC (17-23) |
| 3′-GTAAA (46-50) | 3′-GTCAAGTAAA (41-50) | 3′-GCGTTGGTCA (35-44) | 3′-GCGT (35-38) |
| 5′-ACGTAACACTTTACA (57-71)<br>3′-TGCATTGTGAAATGT (57-71) | 5′-ACGTAACACTTTACA (57-71)<br>3′-TGCATTGTGAA (57-67) | 5′-TCAA (54-57)<br>3′- GTTG (55-58) | 3′-AGTTGCATTG (54-63) |
| 5′-TTTGATATGATGC (83-95)<br>3′- ACTATACTAC (85-94) | 5′-TTTGATAT (83-90)<br>3′- ACTATAC (85-91) | 5′-TAACACT (60-66)<br>3′-ATTGTG (60-65) | 3′-TGTC (69-72) |
| 5′- ATAAGGG (109-115)<br>3′-GCTA (107-110) | 5′- ATAAGGG (109-115)<br>3′-GCTA (107-110) | 5′- AGC (71-73)<br>3′-ATGTC (68-72) | 3′-CCGC (74-77) |
| 5′-AGTA (124-127) | | 5′- GTCAA (79-83)<br>3′-GCGCAGT (76-82) | 3′-ATAC (88-91) |
| | | 5′- GATGCGCC (91-98)<br>3′-ATACT (88-92) | |
| | | 5′-TCCCGAT (104-110) | |
| | | 5′-AGGCCAGTAA (119-128) | |
| | | 5′-ATTA (134-137) | |
| | *ptyr2* Fragment, from pMLB1048 | | |
| 3′-GATGTGTC (20-27) | 3′-GATGTGT (20-26) | 3′-TGT (22-24) | 3′-CTT (30-32) |
| 3′-TTCTATACT (31-39) | 3′-TTCTAT (31-36) | 3′-TCGA (26-29) | 3′-AGCTCT (58-63) |
| 3′-AGAATT (70-75) | 3′-AGAATT (70-75) | 3′-ACGCGCG (40-46) | 3′-TTTC (90-93) |
| 3′-GTG (82-84) | 3′-GTG (82-84) | 3′-ACTGCA (53-58) | 3′-TCGCGCAGAAA (103-113) |
| 3′-GTTTCAT (89-95) | 3′-GTTTCAT (89-95) | 3′-GCACGT (68-73) | |
| | | 3′-AT (87-88) | |
| | | 3′-ATG (94-96) | |
| | | 3′-CC (98-99) | |

[a] The position of the sequence, with reference to the numbering schemes in Figures 8 and 9, is indicated in parentheses. The sites of protection take into account a cross-strand stagger of about 2 bonds in the 3′ direction. The binding sites for the bleomycin–Co complex and phleomycin E were determined from McLean et al. (1989) and Fox et al. (1987), respectively.

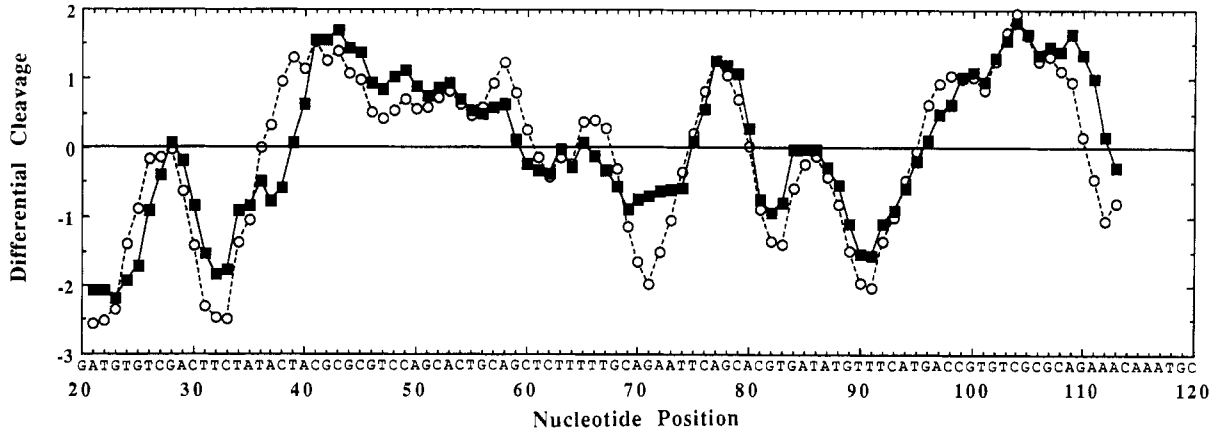



FIGURE 8: Differential cleavage plots showing differences in susceptibility of the *ptyr2* DNA fragment to DNase I in the presence of (■) compound **2** (1 μM) and (○) netropsin (10 μM). Other details as for Figure 6.

intercalate into DNA (Müller et al., 1973) than that reported for proflavin which fully intercalates DNA (Table I). The partial intercalation mode, which we previously postulated using fluorescent- and spin-labeled bithiazole derivatives (Hénichart et al., 1985b), is thus also supported by these ELD measurements. Whatever the exact significance of these negative dichroism signals, it is evident on the basis of the present data that neither complete intercalation between DNA base pairs nor classical minor groove binding (as observed with netropsin) can be proposed for the bithiazole moiety.

Linkage of the bithiazole moiety to the bis-pyrrole unit of netropsin via a flexible alkyl linker may allow the intercalation of the bithiazole between base pairs. The ELD experiments reported here with the compound **1** are not in favor of such a process but, as mentioned above, cannot rigorously exclude it. However, intercalation of the bithiazole moiety of compound **1** is difficult to envision because (i) the weak affinity constant of **1** for CT-DNA does not support this view, (ii) molecular mechanics calculations (Mrani et al., 1991) suggested that the positively charged amino group of the netropsin moiety of **1** anchors deep inside the negatively charged minor groove, thereby forcing the bithiazole moiety to extend away from the groove, and (iii) the footprinting studies detailed below indicate that this ligand covers only 3–4 bp. These different results support the conclusion that the bithiazole moiety does not contribute to the binding of compound **1** to DNA.

The results presented here are consistent with a model in which the netropsin moiety of compound **1** resides in the minor groove of DNA and where the appended bithiazole moiety is projected away from the DNA groove. This hybrid compound has a weak affinity for DNA and shows a strict preference

for A and T stretches. Netropsin, by virtue of its bicationic nature, binds more strongly to homopolymeric (dA)·(dT) sequences than to alternating (dA-dT) sequences (Wähnert et al., 1975), where a higher electronegative potential exists in the minor groove of DNA (Pullman & Pullman, 1981). However, compound **1** is monocationic; therefore, its sharp selectivity for homopolymeric sequences may derive rather from the particular geometry of such sequences than from electrostatic considerations. It is known that phased runs of poly[(dA)·(dT)] give rise to intrinsically bent DNA (Crothers et al., 1990), and a minimum of four consecutive As seems to be necessary and sufficient to form such specific curved structures (Koo et al., 1986; Nelson et al., 1987). These results imply that compound **1** may act as an architectural element recognizing distinct local conformation of DNA and thereby stabilizing DNA bends. This is a reasonable assumption since recent studies have shown that DNA conformation rather than primary structure per se is a major factor determining sequence specificity of binding (Laughton et al., 1990). Bent DNA sequences play a crucial role in the packaging of DNA and in many biological processes (Crothers et al., 1990; Travers, 1989, 1990). For example, a requirement for DNA bending in transcription has been clearly established (Amouyal & Buc, 1987), and bent DNA sequences containing repeated oligo[(dA)·(dT)] tracts might enhance transcription initiation (Collis et al., 1989; Bracco et al., 1989). It has been shown that transcription initiation is activated by netropsin to a greater extent when the promoter bears homopolymeric AT sequences rather than alternating AT sequences (Bruzik et al., 1987). Therefore, drugs such as compound **1**, able to recognize bent DNA sequences and probably, by analogy with netropsin, able to further bend the targeted sequence upon binding, may have interesting biological effects. The different experiments concur in suggesting that the bithiazole moiety of compound **1** does not physically bind to DNA, but we cannot exclude the possibility that the bithiazole group of **1** affects the binding of its netropsin moiety to DNA.

It should also be mentioned that the extent of DNase I-enhanced cleavage in the presence of compound **1** is relatively low and weaker than that produced by compound **2** and netropsin. This may indicate that compound **1** introduces weak (if any) local structural changes in DNA. But examination of the pattern of cleavage enhancements in the DNase I digest of a restriction fragment has shown that this is probably not attributable to the transmission of conformational changes in DNA as a result of drug binding but is most likely due to a redistribution of the enzymic cleaving agent as a result of drug binding (Ward et al., 1988). The high ligand/DNA ratios employed in our experiments increase the likelihood of such an interpretation.

The results obtained with compound **2**, which has been identified as a classical minor groove binder, clearly indicate that the bithiazole moiety of bleomycin contributes significantly to, but is not in itself sufficient to determine, the site recognized along the DNA strand. Recent DNase I cleavage inhibition analysis and sequence-specific cleavage studies led the authors to conclude that "The 2-amino group of guanosine base adjacent to the 5′ side of the cleaved pyrimidine base is one key element of specific 5′G-C or G-T recognition by metallobleomycin, and the bithiazole group probably plays an important role as an anchor on B-DNA" (Kuwahara & Sugiura, 1988). The present results fully support this view and, for the first time, show unambiguously that the bithiazole group of bleomycin acts as a DNA recognition element,

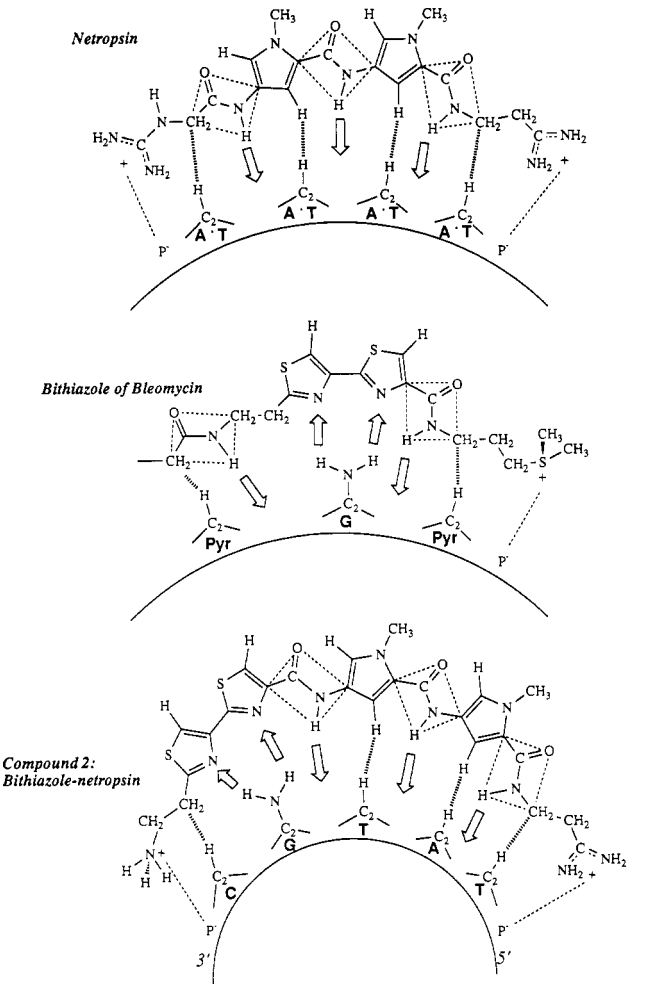



FIGURE 9: Representation of the binding to DNA of netropsin [from Kopka et al. (1985b)], the bithiazole region of bleomycin [in part from Prive et al. (1985)], and the proposed binding model of compound **2** to the sequence 3′-CGTAT. Heavy arrows are hydrogen bonds, from donor to acceptor. Barred lines mark close van der Waals nonbonded contacts between DNA and drug. Dotted lines indicate electrostatic contacts.

offering opportunities to recognize GC bp-containing DNA sequences.

From the footprinting studies and considering also published reports, a model that schematizes the binding of compound **2**, and thus of the bithiazole unit, can be proposed. Figure 9 illustrates the binding of compound **2** to the sequence 5′-TATGC. This model readily explains the experimentally observed specificity of this groove binding agent. However, it is wise to remember that DNase I is an excellent sensitive probe for detecting sites but is poor for defining the precise site size (Dabrowiak & Goodisman, 1989). The DNA fragment 5′-TATGC has been chosen since it contains the sequence 5′-TAT, which is known as a strongly preferred netropsin binding site (Portugal & Waring 1987a,b). Moreover, the crystal structure of the $d(GCGCATATGCGC)_2$–netropsin complex is available, the exact molecular arrangement of netropsin in the minor groove of this duplex fragment is known (Coll et al., 1989), thus providing a solid basis to derive the model. This sequence also contains a pyrimidine-G-pyrimidine motif evidenced in our study as being frequently recognized by the bithiazole as a binding site. It is also worth noting that (i) the stereochemical fitting of metallobleomycin in the duplex DNA fragment $d(ATGCCA)_2$ has revealed that the bithiazole–carboxamide moiety of bleomycin interacts with the guanosine residue of the 5′-TGC motif (Kuwahara &

Sugiura, 1988) and (ii) the consensus tetranucleotide double-stranded sequence (5′-pyrimidine-G-C-purine)·(3′-purine-C-G-pyrimidine) probably represents the optimal sequence site for a bleomycin double-strand break (Mirabelli et al., 1982).

After careful inspection of the catalog of binding sites for compound **2** reported in Tables III and IV, the pentanucleotide 5′-TATGC appears as the sequence which best characterizes the sequence selectivity of compound **2**. However, this sequence, drawn from the comparison of many different binding sites for **2**, does not exist in any of the fragments used in this study and thus cannot be rigorously presented as a specific binding site for compound **2**. However, the sequence 5′-TATGA exists in the *tyrT* fragment (from nucleotide positions 88 to 92) and effectively corresponds to a specific binding site for **2**. The most interesting feature is that this particular sequence is differently recognized by netropsin and compound **2**. Netropsin binds to the tetranucleotide 5′-ATAT (87–90) of the *tyrT* fragment (Table IV) whereas in the presence of compound **2**, it is evident that the DNase I-protected site is 3–4 bp longer and extends to the sequence 5′-GAT flanking the ATAT site on the Watson strand of the *tyrT* fragment. Analysis of the complementary Crick strand also reveals the same binding site and thus confirms this sequence as being a binding site for compound **2**. The sequences 3′-TATCG (versus 5′-ATAGC) and 3′-ATACT (versus 5′-TATGA) found respectively in the 253-mer fragment (positions 87–91) and the *ptyr2* fragment (positions 35–39) are both revealed by DNase I as being potential binding sites for compound **2** and not for netropsin.

Binding of both the pyrrole and thiazole rings of compound **2** in the minor groove of the proposed site sequence should displace the spine of water molecules from the floor of the minor groove and permit additional hydrogen bonds between the nitrogen atom of the bithiazole and the exocyclic N-2 amine hydrogen of a guanine residue. For the two electronegative nitrogen–bithiazole atoms to be oriented into the concave face of the ligand, the bithiazole rings must adopt a syn orientation since the trans orientation may not allow both nitrogens to be offered simultaneously to the DNA upon binding. The syn conformation has been previously proposed (Sakai et al., 1981; Lin & Grollman, 1981) and was again suggested by a study on phototransformed bleomycins which indicated that nitrogens rather than sulfurs of the bithiazole group may participate in the sequence specificity of bleomycin (Kuwahara & Sugiura, 1988). However, bithiazole-containing derivatives whose structures have been solved by X-ray crystallography all appear to exhibit the trans orientation of the two rings (Koyama et al., 1968; Kuroda et al., 1982; Houssin et al., 1986). The different molecular contacts represented in this putative model need to be checked, but this scheme suggests that a sequence-specific interaction between DNA and the bithiazole group has occurred.

The model proposed in Figure 9 is supported by the footprinting data and in particular accounts for the footprints where more nucleotides are protected by **2** than by netropsin. However, some sites are bound identically by netropsin and compound **2** (such as the site around position 63 in Figure 5A). Thus, the bithiazole moiety does not necessarily extend the netropsin footprint even if rigidly attached to the bispyrrole moiety buried in the minor groove of DNA. Thiazole-containing lexitropsins are quite sterically demanding and therefore, when possible, require the wider GC-rich minor groove (Kumar et al., 1991; Plouvier et al., 1991). Indeed, in some cases, the steric demands are such that intercalation of a terminal thiazole moiety results (Kumar et al., 1990). This property gave rise to the concept of base site avoidance and accounts for the 100% AT specificity of lexitropsins bearing thiazole moieties with inward-directed sulfur (Rao et al., 1990). It follows therefore that while compound **2** will recognize mixed sequences readily (Figure 9), if challenged by longer and narrower AT stretches, then some conformational adjustment of the ligand is necessary. The sterically demanding bithiazole moiety is likely to rotate out of the groove in these circumstances and be bound to the DNA only by the AT-recognizing netropsin moiety. The rotation around the amide bond between the bithiazole and the *N*-methylpyrrole ring would project the bithiazole part out of the DNA surface and thus, would not allow it to engage hydrogen bonds with DNA. This situation is analogous to the monodentate binding of rigid cis-linked bis-netropsins, contrasted with the bidentate binding of the trans-linked isomers (Kissinger et al., 1990). The binding site size of the cis isomer is about half that of the trans isomer. Compound **2** in these circumstances would recognize shorter sequences equal to that of netropsin. Thus, it seems that, depending on the sequence flanking the netropsin site, the bithiazole moiety of compound **2** can participate or not to the sequence-specific DNA recognition process.

It is tempting to suppose that the present results are grounds for extrapolating the behavior of the bithiazole moiety of the netropsin–bithiazole lexitropsin **2** to that of bleomycin. However, bleomycin cannot simply be considered as a DNA-binding molecule (the bithiazole) covalently attached to a DNA-cleaving molecule. The metal-binding/oxygen activation domain of bleomycin seems to participate qualitatively in the interaction with DNA (Sugiyama et al., 1986; Levy & Hecht, 1988; Chikira et al., 1991). So no definite conclusion can be drawn concerning the sequence selectivity of the bithiazole group of bleomycin. Further details of the true nature of the bleomycin–DNA interaction await the determination of a crystal structure of bleomycin bound to an oligonucleotide. However, synthetic hybrid molecules such as those described here help to improve our understanding of the processes by which bleomycin can read particular DNA sequences. They may provide important information with respect to (i) the mechanism by which antibiotics of the bleomycin family produce their antitumor effects and (ii) the design of potentially more active structural analogs.

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## REFERENCES

Amouyal, M., & Buc, H. (1987) *J. Mol. Biol. 195*, 795–808.
Bailly, C., & Hénichart, J. P. (1990) *Biochem. Biophys. Res. Commun. 167*, 798–806.
Bailly, C., & Hénichart, J. P. (1991) *Bioconjugate Chem. 2*, 379–393.
Bailly, C., Helbecque, N., Hénichart, J. P., Colson, P., Houssier, C., Rao, K. E., Shea, R. G., & Lown, J. W. (1990a) *J. Mol. Recognit. 3*, 26–35.
Bailly, C., OhUigin, C., Rivalle, C., Bisagni, E., Hénichart, J. P., & Waring, M. J. (1990b) *Nucleic Acids Res. 18*, 6283–6291.
Bailly, C., Collyn-d'Hooghe, M., Lantoine, D., Fournier, C., Hecquet, B., Fosse, P., Saucier, J. M., Colson, P., Houssier, C., & Hénichart, J. P. (1992a) *Biochem. Pharmacol. 43*, 457–466.
Bailly, C., Denny, W. A., Mellor, L. E., Wakelin, L. P. G., & Waring, M. J. (1992b) *Biochemistry 31*, 3514–3524.
Booth, T. E., Sakai, T. T., & Glickson, J. D. (1983) *Biochemistry 22*, 4211–4217.

Bracco, L., Kotlarz, D., Kolb, A., Diekmann, S., & Buc, H. (1989) *EMBO J. 8*, 4289–4296.
Brown, S. J., Hudson, S. E., Stephan, D. W., & Mascharak, P. K. (1989) *Inorg. Chem. 28*, 468–477.
Bruzik, J. P., Auble, D. T., & De Haseth, P. L. (1987) *Biochemistry 26*, 950–956.
Chikira, M., Sato, T., Antholine, W. E., & Petering, D. H. (1991) *J. Biol. Chem. 266*, 2859–2863.
Coll, M., Aymami, J., van der Marel, G. A., van Boom, J. H., Rich, A., & Wang, A. H. J. (1989) *Biochemistry 28*, 310–320.
Collis, C. M., Molloy, P. L., Both, G. W., & Drew, H. R. (1989) *Nucleic Acids Res. 17*, 9447–9468.
Crothers, D. M., Haran, T. E., & Nadeau, J. G. (1990) *J. Biol. Chem. 265*, 7093–7096.
Dabrowiak, J. C., & Goodisman, J. (1989) in *Chemistry and Physics of DNA–Ligand Interactions* (Kallenbach, N. R., Ed.) pp 143–174, Adenine Press, New York.
D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3608–3612.
Dattagupta, N., Hogan, M., & Crothers, D. M. (1980) *Biochemistry 19*, 5998–6005.
Drew, H. R., & Travers, A. A. (1984) *Cell 37*, 491–502.
Fisher, L. M., Kuroda, R., & Sakai, T. T. (1985) *Biochemistry 24*, 3199–3207.
Fox, K. R., & Waring, M. J. (1984) *Nucleic Acids Res. 12*, 9271–9285.
Fox, K. R., & Grigg, G. W. (1988) *Nucleic Acids Res. 16*, 2063–2075.
Fox, K. R., Grigg, G. W., & Waring, M. J. (1987) *Biochem. J. 243*, 847–851.
Fredericq, E., & Houssier, C. (1973) in *Electric dichroism and electric birefringence*, Clarendon Press, Oxford.
Gamcsik, M. P., Glickson, J. D., & Zon, G. (1990) *J. Biomol. Struct. Dyn. 7*, 1117–1133.
Glickson, J. D., Pillai, R. P., & Sakai, T. T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2967–2971.
Hecht, S. M. (1986) *Acc. Chem. Res. 19*, 383–391.
Hénichart, J. P., Bernier, J. L., Houssin, R., Lohez, M., Kenani, A., & Catteau, J. P. (1985a) *Biochem. Biophys. Res. Commun. 126*, 1036–1041.
Hénichart, J. P., Bernier, J. L., Helbecque, N., & Houssin, R. (1985b) *Nucleic Acids Res. 13*, 6703–6717.
Hogan, M., Dattagupta, N., & Crothers, D. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 195–199.
Houssier, C. (1981) In *Molecular Electro-Optics* (Krause, S., Ed.) pp 363–398, Plenum Publishing Corp., New York.
Houssier, C., & O'Konski, C. T. (1981) In *Molecular Electro-Optics* (Krause, S., Ed.) pp 309–339, Plenum Publishing Corp., New York.
Houssin, R., Helbecque, N., Bernier, J. L., & Hénichart, J. P. (1986) *J. Biomol. Struct. Dyn. 4*, 219–229.
Hudson, S. E., & Mascharak, P. K. (1989) *Chem. Res. Toxicol. 2*, 411–415.
Kenani, A., Lohez, M., Houssin, R., Helbecque, N., Bernier, J. L., Lemay, P., & Hénichart, J. P. (1987) *Anti-Cancer Drug Des. 2*, 47–59.
Kenani, A., Bailly, C., Helbecque, N., Houssin, R., Bernier, J. L., & Hénichart, J. P. (1989) *Eur. J. Med. Chem. 24*, 371–377.
Kissinger, K. L., Dabrowiak, J. C., & Lown, J. W. (1990) *Chem. Res. Toxicol. 3*, 162–168.
Koo, H. S., Wu, H. M., & Crothers, D. M. (1986) *Nature 320*, 501–506.
Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., & Dickerson, R. E. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1376–1380.
Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., & Dickerson, R. E. (1985b) *J. Mol. Biol. 183*, 553–563.
Koyama, G., Nakamura, H., Muraoka, Y., Takita, T., Maeda, K., Umezawa, H., & Iitaka, Y. (1968) *Tetrahedron Lett. 44*, 4635–4638.
Kross, J., Henner, W. D., Haseltine, W. A., Rodriguez, L., Levin, M. D., & Hecht, S. M. (1982) *Biochemistry 21*, 3711–3721.
Kumar, S., Jaseja, M., Zimmermann, J., Bathini, Y., Pon, R. T., Sapse, A.-M., & Lown, J. W. (1990) *J. Biomol. Struct. Dyn. 8*, 99–121.
Kumar, S., Bathini, Y., Joseph, T., Pon, R. T., & Lown, J. W. (1991) *J. Biomol. Struct. Dyn. 9*, 1–21.
Kuroda, R., Neidle, S., Riordan, J. N., & Sakai, T. T. (1982) *Nucleic Acids Res. 10*, 4753–4763.
Kuwahara, J., & Sugiura, Y. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2459–2463.
Lamond, A. I., & Travers, A. A. (1983) *Nature 305*, 248–250.
Laughton, C. A., Jenkins, T. C., Fox, K. R., & Neidle, S. (1990) *Nucleic Acids Res. 18*, 4479–4488.
Levy, M. J., & Hecht, S. M. (1988) *Biochemistry 27*, 2647–2650.
Lin, S. Y., & Grollman, A. P. (1981) *Biochemistry 20*, 7589–7598.
Lock, R. B., & Ross, W. E. (1987) *Anti-Cancer Drug Des. 2*, 151–164.
Long, E. C., & Barton, J. K. (1990) *Acc. Chem. Res. 23*, 271–273.
Low, C. M. L., Drew, H. R., & Waring, M. J. (1984) *Nucleic Acids Res. 12*, 4865–4877.
Lown, J. W. (1988) *Anti-Cancer Drug Des. 3*, 25–40.
McLean, M. J., Dar, A., & Waring, M. J. (1989) *J. Mol. Recognit. 1*, 184–192.
Miller, K. J., Lauer, M., & Calocia, W. (1985) *Biopolymers 24*, 913–934.
Mirabelli, C. K., Ting, A., Huang, C.-H., Mong, S., & Crooke, S. T. (1982) *Cancer Res. 42*, 2779–2785.
Morii, T., Matsuura, T., Saito, I., Suzuki, T., Kuwahara, J., & Sugiura, Y. (1986) *J. Am. Chem. Soc. 108*, 7089–7094.
Morii, T., Saito, I., & Matsuura, T. (1987) *J. Am. Chem. Soc. 109*, 938–939.
Mrani, D., Gosselin, G., Bailly, C., Houssin, R., Rao, K. E., Zimmerman, J., Balzarini, J., De Clercq, E., Hénichart, J. P., Imbach, J. L., & Lown, J. W. (1992) *Eur. J. Med. Chem.* (in press).
Müller, W. K., Crothers, D. M., & Waring, M. J. (1973) *Eur. J. Biochem. 39*, 223–234.
Murakami, H., Mori, H., & Taira, S. (1976) *J. Theor. Biol. 59*, 1–23.
Murray, V., & Martin, R. F. (1985) *Nucleic Acids Res. 13*, 1467–1481.
Murray, V., Tan, L., Matthews, J., & Martin, R. F. (1988) *J. Biol. Chem. 263*, 12854–12859.
Nelson, H. C. M., Finch, J. T., Luisi, B. F., & Klug, A. (1987) *Nature 330*, 221–226.
Nielsen, P. E. (1991) *Bioconjugate Chem. 2*, 1–12.
Norden, B., Kubista, M., & Kurucsev, T. (1992) *Q. Rev. Biophys. 25*, 51–170.
Otsuka, M., Kittaka, A., Ohno, M., Sugiura, Y., & Umezawa, H. (1986a) *Tetrahedron Lett. 27*, 3635–3638.
Otsuka, M., Kittaka, A., Ohno, M., Suzuki, T., Kuwahara, J., & Sugiura, Y. (1986b) *Tetrahedron Lett. 27*, 3639–3642.
Otsuka, M., Masuda, T., Haupt, A., Ohno, M., Shiraki, T., Sugiura, Y., & Maeda, K. (1990) *J. Am. Chem. Soc. 112*, 838–845.
Pelton, J. G., & Wemmer, D. E. (1989) *Nucleic Acids Res. 86*, 5723–5727.
Plouvier, B., Bailly, C., Houssin, R., Rao, K. E., Lown, J. W., Hénichart, J. P., & Waring, M. J. (1991) *Nucleic Acids Res. 19*, 5821–5829.
Portugal, J., & Waring, M. J. (1987a) *Eur. J. Biochem. 167*, 281–289.
Portugal, J., & Waring, M. J. (1987b) *FEBS Lett. 225*, 195–200.
Povirk, L. F., Hogan, M., & Dattagupta, N. (1979) *Biochemistry 18*, 96–101.
Prive, G. G., Goodsell, D. S., Yoon, C., & Dickerson, R. E. (1985) in *Fourth Conversation in Biomolecular Stereodynamics*, pp 154–155, New York and Albany.

Pullman, A., & Pullman, B. (1981) *Q. Rev. Biophys. 14*, 289–380.
Rao, K. E., Shea, R. G., Yadagiri, B., & Lown, J. W. (1990) *Anti-Cancer Drug Des. 5*, 3–20.
Riordan, J. M., & Sakai, T. T. (1983) *J. Med. Chem. 26*, 884–891.
Sakai, T. T., Riordan, J. M., Booth, T. E., & Glickson, J. D. (1981) *J. Med. Chem. 24*, 279–285.
Sakai, T. T., Riordan, J. M., & Glickson, J. D. (1982) *Biochemistry 21*, 805–816.
Smith, J. M., & Thomas, D. J. (1990) *CABIOS 6*, 93–99.
Stubbe, J., & Kozarich, J. W. (1987) *Chem. Rev. 87*, 1107–1136.
Suck, D., & Oefner, C. (1986) *Nature 321*, 620–625.
Suck, D., Lahm, A., & Oefner, C. (1988) *Nature 332*, 465–468.
Suga, A., Sugiyama, T., Sugano, Y., Kittaka, A., Otsuka, M., Ohno, M., Sugiura, Y., & Maeda, K. (1989) *Synlett*, 70–71.
Sugiura, Y., & Suzuki, T. (1982) *J. Biol. Chem. 257*, 10544–10546.
Sugiura, Y., Suzuki, T., Otsuka, M., Kobayashi, S., Ohno, M., Takita, T., & Umezawa, H. (1983) *J. Biol. Chem. 258*, 1328–1336.
Sugiyama, H., Kilkuskie, R. E., Chang, L. H., Ma, L. T., Hecht, S. M., van der Marel, G., & van Boom, J. H. (1986) *J. Am. Chem. Soc. 108*, 3852–3854.
Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983–5987.
Travers, A. (1989) *Nature 341*, 184–185.
Travers, A. (1990) *Cell 60*, 177–180.
Wähnert, U., Zimmer, C., Luck, G., & Pitra C. (1975) *Nucleic Acids Res. 2*, 391–404.
Ward, B., Rehfuss, R., Goodisman, J., & Dabrowiak, J. C. (1988) *Nucleic Acids Res. 16*, 1359–1369.
Zimmer, C., & Wähnert, U. (1986) *Prog. Biophys. Mol. Biol. 47*, 31–112.
Zunino, F., & Capranico, G. (1990) *Anti-Cancer Drug Des. 5*, 307–317.