Structure of a Dicationic Monoimidazole Lexitropsin Bound to DNA[†,‡]

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ABSTRACT: An X-ray crystal structure has been solved of the complex of a dicationic lexitropsin with a B-DNA duplex of sequence CGCGAATTCGCG. The lexitropsin is identical to netropsin except for replacement of the first methylpyrrole ring by methylimidazole, converting a =CH− to =N−. Crystals are isomorphous with those of the DNA dodecamer in the absence of drug. Although the =N− for =CH− substitution was intended to make that locus on the drug molecule compatible with a G·C base pair, electrostatic attraction for the two cationic ends of the drug predominates, and this lexitropsin binds to the same central AATT site as does the parent netropsin. But unlike netropsin, this lexitropsin exhibits end-for-end disorder in the crystal. Both orientations were refined separately to completion. Final residual errors at 2.25 Å resolution for the 2358 reflections above 2σ in F are $R = 0.165$ for one orientation (LexA) with 37 water molecules and 0.164 for the inverted drug orientation (LexB) with 40 water molecules. This molecular disorder is probably attributable to a weakening of binding to the AATT site occasioned by the imidazole-for-pyrrole substitution.

The antitumor antibiotic netropsin shows strong and specific binding to B-DNA in regions of at least four contiguous A·T base pairs (Wartell et al., 1974; Burckhardt et al., 1985). NMR and X-ray crystallographic studies (Patel 1982; Kopka et al., 1985a−c; Goodsell et al., 1995) have clearly shown the structural basis for this specificity: the flat, crescent-shaped drug molecule (Figure 1a) fits within the deep and narrow A·T region of the minor groove like hand in glove. Theoretical studies have revealed another component: the electrostatic potential well within the minor groove is deeper for A·T base pairs than for G·C (Pullman 1983; Lavery & Pullman, 1985; Zakrzewska et al., 1987). All of these factors have a structural origin. To a first approximation the minor groove can be narrower in A·T regions of B-DNA because A·T base pairs can adopt a larger average propeller twist, dragging the backbone chains along with them in a manner diagramed by Figure 9 of Fratini et al. (1982). The minor groove is spatially deeper in regions of A·T base pairs because adenine lacks the N2 amino group that extends up from the floor of the groove in G·C base pairs. And even the deeper electrostatic well in A·T regions may be caused by the absence of the electropositive N2 amine group of guanine.

The netropsin crystal structure led to the design and synthesis of *lexitropsins*: netropsin analogues designed to bind to an arbitrary sequence of A·T vs G·C base pairs (Kopka et al., 1985a,b; Lown et al., 1986a; Kopka & Larsen, 1992). The archetypical lexitropsin is similar to netropsin, but with one or both pyrrole rings replaced by imidazole (Figure 1b). Substitution of N for a C−H at a five-membered ring on the concave edge of the drug molecule was intended both to make room for a guanine amine group and to provide an acceptor for a new and stabilizing hydrogen bond from that amine. By this means, the A·T preference at that particular locus could, in principle, be changed to a G·C preference. Unfortunately, electrostatic attraction for the charged guanidinium and amidinium ends dominates in interaction with DNA, and these dicationic lexitropsins show only marginal preference for G·C over A·T (Burckhardt et al., 1989). Longer netropsins and lexitropsins can be synthesized (Youngquist & Dervan, 1987; Wang & Lown, 1992), and the double charge on the ends of the molecule can be modulated by replacing the guanidinium head group by the −CHO of distamycin as in Figure 1c (Kissinger et al., 1987) or by an uncharged five- or six-membered ring (Wade et al., 1992). These monocationic lexitropsins appear to be more GC-specific and to offer more promise in the engineering of base specificity (Kissinger et al., 1987; Krowicki et al., 1988).

In this paper we report the first crystal structure of a synthetic lexitropsin bound to DNA. The molecule is dicationic and is identical to netropsin except for substitution of imidazole for the first pyrrole (Lown et al., 1986a,b). In the shorthand representation of the caption to Figure 1 it can be described as $^{+}$Im−Py^{+}. The DNA to which it is complexed, CGCGAATTCGCG (Drew et al., 1981), possesses an ideal netropsin site in its central AATT region, and the $^{+}$Im−Py^{+} lexitropsin goes to this locus as the site of least resistance. The present structure analysis therefore serves as a control for other studies in which the lexitropsin is denied a long A·T binding site or is made more G·C-favoring by elimination of one of the positive charges.

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[‡] Both final atomic coordinates and F_o intensity data have been deposited with the Brookhaven Protein Data Bank (file 1LEX) and are available for immediate distribution.

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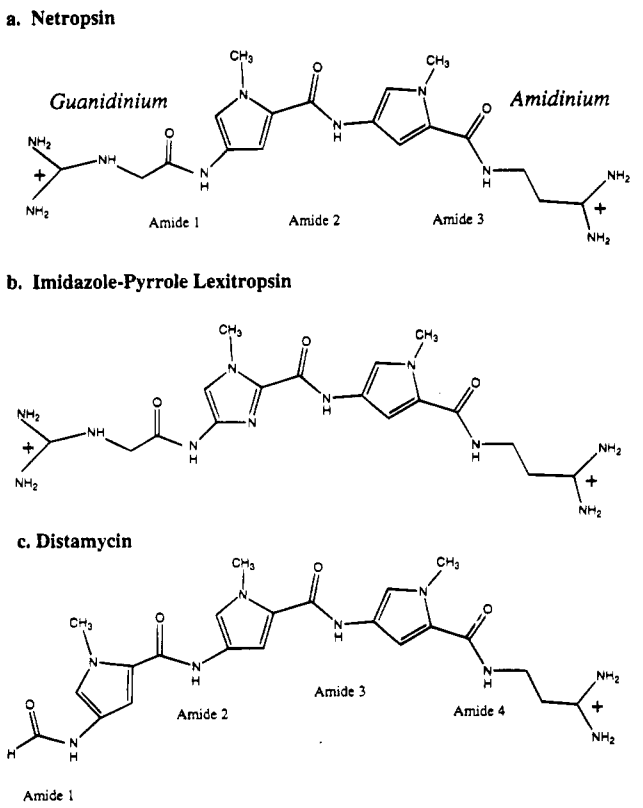


FIGURE 1: Members of the netropsin/lexitropsin/distamycin family. (a) Naturally occurring netropsin with two pyrrole rings, or Nt-2. The guanidinium head at the left and the amidinium tail at the right are both positively charged. In this paper, netropsin and related drugs will be abbreviated by identifying five-membered rings sequentially from the guanidinium "head" to the amidinium "tail". Any charge on either end is given as a superscript. In this representation, native netropsin becomes $^{+}$Py−Py^{+}. (b) $^{+}$Im−Py^{+} lexitropsin, identical to Nt-2 except for replacement of the first pyrrole ring by imidazole. (c) Distamycin with three pyrrole rings, or Dst-3. The uncharged −CHO head is at the left and the charged amidinium tail at the right. Dst-3 will be symbolized by ^{0}Py−Py−Py^{+}, with the leading zero indicating that the head of the molecule is uncharged.

METHODS

Crystals of $^{+}$Im−Py^{+} lexitropsin/DNA were obtained by vapor diffusion of standing drops at 5 °C. A drop originally 0.43 mM in DNA duplex, 6.0 mM in magnesium acetate, 0.2 mM in spermine hydrochloride, 0.73 mM in drug, and 8.8% w/v MPD yielded rectangular crystalline rods after equilibration against a reservoir 40% w/v in MPD. X-ray survey photographs showed that the lexitropsin crystals were isomorphous with the native dodecamer, in space group $P2_12_12_1$, and with cell dimensions a = 24.03 Å, b = 39.26 Å, and c = 66.30 Å. A 0.35 × 0.20 × 0.15 mm crystal was used for data collection on an AFC5R Rigaku diffractometer using Ni-filtered Cu Kα radiation. Data were collected at −25 °C, and Lorentz and absorption factors were applied. Monitoring of three check reflections indicated negligible radiation damage (≤1.0%). The data were 85% complete to 2.25 Å, with 70% observed in the outermost shell (2.39−2.25 Å).

Molecular replacement began with DNA coordinates from PDB entry 1BNA (the "Drew" dodecamer), with all temperature factors set to 20 Å^2. Rigid body refinement in X-PLOR (Brünger, 1993), with progressively 1, 2, 4, 12, and 24 articulated groups, gave a conventional R factor of R = 0.340 for reflections between 8 and 3 Å resolution. (All R factors are calculated using only data above the 2σ level in F.) Positional refinement in NUCLSQ (Hendrickson & Konnert, 1980) reduced this to R = 0.301. Data then were added to 2.25 Å resolution in eight equal-volume shells, with refinement at each step. Following refinement of temperature factors, the model gave R = 0.262. Water molecules were added at peaks that were simultaneously (a) above 1σ in ($2F_o - F_c$) density, (b) above 3σ in ($F_o - F_c$) density, and (c) within proper hydrogen-bonding distance of other atoms. A long continuous ribbon of density down the minor groove was carefully avoided. With 36 water molecules, the residual error stood at R = 0.185.

An initial drug-free difference electron density map then was calculated, subtracting out only the DNA and those well-established water molecules that were not in the immediate vicinity of the putative drug density. This map, shown in Figure 2, is the last completely unbiased view of the drug density. All subsequent difference maps, including the final "omit" map that customarily is published to show how well the drug skeleton fits the electron density, are biased toward the assumed drug structure and are correspondingly of little value in proving the correctness of that structure (Goodsell et al., 1995). The drug image in this initial unbiased difference map of Figure 2 has several prominent features: (a) three major protuberances extending to the right from the convex side of the density crescent near the middle of the image, with smaller projections above and below, and well-defined tails at both ends.

As with netropsin (Kopka et al., 1985a−c; Goodsell et al., 1995), there is no ambiguity about where the lexitropsin skeleton should be placed. The molecule sits squarely in the central AATT region of the helix, in a position enabling it to make bifurcated hydrogen bonds with N and O atoms along the floor of the groove. (These H-bonds will be discussed later.) But we found, as did Tabernero et al. (1993) for netropsin on CGCAAATTTGCG, that the end-for-end orientation of the drug was less certain. We fitted the lexitropsin molecule into the density in two orientations and refined both to completion. In LexA the guanidinium group extends toward DNA base pair C1·G20 at the top of Figure 2a, whereas in LexB the guanidinium points toward G10·C11 at the bottom of Figure 2b. In both orientations the three central protrusions on the difference density are interpreted as the C=O of the central amide group, flanked by methyl groups of the methylpyrrole and methylimidazole rings. In netropsin the methyl protuberances were well-defined and were angled in a particular direction that established the orientation of the drug molecule. [See Figure 6 of Goodsell et al. (1995).] In the present study the protuberances are broader and can accommodate projecting methyl groups in either the LexA or the LexB orientation.

On balance, LexA is favored in the initial difference map for three reasons: (a) the central C=O is somewhat better positioned within the center of its density for LexA than LexB, (b) the upper C=O fits within difference density in LexA but not in LexB, and (c) LexA places the rigid guanidinium group within the slightly better defined upper end of the density image. Each fitting has one flaw: in LexA a methylene elbow from guanidinium sits just outside the 1.5σ contour, and in LexB a carbonyl group projects badly from the density envelope. Both LexA and LexB models were carried through the entire refinement process.

The final refined LexA model with 37 water molecules and with lexitropsin oriented like netropsin has an overall

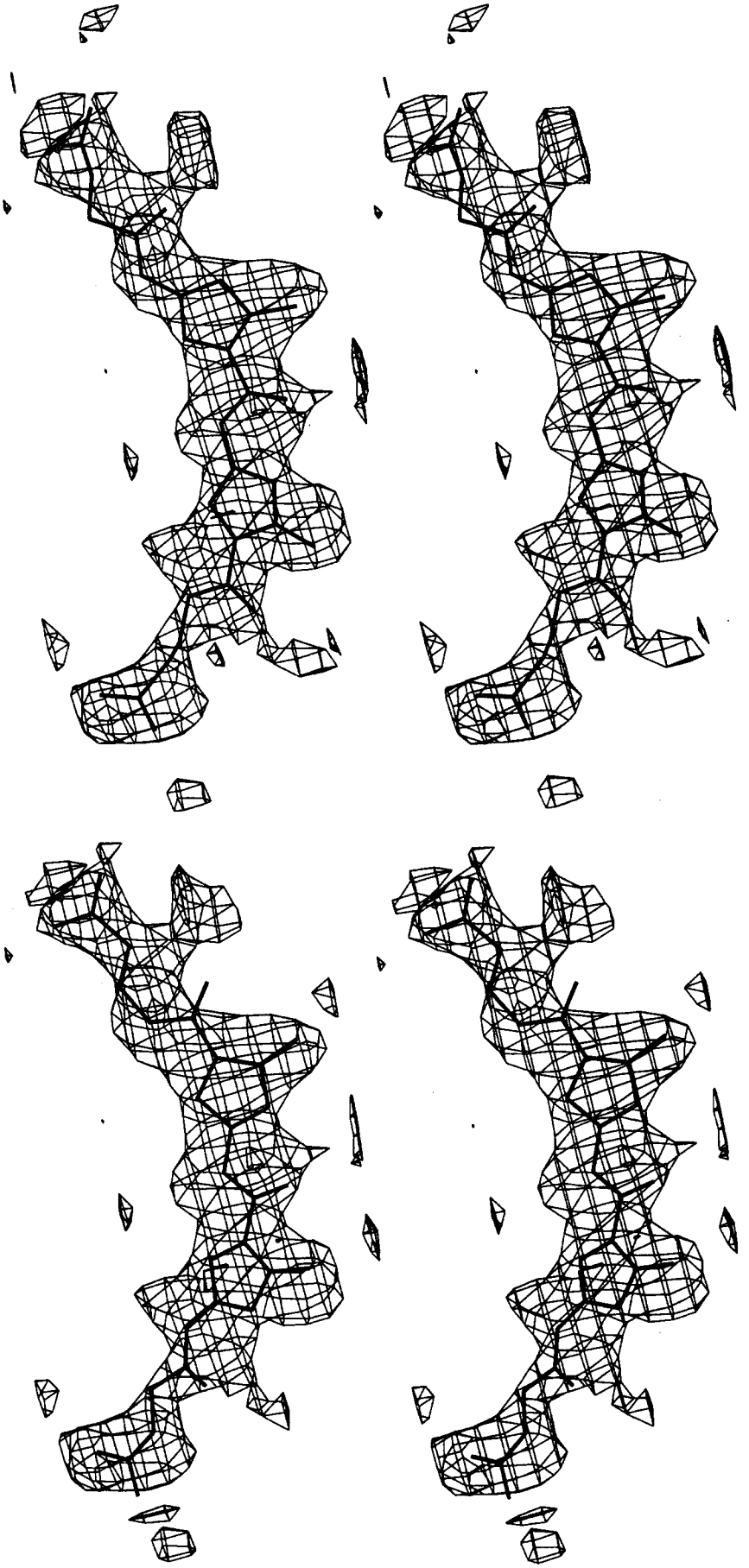


FIGURE 2: Initial difference electron density map of the lexitropsin/DNA complex, after refinement of DNA and 36 water molecules but before making any assumption about the location of the drug molecule. The map is contoured at the 1.5σ level. This density was fitted with lexitropsin in two orientations: (a, top) LexA, with guanidinium at the top, nearer base pair C1·G24, and (b, bottom) LexB, with guanidinium at the bottom, nearer G12·C13. Both models were carried through the entire refinement process.

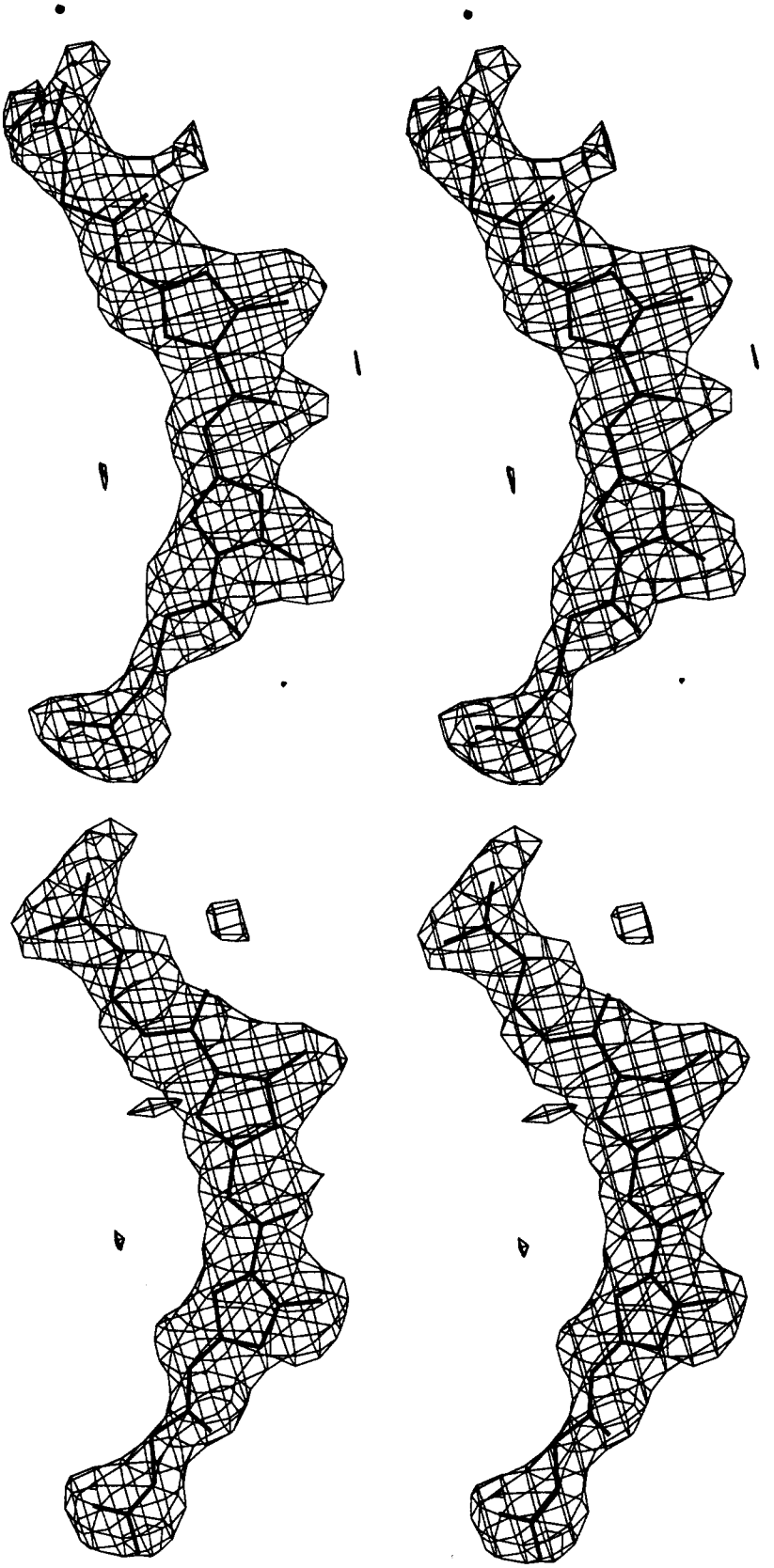
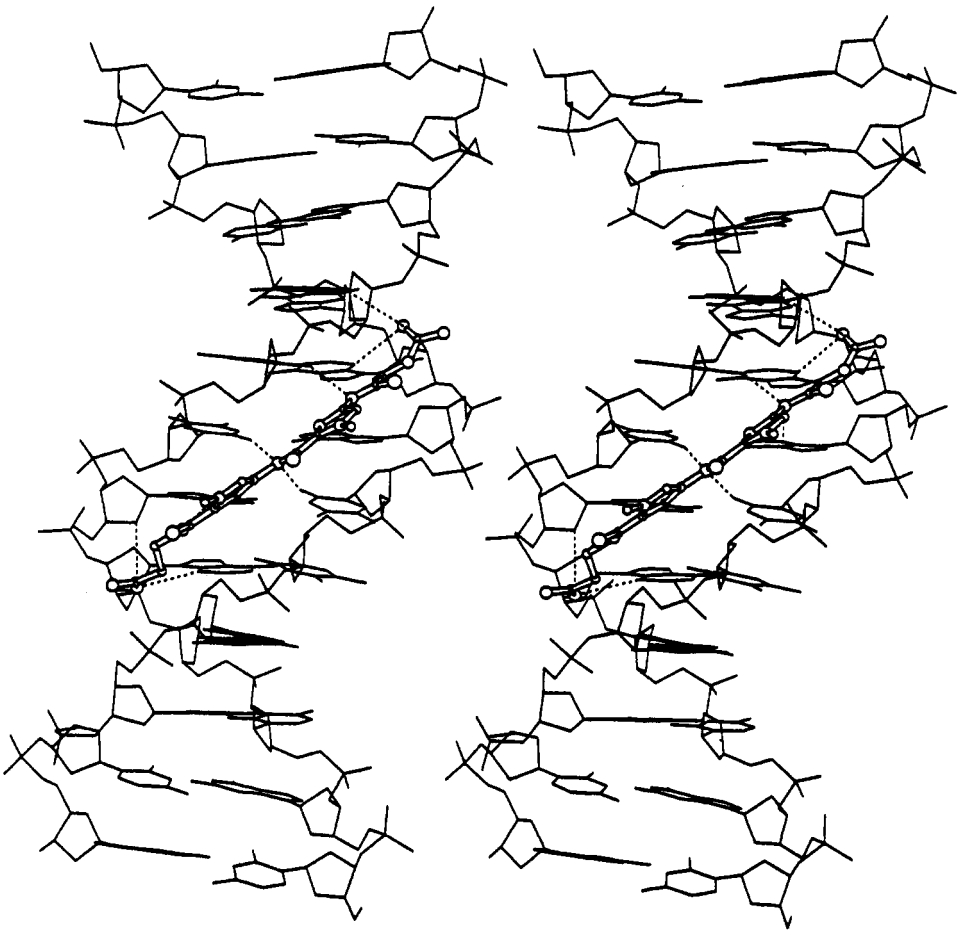
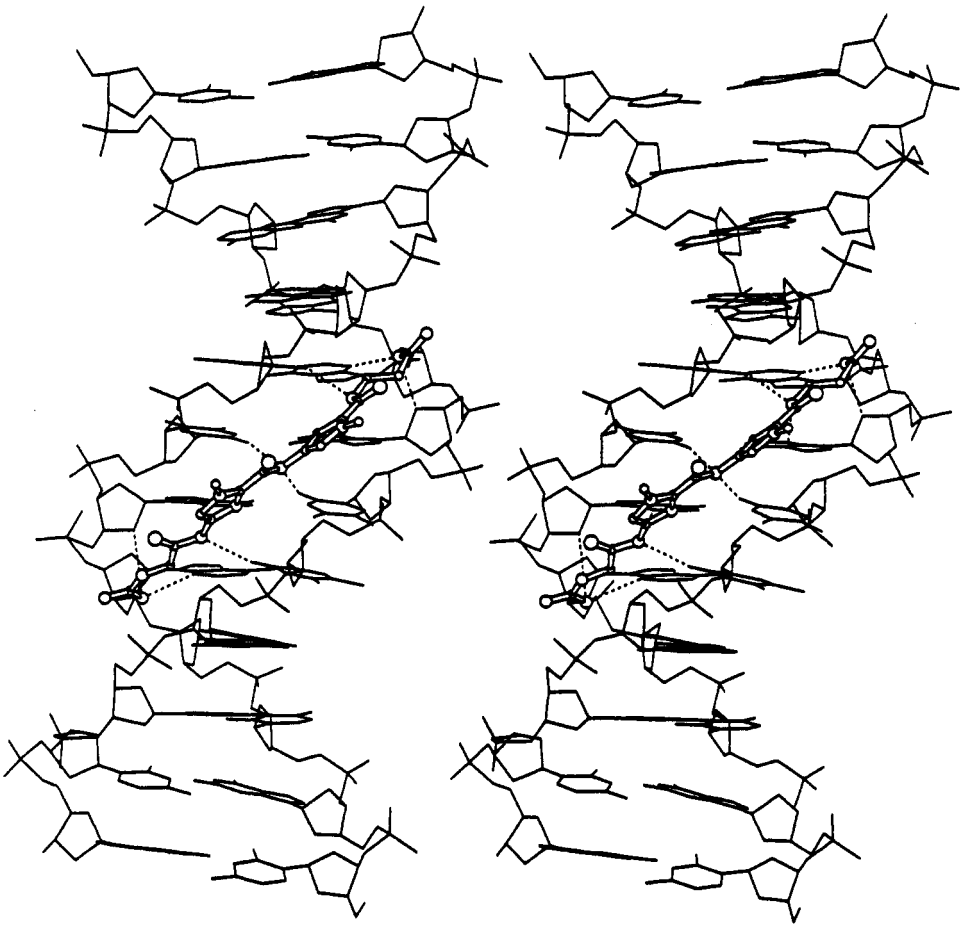


FIGURE 3: Final omit map of the lexitropsin/DNA complex, at the conclusion of refinement. The map is contoured at the 2.0σ level. (a, top) LexA, with guanidinium at the top, nearer base pair C1·G24, and (b, bottom) LexB, with guanidinium at the bottom, nearer G12·C13.

residual error or R factor of 0.165 and a free R factor (10% data sample) of 0.248. The LexB model with 40 waters gives R values of 0.164 (general) and 0.235 (free). Corresponding netropsin values from Goodsell et al. (1995) were 0.163 and 0.252, respectively. At the conclusion of refinement, an omit map was calculated for each orientation, in which the drug was deleted, DNA and solvent were used for phase analysis, and a difference map was calculated to show the image of the missing drug. These omit maps are compared in Figure 3. One can note the self-correcting tendency that makes omit maps worthless as assessments of correctness: the 2.0σ density contour for LexA now encloses the guanidinium





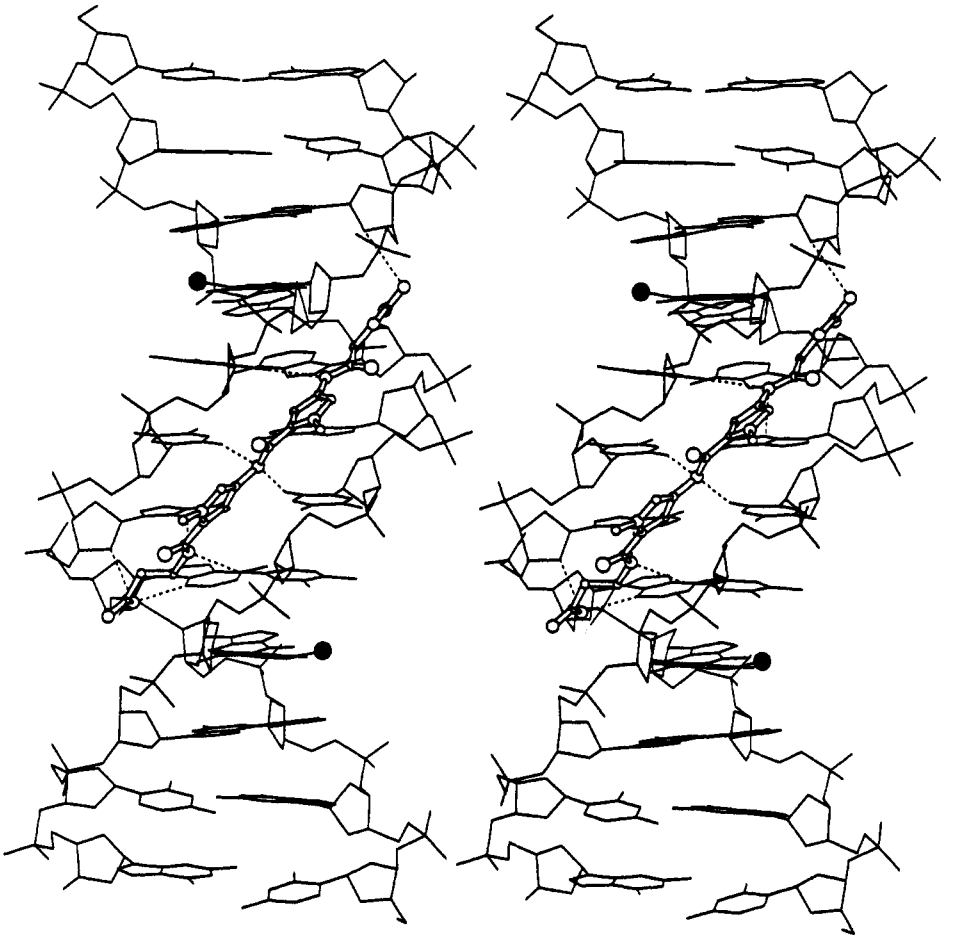


FIGURE 4: Final refined structures for two complexes of lexitropsin with DNA: (a, left page, top) LexA, (b, left page, bottom) LexB, and comparison with (c, above) netropsin (Goodsell et al., 1995).

Table 1: Lexitropsin Refinement Statistics

	LexA	LexB	target σ
geometric constraints			
bond distances			
B, S, L	0.025	0.025	0.030
P	0.024	0.030	0.025
angle distances			
B, S, L	0.046	0.049	0.040
P	0.066	0.067	0.050
planes	0.016	0.015	0.020
chiral centers	0.165	0.144	0.150
single-torsion contacts	0.097	0.107	0.100
multiple-torsion contacts	0.098	0.093	0.100
hydrogen bonds	not constrained		
temperature factor constraints			
across bonds			
B, S, L	5.287	5.480	6.000
P	5.958	5.343	6.000
across angles			
B, S, L	5.938	6.266	6.000
P	5.830	5.843	6.000

methylene, and in LexB the protruding carbonyl group is now wrapped in density. Omit maps adapt to fit the structure whose refinement led to them, whereas initial difference maps are objective.

Table 1 lists final refinement statistics for both orientations. Mean temperature factors or B values for bases, sugars, phosphates, and drug, respectively, are 12.3, 17.9, 27.0, and 44.6 for LexA and 8.1, 13.3, 22.2, and 38.2 for LexB. There is no compelling reason to choose one orientation over the other, and the most probable interpretation is that the drug molecule is disordered within the crystal. But the drug remains centered within the AATT region and is not displaced toward either end by one-half base pair, as had been proposed at one time for netropsin (Sriram et al., 1992).

RESULTS AND DISCUSSION

Table 2 compares the refinement of LexA and LexB with that of netropsin. Refined LexA and LexB structures are shown in Figure 4a,b, for comparison with netropsin in Figure 4c. The DNA duplex is virtually identical in all three structures. In both lexitropsin orientations, as with netropsin, the drug molecule binds to the central AATT region of the helix, with pyrrole and imidazole rings nesting against base pair edges and amide groups between adjacent base pairs. Amide N−H's make bifurcated hydrogen bonds to adenine N3 and thymine O2 atoms on the floor of the minor groove, with N−N and N−O distances as given in Figure 5. In both LexA and LexB, these hydrogen bond distances are somewhat longer at the bottom of the helix, near base pair T8•A17, than at the top.

As with netropsin, in both LexA and LexB each five-membered pyrrole or imidazole ring is packed against a base pair edge. Table 3 gives C−C and N−C distances for both lexitropsin orientations and equivalent distances for netropsin. When pyrrole is replaced by imidazole in lexitropsin, one might have expected the five-membered ring to move closer to the bair pair because of replacement of =CH− by =N−. As Table 3 indicates, this does not happen; the five-membered ring remains roughly in the same place relative to the DNA, probably held there by the bridging hydrogen bonds from amide N−H groups to either side. But the effect

Table 2: Comparison of Netropsin and $^{+}$Im−Py^{+} Lexitropsin Structure Analyses

drug/DNA complex	no. of refl [$F_o > 2\sigma(F)$]	resolution (Å)	final R	no. of waters	reference
Nt/CGCGAATT^{5br}CGCG	2528	2.20	0.211	75	Kopka et al., 1985a−c
Nt/CGCGAATT^{5br}CGCG	2185	2.25	0.163	37	Goodsell et al., 1995
LexA/CGCGAATTCGCG	2358	2.25	0.165	37	this work
LexB/CGCGAATTCGCG	2358	2.25	0.164	40	this work

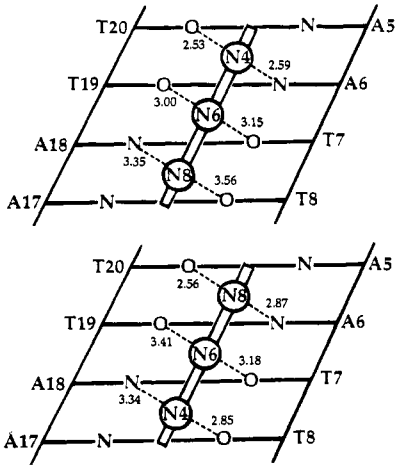


FIGURE 5: Bifurcated hydrogen bond distances in the final refined structures of (a, top) LexA and (b, bottom) LexB. Distances 3.5 Å or less are dashed; longer distances are dotted. Compare these distances with those of netropsin in Figure 4a of Goodsell et al. (1995).

Table 3: Close van der Waals Contact Distances between Drug Ring Atoms and Adenine C2 Atoms

	adenine 6 C2		adenine 18 C2	
	ring and atom	distance (Å)	ring and atom	distance (Å)
netropsin	1 C	3.5	2 C	3.2
LexA	1 N	3.1	2 C	3.6
LexB	2 C	3.5	1 N	3.8

of substituting at least one imidazole for pyrrole is to cause a significant lowering of the DNA/drug binding constant. The K_{app} for binding of netropsin to poly(dA-dT)·poly(dA-dT) is around 10^8 M^{-1}, whereas binding constants for dicationic mono- and diimidazole netropsin analogues—$^{+}$Im−Py^{+}, $^{+}$Py−Im^{+}, $^{+}$Im−Im^{+}—are all 2 orders of magnitude lower regardless of DNA sequence (Burckhardt et al., 1989). This weaker binding of $^{+}$Im−Py^{+} lexitropsin to DNA probably is responsible for the observed disorder, with two drug orientations, LexA and LexB.

The original lexitropsin concept was that replacement of pyrrole by imidazole in netropsin would make that position G·C-selective instead of A·T-selective. Instead, it only made the locus G·C-permissive, or nonspecific. Electrostatic effects dominate in the dicationic lexitropsins, driving them preferentially toward A·T regions, as Burckhardt et al. (1989) have documented. Moreover, the wider minor groove in regions of G·C base pairs would hold the planar drug molecule less securely. Monosubstituted $^{+}$Im−Py^{+} and $^{+}$Py−Im^{+}, like the parent $^{+}$Py−Py^{+} netropsin, continue to form stable complexes with A·T-containing DNA even at 4 M NaCl. But affinity for A·T DNA is lessened by replacement of both pyrroles in $^{+}$Im−Im^{+}, by lengthening the drug molecule as in $^{+}$Im−Im−Im^{+}, or by removing one positive charge as in ^{0}Im−Im^{+}. Both the foregoing two dicationic molecules are driven off A·T DNA by 1 M NaCl.

A similar pattern of DNA interaction is evidenced by DNase I cleavage experiments. Burckhardt et al. (1989) found that $^{+}$Im−Py^{+} provides poly(dA-dT)·poly(dA-dT) the same DNase I cleavage protection as $^{+}$Py−Py^{+} (netropsin), whereas $^{+}$Im−Im^{+} fails to block cleavage and $^{+}$Py−Im^{+} shows intermediate behavior. Against poly(dA-dC)·poly(dG-dT), $^{+}$Py−Py^{+} offers no protection at all. But $^{+}$Im−Py^{+}, $^{+}$Py−Im^{+}, and $^{+}$Im−Im^{+} provide low levels of protection (8%−23%) that presumably reflect the ability of these molecules to bind within a minor groove containing N2 amine groups. This acceptance of guanines is accentuated in the longer $^{+}$Im−Im−Im^{+}, which exhibits 40% protection of poly(dA−dC)·poly(dG-dT).

Evidently, replacement of pyrrole by imidazole is a necessary, but not a sufficient, condition for G·C binding. Reduction of overall positive charge in the monovalent lexitropsins allows more of the G·C-preference of imidazoles to be expressed (Kissinger et al., 1987; Krowicki et al., 1988). The inherent G·C preference of dicationic lexitropsins has been demonstrated recently by Reinert et al. (1995; see their Figure 2), who measure the change of viscosity of a DNA solution as a function of added drug molecules. For general-sequence DNA, netropsin initially produces a more profound change in viscosity than $^{+}$Im−Py^{+} because of netropsin's greater binding constant. Eventually, however, when all of the available A·T regions are saturated, addition of more netropsin produces no further increase in viscosity. In contrast, the more weakly binding $^{+}$Im−Py^{+} produces a smaller initial increase in viscosity per quantity of drug added. But the viscosity continues to rise long past the point where the netropsin viscosity plateaued, presumably because $^{+}$Im−Py^{+}, once it has saturated all the available A·T-rich regions, is still capable of binding to G·C regions as well.

The compound studied here, $^{+}$Im−Py^{+}, is the most similar of all lexitropsins to the parent netropsin, $^{+}$Py−Py^{+}. It seems most important to preserve the integrity of the second half of the molecule: the final pyrrole and the charged propyl-amidinium tail. Substitution of imidazole for pyrrole does indeed confer G·C specificity at particular loci, but that specificity is overwhelmed by electrostatic effects unless the charge on the drug molecule is reduced. Elimination of the charge on one end of the molecule also offers another advantage: it makes possible side-by-side 2:1 drug/DNA complexes within a widened minor groove, which opens up new fields for the design of specificity (Pelton & Wemmer, 1989; Geierstanger et al., 1993, 1994; Mrksich & Dervan, 1993).

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