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DNA minor groove recognition of a non-self-complementary AT-rich sequence by a tris-benzimidazole ligand

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ABSTRACT

The crystal structure of the non-self-complementary dodecamer DNA duplex formed by d(CG[^{5Br}C]ATATTTGCG) and d(CGCAAATATGCG) has been solved to 2.3 Å resolution, together with that of its complex with the tris-benzimidazole minor groove binding ligand TRIBIZ. The inclusion of a bromine atom on one strand in each structure enabled the possibility of disorder to be discounted. The native structure has an exceptional narrow minor groove, of 2.5–2.6 Å in the central part of the A/T region, which is increased in width by ~0.8 Å on drug binding. The ligand molecule binds in the central part of the sequence. The benzimidazole subunits of the ligand participate in six bifurcated hydrogen bonds with A:T base pair edges, three to each DNA strand. The presence of a pair of C-H…O hydrogen bonds has been deduced from the close proximity of the pyrrolidine group of the ligand to the TpA step in the sequence.

INTRODUCTION

The structural features of the interactions of non-covalent minor groove-binding drugs and ligands (typified by netropsin, distamycin, Hoechst 33258, berenil and pentamidine) with DNA have been extensively studied crystallographically (see for example 1–7), as well as by a range of other biophysical methods (see for example 8,9). The crystal structures of these drug–oligonucleotide complexes can be classified into two categories. The first, and largest, comprises 1:1 duplex:drug complexes (1–7). All except one (10) involve dodecanucleotides, with the central sequence 5'-AATT, 5'-TTAA, 5'-AAATTT or 5'-ATAT, where these A/T-selective drugs are bound. The second, numerically smaller, category includes netropsin, distamycin and synthetic polyamide ligands bound to a variety of sequences, with the ligands bound as dimers in the minor groove (11–14). All of the minor groove complexes have self-complementary sequences.

Analysis of these structures (2), together with data from NMR (15) and footprinting (16) studies, have shown that minor groove width is a major determinant of drug binding to particular sequences, together with hydrogen bonding to specific base edges forming the minor groove floor. Several sets of rules based on these factors have been devised (17–20) which have enabled a variety of polyamides to be constructed for the recognition of a wide range of desired sequences. It has proved challenging to extend this recognition beyond a site size of ~6 bp, although sequences in this size range can be very effectively recognised by hairpin polyamide ligands, with nanomolar binding affinities for target sequences *in vitro* (21,22). Sequence specificity to regulatory DNA targets has also been demonstrated in live cells (22,23).

The bis-benzimidazole ligand Hoechst 33258 (Fig. 1) and its analogues interact in the minor groove in a manner analogous to netropsin in that an identical arrangement of hydrogen bonds (from the two benzimidazole groups) to A:T base pair edges is formed (see for example 24–27). The benzimidazole unit is thus a conformationally stable and appropriate platform on which to build further DNA sequence recognition. We have previously determined (28) the crystal structure of the tris-benzimidazole ligand TRIBIZ (Fig. 1) bound to the self-complementary duplex sequence d(CGCAAATTTGCG). This structure showed that the ligand, which covers ~7.5 bp, interacts with the minor groove edges of the four central A:T base pairs by means of three sets of bifurcated hydrogen bonds. Several significant changes were observed in the structure of the oligonucleotide: minor groove width was narrowed and numerous small changes in propeller and helical twist were found. These latter indicated an unwinding of the central AT region by ~15° in total, in order to achieve isohelical register of base pair edges with the hydrogen bond donation from each benzimidazole subunit. Studies on closely related tris-benzimidazoles have confirmed that weak unwinding does occur (29). These have also suggested that the DNA cleavage mediated by DNA topoisomerase I and tris-benzimidazoles is a direct consequence of their minor groove binding (30,31).

We report here the crystal structure analysis of TRIBIZ bound to the sequence d(CGCATATTTGCG)·d(CGCAAATATGCG), as well as that of this native duplex. This is the first report of a minor groove ligand complex involving a non-self-complementary duplex. It was chosen in order to: (i) extend our knowledge of benzimidazole minor groove recognition to

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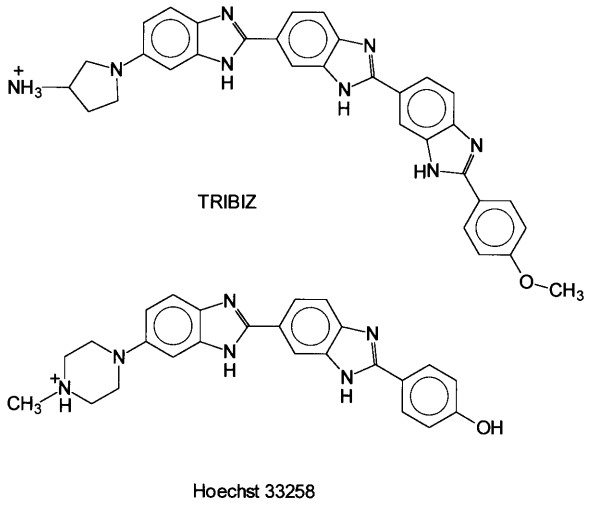


Figure 1. Benzimidazole drug molecules.

a mixed alternating/non-alternating run of A:T base pairs; (ii) examine the role that sequence (and structural) asymmetry play in the recognition process. Few non-self-complementary DNA crystal structures have been reported to date (notably refs 32–34), with disorder being a common problem. In order to unequivocally determine whether the present structures are similarly affected, a 5-brominated cytosine was incorporated at position three on just one strand, i.e. d(CG[^{5Br}C]ATATTTGCG). It was judged that the position of the bromine atom in the major groove would not affect minor groove ligand binding in any way.

MATERIALS AND METHODS

The two DNA dodecamers d(CG[^{5Br}C]ATATTTGCG) and its complementary sequence d(CGCAAATATGCG) were purchased from the Oswel DNA Service (University of Southampton, UK). The two nucleotides were mixed in equimolar amounts and annealed before use at 80°C for 10 min.

Crystallisation and data collection

The hanging drop method was used for crystal growth. Both native and drug complex crystals were grown at 13°C. Droplets contained 1 µl of 75 mM $MgCl_2$, 1.5 µl of 15 mM spermine, 2 µl of 3 mM duplex and 2 µl MPD (25% w/v for the native and 30% for the drug complex), in 40 mM sodium cacodylate buffer, pH 6.5, equilibrated against a 35% w/v MPD reservoir. An aliquot of 2 µl of 3 mM TRIBIZ, as the hydrochloride salt, was added for the drug complex. In both cases, prismatic crystals grew in 3 weeks. Those co-crystallised with TRIBIZ were bright yellow.

Diffraction data were collected on a Rigaku RAXIS-4 image plate detector with CuK_α radiation from a Rigaku RU200 rotating anode generator (100 mA, 50 kV) and a Yale focussing mirror system, set at a crystal to detector distance of 100 cm. Crystals were flash frozen before use and maintained at –173°C using an Oxford Cryostream (Oxford Cryosystems). No additional cryoprotectant was required. One crystal was used for each structure and 100 frames of intensity data were collected using a 1.5º oscillation angle, resulting in the collection of ~16 000 reflections per structure. Data processing was carried out using the DENZO and SCALEPACK packages (35). A total of 3508 unique reflections to 2.2 Å were observed for the native structure (94.1% of the possible reflections were statistically significant to this resolution). Cell dimensions were: a = 25.19 Å, b = 40.58 Å, c = 66.01 Å, space group $P2_12_12_1$, $R_{merge}(I)$ = 6.7% for all data to 2.2 Å. A total of 3281 unique reflections to 2.2 Å were observed for the TRIBIZ complex (87.7% of the possible reflections to this resolution). Cell dimensions were: a = 25.50 Å, b = 40.42 Å, c = 65.55 Å, space group $P2_12_12_1$, $R_{merge}(I)$ = 5.0% for all data to 2.2 Å.

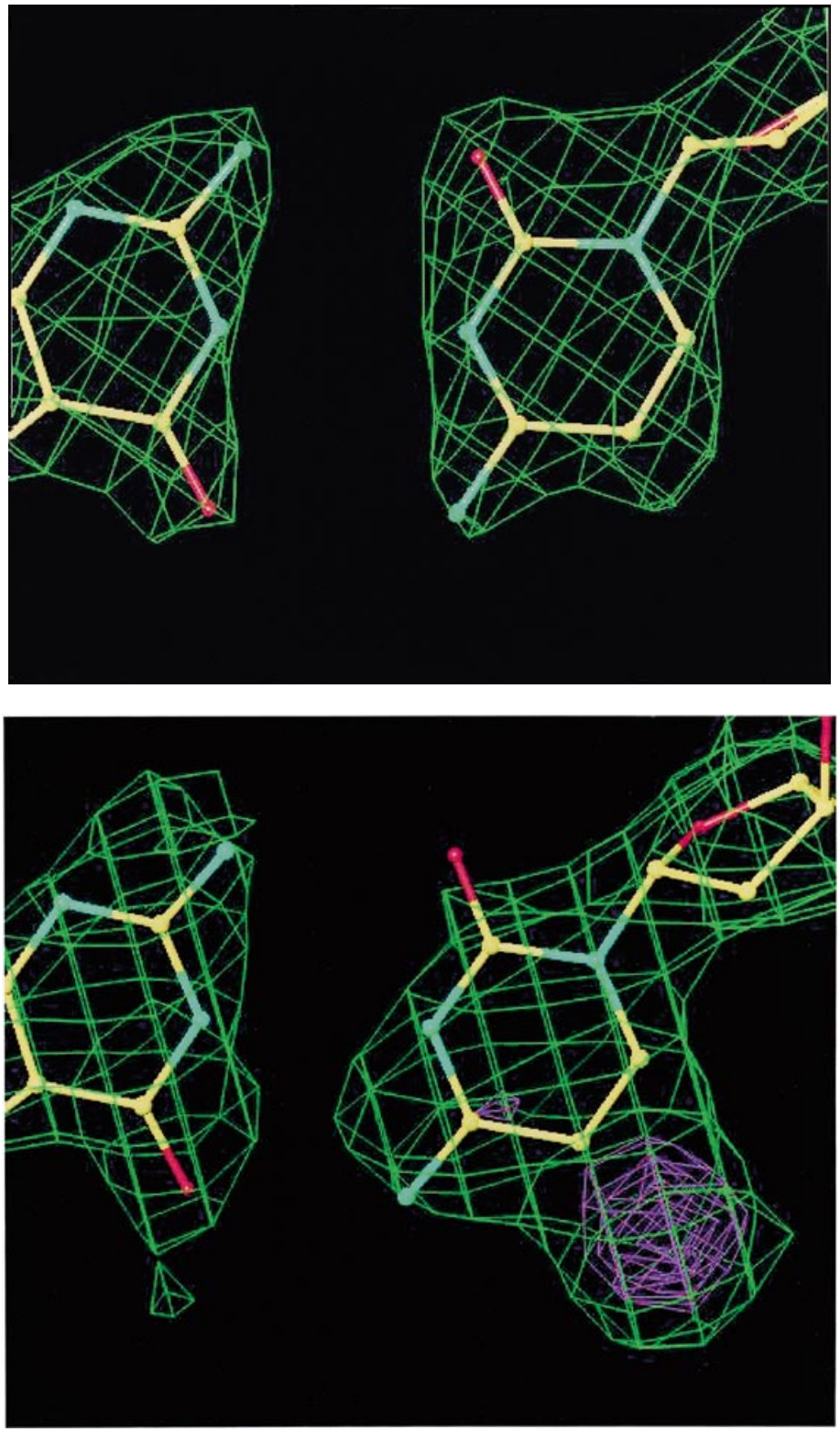
Structure determination and refinement

The atomic coordinates for the DNA from the d(CGCAAATTTGCG)–distamycin complex (36) were used as the starting point for molecular replacement solution of both structures. It was assumed that both structures are isomorphous with this structure and with other dodecanucleotide structures.

The native structure refinement was initiated with the model as a rigid body, using the X-PLOR v.3.1 program (37). The initial R factor was 28.7% (R_{free} = 33.8%) using data from 8 to 4 Å. The model was partitioned into a successively greater number of groups, 2, 8, 12 and 24, whilst increasing the resolution to 2.5 Å. After changing to the correct sequence followed by positional and temperature factor refinement, R decreased to 30.0% (R_{free} = 41.3%) for reflections with $F_o > 4\sigma(F_o)$ in the range 8–2.5 Å and 35.7% (R_{free} = 44.9%) in the range 8–2.2 Å. An $F_o - F_c$ map at this point clearly showed a single position for the bromine atom, close to the C5 atom of cytosine 3: no peak was observed close to cytosine 15 on the second strand (Fig. 2a). This unequivocally showed that the DNA duplex is positioned in only one of its two possible orientations in the crystal lattice. Subsequent refinement was conducted with the SHELX-97 program (38), using a standard library of DNA geometric parameters (39). A total of 78 water molecules were located in successive $F_o - F_c$ maps. The final R factor was 20.7% for all data (2756 reflections) in the range 8–2.3 Å. R_{free} was 30.3%.

The TRIBIZ complex was refined, starting with the model as a rigid body, which gave an initial R factor of 35.9% (R_{free} = 43.7%) with the data from 8 to 4 Å. The model was divided successively into 2, 8, 12 and 24 groups, increasing the resolution to the range 8–2.5 Å. At this stage R was 39.9% (R_{free} = 50.2%). The sequence was corrected and 20 water molecules outside the minor groove were located in a $F_o - F_c$ map. After positional and temperature factor refinement R was 31.2% (R_{free} = 42.9%) for all data in the range 8–2.3 Å. A subsequent difference map (Fig. 2b) showed clear density in the minor groove consistent with the dimensions of the ligand. A significant peak was also observed close to cytosine 3, adjacent to atom C5. This was assigned as a bromine atom, again confirming only one orientation for the duplex in the crystal. Subsequent SHELX-97 refinement included the bromine atom and examined both possible orientations for the ligand. The orientation of the ligand was not convincingly apparent from the difference map, although the shape of the density at one end better matched the shape of the phenol group and its methoxy substituent. After 30 cycles of refinement both orientations gave the same R value, 28.5% (for data for 8–2.4 Å). One orientation resulted in an R_{free} of 36.1%, with very acceptable density for the ends of the molecule apparent in the resulting $2F_o - F_c$ map; however, the other

a



orientation resulted in an R_{free} value of 37.2%, with the density in the $2F_o - F_c$ map failing to cover all the terminal atoms of the ligand. A total of 90 water molecules were subsequently located in a series of $F_o - F_c$ maps. The water arrangement around the termini of the TRIBIZ molecule was consistent with the selected orientation. The final *R* factor was 20.9 for all data (2695 reflections) in the range 8–2.3 Å. R_{free} was 28.3%. Final atomic coordinates and structure factors for both structures have been deposited in the Nucleic Acid Database as entry numbers BD0011 and DD0014.

b

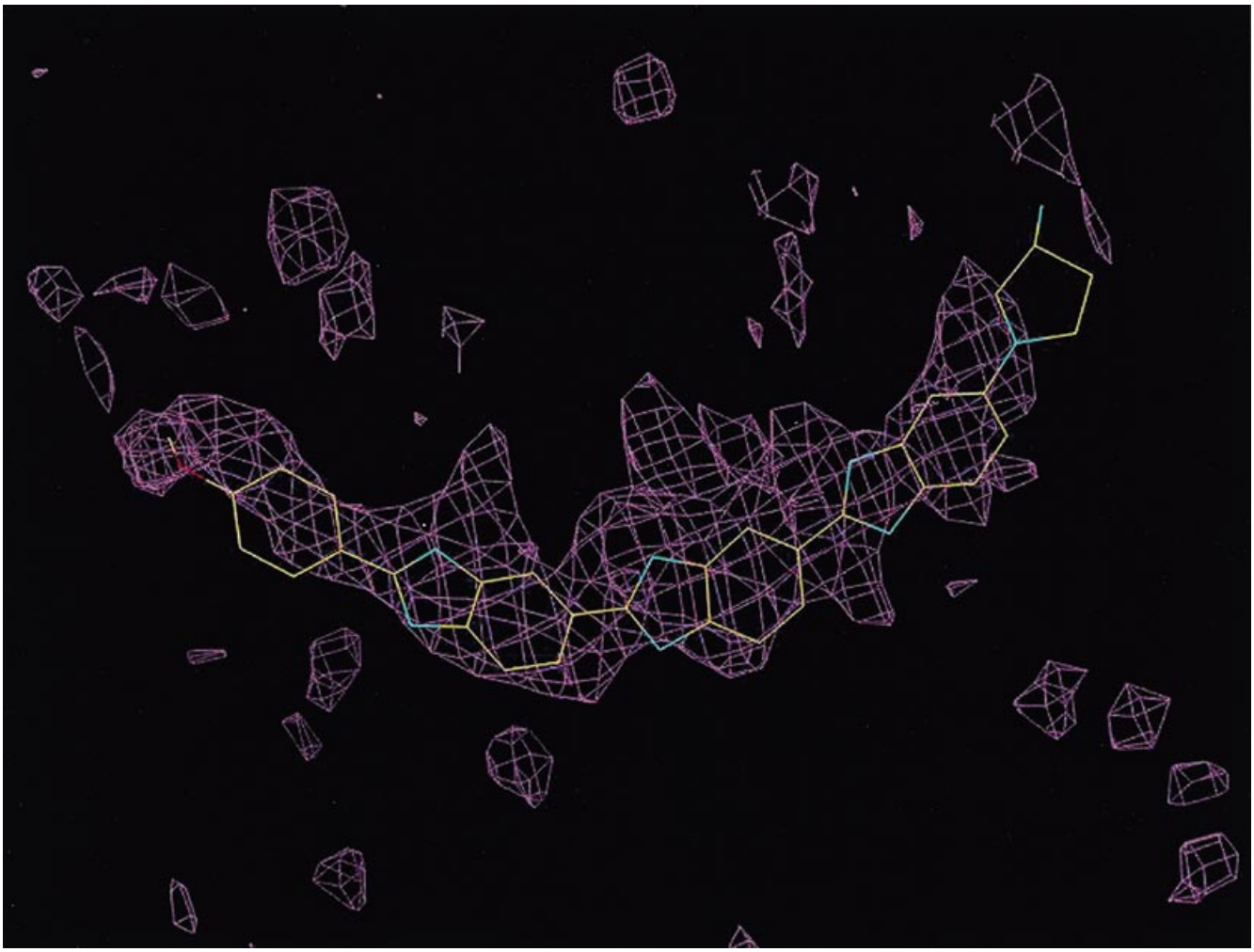


Figure 2. (Previous page and above) (**a**) Base pairs ^{5Br}C3-G22 and C15-G10 in the native structure. $F_o - F_c$ difference density (2.5σ level) is shown in mauve and $2F_o - F_c$ (1σ level) in green, after X-PLOR refinement but before the inclusion of the bromine atom or water molecules. (**b**) $2F_o - F_c$ difference density map for the TRIBIZ molecule (1σ level), calculated at the end of the refinement.

RESULTS

DNA structure

The two oligonucleotide structures determined here are at the same resolution and have been refined by identical procedures, enabling detailed comparisons to be made between them. The employment and subsequent location of a single brominated strand in each structure, as detailed above, has enabled the possibility of duplex disorder to be decisively rejected.

Minor groove width in these two structures has been calculated in terms of C4'…C4' inter-strand separations (using the CURVES program; 40), since C4' rather than P atoms are directed into the groove and contact the drug molecule. The native duplex structure is remarkably narrow over most of the A/T region (Fig. 3a). The narrow region has a minimum width of 2.5–2.6 Å extending over ~4 bp and is slightly displaced in the 5' direction. This width is ~0.7 Å less than the comparable region in the crystal structure of the closely related self-complementary $d(CGCAAATTTGCG)_2$ duplex (41). In contrast, the alternating duplex d(CGCATATATGCG) has a minimum width in the A/T region (again calculated with C4'…C4' distances) of 3.3 Å (42). Exceptionally narrow minor grooves, of 2.7–2.8 Å, have also been reported in A-tract crystal structures (32–34), although the disorder present in some of them may diminish their accuracy.

The seven central base pair steps in the structure have marked alternation of local helical twist angles (Fig. 3b). The TpA step has an exceptionally small value (25º), which is counterbalanced by high twist values for the ApT steps on either side, of 45 and 49º (Fig. 3b). The base pairs at the TpA step are almost entirely unstacked. The twist alternation extends into the 5'-ATTT region, in contrast to the more even spread of values in the non-alternating structures (32,33,41). There is also an unusually small twist value for the TpG step (of 25º) at the point where the groove is 1.5 Å wider. This suggests that it is the consequence of the unwound and unstacked TpA step within the sequence context of overwound A:T base pairs on either side which results in exceptional groove narrowing. Values for propeller twist, in contrast, are consistently lower (Fig. 3d) than in other structures with either

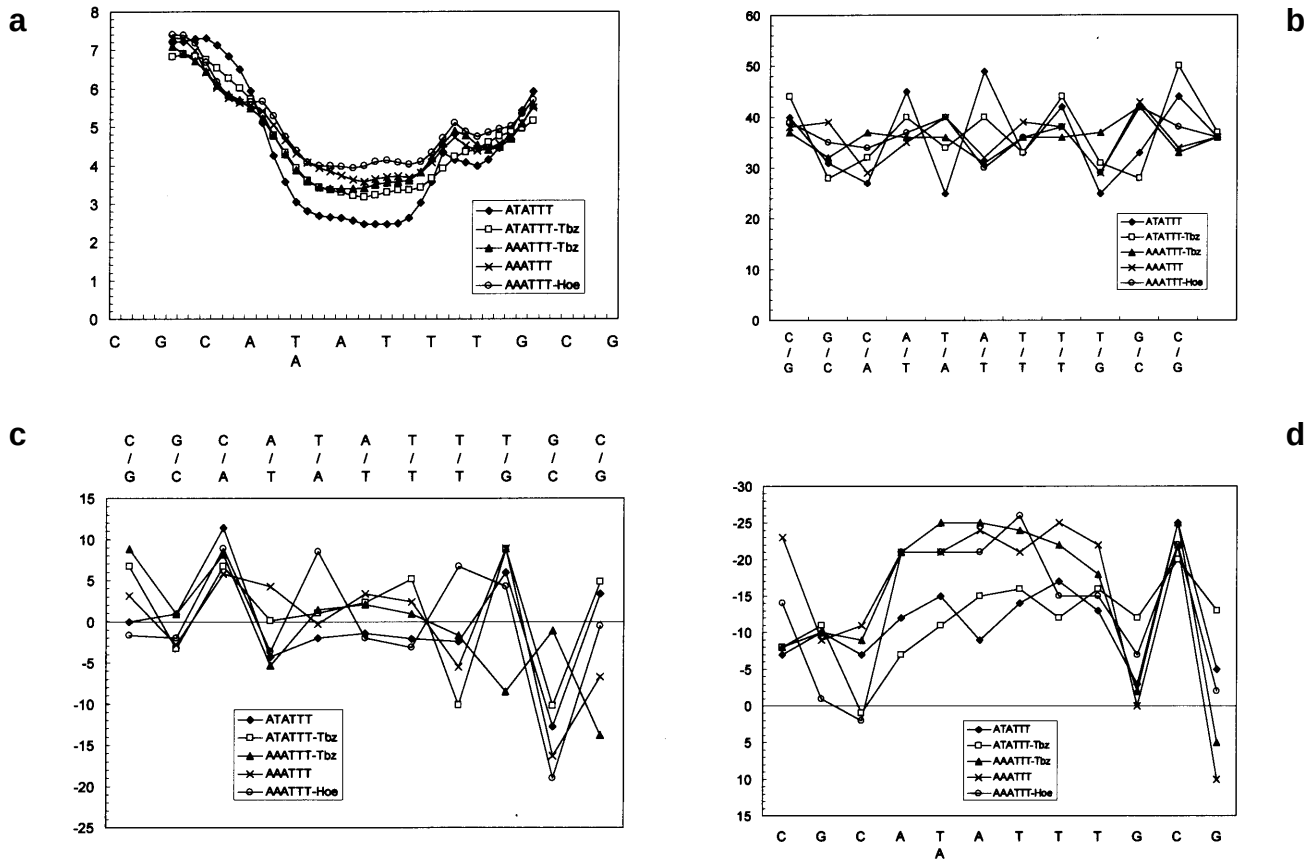


Figure 3. Various helical and base morphology parameters calculated with CURVES (40) for the present native structure and its TRIBIZ complex, for native d(CGCAAATTTGCG) (41) and for its TRIBIZ (28) and Hoechst 33258 (24) complexes. (**a**) Minor groove width, calculated using C4' atoms; (**b**) local helical twist; (**c**) local roll; (**d**) propeller twist.

alternating or non-alternating A:T base pairs (32–34,41–43). It appears that, at least in the present structure, narrow groove width is not directly correlated with propeller twist. The five central steps in the structure all have negative roll values (Fig. 3c). The TpA step has a small negative roll, of –2º, although the total roll over these steps is –12º, which opens up the major groove and compresses the minor groove and so may contribute to its narrow width.

An incomplete minor groove pattern of hydration is observed in this structure, possibly on account of the only moderate resolution of the structure. The T5-A6 step itself is fully hydrated, as is the preceding A4-T5 step (Fig. 4a), with in each case a water molecule bridging between two donor atoms of the base pair. In the case of the ApT step, the water bridges O2 thymine atoms as well as to the O4' atom of A6. This water also contacts two other waters (one of which then bridges between adenine N3 and thymine O2 of the adjacent TpA step). The ApT step water molecule is penta-coordinated, suggesting that it may be a partial occupancy sodium or potassium ion. The ability of these 'water' molecules to bridge between the two strands is illustrative of the narrowed minor groove at this point. It is at the centre of a network of six water molecules, which make a total of three hydrogen bonds to O4' atoms and five to base edges.

The ligand complex

The TRIBIZ molecule is bound in the minor groove (Fig. 4b), with the pyrrolidine ring of the drug at the 5'-end of the binding site, i.e. in the same orientation as in the earlier TRIBIZ complex (28). The inner facing nitrogen atom on all three benzimidazole groups each donates a hydrogen bond to a pair of A:T base pairs (Fig. 5), in bifurcated hydrogen bond arrangements. Three each are to N3 of adenines and to O2 of thymines. The TpA step participates in one of these bifurcated interactions. The six hydrogen bond lengths are all well within accepted values and the arrangement is closely similar to that in the TRIBIZ complex with the 5'-AAATTT sequence (28) in the self-complementary dodecamer d(CGCAAATTTGCG), although here there are hydrogen bonds to four O2 thymines and only two to N3 of adenines. The TRIBIZ molecule is thus in an equivalent position along the sequence in both its complexes. In both cases the four central A:T base pairs are involved in this hydrogen bonding network to the drug, with interactions to both adenines and thymines in the inner-most 2 bp. These 2 bp are propeller twisted such that the major groove edges of the adenines approach close together and the N6…N6 separation is 3.2 Å.

The minor groove width is significantly widened along most of the A/T stretch, by ~0.8 Å, compared to the native structure

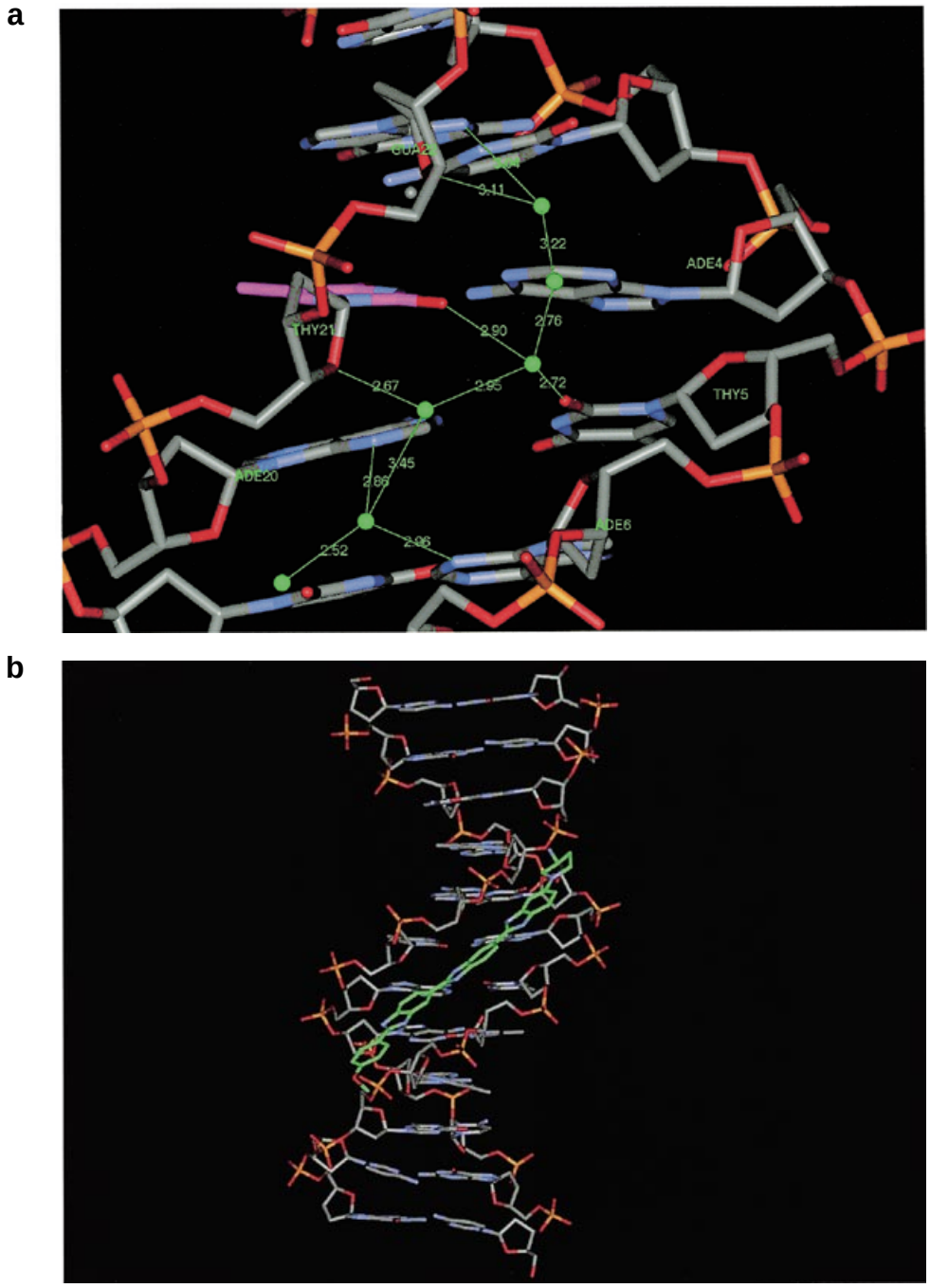


Figure 4. (**a**) View into the minor groove of the native structure, in the region around the TpA step, showing the six water molecules situated in the groove. (**b**) Overall view of the TRIBIZ complex.

(Fig. 3a). The groove dimensions in the ligand complex closely resemble that of the earlier TRIBIZ complex (28), which shows a decrease in groove width compared to the native structure. Thus, regardless of the structural features of the native sequence, binding of this ligand has induced conformational change in order to maximise close contacts with the groove floor and walls. The extent of change in the present structure on binding TRIBIZ is seen in Figure 3b–d. Thus, the marked alternation in helical twist angles is no longer apparent and the tract of negative roll angles becomes slightly positive. However, the TpA step retains its unstacked feature, as seen in the native structure. It is remarkable that the six N3 and O2 base edge atoms involved in ligand hydrogen bonding are preserved in their position since their r.m.s.d. between the native and ligand complex is only 0.3 Å.

The TRIBIZ molecule is highly twisted along its length (Table 1), with a total twist of 47°, comparing well with that found in the self-complementary complex (28), of 41°. This amount of total twist is required for the ligand to achieve helical register with all 4 bp in the binding site. It has been

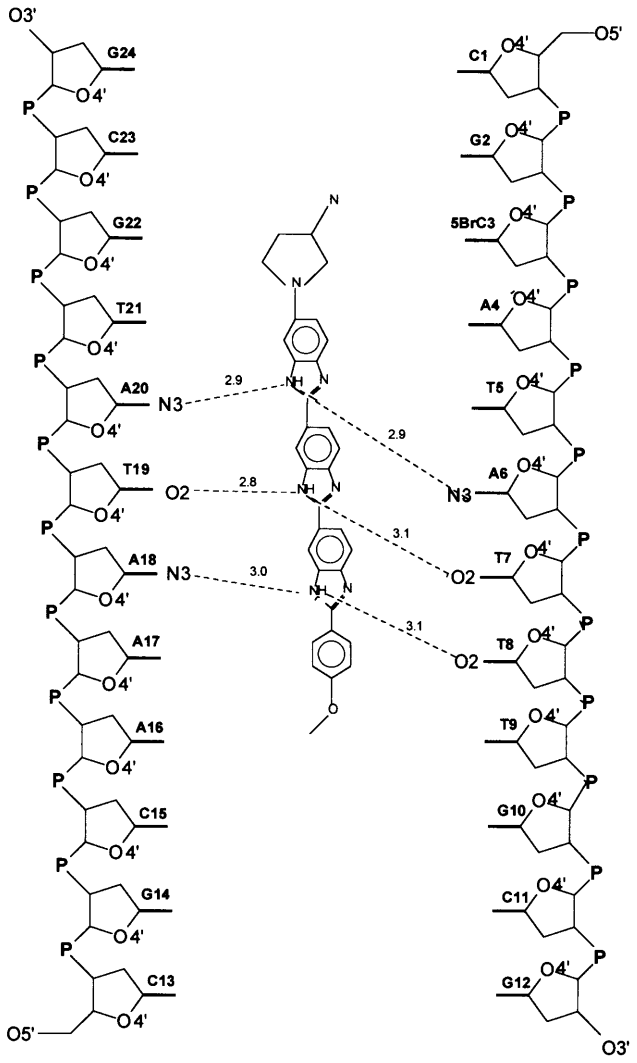
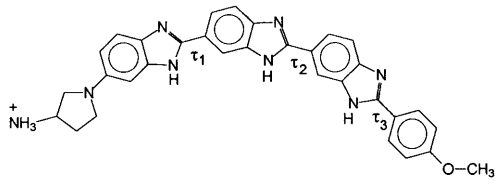


Figure 5. Schematic of the DNA–TRIBIZ complex showing the six hydrogen bonds between benzimidazole subunits and DNA.

previously noted (24,45) that the bis-benzimidazole compound Hoechst 33258 can readily adopt a range of non-planar conformations, dependent on the minor groove environment. Such conformations are also available in free solution. In this instance the shape of the groove combines with the positions of the six hydrogen acceptor atoms to maintain the TRIBIZ molecule in a highly twisted state.

There are a large number of van der Waals close contacts between the ligand molecule and the atoms forming the walls of the minor groove. The majority involve C4' backbone atoms, although C5' (and very rarely O4' atoms) are sometimes involved. These contacts are to all benzimidazole subunits, with closest contacts to the imidazole moieties. The methoxyphenyl ring contacts a deoxyribose ring, on strand 2. In contrast, the pyrrolidine ring at the other end of the ligand is not in close contact with the walls of the minor groove, which is not at its narrowest in this region. There are two very close contacts (2.8 and 3.0 Å) between the inner facing carbon atom of the pyrrolidine ring and the O2 atoms of the thymines at the 5'-end ApT step. Their geometries correspond to C-H…O hydrogen bonds, with near linearity in both instances.

Table 1. Torsional twist between benzimidazole subunits in minor groove complexes



	τ_1	τ_2	τ_3
TRIBIZ in the present structure	13	37	-3
TRIBIZ (28)	21	16	4

Each torsion angle shown is defined by the four atoms facing into the minor groove

DISCUSSION

We have shown here that a non-self-complementary DNA sequence containing (an albeit short) hybrid A/T stretch of alternating and non-alternating A:T base pairs has an exceptionally narrow minor groove, which is not confined to the region immediately around the unstacked TpA step. It may be premature to generalise this finding to other related A/T sequences, in view of their flexibility and potential ability to adopt a variety of B-type polymorphs with varying groove widths. We can confidently discount any significant role for crystal packing effects in changing the minor groove width in the A/T region (46). All of the dodecanucleotide duplexes discussed and referenced here (and almost all others) crystallise in the same space group, yet have wide variations in parameters such as groove width. This is understandable in view of the fact that the A/T region in these crystal structures is never directly involved in duplex–duplex intermolecular contacts.

The majority of monomeric DNA minor groove-binding drugs and ligands have a small binding site, of 3–5 bp. Even so, they can show a surprising ability to discriminate between different A/T sequences (16), with Hoechst 33258 showing a 50-fold difference in affinity between the sites 5'-AATT and 5'-TATA. This has been presumed to be likely due to the presence of the unstacked and unwound TpA steps (Fig. 3a and b), which would increase minor groove width. It is reasonable to assume that TRIBIZ, with its greater binding site size, would have at least as stringent a sequence requirement. The present structure shows that one such TpA step, embedded in an otherwise high affinity site, does not have such an effect. On the contrary, its ability to induce complementary overwinding and stacking in adjacent steps appears to result in decreased minor groove width along much of the length of the A/T sequence. The flexibility of A/T sequences in general means that a relatively large molecule such as TRIBIZ is able to bind in the groove, increasing the width appropriately. The energy cost of

the conformational changes required for this are compensated for upon binding by the large number of van der Waals interactions formed with the hydrophobic atoms lining the groove walls. An additional contribution to ligand stabilisation arises from the pair of short C-H…O hydrogen bonds from the pyrrolidine group of the ligand, which inter-strand straddles the two thymine O2 atoms at the 5'-end ApT step. This would not be possible in the 5'-AAATTT binding site (28) and the pyrrolidine group in this structure adopts a distinct orientation, with no hydrogen bonded close contacts to the DNA. Thus, the presence of the TpA step in the present sequence may confer a modest amount of additional stabilisation to the TRIBIZ complex.

The relative roles played by the various forces involved in stabilising minor groove complexes with small molecules continue to be the subject of debate. In particular, there is controversy concerning the importance of hydrogen bonding to base edges compared to van der Waals interactions. Recent calculations of the absolute free energy of association of netropsin with an A/T sequence (47) show that, for this drug at least, van der Waals forces dominate. Binding free energy data on analogues of the bis-benzimidazole ligand Hoechst 33258 (48) support this view. The present crystal structure of a TRIBIZ complex shows a large number of van der Waals contacts between ligand and DNA, which, numerically at least, greatly outnumber the (six) base–ligand hydrogen bonds. However, we also observe conservation of the relative positions of all six hydrogen bond acceptor atoms on the DNA, comparing the native and ligand-bound oligonucleotide, in spite of significant structural changes elsewhere in the structures. This suggests that although hydrogen bonding is not the dominant force stabilising the complex, it plays a key steering role in optimally positioning this ligand in its binding site.

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