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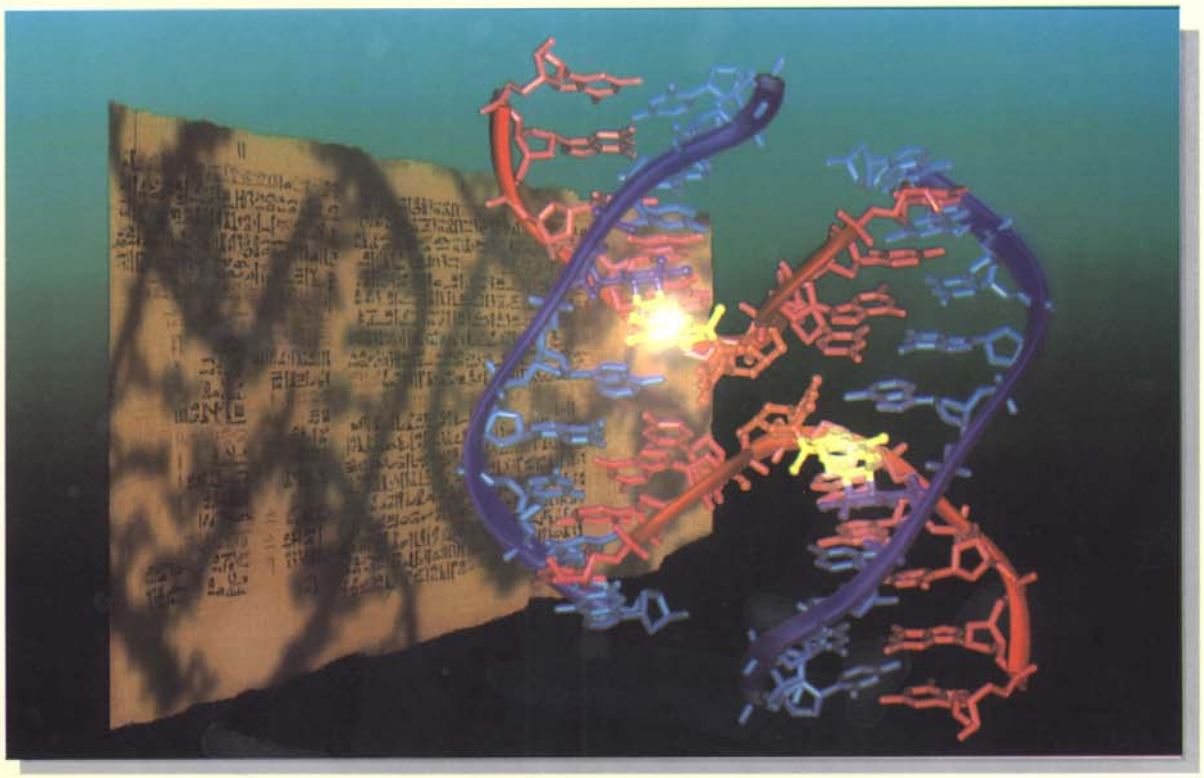

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The Crystal Structures of Psoralen Cross-linked DNAs: Drug-dependent Formation of Holliday Junctions

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The single-crystal structures are presented for two DNA sequences with the thymine bases covalently cross-linked across the complementary strands by 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT). The HMT-adduct of d(CCGCTAGCGG) forms a psoralen-induced Holliday junction, showing for the first time the effect of this important class of chemotheraputics on the structure of the recombination intermediate. In contrast, HMT-d(CCGGTACCGG) forms a sequence-dependent junction. In both structures, the DNA duplex is highly distorted at the thymine base linked to the six-member pyrone ring of the drug. The psoralen cross-link defines the intramolecular interactions of the drug-induced junction, while the sequence-dependent structure is nearly identical to the native Holliday junction of d(CCGGTACCGG) alone. The two structures contrast the effects of drug- and sequence-dependent interactions on the structure of a Holliday junction, suggesting a role for psoralen in the mechanism to initiate repair of psoralen-lesions in mammalian DNA.

Keywords: DNA-binding drug; DNA structure; Holliday junction; psoralen; recombination

Introduction

Psoralens are a class of furocoumarin compounds that have been applied as photochemotherapeutic treatments for skin ailments, and as powerful tools for studying DNA structure and function (reviewed by Cimino *et al.*, 1985). The use of psoralen containing plants to treat psoriasis, vitiligo, and other skin disorders were reported as early as ~2000 B.C. (reviewed by Bethea *et al.*, 1999; Helm *et al.*, 1991), making this one of the oldest classes of chemotheraputics in recorded history. Psoralens have been shown to inhibit replication (Baden *et al.*, 1972; Lüftl *et al.*, 1998; Trosko & Isoun, 1971) and transcription (Sastry & Hearst, 1991a,b; Shi *et al.*, 1988a) by covalently modifying DNA, and can act as photosensitizers to affect cell differentiation through interaction with cell surface membranes (Lanskin, 1994; Lanskin & Lee, 1991). In addition, psoralen adducts are highly recombinagenic in cells (Cassier *et al.*, 1984; Cole, 1973; Vos & Hanawalt, 1989). The detailed structures of psoralen-DNA adducts have been of great interest because of the drug's ability to cross-link the DNA duplex. Here, we present the single crystal structures of two psoralen cross-linked DNA sequences which, interestingly, form unique four-way Holliday junctions. This study shows for the first time how covalent modification by psoralen affects the structure and formation of the intermediate involved in recombination.

Psoralen preferentially forms interstrand DNA cross-links at d(TpA) dinucleotides upon exposure to light (Scheme 1). In this mechanism (Isaacs *et al.*, 1977), the drug initially intercalates noncovalently between base-pairs of the DNA duplex. Upon absorption of a single long wavelength photon (320-410 nm), either the furan-side or pyrone-side of the drug reacts with a pyrimidine base to form a cyclobutanyl monoadduct. Furan-side monoadducts can absorb a second photon to cross-link the

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Abbreviations used: HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; MPD, 2-methyl-2,4-pentanediol.

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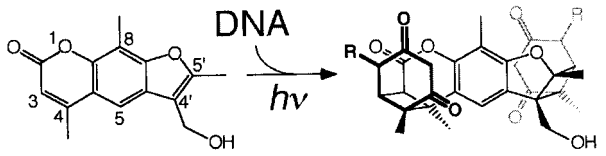
thymine base on the complementary strand of the DNA duplex.



Scheme 1.

The effect of a psoralen cross-link on the conformation of DNA remains unresolved. In NMR structures of deoxythymidine monoadducts, the thymine bases were in *cis-syn* geometry relative to psoralen (Kanne *et al.*, 1982b; Straub *et al.*, 1981). This stereochemistry was also seen in the NMR structure of two deoxythymidine nucleosides cross-linked by psoralen derivatives (Kanne *et al.*, 1982a) and in the crystal structure of a thymine base bonded to the furan-side of 8-methoxypsoralen (Peckler *et al.*, 1982). Models constructed from these simple structures suggested that the drug could kink the DNA double-helix by as much as 70°. Indeed, the first NMR structure of a psoralen cross-linked octanucleotide duplex reported a 53° helical bend toward the major groove of the DNA duplex (Tomic *et al.*, 1987). Furthermore, cross-linked, but not furan-side monoadducted DNAs were observed by electron microscopy to be significantly kinked (Shi *et al.*, 1988b). However, studies that probe the ability of the drug to kink or bend DNA using anomalous gel migration (Sinden & Hagerman, 1984) and differential decay of birefringence methods (Haran & Crothers, 1988) showed the DNA duplex to remain essentially straight, and more recently determined NMR structures of psoralen-modified octanucleotides indicate that psoralen cross-links do not place a kink in the DNA at the site of intercalation (Hwang *et al.*, 1996; Spielmann *et al.*, 1995). Therefore, the structural studies to date have focussed primarily on how the drug affects the structure of the DNA duplex.

Psoralen lesions in cells, however, have been shown to be highly recombinagenic. Genetic recombination plays an important role in the cellular response to and repair of psoralen cross-links in bacterial (Cole, 1973; Sinden & Cole, 1978), yeast (Cassier *et al.*, 1984; Saffran *et al.*, 1991, 1994), and mammalian cells (Liu-Lee *et al.*, 1984; Vos & Hanawalt, 1989). Double-strand DNA damage as psoralen cross-links and as double-strand breaks induce more recombination events with reciprocal exchange than do the analogous single-stranded lesions or adducts (Saffran *et al.*, 1994). The mechanism to remove psoralen cross-links in *Escherichia coli*, first proposed by Cole (1973), has been confirmed to consist of sequential nucleotide excision and recombination events (Cheng *et al.*, 1991; Sladek *et al.*, 1989). In yeast, repair of psoralen cross-links involve Rad51, a protein also required for radiation-induced recombination (Jachymczyk *et al.*, 1981). Although the repair of psoralen cross-links in mammalian cells is not as well understood, mutations in the human XRCC2 and XRCC3 genes, members of the RAD51 family, result in sensitivity to cross-linking agents. This suggests a role for these gene products in recombination-coupled repair (reviewed by Thompson, 1996).

In an attempt to detail the effect that psoralen cross-links have on the DNA conformation, we studied the single crystal structures of the sequences d(CCGGTACCGG) and d(CCGCTAGCGG) cross-linked at the thymine bases by 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT). It is interesting that the cross-linked sequences crystallized as distinct four-stranded Holliday junctions (Figures 1 and 2). Interstrand cross-links in themselves have not been shown to induce junctions. The sequence d(CGCGAATTCGCG) with a dithiobis-propane cross-link (Chiu *et al.*, 1999) and the sequence d(CCTCGCTCTC)/d(GAGAGCGAGG) containing a cisplatin interstrand cross-link (Coste *et al.*, 1999) both remain *B*-type duplexes. Comparing the junctions presented here with structures of their respective unmodified sequences (Eichman *et al.*, 2000) shows that the junction formed by d(CCGCTAGCGG) is induced by the drug, while that of d(CCGGTACCGG) is a consequence of the DNA sequence. The drug-induced junction structure represents the most dramatic perturbation to the DNA helix by an intercalator observed in a crystal structure to date, and suggests a mechanism by which the drug destabilizes the DNA duplex to allow initiation of cross-link repair in mammalian systems.

Results

The psoralen-induced Holliday junction in HMT-d(CCGCTAGCGG)

The psoralen adduct of d(CCGCTAGCGG) was initially crystallized to study the effects of the drug on a DNA double helix. The structure unexpectedly was solved as a Holliday junction (Figure 1). Two symmetry-related molecules assembled in the crystal to form this four-stranded complex in an anti-parallel stacked-X structure. Since this unmodified sequence crystallizes as a regular *B*-DNA double helix (Eichman *et al.*, 2000), the junction in HMT-d(CCGCTAGCGG) must be a direct consequence of the drug cross-link.

In this structure, the psoralen cross-link is seen to destabilize the DNA duplex at the strand linked to the HMT pyrone-side, thereby allowing this strand to exchange across duplexes (Figure 1(a)). This destabilization directly results from distortions to the base-pair with its thymine linked to the drug's six-membered pyrone-ring. It was possible that the asymmetric psoralen intercalated into a symmetric DNA duplex would be disordered; however, the HMT molecule is well ordered in one

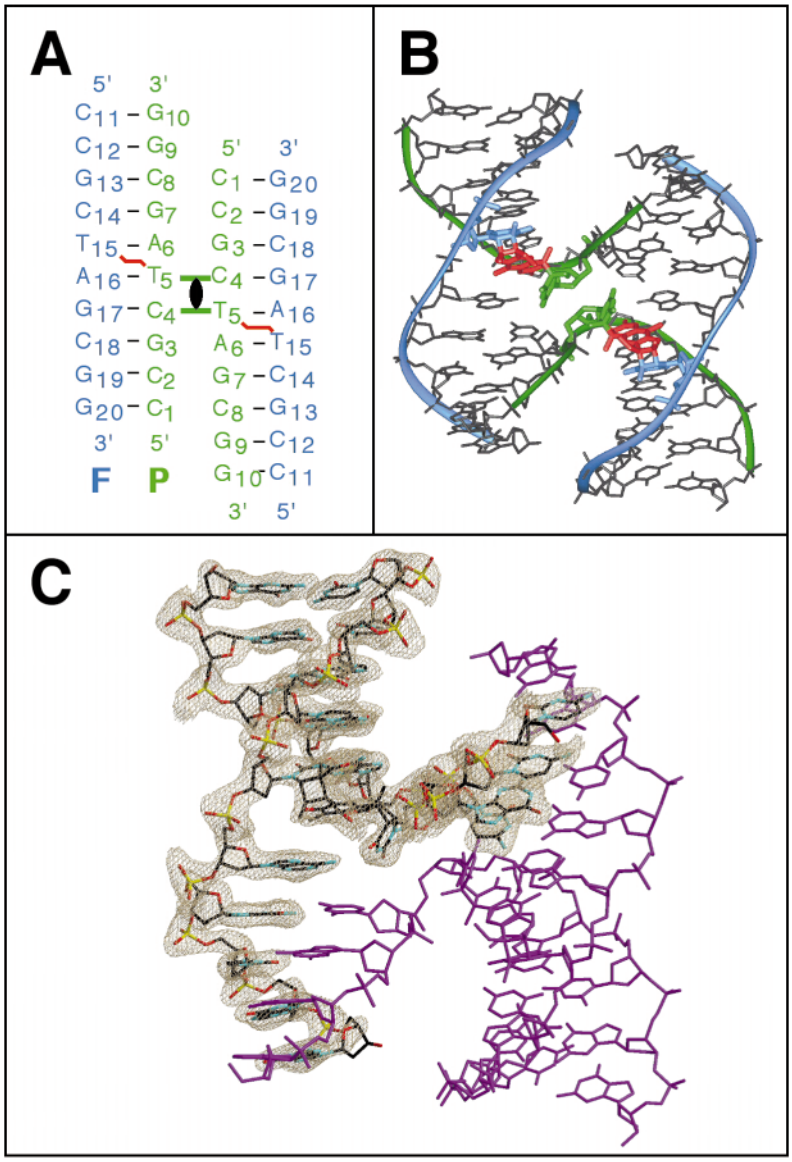


Figure 1. Single crystal structure of the Holliday junction induced in HMT-d(CCGCTAGCGG). (a) The assembly of four DNA strands form a junction, with two strands crossing over to connect the stacked helical arms. The strand that is adducted to the pyrone-side of the psoralen drug is labeled P, and this DNA strand is designated the P-strand (green). In a similar way, the DNA strand containing the furan-side adducted thymine is the F-strand (blue). The two stacked DNA helical arms are related by crystallographic 2-fold symmetry so that two cross-linked strands define the asymmetric unit in the crystal. (b) Model of the HMT-adducted structure is shown with the drug (red) covalently bound to the thymine bases along the P and F-strands of the DNAs. (c) The electron density contoured at 1σ in a $2F_o - F_c$ map is shown for the psoralenated strands of the asymmetric unit. The electron densities of the symmetry-related strands (purple) are omitted for clarity.

orientation in the crystal. The $F_o - F_c$ annealed omit electron density map clearly distinguishes the two sides of the drug (Figure 3(a)), with both the pyrone-side keto oxygen and the hydroxymethyl group at the furan 4′ carbon well resolved in the maps. The electron density maps also clearly show cyclobutanyl rings linking the HMT to the thymine bases (Figure 1(c)), with carbon atoms C5 and C6 of the thymine bases bonded to carbon atoms C4 and C3 of the pyrone ring, and to carbons C4′ and C5′ of the furan ring of HMT. Thus, the structure at the present resolution allows us to define the orientation of the psoralen and resolve its effects on the DNA strands at the pyrone-side (P-strand) and at the furan-side (F-strand) of the drug.

The d(T·A) base-pair with the thymine on the DNA F-strand shows the least distortion by the drug. Both hydrogen bonds remain within this base-pair, even though the thymine base shows a large increase in the inclination angles (η) between the native and HMT-cross-linked sequences ($\Delta\eta = 34.1\,^\circ$). This furan-side base-pair also shows strong π-stacking interactions between the adenine base and the HMT pyrone ring.

The most dramatic distortion is seen as a large (55.8 °) buckle in the d(T·A) base-pair with the thymine on the P-strand (Figure 1(b)), resulting from a steric collision between the O4 keto oxygen of the thymine base and the HMT pyrone ring. As a consequence, the base-pair no longer maintains two Watson-Crick hydrogen bonds. The thymine O4 keto to the adenine N6 amino hydrogen bond remains. However, the hydrogen bond from thymine N3 to adenine N1 is lost and replaced by a compensating water bridge (Figure 3(a)). Furthermore, there is virtually no stacking interactions between the adenine of this base-pair and the HMT furan ring.

The perturbations at the pyrone-side d(T·A) base-pair account for the destabilization of the

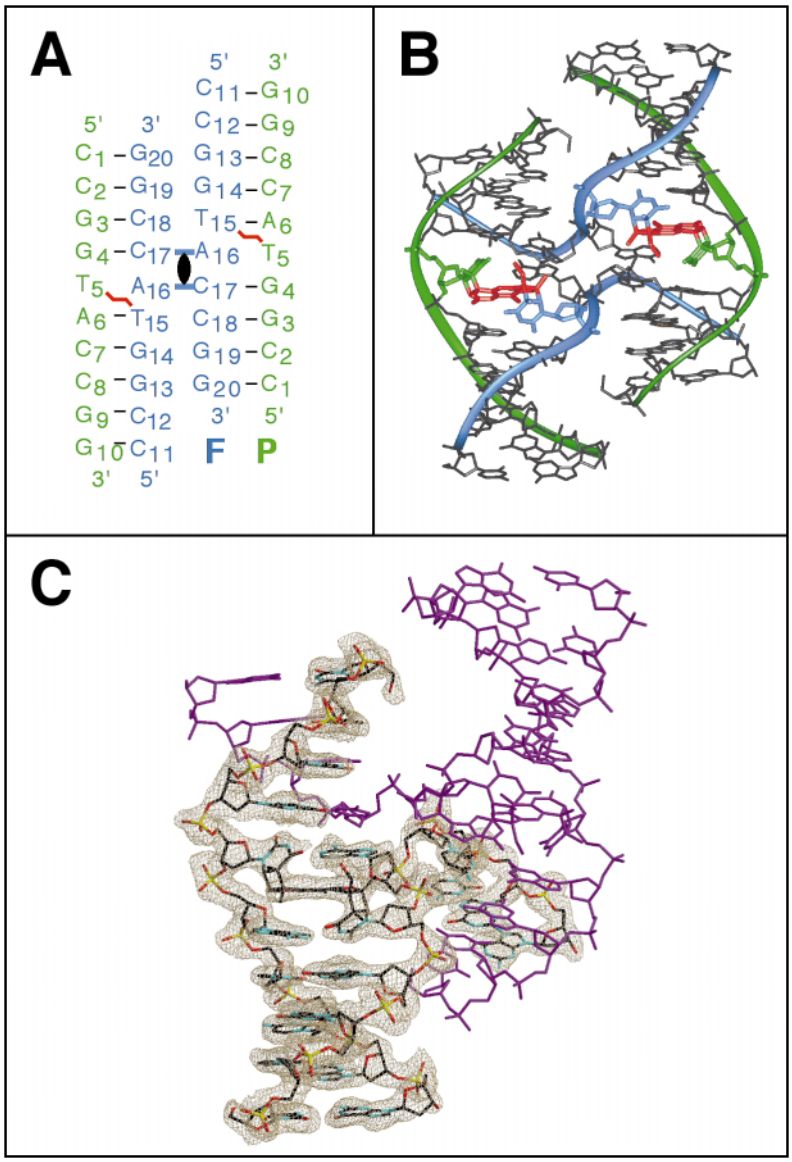


Figure 2. Single crystal structure of the sequence-dependent Holliday junction in HMT-d(CCGGTACCGG). (a) The assembly of four DNA strands form a junction, with two strands crossing over to connect the stacked helical arms. The strand designations and colors are identical to those in Figure 1. (b) Model of the HMT-adducted structure is shown with the drug covalently bound to the thymine bases along the P and F-strands of the DNAs, as in Figure 1. (c) The electron density contoured at 1σ in a $2F_o - F_c$ map is shown for the psoralenated strands of the asymmetric unit. Again, the electron densities of the symmetry-related strands (purple) are omitted for clarity.

DNA duplex at the P-strand in the HMT adduct as compared to the native *B*-DNA structure of d(CCGCTAGCGG). In the HMT structure, the direction of the phosphoribose backbone of the crossed-over P-strand deviates from normal *B*-DNA starting at the ribose of the thymidine nucleotide. As a direct result of the inclined thymine base, the deoxyribose ring is rotated outward, moving C4′ away from the helix axis. This displaces the 5′-end of the P-strand away from its normal helical trajectory, with the thymidine phosphate pushed $\geqslant$3.7 Å away from its position in the native duplex structure. Therefore, the distortion to the pyrone-side thymine base is propagated to the DNA backbone, consequently disrupting the double-helix in this region, and allowing the resulting frayed ends of two molecules to associate into a Holliday junction in the crystal.

Although the structure shows that a Holliday junction is induced by psoralen, are the physical characteristics of the junction defined by the drug? If the cross-over of the junction were not at the drug cross-linked base-pairs, would the DNA duplex be similarly affected? These questions are addressed by comparing the psoralen-induced junction to the sequence-dependent Holliday junction of HMT-d(CCGGTACCGG).

Sequence-dependent Holliday junction in HMT-d(CCGGTACCGG)

The sequence d(CCGGTACCGG) cross-linked with HMT also crystallized as a four-stranded Holliday junction (Figure 2). We had previously shown that the unmodified d(CCGGTACCGG) sequence crystallizes in a stacked-X structure (Eichman *et al.*, 2000), and is stabilized by hydrogen bonding and base stacking interactions at the central ACC trinucleotide that forms the stable core of this native junction. The four-stranded

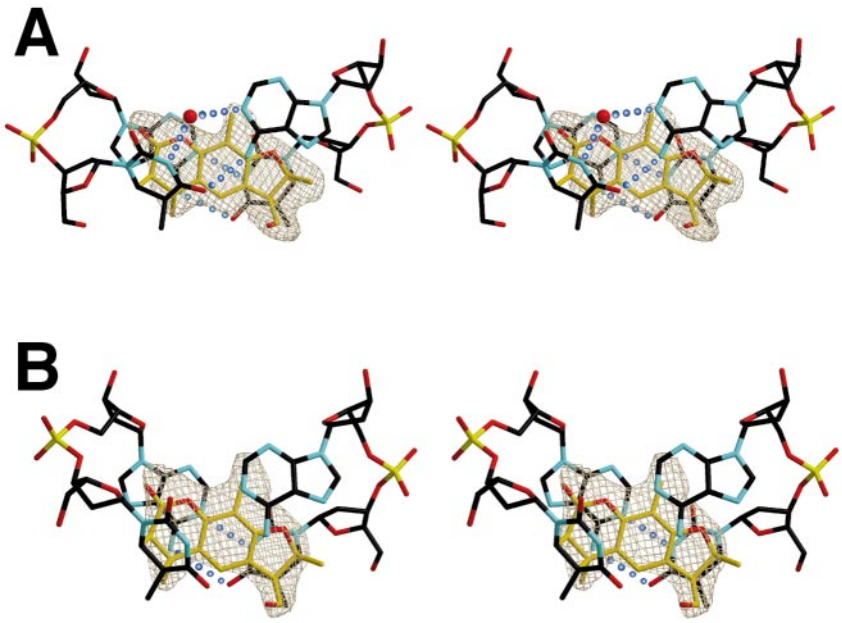


Figure 3. Stereodiagram of the HMT-adducted d(T·A) base-pairs in the d(CCGCTAGCGG) (a) and d(CCGGTACCGG) (b) structures. The annealed $F_o - F_c$ omit electron density map (contoured at 3σ) around the HMT molecule shows the exocyclic substituents used to orient the drug in both DNA structures. A single water molecule (red sphere) is shown bridging the N3 nitrogen of the pyrone-side thymine with the N1 nitrogen of the complementary adenine base in HMT-d(CCGCTAGCGG).

Holliday junction and the stabilizing interactions at the cross-over strands formed from two symmetry-related molecules in the HMT-d(CCGGTACCGG) structure are nearly identical to that formed by the native junction. In particular, we see a direct hydrogen bond from the N4 amino nitrogen of cytosine 18 to the O2P phosphate oxygen of cytosine 17, as well as water-mediated hydrogen bonds from the O6 keto oxygen of guanine 3 to the O2P phosphate oxygen of adenine 16 within the stabilizing ACC core. Also, the two stacked arms flanking the cross-link are indistinguishable from a regular *B*-DNA duplex in terms of phosphate positions and base stacking, except at the strand cross-overs and at the psoralen cross-links. It is clear that the junction in HMT-d(CCGGTACCGG) results from the inherent ability of this DNA sequence to form a junction and, in this sequence, cross-linking the thymine bases with HMT does not significantly distort the overall conformation of the junction.

As in the psoralen-induced junction structure the cross-linked thymidine nucleotides at the furan- and pyrone-sides of the drug show different degrees of distortion. Both hydrogen bonds remain within the furan-side d(T·A) base-pair, although it is slightly buckled as a result of the cross-link ($\Delta\kappa = 9.6^\circ$ between the native and cross-linked structures). The highly inclined and buckled P-strand thymine base, however, is no longer base-paired with its adenine, but shows an elongated 3.2 Å hydrogen-bond from the O4 keto oxygen to the hydroxymethyl O14 oxygen atom of psoralen. This now unpaired adenine is slightly more inclined and tipped than it is in the native d(CCGGTACCGG) structure, and shows only minimal stacking against the psoralen furan ring.

Comparison of the sequence- and drug-dependent Holliday junctions

How do these two psoralen cross-linked Holliday junctions compare? In both structures, the strand cross-overs of the Holliday junction sit adjacent to the d(T·A) base-pair cross-linked to the pyrone-side of the drug. However, the strands that form the cross-over of the junction are switched between the two HMT sequences (Figures 1 and 2). In the HMT-d(CCGGTACCGG) structure, the junction is 3′ to the adenine base on the F-strand (Figure 2), placing one residue and two phosphates between the psoralen cross-link and the junction cross-over. In contrast, the junction in the HMT-d(CCGCTAGCGG) structure is immediately 5′ to the P-strand thymine base, placing the backbone of this thymine residue directly at the cross-over (Figure 2). The closest atomic interaction at this junction is between two symmetry-related thymine ribose O4′ oxygen atoms. Therefore, the junctions are conformational isomers (or conformers) of each other, and can be interconverted by switching the arms that are paired to form the stacked duplexes on each side of the junction.

As a result of the strand cross-overs occurring at different positions in the nucleotide sequences, the specific contacts at the junctions are very different between the two structures (Figure 4). In contrast to the more rigid ACC junction, the drug-induced junction is stabilized by relatively weak interactions. Two contacts occur between the O2 keto oxygens and the C5′ carbons of the symmetry related pyrone-side thymine bases, and between the C5M methyl carbon of this thymine and the O5′ oxygen of cytosine C14 on the same P-strand (Figure 4(a)). Both of these weaker interactions (3.2-3.3 Å) can be considered C-H···O hydrogen-

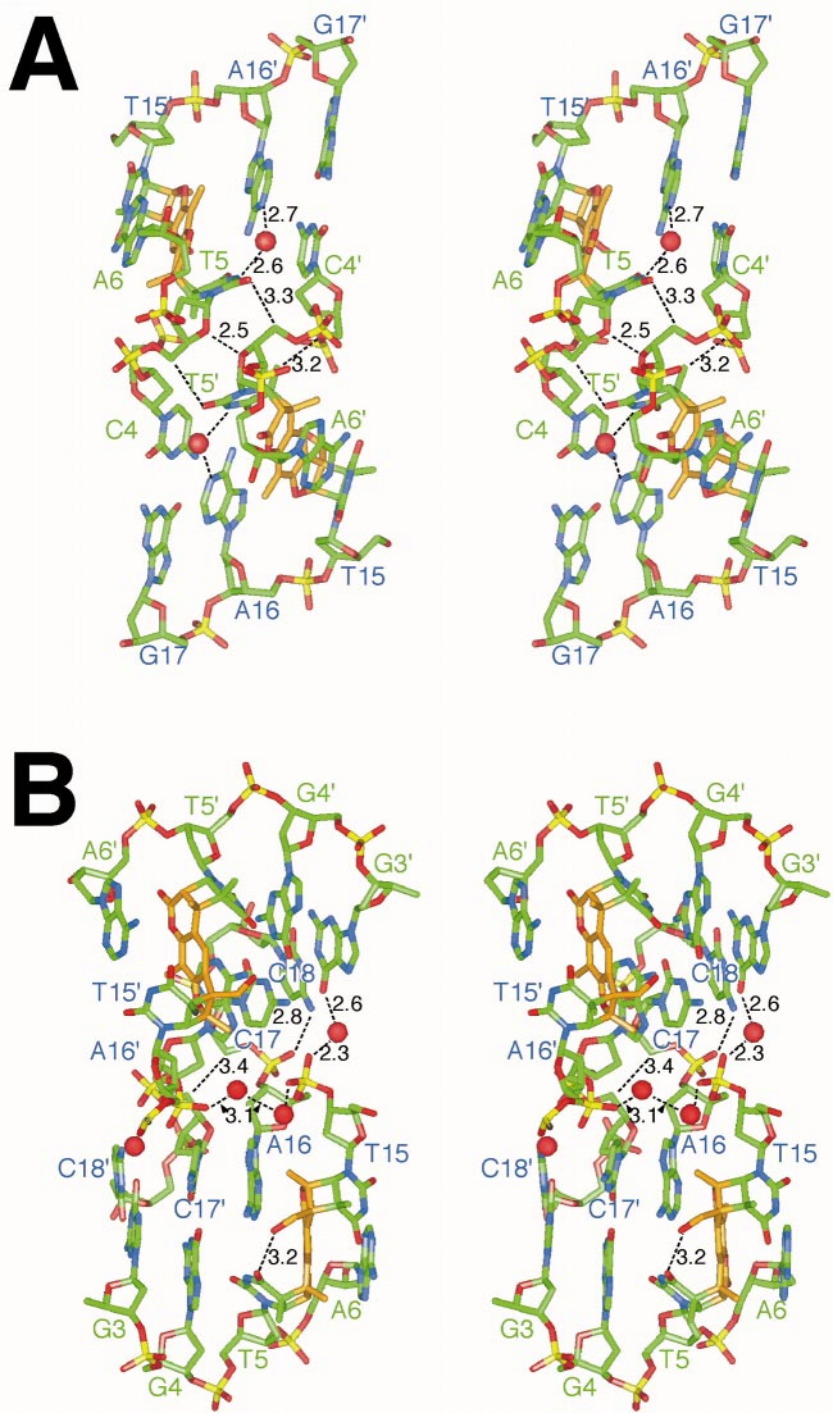


Figure 4. Comparison of the interactions at the HMT-d(CCGCTAGCGG) (a) and HMT-d(CCGGTACCGG) (b) Holliday junctions. Both models are colored by atom type (green, carbon; blue, nitrogen; red, oxygen; yellow, phosphorus). Psoralen carbon atoms are colored gold, and water molecules are shown as red spheres. Nucleotides in each strand are labeled and colored as in Figure 1. Distances between atoms are in Ångstroms.

bonds (Taylor & Kennard, 1982). The contact between the O4′ ribose oxygen atoms of the symmetry-related pyrone-side thymine bases across the junction is most likely a longer van der Waals interaction, which is not represented by the current model. Although the electron density at the junction clearly shows the sharp turns in the cross-over strands, the density at the current resolution accommodates a 2.5-3.3 Å range between the O4′ oxygen atoms. The contacts at the sequence-dependent junction in HMT-d(CCGGTACCGG) are those expected for the ACC core sequence (Eichman *et al.*, 2000), with one exception (Figure 4(b)). The bridging sodium ion between phosphates across the cross-over strands in the native sequence is occupied by two hydrogen-bonded, phosphate-bridging waters in the current HMT structure. In addition to the hydrogen-bonds at this junction, a van der Waals contact is formed between the C5′ carbon atoms of the F-strand cytosine atoms across the symmetry-related cross-over strands. Therefore, both structures are right-handed antiparallel Holliday junctions, but are conformers with different stabilizing contacts at the strand cross-overs.

As expected for a DNA-intercalating drug, psoralen unwinds and extends the helix by the equivalent of one base-pair; the differences in helical twist and length of each decanucleotide duplex between the native and HMT structures is −35.7° and 3.4 Å. The immediate effects of psoralen on the cross-linked thymine bases and their paired adenines are significant, but are very similar between the two sequences (RMSD = 0.76 Å for the atoms of psoralen and the thymine and adenine bases between these structures). Thus, although the two HMT-DNA sequences form different isomeric forms of the Holliday junction, the local perturbations to the DNA are nearly identical. In the context of a sequence-dependent structure such as the ACC core-stabilized Holliday junction, psoralen perturbs the DNA duplex only locally, and interactions that stabilize the Holliday junction in the sequence-dependent structure overshadow the destabilization to the *B*-DNA helix induced by the drug. However, in the absence of such sequence-dependent stabilization, the local distortions can propagate to longer range effects to destabilize the DNA double-helix at the P-strand, which then allows for strand exchange and the formation of a drug-induced junction.

Discussion

The crystal structures presented here show that psoralen is capable of inducing both very minor and very dramatic distortions to the double helix, depending on the DNA sequence. The structure of the HMT-adduct of d(CCGCTAGCGG) shows that psoralen destabilizes the DNA duplex at the P-strand, thereby inducing the displacement of four nucleotides from one duplex to cross-over to an adjacent duplex to form a four-stranded complex. In contrast, the sequence d(CCGGTACCGG) forms nearly identical Holliday junctions whether in the presence or absence of the drug, suggesting that the interactions at the ACC-core overrides the effect of the drug. The structures presented here, therefore, reveal: (i) how psoralen cross-links perturb the overall structure of a DNA four-way junction, (ii) how a junction induced by the drug may affect the cellular mechanisms for the repair of such adducts, and iii) how the drug cross-link affects the structure of a stable DNA junction, leading to a model for the psoralen adduct of a *B*-DNA double-helix.

Effect of psoralen cross-links on the geometry of four-way junctions

The HMT-adducts of d(CCGGTACCGG) and d(CCGCTAGCGG) expands to four the number of crystal structures of DNA junctions, and to six the number of DNA containing junctions with the inclusion of the DNA/RNA junctions (Ortiz-Lombardía *et al.*, 1999, Eichman *et al.*, 2000, Nowakowski *et al.*, 1999, 2000). Together, these structures may lead us to a better understanding of the compact stacked-X form of the junction. In the four DNA structures, the stacked duplex arms are related in a right-handed sense across the junction, with interhelical angles that are more shallow than previously observed by anomalous gel migration (Cooper & Hagerman, 1987, Duckett *et al.*, 1988), electric birefringence (Cooper & Hagerman, 1989), fluorescent resonance energy transfer (Murchie *et al*, 1989), or atomic force microscopy (Mao *et al.*, 1999). In addition, the interhelical angles of 36.4° for HMT-d(CCGGTACCGG) and 37.2° for HMT-d(CCGCTAGCGG) are more shallow than the 41.4° and 40.6° angles of the unmodified d(CCGGTACCGG) and d(CCGGGACCGG) sequences, respectively. These differences may result from the interactions within the junction, from crystal packing distortions, or from some other physical effect. The near identical junctions in the three common ACC-type structures suggest that the interactions at the junction itself do not directly define the angle relating the duplex arms. In addition, the *C*2 space group of the crystals for all four DNA junctions can accommodate other angles relating resolved *B*-DNA helices (Eichman *et al.*, 2000). Therefore, it is unlikely that the crystal lattice is itself restricting the geometry of these structures.

We suggest that the interhelical angle of these junctions is defined by interactions between the stacked duplex arms that are distant from the strand cross-overs. In support of this, we see that the intercalated drug extends the stacked arms of both psoralenated structures by the equivalent of one base-pair and, even though they differ at the junction and in their isomer conformation, the interhelical angles are nearly identical. In contrast, each of the DNA/RNA structures has one of the four arms with only a single Watson-Crick base-pair, which is too short to provide any long range interaction. Indeed, these DNA/RNA junctions show great variability in their interhelical angles, with the first structure having an interhelical angle of 55° (Nowakowski *et al.*, 1999)., and the more recent structure showing a −80° angle that relates the stacked duplexes in a left-handed sense (Nowakowski *et al.*, 2000).

The two psoralen structures are conformers of each other, with different DNA strands crossing-over between duplexes (the strand at the furan-side in HMT-d(CCGGTACCGG) but the pyrone-side strand in HMT-d(CCGCTAGCGG)), and different length arms forming the stacked duplexes (four base-pairs over six in HMT-d(CCGGTACCGG) and six over four in HMT-d(CCGCTAGCGG)). Why are they different? It is clear that the thymine covalently linked to the pyrone-side of the drug is destabilizing, and the differences in the structures may relate to how each accommodates this destabilizing effect. In HMT-d(CCGGTACCGG), the hydrogen bonds in the pyrone-side d(T·A) base-pair are apparently lost to maintain the duplex past the drug (Figure 3(b)). In HMT-d(CCGCTAGCGG), however, these thymine

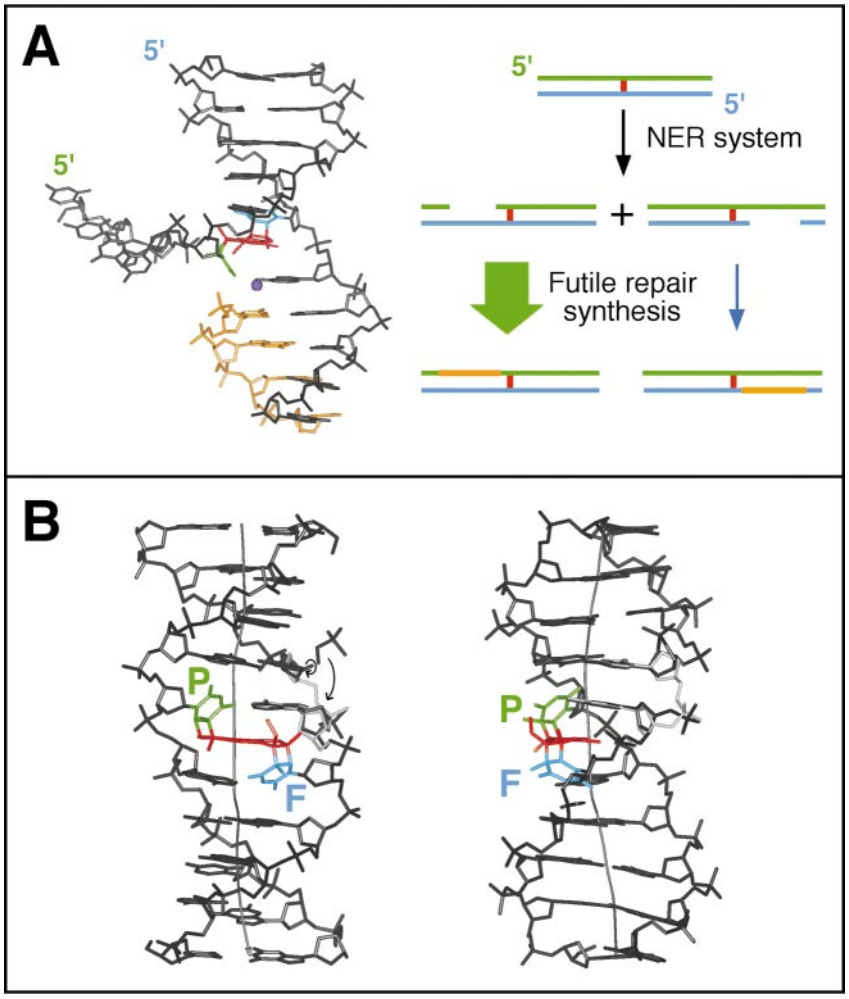


Figure 5. Models of the psoralen adducted DNAs in their potentially biologically relevant states. In each panel, green thymine bases and DNA strands correspond to the pyrone-side, and blue to the furan-side of the drug. The psoralen molecule is colored red. (a) The model of an HMT duplex (left), with its destabilized P-strand allowing the single F-strand to serve as a temple for synthesis of a new DNA strand (gold), was constructed from the two strands of the asymmetric unit of HMT-d(CCGCTAGCGG) and nucleotides C1 to G4 from the symmetry-related molecule. The position of the water mediating the hydrogen bonds of the P-strand d(T·A) base-pair is shown in purple. This water as well as the adducted thymine residue would be displaced by extension of the newly formed third strand. A possible mechanism for initiation of psoralen cross-link repair in mammalian systems is shown on the right. The nucleotide excision repair (NER) system creates gaps 5′ to the cross-link (red bar) on either strand with equal efficiency (Bessho *et al.*, 1997). Futile repair synthesis to fill the gap occurs 10 times more efficiently on the pyrone-side (Mu *et al.*, 2000). (b). A duplex model of a DNA cross-linked by HMT was constructed by rotating the C4′-C5′ bond of cytosine C17 in the crystal structure of HMT-d(CCGGTACCGG) (dark bonds). The subsequent structure was geometry optimized to form a duplex having a continuous phosphodeoxyribose backbone (light bonds). The resulting model is shown looking into (left) and perpendicular to (right) the major groove of the DNA helix. The helix axis was calculated using the program CURVES 5.2 (Lavery & Sklenar, 1989).

and adenine bases remain as a hydrogen bonded pair, but at the expense of the duplex structure, with the pyrone-side strand displaced to form the cross-overs of the junction. Therefore, the interactions at the ACC-trinucleotide junction are sufficiently stable to define the location and the interactions of cross-over strands, and this overcomes any destabilizing effects of the drug on the duplex. Conversely, in the absence of the stable ACC-core, the destabilization of the DNA duplex by the drug becomes the defining interaction at that junction.

The drug-induced junction in HMT-d(CCGCTAGCGG)

In the drug-induced Holliday junction of HMT-d(CCGCTAGCGG), we see that the DNA strand cross-linked to the pyrone side of psoralen becomes displaced from the duplex. The four-stranded complex thus is likely a result of having high concentrations of such frayed molecules in the crystallization solutions. Still, formation of the junction does mirror what is generally observed as the cellular response to the drug. Psoralen cross-links are highly recombinagenic in bacterial (Cole, 1973), yeast (Cassier *et al.*, 1984; Saffran *et al.*, 1991, 1994), and mammalian cells (Liu-Lee *et al.*, 1984; Vos & Hanawalt, 1989), with these events playing important roles in the repair of these lesions (Cheng *et al.*, 1991; Cole, 1973; Jachymczyk *et al.*, 1981; Sladek *et al.*, 1989). Holliday junctions are the principle DNA intermediates formed during a wide range of recombination processes (Holliday, 1964; Orr-Weaver *et al.*, 1981; Potter & Dressler, 1976; Sigal & Alberts, 1972). Therefore, the structure of HMT cross-linked d(CCGCTAGCGG) can serve as a model to explain how psoralen lesions can promote recombination events, thus facilitating its own repair. For example, the distortion of the P-strand oligonucleotide is supported by the mechan-

ism to initiate repair of psoralen damaged DNAs in mammalian cells.

The junction formed by the HMT-d(CCGCTAGCGG) sequence can be construed to be a model of a DNA fragment that has been incised on either side of the psoralen cross-link, i.e. the short F-strand represents incisions at the furan side of the drug and the P-strand with incisions at the pyrone-side (Figure 5(a)). The repair of psoralenated adducts in both *E. coli* and mammalian systems is initiated by incisions to one DNA strand. In a situation where a psoralenated DNA sequence can be incised at either DNA strand, this structure tells us that the P-strand is unstable in the duplex and can be displaced. Mammalian cell extracts show no preference as to which strand is initially incised (Bessho *et al.*, 1997). Despite this, the incised P-strand shows a tenfold higher probability of initiating futile repair of the adduct (Mu *et al.*, 2000). The current structure suggests that the drug-induced destabilization of the P-strand can facilitate repair from this side of the drug by allowing the complementary F-strand to be recognized as a template for the mammalian repair system (Figure 5(a)). Therefore, following its incision during the initiation of repair in mammalian systems, the destabilized P-strand alone may serve as a signal for enzymes to complete the repair process.

Effect of psoralen cross-links on a stable DNA junction

In contrast, the psoralenated sequence d(CCGGTACCGG) forms a junction not because of the drug, but in spite of it. This DNA sequence had been shown to crystallize as Holliday junctions in its unmodified form (Eichman *et al.*, 2000), even with two d(G·A) mismatch base-pairs located across the junction (Ortiz-Lombardía *et al.*, 1999). In the current structure, the junction remains stable and effectively unaltered despite the cross-linked thymines. This further supports the model that the ACC trinucleotide is the stabilizing core of the junction in this sequence motif (Eichman *et al.*, 2000). More importantly for this study, we see that the psoralen drug has virtually no effect on the structure of the junction or the double-helical arms that extend from the junction. In particular, we see that the trajectory of the helical arms are not kinked or bent. Even though the psoralenated thymines sit adjacent to the cross-over of the junction, the d(T·A) base-pair on the furan-side of the drug remains effectively as a hydrogen bonded pair, and the base planes of the adenine nucleotides are lying nearly perpendicular to the axis of the stacked helical arms. Thus, although we did not explicitly determine the structure of a psoralen-adducted DNA resolved as a duplex, the current structure strongly suggests that the helical trajectory of the DNA is not dramatically perturbed by the drug.

A model for a psoralenated DNA duplex can be readily constructed by simply breaking the phosphate bond between the A16 and C17 nucleotides of one cross-over strand, rotating ~180° about the C4′-C5′ bond, and reforming the phosphodiester bond (Figure 5(b)). The resulting duplex formed from the two stacked arms of the four-stranded assembly is now resolved into two distinct DNA duplexes, with each duplex showing an unbent trajectory of the helical axis. The double-helical model and the structure of the psoralenated junction in d(CCGGTACCGG) simply show that the drug need not induce a bend, i.e. a straight DNA duplex can readily accommodate the distortions resulting from the cross-link. This is consistent with the more recent unbent NMR structures of the psoralenated DNA duplexes (Hwang *et al.*, 1996; Spielmann *et al.*, 1995). Therefore, the psoralenated DNA duplex apparently presents no bend or kink that can serve as a signal for recognition by proteins. However, the drug's effect on the stability of the duplex, as seen in the d(CCGCTAGCGG) junction, may provide such a signal for repair enzymes.

Materials and Methods

Crystallization and X-ray data collection

DNA sequences were synthesized on an Applied Biosystems DNA synthesizer, purified by HPLC, and photo-cross-linked with HMT according to the published protocol (Spielmann *et al.*, 1992), with the following modifications. The DNA-psoralen solutions were irradiated using either an argon (360 nm) or a krypton laser (336 and 356 nm) operating at 200 mW for three hours using a flow cell while stirring in an ice bath. The cross-linked DNA was extracted three times with 0.25 to 0.5 volume chloroform and ethanol precipitated after removal of chloroform. The sample volume was reduced by rotary evaporator, and the cross-linked oligonucleotides further purified by HPLC.

Very thin diamond-shaped crystals of HMT-d(CCGGTACCGG)$_2$ (400 μm × 200 μm × 20 μm) were grown at 4 °C from solutions containing 0.5 mM DNA, 30 mM sodium cacodylate buffer (pH 7), 150 mM $MgCl_2$, 0.5 mM spermine·4HCl, 10 % (v/v) 2-methyl-2,4-pentanediol (MPD) and equilibrated against 20 % (v/v) MPD. Slightly thicker crystals of HMT-d(CCGCTAGCGG)$_2$ (300 μm × 150 μm × 50 μm) were obtained from solutions containing 0.5 mM DNA, 30 mM sodium cacodylate buffer (pH 7), 50 mM $CaCl_2$, 10 % MPD and equilibrated against 20 % (v/v) MPD. Both crystals are in the monoclinic *C*2 space group, with unit cell dimensions of $a = 72.1$ Å, $b = 23.7$ Å, $c = 36.2$ Å, and $\beta = 112.6°$ (HMT-CCGGTACCGG), and $a = 70.7$ Å, $b = 23.8$ Å, $c = 44.3$ Å, and $\beta = 130.1°$ (HMT-CCGCTAGCGG). X-ray diffraction data were collected at liquid nitrogen temperatures using 1.1 Å radiation on beamline 5.0.2 at the Advanced Light Source in Berkeley, CA. Diffraction images were processed and reflections merged and scaled using DENZO and Scalepack from the HKL package (Otwinowski & Minor, 1997) (Table 1).

Table 1. Data collection and refinement statistics

	HMT-d(CCGCTAGCGG)	HMT-d(CCGGTACCGG)
A. *Data collection*		
Resolution Range (Å)	34.7-2.2	33.4-2.2
Measured (unique) reflections	10418 (2937)	9596 (2900)
Completeness (%)[a]	99.2 (99.3)	96.7 (98.3)
R_{merge} (%)[a,b]	5.9 (8.0)	6.7 (10.0)
$\langle I/\sigma_I \rangle$[a]	16.9 (8.2)	12.3 (8.1)
B. *Refinement*		
Resolution Range (Å)	34.7-2.2	33.4-2.2
$\langle R_{cryst} \rangle$	24.5(±0.2)	22.2(±0.1)
$\langle R_{free} \rangle$[c]	28.3(±0.6)	28.4(±1.1)
DNA atoms (solvent molecules)	423 (22)	423 (41)
Ave. *B*-factors (Å^2)		
DNA atoms	32.3(±1.7)	37.5(±1.9)
Water atoms	48.6(±10.1)	60.7(±9.8)
RMSD from ideality		
Bond lengths (Å)	0.013	0.005
Angles (deg.)	1.717	1.229

[a] Values in parentheses refer to the highest resolution shell.

[b] $R_{merge}(I) = \Sigma_{hkl}\ \Sigma_i |I_{hkl,\ i} - \langle I \rangle_{hkl}| / \Sigma_{hkl}\ \Sigma_i |I_{hkl,\ i}|$ where I_{hkl} is the intensity of a reflection and $\langle I \rangle_{hkl}$ is the average of all observations of this reflection and its symmetry equivalents.

[c] $R_{cryst} = \Sigma_{hkl} |F_{obs} - kF_{calc}| / \Sigma_{hkl} |F_{obs}|$. $R_{free} = R_{cryst}$ for 10 % of reflections that were not used in refinement (Brünger, 1992a). The average values $\langle R_{cryst} \rangle$ and $\langle R_{free} \rangle$ and their standard deviations were calculated from the multiple cross-validation procedure as described in the text.

Structure solution

The HMT-d(CCGGTACCGG) structure was solved using a directed real space translation/rotation/rigid body search (X-PLOR 3.851 (Brünger, 1992b) script written in this lab). In brief, the structure of d(CCGGCGCCGG) (Heinemann *et al.*, 1992) was used as a search model by modifying the central d(CpG) dinucleotide to d(TpA), separating the two pentamer halves apart by 3.4 Å to accommodate the drug, and deleting the phosphate groups at this central dinucleotide step. The center of mass of this model was positioned in the unit cell at $x = 0$, $y = 0$, and $z = 1/4$ and inclined 6° from the xy plane and 17° from the xz plane as indicated by the native Patterson maps. The model was incrementally translated in the x direction, rotating the whole duplex and each pentamer about the helical axis, and performing rigid body refinement against 2.5 Å data at each step. A clear solution was obtained with an R_{cryst} of 48.2 % and R_{free} of 48.3 %. With the duplex properly positioned, the HMT was unambiguously modeled into 2σ $F_o - F_c$ density. A break in the electron density was observed between adenine 16 and cytosine 17, but was continuous between symmetry-related residues, as previously observed in the structure of the unmodified sequence (Eichman *et al.*, 2000). The model was thus rebuilt with the strand cross-overs of a junction.

The structure of HMT-d(CCGCTAGCGG) was solved by molecular replacement using EPMR (Kissinger *et al.*, 1999). Two stacked *B*-DNA arms from the refined HMT-d(CCGGTACCGG) structure were used as a search model against 2.8 Å data, resulting in a correlation of 63 % and an *R*-factor of 49 %. Simulated annealing against 2.2 Å data resulted in an R_{cryst} of 30.7 % and R_{free} of 37.5 %. At this stage the drug could be unambiguously positioned into $F_o - F_c$ density. Again, the electron density was broken between cytosine 4 and thymine 5 on one strand, and was continuous between these residues on symmetry-related strands. The model was thus rebuilt to reflect the trace of the electron density in the maps.

Structure refinement

Both structures were refined to a nominal resolution of 2.2 Å using X-PLOR 3.851 (Brünger, 1992b) with no σ-cutoffs applied to the data (Table 1). Although the overall diffraction of both crystals extended to better than 1.7 Å resolution, the data were highly anisotropic, with the intensity of reflections in the b direction falling to a minimum at 2.2 Å. Therefore, the resolution was limited to 2.2 Å for the refinements and anisotropic *B*-factor scaling was applied to properly weigh the calculated (F_c) to the observed structure factors (F_o) during refinement (X-PLOR 3.851 script written by Ethan Merritt). Differences in the degree of anisotropy in two crystals are reflected in the differences in the refined *B*-factors of the DNA and the solvent for the two sequences.

In the course of these refinements, we observed that values of R_{free} were highly dependent on how the test set was sequestered, thus reducing the rigor of cross validation by R_{free}. To account for this, we generated 10 different data sets for each structure, each associated with different test sets in which 10 % of the original reflections were randomly sequestered. The two structures were refined against all ten of their respective data sets to determine average values $\langle R_{cryst} \rangle$ and $\langle R_{free} \rangle$ (Table 1). These average values are more robust than those from any single combination of working and test sets, and the standard deviations reflect the degree to which the test sets affect the variation in these values.

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