Molecular Recognition

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O-Glycoside Orientation Is an Essential Aspect of Base J Recognition by the Kinetoplastid DNA-Binding Protein JBP1**

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Leishmaniasis (cutaneous or visceral, *Leishmania* sp.), African trypanosomiasis (sleeping sickness, *Trypanosoma brucei*), and American trypanosomiasis (Chagas' disease, *T. cruzi*) are devastating diseases in the developing world. Present pharmaceuticals against these parasites are limited in terms of effectiveness, increasing drug resistance, and inherent drug toxicity. Therefore, there is a clear need for elucidation of parasite-specific biological targets that would be amenable to new therapeutic approaches.

In this regard, Borst and co-workers[1–3] made a landmark contribution when they discovered that DNA from kinetoplastida and *Euglena*,[4] but not other eukaryotes, contains a modified nucleobase, 5-(β-D-glucopyranosyloxymethyl)-2′-deoxyuridine (**1a**), called nucleoside dJ or base J (Figure 1). Base J is found in telomeric repeats of all kinetoplastida[1] and in inactive telomeric variant surface glycoprotein (VSG) gene expression sites in *T. brucei*.[5–9] Borst and co-workers[10] subsequently found that extracts of several kinetoplastid flagellates contain a DNA-binding protein that specifically binds to dJ-containing duplex DNA. The role of this dJ-binding protein 1 (JBP1) is yet to be clearly defined, but *JBP1* gene knock-outs of *Leishmania tarentolae* have revealed that JBP1 is essential for its survival,[11] hinting that inhibition of JBP1/dJ-DNA binding may offer merit as a new therapeutic strategy against *Leishmania*.

The design of molecules that may disrupt this interaction would clearly benefit from structural information, at atomic resolution, regarding the nature of this relatively rare molecular interaction in biological systems—that between a glycosylated oligonucleotide and a protein. However, at present, there is no X-ray structural analysis of either JBP1 alone or in complex with dJ-containing DNA to guide library

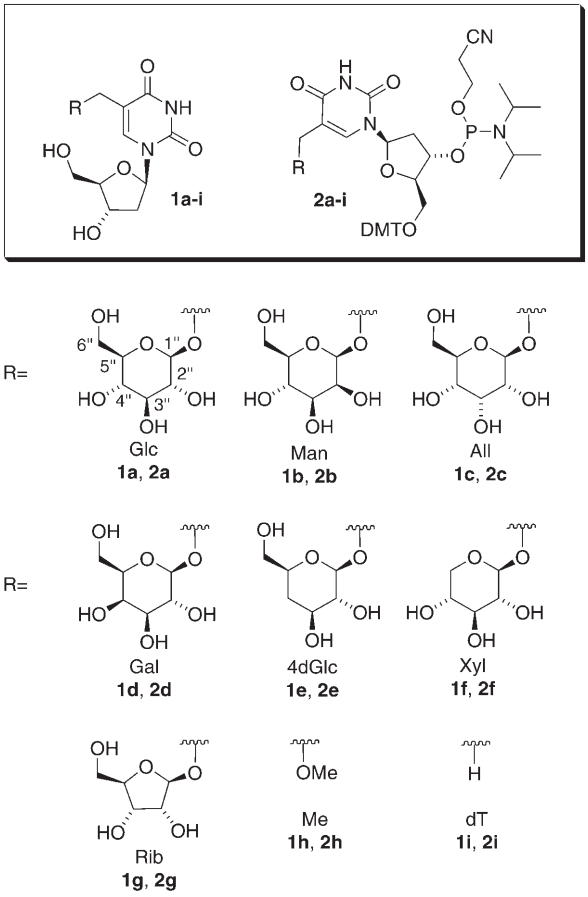

Figure 1. Base dJ (**1a**) and HMdU O-glycoside analogues (**1b–h**) were synthesized as their peracetylated 5′-*O*-dimethoxytrityl-3′-*O*-(2-cyanoethyl-*N*,*N*′-diisopropyl)phosphoramidite derivatives (**2a–h**) for incorporation into the telomeric 16-mer oligonucleotide sequence (tel-J*) shown. **J*** is the locus of incorporation of **1a–i**. DMT = dimethoxytrityl.

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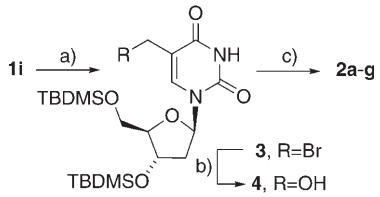
design. And, while the amino acid sequence of JBPs from several kinetoplastids are known and all share significant degrees of homology (43–67%),[10] the unique nature of these sequences has, thus far, meant that homology modeling has proven unsuccessful.

Previous studies by Sabatini and co-workers have delineated the oligonucleotide requirements for the binding of JBP1 to dJ-containing DNA.[12,13] These studies reveal that JBP1 binds only to double-stranded dJ-containing DNA and does so optimally when the dJ base is flanked by at least five other nucleotide residues in a telomeric sequence ($K_D \approx 40$ nM, measured using gel shift assays). DNA footprinting techniques further reveal that JBP1 does not make any sequence-specific contacts with the nucleosides adjacent to base J and that the only critical contacts between JBP1 and dJ-DNA occur on the dJ-containing oligonucleotide. These contacts consist of thymine-dependent major- and minor-groove contacts at base dJ and a sequence-independent major-groove contact at the nucleotide 5′ to J (termed the J-1 nucleotide). When dJ is replaced with dT, the nucleobase considered to be the biosynthetic precursor of genomic dJ, Sabatini et al.[12] showed that JBP1 binding is reduced to a level undetectable by gel shift assay.

Herein, we report the first in-depth analysis of the molecular recognition between the O-linked glycoside component of dJ in telomeric dJ-containing double-stranded (ds)-DNA and JBP1. Comparison between the molecular dynamics (MD) snapshots and the $\Delta\Delta G$ values of the binding of JBP1 to the duplex tel-J* oligonucleotides **1a**–**h** reveals that JBP1 binding to dJ-containing oligonucleotides occurs preferentially when the β-D-glucopyranosyl moiety adopts a conformation within the major groove wherein the C2 and C3 hydroxy groups of the glucoside form hydrogen bonds with the nonbridging *pro-R* phosphoryl oxygen of the J-1 nucleotide's phosphate group. If this orientation is perturbed even slightly, then JBP1 binding affinity drops to the level of that when dJ is replaced by dT (100-fold, ≈ 11 kJ mol^{-1}).

The N^3-unprotected phosphoramidite analogues **2b**–**h** (Figure 1) were prepared based upon an original method for the N^3-unprotected phosphoramidite **2a**, developed by van Boom and co-workers (Scheme 1).[14–16] Thus, bis-silylation of dT (**1i**), followed by radical allylic bromination gave allylic bromide **3** in good yield (67% over two steps). Nucleophilic displacement of the crude alkyl bromide **3** with cesium methoxyacetate in DMF followed by saponification with K_2CO_3 in methanol gave bis-silylated 5′-hydroxmethyl deoxyuridine (HMdU) **4** in 72% yield (a considerable improvement over the published yield of 51%).[15] The critical glycosylation of **4** with the corresponding sugars required significant optimization. Optimal glycosylation of **4**, coupled with β-anomeric selectivity, was achieved by means of a Schmidt condensation[17] in the presence of trimethylsilyltriflate (TMSOTf) at −25 °C, with the sugars as their corresponding perbenzoylated α-trichloroacetimidate esters. These conditions not only served to minimize formation of the corresponding and unwanted α anomer but also prevented HMdU dimerization.

Conversion of the resultant perbenzoylated HMdU O-glycosides into their more ammonia-labile peracetylated



Scheme 1. Synthesis of glycosylated phosphoramidites **2a**–**h**. a) 1. TBDMS-Cl, imidazole, DMF; 2. NBS, AIBN, C_6H_6, 67% over two steps; b) 1. cesium methoxyacetate, DMF; 2. K_2CO_3, CH_3OH, 71% over two steps; c) 1. perbenzoylated α-trichloroacetimidate ester sugars, TMSOTf, −25 °C, DCE; 2. NaOMe, CH_3OH; 3. Ac_2O, Pyr, 94% over two steps; 4. $Et_3N{\cdot}3HF$, Pyr; 5. DMTCl, Pyr, RT; 6. P(OCE)-(N*i*Pr$_2$)Cl/Et_3N. Note **2h** is prepared by treatment of **3** with NaOMe, 95% followed by c) steps 5 and 6. AIBN = azobisisobutyronitrile, DCE = 1,2-dichloroethane, DMF = dimethylformamide, NBS = *N*-bromosuccinimide, Pyr = pyridine, TBDMS = *tert*-butyldimethylsilyl.

analogues, for the purposes of oligonucleotide synthesis,[15] was then achieved by treatment with NaOMe/MeOH followed by acetic anhydride to give the peracetylated sugar-HMdU β-glycosides in high yield (94% over two steps). Desilylation, followed by protection at 5′-O with a dimethoxytrityl (DMT) group and conversion into the 3′-*O*-(2-cyanoethyl)(N*i*Pr$_2$)phosphoramidite gave the nucleoside building blocks **2a**–**g** required for DNA synthesis.

The modified base, J*, was then introduced into the telomeric oligonucleotide sequence, 5′-CCCTAACCC-**J***AACCCT-3′, using conventional phosphoramidite solid-phase DNA synthesis methods. All the telomeric J*-containing DNA sequences were purified using denaturing poly(acrylamide) gel electrophoresis (PAGE) and purity (> 95%) was confirmed by reversed-phase (RP)-HPLC and denaturing PAGE. The complementary sequence, 5′-NH_2-$(CH_2)_6$AGGGTTAGGGTTAGGG-3′, was prepared and purified as described above, and then coupled with Cy5-*N*-hydroxysuccinimidyl ester and purified by RP-HPLC. DNA annealing between the J*-containing oligonucleotides and the Cy5-labeled counterstrand was performed by heating a 1:2 mixture of the Cy5-labeled strand and the J*-base strand to 95 °C for 5 min in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (20 mM, pH 7.9) containing KCl (50 mM) followed by slow cooling to 4 °C. Duplex formation was verified by native 20% PAGE.

Recombinant JBP1 from the kinetoplastid *Crithidia fasciculata* containing a His_{10} tag was expressed in *E. coli* and purified by metal ion affinity column as previously described[12] and shown to be over 98% pure by sodium dodecylsulfate (SDS) gel electrophoresis. Prior to the binding assays, the protein was dialyzed into binding buffer, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; 35 mM, pH 7.9), ethylenediaminetetraacetic acid (EDTA; 1 mM), KCl (50 mM), $MgCl_2$ (5 mM), and 1,4-dithiothreitol (DTT; 1 mM). Isotherms for the binding of recombinant JBP1 to the tel-J*-oligos (20 nM) were then measured by fluorescence anisotropy (excitation: 590 nm, emission: 680 nm) at 20 °C. The data were fit to a model for 1:1 association[18] to determine the dissociation constant, K_D (Table 1).

The measured K_D value for the binding of JBP1 to tel-J* (where J* is **1a**) is $K_D(\mathbf{1a}) = (13 \pm 8)$ nM, and when dJ (**1a**) is

replaced with dT (**1i**), the measured K_D drops 105.4-fold [K_D(**1i**) = (1370 ± 15) nM]. This is equivalent to a free energy difference ($\Delta\Delta G$) between dJ and dT of 11.35 kJ mol^{-1}. The measured binding constants for JBP1 and tel-J* oligos containing the dJ analogues **1b–h** all lie between the measured values for **1a** and **1i**. What is immediately striking, is that epimeric modifications of single hydroxy groups around the pyranosyl core of dJ can cause a reduction in JBP1 binding that is equivalent to that observed for the dJ-to-dT modification (see above). Thus, the stepwise equatorial-to-axial epimerization of hydroxy groups of C2, C3, and C4—replacing the Glc of dJ (**1a**) with Man (**1b**), All (**1c**), and Gal (**1d**)—results in a loss in binding affinity for JBP1 of 102.3-, 93.8-, and 95.4-fold, respectively (Table 1). Other structural modifications that cause significant reductions in JBP1 binding are the replacement of the O-glycoside of dJ with Me (**1h**) or Rib (**1g**); these changes cause a 91.5- and 88.5-fold drop in JBP1 binding affinity, respectively. There are structural modifications to dJ that do not have such a large impact on JBP1 binding. Deletion of the hydroxy group on C4 or the hydroxymethyl group on C5 of the β-D-glucopyranosyl ring to give the 4dGlc (**1e**) and Xyl (**1f**) analogues results in a reduction in JBP1 binding affinity of only 20.8- and 4.4-fold, respectively.

Table 1: Binding data for JBP1 (*Crithidia fasciculata*) to dsDNA containing base dJ (**1a**), analogues **1b–h**, and dT (**1i**).

Entry	J*	K_D[a] [nM]	f[b]	$\Delta\Delta G$[c] [kJ mol^{-1}]
1	Glc (**1a**)	13 ± 8	1.0	0.00
2	Man (**1b**)	1330 ± 50	102.3	11.27
3	All (**1c**)	1220 ± 44	93.8	11.06
4	Gal (**1d**)	1240 ± 44	95.4	11.10
5	4dGlc (**1e**)	270 ± 10	20.8	7.39
6	Xyl (**1f**)	57 ± 8	4.4	3.61
7	Rib (**1g**)	1150 ± 36	88.5	10.92
8	Me (**1h**)	1190 ± 43	91.5	11.00
9	dT (**1i**)	1370 ± 15	105.4	11.35

[a] Binding titrations for each modified base were performed multiple times, using different batches of protein, and K_d values are reported as the mean (± standard deviation) of at least three measurements. [b] $f = K_D$(**1b–h**)/K_D(**1a**). [c] $\Delta\Delta G = -RT\ln f$.

To gain insight into how structural modifications to the HMdU O-linked glycosides **1a–h** may be impacting JBP1 binding by means of changes in the conformation adopted by the glycoside within the major groove of tel-J*, we studied the conformational dynamics of the tel-J* ds-DNA oligonucleotides. A 5- to 10-ns molecular dynamics (MD) simulation was performed for each of the oligonucleotide tel-J* sequences containing **1a–h**. The AMBER 8[19] suite of programs with an all-atom force field (ff99)[20,21] and the GLYCAM carbohydrate force field (glycam04)[22–24] were used to build the initial duplex structures of hydrated tel-J* DNA sequences and to run all MD simulations. The MD simulation of the native dJ (**1a**) containing tel-J* sequence shows that the β-D-glucopyranosyl ring adopts a chair conformation and extends into the major groove (Figure 2a). The glucose is anchored to the DNA backbone by two hydrogen bonds between the equatorial hydroxy groups on C2 and C3 of the pyranosyl ring and the non-bridging *pro-R* phosphoryl oxygen of the adjacent 5′-nucleotide residue (cytidine-9) (Figure 2b). The orientation of the pyranosyl ring is roughly parallel to the axis of the duplex and perpendicular to the plane of the major groove. No other significant contacts are made between the glycoside and the DNA, and the hydroxymethyl group on C5 of the pyranosyl ring is seemingly free to rotate within the major groove.

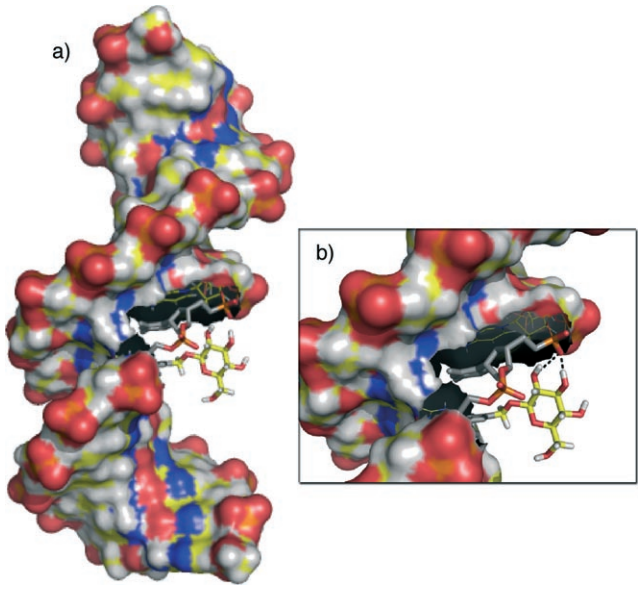


Figure 2. MD snapshot of tel-J* (surface rendering) showing the β-D-glucopyranosyl ring of **1a** (stick representation) occupying the major groove. a) Overall view, showing the complete telomeric sequence; b) zoom view, focusing on the orientation of the glucoside within the major groove. Note the two hydrogen bonds (shown as dotted lines) between the hydroxy groups of C2 and C3 of the glucopyranoside and the nonbridging *pro-R* phosphoryl oxygen of the J-1 nucleotide. The nucleotides J and J-1 are colored gray with the exception of the DNA-backbone phosphorus atoms of the J and J-1 nucleotides, which are orange, and the phosphoryl oxygen atoms, which are red. O-glycoside oxygen atoms (red), carbon atoms (yellow), hydrogen atoms (white). Hydrogen bonds are shown as dotted lines.

Inspection of the MD simulations of the tel-J* double-stranded DNA analogues **1e** and **1f** reveals that the hydroxy groups of C2 and C3 of the pyranosyl ring form hydrogen bonds with the J-1 *pro-R* phosphoryl oxygen and the glycoside is locked in a conformation almost identical to that for the native dJ (**1a**) (Figure 3a, e, f). In contrast, for analogues **1b**, **1c**, **1d**, and **1g** these hydrogen bonds between the hydroxy groups of C2 and C3 and the DNA backbone are disrupted and the conformation of the respective glycosides in the major groove differs considerably from that observed for the dJ-containing oligonucleotide (Figure 3b–d). Man(**1b**)-containing tel-J* forms no apparent hydrogen bonds with the tel-J* DNA backbone (Figure 3b). The All (**1c**) O-glycoside forms a hydrogen bond between the equatorial hydroxy group on C4 and the *pro-R* phosphoryl oxygen of the J-1 nucleotide, with the axial hydroxy group on C3 being rotated away from the DNA backbone (Figure 3c). In the case of Gal (**1d**), the axial hydroxy group on C4 makes a hydrogen bond with the J-

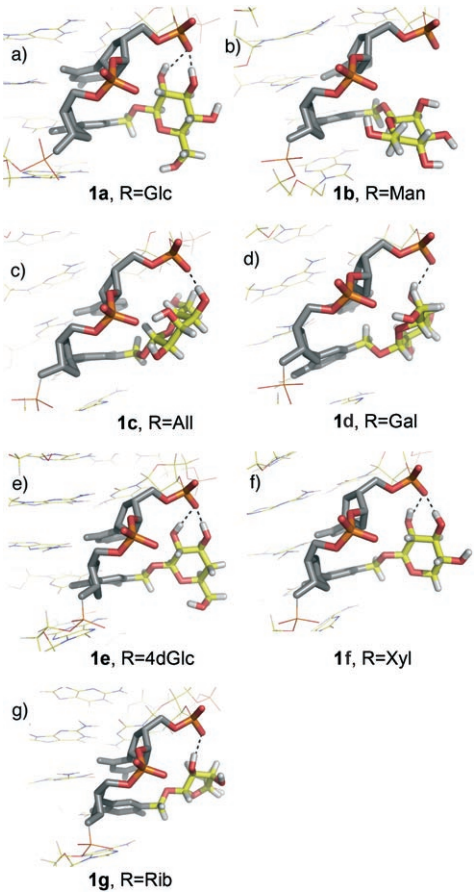


Figure 3. Images of the MD snapshots of the minimum-energy, most populated conformations of the HMdU O-glycosides **1a–g** within the major groove of tel-J* ds-DNA.

1 phosphate in the DNA backbone but at the expense of the equatorial hydroxy groups on C2 and C3. In Rib (**1g**) the ring pucker of the furanose ring in the major groove corresponds to a C2 *endo* envelope conformation, with the C2 hydroxy group forming a hydrogen bond with the *pro-R* phosphoryl bond of the J-1 nucleotide.

When the MD simulations of the tel-J* oligonucleotides are compared to their respective JBP1 binding affinities (Table 1), it becomes clear that while JBP1 binds to all the tel-J* sequences **1a–i**, it does so with the highest affinity when the HMdU O-linked glycoside can adopt a conformation that is locked by hydrogen bonds, established between the hydroxy groups of C2 and C3 of the pyranosyl ring and the *pro-R* phosphoryl oxygen of the J-1 nucleotide. This H-bond network and locked conformation of the β-D-pyranosyl ring is observed with native Glc (**1a**), 4dGlc (**1e**), and Xyl (**1f**) (Figure 3). When the pyranoside can adopt this H-bond-locked conformation within the major groove, the binding data with Xyl (**1f**) and 4dGlc **1e** reveals that deletion of the C5 hydroxymethyl group of **1a** costs 3.61 kJ mol^{-1} in binding energy (equivalent to one typical-strength hydrogen bond) to JBP1, and deletion of the C4 hydroxy group of **1a** costs 7.39 kJ mol^{-1} of binding energy (equivalent to one to two hydrogen bonds) to JBP1. This data suggests that the hydroxy groups of C4 and C6 of **1a** are bound by groups within JBP1 once the orientation optimal for binding of these hydroxy groups is established by locking of the pyranosyl ring.

If the HMdU O-glycoside cannot adopt this edge-on conformation, the loss in binding energy of the O-glycoside is almost equivalent to that observed for replacement of dJ with dT ($\Delta\Delta G = 11.35$ kJ mol^{-1}). This is seen clearly when one compares the binding constants of the sequence containing dT (**1i**) [K_D(**1i**) = 1370 ± nM] with those containing Man (**1b**) [K_D(**1b**) = 1330 ± 50 nM], All (**1c**) [K_D(**1c**) = 1220 ± 44 nM], Gal (**1d**) [K_D(**1d**) = 1240 ± 44 nM], and Rib (**1g**) [K_D(**1g**) = 1150 ± 36 nM]. It is hard to rationalize how the major changes in the orientation of the O-glycoside of **1b**, **1c**, **1d**, and **1g** within the major groove (Figure 3) can correspond to such minor changes in JBP1 affinity unless it is the case that JBP1 has an alternative low-affinity binding site on the tel-J* oligonucleotides that is remote from the nucleotide J*. This observation is new in the context of DNA binding by JBP1 and one that warrants further study.

A question unanswered in previous studies of JBP1/dJ binding is the nature of the interaction between the 5′ (J-1) nucleotide and JBP1. As described above, Sabatini et al.[13] showed that this interaction is nucleotide independent. What our study reveals is that the critical molecular component of the J-1 nucleotide interaction seems to be the *pro-R* non-bridging phosphoryl oxygen atom. This phosphoryl group locks the pyranosyl group into the edge-on conformation necessary for optimal JBP1 binding.

Our study also has two reasonable implications from a drug-design perspective. First, there may be a site within the DNA-binding domain of JBP1 that accepts the edge-on conformation of the β-D-glucopyranosyl ring complexed with the DNA backbone phosphoryl atom. From the viewpoint of molecular recognition this locus should be a slot or crevice in the protein orthogonal to the plane of DNA binding. Second, disruption of the edge-on conformation of the glucose moiety in dJ (**1a**) at the genomic level, by disengaging the hydrogen-bonding network should also prevent JBP1 binding. Small molecules that bind to DNA and, in doing so, modulate DNA structure, such as major- or minor-groove binders or intercalators, may all promote this effect. An X-ray crystal study by Wang and co-workers[25] of a β-D-glucosylated DNA hexamer complexed with the DNA intercalator daunorubicin hints that this approach may be effective. In this DNA–daunorobicin complex, the glucoside does not adopt the edge-on conformation we have shown to be important for optimal JBP1 binding. More recently, Sabatini et al.[13] have shown that the minor-groove binder chromomycin A_3[26] blocks JBP1 binding to telomeric dJ-containing DNA, although it is not clear whether this effect arises from direct inhibition of the minor-groove JBP1 nucleotide binding or indirect inhibition by a pertubation of the edge-on conformation of the O-glycoside within the major groove by chromomycin A_3. Further studies are warranted to answer this question.

In conclusion, a comparison of equilibrium binding constants and MD simulations of a panel of telomeric ds-DNA sequences containing HMdU O-glycosides suggests that the protozoan DNA-binding protein JBP1 binds to a preferred conformation of β-D-glucopyranoside within the major groove of dJ-containing oligonucleotides. This con-

formation is established by hydrogen bonds between the equatorial C2 and C3 hydroxy groups of the sugar and the *pro-R* phosphoryl oxygen of the J-1 nucleotide. This observation has clear ramifications for drug design against eukaryotic parasites that are a truly global menace.

Experimental Section

The fluorescence anisotropy of the Cy5-labeled DNA duplex (20 nM) in binding buffer (Hepes buffer (35 mM, pH 7.9), EDTA (1 mM), KCl (50 mM), $MgCl_2$ (5 mM) and DTT (1 mM)) was measured in an SLM 8100 spectrofluorimeter equipped with a thermostatted cuvette holder (20 °C). The sample was excited at 590 nm and emission measured at 680 nm, with correction for both the buffer background and G-factor of the instrument. JBP1 was titrated into the sample, and emission spectra (at the magic angle) and anisotropy were measured after each addition. The emission spectra showed only marginal changes in intensity and wavelength maximum on protein binding, whereas the anisotropy changes were significant. The resulting binding isotherms (anisotropy vs. JBP1 concentration) were fit to a 1:1 association model for anisotropy, according to Equation (19) in the article by Bailey et al.[18]

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