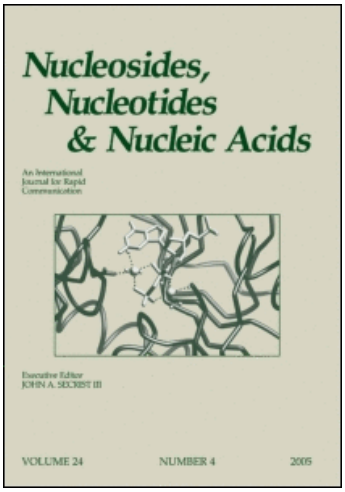

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DUPLEX STABILIZATION BY DNA-HOECHST 33258 CONJUGATES: EFFECTS OF BASE PAIR MISMATCHES

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ABSTRACT

Three types of DNA-Hoechst conjugates are examined for duplex stability in the presence of single-site base pair mismatches. Two of the complexes have the Hoechst ligand tethered to an internal phosphate residue, while the third complex employs terminally labeled conjugate. The presence of the mismatched base pair reduces the T_m values for the non-conjugate duplexes as is expected. Addition of the conjugated Hoechst ligand enhances the T_m values for the mismatched duplexes, but these values remain significantly below that obtained for the native conjugated duplex. While stabilization by the tethered ligand is observed for both cognate and non-cognate sequences, the differences suggest that significant sequence selectivity is still apparent for these conjugates.

The minor groove structure of B-form duplex DNA has been shown to function as an effective ligand binding site for a number of heterocyclic compounds, for example the oligopyrroles or the bisbenzimidazoles, both of which are capable of adopting a "banana-like" conformation, and upon binding become isohelical with the DNA double helix. In addition to DNA binding by free minor groove ligands, DNA sequences tethering such minor groove ligands have been shown to be effective in stabilizing both DNA duplexes[1-5] and DNA triplexes.[6-8] The conjugates examined to date include tethered derivatives of Hoechst 33258,[2,3,5,7,8] CDPI-based structures[4,9] and the oligopyrroles.[6] Most minor groove ligands bind preferentially at dA-dT rich sequences,[10] although the bisbenzimidazoles have been altered to target dG-dC rich sequences[11] and more recent developments of mixed oligopyrrole/imidazole agents[12] have resulted in ligands that will bind regions of dG-dC rich base pairs in a sequence-specific manner.

With DNA duplexes, the hybridization event creates the target minor groove structure and triggers the binding event by the tethered ligand. With DNA triplexes the binding mechanism may be less clear, although in one case[8] it appears that ligand binding

to the duplex minor groove by a tethered Hoechst 33258 analogue precedes triplex formation by the conjugated third strand.

Enhanced stability of DNA hybridization complexes is important for PCR primers as well as the development of DNA-based antisense or anti-gene agents. However, enhanced stability might occur at the expense of sequence selectivity – the differences in stability for complexes formed at the target sequence vs. those formed at target-like sequences (those containing at least one incorrect base residue) might be masked by the enhanced binding energy resulting from the tethered minor groove ligand. To probe such effects, we have prepared a series of mismatched duplexes, those containing a single incorrect base pair at various positions within the target site, and examined the results of hybridization to such sequences with a tethered ligand probe.

EXPERIMENTAL

Preparation of the Hoechst-Conjugates

Internal-[2,3] and terminal-[5]conjugated sequences were prepared according previously published procedures. The internal conjugates were prepared by post-synthetic reaction of a thiol-containing linker with two different Hoechst 33258 analogues functionalized as bromoacetamides. The terminal conjugate was prepared during the assembly of the DNA synthesis by a phosphoramidite-based coupling between a Hoechst derivative tethered to a hexa(ethylene glycol) linker.

Thermal Denaturation Studies

Thermal denaturation studies were performed in 21 mM HEPES pH 7.5, 100 mM NaCl and 20 mM $MgCl_2$ at duplex concentrations of ~1 μM. Absorbance (260 nm) and temperature values were measured with an AVIV 14DS UV/Visible spectrophotometer equipped with digital temperature control. The temperature of the cell compartment was increased in 1.0 °C steps (from 0 to 95 °C) and when thermal equilibrium was reached, temperature and absorbance data were collected. T_m values were determined both from first-order derivatives and by graphical analysis of the Absorbance vs. Temperature plots.

Fluorescence Studies

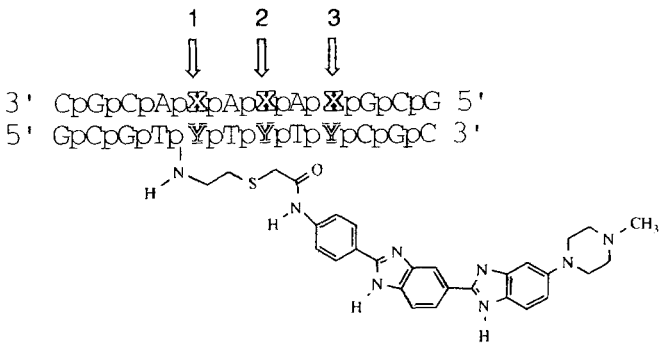
Emission spectra were collected on a Shimadzu RF5000U fluorescence spectrophotometer containing a Shimadzu DR-15 microprocessor and graphics display terminal. Fluorescence measurements were made in 21 mM HEPES pH 7.5, 100 mM NaCl and 20 mM $MgCl_2$ at duplex concentrations of ~1 μM. All measurements were performed with the following list of parameters, Slit width: Ex/Em = 10 nm/10 nm, low sensitivity, medium speed. Samples were introduced into a 1.25 ml cell thermally isolated with a water jacket. Temperature was controlled with a recirculating water bath. Excitation wavelength = 342 nm, Emission range = 380 - 600 nm.

RESULTS

We have reported[2] that the presence of a Hoechst 33258 ligand tethered to an internucleotide phosphate of a double-stranded DNA 12-mer results in approximately a 20 °C increase in T_m value when the phosphoramidate linkage is of the *Rp* configuration (see also Tables 1 and 2). More moderate increases were observed when the ligand was tethered to the *Sp* diastereomer. In a related study,[5] tethering the Hoechst ligand to the 5'-terminus of a 15-residue oligonucleotide (see also Table 3) resulted in increased T_m values of 10 - 15 °C depending upon the position and character of dA-dT rich ligand target site. Although the binding effects of the tethered ligand contribute to the overall complex stability of relatively short DNA duplexes, such additional binding energy could, in principle, be counterproductive if it masked destabilizing effects due to the presence of base pair mismatches. The result of masking such effects would be an overall loss of sequence specificity with the conjugate capable of forming complexes of similar stability at both cognate and non-cognate sequences. To probe the effects of such conjugates on complexes containing single mismatched base pairs, we prepared groups of dodecamers each of which contained a single non-cognate base pair. The native sequence contained a central binding site composed of six dA-dT base pairs, flanked by three dG-dC base pairs. The mismatched bases were introduced in each case for the dA residue of a single dA-dT base pair. Position 1 occurred immediately adjacent to the phosphoramidate linker, while position 2 was removed an additional two base pairs (at the center of the binding site), and position 3 was four base pairs from the site of the linker. When the mismatched base pair was present at position 1 or 3, a dA-dT binding site of four or five base pairs remained. Even when the mismatch was present at position 2, a putative ligand binding site of three base pairs was still available. For each position, three complexes were prepared containing dG-dT, dC-dT and dT-dT base pair mismatches in addition to the sequence containing the cognate dA-dT base pair.

Table 1 illustrates the T_m data obtained for the **B**romo**A**cet**A**mido (BAA) Hoechst derivative in which the Hoechst ligand is tethered through a seven atom linker to a chiral internucleotide phosphoramidate residue. The T_m for the Hoechst-conjugated native sequence is increased by 20 °C for the *Rp* diastereomer and 10 °C for the *Sp* isomer relative to the unconjugated sequence. Introducing a base pair mismatch at any of the three positions in the native sequence results in an 8 - 18 °C decrease in T_m values for the *Rp* diastereomers, generally most dramatic for the dC-dT mismatch, and less significant for the dG-dT base pair – the latter is capable of forming a Watson-Crick wobble base pair. Introduction of the Hoechst ligand to the mismatched duplexes results, in all cases, in significant increases in T_m values – as little as 10 °C for the dG-dT mismatch at position 1, and as much as 25 °C for the dT-dT mismatches at positions 2 and 3 of the *Rp*

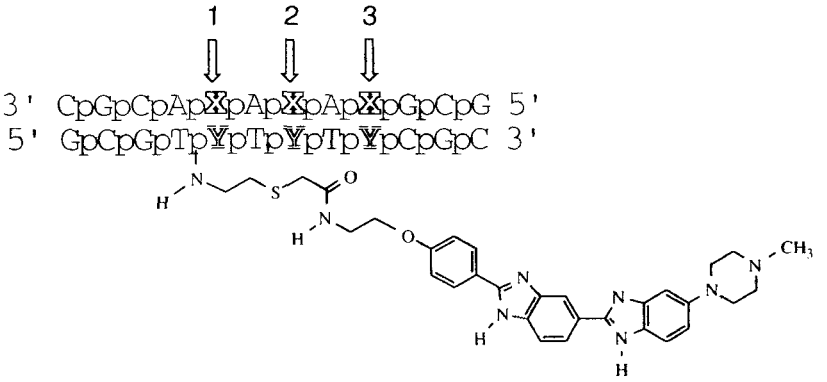
Table 1. T_m and Fluorescence Characteristics of BAA-Hoechst DNA conjugates



Mismatch Complexes		Duplexes	Conjugated Duplexes			
			(Rp)		(Sp)	
Sequence Position		$T_m(C)^1$	$T_m(C)^1$	ΔF^2	$T_m(C)^1$	ΔF^2
Native Sequence	XY = AT	56	76	9	66	9
Postion 1	XY = GT	48	58	10	55	9
	XY = CT	41	59	4	54	5
	XY = TT	39	59	7	55	5
Position 2	XY = GT	45	61	10	52	10
	XY = CT	38	62	6	54	8
	XY = TT	42	67	7	58	7
Postion 3	XY = GT	48	69	8	60	9
	XY = CT	41	64	8	55	8
	XY = TT	40	65	8	56	10

1. **Tm** values are estimated to be +/- 1 °C.
2. **ΔF** = the fold enhancement in fluorescence upon addition of the complementary sequence to the single-stranded conjugate.

Table 2. T_m and Fluorescence Characteristics of BAE-Hoechst DNA conjugates



Mismatch Complexes		Duplexes	Conjugated Duplexes			
			(Rp)		(Sp)	
Sequence Position		$T_m(C)^1$	$T_m(C)^1$	ΔF^2	$T_m(C)^1$	ΔF^2
Native Sequence	XY = AT	56	74	24	65	24
Postion 1	XY = GT	48	59	17	53	17
	XY = CT	41	56	13	51	13
	XY = TT	39	60	16	55	16
Position 2	XY = GT	45	60	20	51	14
	XY = CT	38	59	15	52	15
	XY = TT	42	66	13	57	16
Postion 3	XY = GT	48	69	19	59	19
	XY = CT	41	63	19	55	17
	XY = TT	40	65	17	55	19

1. T_m values are estimated to be +/- 1 °C.
2. ΔF = the fold enhancement in fluorescence upon addition of the complementary sequence to the single-stranded conjugate.

diastereomers (see Table 1). The effects observed for the *Sp* diastereomers are similar but less dramatic with enhancements in T_m values varying from 7 to 16 °C.

The Hoechst dyes are largely non-fluorescent due in part to relaxation of the excited state by collisional processes, primarily involving water. Upon binding to double stranded DNA they become brightly fluorescent as the tight binding complex impedes the accessibility of water to the dye excited state. The native complexes conjugated to the BAA-Hoechst derivative exhibit roughly a ten-fold enhancement in the quantum yield in the fluorescence emission spectrum (Table 1). All of the dG-dT mismatch complexes exhibit a similar effect, while the dC-dT and dT-dT mismatch complexes are all reduced somewhat in quantum yield, suggesting some disruption of the groove structure and better accessibility of the excited state to collisional process with water.

Table 2 illustrates the T_m data obtained for the **B**romo**A**cetamido**E**thanolamine (BAE) Hoechst derivative in which the linker tethering the ligand has been increased by 3 atoms through the insertion of an ethanol moiety. T_m values for the native conjugate are increased by 18 °C for the *Rp* diastereomer and 9 °C for the *Sp* isomer. Introduction of the mismatched bases results in decreased T_m values for the unconjugated duplexes as noted above. As with the BAA-Hoechst derivatives, conjugation of the duplexes resulted in enhanced T_m values. The *Rp* diastereomers all exhibited T_m values increased by at least 11 °C, and by as much as 25 °C. However, the conjugated mismatch duplexes exhibited T_m values that generally remained 10 °C or more below that observed for the native conjugate (with the exception of the dG-dT mismatch at position 3). Similar but more moderate effects were observed for the conjugated *Sp* isomers (Table 2).

The fluorescence properties of both diastereomers of the conjugates were also affected somewhat by the presence of the single base pair mismatches. The native complex exhibits a quantum yield increase of some 24-fold - very similar to what has been observed for the native untethered Hoechst 33258 dye. The complexes containing the dG-dT mismatches (wobble base pair) are still quite fluorescent with quantum yield increases of about 20-fold, while with the pyrimidine-pyrimidine mismatches these values decrease somewhat. These results parallel those of the BAA Hoechst conjugates and suggest that the mismatches introduce some distortion of the groove structure that permits water to access the excited Hoechst dye.

The final set of conjugates that we examined include those in which the dye has been tethered to the terminus of the dodecameric sequence through a hexa(ethylene glycol) linker. The longer linker present in these conjugates increases the flexibility of the ligand binding site since the Hoechst ligand can "reach" beyond the site of linker attachment and bind to a dA-dT sequence removed some distance from the terminus of the sequence. As an example, we prepared the dodecamer containing a similar $(dA\text{-}dT)_6$ binding site

removed from the 5'-terminus by three intervening dG-dC base pairs (see Table 3). The T_m value for the native conjugated duplex is increased by 9 °C relative to the unconjugated duplex. This value is somewhat lower than that observed for the internally tethered conjugates (see Tables 1 and 2) and likely reflects an unfavorable entropy contribution to the binding event introduced with the long disordered hexa(ethylene glycol) linker. The mismatch duplexes also exhibit similar effects of enhanced stability with T_m values for the conjugates generally increased by some 9 - 11 °C (Table 3). However, with the exception of the sequences containing the dG-dT wobble base pair mismatches, the conjugated mismatched duplexes exhibit T_m values that remain some 10 - 15 °C below that of the corresponding native conjugate.

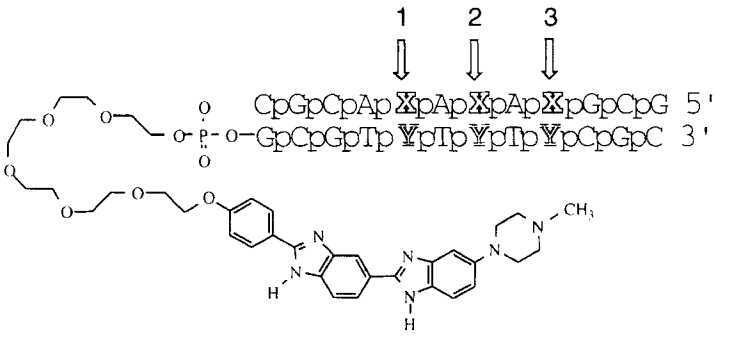
The fluorescence data observed is in general agreement with that observed in Tables 1 and 2. The native conjugate exhibits a quantum yield enhancement of some 16-fold, and the presence of single mismatched base pairs reduces these effects such that no more than a 13-fold enhancement in quantum yield is measured.

DISCUSSION

For all the conjugated duplexes examined, each of which contained a single base pair mismatch, increased T_m values (relative to the native mismatched sequence) were observed. Although the increased T_m values are not dramatically different for complexes in which either the mismatch or the position of the mismatch varied, there are some notable differences. The simple duplexes containing a dG-dT mismatch always exhibited the highest T_m value in each group of mismatch sequences. This result is not surprising since a wobble base pair can be formed in these cases. However, when the *Rp* internally conjugated duplexes were examined, these same sequences exhibited the smallest ΔT_ms (conjugate T_m - duplex T_m) varying from about 10 °C (for position 1) to 21 °C for position 3 (the *Sp* diastereomers exhibited smaller ΔT_m values in all cases) (see both Table 1 and Table 2). Both the dC-dT and dT-dT containing conjugates exhibited more significant ΔT_m values at each position. These effects suggest that the minor groove ligand is reasonably effective in providing additional helix stability, at least for sequences containing the pyrimidine-pyrimidine mismatches (the corresponding purine-purine mismatches were not examined in this study).

In spite of the increased T_m values for mismatched conjugates, these complexes still exhibit T_m values that lie below that of the cognate complex. The differences in T_m values for the BAA conjugates of the mismatched duplexes, vs. the conjugate of the cognate duplex were at least 7 °C (for the *Rp* diastereomers), although this value varied with the position of the mismatch and one often much more significant. At position 1, nearest the site of the tether, these differences were about 17 °C for the BAA Hoechst derivative (Table

Table 3. T_m and Fluorescence Characteristics of Hexa(ethylene glycol) tethered Hoechst-DNA Conjugates



Mismatch Complexes		Duplexes	Conjugated Duplexes	
Sequence Position		T_m(°C)[1]	T_m(°C)[1] [1]	ΔF[2]
Native Sequence	XY = AT	56	65	16
	XY = GT	48	58	10
Postion 1	XY = CT	41	50	10
	XY = TT	39	50	13
	XY = GT	45	50	6
Position 2	XY = CT	38	47	10
	XY = TT	42	55	13
	XY = GT	48	58	13
Postion 3	XY = CT	41	50	10
	XY = TT	40	50	9

1. T_m values are estimated to be +/- 1 °C.
2. ΔF = the fold enhancement in fluorescence upon formation of the double-stranded conjugate from the single-stranded conjugate

1) and slightly less for the BAE Hoechst derivative (Table 2). As the mismatched was moved further from the site of the linker these differences between the cognate and non-cognate conjugates tended to decrease.

Tethering the Hoechst derivative to the 5'-terminus of the DNA sequence through a hexa(ethylene glycol) linker also permits minor groove binding by the ligand triggered by the hybridization event. However the enhancement in T_m value for the native sequence is not as dramatic as for the internally tethered ligands (~10 °C). The mismatched duplexes

also exhibit increased Tm values when the conjugate is present. With the mismatch at any of the three positions roughly a 10 °C increase in T_m was observed (the exception being the presence of dG-dT at position 2). The relatively long hexa(ethylene glycol) linker may permit the Hoechst derivative to "slide" more easily along the groove and optimize its binding site. The presence of a dG-dT base pair (with the N^2-amino group present in the minor groove) in the middle of the binding site may result in a less than optimal binding site with only a 5 °C increase in T_m observed (Table 3).

Although more difficult to interpret, the fluorescence data suggests that in all cases when mismatched bases are present, the result binding site for the Hoechst ligand is less than optimal and while all the complexes remain brightly fluorescent, the quantum yields for the complexes are generally reduced by 30 - 40%.

CONCLUSIONS

The Hoechst-tethered DNA conjugates are capable of binding to various target sequences, each containing a single mismatched base pair. While binding of the conjugate to the target-like sequences results in enhanced T_m values, the overall stability of these complexes, as measured by T_m values, remains significantly below that of the cognate complex. In this respect, the ligand binding event does not appear to overwhelm the destabilizing effects of mismatched base pairs, and hybridization probes that incorporate a tethered ligand should function to discriminate cognate from non-cognate sequences.

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