# Stable Triple Helices Formed by Acridine-Containing Oligonucleotides with Oligopurine Tracts of DNA Interrupted by One or Two Pyrimidines

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**Abstract:** Oligopurine·oligopyrimidine sequences of DNA can be recognized by oligonucleotides forming intermolecular triple helices. These triple-helical complexes are strongly destabilized by base pair inversions in the target sequence, i.e., interruption of the oligopurine tract by one or more pyrimidines. Here we show that an acridine derivative incorporated within the sequence of an oligopyrimidine third strand strongly stabilizes the triple-helical complexes. The stability depends on the nature of the base facing the base pair inversion site and the position of the acridine with respect to this base. Fluorescence studies provide evidence that the acridine is intercalated into the triplex. These results show that an extension of DNA target sequences for stable triple helix formation can be obtained with oligonucleotides containing internally-incorporated intercalator(s).

## Introduction

Oligonucleotide-directed triple helix formation has been previously described as an elegant and versatile approach to recognize specifically a duplex DNA sequence[1−3] and to interfere with gene expression at both transcriptional and replicational levels.[4−11] This so-called "*antigene strategy*" has stimulated intense research due to its fundamental and potential therapeutic applications. One of the major restrictions of the triple helix approach remains on the requirement of a long-enough oligopurine·oligopyrimidine tract on the target DNA sequence. The interruption of the stretch of purines by one or more pyrimidines leads to base triplet mismatches and results in substantial destabilization of triplexes. Mismatched A·T × G and G·C × Y base triplets (Hereafter the symbols · and × indicate Watson−Crick and Hoogsteen hydrogen bonds, respectively; R and Y are purine and pyrimidine, respectively) are the least destabilizing triplets in triple helices involving Hoogsteen hydrogen bonds,[12−16] but the extent of destabilization depends on nearest-neighbor triplets.[17] The present work shows that the loss in triplex stability at the mismatch sites can be compensated by incorporating an intercalator at an internal site of the third-strand oligonucleotide. The rational was to take advantage of the additional binding free energy due to intercalation within the mismatched triplex, in a way similar to what was previously done when an intercalator was tethered to the ends (most efficiently to the 5′ end) of the third-strand oligonucleotide.[18,19]

## Experimental Section

**Oligonucleotides.** Oligonucleotides and acridine-containing oligonucleotides were synthesized by Eurogentec (Belgium), except one of them (Z = Acr in Figure 2) which was a gift from Clontech. Acridine-ON phosphoramidite (Glen Research) was internally incorporated into oligonucleotides as shown in Figure 1 where the natural 3-carbon internucleotide phosphate distance is maintained. The quality of oligonucleotides was controled by gel electrophoresis using the "UV shadow effect" of the oligonucleotide and the fluorescence of incorporated acridine. They were used without further purification. The concentration of oligonucleotides or acridine-containing oligonucleotides was determined spectrophotometrically using the extinction coefficients calculated by nearest-neighbor methods,[20,21] or an extinction

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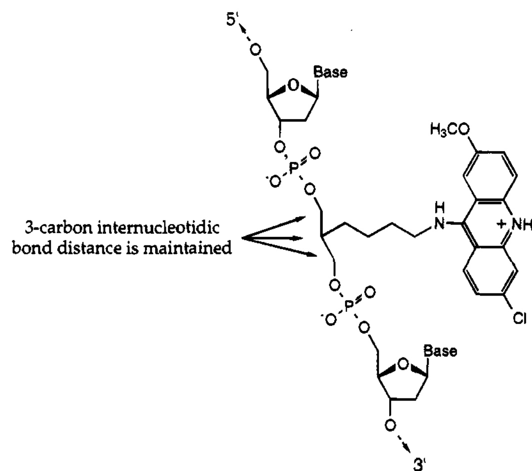
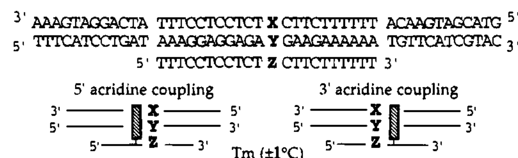



**Internal incorporation of acridine**

**Figure 1.** Chemical structure of the internally-incorporated acridine within an oligonucleotide in which the 3-carbon internucleotide phosphate distance is maintained.




| X•Y = | T•A | | | A•T | | | G•C | | | C•G | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Position of acridine with respect to Z | | 5' | 3' | | 5' | 3' | | 5' | 3' | | 5' | 3' |
| Z = A | | 28 | 30 | | 27 | 28 | | 25 | 27 | | 28 | 29 |
| C | | 28 | 31 | | 29 | 30 | 27 | 29 | 31 | 35 | 32 | 31 |
| G | | 32 | 32 | 27 | 28 | 28 | | **33** | 29 | | 31 | **35** |
| T | 37 | **36** | **35** | | 26 | 29 | 27 | 26 | 30 | | 28 | 30 |
| I | | 30 | 34 | | 26 | 29 | | 28 | 31 | | 29 | **36** |
| N | | 29 | 33 | | 27 | 30 | | 27 | 30 | | 28 | 31 |
| L | | 29 | 33 | | 28 | **32** | | 26 | 32 | | 28 | 31 |
| None | | 30 | | | 30 | | | 29 | | | 30 | |

**Figure 2.** Melting temperatures (±1 °C) of the triplexes described at the top of the figure upon binding of acridine-containing oligonucleotides to a DNA duplex containing a 22-bp oligopurine•oligopyrimidine tract in which a single base pair inversion was introduced. The intercalation site of acridine (hatched rectangle) and its position with respect to Z are indicated. The best combinations in each case are highlighted in the table. The underlined values correspond to the $T_m$ of triplex with canonical triplets or the least destabilizing mismatched triplets in the absence of acridine (see text for details and ref 14 for other $T_m$ values at mismatch sites). "Z = None" indicates that no nucleotide or linker except acridine was added facing the base pair inversion site. In the text, acridine is designated as W.

coefficient of 8845 $M^{-1}\cdot cm^{-1}$ for acridine at 424 nm,[22] respectively.

**Thermal Denaturation Experiments.** Thermal denaturation experiments were carried out on a Uvikon 940 spectrophotometer, interfaced to an IBM-AT computer for data collection and analysis. Temperature control of the cell holder was achieved by a Haake P2 circulating water bath. The temperature of the water was varied at a rate of 0.1 °C/min by a Haake PG20 thermoprogrammer, and the absorbance at 260 or 424 nm was recorded every 8 min. Under the experimental conditions (10 mM cacodylate buffer, pH 7, 100 mM NaCl, 0.2 mM spermine), all melting profiles were reversible. The melting temperature $T_m$ (±1 °C) was determined as the maximum of the first derivative of melting curves. Identical $T_m$ values were obtained from absorbance at either 260 or 424 nm (where acridine absorbs). The triplex concentration was 1 $\mu$M.

**Static Fluorescence Measurements.** Fluorescence emission spectra were recorded with a Spex Fluorolog F1T11T spectrofluorimeter using quartz suprasil microcells of 1-cm pathway. They were corrected for the wavelength dependence of the transmission and detection systems. The areas under the fluorescence spectra were integrated to estimate the relative fluorescence quantum yields. The excitation wavelength was set at 424 nm. Temperature of the cell holder was controled by a Huber Ministat circulating water bath. Fluorescence melting profiles were obtained by measuring the ratio of the fluorescence quantum yields of the acridine-containing oligonucleotide in the presence and in the absence of target double helix at various temperatures, as previously described.[18,23]

## Results and Discussion

This work was carried out with a DNA fragment containing a 22-bp oligopurine•oligopyrimidine sequence in which a single or double base pair inversion was introduced at the central position. Our previous work has characterized the effect of single mismatches on the stability and kinetics of triplex formation on the same sequence.[14,24] The single mismatch was flanked by T•A × T and C•G × C+ triplets on the 5'- and 3'-sides, respectively (Figure 2). An intercalator, 2-methoxy-6-chloro-9-aminoacridine (W), was incorporated on either the 5'- or 3'-side of the nucleotide (A, C, G, T, I (inosine), N (nebularin)) or the propanediol linker (L) facing the inverted base pair. The thermal stability of these triplexes was assessed by DNA thermal denaturation experiments.

**UV Thermal Denaturation.** A single base pair inversion (A•T or G•C) in the polypyrimidine•polypurine sequence resulted in a decrease of 8−10 °C of the melting temperature as compared to the related canonical triplexes. In general, incorporation of the acridine derivative at an internal site tends to stabilize these triplexes. At the A•T inversion site, the highest stabilization was obtained when acridine was incorporated on the 3'-side of a propanediol linker (5'-LpW-3', $T_m$ = 32 °C) (Figure 2). At the G•C inversion, the best choice was an acridine on the 5'-side of a guanine residue (5'-WpG-3', $T_m$ = 33 °C). These values should be compared with the least destabilizing mismatched triplets, A•T × G and G•C × Y, in the absence of intercalator. Triplex stabilization was increased by 5° and 6°, respectively (Figure 2). In the absence of base pair inversion, incorporation of the acridine derivative on the 5' or 3'-side of the canonical base in the third-strand oligonucleotide did not alter significantly the triplex stability at a T•A base pair or slightly reduced stability at a C•G site. This result was expected since acridine is not a triplex-specific intercalator and should exhibit electrostatic repulsion with a positively charged C•G × C+ triplet.

When a double base pair inversion was introduced (Figure 3), the combination 5'-TpWpG-3' was the best among the tested combinations 5'-TpWpZ-3' or 5'-TpZpW-3' which were primarily synthesized for evaluation of single mismatches as described above. The corresponding $T_m$ value (26 °C) should be compared with that of the third-strand oligonucleotide containing 5'-TpG-3' which forms the least destabilizing mismatched G•C × T and A•T × G triplets at the G•C and A•T single base pair inversion sites, respectively ($T_m$ = 9 °C), and that of the oligonucleotide made by the linkage of two seperate oligonucleotides through a hairpin loop ($T_m$ = 6 °C), a strategy similar to that previously described for two oligonucleotides binding cooperatively to adjacent sites through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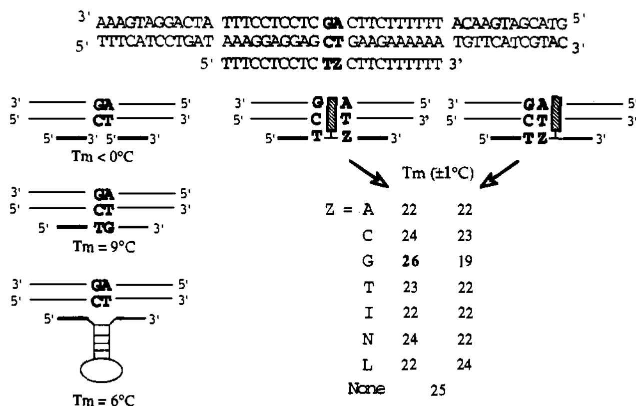


**Figure 3.** Melting temperatures of some of the triplexes obtained when acridine-containing oligonucleotides (center and right panels) bind to a DNA fragment containing a double base pair inversion (see sequence at the top). $T_m$ values of other triplexes in the absence of acridine are also given (left panel) (see text for details). The sequence of the hairpin third strand (lower left) is 5'-**TTTCCTCCTC**CGCTGGC*TTTT*GC-CAGC*C***CTTCTTTTTT**-3' (where the bold sequences represent triplex-forming domains, the underlined sequences form the stem, and bases in italic indicate the linker and loop region). A schematic representation of intercalated complexes is also given (as in Figure 2).

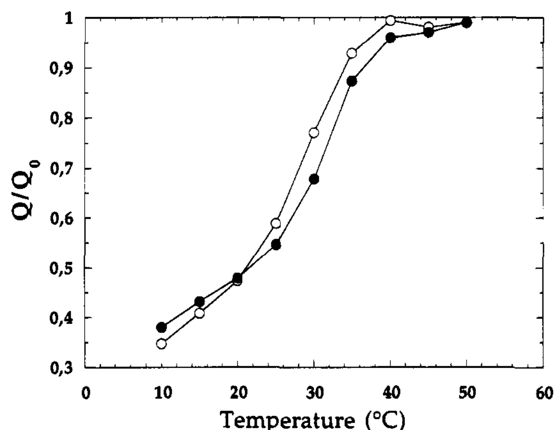


**Figure 4.** Temperature dependence of the ratio of the fluorescence quantum yield ($Q$) of the complex formed between the 46-bp duplex with an A·T base pair inversion at the center (see sequence in Figure 2) and 5'-TTTCCTCCTCTCWCTTCTTTTTT-3' (open circles) or 5'-TTTCCTCCTCTLWCTTCTTTTTT-3' (filled circles), relative to that of the free third strands ($Q_0$).

Waston−Crick base pair formation.[25] The two separated oligonucleotides did not form triplexes. Further study will show whether other combinations such as 5'-$Z_1$p$W$p$Z_2$-3', 5'-$Z_1$p$Z_2$pW-3' ($Z_1 \neq$ T) or 5'-Wp$Z_1$p$Z_2$-3' provide higher stability than that of 5'-TpWpG-3'.

**Fluorescence Quenching.** The fluorescence properties of acridine were used to study triple helix formation. As shown previously,[18,23] the fluorescence of acridine is quenched upon stacking with guanine. It was observed that the fluorescence of acridine strongly decreased when the oligonucleotide containing a 5'-ZpW-3' sequence was bound to the duplex with the inverted A·T base pair. The excitation wavelength (424 nm) was not chosen at the isosbestic point observed when acridine intercalates in double and triple helices. Hypochromism at 424 nm was previously reported to be ∼30%.[18] The observed fluorescence decrease (∼70%, see Figure 4) revealed a quenching of the acridine fluorescence upon intercalation of the oligonucleotide with its target DNA sequence. Fluorescence quenching was totally relieved when the triple helix was dissociated at high temperature. It should be noted that the melting profiles obtained by fluorescence measurements (Figure 4) gave the same $T_m$ values as those obtained in the absorption studies. No quenching was observed when acridine was incorporated on the 5' side (in 5'-WpZ-3' sequences) as expected because a T·A × T base triplet is located on the 5'-side of the inverted A·T base pair (data not shown). These results provided evidence that acridine was stacked with the adjacent C·G base pair located on the 3'-side of the inverted A·T base pair. Therefore the acridine is likely intercalated within the triplex at the mismatch site.

**Sequence Effects.** When a pyrimidine is inserted in a polypurine sequence, a 5'-YpR-3' step is created which is known to be a favored intercalation site for many intercalators,[26−28] including acridine derivatives. Griffin *et al.*[29] previously reported the synthesis of a nucleoside analog, named D3, aimed at recognizing A·T and G·C base pair inversions. But later NMR experiments[30] showed that D3 was intercalated between the inverted A·T or G·C base pair and the base triplet on the 3'-side. In the present study, acridine incorporation on the 3'-side of the nucleotide facing the inverted base pair gave a better stabilization than that on the 5'-side. The only exception is 5'-WpG-3' where the acridine on the 5'-side of guanine gave the highest stabilization. However, for this sequence, it seems that the 2-amino group of guanine in the third strand plays a critical role in the recognition of the G·C inversion site, since replacement of guanine by inosine results in a loss of 5 °C in stability and an acridine gives a higher stabilization on the 3'-side of inosine than on the 5'-side. The same result was obtained with a double base pair inversion where the highest stabilization was obtained with 5'-TpWpG-3' (i.e., acridine on the 5'-side of G) and again inosine substitution decreased the $T_m$ by 4 °C.

In both the single and the double base pair inversions, selectivity of the 5'-WpG-3' sequence to recognize a G·C base pair might be dependent on nearest neighbors. A positive charge carried by a C·G × C+ base triplet (which is present on the 3'-side of G in the third strand) might produce electrostatic repulsion with the positively charged intercalator. A preliminary study in which two T·A × T base triplets were placed on both sides of the inversion site showed that the 5'-YpW-3' sequence actually gives better stabilization than the 5'-WpG-3' sequence facing a G·C inversion site (data not shown).

It should be noted that the use of an intercalator, in some cases, reduces sequence selectivity. For instance, an 5'-LpW-3', which gave the highest stabilization at an A·T inversion site, did not discriminate between the four base pairs facing the propanediol linker L (Figure 3). The absence of selectivity was anticipated due to the absence of any recognition element. In contrast, the sequence 5'-WpG-3' provides a better selectivity with respect to a G·C rather than an A·T inverted base pair which probably could be ascribed to the presence of the 2-amino group of guanine as a recognition element (see above).

## Conclusion

What has emerged from the present study is that an appropriately incorporated acridine within an oligonucleotide

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sequence allows stable triplex formation even in the presence of single and/or consecutive base pair inversions in the target sequence, with limited destabilization as compared with the triple helix formed with the related polypurine•polypyrimidine target sequence. Unexpectedly, the introduction of an acridine in the vicinity of the A•T × G and G•C × Y triplets (which were found to be the least destabilizing mismatched triplets in the absence of intercalator) did not give the highest stabilization. Preliminary analysis revealed nearest-neighbor effects. When the single base pair inversion was flanked by T•A and C•G base pairs on the 5′- and 3′-sides, respectively, the best combinations involved an acridine (W) incorporated on the 3′-side of a propanediol linker (5′-LpW-3′) or on the 5′-side of a guanine (5′-WpG-3′) with the propanediol linker (L) and guanine (G) facing the inverted A•T and G•C base pairs, respectively. Therefore, the present work points to the new possibility of extending the range of DNA sequences which can be recognized by intercalator-containing oligonucleotides. Preliminary biological assays of oligonucleotides targeted to the HIV proviral genome fulfill this expectation. Work is under way to incorporate triplex-specific intercalators, e.g., benzopyridoindole derivatives,[31−33] in order to enhance the stabilizing effect.

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