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Binding Affinities of Oligonucleotides and PNAs Containing Phenoxazine and G-Clamp Cytosine Analogues Are Unusually Sequence-Dependent

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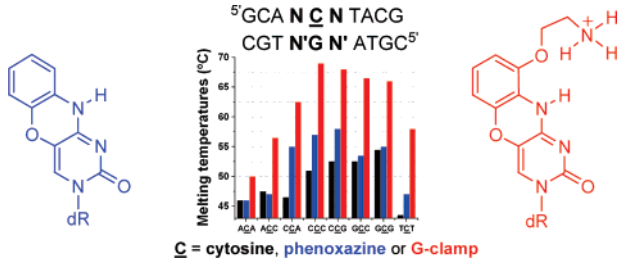
ABSTRACT



Melting temperatures of DNA duplexes containing the phenoxazine (P) and G-clamp (X) cytosine analogues exhibited a strong and unusual dependence on the nucleoside flanking the modified nucleobase, and the same trend was observed in PNA–DNA duplexes incorporating X in the PNA chain. Molecular dynamics simulations of the DNA duplexes show that generalized stacking (including secondary interactions of the ammonium group of X) and hydrogen bonding are good descriptors of the different duplex stabilities.

Numerous biotechnological, biomedical, and material sciences applications of oligonucleotides (ODNs) and analogues would significantly benefit from enhanced binding affinities in the formation of secondary structures with their complementary sequences.[1] Nucleobase analogues that maximize the stacking interactions and increase the number of hydrogen bonds between base pairs provide a means for enhancing duplex stability. The tricyclic cytosine analogues phenoxazine (P) and 9-(2-aminoethoxy)phenoxazine (G-clamp or amino-G-clamp, X) have emerged as powerful tools to reach this goal (Figure 1).[2] The G-clamp nucleoside phosphoramidite has been recently made commercially available to allow introduction in synthetic oligonucleotides.[3] The extended π system of the phenoxazine ring is thought to be responsible for the increase in the binding affinity, and the G-clamp, in addition to its enhanced stacking, is believed to simultaneously recognize both the Watson−Crick and Hoogsteen faces of guanine through the formation of four hydrogen bonds. ODNs containing the phenoxazine and G-clamp cytosine analogues have shown interesting antisense activities

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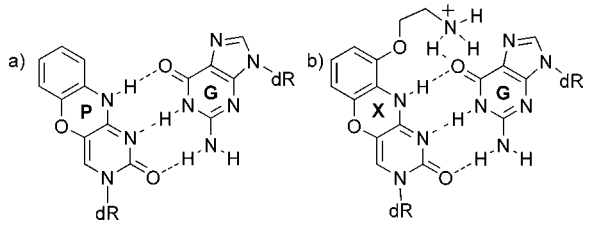

Figure 1. Hydrogen bonding in the base pairs of guanine with the tricyclic cytosine analogues: (a) phenoxazine and (b) G-clamp.

and 3′-exonuclease resistance.[4] Moreover, G-clamp 2′-*O*-methyl ODNs have also been shown to inhibit the HIV-1 Tat-TAR interaction more effectively than other ODNs.[5] It is also worth mentioning that tricyclic cytosine analogues exhibit fluorescent properties and can be exploited as reporter groups. The high fluorescent quantum yields of the phenothiazine ring are in particular largely insensitive to interactions with neighboring nucleobases in ODNs or duplexes.[6]

Although the binding studies of ODNs incorporating the phenoxazine analogue showed only moderate increases in Tm of 3−7 °C, a single incorporation of a G-clamp was found to increase the Tm of a polypyrimidine DNA decamer by 18 °C.[2] Duplexes containing the 2′-*O*-methyl-G-clamp analogue showed Tm increases ranging from 4 to 17 °C, and the magnitude of the increase was attributed to the position of the G-clamp within the sequence.[5] Phenothiazine-containing ODNs and peptide nucleic acids (PNAs) also form more stable duplexes than the unmodified ones, and DNA duplexes showed an unperturbed B-DNA conformation.[6b] Surprisingly, ODNs incorporating another phenoxazine-derived cytosine analogue, the guanidino-G-clamp, showed lower melting temperatures (ΔTm = 6 °C) than the amino-G-clamp ODNs, although five hydrogen bonds between the guanidino-G-clamp nucleobase and guanine had been observed by X-ray crystallography.[7]

The close similarities between the studied sequences incorporating these cytosine analogs[2] prompted us to investigate whether the thermal stability of the duplexes varied depending on the sequence context, in particular the residues flanking the modified nucleoside. In contrast with the original claim,[2b] we now report that the binding affinities of ODNs and PNAs modified with these base analogs exhibit a strong sequence-dependence, although both the CD spectra and molecular dynamics simulations indicate the formation of a B-DNA duplex upon hybridization with their complementary strands.

The synthesis of the phenoxazine- and G-clamp-protected nucleoside phosphoramidites was performed essentially as previously described.[2] However, in our hands the syntheses were not at all straightforward, and careful adjustments in the experimental conditions had to be made to obtain the desired products and optimize the yields (Supporting Information). Phosphoramidites were subsequently used in solid-phase ODN assembly. It should be mentioned that ODNs containing the phenoxazine and amino-G-clamp analogues are light sensitive and start decomposing when exposed to daylight at room temperature in less than 24 h, but they are stable enough for large periods when stored lyophilized and in the freezer. A series of natural and modified 10-mer ODNs having the general sequence 5′GCA**N*C*N**TACG3′ (where ***C*** denotes either cytosine (C), phenoxazine (P), or G-clamp (X), and **N** any nucleoside) were obtained, hybridized to their complementary strands, and used in UV melting experiments (Table 1). In the vaste majority of reported sequences, P or X were flanked by two cytosines. Our sequences were selected so that either cytosine was combined with a purine at the 5′ or 3′ side or the two flanking nucleobases **N** were identical.

Table 1. Hybridization Properties of Oligonucleotides[a]

nucleobase	sequence	Tm[b] (°C)	ΔTm[c] (°C)	ΔΔTm[d] (°C)
cytosine (C)	GCA**ACA**TACG	46.0		
	GCA**ACC**TACG	47.5		
	GCA**CCA**TACG	46.5		
	GCA**CCC**TACG	51.0		
	GCA**CCG**TACG	52.5		
	GCA**GCC**TACG	52.5		
	GCA**GCG**TACG	54.5		
	GCA**TCT**TACG	43.5		
phenoxazine (P)	GCA**APA**TACG	46.0	0	
	GCA**APC**TACG	47.0	−0.5	
	GCA**CPA**TACG	55.0	8.5	
	GCA**CPC**TACG	57.0	6.0	
	GCA**CPG**TACG	58.0	5.5	
	GCA**GPC**TACG	53.5	1.0	
	GCA**GPG**TACG	55.0	0.5	
	GCA**TPT**TACG	47.0	3.5	
G-clamp (X)	GCA**AXA**TACG	50.0	4.0	4.0
	GCA**AXC**TACG	56.5	9.0	9.5
	GCA**CXA**TACG	62.5	16.0	7.5
	GCA**CXC**TACG	69.0	18.0	12.0
	GCA**CXG**TACG	68.0	15.5	10.0
	GCA**GXC**TACG	66.5	14.0	13.0
	GCA**GXG**TACG	66.0	11.5	11.0
	GCA**TXT**TACG	58.0	14.5	11.0

[a] Experiments were performed with ODNs at 5 μM concentration in 5 mM NaH_2PO_4/Na_2HPO_4, 1 mM $MgCl_2$, and 140 mM KCl buffer, at pH 7.2. [b] The error in Tm values was ± 0.5 °C. [c] Differences between Tm values of phenoxazine- or G-clamp-modified and unmodified ODNs. [d] Differences between Tm values of G-clamp- and phenoxazine-containing ODNs.

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Phenoxazine-containing ODNs showed striking sequence-dependent differences in their ability to stabilize the duplexes with respect to the unmodified ODNs. In the sequences where the 5′ flanking nucleobase was a purine (APA, APC, GPG, and GPC), the phenoxazine nucleobase produced little or no stabilizing effect (ΔTm ± 1 °C). The most stabilized duplexes (ΔTm = 5.5−8.5 °C) were those in which phenoxazine was flanked by a 5′ cytosine (CPA, CPC, and CPG), while the TPT duplex was moderately stabilized (ΔTm = 3.5 °C). These results are in good agreement with those reported for DNA duplexes incorporating the phenothiazine analog.[6b]

As a general trend, G-clamp-containing duplexes were much more stable than those containing either cytosine or phenoxazine, thus confirming the outstanding stabilizing properties of this cytosine analog. However, a noticeable sequence-dependent effect was again observed that shares some common trends with phenoxazine-containing duplexes. The most stabilized duplexes (ΔTm = 15.5−18 °C) were those in which the G-clamp was flanked by a 5′ cytosine (CXA, CXC, and CXG). On the contrary, the lowest stabilizing effects were observed when the G-clamp was flanked by a 5′ purine (AXA, AXC, GXG, and GXC), 5′ guanines being significantly more stabilizing (ΔTm = 11.5−14 °C) than 5′ adenines (ΔTm = 4−9 °C). Another clear effect is that, for a given 5′ residue, the presence of cytosine at the 3′ side of the G-clamp was more stabilizing than that of a purine, especially when the 3′ purine was adenine (AXC vs AXA and CXC vs CXA) and much less when it was guanine (GXC vs GXG and CXC vs CXG). This behavior can be attributed to the aminoethoxy arm of the G-clamp, since it was not observed in phenoxazine-containing duplexes, where the differences between these pairs of triplets were less significant. The consequence for the less stable 5′ purine G-clamp sequences was that the presence of a 3′ cytosine partially counterbalanced the negative impact of the 5′ purine on the binding affinity (AXC vs AXA and GXC vs GXG).

Overall, the ΔTm values shown in Table 1 indicate that the increase in the binding affinities of oligonucleotides modified with phenoxazine or G-clamp is far from being homogeneous. Table 2 depicts the relative order (in parentheses) of duplex stabilities of the unmodified and P or X modified sequences, showing that the non-homogeneous stabilizing properties of the nucleobase modifications lead to interesting inversions in the order of stability of certain pairs of triplets. For example, the GCG triplet gives more stable duplexes than GCC, but their stabilities are almost identical when the central C is replaced by X. An inversion in stability (Tm) is observed for other pairs of triplets: CCG > CCC but CXC > CXG; ACC > CCA but CXA > AXC; etc.

Table 2. Relative Order of Thermal Stabilities of the Duplexes (in Parentheses) Formed by the Natural Sequences and the P− or X-Modified Oligonucleotides

cytosine (C)	phenoxazine (P)	G-clamp (X)
GCG (1)	GPG (3)	GXG (4)
GCC (2)	GPC (5)	GXC (3)
CCG (2)	CPG (1)	CXG (2)
CCC (4)	CPC (2)	CXC (1)
ACC (5)	APC (6)	AXC (7)
CCA (6)	CPA (4)	CXA (5)
ACA (7)	APA (8)	AXA (8)
TCT (8)	TPT (6)	TXT (6)

ΔΔTm values (Table 1) were calculated to evaluate the contribution of the appending aminoethoxy arm with respect to that provided by the phenoxazine ring. These values clearly reveal that the stabilizing effect of the additional hydrogen bond formed by the protonated amino group is higher than that resulting from the stacking interactions of the phenoxazine ring. Moreover, from the comparison of ΔTm and ΔΔTm values it can be inferred that the aminoethoxy arm of the G-clamp is the main contributor to the enhanced binding affinity of the sequences where the G-clamp is flanked by a 5′ purine.

The melting temperatures of the CCC, CPC, and CXC duplexes were also determined at different salt concentrations (70, 140, and 280 mM KCl). As expected, the Tm values increased with ionic strength because of the decrease in the repulsion between phosphate groups (Supporting Information). However, the increase in Tm values was the same for the three sequences, irrespective of whether C, P, or X was present, indicating that the enhanced affinity of the G-clamp-modified ODNs cannot be attributed to an ionic interaction between the protonated amino group of X and a phosphate group of the complementary strand.

It is also worth noting that the CD spectra of the phenoxazine- and G-clamp-containing duplexes showed the characteristic profile of a B-form DNA, and did not exhibit significant differences compared with unmodified duplexes. In all cases, a positive band centered at 280 nm and a negative band at 250 nm were observed (Supporting Information).

The amino-G-clamp cytosine analog has also been introduced in PNA sequences and reported to produce a dramatic increase in the stabilities of PNA−DNA and PNA−RNA duplexes when placed between two cytosines (ΔTm = 18.2 and 23.7 °C, respectively).[8] In order to check whether this stabilizing effect was maintained in our sequences, we decided to study two PNAs with the same sequence as the DNA strands exhibiting the most extreme behavior, those in which the G-clamp was flanked by two cytosines or two adenines: H-gca**cxc**tacg-NH_2 and H-gca**axa**tacg-NH_2. PNA monomers were obtained as previously described with minor modifications,[9] and introduced in PNA sequences using standard solid-phase procedures. The UV melting curves of PNA-DNA duplexes showed, again, the remarkable stabilizing effect of the G-clamp (Supporting Information). The Tms of the PNA−DNA duplexes formed by H-gca**cxc**tacg-NH_2 and H-gca**axa**tacg-NH_2 were 16 and 8.5 °C, respectively,

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higher than those of the unmodified ones. It is noteworthy that the difference in ΔTm between the sequences containing the **cxc** and **axa** triplets was less pronounced than in DNA−DNA duplexes. We hypothesize that the more flexible polyamide backbone of PNA, as compared with DNA, allows to partially offset the lower stabilizing effect when the G-clamp is flanked by two adenines.

To gain a deeper insight into the reasons underlying the sequence dependence of the duplex stability of ODNs containing these cytosine analogues, molecular dynamics simulations of all 24 duplexes were performed (Supporting Information).[10] All trajectories were stable, sampling regions close to the canonical B-form and maintaining well the expected pattern of hydrogen bonds. P or X analogues did not introduce significant changes in the structure. Both P and X derivatives made the expected interactions; in particular, the ammonium−guanine interactions were very strong in all of the X-duplexes with interaction distances N-O^6 and N-N^7 corresponding to those of strong hydrogen bonds.

The melting temperature is linearly related ($r = 0.94$) to the generalized stacking and hydrogen bond energies in the central 5 (or 3)-pairs segment (Supporting Information), showing that these two terms are good descriptors of the DNA stability. The introduction of the P derivative slightly increases (around 1 kcal/mol) the hydrogen-bond energy and clearly improves by 5−10 kcal/mol the stacking energy (Supporting Information). The sequences where the gains in stacking are larger are those for which greater increases in melting temperatures are found (melting temperature and the gain in melting temperature upon C→P substitution correlate linearly; $r = 0.8$). Clearly, the sequence-dependence of stacking explains the different gains in stability upon C→P substitution in different sequences.

The addition of the charged moiety to phenoxazine ring in X leads to a large gain in hydrogen bonding (between 25 and 30 kcal/mol) and also to an unexpected gain (from 18 to 34 kcal/mol) in generalized stacking (Supporting Information), which is largely a consequence of the secondary interactions of the ammonium group with hydrogen bond acceptors of bases different from the paired guanine (Figure 2). Note that these secondary interactions (captured in the generalized stacking term) can be large and are strongly sequence-dependent, and when combined with the direct hydrogen bond term are able to reproduce well ($r = 0.93$) the sequence dependent increase in melting upon P→X substitution, confirming the goodness of the simple descriptors used here.

In summary, atomistic simulations suggest that flat pictures as those in Figure 1 might be very useful to underline desired interactions. However, once the modified nucleobase is inserted in DNA, many other interactions may be established. Experimental data and theoretical calculations are in good agreement and show that ODNs incorporating the tricyclic cytosine analogues P and X form duplexes with a practically unperturbed B-DNA conformation. However, unexpected interactions with neighboring bases give rise to profound and striking differences in the stability of the resulting duplexes depending on the sequence context of the modified nucleobase. These differences should be taken into account when designing the incorporation of P and X into ODNs and PNAs in order to enhance their binding affinities. From our study it can be concluded, as a general rule, that the substitution of cytosine by phenoxazine does not enhance duplex stability if the 5′ flanking nucleobase is a purine, while some stabilization may be gained if a 5′ cytosine flanks the phenoxazine ring. When replacing cytosine by the G-clamp, the highest binding affinities will be obtained if the 5′ nucleobase can be a cytosine. If the 5′ nucleobase has to be a purine, then guanine is preferred to adenine. The 3′ flanking nucleobase of the G-clamp also has some effect on duplex stability and we would recommend cytosine rather than a purine at this position. Sequences with only one T flanking P or X were not studied. Although firm predictions cannot be made, from the comparison of the Tms of C*C*C and T*C*T we would expect duplex stability to slightly decrease when replacing a single C by a T.

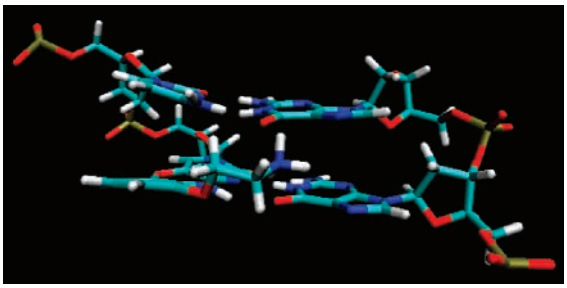

Figure 2. Snapshot of $^{5'}$CX•$^{3'}$GG tract obtained by molecular dynamics, showing the secondary interactions of ammonium group of X with the paired and nonpaired guanines in the opposite strand.

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Supporting Information Available: Details on the synthesis of phenoxazine and G-clamp building blocks, ODNs, and PNAs. UV melting profiles, CD spectra, and theroretical calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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