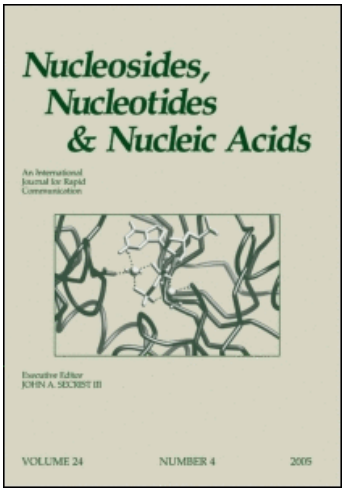

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 06 January 2004

To cite this Article Ryabinin, Vladimir A. , Boutorine, Alexandre S. , Hélène, Claude , Pyshnyi, Dmitrii V. and Sinyakov, Alexandre N.(2004) 'Oligonucleotide-Minor Groove Binder Conjugates and Their Complexes with Complementary DNA: Effect of Conjugate Structural Factors on the Thermal Stability of Duplexes', Nucleosides, Nucleotides and Nucleic Acids, 23: 5, 789 — 803
To link to this Article: DOI: 10.1081/NCN-120039358
URL: http://dx.doi.org/10.1081/NCN-120039358

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, No. 5, pp. 789–803, 2004

Oligonucleotide–Minor Groove Binder Conjugates and Their Complexes with Complementary DNA: Effect of Conjugate Structural Factors on the Thermal Stability of Duplexes

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ABSTRACT

Synthetic polycarboxamide minor groove binders (MGB) consisting of N-methylpyrrole (Py), N-methylimidazole (Im), N-methyl-3-hydroxypyrrole (Hp) and β-alanine (β) show strong and sequence-specific interaction with the DNA minor groove in side-by-side antiparallel or parallel orientation. Two MGB moieties covalently linked to the same terminal phosphate of one DNA strand stabilize DNA duplexes formed by this strand with a complementary one in a sequence-specific manner, similarly to the corresponding mono-conjugated hairpin structures. The series of conjugates with the general formula Oligo-(L-MGB-R)$_m$ was synthesized, where

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DOI: 10.1081/NCN-120039358
1525-7770 (Print); 1532-2335 (Online)

m = 1 or 2, L = linker, R = terminal charged or neutral group, MGB = $-(Py)_n-$, $-(Im)_n-$ or $-[(Py/Im)_n-(CH_2)_3CONH-(Py/Im)_n-]$ and $1 < n < 5$. Using thermal denaturation, we studied effects of structural factors such as m and n, linker L length, nature and orientation of the MGB monomers, the group R and the backbone (DNA or RNA), etc. on the stability of the duplexes. Structural factors are more important for linear and hairpin monophosphoroamidates than for parallel *bis*-phosphoroamidates. No more than two oligocarboxamide strands can be inserted into the duplex minor groove. Attachment of the second sequence-specific parallel ligand $[-L(Py)_4R]$ to monophosphoroamidate conjugate $CGTTTATT-L(Py)_4R$ leads to the increase of the duplex Tm, whereas attachment of $[-L(Im)_4R]$ leads to its decrease. The mode of interaction between oligonucleotide duplex and attached ligands could be different (stacking with the terminal A:T pair of the duplex or its insertion into the minor groove) depending on the length and structure of the MGB.

Key Words: Oligonucleotide; Minor groove binder; Conjugate; Duplex stability; Sequence-specific recognition.

INTRODUCTION

Polycarboxamide minor groove binders (MGB) that recognize the minor groove of B-DNA in a sequence-specific manner have been intensively studied the last years because of their potential to open new ways for sequence-specific control of gene expression. The group of P. Dervan formulated the principles of design of the hairpin polyamides capable of sequence-specific binding to DNA[1–6] based on the fact that two antiparallel oligocarboxamides can insert into the same DNA minor groove.[7–9] These polyamides contain two mixed blocks of N-methylpyrrole carboxamide (Py), N-methyl-3-hydroxypyrrole carboxamide (Hp) and N-methylimidazole carboxamide (Im) units as monomers for ligand construction separated by a linker, and form a ''head-to-tail'' hairpin construction. A code for the binding of hairpin polyamides has been proposed where Im/Py, Py/Im, Py/Hp and Hp/Py pairs recognize GC, CG, AT and TA base pairs, respectively.[10–13] Replacement of certain units by a more flexible β-alanine residue allowed for increase in the affinity and sequence selectivity of specific polycarboxamides.[14]

Conjugates of minor groove binders with oligonucleotides appeared to be most promising. They provide increased binding specificity to single-stranded DNA due to participation of both the oligonucleotide and the ligand components.[15–20] For example, the use of oligonucleotide–MGB conjugate as a primer led to increased stability and specificity of primer binding to target DNA.[21] Simultaneous specific interactions of conjugates with both major and minor grooves of the double-stranded DNA allowed for stabilization of DNA triple helices.[22–25] Recently new conjugates containing two minor groove binder moieties attached to the same terminal phosphate of oligonucleotides were described.[22,26] In our previous work we demonstrated high and sequence-specific stabilization of short DNA duplexes if one of the strands contained two parallel tetracarboxamide MGB moieties attached to the same 3′-terminal phosphate.[26] The extent of stabilization was comparable (if not better) with that of hairpin antiparallel MGBs. For optimization of conjugate construction, several structural factors could be varied: 1) the length of the linker between oligonucleotide and

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ligand, 2) the number of carboxamide units in each MGB strand as well as their chemical nature, 3) orientation of MGB strands (N→C or C→N), 4) nature of the C-terminal group (either neutral ester group or positively charged amino group) and 5) chemical nature of target nucleic acid backbone (DNA, RNA, etc.).

In the present work, a series of conjugates with the general formula Oligo-(L-MGB-R)$_m$ was synthesized, where m = 1 or 2, L = linker (γ-aminobutyric or ε-aminocaproic acid residue), R = terminal charged or neutral group, MGB = $-(Py)_n-$, $-(Im)_n-$ or $-[(Py/Im)_n-(CH_2)_3CONH-(Py/Im)_n-]$ and $2 \leq n \leq 4$. Effects of the number of carboxamide units (n) and their nature, the linker L length, the nature of the group R, the nature of the backbone (DNA or RNA) on the stability of their complexes with complementary oligonucleotides were studied. As a basic experimental model, a 3′-conjugate of one or two tetrapyrrole carboxamides with a short oligonucleotide containing an AT tract at the modified terminus was used.

MATERIALS AND METHODS

Reagents

All reagents and solvents were purchased from Sigma-Aldrich-Fluka and SDS. The model oligonucleotides were synthesized by the Oligonucleotide Synthesis Group in Novosibirsk Institute of Bioorganic Chemistry or by Eurogentec.

MGB Synthesis

Synthesis, purification, NMR and mass-spectrometry analysis of amino-functionalized minor groove binders were carried out as described previously.[16,17,26–28]

Synthesis of Oligonucleotide–MGB Conjugates

For covalent coupling of one MGB molecule to the oligonucleotide we used terminal phosphate activation as described in previous papers.[26,29,30] The phosphate activation step was done in organic solvent (DMSO) and the attachment of the ligand in water–DMSO mixture.

For synthesis of 2:1 MGB:oligonucleotide conjugate, 0.05–0.5 μmoles of 1:1 conjugate were precipitated by CTAB, dried, dissolved in DMSO, its phosphoroamidate phosphate activated again with triphenylphosphine/dipyridyldisulfide/dimethylaminopyridine as it was described previously,[29] excess of the second ligand (2–3 mg in DMSO) added directly to the reaction mixture and the mixture incubated at least 2 hours (preferably overnight) at room temperature.

The conjugates were purified by HPLC on a C-18 X-Terra column (Waters, 7 μm, 300 × 7.8 mm) in a 5–40% acetonitrile linear gradient (elution rate 2.0 ml/min) in 0.02 M ammonium acetate using the 1100 chromatography system from Agilent Technologies.

Oligonucleotide conjugates were analyzed by gel electrophoresis, UV spectrophotometry and mass spectrometry on Q-Star instrument from Applied Biosystems as it was described before.[22,26–28]

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Thermal Denaturation Assay

The thermal denaturation and renaturation studies of duplexes were carried out on a Kontron Uvikon 940 spectrophotometer (BioTek) with thermostated cuvettes of 1-cm optical pathlength. The concentrations of the duplex strands in each sample were 1.5 μM, in 10 mM phosphate buffer pH 7.3, 0.1 M NaCl, 0.1 mM EDTA. The sample temperature was changed at 0.2°C/min and the absorption was recorded every 200 s. The treatment of the melting curves was carried out using KaleidaGraph and Microsoft Excel softwares. In certain experiments, the melting curves of the duplexes were recorded in a specially constructed thermostated microcuvette of the liquid microchromatography system ''Millichrom'' (Novosibirsk Institute of Bioorganic Chemistry, Russia) according to the method described in a previous report.[15]

RESULTS AND DISCUSSION

Synthesis of Conjugates

For covalent attachment of minor groove binders containing a primary aminoalkyl group at the N-terminus via phosphoroamidate formation, we used a simple method (proposed by Zarytova et al.,)[31] of oligonucleotide terminal phosphate activation with Mukaiyama reagents (triphenylphosphine–dipyridyldisulfide–dimethylaminopyridine)[32] in dry polar organic solvents. The coupling reaction in the aqueous solutions leads to attachment of only one ligand,[29] while in water-free organic solvents (DMSO, DMF) in excess of ligands and activating agents, two aminoalkyl groups can be

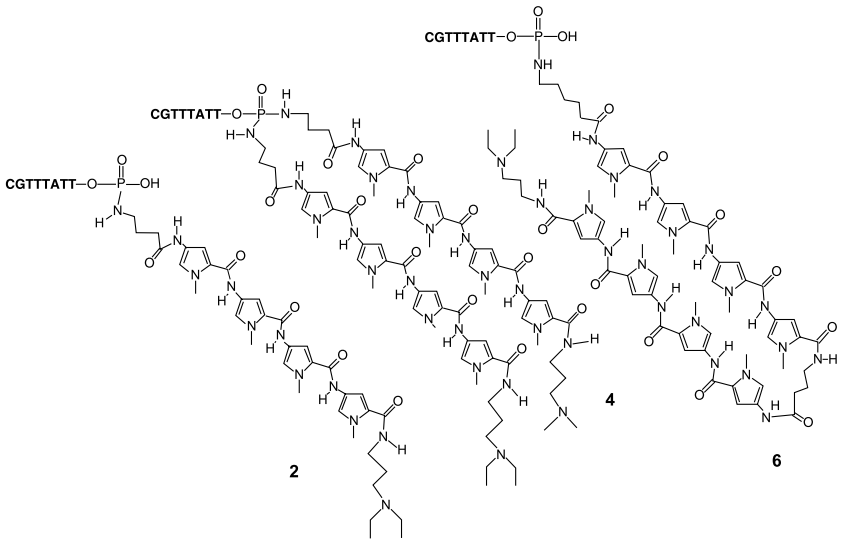

Figure 1. Examples of oligonucleotide CGTTTTATTp—minor groove binder conjugates. Number of conjugate indicates number of duplex where it was used according to Table 1.

attached to the same phosphate, thus *bis*-phosphoroamidate derivative can be formed.[26,33]

In the present work we used mainly oligopyrrole carboxamide ligands with γ-aminobutyric or ε-aminocaproic acid as N-terminal unit and short oligonucleotide duplexes containing A:T tracts (Fig. 1). As it was demonstrated, when *bis*-phosphoroamidate oligonucleotide–MGB conjugates form duplexes with complementary oligonucleotide sequences, both ligands insert into the minor groove and strongly stabilize complex formation similarly to classical hairpin MGB structures with antiparallel strand orientation.[26]

Thermal Denaturation Method

The stability of duplexes formed by oligonucleotides modified by terminally attached ligands with complementary sequences was determined by thermal denaturation method. We followed optical densities of preformed duplexes at two wavelengths: 260 nm and 330 nm. These wavelengths are close to the absorption maximums of the oligonucleotide and MGB parts of the complex, respectively. As it was shown in a previous paper,[26] the interaction of the MGB with the minor groove of DNA leads to a red shift of the absorption maximum of the carboxamide (from 305–310 nm to 320–325 nm). Thus, melting of the DNA-MGB complex is accompanied by a decrease in the optical density at 330 nm, and the negative peak on the melting curve at this wavelength is considered as a clear indication of interaction between duplex and MGB. All the results are summarized in Table 1 (in the following text, the numbers are attributed to duplexes according to this table).

The nonmodified model duplex 1 dissociates at 20°C. As it was already shown, addition of only one tetrapyrrole ligand to the 3′-end of the AT-tract of short oligonucleotide led to the significant increase of its melting temperature with complementary oligonucleotide resulting in ΔTm from 10°C for neutral ligand up to 26°C for positively charged one (compare for example duplex 1 to 3, 9 and 11). Duplexes with a strand conjugated to two parallel tetrapyrrole ligands were much more stable; their melting temperatures were about ΔTm = 40°C higher than those of nonmodified duplexes (compare for example 1 to 4, 5, 10, 12 and 16). For conjugates with a hairpin MGB consisting of two antiparallel strands, the difference in melting point could vary in a wide range depending on the structure of the conjugate (duplexes 6, 13).

Effect of Linker Length

We compared stability of duplexes linked to one tetrapyrrole residue via γ-aminobutyric and ε-aminocaproic acid. For 1:1 conjugates, longer linker provided slightly better stability of the duplex independently of the charged (duplexes 2 and 3) or uncharged (duplexes 9 and 11) C-terminal groups. The difference was between 5° and 9°C. When two parallel tetrapyrrole residues were attached, no evident difference was detected (compare duplexes 4 and 5, 10 and 12).

The majority of the experiments have been done with γ-aminobutyric acid linker between oligonucleotide and tetrapyrrole residue.

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Table 1. Thermal denaturation temperatures of the duplexes formed by oligonucleotides–MGB conjugates with their complementary sequences.

Expt n°	Duplex[a]	X_1	X_2	T_m, °C
1*	A	~ O^-	~ O^-	20
2	A	~ O^-	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	37
3*	A	~ O^-	~ $NH(CH_2)_5CO(Py)_4$-$NH(CH_2)_3NEt_2$	46
4	A	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	61
5*	A	~ $NH(CH_2)_5CO(Py)_4$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_5CO(Py)_4$-$NH(CH_2)_3NEt_2$	60
6*	A	~ O^-	~ $NH(CH_2)_5CO(Py)_4$–$-NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	58
7	A	~ $NH(CH_2)_5CO(Py)_4$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_5CO(Py)_4$–$-NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	37
8	A	~ $NH(CH_2)_5CO(Py)_4$–$-NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_5CO(Py)_4$–$-NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	37
9	A	~ O^-	~ $NH(CH_2)_3CO(Py)_4OEt$	30
10	A	~ $NH(CH_2)_3CO(Py)_4OEt$	~ $NH(CH_2)_3CO(Py)_4OEt$	58
11*	A	~ O^-	~ $NH(CH_2)_5CO(Py)_4OEt$	35
12*	A	~ $NH(CH_2)_5CO(Py)_4OEt$	~ $NH(CH_2)_5CO(Py)_4OEt$	60
13*	A	~ O^-	~ $NH(CH_2)_5CO(Py)_4$–$-NH(CH_2)_3CO(Py)_4OEt$	49
14	A	~ $NH(CH_2)_3CO(Py)_4$–$-NH(CH_2)_3CO(Py)_4OEt$	~ $NH(CH_2)_3CO(Py)_4$–$-NH(CH_2)_3CO(Py)_4OEt$	38
15	A	~ O^-	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3N^+Me_3$	31

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16	A	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3N^+Me_3$	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3N^+Me_3$	63
17	A	~ O^-	*$Py(Py)_3NH(CH_2)_3NH$ ~	31
18	A	*$Py(Py)_3NH(CH_2)_3NH$ ~	*$Py(Py)_3NH(CH_2)_3NH$ ~	34
19	A	~ O^-	~ $NH(CH_2)_3CO(Py)_3$-$NH(CH_2)_3NEt_2$	37
20	A	~ $NH(CH_2)_3CO(Py)_3$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_3CO(Py)_3$-$NH(CH_2)_3NEt_2$	61
21	A	~ O^-	~ $NH(CH_2)_3CO(Py)_2$-$NH(CH_2)_3NEt_2$	30
22	A	~ $NH(CH_2)_3CO(Py)_2$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_3CO(Py)_2$-$NH(CH_2)_3NEt_2$	46
23	A	~ O^-	~ $NH(CH_2)_3CO(Py)_2OEt$	26
24	A	~ $NH(CH_2)_3CO(Py)_2OEt$	~ $NH(CH_2)_3CO(Py)_2OEt$	33
25	A	~ O^-	~ $NH(CH_2)_3CO(Py)_2NHBu$	27
26*	A	~ O^-	~ $NH(CH_2)_5CO(Im)_4$-$NH(CH_2)_3NEt_2$	23
27*	A	~ $NH(CH_2)_5CO(Im)_4$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_5CO(Im)_4$-$NH(CH_2)_3NEt_2$	24
28*	A	~ $NH(CH_2)_5CO(Py)_4$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_5CO(Im)_4$-$NH(CH_2)_3NEt_2$	30
29	B	~ O^-	~ O^-	16
30	B	~ O^-	~ $NH(CH_2)_3CO(Py)_4$-NH $(CH_2)_3NEt_2$	35
31	B	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	58
32	C	~ O^-	~ O^-	15
33	C	~ O^-	~ $NH(CH_2)_3CO(Py)_4$-NH $(CH_2)_3NEt_2$	46
34	C	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	~ $NH(CH_2)_3CO(Py)_4$-$NH(CH_2)_3NEt_2$	51
35	C	~ O^-	~ $NH(CH_2)_5CO(Py)_4-$	55

(*continued*)

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Table 1. Continued.

Expt n°	Duplex[a]	X_1	X_2	T_m, °C
			$-NH(CH_2)_3CO(Py)_4$- $NH(CH_2)_3NEt_2$	
36	D	$\sim O^-$	$\sim O^-$	20
37	D	$\sim O^-$	$\sim NH(CH_2)_3CO(Py)_4$- $NH\ (CH_2)_3NEt_2$	26
38	D	$\sim NH(CH_2)_3CO(Py)_4$- $NH(CH_2)_3NEt_2$	$\sim NH(CH_2)_3CO(Py)_4$- $NH(CH_2)_3NEt_2$	24
39	E	$\sim O^-$	$\sim O^-$	20
40	E	$\sim O^-$	$\sim NH(CH_2)_3CO(Py)_4$- $NH\ (CH_2)_3NEt_2$	63
41	E	$\sim NH(CH_2)_3CO(Py)_4$- $NH(CH_2)_3NEt_2$	$\sim NH(CH_2)_3CO(Py)_4$- $NH(CH_2)_3NEt_2$	69
42	F	$\sim O^-$	$\sim O^-$	20
43	F	$\sim O^-$	$\sim NH(CH_2)_5CO(Py)_4-$ $-NH(CH_2)_3CO(Py)_4$- $NH(CH_2)_3NEt_2$	67
44	G	$\sim O^-$	$\sim NH(CH_2)_5CO(Py)_4-$ $-NH(CH_2)_3CO(Py)_4$- $NH(CH_2)_3NEt_2$	68

*Data from our previous article (submitted to Nucleosides, Nucleotides and Nucleic Acids).

A: 5′-CGTTTATT-pX_1X_2, 3′-GCAAATAA-5′

B: 5′-X_1X_2p-GTATTTG, 3′-CATAAAC-5′

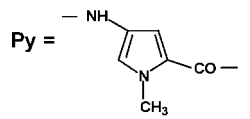
C: 5′-GAAAATT-pX_1X_2, 3′-CTTTTAACC

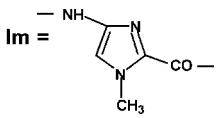
D: 5′-TTTTTTTT-pX_1X_2, 3′-poly(rA)

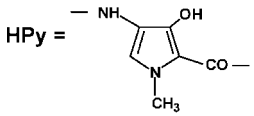
E: 5′-TTTTTTTT-pX_1X_2, 3′-poly(dA)

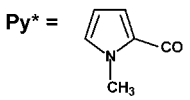
F: 5′-TTTTTTTT-pX_1X_2, 3′-AAAAAAAA

G: 5′ X_1X_2p-TTTTTTTT-pX_1X_2, 3′-AAAAAAAA









β = —HNCH$_2$CH$_2$CO —

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Effect of a Positive Charge at the C-Terminal Group

Three different C-terminal groups of the MGB residues were tested: neutral carboethoxy group, ternary diethylamino group that must be protonated under physiological conditions and positively charged quaternary trimethylammonium group. For 1:1 conjugates connected by γ-aminobutyric acid, the best stabilizing effect was observed with the ternary diethylamino group (ΔTm = 17°C, compare duplexes 1 and 2). The effect observed for trimethylammonium group was less pronounced (maybe due to steric hindrance), ΔTm = 11°C (compare duplexes 1 and 15), while the lowest effect was shown for neutral carboethoxy group (ΔTm = 10°C, compare duplexes 1 and 9). With the longer ε-aminocaproic linker, this difference between charged and uncharged terminal group was even stronger: ΔTm = 26°C and 15°C, for diethylamino- and carboethoxy groups, respectively (duplexes 3 and 11).

A terminal positive charge was considered as an important element for design of hairpin MGB.[11,13,34,35] In our experiments, we also observed more stable duplexes if they were formed by 1:1 conjugates with a hairpin octapyrrole and C-terminal positively charged group (compare duplexes 6 and 13, ΔTm = 38°C and 29°C, respectively). In contrast, low effect of C-terminal positive charge was observed for duplexes with parallel tetrapyrrole ligands (duplexes 4,5,10,12,16). For all these duplexes, ΔTm was around 40°C; it was almost independent of the terminal charge and only slightly dependent on the linker length. Thus, a structure with two minor groove binder strands attached to the same phosphate group seems to have better affinity to the target DNA than a hairpin structure. Two positive charges of both ligands in complexes 4,5 and 16 must theoretically stabilize the complex formation even more, but this effect could be compensated by their electric repulsion due to close location of amino groups in the parallel conjugate.

Effect of Oligocarboxamide Chain Length

A tripyrrole is a minimal size ligand for effective insertion into the DNA minor groove. Thus, for 1:1 conjugates with γ-aminobutyric linker between two components and a diethylamino group at the C-terminus, ΔTm is 10, 17 and 17°C for di-, tri- and tetrapyrrole ligands, respectively (duplexes 21, 19 and 2). For strands with two attached ligands, similar changes have been shown (26, 41 and 41°C, duplexes 22, 20 and 4). A similar tendency is observed for conjugates with neutral C-terminal groups (duplexes 23 and 9, 24 and 10). As it has already been demonstrated, the character of the interaction with duplexes is different for tetrapyrrole and dipyrrole fragments. Tetrapyrrole carboxamides (in both 1:1 and 2:1 conjugates) interact with the DNA minor groove [1–6,19,submitted]. Short dipyrrole ligands cannot provide strong enough interaction with a minor groove. According to NMR data,[27] the conjugated carboxamide residue can either interact with the terminal A:T pair by stacking (form 1), or insert into the minor groove (form 2). For duplex 23 (dipyrrole) the form 1 is preferable.[27] This interaction leads to its higher thermal stability with a difference of ΔTm = 6°C. A similar effect was observed when a butylamino group was present at the C-terminus of the attached ligand (duplex 25, ΔTm = 7°C). The presence of a diethylamino group at the C-terminus leads to an increase of ΔTm up to 10°C (duplex 21).

Our hypothesis is that the two forms are in a dynamic equilibrium. Preference for form 1 or 2 depends on the nature of the attached ligands and their C-terminal group.

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Since oligoimidazole derivatives cannot insert into the minor groove of A:T-containing sequences (in confirmation, no changes of optical density at 330 nm is observed during melting for duplexes 26 and 27 with one or two tetraimidazole residues conjugated to one strand), slight stabilization of the duplex can be explained only by stacking interaction. It is worth noting that almost the same $\Delta Tm = 3-4^{\circ}C$ was observed for both one and two conjugated oligoimidazoles. So this ΔTm could be considered as a difference in the thermal stability provided just by stacking.

For two tetrapyrrole residues where interaction with the minor groove is highly preferable, ΔTm does not depend on the nature of the C-terminal group (see above). However, for two dipyrrole residues, ΔTm is $13^{\circ}C$ for neutral C-terminal group (duplex 24) and $26^{\circ}C$ for positively charged C-terminal group (duplex 22), which reflects an increase of the form 2 (minor groove binding) contribution into stabilization that depends on the C-terminal group of attached MGB.

These data together with published data[27] also indicate that depending on the structure of two parallel attached ligands, different variants of interaction can be realized: stacking interaction, insertion of only one or both residues into the minor groove of the duplex and dynamic intermediate states. This hypothesis, however, needs additional studies and confirmations.

Sequence Specificity of Duplex Stabilization

When one of the conjugated ligands is tetrapyrrole and the second tetraimidazole, the difference is $\Delta Tm = 10^{\circ}C$ (duplex 28), which reflects a contribution of binding of only one tetrapyrrole residue to the minor groove. However, this effect is lower compared to that of only one tetrapyrrole strand ($\Delta Tm = 26^{\circ}C$). Comparison of duplexes 3, 26, 5, 27 and 28 indicates that the presence of a second parallel ''cognate'' (A:T-specific) MGB residue strongly stabilizes the interaction with the minor groove of the duplex, whereas a second ''noncognate'' MGB strongly destabilizes it. This destabilization provides additional sequence specificity for selective recognition of DNA by parallel double-stranded minor groove binders.

Maximal Capacity of the Minor Groove

Several conjugates were synthesized where ligands could theoretically form more than a two-stranded oligopyrrole complex. Two hairpin ligands (duplex 8) as well as one hairpin and one linear ligand (duplex 7) were attached to the same strand of the duplex A. As it is seen from the table, addition of a third and fourth strand decreased the stability of the duplex. In both cases, $\Delta Tm = 17-18^{\circ}C$ was observed compared to $\Delta Tm = 38^{\circ}C$ for hairpin octapyrrole (duplex 6) and $40^{\circ}C$ for two parallel tetrapyrroles (duplex 5). Similar results were obtained with the same model possessing neutral ester group at the C-terminus of the ligands (duplex 14, $\Delta Tm = 18^{\circ}C$). Thus, we conclude that only two parallel or antiparallel oligopyrrole strands can insert into the DNA minor groove. According to our data, formation of 3:1 and 4:1 complexes (reported in Refs. [36,37]), in the case of attached oligopyrrole ligands, has a low probability.

Other Structural Features

The protruding end of the complementary oligonucleotide strand does not hinder interaction of the attached ligands with the duplex. Stabilization is observed after

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attachment of one (duplex 33; ΔTm = 31°C) and two tetrapyrrole residues in parallel (duplex 34, ΔTm = 36°C) or antiparallel orientation (duplex 35; ΔTm = 40°C) to the oligonucleotide GAAAATTp when it forms a duplex with complementary sequence CCAATTTTC elongated by two cytosines from 5′-terminus.

Moreover, the replacement of A:T pair by G:C pair at the terminus of the duplex does not prevent insertion of the ligands into the minor groove (ΔTm = 19°C for 1:1 conjugate and 42°C for 1:2 conjugate, duplexes 30 and 31, respectively), even with a short γ-aminobutyric acid linker. This tolerance for terminal mismatch is quite surprising and could be explained by ''breathing'' of the terminal base pair and interaction with only 3 side-by-side elements.

It must be noted that in duplexes 30 and 31, MGBs are attached to the 5′-terminus of the duplex strand and not to the 3′-terminus as in the duplex A. So, no effect of DNA attachment terminus on the stability of the duplex was observed. In contrast, for one and especially for two parallel tetrapyrrole ligands, the N→C orientation of the MGB is preferable compared to C→N orientation (compare 11, 12, 17 and 34).

Interaction with RNA Targets

Octathymidylate conjugates with one or two tetrapyrrole carboxamides were tested for their binding the poly(rA) (dupl. 36–38). With only one residue, stabilization effect was quite low (ΔTm = 6°C). Addition of the second residue even destabilized modified duplex (ΔTm = 4°C). However, slight stabilization (possibly due to stacking interactions) compared to nonmodified heteroduplex is observed. This result is different from those obtained for octathymidylate/poly(dA) (dupl. 40–41, ΔTm = 43° and 49°C). It demonstrates that attachment of MGB to the DNA strand does not improve the adaptation of the ligand to the DNA–RNA heteroduplex that has different structure compared to DNA–DNA duplexes.

Close stability of the duplexes 40 and 41 even when only one tetrapyrrole is attached to oligo(dT) strand was explained by formation of 2:1 MGB:dsDNA complexes by insertion into the same minor groove of two MGB moieties attached to the neighboring octathymidylates on the poly(dA) matrix.[38]

CONCLUSION

Covalent attachment of one and two oligopyrrole ligands to one of the strands of A:T-containing duplex increases interaction of the ligands with DNA and strongly stabilizes the duplex. The mechanism of interaction and the extent of stabilization do not depend on the attachment site (3′- or 5′) of the MGB residues, but on the structural factors such as the length of oligocarboxamide part of the conjugate, the nature of the linker between oligonucleotide and ligand(s), the nature of the C-terminal group of the ligand(s), and the MGB residue orientation. The effect of structural factors is more important for monophosphoroamidate conjugates in both linear and hairpin forms. For duplexes containing a strand with two attached ligands, the effect of structural factors, especially C-terminal charge, is less pronounced. In the case of *bis*-phosphoroamidate, conjugates' presence of second ''noncognate'' MGB residue impedes interaction of the first ''cognate'' MGB strand with the DNA minor groove, which could be considered as an additional factor increasing sequence specificity of the conjugates. However, the

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interaction between two oligopyrrole ligand strands and DNA minor groove tolerates G:C mismatch at the duplex terminus.

ABBREVIATIONS

MGB	Minor groove binder
Py	N-methylpyrrole-2-carboxamide
Im	N-methylimidazole-2-carboxamide
Hp	N-methyl-3-hydroxypyrrole-carboxamide
β	β-alanine

ACKNOWLEDGMENTS

This work was supported by INSERM, European Community (grant INTAS 01-0638), French Ministry of Foreign Affairs (travel grant EGIDE 04542ND), Russian Foundation for Fundamental Researches (project 04.70043-69) and by short-term grants from Centre National de la Recherche Scientifique, France (''poste rouge'' for A.N.S.) and Muséum National d'Histoire Naturelle, Paris, France (invited researcher position for V. A. R.). Authors are grateful to C. Caux and A. Blond for NMR spectra and to Région Ile-de-France, the French Ministry of Research and Technology and CNRS for financial support in the acquisition of the NMR spectrometer.

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Received October 10, 2003
Accepted March 22, 2004

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