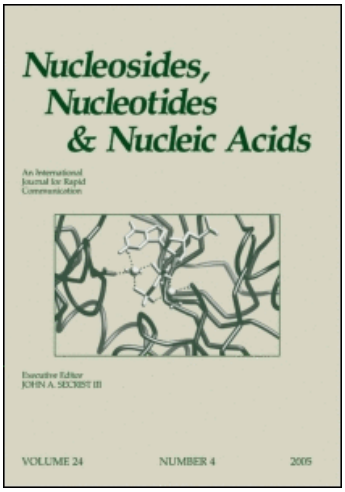

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The Synthesis of 2′-O-Methyl G-Clamp Containing Oligonucleotides and Their Inhibition of the HIV-1 Tat-TAR Interaction

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ABSTRACT

We have synthesised a 2′-O-methyl riboside phosphoramidite derivative of the cytosine analogue 9-(2-aminoethoxy)-phenoxazine ('G-clamp') and successfully incorporated it into a series of small steric blocking 2′-O-methyl oligonucleotides targeting the stem-loop region of HIV-1 TAR RNA. The 'G-clamp' containing oligonucleotides show significant increases in binding to a model TAR RNA system when the 'G-clamp' is positioned opposite the loop region. The oligonucleotides also display dose-dependent inhibition of Tat-dependent transcription of an HIV DNA template in HeLa cell nuclear cell extract.

Key Words: Steric block; Oligonucleotides; G-clamp; Nucleoside analogue.

HIV-1 transcription is regulated by the trans-activator protein, Tat, binding to its RNA recognition sequence, TAR, a stem loop structure that occurs at the 5′-end of all HIV RNA transcripts.[1] As such, the Tat-TAR interaction has become an important drug target and model system for the development of RNA-protein inhibitors.

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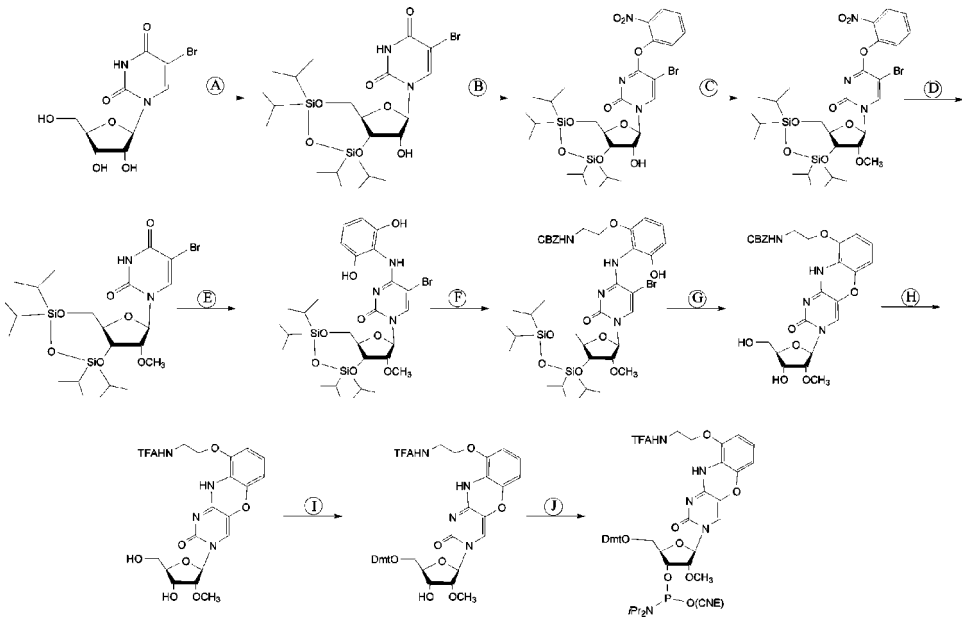

Scheme 1. Synthesis: A: Markiewicz Reagent, Pyridine (95% yield); B: i. TMSCl, Et_3N, DCM. ii. MesCl, Et_3N, DMAP. iii. σ-NO_2-phenol, DBO. iv. 2% Tosic acid, DCM (80%) C: MeI, Ag_2O, Acetone (85%) D: 4-nitrobenzaldoxime, tetramethylguanidine, Dioxane/Water (75%) E: i. CCl_4, PPh_3, DCM, ii. 2-aminoresorcinol (75%) F: Benzyl-N-(2-hydroxyethyl)carbamate, DEAD, PPh_3 (75%) G: KF, EtOH (60%) H: i. H_2, Pd/C, DMF ii. Ethyltrifluoroacetate, DMAP (65%) I. DmtCl, Pyr. (85%) J. Phosphitylating agent, DIPEA, DCM (85%).

Small molecules and peptidomimetics have been previously shown to inhibit the Tat-TAR interaction by binding to TAR-RNA in the region of a U-rich bulge.[2] Furthermore, the system has been used to investigate the ability of short oligonucleotides (and analogues) to strand invade and sterically block TAR RNA function.[3–5]

A recent development within the field of steric blocking and antisense molecules has been the synthesis of a cytosine analogue, 9-(2-aminoethoxy)-phenoxazine ('G-clamp'), capable of enhanced binding and specificity towards its complementary target.[6] A single analogue incorporated into a decamer DNA sequence was found to increase the T_m of its duplex with complementary DNA by 18°C.

We have chemically synthesised a novel 2′-O-methyl riboside phosphoramidite derivative of the 'G-clamp' (Sch. 1) and incorporated it into a series of small steric blocking 2′-O-methyl oligonucleotides targeting the stem-loop region of TAR RNA by solid phase methods previously described.[4,5]

The results from a gel mobility shift assay indicated that the 2′-O-methyl 'G-clamp' containing oligonucleotides showed significant increases in binding to a model TAR RNA system when the base analogue was positioned opposite the TAR RNA loop region. However, positioning the 'G-clamp' analogue to target guanosine residues in the duplex region of TAR RNA resulted in poorer binding than that obtained using an unmodified 2′-O-methyl oligoribonucleotide (Table 1).

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Table 1. 2′-O-methyl oligonucleotide binding to TAR RNA (Kd) and to complementary DNA and RNA (T_m).

Name	Sequence 5′-3′ 'G-clamp' analogue = **C**	Gel mobility shift binding assay Kd[a]	Gel mobility shift binding assay Kd[b]	Tm[c] DNA (°C ± 1)	Tm[c] RNA (°C ± 1)
12 TAR OMe	5′- CUC CCA GGC UCA	12 ± 1.5	3.2 ± 0.7	58	79
12 TAR P1	5′- **C**UC CCA GGC UCA	20 ± 0.8	3.3 ± 1.6	64	85
12 TAR P3	5′- CU**C** CCA GGC UCA	6.5 ± 0.6	1.5 ± 0.4	71	90
12 TAR P4	5′- CUC **C**CA GGC UCA	1.0 ± 0.1	0.8 ± 0.2	75	94
12 TAR P5	5′- CUC C**C**A GGC UCA	0.9 ± 0.4	0.2 ± 0.1	73	94
12 TAR P9	5′- CUC CCA GG**C** UCA	15 ± 2.0	2.4 ± 1.0	70	87
12 TAR P11	5′- CUC CCA GGC U**C**A	27 ± 1.1	3.1 ± 0.7	67	88

Gel Mobility Shift Assay Buffer Conditions:
[a](TK80 buffer) 50 mM Tris HCl pH 7.4, 80 mM KCl.
[b](Transcription buffer) 20 mM HEPES, 2 mM DTT, 10 μM $ZnSO_4$, 80 mM KCl, 3 mM $MgCl_2$, 10 mM creatine phosphate.
Thermal Melting experiment conditions:
[c]20 mM KCl, 5 mM Na_2HPO_4 pH 7.2, 10 mM $MgCl_2$.

The stabilities of duplexes formed by the modified oligonucleotides with their complementary DNA or RNA targets were also investigated. All duplexes containing a 2′-O-methyl 'G-clamp' analogue showed T_m increases ranging from 4–17 degrees. The magnitude of the increase is dependent on the position of the 'G-clamp' within the duplex and the most stable duplexes are those where the analogue is placed in the interior of the oligonucleotide.

The series of 2′-O-methyl 'G-clamp' containing oligonucleotides all displayed dose-dependent inhibition of Tat promoted transcription of an HIV DNA template in HeLa cell nuclear cell extract.[5] Fifty percent transcription inhibition occurred at between 50-200 nM oligonucleotide concentration with the oligonucleotide 12BRU P5 having the greatest inhibition of in vitro transcription. The investigation revealed a complex relationship between the binding strength of an oligonucleotide and its ability to inhibit in vitro transcription.

We are currently investigating the incorporation of multiple 'G-clamp' substitutions into an oligonucleotide and its effect on binding strength and inhibition of in vitro transcription. The ability of 2′-O-methyl 'G-clamp' modified oligonucleotides to inhibit Tat-dependent transcription in cells is also under investigation.

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