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The Biochemical Impact by Covalent Shielding of the Anionic Oxygen of the Phosphate Group in DNA and RNA as Methylated Phosphotriester Linkage on the Inhibition of DNA Duplication and on HIV-1 RNA Viral Infectivity Has Been Seriously Overlooked

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Abstract

With the help of model experiments, we are able to offer a detailed proposal for the inhibition of DNA duplication and no inhibition of RNA viral infectivity. As a backbone, we introduced methyl phosphotriester (MPTE). Duplex formation according to the traditional Watson and Crick base-pairing: $[(MPTE)_{n-1}$ DNA] * DNA and $[(MPTE)_{n-1}$ DNA] * RNA, where n = number of DNA and RNA bases. However, in the latter case, inhibition is obtained by reduction of the number of MPTE linkages, as is confirmed with model experiments *and* under biological conditions with micro (mi)RNA substrates. The latter results have recently been published. One or more single MPTEs are disseminated over different places of DNA without neighbour MPTEs (Prof. Wen-Yih Chen and his group, Taiwan).

Keywords

Methylated Phosphotriester (MPTE) DNA, Partially MPTE DNA, Model Inhibition Experiments, Micro (mi)RNA, HIV-1 RNA, Conformational Transition

1. Introduction

The most exciting conformational transition is found in the observation of a right-handed into a left-handed helix indicated as B-Z DNA isomerization for

repeating CpG sequences under high-salt conditions. We found that under dimer conditions, methylation of the anionic oxygen as OCH_3 group of the phosphate linkage also results in the B-Z isomerization under the absence of salts [1]. This has been established with CD, NMR and melting-point transitions. Recently, it was observed that DNA binding force facilitates Z-DNA formation under physiological salt conditions [2]. Generally, the impact of the presence of methyl phosphotriester (MPTE) linkages of the DNA backbone results in higher nuclease stability and the benefit of lipophilic character as has been demonstrated under biological conditions [3]. The MPTE linkages also improve the duplex stability due to the reduction of the electrostatic repulsion between the strands by eliminating the negative charge in one strand [4].

Interestingly, the investigation of phosphate methylation has also resulted in the formation of parallel DNA duplexes exclusively for the pyrimidine bases C and T under the control of the chirality of phosphorus. The combination T-T occurs for both configurations, whereas for C-C the *S* configuration is necessary. These results could be confirmed with X-ray for T-T and AMBER molecular mechanical calculations for both duplexes demonstrating the role of *accommodation* of the methyl group into the helix groove [5] [6]. Similar results were obtained for the corresponding MPTE RNA dimers with the exception that $r(C_pG)$ is *right-handed* [7] [8]. From model consideration and NMR measurements, it can be concluded that the puckering in the ribose ring results in C(3)-*endo* (A-form) and for the corresponding DNA dimers C(2)-*endo* (B-form). Change in adaptation is hindered by unfavourable steric interactions through the introduction of the methyl group. The (natural) RNA molecule is conformationally rigid and always assumes an A-geometry, whereas the MPTE DNA lacks the flexibility to adopt an A-geometry.

Under peptide-induced conditions, similar observations for the parallel pyrimidine-pyrimidine bases were obtained [9]. In these systems, the role of the protein has a stereo-selective character that reflects the combination of the phosphate shielding with a stereo-chemical fit within the duplex. This process of bioconjugation may be considered as an accommodation of autonomic properties resulting in a parallel duplex. For the natural DNA oligomers dC_{10}, $d(C_6T_6)$, and $d(T_6C_2T_2)$, only the cationic oligopeptide octadeca(L-lysine)(Lys_{18}) is able to induce the formation of parallel duplexes with C-C and T-T base pairs. With octadeca(L-ornithine)(Orn_{18}), unfavourable steric interactions are present with C-C base pairs as has been concluded from model studies. There is an analogy with the corresponding MPTE DNAs controlled by the *S* and *R* configurations of phosphorus. Thus, when cytosine bases are present, parallel duplex formation in both protein-complexated and MPTE oligomers is possible when the shielding group links to the sterically most available oxygen atom in the phosphate group corresponding with the *S* configuration.

Between 1985 and 1990, my group at Eindhoven University of Technology was involved in the HIV-1 RNA research of the preparation of MPTE antisense DNA

with 20 nucleotides for inhibition of target regions of the virus with hairpin loop (single-stranded) structures. A highly favoured structure is TAR (trans-activation response element) with six bases in the loop which was established by X-ray [3]. Unfortunately, the general opinion at that time was that specific inhibition should be more effective with an *increase* of MPTE linkages, precisely in contradiction with the model experiments and results as described in the next section.

2. Results and Discussion

2.1. Model Experiments

The most far-reaching overview of my group was drawn from an article in *Bioconjugate Chemistry* of May/June 1990, Vol. 1, Nr. 3 by John Goodchild. In comparison with *methyl phosphonate* DNA systems, the P-C bond disturbs the helix conformation for stereo-electronic reasons, resulting in a weaker hybridization with DNA and RNA for longer strands. However, MPTE DNA is a superior antisense agent with a high melting temperature that is not influenced by the chirality of phosphorus. An illustration of this hybridization is given in **Figure 1**. However, the MPTE DNA hybridizes poorly with RNA, the usual target for antisense inhibition.

The results for hybridization with the melting point transitions (T_m) for short and complete MPTE DNAs complementary with natural DNA and RNA [10] [11] are given in **Table 1**.

From the melting point transitions, it is clear that *increase* of MPTE linkages

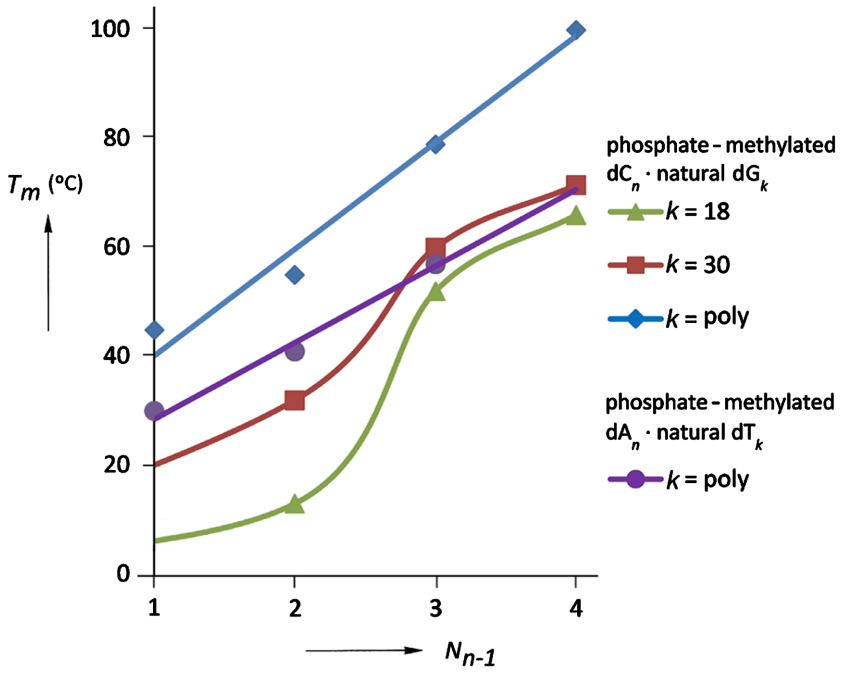

Figure 1. Melting temperature (T_m) of phosphate-methylated dC_n with MPTE (N_{n-1}) values for n = 2 - 5 bases hybridized with its natural complement dG_k for different k values (the numbers are shown in the column) and of phosphate-methylated dA_n for n = 2 - 5 bases with poly (dT). Both T_m values for n = 5 with k = poly are obtained by extrapolation.

Table 1. T_m values (˚C) for hybrids between MPTE (P) oligonucleotides and natural DNA and RNA.

Duplex	T_m	Duplex	T_m
d(ApA).poly(dT)	30	d(ApA).poly(U)	13
d(ApApA).poly(dT)	41	d(ApApA).poly(U)	<10
d([Ap]$_3$A).poly(dT)	57	-	-
d(CpC).poly(dG)	45	d(CpC).poly(rG)	28
d(CpCpC).poly(dG)	55	d(CpCpC).poly(rG)	12

of DNA favours the inhibition with natural DNA and disfavours the inhibition with natural RNA. These measurements at least suggest that complete phosphate methylation of DNA with natural RNA does not result in inhibition of natural RNA for selected regions whereas *decrease* of MPTE DNA favours RNA inhibition.

In biological experiments, a corresponding result was obtained. For rat vibroblast cells, it was found that 10 micro Mol complete MPTE timer d(A_3) exclusively reduces DNA synthesis (monitored by (^{3}H)-thymidine uptake) and that the protein synthesis (monitored by (^{35}S)-methionine uptake) was found to be essentially unaffected. The experiments were carried out by prof Van der Eb and dr Bos (University of Leiden, Medical Biochemistry Sylvius Laboratories) [12].

In fact, this result should have contributed to our fundamental knowledge as start for the virological experiments with prof Goudsmit (University of Amsterdam, AMC) as demonstrated in the next section.

2.2. HIV-1 Experiments

The HIV-1 constructs under investigation were selected TAR (trans-activation response element), PBS (primer binding site), NEF (negative regulatory factor), and VIF (viral infectivity factor) targets with specific loop structures for inhibition with MPTE DNAs consisting of 20 nucleotides. The *initial* experiments showed *no* viral inhibition (unpublished results). This result corresponded with our model experiments. Unfortunately, in *the Science paper*, *only little attention was given to the model experiments* [13].

Finally, it was suggested that the degree of demethylation varied greatly under the various factors mainly based on the deprotection of the bases in the synthesis accompanied with demethylation of the MPTE linkages, specifically for the long oligonucleotides. *This results in a reduction of the number of MPTE linkages and thus favouring HIV-1 inhibition*.

Recently, experimental results corresponding with the model experiments (**Table 1**) have been published by Po-Hsiang Wang *et al.* in their study on the inhibition of mi-RNA with (strongly) reduced MPTE DNA linkages [14]. In the next section, we focus the attention on the degree of MPTE in the DNA backbone for their inhibition with RNA.

2.3. Degree of Methylation

The impact of methylation as MPTE DNA on its role as inhibitor of mi-RNA substrates has been well described by Po-Hsiang Wang *et al.* [14]. As target for inhibition, miR-21 was used because of their relatively short 19 - 24 nucleotides and it has been proposed as a critical diagnostic biomarker for various cancers and is present in particular human colorectal cancer.

For their research, they used partially MPTE antisense DNA with *four* MPTE linkages in comparison with the effectivity of natural DNA, LNA (locked nucleic acid) with A-form, and more MPTEs until complete MPT linkages. In practice, LNA may be considered as the *golden standard* for the inhibition experiments [15]. Summarizing, the experimental inhibition experiments of Po-Hsiang Wang *et al.* [14] are in agreement with our model experiments in **Table 1**, *i.e.* full MPTE DNA cannot hybridize with miR-21 in contrast with partially MPTE DNA. The MPTE positions for hybridization with RNA may not be arranged consecutively as follows in **Table 1**.

3. Conclusions

Methylated phosphotriester linkages in DNA play a selective role in the recognition of DNA and (viral) RNA for the formation of duplex structures following the Watson and Crick base-pairing. The model experiments under non-biological conditions give a good insight into the degree of methylation and their different locations on the choice of modified DNA-DNA or modified DNA-RNA duplex formation. For the latter duplex, we are dealing with partially methyl phosphotriester DNAs. An extensive study has been published by Po-Hsiang Wang *et al.* [14]. Different methyl locations have been shown. Fully methylated DNA has a preference for DNA and not for RNA, *vide supra*. An interesting aspect is the allosteric-like effect demonstrated with a sharp sigmoidal transition in **Figure 1**. This means that MPTE DNA binds cooperatively to its natural complementary DNA which involves that the natural strand adapts the modified conformation during hybridization. This effect is absent where sugar puckering differs substantially from the complementary natural one as in the case of MPTE DNA, in which the MPPTE groups are not consecutively arranged, with natural RNA. The picture that partially MPTE DNA may result in hybridization with RNA might be the result of the benefits of the localized absence of the Coulomb repulsion and the advances of higher stability under biochemical conditions. If there is a preference for specific locations of the MPTEs, molecular mechanics calculations may give a qualitative answer.

Finally, we focus the attention on a study of the synthesis of neutral phosphate triester backbone modifications as short-interfering RNA (si-RNA) which displays gene-silencing activity [15]. Although we are dealing with hydrophobic tails improving the hybridization affinity and stability, their geometric composition differs greatly from the natural si-RNA.

Other examples of MPTEs ligands are given in **Figure 2**. In contrast with the

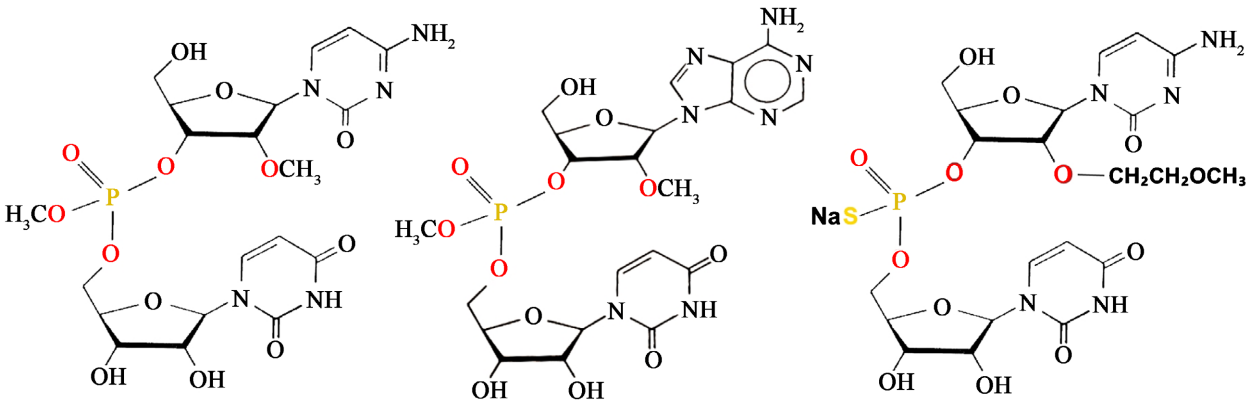

Figure 2. Modified phosphate-methylated RNA dimers (left, center with separate S_P and R_P configurations of phosphorus) and the modified phosphorothioate RNA dimer (right) [3].

models given in the article, we are dealing with RNA-RNA hybrids instead of DNA-RNA inhibition.

This model in a *simplified* version has been used in the fight against progressive neurological diseases [3]. In our opinion, the MPTE DNAs with variety in the number of MPTE are strongly favoured over the different models as a publisher over a long period.

Summarizing, the results of 2.1. Model Experiments, 2. Results and Discussion in combination with the 2.2. HIV-1 Experiments as described in this article, are not correctly presented in the original 1990—*Science* article [13]. Our Model Experiments published *before* the *Science* article match with the vital results of the recent paper by the group of Wen-Yih Chen [14].

4. Synthesis

4.1. Synthesis of MPTE DNAs

The synthesis of MPTE DNAs with 2 - 5 bases consisting of A, T, G, and C is based on a stepwise route following the standard phosphoramidite chemistry of Beacage *et al.* [16]. As a protective agent for the bases A, G, and C, we used the 9-fluorenylmethoxycarbonyl (Fmoc) group [17]. After the isolation of the phosphites, the base-protected triester was obtained through oxidation with *t*-butyl hydroperoxide. For the removal of Fmoc, triethylamine was used. For longer fragments, we used the natural one as starting point. After base protection, the phosphodiester backbone was methylated as described by Miller *et al.* [18] followed by base deprotection. In the context of a recombinant DNA study, we used the *E. coli pab B* gene, coding for para aminobenzoate synthetase, inserted in the *M13mp18* phage selecting 2 templates for relative synthesis activity of complementary phosphatemethylated 8- and 18-mers along the template position [3].

4.2. Synthesis of MPTE RNAs

A number of MPTE RNAs had been prepared on the dinucleotide level in which

the 2'-OH group was substituted by OCH_3. This is necessary in order to prevent an intramolecular interaction with the phosphorus in the MPTE linkage via a trigonal bipyramidal state resulting in chain scission.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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