Retracted HIV Study Provides New Information about the Status of the *in Vitro* Inhibition of DNA Replication by Backbone Methylation

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Abstract

In this publication attention is given to a retracted article in *Science* at the end of 1990 concerning the HIV-1 inhibition by a modified backbone DNA as the phosphatemethylated DNA. A disproportion in the presented data resulted in a faulty generalization of the (bio)chemical characteristics of the phosphatemethylated DNA (18- and 20-nucleotides). In the confusion and the outside pressure a related study in *Nucleic Acids Research* on the *in vitro* dynamics of a regiospecific inhibition of DNA duplication with long (20- and 18-nucleotides) and short (8-nucleotides) phosphatemethylated DNA was completely ignored. A restoration will be given based on a comprehensive view demonstrating the unique molecular and conformational properties of phosphatemethylated DNA in their (bio)chemistry towards natural DNA and RNA (HIV-1 RNA loops).

Keywords

Phosphatemethylated DNA, Conformational Study, HIV-1, *E. coli pab B* Gene, *in Vitro* Inhibition of DNA Replication

1. Introduction

After the publication in *Science* entitled *Inhibition of HIV-1 Infectivity by Phosphate-Methylated DNA*: *Retraction* of Moody *et al.* [1] a number of articles have been published in order to reduce the imbalance between the various passages in the Retraction (presented as a Technical Comment) and the "meltdown" of the bio- and physical chemical properties of phosphatemethylated DNA [2]-[6]. The Retraction mainly focused on the small

degree of methylation of the 20-nucleotide DNA's. These results conflict with their achievement in the *in vitro* regiospecific inhibition of DNA duplication with long and short fragments, prepared in a similar manner as in the HIV-study [7]. The suppression of the DNA polymerase I (Klenow fragment) in the inhibition experiments with the *neutrally* backbone-modified DNA's as inhibitors, has been explicitly demonstrated and may be considered as an additional support for the synthesis of the methylated DNA backbones [7].

2. Methods and Materials

After the HIV-publication in *Science* [8], HPLC analysis was carried out to determine the degree of backbone-methylation. The procedure for relatively long fragments was developed by Koole *et al.* [7] [9] and based on the following reaction sequence:

natural DNA → base-protection → phosphate-methylation → base-deprotection
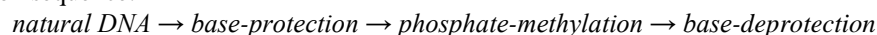
The base-protection was carried out with the 9-fluorenylmethoxycarbonyl (*Fmoc*) group, the methylation with *p*-toluenesulphonyl chloride and methanol, and the base-deprotection with triethylamine. In the Retraction it was concluded that the protection of the bases resulted in an (almost) insoluble mixture leading to low yields in the following steps [1]. As a consequence of the individual organization around the Retraction, the attention was focused on the apparent failure of the phosphate methylation of the natural DNA.

In the proofs, the *selectivity* of the HIV-1 viral inhibition was maintained [10] but removed in the final text of the Retraction: "There is no evidence to suggest that the observed antiviral effects should be ascribed to the phosphate-methylation of natural DNA".

The correctness of the synthetic part of the Technical Comment obtains a doubtful or negative assessment with the quote: "This result contrasts with *earlier* statements that the degree of phosphate-methylation of tested DNA was 90% to 100%" (see Regiospecific inhibition of DNA duplication by antisense phosphate-methylated oligo-nucleotides of Moody *et al.* [7]). These experiments were carried out with long and short modified DNA's (18- and 8-mers), prepared according to Koole's procedure.

Besides, the specific conformational aspects of phosphatemethylated DNA were left behind in the Retraction. It should be assigned that we are dealing with a high selectivity and enhanced duplex formation of phosphate-methylated DNA (a *fixed* B-conformation) for its complementary natural DNA (A- and B-conformation) which is in sharp contrast with the poor hybridization affinity at the RNA level (A-conformation). The established selectivity and pronounced duplex formation for natural DNA in contrast to RNA has been concluded from model studies for short phosphatemethylated DNA fragments with 2 - 5 nucleotides, *vide infra* [2]-[6].

Actually, the absence of a regioselective inhibition of four selected HIV-1 target regions TAR, PBS, NEF, and VIF in the *Science*-study, as confirmed in a subsequent report [10], is in excellent correspondence with these model studies. Additionally, negative results, *i.e.* the *absence of inhibition* were omitted in the Retraction.

In a private communication, Iyer confirms the *absence* of anti-HIV-1 activity with phosphatemethylated DNA's [5] [11]-[14].

2.1. Inhibition of DNA Duplexation by Phosphatemethylated Oligodeoxynucleotides

From the biological model proofs, *i.e.* the regiospecific inhibition of DNA duplication and the 1H NMR data, there is a distinct indication that the phosphate-backbone in the natural DNA was methylated [7].

The *E. coli pab B* gene, coding for *para*-aminobenzoate synthetase, inserted in the M13mp18 phage was used as template for a phosphatemethylated 18- and 8-mer (concentration 3 μM), complementary to a pair of selected template regions [15]. The relative synthesis activity along the template strand shows a sharp decrease precisely at the selected region(s) downstream of natural d(AGTAATCACAGCGGGAGA), complementary to the 14 - 31 region: -TCTCCCGCTGTGATTACT-, used as the primer for the sequencing reaction. The corresponding phosphatemethylated DNA was unable to act as a primer for Klenow DNA polymerase I. Thus, the Klenow fragment fails to recognize the modified DNA as primer. An arbitrary selected phosphatemethylated d(CTGCTAGAG-ATTTTCCACAC) has, as to be expected, no impact on the progress of the synthesis. The activity as measured for the 18- and 8-mer at room temperature and 0°C respectively, is given in **Figure 1**. The 18-mer d(CACTC-ACCCATGAACAGC) is complementary to the nucleotide sequence for the −110 - −93 region: -GCTGTTCATGGGTGAGTG- and the 8-mer d(AGCCTGAC) is complementary to the 1455 - 1462 region: -GTCAGGCT-. In order to quantify the effect of the inhibition, the density of each band of the sequencing
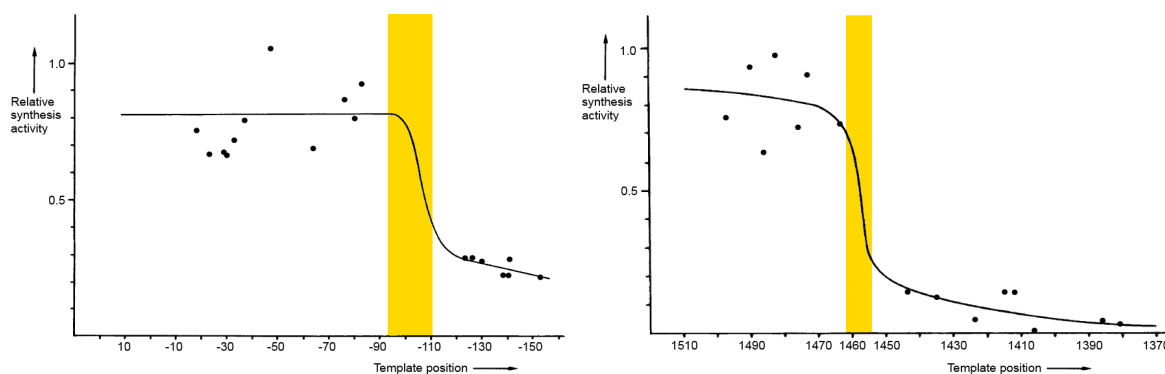
Figure 1. Relative synthesis activity along the template strand. The positions of the phosphatemethylated 18-mer d(CACT-CACCCATGAACAGC) complementary to the −110 - −93 region at room temperature (left) and the 8-mer d(AGCCTGAC) complementary to the 1455 - 1462 region at 0˚C (right) are indicated with the yellow segment. The method for the determination of the relative synthesis activity along the template strand is given in the text.

pattern was measured optically. Eliminating the normally occurring density variation due to sequence-dependent strong and weak dideoxy stops, the ratio of the absorbance of the sequencing bands of this pattern with inhibitor and the corresponding bands of the reference pattern was used as a measure for the relative synthesis activity along the template strand. The stop of any synthesis activity after the inhibition region is the result of the absence of a natural primer. For the progress of the duplication after the inhibited regions, the phosphatemethylated DNA should be coupled with a natural fragment complementary with the nucleotide sequence of the template. This aspect that obtained not much attention in the anti-sense dynamics, may have its repercussions for other neutral backbone-modified DNA's as, e.g., methylphosphonates.

In that respect it is of interest to take notice of recent investigations of Song *et al.*, based on structural/sequence motifs in interruption processes of duplication [16], and former conformational studies concerning the allosteric behaviour induced by phosphatemethylated DNA on its natural complementary strand [2]-[6].

These results based on a site-specific inhibition of the DNA replication process were omitted in the Retraction. Generally, the melting temperatures of the modified 6-, 8-, 12-, and 18-mers showed sharp transitions. Obviously, the 8-mer was too short to prevent the action of Klenow polymerase I at room temperature.

2.2. 1H NMR Spectroscopy of Phosphatemethylated 18-Mer d(CACTCACCCATGAACAGC) Used in the Inhibition of DNA Duplexation

The methylation of the phosphate backbone was confirmed with 1H NMR. The assignment was based on the position of the exclusivity of the POCH_3 chemical shift value in combination with an estimate of the percentage of methylation. The 1H NMR spectrum of the 18-mer, used as the complementary site of the −110 - −93 part of the *E. coli pab B* gene, is given in **Figure 2**. The 31P NMR spectra for the phosphatemethylated DNA and the corresponding natural DNA are given in the text.

The 1H NMR 200 MHz subspectrum, the crucial part of the spectrum for the assignment of the backbone-methylation, is composed of a diastereomeric mixture. Although the POCH_3 signals are complex too, they are distinct from the other proton resonances. This is clearly demonstrated for a number of phosphatemethylated dimers as shown in **Figure 3** with the 1H NMR 600 MHz spectrum of the S_P phosphatemethylated dimer d(ApC) [9]. Initially, the dimers were obtained as a mixture of R_P and S_P isomers. For their separation reversed-phase HPLC was used. A number of these dimers were used in self-complementary studies. The most interesting ones are the self-complementary phosphatemethylated Z-d(CpG) and B-d(GpC) for both diastereoisomers [2]-[5]. The left- and right handed miniduplexes, respectively, were clearly demonstrated with circular dichroism and NMR spectroscopy. These studies were helpful for our molecular understanding of one of the fundamental aspects in the dynamics of gene expression caused by epigenetics. In these studies the methyl group plays a dominant role [5] [6].

The POCH_3 doublet is explicitly indicated. These compounds were prepared via the step-wise phosphoramidite procedure with *Fmoc*-protected nucleotides with 2 - 5 nucleotides, *vide supra*. Based on a comparison of the
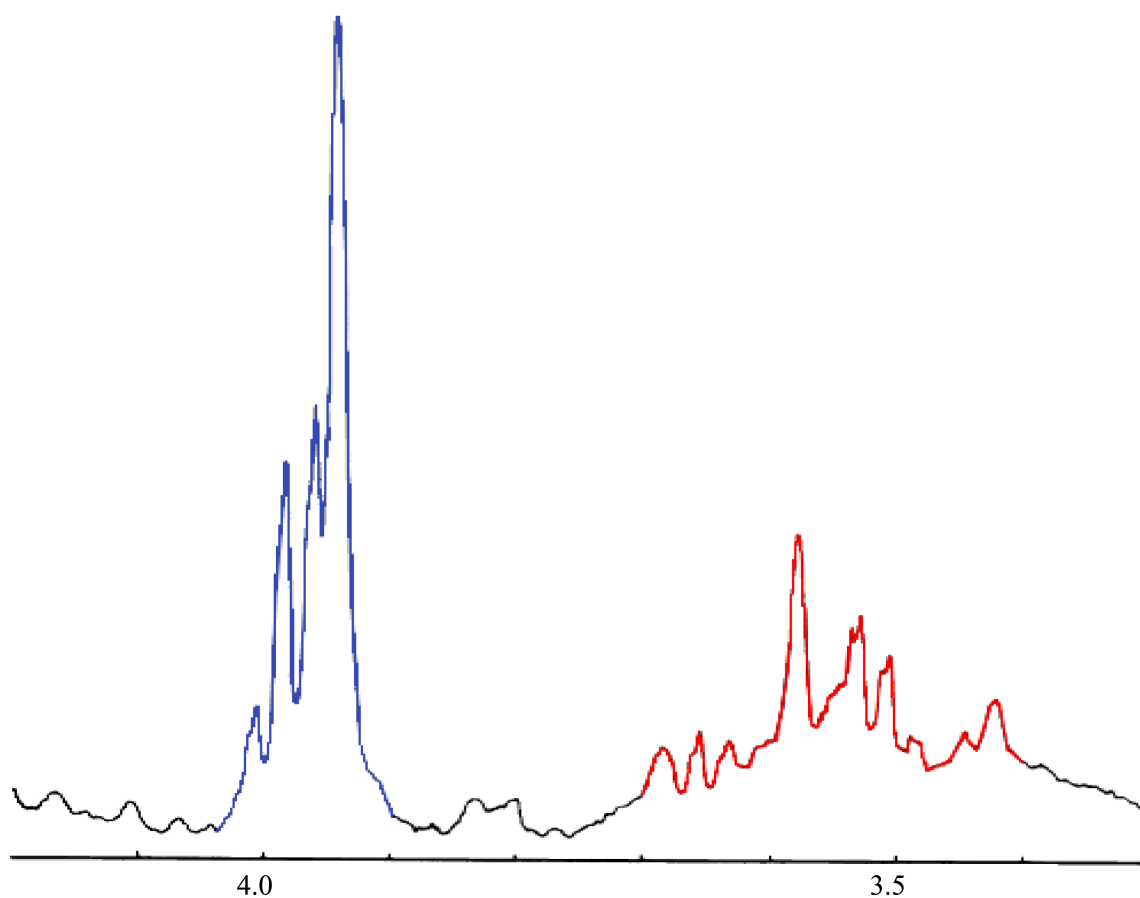
Figure 2. 1H NMR subspectrum of phosphatemethylated d(CACTCACCCATGAACAGC) for the POCH_3- and 5'-end $H_{5'}/H_{5''}$ resonances (chemical shifts in ppm) in the 3.7 - 3.4 region. These regions are indicated with blue and red, respectively.
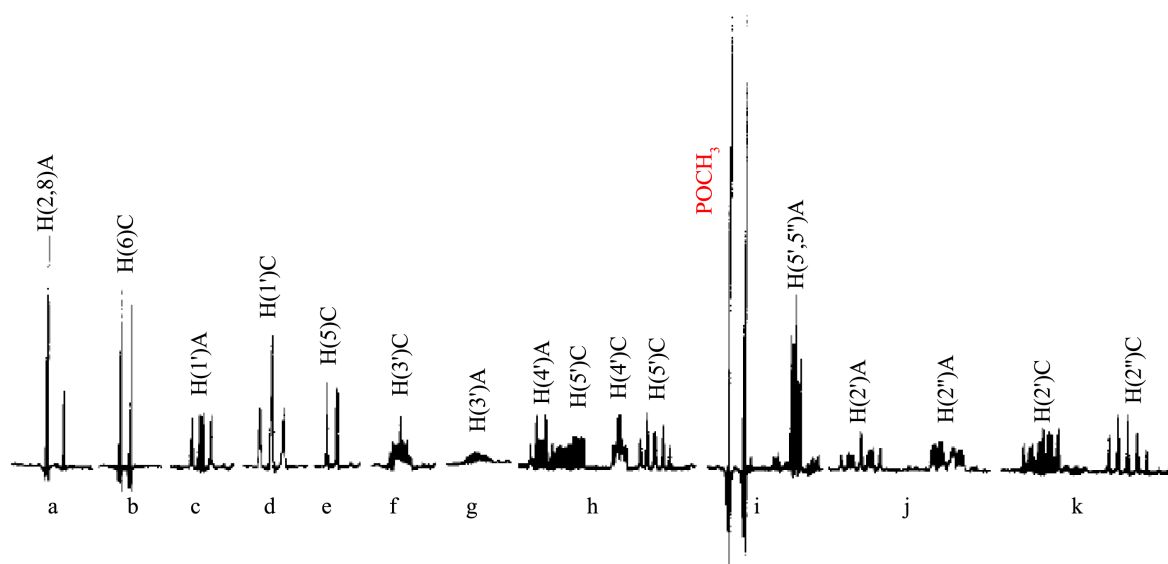

Figure 3. 600 MHz 1H NMR spectrum of the S_P isomer of phosphatemethylated d(A_PC). Regions (chemical shift in ppm): (a) 8.17 - 8.07; (b) 7.57 - 7.49; (c) 6.38 - 6.30; (d) 6.15 - 6.09; (e) 5.76 - 5.70; (f) 5.30 - 5.24; (g) 5.17 - 5.09; (h) 4.46 - 4.24; (i) 3.84 - 3.70; (j) 2.90 - 2.64; (k) 2.38 - 2.16.

surface area of the POCH_3 and 5'- end H5'/H5" resonances in the 3.7 - 3.4 ppm region with other proton resonances e.g., the combined H1' and cytosine H5 signals, an overall methylation of the backbone of at least 90% was abstracted. For the deprotection of the *Fmoc*-protected amino bases three hours were included. Model experiments demonstrated approximately 5% demethylation [17]. The 31P NMR data of the phosphatemethylated DNA 18-mer at 2.0 ppm showed one single broad resonance spectrum, due to the complexity of the diastereomeric mixture. In the corresponding natural DNA there is no significant chemical shift compared with the methylated one. In the absence of chiral phosphates separate peaks are identifiable.

3. General Remarks Concerning the Duplication Study

The general procedure concerning the DNA phosphate-backbone methylation, *vide supra*, was started via a DNA synthesizer for the preparation of natural DNA's with the following sequences d(CGAATC), d(AGCCTGAC), d(GGAATCCTGCAG), and d(CACTCACCCATGAACAGC). The latter one was used to describe the follow-up of the synthesis. The temporary protection of the bases with the *Fmoc* group and the methylation of the phosphate groups were accomplished in solution etc. This is in disagreement with the text of the Retraction: "It was concluded that the protection of the bases resulted in an (almost) insoluble mixture leading to low yields in the following steps".

The melting temperatures of the modified 6-, 8-, 12-, and 18-mers showed sharp transitions varying from 27°C to 75°C. For the complete preparation and purification see Refs. [7] and [9].

After the publication of the Retraction, an explanation for the inhibition of the DNA replication of selected regions at the *E. coli pab B* gene was suggested by the presence of triethyl ammonium- and possibly triethylmethyl ammonium cations (methyl transfer via the methylating agent) as by-products of the synthesis, *vide supra* [3]. The latter duplex is faclitated by electrostatic interaction between the positively charged triethylmethyl ammonium groups and the negatively charged phosphate groups in combination with the amphipathic character of the former group [3] [5] [19]. Through hydrogen bridging and (partial) methyl shielding of the phosphates, the duplex formation should be increased. For the success of the *in vitro* experiments covalent shielding, *i.e.*, formation of a POCH_3 bonding, is a prerequisite. The various DNA models are given in **Figure 4**.

4. Conclusion

Taking into account the contradictions in the experimental data and their corresponding interpretations, a disqualification of the scientific research with phosphatemethylated DNA and its corresponding abrupt ending [1] [8] [10] [18] cannot be regarded as credible or trustworthy.
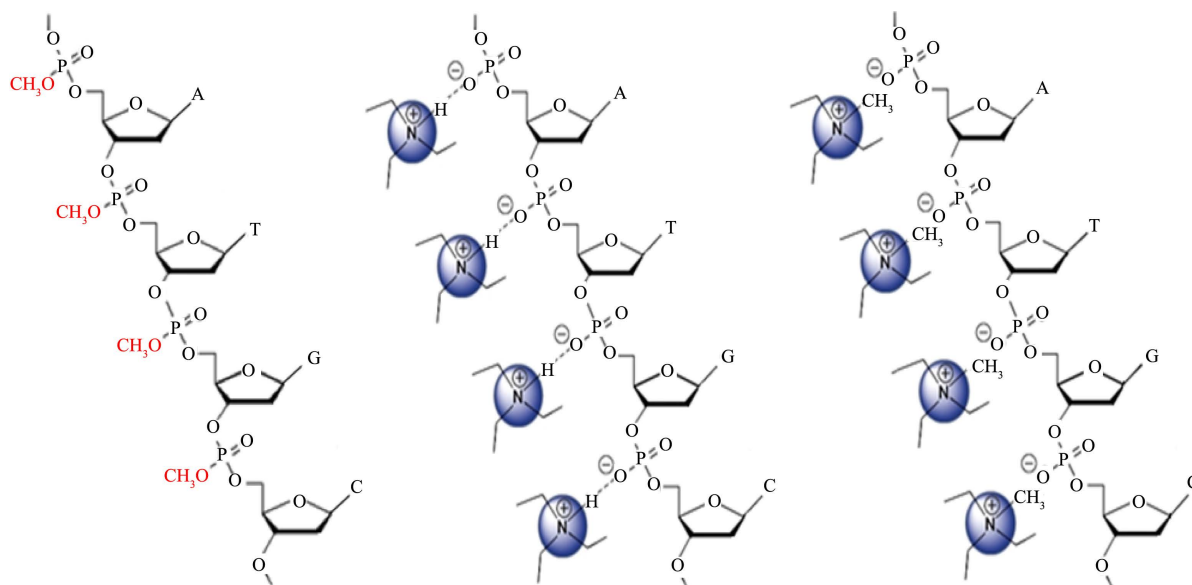

Figure 4. Backbone-modified DNA models. Covalent shielding with PO-CH_3 as methylphosphotriester (left), hydrogen-bridging with triethyl ammonium cation (center) and cationic shielding with triethylmethyl ammonium ion (right).

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