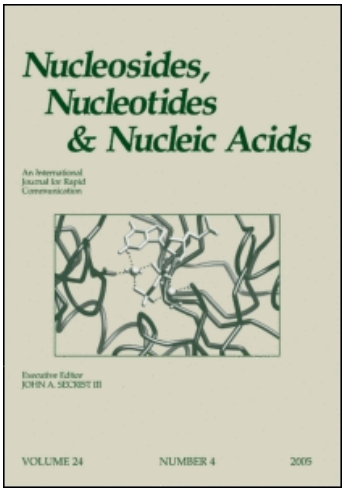

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Studies on the Interaction between HIV-1 Reverse Transcriptase and Templateoligo α-Thymidylate Duplexes

Georges Maury[a]; Bernard Rayner[a]; Jean-Louis Imbach[a]; Barbara Müller[b]; Tobias Restle[b]; Roger S. Goody[b]
[a] Université de Montpellier II, Labomtoire de Chimie Bioorganique, Montpellier Cedex 5, France [b] Abteilung Biophysik, Max-Planck-lnstitut für medizinische Forschung, Heidelberg, F.R.G.

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STUDIES ON THE INTERACTION BETWEEN HIV-1 REVERSE TRANSCRIPTASE AND TEMPLATE:OLIGO α-THYMIDYLATE DUPLEXES

Georges Maury[1], Bernard Rayner[1], Jean-Louis Imbach[1], Barbara Müller[2], Tobias Restle[2] and Roger S. Goody[2]

[1]Université de Montpellier II, Laboratoire de Chimie Bioorganique, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France, and [2]Abteilung Biophysik, Max-Planck-Institut für medizinische Forschung, Jahnstraße 29, 6900 Heidelberg, F.R.G.

Abstract: Two methods have been used to determine the affinity to HIV-1 reverse transcriptase of duplexes between α-dT_n and an RNA template. In one of these, competition between the duplexes and a fluorescently labeled template:primer complex was used to provide equilibrium affinity data. In the second method, the duplexes were used as competitors for polyA:oligo dT in a standard reverse transcriptase assay. Both approaches indicated that substitution of β-nucleoside units by their α-analogs led to a 2-10 fold lowering of the affinity to HIV-1 reverse transcriptase.

It is now firmly established that α-anomeric oligonucleotides display remarkable differences in properties when compared to the corresponding β-isomers. The most noteworthy are resistance to nuclease catalyzed hydrolysis (1,2) and the relative stability of the (parallel) hybrids with single stranded DNA or RNA (3,4). This has led to the use of α-oligomers to inhibit DNA polymerase activity, including the reverse transcriptases of Moloney leukemia virus (5) and of human immunodeficieny virus (HIV-RT; ref. 6). However, the results of these two studies do not provide a precise and direct comparison of the affinities of the template:β-oligomer and the corresponding template:α-oligomer duplexes to reverse transcriptase. The aim of our study was to obtain these comparative data for HIV-RT using highly homogeneous samples of heterodimeric recombinant enzyme (7) in an efficient method based on the use of fluorescent chain terminators (8).

It was shown earlier that the fluorescent nucleotide analog succinylfluorescein dideoxythymidine triphosphate (9) is a substrate for HIV-RT and can be added to the 3'-end of, for example, the 36/18 DNA:DNA template:primer complex shown in I (8). The resulting 36/19 complex shows a large fluorescence change on binding to reverse transcriptase. This allows the affinity of the fluorescent complex to HIV-RT to be determined, after which competitive titrations with non-fluorescent complexes can be analyzed to obtain their affinities.

5'TCCCTGTTCGGGCGCCAC 3'
3'AGGGACAAGCCCGCGGTGACGATCTCTAAAAGGTGT 5'

I

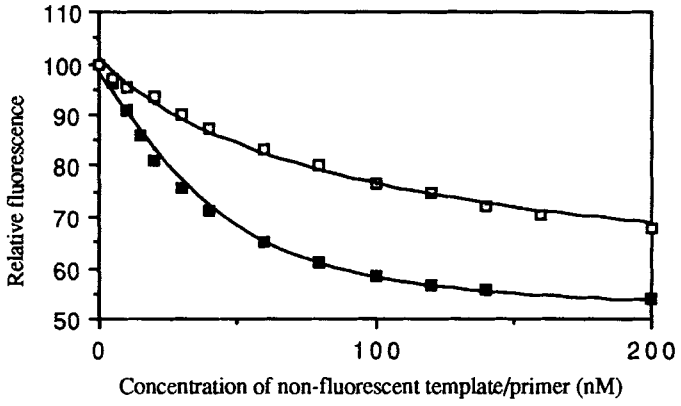

Figure 1 Displacement of the fluorescent 36/19 hybrid from its 1:1 complex with HIV 1 RT (filled squares 60 nM, empty squares 90 nM) by poly A:β-dT_{12} (filled squares) or poly A:α-dT_{12} (empty squares) hybrids. Each addition of non-fluorescent hybrid was followed by an equilibration period of 5 min. before the fluorescence intensity was measured. The curves are the computer fits to the data using the quadratic equation describing the displacement process assuming that complex formation was initially 100%.

The complex between HIV-RT and the fluorescent 36/19 duplex was found to have a dissociation constant (in the absence of nucleoside triphosphates) of ca. 3 nM at 25°C. Competitive titrations with poly A:β-dT_{12} and poly A:α-dT_{12} are shown in Fig. 1. From the results of several such experiments under different concentration conditions, the dissociation constant for the β-dT containing hybrid was estimated to be in the range of 0.4-0.7 nM, that of the corresponding α-dT containing complex 3-8 nM. Similar titrations using non hybridized (i.e. single stranded) template or primer led to a negligible reduction of the fluorescence in the concentration range of Fig.1, indicating that binding to single stranded RNA or DNA is much weaker than to double stranded, or in particular to such complexes with overhanging 5'-ends.

It was of interest to compare the results obtained using the fluorescence method with the behaviour of the α-oligomers in the standard reverse transcriptase assay. Thus, the kinetics of RT inhibition by poly A:α-dT_{12} (base ratio 5:1) were studied using the corresponding poly A:β-dT_{12} as template:primer ("substrate" in the steady state kinetic sense). In each series of experiments, the concentration of the latter and of the triphosphate substrate ([methyl-^{3}H]dTTP) were held constant (0.1-2 μM and 16 μM, respectively), and the concentration of the competing α-hybrid was varied in the range of 0.025-10 μM. Initial reaction rates were determined at 37 °C, pH 8.0 and 0.2 μg RT/ml. Incorporation of acid precipitable (5% trichloroacetic acid) polymers was determined after 20 min. From each set of data, an apparent dissociation constant, K_d, for the α-oligomer:template complex was calculated. Assuming competitive inhibition, this constant is described by the equation

$$K_d = K_\alpha(1 + [\beta]/K_\beta)$$

where K_α and K_β are the dissociation constants (in the case of the β-oligomer complex this is not the actual dissociation constant but a K_m value for this substrate). The relationship between the K_d and [β] was indeed linear over the range of concentrations used (0.1-2 μM), confirming that there is competitive inhibition between the two species, and the value of K_α/K_β was found to be 2.3.

A different pattern of inhibition was observed, as expected, using non-hybridized oligo α–dT_{12}. A reciprocal plot of the rate of polymerisation against increasing concentration of α-oligomer is linear in the range up to about twice the concentration of the β-primer, after which there is an abrupt decrease in rate. This results from filling up of the available free stretches on the template, and thus does not represent an inhibition of the actual enzyme activity, but a depletion of available template sites.

A third type of inhibition which can occur with the α-oligomers is that responsible for the results described by Pauwels et al. (6). In this type of experiment, template is hybridized simultaneously with a mixture of α- and β-oligomers. The results obtained thus reflect the relative affinities of α- and β-oligomers for template, but do not provide information on the interaction of the α-oligomers with RT, except that the fully competitive nature of the inhibition seen confirms that the α-oligomers cannot prime DNA synthesis.

In conclusion, there appear to be at least three possible modes of inhibition of reverse transcriptase activity by α-oligonucleotides. Firstly, as shown previously, they can compete with template binding of natural primer molecules with the β-configuration. Secondly, they can occupy stretches of the template downstream from the primer and thus inhibit polymerisation. Thirdly, as shown in this contribution, hybrids between α-oligomers and RNA have a high affinity for reverse transcriptase, and this can lead to inhibition of reverse transcriptase activity (and presumably in other systems of other DNA-polymerase activity) by competing for the pool of available enzyme. The relative importance of the 3 types of inhibition will depend on the exact conditions of the in vitro or in vivo situation under investigation.

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