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4′*C*-Ethynyl-thymidine acts as a chain terminator during DNA-synthesis catalyzed by HIV-1 reverse transcriptase

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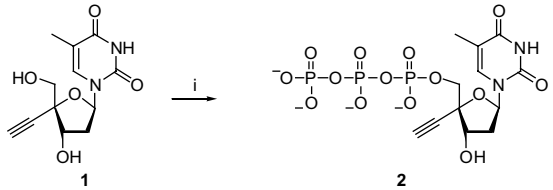
Abstract—Recently, 4′*C*-ethynyl nucleoside analogues have been identified as highly potent agents against HIV-1, including several multidrug-resistant strains. In contrast to most known nucleoside inhibitors 4′*C*-ethynyl nucleoside analogues possess a 3′-hydroxyl function. Here we show that the 5′*O*-triphosphate of 4′*C*-ethynyl thymidine gets readily incorporated into a nascent DNA strand by HIV-1 reverse transcriptase and significantly inhibits further post-incorporation chain extension by the enzyme.

Most known nucleoside reverse transcriptase inhibitors (NRTI) like 3′*C*-azido-3′-deoxythymidine (AZT) are first phosphorylated by cellular kinases to yield the respective 5′*O*-triphosphates. These activated nucleotides bind competitively to the HIV-1 reverse transcriptase active site and cause chain termination after incorporation into the nascent DNA strand due to a lack of a 3′-hydroxyl function.[1] Recently, 4′*C*-ethynyl nucleoside analogues have been identified as potent agents against HIV-1, including several multidrug-resistant strains.[2–5] Particularly, this new class of NRTI exhibits potent activity against several multidrug-resistant (MDR) HIV-1 strains that represent a severe restriction to therapy. In contrast to common NRTIs, 4′*C*-ethynyl nucleoside analogues possess a 3′-hydroxyl group. However, the exact mode of action of these analogues is still unclear. It has been speculated that the mechanism of inhibition of HIV-1 reverse transcriptase (RT) is similar to that of AZT.[5] Thus, 5′*O*-triphosphate analogues generated by cellular kinases are thought to get incorporated into a nascent DNA strand by HIV-1 RT, which in turn results in termination of further chain extension despite the presence of a free 3′-hydroxyl group. However, hitherto no studies with a respective 4′*C*-ethynyl nucleoside-5′*O*-triphosphate have been described that indeed show such kind of mechanism of action. Thus, we set out to synthesize a 4′*C*-ethynyl nucleoside-5′*O*-triphosphate in order to evaluate its action on HIV-1 RT in vitro. Here we describe comparative studies of 4′*C*-ethynyl thymidine-5′*O*-triphosphate with the respective AZT derivative that unambiguously show that 4′*C*-ethynyl thymidine acts as a chain terminating nucleoside after incorporation into DNA by HIV-1 RT.

First we synthesized 4′*C*-ethynyl thymidine **1** following a described route.[2] **1** was subsequently converted to the respective 5′*O*-triphosphate **2** (Scheme 1) using a multistep one-pot reaction sequence.[6]

To evaluate the potential of **2** to act as an inhibitor of HIV-1 RT promoted DNA synthesis we used an assay format recently developed to measure DNA polymerase activity in real time.[7] In this assay format a fluorescence increase is generated by HIV-1 RT promoted DNA synthesis that results in opening of a template containing a molecular beacon construct. By time-resolved observation of fluorescence increase, reaction velocities can be



1 2

Scheme 1. Reagents: (i) $POCl_3$, 1,8-bis-(dimethylamino)-naphthalene, then $(n\text{Bu}_3\text{NH})_2\text{H}_2\text{P}_2\text{O}_7$, n-Bu_3N, DMF, then 0.1 M aqueous $(\text{Et}_3\text{NH})\text{HCO}_3$, 9%.

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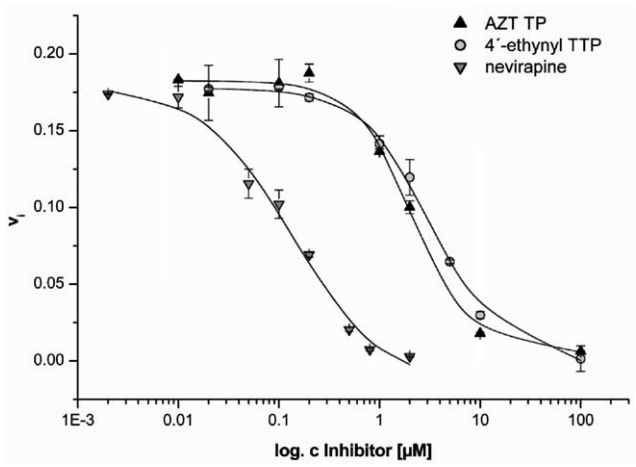

Figure 1. Inhibition profiles of HIV-1 RT inhibitors. Data for nevirapine and AZT TP was from Ref. 7. V_i = fluorescence [au]/min.

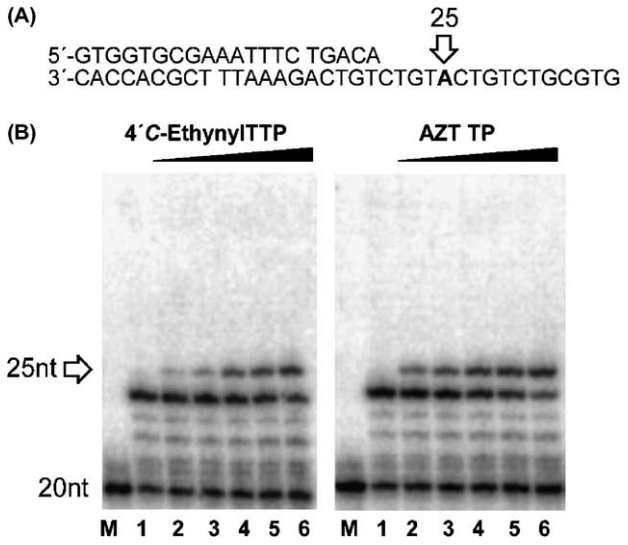

Figure 2. Insertion catalyzed by HIV-1 RT of 4′C-ethynyl TTP **2** in comparison to AZT TP. (A) Primer template complex sequences employed in this study. (B) Insertion of **2** and AZT TP. Conditions: primer template complex (120 nM), HIV-1 RT (8.6 nM), 37 °C, 5 min in 50 mM Tris–HCl pH 8.0, 10 mM MgCl, and 50 mM KCl and 10 μM each of dGTP, dATP, dCTP. Analysis was performed by 14% denaturating PAGE and subsequent phosphorimager analysis. M: Marker (primer template complex), lane 1: no TTP analogue, 2: 0.05 μM, 3: 0.1 μM, 4: 0.5 μM, 5: 1 μM, 6: 5 μM either **2** or AZT TP as indicated in the figure.

deduced. When testing **2** we observed decreasing activity with increasing concentrations of the analogue. The results obtained for **2** were compared to those derived from known HIV-1 RT inhibitors that is AZT TP and nevirapine (Fig. 1). Figure 1 shows that the IC_{50}-value of **2** (3.28 ± 0.21 μM) is well in the range of that of AZT TP (2.10 ± 0.22 μM). These experiments clearly indicate that **2** is able to inhibit DNA synthesis catalyzed by HIV-1 RT.

To gain insights into the mechanism of action of **2** on HIV-1 RT we performed primer extension reactions using a radioactively labeled primer strand, subsequent analysis by high-resolution denaturating PAGE and autoradiography. We designed a primer template complex in a way that after extension of the primer strand by four nucleotides a single dA-moiety in the template at position 25 calls for incorporation of a thymidine analogue by HIV-1 RT (Fig. 2A). In a first set of experiments we performed primer extension reactions using dATP, dCTP, dGTP, and **2** at varied concentrations. For comparison we performed reactions under the same conditions using AZT TP instead of **2** (Fig. 2B). When neither **2** nor AZT TP were present the reactions arrest after yielding a 24nt long reaction product due to the absence of any thymidine analogue (Fig. 2B, lanes 1). With increasing amounts of **2** or AZT TP 25nt long reaction products were detected (Fig. 2B, lanes 2–6). However, no further reaction occurs after incorporation of a 4′*C*-ethynyl thymidine under the applied condition. This is in particular interesting since a free 3′-hydroxyl function at the 3′-primer terminus is present that in principle should be available for further chain extension. Nevertheless, further chain extension is blocked by addition of a 4′*C*-ethynyl group in comparison to an unmodified DNA primer strand. Thus, the chain terminating action of **2** on HIV-1 RT catalyzed DNA synthesis resembles the action of AZT TP, even both molecules differ in respect to the presence of a free 3′-hydroxyl group.

These results are similar to those obtained with other known 4′*C*-modified nucleoside-5′*O*-triphosphates. 4′*C*-azido-,[8] 4′*C*-acyl-,[9,10] and 4′*C*-alkyl-thymidine-5′*O*-triphosphates[11–13] have been shown to exert strong blockage of post-incorporation chain extension by various DNA polymerases including HIV-1 RT. This feature is presumably caused through unfavorable interaction of the added 4′*C*-group with functionally important HIV-1 RT motifs such as the so called 'primer grip', which contacts the primer 3′-terminus.[14–16] Thus, presence of the additional bulk hampers the formation of productive enzyme substrate complexes resulting in blockage of further chain extension.

Next, we investigated whether **2** is able to compete with incorporation with unmodified TTP by HIV-1 RT. As seen above, incorporation of a 4′*C*-ethynyl thymidine moiety blocks further HIV-1 RT promoted DNA synthesis. Thus, incorporation of **2** in the presence of natural TTP should result in the accumulation of 25nt long reaction products if **2** is indeed a substrate for the enzyme under these conditions. In the absence of any thymidine analogue **2** or AZT TP and in the presence of TTP full length reaction product formation was observed (Fig. 3, lanes 1).

With increasing amounts of **2** accumulation of a 25nt long reaction product was observed accompanied by a decreasing amount of reaction products longer than 25nt (Fig. 3, lanes 2–6). Similar observations were made when AZT TP was used instead of **2**. These results indicate that **2** is a suitable substrate for incorporation into a growing DNA strand by HIV-1 RT even in the presence of unmodified TTP. Incorporation of a 4′*C*-ethynyl thymidine moiety results in blockage of further chain

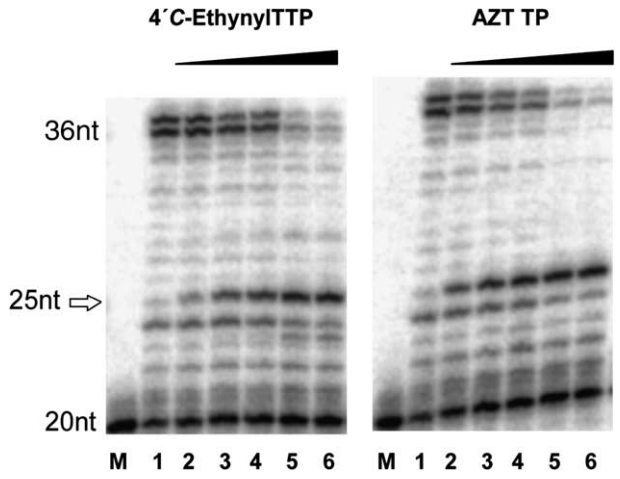

Figure 3. Insertion catalyzed by HIV-1 RT of 4′C-ethynyl TTP **2** in comparison to AZT TP in the presence of TTP. Conditions as described in Figure 2 including 10 μM of TTP. M: Marker (primer template complex), lane 1: neither of **2** and AZT TP, 2: 1 μM, 3: 5 μM, 4: 10 μM, 5: 50 μM, 6: 100 μM either **2** or AZT TP as indicated in the figure.

extension by HIV-1 RT and leads to post-incorporation chain termination.

Our experiments unambiguously show for the first time that the 5′*O*-triphosphate of 4′*C*-ethynyl thymidine **1**, a member of a family of promising antiviral nucleoside derivatives, is an inhibitor of HIV-1 RT catalyzed DNA synthesis and gets readily incorporated by the enzyme into a growing DNA strand. Interestingly, after incorporation of a 4′*C*-ethynyl thymidine moiety DNA synthesis promoted by HIV-1 RT arrests despite the presence of a free 3′-hydroxyl group at the primer terminus, which in principle should allow proceeding DNA synthesis. Furthermore, our experiments indicate that **2** can compete with TTP as a substrate for HIV-1 RT since **2** gets readily incorporated into a growing DNA strand even in the presence of unmodified TTP.

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