An HIV Reverse Transcriptase-Selective Nucleoside Chain Terminator

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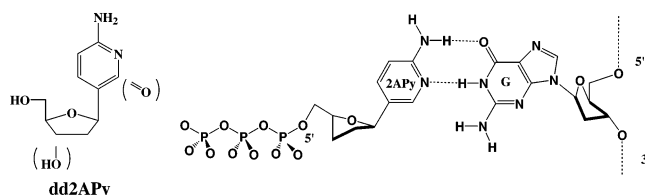
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The mode of action of anti-retroviral (e.g., anti-HIV) nucleoside chain terminators such as AZT, ddI, and ddC involves their in vivo conversion to the corresponding 5′-triphosphates, which enables them to function as substrates for retroviral reverse transcriptases (RT). In the absence of a 3′-hydroxyl, chain termination results after the incorporation of such derivatives and DNA synthesis of the viral cDNA genome ceases. A common problem with antiviral therapies involving chain-terminating nucleosides is both their immediate and delayed toxicity effects. Delayed toxicity most commonly takes the form of peripheral neuropathy, myopathy, or pancreatitis, but the nature of toxicity depends on the specific analogue.[1-4] A variety of mechanisms can account for toxicity effects, but the most obvious involve substrate activity with host polymerases. In proliferating cells, toxicity effects (such as the bone marrow toxicity of AZT) may be due to the actions of human chromosomal polymerases such as Pol β involved in DNA repair.[5] In nonproliferating cells toxicity appears to be correlated with mitochondrial DNA synthesis (the role of Pol γ).[5-10] In fact, many of the symptoms resulting from antiviral nucleosides are similar to the dysfunctions reported for mitochondrial genetic disorders.[6-9] Human mitochondrial polymerase γ appears to be the most sensitive to the presence of some antiviral nucleosides.[10-12]

Our design of an HIV-selective nucleoside chain terminator relied upon recent work suggesting that some mammalian polymerases make contacts to the minor groove functional groups of base residues in both the primer and template strands.[13,14] Similarly, studies of dNTP building blocks designed on the basis of geometric shape have suggested that the loss of functionality can result in reduced rates of incorporation by some mammalian polymerases.[15-17] The described nucleoside analogue (dd2APy, Figure 1) incorporated two design features; one was a modification of the nucleobase residue to eliminate the O2-carbonyl, and the second was the use of a 2′,3′-dideoxycarbohydrate to eliminate the 3′-hydroxyl. In other respects the derivative was designed to form a bidentate Watson−Crick base pair with dG (Figure 1), but to maintain the correct tautomeric form for base pairing, the derivative was prepared as a C-nucleoside. Nearly all of the current nucleoside antivirals contain essentially native nucleobases coupled to appropriately modified carbohydrate residues. The described modifications were implemented to result in a derivative in which the heterocyclic nucleobase could provide the desired polymerase selectivity (e.g., HIV vs mammalian polymerases), while the 2′,3′-dideoxy sugar would ensure chain termination of the elongating strand.

The 2′,3′-dideoxy nucleoside chain terminator (7) could be prepared in 60% overall yield from a simple five-step synthesis (Scheme 1). After iodonation of 2-aminopyridine (1), the product (2) was coupled to the silyl-protected glycal (3) by a Heck-type reaction[18] to generate solely the β-nucleoside (4). After deprotection

Figure 1. Structure of dd2APy (left). Watson−Crick base-pairing scheme for an incoming dd2APy triphosphate analogue and template dG (right).

of the nucleoside to generate 5, the carbonyl was eliminated by conversion to the p-toluylsulfonyl hydrazone (6) and then smoothly reduced to the 2′,3′-dideoxynucleoside (7). This latter transformation is based upon earlier work[19,20] with α,β-unsaturated steroids. A small portion of 7 was converted to the corresponding 5′-triphosphate (dd2APyTP) by first protection of the exocyclic amino group as the *N,N*-dimethylamidine followed by conventional phosphorylation/pyrophosphate treatment and finally removal of the amidine in aqueous ammonia.

The dideoxy nucleoside 7 as the 5′-triphosphate derivative (dd2APyTP) was incubated with either calf thymus DNA polymerase α or human DNA polymerase β over a range of concentrations (50−1000 μM) for a 45-min period with a primer/template complex. No significant elongation of the primer strand was observed (Figure 2). In a control reaction both enzymes effectively use dCTP as a substrate. In related control reactions, incorporation of dCTP by DNA polymerase β at low (2 μM) or relatively high (100 μM) concentrations was not substantially inhibited by the presence of dd2APyTP ($\leq$1 mM). By comparison at low concentrations of dCTP (2 μM), DNA polymerase α could be inhibited by 0.5 and 1.0 mM concentrations of dd2APyTP. This experiment suggests that in the absence of the O2-carbonyl, the dd2APyTP derivative does not have significant binding activity with either of these mammalian enzymes, except at very high concentrations.
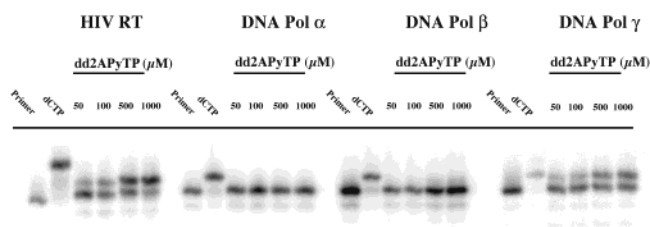
Scheme 1[a]
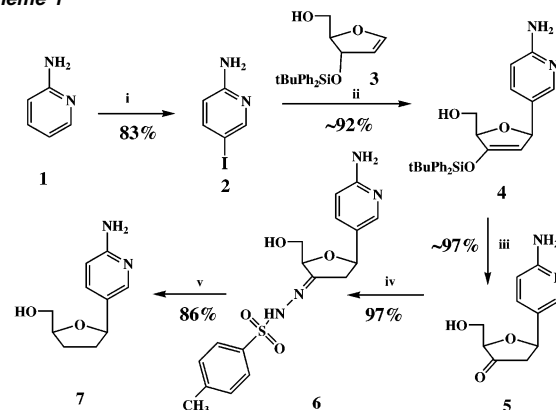
[a] Conditions: i. I$_2$/HIO$_4$/H$_2$SO$_4$/CH$_3$COOH; ii. (dba)$_2$Pd^0/Ph$_3$P/*i*-Pr$_2$EtN/CH$_3$CN; iii. *n*-Bu$_4$N$^+$F$^-$/CH$_3$COOH/THF; iv. *p*-toluylsulfonylhydrazide; v. Na(OAc)$_3$BH, CH$_3$COOH/CH$_3$CN, H$_2$O quench.

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Polymerase Catalyzed Incorporation of dd2APyTP

5′ CAA TAG GAA CCC ATG TAC CGT AA
3′ GTT ATC CTT GGG TAC ATG GCA TTG TCA CTC

Figure 2. Primer extension reactions with dd2APy triphosphate were performed for the enzymes, HIV RT, calf thymus DNA polymerase α, human DNA polymerase β, and human mitochondrial DNA polymerase γ, each under its optimal pH conditions. For Pol γ the primer contains a phosphorothioate diester at the 3′-d(ApA).

This observation is consistent with the results reported for hydrophobic bases that also lack minor groove functionality and are reported to be inactive with these enzymes.[15] Initial work with human mitochondrial DNA polymerase γ[21] resulted largely in primer degradation by the exonuclease activity of the enzyme. To better assess analogue incorporation, we altered the primer to contain a 3′-phosphorothioate diester, a derivative known to be refractory to nuclease activity.[22] Under these conditions we could observe moderate elongation by the mitochondrial enzyme. In a time-course assay (100 μM dd2APy triphosphate) roughly 11% of the primer was elongated with the analogue by human mitochondrial polymerase γ, while under the same conditions HIV RT elongated 50% of the primer.

Analogue triphosphates capable of selective termination of the replication process catalyzed by HIV RT have the potential to become effective antivirals. On the other hand, similar termination of replication of the mitochondrial genome has been linked to toxicity effects. Limited toxicity may be related in part to the extent of exonucleolytic removal of the incorporated dideoxy derivative.[11] To determine whether incorporation of dd2APy by the mitochondrial polymerase could be reversed, we incubated the dd2APy-elongated primer with the human mitochondrial polymerase. Over a period of 12 h the analogue was removed ($k_{obs} = 0.02$ min^{-1}). This rate is commensurate with the removal rate for the dideoxy derivative 3TC and significantly faster than that observed for ddC.[11]

The observed polymerase selectivity can be explained by two possible mechanisms: (i) Duplexes containing dG-d2APy base pairs appear to be destabilized more than what would be expected for the simple loss of an interstrand hydrogen bond.[21] A mispairing effect may be present and may alter the relative positions of the α-phosphate of the incoming dNTP and 3′-hydroxyl of the 3′-primer residue. Such relative changes in geometry may be more significant for the mammalian polymerases than for the HIV RT. (ii) Simple hydrophobic isosteric triphosphates in the absence of a 3′-minor groove functional group are generally poor substrates[13] for mammalian polymerases. These reports suggest that a minor groove functional group on the incoming dNTP is a prerequisite for effective substrate activity,[13] although this requirement varies with the specific polymerase.[15] The critical minor groove functional group of dCTP is the O2-carbonyl, a residue absent in the dd2APy derivative (Figure 1). Its absence may be most significant for the mammalian polymerases α and β where essentially no incorporation of the dd2APy analogue is observed (Figure 2). Human mitochondrial polymerase γ exhibits somewhat less dependence upon the presence of the minor groove O2-carbonyl, but the analogue dd2APyTP is still at best a relatively poor substrate for this

polymerase. HIV RT is the enzyme in this study that exhibits the least dependence upon the O2-carbonyl and will incorporate the analogue dideoxy chain terminator much more efficiently than any of the other three enzymes.

Effective antiviral activity requires that the analogue be converted to the triphosphate. Initial experiments with deoxycytidine kinase indicate that the dd2APy derivative is a poor substrate for this enzyme, but this initial step in the pathway to the triphosphate can be circumvented by preparation of neutral phosphate analogues designed to permit intracellular release of nucleoside monophosphates.[24,25]

Although the analogue described here is not as effective a substrate as is dCTP, incorporation of a single chain-terminating derivative during the course of viral genome replication is in principle sufficient for chain termination. The absence or low activity with human polymerases could result in reduced toxicity effects for this class of derivatives.

Supporting Information Available: Synthetic procedures for the analogue nucleoside, procedures for enzyme assays and selected gels (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA020639Y