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# Molecular basis for the enantioselectivity of HIV-1 reverse transcriptase: role of the 3′-hydroxyl group of the L-(β)-ribose in chiral discrimination between D- and L-enantiomers of deoxy- and dideoxy-nucleoside triphosphate analogs

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## ABSTRACT

**In order to identify the basis for the relaxed enantioselectivity of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) and to evaluate possible cross-resistance patterns between L-nucleoside-, D-nucleoside- and non-nucleoside RT inhibitors, to be utilised in anti-HIV-1 combination therapy, we applied an *in vitro* approach based on the utilisation of six recombinant HIV-1 RT mutants containing single amino acid substitutions known to confer Nevirapine resistance in treated patients. The mutants were compared on different RNA/DNA and DNA/DNA substrates to the wild type (wt) enzyme for their sensitivity towards inhibition by the D- and L-enantiomers of 2′-deoxy- and 2′,3′-dideoxynucleoside triphosphate analogs. The results showed that the 3′-hydroxyl group of the L-(β)-2′-deoxyribose moiety caused an unfavourable steric hindrance with critic residues in the HIV-1 RT active site and this steric barrier was increased by the Y181I mutation. Elimination of the 3′-hydroxyl group removed this hindrance and significantly improved binding to the HIV-1 RT wt and to the mutants. These results demonstrate the critical role of both the tyrosine 181 of RT and the 3′-position of the sugar ring, in chiral discrimination between D- and L-nucleoside triphosphates. Moreover, they provide an important rationale for the combination of D- and L-(β)-dideoxynucleoside analogs with non-nucleoside RT inhibitors in anti-HIV chemotherapy, since non-nucleoside inhibitors resistance mutations did not confer cross-resistance to dideoxynucleoside analogs.**

## INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) high mutation rate constitutes a major obstacle in the development of effective drugs and vaccines. Error-prone DNA synthesis by HIV-1 reverse transcriptase (RT) and high viral turnover *in vivo* are responsible for the emergence of cross-resistance between and within classes of anti-retroviral drugs, either nucleoside inhibitors (NI) or non-nucleoside inhibitors (NNI) (1–6). Recently, the combination of two NI, the thymidine analog 3′-azido-2′,3′-dideoxythymidine (AZT) and the new compound L-(β)-2′,3′-dideoxy-3′-thiacytidine (3TC; Lamivudine), has proved to be particularly effective with respect to AZT monotherapy (7–9) since 3TC resistance not only did not confer cross-resistance to AZT, but was also able to restore AZT sensitivity (10–12). 3TC is an L-nucleoside, its sugar moiety having the unnatural L-configuration, opposite to the D-configuration of the natural nucleoside analogs such as AZT. D-(β)- and L-(β)-nucleotides have almost superimposable nucleobase and α-phosphorus, differing only in the mutual orientation of the sugar ring (13). HIV-1 RT, contrary to cellular DNA polymerases, has been shown to be able to bind and incorporate L-2′-deoxythymidine triphosphate (L-dTTP) when acting on a homopolymeric template–primer (TP) (14,15), as well as the L-enantiomers of 2′,3′-dideoxynucleoside triphosphate analogs such as L-2′,3′-dideoxythymidine triphosphate (L-ddTTP), L-2′,3′-dideoxycytidine triphosphate (L-ddCTP) and L-2′,3′-dideoxy-5-fluorocytidine triphosphate (L-FddCTP) (16,17). Moreover, L-2′,3′-dideoxycytidine (L-ddC) and L-2′,3′-dideoxy-5-fluorocytidine (L-FddC) showed potent anti-HIV activity on infected cells (18). Clinical trials are currently underway which explore the efficacy of multiple drug therapy protocols, involving the combined use of NI such as AZT and 3TC together with NNI (19). In light of the potential use of L-nucleoside

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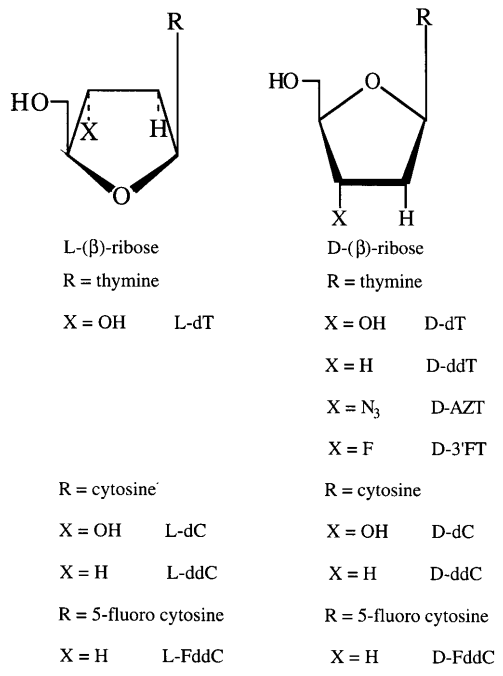



**Figure 1.** Structure of the D-(β)- and L-(β)-nucleosides and their analogs used in this study.

analogs in combination therapy, we have used an *in vitro* approach to study the sensitivity of HIV-1 RT mutants known to confer NNI resistance to treated patients, to the D- and L-enantiomers of deoxy- and dideoxynucleoside triphosphates and their analogs (Fig. 1), in order to: (i) identify structural determinants responsible for the interaction of HIV-1 RT with L-deoxy- and -dideoxynucleoside triphosphates in comparison with the corresponding D-enantiomers; and (ii) evaluate whether NNI resistance mutations, which appear very rapidly during NNI monotherapy (20–23), could confer cross-resistance to NI and in particular to L-nucleoside analogs. This is because, even if NNI resistance mutations involve residues outside the RT active site (24), it has been shown that some of them can affect both binding and incorporation of a nucleotide substrate (25). Our present results provide a rationale for the utilisation of L-dideoxynucleoside analogs in combination with NNI during multi-drug chemotherapy of HIV-1 infections.

## MATERIALS AND METHODS

### Chemicals

[$^3$H]dTTP or 2′-deoxycytidine triphosphate (dCTP) (40 Ci/mmol) and [γ-$^{32}$P]ATP (3000 Ci/mmol) were from Amersham. L-dTTP, L-dCTP, L-ddCTP and L-FddCTP were prepared by a standard phosphorylation method from their corresponding nucleosides. The 5′-triphosphate derivatives were fully characterised by nuclear magnetic resonance ($^1$H, $^{31}$P) and fast atom bombardment mass spectrometry and their purity was ascertained by high performance liquid chromatography and UV spectroscopy. The 66mer and 24mer oligodeoxynucleotides and unlabelled dNTPs were from Boehringer. Whatman was the supplier of the GF/C filters. All other reagents were of analytical grade and purchased from Merck or Fluka.

### Nucleic acid substrates

The homopolymers poly(rA), poly(dA) or poly(dI) (Pharmacia) were mixed at weight ratios in nucleotides of 10:1, to the complementary oligomer oligo(dT)$_{12–18}$ or oligo(dC)$_{12–18}$ (Pharmacia) in 20 mM Tris–HCl (pH 8.0), containing 20 mM KCl and 1 mM EDTA, heated at 65°C for 5 min and then slowly cooled at room temperature. For the preparation of the 5′-end-labelled sp d24:d66mer DNA template, the d24mer primer was labelled with T4 polynucleotide kinase (Ambion) and [γ-$^{32}$P]ATP, according to the manufacturer's protocol. After removal of unincorporated ATP on a Sephadex G-25 column, the 5′-end-labelled d24mer primer was mixed at 1:1 molar ratio with the d66mer template in 20 mM Tris–HCl (pH 8.0), containing 20 mM KCl and 1 mM EDTA, heated at 65°C for 5 min and then slowly cooled at room temperature. The concentrations of the d66mer and d24mer were calculated according to their molar extinction coefficients (758 760 l mol$^{-1}$ cm$^{-1}$ and 250 950 l mol$^{-1}$ cm$^{-1}$, respectively).

### Expression and purification of recombinant HIV-1 RT forms

The coexpression vectors pUC12N/p66(His)/p51with the wild type (wt) or the mutant forms of HIV-1 RT p66 were kindly provided by Dr S. H. Hughes (NCI-Frederick Cancer Research and Development Center). Protein expression and purification was as described (25). All enzymes were purified to >95% purity.

### HIV-1 RT DNA polymerase activity assay

RNA- and DNA-dependent DNA polymerase activities were assayed as described (25) in the presence of 0.5 µg of either poly(rA)/oligo(dT)$_{10:1}$, poly(dA)/oligo(dT)$_{10:1}$ or poly(dI)/oligo-(dC)$_{10:1}$ (0.3 µM 3′-OH ends), 10 µM [$^3$H]dTTP or [$^3$H]dCTP (1 Ci/mmol) and 2–4 nM RT. When the 5′-$^{32}$P-labelled d24:d66mer template was used, a volume of 10 µl contained 0.05 µM (3′-OH ends) of the DNA template. After incubation at 37°C, samples were mixed with denaturing gel loading buffer, boiled for 3 min and the products of the reaction were separated on a 7 M urea–20% polyacrylamide sequencing gel. Quantification of the products was performed by scanning of the gel with a Molecular Dynamics PhosphorImager and integration with the program ImageQuant.

### Steady-state kinetic measurements

Reactions were performed under the conditions described for the HIV-1 RT RNA- and DNA-dependent DNA polymerase activity assay. Time-dependent incorporation of radioactive nucleotides into the different TPs at different nucleotide substrate concentrations was monitored by removing 25 µl aliquots at 2 min time intervals. Initial velocities of the reaction were then plotted against the corresponding substrate concentrations. When the 5′-$^{32}$P-labelled d24:d66mer template was used, initial velocities after 10 min incubation at 37°C in the presence of different substrate concentrations were calculated from the integrated gel band intensities (see also below). For determination of the $K_m$ and $k_{cat}$ values, an interval of substrate concentrations from 0.2 to 10 $K_m$ was used. For $K_i$ determination, an interval of inhibitor concentrations between 0.2 and 5 $K_i$ was used in the inhibition assays. Data were then plotted according to Lineweaver–Burke and Dixon.

### Kinetic parameters calculation

All values were calculated by non-least squares computer fitting of the experimental data to the appropriate rate equations. $K_m$, $V_{max}$ and $k_{cat}$ values were determined according to the Michaelis–Menten equation. $K_i$ values were calculated according to the equation for competitive inhibition.

The stereoselectivity index (S.I.) was calculated as the ratio: $(k_{cat}/K_m)_{D\text{-nucleotide}}/(k_{cat}/K_m)_{L\text{-nucleotide}}$

According to the minimal kinetic pathway proposed for both processive and forced termination DNA synthesis catalysed by HIV RT (26) it can be shown that: $k_{cat} = k_{pol}$; $K_m = K_d$ for processive synthesis; $k_{cat} = k_{pol}k_{off}/(k_{pol} + k_{off})$; $K_m = K_dk_{off}/(k_{pol} + k_{off})$ for non-processive synthesis.

For both processive and non-processive DNA synthesis then: $k_{cat}/K_m = k_{pol}/K_d$. Accordingly, S.I. = $(k_{polD\text{-nucleotide}}K_{dL\text{-nucleotide}})/(k_{polL\text{-nucleotide}}K_{dD\text{-nucleotide}})$; $K_i/K_m$ = S.I.$(k_{polL\text{-nucleotide}}/k_{polD\text{-nucleotide}})$ for processive synthesis; $K_i/K_m$ = S.I.$(k_{offL\text{-nucleotide}}/k_{offD\text{-nucleotide}})$ for non-processive DNA synthesis.

Since $k_{polL\text{-nucleotide}} \leq k_{polD\text{-nucleotide}}$, and $k_{offL\text{-nucleotide}} \leq k_{offD\text{-nucleotide}}$ (see Results) the $K_i/K_m$ value derived from inhibition studies can be considered a lower limit estimation of the true S.I.

The values of integrated gel band intensities in dependence of the nucleotide substrate concentrations were fitted to the equation (27):

$I^*_T/I_{T-1} = V_{max}[dNTP]/(K_m + [dNTP])$

where: T = target site, the template position of interest; $I^*_T$ = the sum of the integrated intensities at positions T, T + 1 ... T + n.

## RESULTS

### Nevirapine-resistance mutations of HIV-1 RT result in higher sensitivity to 3′-azido-2′,3′-dideoxythymidine triphosphate (AZTTP) and 3′-fluoro-2′,3′-dideoxythymidine triphosphate (FTTP)

In order to evaluate whether NNI-resistance mutations could alter the recognition of NI, the effect of different dTTP analogs on the RDS catalysed by recombinant HIV-1 RT mutants L100I, K103N, V106A, V179D, Y181I and Y188L, known to confer NNI resistance in both infected cells and treated patients (28), was analysed. The results are summarised in Table 1. Most of the mutations did not significantly affect dTTP utilisation by HIV-RT, with the exception of the mutant Y181I which showed a 5-fold increase in the $K_m$ value. Elimination of the 3′-hydroxyl group of dTTP significantly improved binding, but the mutant Y181I displayed again a similar increase (4-fold) in the $K_i$ for ddTTP, compared to wt. On the other hand, an azido- or fluoro-substituent at the 3′ position of the ribose significantly improved the relative affinity to the Y181I mutant, which showed a $K_i$ value similar to the wt enzyme and displayed a 3.5-fold higher preference for both AZTTP and 3′FTTP over dTTP when compared to wt, as shown by the increase in the $K_m/K_i$ values. The mutant L100I also displayed an ~2-fold reduction in dTTP and ddTTP affinity with respect to wt, which corresponded to a proportional increase in preferential AZTTP and 3′FTTP binding over dTTP. These results indicated that NNI-resistance mutations did not cause significant cross-resistance towards NI. Indeed, 3′-substituted NI such as AZT and 3′-fluoro-2′,3′-dideoxythymidine (3′-FT) could have an increased therapeutic potential towards the Y181I and L100I mutants.

**Table 1.** Effects of deoxy- and dideoxy-TTP analogs on RNA-dependent DNA synthesis catalysed by HIV-1 RT wt and mutants[a]

| RT | dTTP | | ddTTP | | | AZTTP | | | 3'FTTP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $K_{mM}/K_{mwt}$ | $K_i$ (nM) | $K_m/K_i$ | $K_{iM}/K_{iwt}$ | $K_i$ (nM) | $K_m/K_i$ | $K_{iM}/K_{iwt}$ | $K_i$ (nM) | $K_m/K_i$ | $K_{iM}/K_{iwt}$ |
| wt | 2 | - | 18 | 111 | - | 0.75 | 2666 | - | 16 | 125 | - |
| L100I | 4.8 | 2.4 | 38 | 126 | 2.1 | 0.81 | 5925 | 1.1 | 23 | 208 | 1.4 |
| K103N | 1.3 | 0.6 | 15 | 87 | 0.8 | 0.66 | 1970 | 0.9 | 19 | 67 | 1.2 |
| V106A | 2.3 | 1.1 | 15 | 153 | 0.8 | n.d.[b] | n.d. | n.d. | n.d. | n.d. | n.d. |
| V179D | 1.2 | 0.6 | 25 | 48 | 1.4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Y181I | 10 | 5 | 75 | 133 | 4.2 | 1.3 | 7692 | 1.7 | 27 | 370 | 1.7 |
| Y188L | 4 | 2 | 25 | 160 | 1.4 | 2.6 | 1538 | 3.5 | 23 | 173 | 1.4 |

[a]Assays were performed with poly(rA)/oligo(dT)$_{10:1}$ as described in Materials and Methods. Experimental errors (±S.D.) between three independent experiments were ≤5%.
[b]n.d., not determined.

### Stereoselectivity of HIV-1 RT towards L-dTTP is differentially affected by NNI-resistant mutations during RNA- and DNA-dependent DNA synthesis

In order to test whether NNI-resistance mutations could affect the recognition of L-nucleotides by RT, the inhibition by L-dTTP of both RNA- and DNA-dependent DNA synthesis (RDS and DDS, respectively) catalysed by HIV-1 RT wt and Nevirapine-resistant mutants was studied. As summarised in Table 2, in most cases the variations in stereoselectivity (expressed as the ratio between the $K_i$ for L-dTTP and the $K_m$ for dTTP) observed for the different mutants with respect to RT wt correlated well with the corresponding changes in the relative affinities for dTTP ($K_{mM}/K_{mwt}$ values) and L-dTTP ($K_{iM}/K_{iwt}$ values) on both RDS and DDS, so that the $K_i/K_m$ values decreased from RDS to DDS both for the mutants and for the wt. A relevant exception was the mutant Y181I on DDS, which did not show any significant change in the relative affinity for dTTP, but nevertheless was 6-fold more stereoselective than RT wt. Thus, (i) stereoselectivity decreased from RDS to DDS, as shown by the lower $K_i/K_m$ values and (ii) the mutation Y181I specifically affected recognition of L-dTTP on a DNA template.

### Inhibition of the Y181I mutant by L-dCTP is influenced by the 3′-position of the sugar ring

Next, the effect of L-dCTP and L-ddCTP on DDS catalysed by HIV-1 RT wt and the Y181I mutant was studied. The results are summarised in Table 3. The Y181I mutant was 5-fold more stereoselective than wt towards L-dCTP ($K_i/K_m$ values), without showing significant differences in the relative affinity for dCTP ($K_{iM}/K_{iwt}$ values). However, when L-ddCTP was tested under the same conditions, Y181I showed no differences in both the relative affinity and stereoselectivity with respect to wt. Affinity for HIV-1 RT improved 8-fold from L-dCTP to L-ddCTP in case of the wt enzyme and >20-fold in case of the Y181I mutant. Both enzymes were no longer able to discriminate between the D- and L-enantiomers of ddCTP. These results indicated that the presence of a 3′-hydroxyl group in the L-ribose sugar moiety negatively influenced binding of L-nucleotides to HIV-1 RT and this effect was enhanced by the Y181I mutation.

**Table 2.** Inhibition by L-(β)-dTTP of RNA- and DNA-dependent DNA synthesis catalysed by HIV-1 RT wt and mutants[a]

| | RDS[b] | | | | | DDS[b] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| RT | dTTP | | L-dTTP | | | dTTP | | L-dTTP | | |
| | $K_m$ (μM) | $K_{mM}/K_{mwt}$ | $K_i$ (μM) | $K_{iM}/K_{iwt}$ | $K_i/K_m$ | $K_m$ (μM) | $K_{mM}/K_{mwt}$ | $K_i$ (μM) | $K_{iM}/K_{iwt}$ | $K_i/K_m$ |
| wt | 2 | - | 21 | - | 10.5 | 14 | - | 47 | - | 3.3 |
| L100I | 4.8 | 2.4 | 20 | 0.9 | 4.2 | 7.2 | 0.5 | 8 | 0.2 | 1.1 |
| K103N | 1.3 | 0.6 | 14 | 0.6 | 10.7 | 6.5 | 0.4 | 34 | 0.7 | 5.2 |
| V106A | 2.3 | 1.1 | 52 | 2.5 | 22.6 | 2.5 | 0.2 | 16 | 0.3 | 6.4 |
| V179D | 1.2 | 0.6 | 47 | 2.2 | 39.2 | 21 | 1.5 | 28 | 0.6 | 1.3 |
| Y181I | 10 | 5 | 175 | 8.3 | 17.5 | 10 | 1.4 | 183 | 3.9 | 18 |
| Y188L | 4 | 2 | 63 | 3 | 15.7 | 35 | 2.5 | 250 | 5.3 | 7.1 |

[a]Assays were performed with poly(rA)/oligo$(dT)_{10:1}$ or poly(dA)/oligo$(dT)_{10:1}$ as described in Materials and Methods. Experimental errors (±S.D.) between three independent experiments were ≤5%.
[b]RDS, RNA-dependent DNA synthesis; DDS, DNA-dependent DNA synthesis.

**Table 3.** Effect of L-(β)-dCTP and L-(β)-ddCTP on DNA-dependent DNA synthesis catalysed by HIV-1 RT wt and Y181I mutant[a]

| RT | dCTP | | L-dCTP | | | ddCTP | | L-ddCTP | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $K_{mM}/K_{mwt}$ | $K_i$ (μM) | $K_{iM}/K_{iwt}$ | $K_i/K_m$ | $K_i$ (μM) | $K_{iM}/K_{iwt}$ | $K_i$ (μM) | $K_{iM}/K_{iwt}$ | $K_i/K_m$ |
| wt | 3.6 | - | 8 | - | 2.2 | 0.8 | - | 1.2 | - | 1.5 |
| Y181I | 4.5 | 1.1 | 45 | 5.6 | 10 | 0.8 | 1 | 1.9 | 1.5 | 2.3 |

[a]Assays were performed with poly(dI)/oligo$(dC)_{10:1}$ as described in Materials and Methods. Experimental errors (±S.D.) between three independent experiments were ≤5%.

## HIV-1 RT wt and mutants can incorporate both D- and L-enantiomers of dTTP and dCTP on a DNA template

The stereoselectivity index $K_i/K_m$ derived from inhibition studies was only an approximation of the true S.I. value (Materials and Methods). To better evaluate the differences in stereoselectivity between HIV-1 RT wt and mutants observed during DDS, the incorporation of both D- and L-enantiomers of dTTP and dCTP by HIV-1 RT was studied. In order to provide a more 'physiological' sequence context for HIV-1 RT, a synthetic 66mer oligodeoxynucleotide corresponding to nt 1006–1071 of the HIV-1 *pol* gene (codons 169–190 of the RT coding sequence) was used as a template, in combination with a complementary 24mer oligodeoxynucleotide primer. Figure 2a shows the products of the reactions catalysed by HIV-1 RT wt, L100I and Y181I, in the presence of D- and L-dTTP, resolved by electrophoresis on a 7 M urea–20% polyacrylamide gel. Under the conditions used, HIV-1 RT wt showed significant misincorporation in the presence of dATP, dCTP and dGTP (Fig. 2a, lane 1). However, only when the reaction was complemented with either dTTP (lane 2) or with increasing amounts of L-dTTP (lanes 3–5), full-length products appeared in a dose-dependent manner. The same was observed with the L100I (lanes 6–10) and Y181I (lanes 12–16) mutants. However, the Y181I mutant was less efficient in L-dTTP incorporation than RT wt and L100I (compare lane 15 to lanes 3 and 8). Similar experiments with the D- and L-enantiomers of dCTP were performed and the results are shown in Figure 2b. In the presence of increasing amounts of dCTP (Fig. 2b, lanes 2–4), HIV-1 RT wt showed some misincorporation, whereas in the presence of corresponding L-dCTP concentrations (lanes 5–7) synthesis stopped after the first incorporation event, as expected from the template sequence. When dTTP (the next encoded nucleotide) was added in combination with dCTP, RT wt showed again misincorporated products (lane 8), whereas in the presence of a corresponding amount of L-dCTP, most of the synthesis stopped after the second position (lane 9), indicating that misincorporaton of an L-nucleotide is catalytically unfavoured. The mutant Y181I showed a similar pattern (lanes 11–18). In order to achieve significant incorporation, the Y181I mutant was used at a 5-fold higher concentration than in the experiment shown in Figure 2a. This confirmed that Y181I was severely impaired in L-nucleotides incorporation.

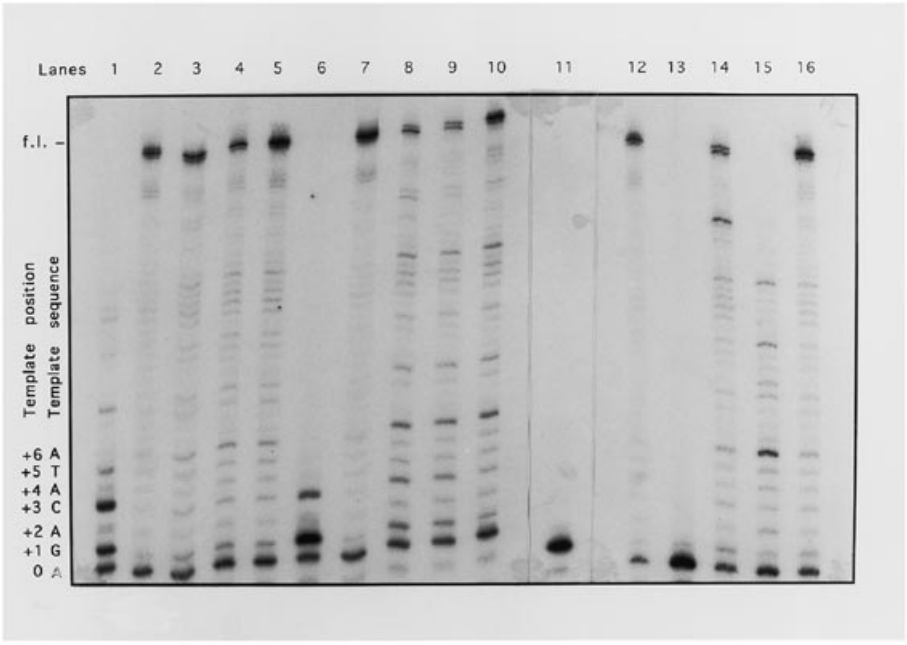
## Kinetic parameters for the incorporation of D- and L-enantiomers of dTTP and dCTP catalysed by HIV-1 RT wt and mutants during DDS

Substrate titration experiments similar to those shown in Figure 2 were performed with RT wt and mutants and the corresponding $K_m$ and $k_{cat}$ values for D- and L-nucleotide incorporation were calculated as described in Materials and Methods. Each experimental point was in duplicate. Errors (±S.D.) were ≤10% in all the experiments. The results are summarised in Table 4. In general, the major contribution to the stereoselectivity came from the low $k_{cat}$ values displayed for L-dTTP incorporation by HIV-1 RT wt and mutants in comparison with dTTP. S.I. values were similar between RT wt and the mutants. The only exception was the mutant Y181I, which showed a specific impairment in L-dTTP incorporation with a 70-fold lower $k_{cat}/K_m$ value and a 50-fold higher S.I. value with respect to wt. When HIV-RT wt and the Y181I mutant were compared for L-dCTP incorporation, a similar result was obtained with a 20-fold lower $k_{cat}/K_m$ value for L-dCTP incorporation by Y181I with respect to wt. In this case the difference in the S.I. value between the mutant and the RT wt was only 8-fold. This was due to higher $k_{cat}$ value for dTTP, which reflected full-length synthesis, whereas the $k_{cat}$ value for dCTP was measured under single nucleotide incorporation conditions and therefore corresponded to the $k_{off}$ for dissociation of the enzyme–primer complex (see also Material and Methods). These results confirmed that the Y181I mutation specifically decreased the affinity of HIV-1 RT wt for L-nucleotides during DDS.

## Impaired incorporation of L-deoxynucleoside triphosphates by the Y181I mutant is rescued by elimination of the 3′-hydroxyl group of the sugar ring

Next, the incorporation of the D- and L-enantiomers of ddCTP and FddCTP catalysed by HIV-1 RT wt and Y181I during DDS was studied. The calculated kinetic parameters are summarised in Table 5. The Y181I mutant showed only a slightly reduced $k_{cat}/K_m$ value for both D- and L-enantiomers of ddCTP and FddCTP with respect to wt. Moreover, both enzymes were unable to discriminate between the two enantiomeric forms, incorporating D- or L-nucleotides with almost the same efficiency as indicated

a




b

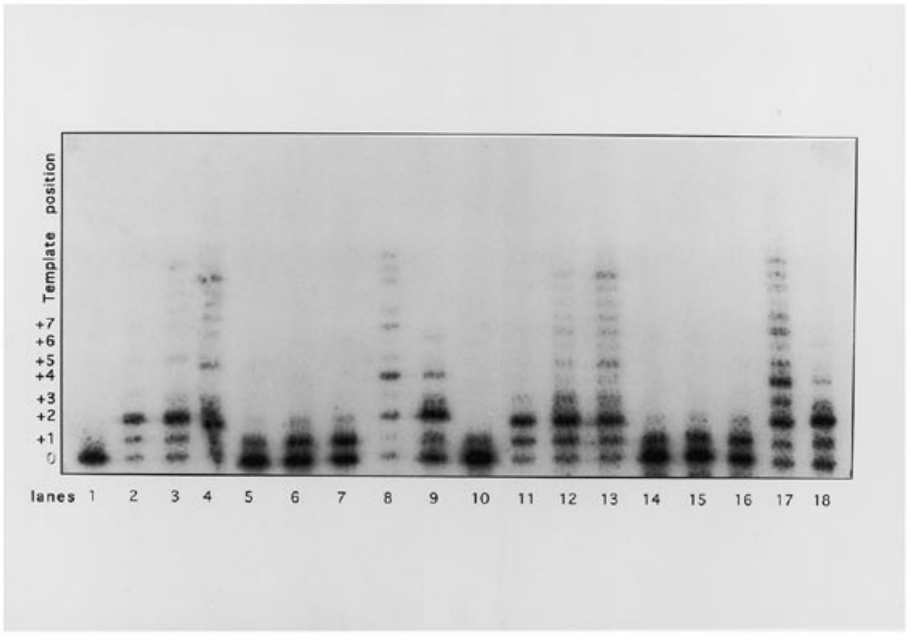



**Figure 2.** Incorporation of D- and L-enantiomers of deoxynucleoside triphosphates by HIV-1 RT wt and mutants on the d24:d66mer DNA template. (**a**) Incorporation of D- and L-dTTP by HIV-1 RT wt and mutants on the d24:d66mer DNA template. Reactions were performed as described in Materials and Methods in the presence of 20 nM HIV-1 RT wt (lanes 1–5), L100I (lanes 6–10) or Y181I (lanes 12–16). Reaction mixtures were supplemented with no dNTPs (lane 11); 5 μM dATP, dCTP and dGTP (lanes 1, 6 and 13); 5 μM dATP, dCTP, dGTP, dTTP (lanes 2, 7 and 12); 5 μM dATP, dCTP, dGTP and 17 μM L-TTP (lanes 3, 8 and 15) or 72 μM L-TTP (lanes 4, 9 and 14) or 360 μM L-TTP (lanes 5, 10 and 16); f.l., full length 66mer product. (**b**) Incorporation of D- and L-dCTP by HIV-1 RT wt and Y181I on the d24:d66mer DNA template. Reactions were performed as described in Materials and Methods in the presence of 20 nM HIV-1 RT wt (lanes 1–9) and 100 nM Y181I (lanes 10–18). Reaction mixtures were supplemented with no nucleotides (lanes 1 and 10); 20 μM dCTP (lanes 2 and 11); 50 μM dCTP (lanes 3 and 12); 200 μM dCTP (lanes 4 and 13); 20 μM L-dCTP (lanes 5 and 14); 50 μM L-dCTP (lanes 6 and 15); 200 μM dCTP (lanes 7 and 16); 200 μM dCTP and 200 μM dTTP (lanes 8 and 17); 200 μM L-dCTP and 200 μM dTTP (lanes 9 and 18).

**Table 4.** Kinetic parameters for the incorporation of D- and L-(β)-dTTP and D- and L-(β)-dCTP catalysed by HIV-1 RT wt and mutants[a]

| RT | dTTP | | | L-dTTP | | | | dCTP | | | L-dCTP | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}s^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}s^{-1}$) (x$10^{-4}$) | S.I.[b] | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}s^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}s^{-1}$) (x$10^{-4}$) | S.I. |
| wt | 0.4 | 0.5 | 1 | 38 | 0.05 | 14 | 724 | 9.5 | 0.05 | 0.005 | 47 | 0.03 | 6.4 | 8 |
| L100I | 0.13 | 0.02 | 0.15 | 23 | 0.008 | 3.4 | 382 | n.d.[c] | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| K103N | 0.33 | 0.22 | 0.67 | 34 | 0.08 | 7.1 | 943 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| V106A | 0.1 | 0.07 | 0.63 | 73 | 0.03 | 4.1 | 1547 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Y181I | 0.4 | 0.3 | 0.75 | 78 | 0.002 | 0.2 | 37500 | 13 | 0.03 | 0.002 | 80 | 0.003 | 0.3 | 66 |

[a]Assays were performed with the oligodeoxynucleotide d24:d66mer as the template as described in Materials and Methods. Experimental errors (±S.D.) between two independent experiments were ≤10%. Background subtraction and linearity correction were performed with the program ImageQuant.
[b]S.I., stereoselectivity index calculated according to the equation: $(k_{cat}/K_m)_D/(k_{cat}/K_m)_L$.
[c]n.d., not determined.

by the similar S.I. values. Thus, elimination of the 3′-hydroxyl group of the sugar ring increased >150-fold the affinity of L-ddCTP for HIV-1 RT wt and Y181I with respect to L-dCTP on this template and completely abolished the higher stereoselectivity observed for the Y181I mutant in the case of L-dTTP and L-dCTP incorporation.

**Table 5.** Kinetic parameters for the incorporation of D- and L-(β)-ddCTP and D- and L-(β)-FddCTP catalysed by HIV-1 RT wt and the Y181I[a]

| RT | ddCTP | | | L-ddCTP | | | | FddCTP | | | L-FddCTP | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}s^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}s^{-1}$) | S.I.[b] | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}s^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}s^{-1}$) | S.I. |
| wt | 0.2 | 0.05 | 0.25 | 0.3 | 0.05 | 0.17 | 1.4 | 0.6 | 0.05 | 0.08 | 1.1 | 0.05 | 0.04 | 2 |
| Y181I | 0.4 | 0.02 | 0.05 | 0.5 | 0.02 | 0.04 | 1.2 | 2 | 0.03 | 0.015 | 3 | 0.03 | 0.01 | 1.5 |

[a]Assays were performed with the oligodeoxynucleotide d24:d66mer as the template as described in Materials and Methods. Experimental errors (±S.D.) between two independent experiments were ≤10%. Background subtraction and linearity correction were performed with the program ImageQuant.
[b]S.I., stereoselectivity index calculated according to the equation: $(k_{cat}/K_m)_D/(k_{cat}/K_m)_L$.

## DISCUSSION

HIV-1 RT, contrary to cellular DNA polymerases, is able to incorporate both D- and L-enantiomers of nucleotides (14,15) as well as the triphosphates of nucleoside analogs such as L-ddC and L-FddC, extremely potent *in vitro* as RT inhibitors (17) and in infected cells against HIV-1 proliferation (18). Phosphorylation of nucleoside analogs to their monophosphate forms is the first and often the rate limiting step of their activation. L-nucleosides can be activated and exert their antiviral effects, due to the lack of enantioselectivity of the cellular deoxycytidine kinase, which, contrary to other nucleoside kinases, has been shown to phosphorylate L-deoxy- and dideoxycytidine analogs (13). Further activation to the metabolically active triphosphate form is catalysed by cellular nucleotide kinases. In light of the potential application of these L-nucleosides in combination therapy regimens together with other NI and NNI, we wanted to investigate possible patterns of cross-resistance between these different drugs. Thus, recombinant HIV-1 RT containing the single substitutions L100I, K103N, V106A, V179D, Y181I and Y188L, known to confer NNI resistance (28), were tested for their sensitivity to inhibition by D- and L-nucleoside triphosphate analogs during RDS and DDS. None of the mutations tested induced significant cross-resistance to ddTTP, AZTTP and 3′FTTP. Remarkably, mutants L100I and Y181I showed a 2–3-fold increased preference for AZTTP and 3′FTTP over dTTP with respect to wt (Table 1). The only mutation which showed significantly increased stereoselectivity towards L-dTTP and L-dCTP with respect to RT wt was the Y181I substitution on DDS (Tables 2 and 4). However, this enhanced discrimination between D- and L-enantiomers was completely abolished by elimination of the 3′-hydroxyl group of the sugar ring. In fact the Y181I mutant, similarly to RT wt, was completely unable to discriminate between the D- and L-enantiomers of ddCTP and FddCTP, incorporating both with the same efficiency (Tables 3 and 5). The L-ribose should bind in the enzyme active site with an opposite orientation with respect to the D-enantiomer. The fact that affinity increased from the L-2′-deoxy- to the L-2′,3′-dideoxynucleoside triphosphate analogs for both RT wt and Y181I, suggest that the hydroxyl group at the 3′ position of the sugar moiety makes unfavourable steric hindrances with residues in the RT nucleotide binding site (16). The Y181I mutation lies outside, but very close to, the enzyme active site (24). This mutation has been already shown to affect nucleotide incorporation (25). As shown in Tables 4 and 5, the $k_{cat}$ values of the Y181I mutant for both L-dTTP and L-dCTP incorporation were 10-fold lower than those observed for dCTP and the D- and L-enantiomers of ddCTP and FddCTP. It is then possible that, once an unnatural L-deoxynucleotide is bound to the Y181I mutant, the rate of the conformational change preceding the catalytic step, which is the rate limiting step for polymerisation by HIV-1 RT (29), is considerably slowed down, thus explaining the reduction in the $k_{cat}$ value and the impaired recognition of L-deoxynucleotides. The results of our study clearly show that HIV-1 RT is able to incorporate L-deoxy- and dideoxynucleotides, albeit with very different efficiencies, in agreement with previous work (14,15). Recently, another study (30) failed to detect incorporation of L-deoxynucleotides by HIV-1 RT on a singly-primed circular DNA template. However, the differences in both enzyme sources and substrate structure and concentration between that study and our one, could likely account for these discrepancies.

In conclusion, our present results indicate that the 3′-position of the sugar moiety is critical for stereoselectivity and that azido- or fluoro-substitutions at this position of D- and L-dideoxynucleoside analogs, could confer an increased therapeutic potential towards NNI-resistant HIV-1 RT mutants. Moreover, we have provided an important rationale for the utilisation in anti-HIV chemotherapy of D- and L-dideoxynucleotides analogs in combination with NNI, due to the lack of significant cross-resistance.

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