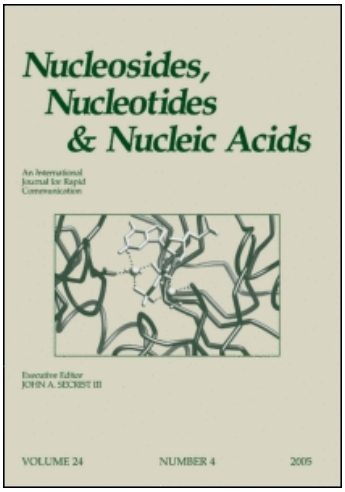

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Structural Determinants of HIV-1 Reverse Transcriptase Stereoselectivity Towards (β)-L-Deoxy- and Dideoxy-Pyrimidine Nucleoside Triphosphates: Molecular Basis for the Combination of L-Dideoxynucleoside Analogs with Non-nucleoside Inhibitors in Anti HIV Chemotherapy

G. Maga[a]; M. Amacker[b]; U. Hübscher[b]; G. Gosselin[c]; J-L. Imbach[c]; C. Mathé[c]; A. Faraj[d]; J. P. Sommadossi[d]; S. Spadari[a]
[a] Consiglio Nazionale delle Ricerche, Istituto di Genetica Biochimica ed Evoluzionistica, Pavia, Italy [b] Institute of Veterinary Biochemistry, University of Zürich-Irchel, Zürich, Switzerland [c] Laboratoire de Chimie Bioorganique, UMR CNRS-USTL 5625, Université Montpellier II, Montpellier Cedex 5, France [d] Department of Pharmacology, University of Alabama at Birmingham, Birmingham, AL, USA

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STRUCTURAL DETERMINANTS OF HIV-1 REVERSE TRANSCRIPTASE STEREOSELECTIVITY TOWARDS (β)-L-DEOXY- AND DIDEOXY - PYRIMIDINE NUCLEOSIDE TRIPHOSPHATES: MOLECULAR BASIS FOR THE COMBINATION OF L-DIDEOXYNUCLEOSIDE ANALOGS WITH NON-NUCLEOSIDE INHIBITORS IN ANTI HIV CHEMOTHERAPY.

G. Maga*[1], M. Amacker[2], U. Hübscher[2], G. Gosselin[3], J.-L. Imbach[3], C. Mathé[3], A. Faraj[4], J.P. Sommadossi[4] and S. Spadari[1]

[1] Istituto di Genetica Biochimica ed Evoluzionistica, Consiglio Nazionale delle Ricerche, I-27100, Pavia (Italy)
[2] Institute of Veterinary Biochemistry, University of Zürich-Irchel, CH-8050, Zürich (Switzerland)
[3] Laboratoire de Chimie Bioorganique, UMR CNRS-USTL 5625, Université Montpellier II, F-34095, Montpellier Cedex 5 (France)
[4] Department of Pharmacology, University of Alabama at Birmingham, AL-35294, Birmingham (USA)

ABSTRACT: We have compared the HIV-1[1] RT mutants containing the single substitutions L100I, K103N, V106A, V179D, Y181I and Y188L, known to confere NNI-resistance in treated patients, to HIV-1 RT wt for their sensitivity towards inhibition by D- and L-deoxy- and dideoxy-nucleoside tiphosphates. The results showed a differential effect of the substitutions on the affinity for both D- and L-enantiomers of deoxy- and dideoxy-nucleoside triphosphates and provide a rationale for the utilization of L-dideoxynucleoside analogs with NNI in combination chemotherapy.

INTRODUCTION

The high viral turnover and mutation rate of HIV-1 create the potential for drug resistant viruses to appear rapidly under the selective pressure of antiretroviral therapy (1). Thus, detection of viral resistance to one or more drugs used in combination therapy may represent an early marker of therapy failure (2). For this reasons, information on patterns of cross-resistance between antiretroviral drugs is of great importance in order not to limit therapeutic options by using drug combinations early in the treatment which select

for cross-resistant viral variants. In order to try to provide the clinicians with useful indications for the optimization of therapy protocols, we focused our attention on the possible effects of the Nevirapine resistance mutations L100I, K103N, V106A, V179D, Y181I and Y188L (3, 4) on the stereoselectivity of HIV-1 RT, namely the ability to recognize as substrates both optical D- and L-enantiomers of nucleoside triphosphates (5, 6, 7). This, because the recent introduction in anti-HIV chemotherapy of a L-deoxycytidine analog, 3TC, which proved to be particularly effective in combination with AZT (8), indicates that L-nucleosides analogs might be a very promising class of RT inhibitors and anti-HIV drugs (9, 10, 11). However, very little is known about their possible cross-resistance with other classes of anti-RT drugs, such as the non-nucleoside inhibitors.

RESULTS AND DISCUSSION

Location of the mutations analysed in the crystal structure of HIV-1 RT.

For this study, we used six different recombinant HIV-1 RT carrying single point mutations known to confer Nevirapine resistance *in vivo*. All the mutations are located in different β-sheets or connecting loops within the so-called NNI-binding pocket (12). The structure of this pocket, as well as the position of the secondary structure elements containing the mutations analysed are indicated in **FIG.** 1. The mutations L100I and K103N are in the β5a- β6 loop of the p66 subunit of HIV-1 RT. Mutation V106A is part of the β6, whereas mutations V179D and Y181I affect amino acids of the β9 structural element. Finally, mutation Y188L is within the β10. All these mutations are outside, but very close to the active site of HIV-1 RT, which is constituted by D110 on β6, Y183 on β9, M184 on the β9- β10 connecting loop and by D185 and D186 on β10.

NNI-resistance mutations differentially affect D-nucleoside triphosphate binding by HIV-1 RT during RNA- or DNA-dependent DNA synthesis.

The different HIV-1 RT mutants were compared to RT wt for their ability to utilize as substrate the D-enantiomer of dTTP (D-dTTP) on poly(rA)/ or poly(dA)/oligo(dT) template-primer homopolymers. **FIG.** 2 illustrates the results of the experiments and the corresponding K_m, K_i and K_i/K_m values are summarized in **TABLE** 1. The affinity for D-

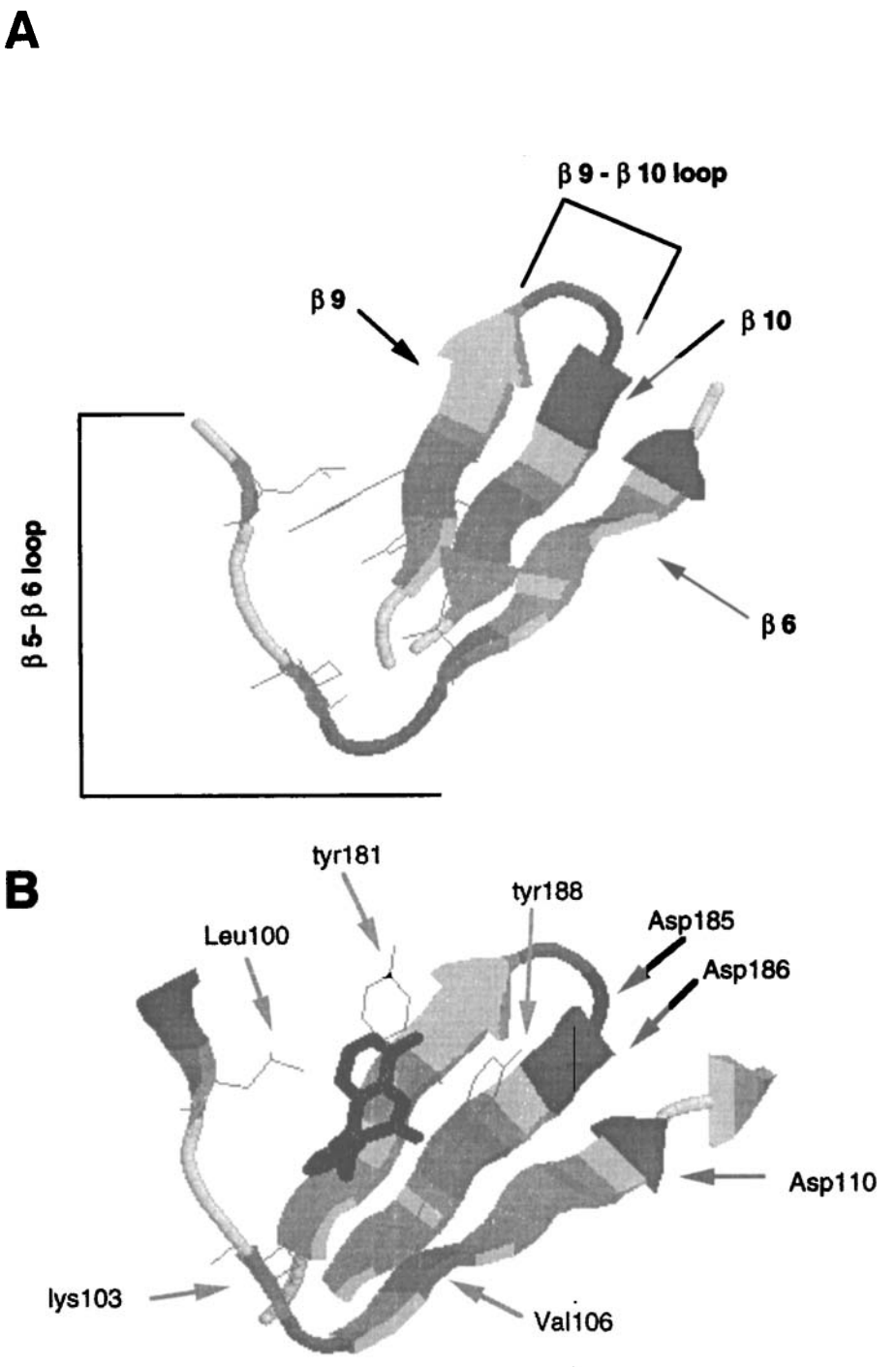

FIGURE 1. Structure of the NNI binding site of the p66 subunit of HIV-1 RT. A: secondary structure elements of the NNI binding site in the unliganded HIV-1 RT. B: position of the NNI-resistance mutations in the structure of the Nevirapine-RT complex according to Ren et al. (12).

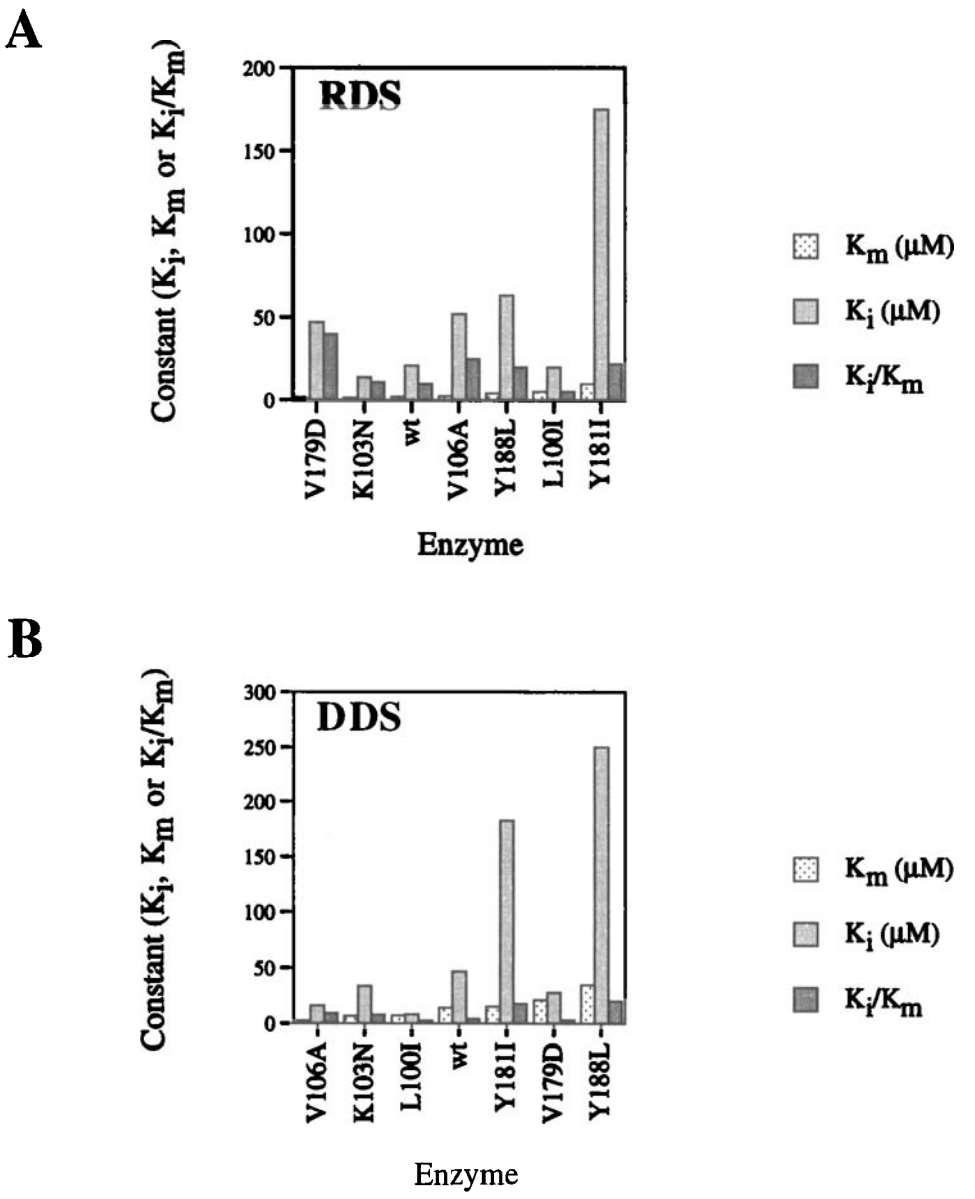

FIGURE 2 . Effect of NNI-resistance mutations on the utilisation of D- and L-dTTP by HIV-1 RT. A: kinetic parameters for D- and L-dTTP utilisation by HIV-1 RTwt and mutants on RNA-dependent DNA synthesis. B: kinetic parameters for D- and L-dTTP utilisation by HIV-1 RTwt and mutants on DNA-dependent DNA synthesis.

Table 1. Sensitivity of HIV-1 RT wt and mutants[a] to D- and L-(β)-dTTP on RNA- and DNA-dependent DNA synthesis.

	RDS[b]			DDS[b]		
RT	dTTP	L-dTTP		dTTP	L-dTTP	
	K_m (μM)	K_i (μM)	K_i/K_mK_m	K_i (μM)	K_i/K_m (μM)	
wt	2	21	10.5	14	47	3.3
L100I	4.8	20	4.2	7.2	8	1.1
K103N	1.3	14	10.7	6.5	34	5.2
V106A	2.3	52	22.6	2.5	16	6.4
V179D	1.2	47	39.2	21	28	1.3
Y181I	10	175	17.5	10	183	18
Y188L	4	63	15.7	35	250	7.1

[a]. Assays were performed with poly(rA)/oligo(dT)$_{10:1}$ or poly(dA)/oligo(dT)$_{10:1}$ as described in Material and Methods.
[b]. RDS, RNA-dependent DNA synthesis; DDS, DNA-dependent DNA synthesis.

dTTP on the RNA/DNA template-primer was not affected by the mutations analysed with the exceptions of the mutants L100I and Y181I, which showed an increase in the K_m value with respect to RT wt of 2.5 and 5-fold, respectively, and the mutants V179D and K103N, which showed an almost 2-fold increased affinity for D-dTTP in comparison to RT wt (**FIG.** 2 A). When a DNA/DNA template-primer was used, the affinity for D-dTTP was lower for all the enzymes tested with the exception of mutants V106A and Y181I, which showed no differences (**FIG.** 2 B). Mutants L100I, K103N and V106A showed an increase in the affinity for D-dTTP with respect to RT wt from 2-fold to 5.5-fold respectively, whereas the mutant Y181I did not show significant differences in the K_m for D-dTTP when compared to RT wt on this template (**TABLE** 1). These results then suggested that the β5a- β6 loop is important for nucleoside triphosphate recognition,

since the mutation K103N resulted in an increase in D-dTTP affinity on both RDS and DDS. The mutation L100I located in the same loop, on the other hand, showed opposite effects depending on the structure of the template-primer, suggesting a repositioning of the L100 side chain during the switch from RDS to DDS. A critical role of the L100 residue for efficient catalysis has been already suggested (4). The observed increased in affinity for D-dTTP of the V106A mutant during DDS but not RDS, suggests a possible rearrangement of the position of the β-sheet β6, depending on the template-primer structure. The Y181I mutation located on β9 seemed to affect D-dTTP affinity specifically during RDS, whereas the Y188L mutation on β10 showed an opposite behaviour. The two β-sheets β9 and β10 in the p66 subunit of HIV-1 RT comprise the highly conserved motif YMDD (aa 183-186) essential for catalysis (12). Thus, it is conceivable that mutations very close to this motif would also affect nucleoside triphosphate incorporation.

Mutations in defined secondary structure elements of HIV-1 RT p66 differentially affect stereoselectivity depending on the nature of the template-primer.

Similar experiments were performed with the L-enantiomer of dTTP (L-dTTP). The results are illustrated in **FIG.** 2 and the corresponding K_m, K_i and K_i/K_m values are summarized in **TABLE** 1. Affinity for L-dTTP on an RNA/DNA template-primer decreased in the order wt < (i.e. lower Ki value) V179D < V106A < Y188L < Y181I (**FIG.** 2 A). When a DNA/DNA template-primer was used (**FIG.** 2 B), mutations L100I, V106A and showed respectively a 6-fold 3-fold and 1.5-fold increased affinity for L-dTTP with respect to RT wt, whereas mutations Y181I and Y188L showed again 5- and 6-fold reduced affinity, respectively (**TABLE** 1). As observed in the case of D-dTTP, mutations affecting the residues Y181 and Y188 on the β9 and β10 structural elements negatively influenced L-dTTP recognition. However, it must be noted that the Y181I mutant showed a remarkable decrease in the affinity for L-dTTP on a DNA/DNA template-primer, whereas this mutation had almost no effect on the affinity for D-dTTP on the same template, suggesting a specific impairment of the Y181I mutant in recognition of L-deoxynucleotides. Mutants L100I and V106A showed an increased affinity for L-dTTP on DDS but not on RDS, as observed with D-dTTP, again suggesting

a role for the β5a- β6 loop and β6 elements in nucleoside triphosphate recognition and template repositioning in the switch from RDS to DDS. In general, the stereoselectivity indexes (K_i/K_m values) of the enzymes tested decreased from RDS to DDS. The mutant V179D was 4-fold more stereoselective than wt on a RNA/DNA template-primer (**FIG.** 2 A) and 3-fold less stereoselective on a DNA/DNA template-primer (**FIG.** 2 B). Mutant L100I, on the other hand, showed a 2.5- to 3-fold lower K_i/K_m values than RT wt on both templates. Conversely, mutant V106A showed a 2-fold increase in stereoselectivity with respect to RT wt on both RDS and DDS (**FIG.** 2 A and B). In all these cases, the stereoselectivity indexes reflected the different effects on the affinity on both D- and L-dTTP observed with these mutants. The only mutation which seemed to affect specifically L-dTTP recognition, without any effect on D-dTTP binding, was the Y181I substitution on DDS, which showed a 6-fold increase in the K_i/K_m value when compared to RT wt. Thus, stereoselectivity of HIV-1 RT seemed to be affected by mutations on the β5a- β6 connecting loop and the β-sheets β6 and β9.

The 3' hydroxyl group in the sugar moiety of the nucleoside triphosphate substrate can influence the stereospecific recognition of D- and L-nucleoside triphosphates by HIV-1 RT wt and the Y181I mutant.

In order to identify possible structural elements in the nucleoside triphosphate substrate important for stereoselective recognition, RT wt and the Y181I mutant were tested for their ability to recognize the D- and L-enantiomers of dCTP and its 2',3' dideoxy derivative, ddCTP. The results are shown in **FIG.** 3. Both RT wt and the Y181I showed preference for the D-enantiomer of dCTP, with Y181I being 5-fold more stereoselective than RT wt (**FIG.** 3 A). However, both enzymes showed increased affinity for the D- and L-enantiomers of ddCTP, with Y181I recognizing the L-2',3'-dideoxy derivative almost 40-fold better than the corresponding L-2'deoxynucleotide. Remarkably, in the case of ddCTP, the stereoselectivity indexes were comparable for the two enzymes, which proved to be able to recognize D- and L-ddCTP with almost the same efficiency (**FIG.** 3 B). These results suggested that the 3' hydroxyl group in the sugar moiety of L-nucleoside triphosphates is one major structural determinant of the stereoselectivity observed for HIV-1 RT wt and the Y181I mutant.

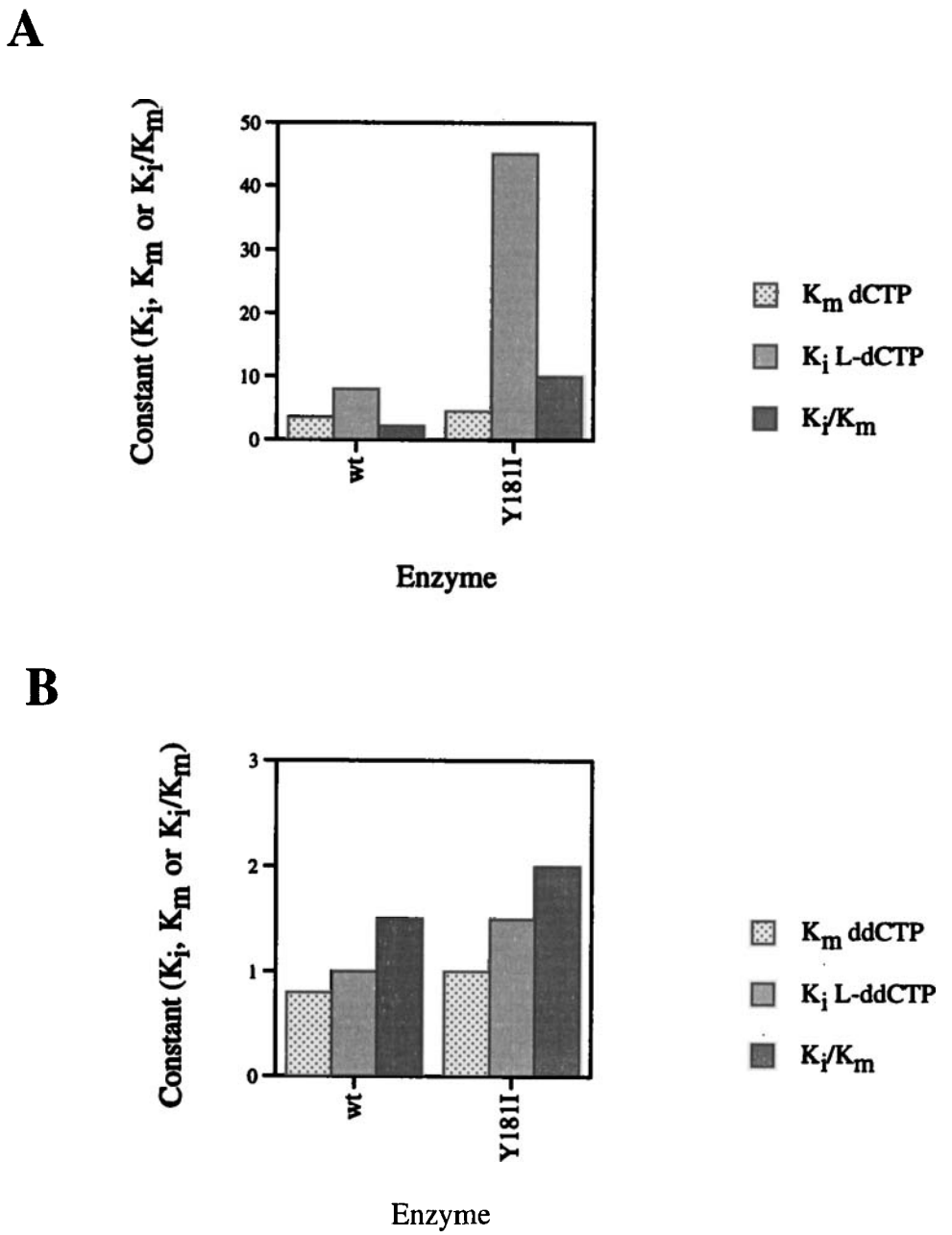

FIGURE 3. Sensitivity of HIV-1 RTwt and Y181I towards the D- and L-enantiomers of dCTP and ddCTP on DNA-dependent DNA synthesis. A: kinetic parameters for D- and L-dCTP utilisation. B: kinetic parameters for D- and L-ddCTP utilisation.

In summary, the present study showed that defined structural elements in the region of the RT active site play a role in modulating nucleoside triphosphate recognition and stereoselective discrimination. However, our results also suggest that no significant cross-resistance of NNI-resistant RT mutants has to be expected towards L-2'3' dideoxynucleosides, thus providing a rationale for their utilization in anti HIV-1 combination therapy[2].

EXPERIMENTAL SECTION

Chemicals. [^{3}H] dTTP or dCTP (40 Ci/mmol) were from Amersham. L-dTTP, L-dCTP and L-ddCTP were prepared by a standard phosphorylation method from their corresponding nucleosides. The 5'-triphosphates derivatives were fully characterized by nuclear magnetic resonance (^{1}H, ^{31}P), fast atom bombardment mass spectrometry, high performance liquid chromatography and UV spectroscopy. Unlabelled dNTP's 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 nucleoside triphosphates of 10:1, to the complementary oligomer oligo(dT)$_{12\text{-}18}$ or oligo(dC)$_{12\text{-}18}$ (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.

HIV-1 RT DNA polymerase activity assay. RNA- and DNA-dependent DNA polymerase activities were assayed as follows: a final volume of 25 µl contained reaction buffer (50 mM Tris-HCl pH 7.5, 1 mM DTT, 0.2 mg/ml BSA, 4% glycerol), 10 mM $MgCl_2$, 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. Reactions were incubated at 37°C for 15 min. 20 µl-aliquots were then spotted on glass fiber filters GF/C which were immediately immersed in 5% ice-cold TCA. Filters were washed twice in 5% ice-cold TCA and once in ethanol for 5 min, dried and acid-precipitable radioactivity was quantitated by scintillation counting.

Steady-state kinetic measurements and kinetic parameters calculation. 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 dTTP or dCTP into poly(rA)/oligo(dT), poly(dA)/oligo(dT) or poly(dI)/oligo(dC) at different nucleoside triphosphate substrate concentrations was monitored by removing 25 µl-aliquots at 2-min time intervals. Initial velocities of the reaction, determined by linear regression analysis of the data, were then plotted against the corresponding substrate concentrations. Inhibition assays were performed under the conditions described for the HIV-1 RT DNA- and RNA-dependent DNA polymerase activity assay in the presence of increasing amounts of the inhibitors. 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. Data were then plotted according to Lineweaver-Burke and Dixon.

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FOOTNOTES

[1]. Abbreviations used were:

HIV-1, human immunodeficiency virus type 1

RT, reverse transcriptase

NNI, non-nucleoside inhibitors

DDS, DNA-dependent DNA synthesis

RDS, RNA-dependent DNA synthesis

dTTP, deoxythymidine triphosphate

dCTP, deoxycytidine triphosphate

ddCTP, 2',3'-dideoxycytidine triphosphate

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