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Enzymatic properties and sensitivity to inhibitors of human immunodeficiency virus Type 1 (HIV-1) reverse transcriptase with Glu-138→Arg and Tyr-188→His mutations

Hong Zhang[a,c], Lotta Vrang[a], Kristina Bäckbro[b], Torsten Unge[b], Rolf Noréen[a], Bo Öberg[a,c,*]

[a]*Medivir AB, Lunastigen 7, S-141 44 Huddinge, Sweden*
[b]*Department of Molecular Biology, BMC, Box 590, S-751 24 Uppsala, Sweden*
[c]*Department of Virology, Karolinska Institute, c/o SBL, S-105 21 Stockholm, Sweden*

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Abstract

Two mutants of HIV-1 reverse transcriptase (RT), Tyr-188→His and Glu-138→Arg have been prepared and their catalytic properties and sensitivities to inhibitors studied. As compared to wild type RT, a reduction in catalytic efficiency and turn over number was observed, especially for the Tyr-188→His mutant. The non-nucleoside inhibitors nevirapine, L-697,661 and 9-Cl-TIBO caused a mixed type of inhibition of RT (Arg-138) with respect to substrate, and with the exception of a non-competitive inhibition by nevirapine, also a mixed type of inhibition of RT (His-188). Foscarnet (PFA) caused a non-competitive type of inhibition of RT (Arg-138) and a mixed inhibition of RT (His-188). The inhibition by ddG-TP was competitive with both mutant RTs. Inhibition by nevirapine gave IC_{50} values of 0.15, 0.23 and 0.72 μM; by 9-Cl-TIBO of 0.20, 2.50 and 10.3 μM; by L-697,661 of 0.064, 0.28 and 0.60 μM; by ddGTP of 0.13, 0.14 and 0.02 μM; by PFA of 17.0, 48.0 and 15.0 μM for RT wt, RT (Arg-138) and RT (His-188), respectively.

Key words: HIV reverse transcriptase; Mutant; Enzyme kinetics; Inhibitor

*Corresponding author. Fax: +46 8 774 95 19.

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1. Introduction

Several chemically distinct but mechanistically similar non-nucleoside inhibitors acting directly on HIV-1 reverse transcriptase (RT) have been reported, such as 9-Cl-TIBO (Pauwels et al., 1990), nevirapine (BI-RG-587) (Merluzzi et al., 1990), L-697,661 (Goldman et al., 1991), BHAP (Romero et al., 1991), α-APA (Pauwels et al., 1993), TSAO (Balzarini et al., 1992a), and PETT (Ternansky et al., Sixth International Conference on Antiviral Research, Venice, 1993). These compounds inhibit HIV-1, but in most cases not HIV-2, non-competitively with high potency and low toxicity. However, many have caused a rapid selection of resistant virus. This type of inhibitors has recently been reviewed by De Clercq (1993) and Young (1993).

Many observations have indicated that the non-nucleoside inhibitors bind to HIV-1 RT at a common binding pocket and that resistance to one compound results in cross-resistance to other inhibitors (Grob et al., 1992; Sardana et al., 1992; Shih et al., 1991; Boyer et al., 1993). Changes in amino acids 181 (Tyr→Cys, Ser, His) and 188 (Tyr→His, Cys) in RT result in resistance to several non-nucleoside RT inhibitors (Sardana et al., 1992) and changes in amino acid 138 (Glu→Arg, Lys) have been observed in virus grown in the presence of a TSAO compound (Balzarini et al., 1993a and b), and one PETT compound (L. Vrang, unpublished observation).

We have prepared different HIV-1 reverse transcriptases with point mutations in an attempt to rapidly identify the mechanism of action of new inhibitors and to guide the rational design of inhibitors active against various mutants of HIV-1. HIV-1 RT preparations with mutations Leu-100→Ile and Tyr-181→Cys have earlier been prepared and used in the design and evaluation of new inhibitors (Zhang et al., 1993). Goldman et al. (1993) have reported the use of RT mutants in the development of new specific non-nucleoside RT inhibitors with increased potencies by using RT mutants Lys-103→Asp and Tyr-181→Cys and double-mutants. RT mutants with resistance against non-nucleoside inhibitors have recently been summarized by De Clercq (1993).

In this paper we report the construction of the HIV-1 RT mutants Glu-138→Arg, Lys- and Tyr-188→His and the evaluation of their enzymatic properties and sensitivity to several inhibitors. The reasons for studying these RT mutants are the importance of the Tyr-188→His mutation for resistance against several inhibitors and the surprising location of the Glu-138→Arg, Lys mutation.

2. Materials and methods

2.1. Materials

The template-primers $(rA)_n \cdot (dT)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ were purchased from Pharmacia LKB Biotechnology AB, Uppsala, Sweden. 16S and 23S ribosomal RNA templates and specific 15-mer primer (5′-TAACCTTGCGGCCGT-3′) were generous gifts from Dr. Richard Jaskunas, Eli Lilly Co., Indianapolis, USA. Activated calf thymus DNA was prepared by treating calf thymus DNA with pancreatic DNase as described by Schlabach et al. (1971).

Tritium-labelled deoxyribonucleotides, dGTP and dTTP, were obtained from Amersham, UK. The specific activities were 37 Ci/mmol and 110 Ci/mmol, respectively. The four deoxyribonucleoside triphosphates (dATP, dCTP, dTTP, dGTP), trisodium phosphonoformate (PFA), and 2′,3′-dideoxyguanosine triphosphate (ddGTP) were purchased from Sigma Chemicals, St. Louis, MO, USA.

9-Cl-TIBO was purchased from Pharmatech Int. Inc., New Jersey, USA, L-697,661 (3-{[(4,7-dichloro-1,3-benzoxazol-2-yl)methyl]amino}-5-ethyl-6-methylpyridin-2(1H)-one) was kindly supplied by Dr. M.E. Goldman at Merck, Sharp and Dohme Research Laboratories, Rahway, NY, USA. Nevirapine was synthesized according to published methods. Stock solutions of 9-Cl-TIBO, L-697,661 and nevirapine were made in dimethyl sulfoxide (DMSO).

2.2. Enzyme preparations and assays

Construction of expression systems and preparation of HIV-1 RT have been described by Zhang et al. (1993). Briefly, single-stranded DNA was isolated from the plasmid pRT.BS, which contains the RT sequence. Oligo-directed site-specific mutagenesis was done according to the method by Sayers et al. (1988). Glu-138 (GAG) was mutated to Lys (AAG) and to Arg (AGG). Tyr-188 (TAT) was mutated to His (CAT). The mutations were confirmed by sequence analysis. The expression vectors for the mutant forms of RT were constructed by ligation of the mutated RT sequences to the plasmid pET11a using the *Nde*I and *Bam*HI sites for directional ligation (Studier et al., 1990). Expression was performed in the *E. coli* strain BL21(DE3). Transformed cells were grown to $OD_{550} = 1.0$ before induction with IPTG. The cells were harvested after 3 h of induction. The mutant RT p66/p51 heterodimer was purified using Q Sepharose, heparin-Sepharose and S Sepharose from Pharmacia (Bhikhabhai et al., 1992). The RT material was analyzed by SDS-PAGE and found to be more than 90% pure.

The RT assay using the homopolymeric template primers $(rA)_n \cdot (dT)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ contained 100 mM Tris-HCl (pH 7.8), 100 mM KCl, 4 mM DTT, 4 mM $MgCl_2$ and 250 μg/ml bovine serum albumine. The template primer concentrations were saturated at 20–25 μg/ml. The DMSO concentration in the assays, originating from dissolved inhibitors, was 1%.

Assay conditions using the heteropolymeric template-primers, 10 μg/ml ribosomal RNA-oligo DNA or 125 μg/ml activated calf thymus DNA, were the same as for homopolymeric template primers with the exception that 50 mM KCl and 6 mM $MgCl_2$ were used for the RNA template-primer, and 100 mM KCl and 20 mM $MgCl_2$ for the activated DNA template-primer.

Reactions were started by addition of enzyme to the final concentrations of 250 ng/ml of RT (wt); 250 ng/ml of RT (Arg-138) and 375 ng/ml of RT (His-188). After 30 min incubation at 37°C, 45 μl reaction mixture was spotted onto filter discs (Munktell, Sweden), washed in 5% TCA and ethanol, dried and counted in a scintillation counter.

2.3. Enzyme kinetics

Enzyme kinetic properties were evaluated under apparent first-order conditions.

The initial velocities of incorporation of deoxynucleotides were linear with respect to time. Enzyme kinetic constants were determined from Lineweaver-Burk transformation plots by $1/V$ vs. $1/S$, where V is velocity and S substrate concentration and expressed as K_m (Michaelis constant) and k_{cat}/K_m (catalytic efficiency), [$V_{max} = k_{cat}$ (Eo)].

3. Results

3.1. Expression of mutant enzymes

The three mutants, RT (Lys-138), RT (Arg-138) and RT (His-188), were expressed to the same level in the bacteria, as determined by SDS-PAGE (not shown). The mutants, RT (Arg-138) and RT (His-188) could be purified to better than 90% homogeneity, but the mutant RT (Lys-138) could not be isolated because of sensitivity to degradation.

3.2. Characterization of the enzymatic properties of wild type and mutant RT preparations

Enzyme kinetic studies were carried out under apparent first order conditions following Michaelis-Menten kinetics. The RT catalytic velocity was expressed as a function of substrate concentration and highly dependent upon the template-primer used. The enzymatic properties of RT (wt), RT (Arg-138) and RT (His-188) were expressed as k_{cat}/K_m (catalytic efficiency) and K_m (substrate affinity) using different template-primers and the results are summarized in Tables 1 and 2.

Using $(dC)_n(dG)_{12-18}$ as template-primer it was observed that the RT preparations had almost the same k_{cat}/K_m values. When $(rC)_n(dG)_{12-18}$ was used RT (His-188) showed a lower catalytic efficiency than RT (wt) and RT (Arg-138). The catalytic efficiency using $(rA)_n(dT)_{12-18}$ was also highest for RT (wt) and lowest for RT (His-188).

Table 1
Enzyme-kinetic constants for reverse transcriptases using homopolymeric template-primer

Reverse transcriptase	Template-primer	Substrate	K_m (μM)	k_{cat} (S^{-1})	k_{cat}/K_m ($S^{-1}\ \mu M^{-1}$)
HIV-1 RT (wt)	rAdT	dTTP	0.79	0.65	0.82
HIV-1 RT (Arg-138)	rAdT	dTTP	1.60	0.50	0.31
HIV-1 RT (His-188)	rAdT	dTTP	2.40	0.08	0.033
HIV-1 RT (wt)	rCdG	dGTP	0.22 ± 0.06[a]	0.08 ± 0.02	0.38 ± 0.06
HIV-1 RT (Arg-138)	rCdG	dGTP	0.41 ± 0.23	0.054 ± 0.01	0.15 ± 0.04
HIV-1 RT (His-188)	rCdG	dGTP	0.32 ± 0.13	0.012 ± 0.006	0.04 ± 0.005
HIV-1 RT (wt)	dCdG	dGTP	3.85 ± 1.30	1.76 ± 0.75	0.45 ± 0.04
HIV-1 RT (Arg-138)	dCdG	dGTP	6.10 ± 1.70	3.75 ± 0.85	0.62 ± 0.04
HIV-1 RT (His-188)	dCdG	dGTP	0.27 ± 0.02	0.14 ± 0.005	0.50 ± 0.02

[a] ± S.D. of at least two independent experiments.

Table 2
Enzyme-kinetic constants for reverse transcriptases using heteropolymeric template-primer

Reverse transcriptase	Template-primer	Substrate	K_m (μM)	k_{cat} (S^{-1})	k_{cat}/K_m (S^{-1} μM^{-1})
HIV-1 RT (wt)	rRNA	dGTP	0.25 ± 0.05[a]	0.05 ± 0.02	0.19 ± 0.06
HIV-1 RT (Arg-138)	rRNA	dGTP	0.44 ± 0.10	0.06 ± 0.02	0.12 ± 0.03
HIV-1 RT (His-188)	rRNA	dGTP	0.32 ± 0.04	0.01 ± 0.0004	0.03 ± 0.005
HIV-1 RT (wt)	rRNA	dTTP	0.12 ± 0.07	0.065 ± 0.02	0.54 ± 0.01
HIV-1 RT (Arg-138)	rRNA	dTTP	0.58 ± 0.05	0.11 ± 0.009	0.19 ± 0.03
HIV-1 RT (His-188)	rRNA	dTTP	0.43 ± 0.04	0.011 ± 0.001	0.025 ± 0.001
HIV-1 RT (wt)	act DNA	dGTP	0.17 ± 0.03	0.073 ± 0.009	0.45 ± 0.07
HIV-1 RT (Arg-138)	act DNA	dGTP	0.69 ± 0.33	0.16 ± 0.073	0.23 ± 0.00
HIV-1 RT (His-188)	act DNA	dGTP	0.72 ± 0.08	0.015 ± 0.005	0.02 ± 0.005
HIV-1 RT (wt)	act DNA	dTTP	0.31 ± 0.06	0.17 ± 0.04	0.55 ± 0.04
HIV-1 RT (Arg-138)	act DNA	dTTP	1.19 ± 0.02	0.41 ± 0.08	0.35 ± 0.07
HIV-1 RT (His-188)	act DNA	dTTP	0.57 ± 0.06	0.04 ± 0.005	0.062 ± 0.002

[a] ± S.D. of at least two independent experiments.

When using dTTP the substrate affinity was highest for RT (wt) and lowest for RT (His-188). The affinity of dGTP to the enzyme was dependent both on template-primer and enzyme, the highest affinity shown by RT (wt) using $(rC)_n(dG)_{12-18}$ and the lowest for RT (Arg-138) using $(dC)_n(dG)_{12-18}$.

With the heteropolymeric templates (Table 2), ribosomal RNA and activated calf thymus DNA, the highest k_{cat}/K_m was seen for RT (wt) and the lowest for RT (His-188). The rate of incorporation of dTTP did not differ appreciably from that of dGTP. The K_m values for RT (wt) were lower than those of the mutants for all template-primers used.

3.3. Inhibition of HIV-1 RT by nevirapine, 9-Cl-TIBO and L-697,661

Several structurally distinct but functionally similar non-nucleoside RT-specific inhibitors have been reported and some were used here to characterize the different HIV RT preparations.

Nevirapine caused mixed and non-competitive types of inhibition of RT (Arg-138) (Fig. 1) and RT (His-188) (not shown) and the kinetic constants for these enzymes as well as for wild type RT are summarized in Table 3.

RT (Arg-138) and RT (His-188) were 6.8- and 2.8-fold less susceptible than RT (wt) to inhibition by nevirapine when using $(rC)_n(dG)_{12-18}$ as primer-template as shown in Table 3. RT (Ile-100) and RT (Cys-181) were 34- and 330-fold less susceptible to nevirapine than RT (wt) when the K_{is} values were compared.

The inhibition of RT (Arg-138) (Fig. 2) and RT (His-188) (not shown) by 9-Cl-TIBO resulted in a mixed type of inhibition with regard to dGTP, which is summarized in Table 4. 9-Cl-TIBO showed strongly increased K_i values, 80-fold for K_{is} and

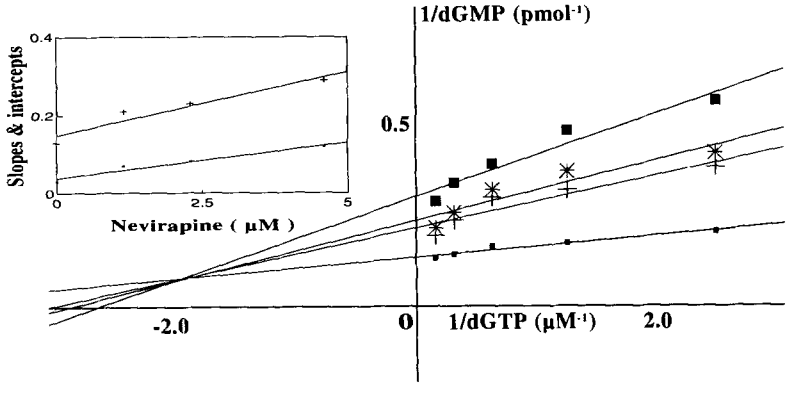

Fig. 1. Inhibition of HIV-1 RT (Arg-138) by nevirapine. Double-reciprocal plots of initial velocities of HIV-1 RT (Arg-138) using $(rC)_n(dG)_{12-18}$ as primer template in the presence of nevirapine at 0.0 (■–■), 1.4 (+–+), 2.28 (*–*) and 4.56 μM (■–■) give $K_m = 0.22$ μM for dGTP. The insert replot of slope (■–■) and intercept (+–+) vs. nevirapine gives $K_{is} = 2.05$ μM and $K_{ii} = 4.56$ μM.

100-fold for K_{ii} for RT (His-188) as compared to RT (wt).

The RT inhibitor L-697,661 caused a mixed type inhibition of both of RT (Arg-138) (Fig. 3) and RT (His-188) (not shown) and a non-competitive inhibition of RT (wt) (not shown) when $(rC)_n(dG)_{12-18}$ and dGTP were used (Table 5). Both RT (Arg-138) and RT (His-188) were partially resistant to L-697,661 as compared to the sensitivity of RT (wt).

For comparison the mixed inhibition by nevirapine, 9-Cl-TIBO and L-697,661 of RT preparations with two other point mutations, (Leu-100→Ile) and (Tyr-181→Cys) reported by Zhang et al. (1993), are also given in Tables 3–5.

Table 3
Inhibition of HIV-1 (wt) and mutant RT by nevirapine using $(rC)_n(dG)_{12-18}$ and ^{3}H-dGTP

Reverse transcriptase	K_m (μM)	K_{is} (μM)	K_{ii} (μM)	IC_{50} (μM)	Mode of inhibition
HIV-RT (wt)	0.61	0.30	0.31	0.15 ± 0.04[a]	non-competitive
HIV-RT (Ile-100)[b]	0.55	10.2	23.0	11.8	mixed
HIV-RT (Arg-138)	0.22	2.05	4.56	0.27 ± 0.05	mixed
HIV-RT (Cys-181)[b]	0.20	99.0	228	201 ± 23.3	mixed
HIV-RT (His-188)	0.40	0.84	0.91	0.72 ± 0.04	non-competitive

[a] ± S.D. of at least two independent experiments.
[b] Data from Zhang et al. (1993).

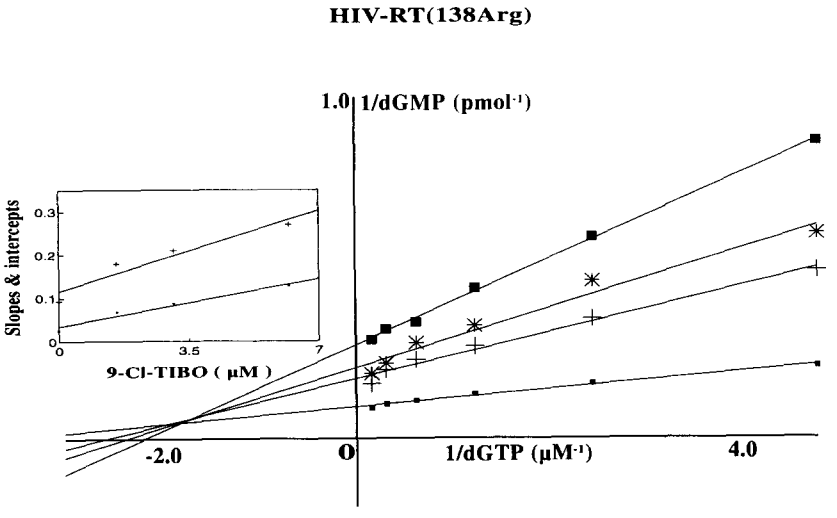

Fig. 2. Inhibition of HIV-1 RT (Arg-138) by 9-Cl-TIBO. Double-reciprocal plots of initial velocities of HIV-1 RT (Arg-138) using $(rC)_n(dG)_{12-18}$ as primer-template in the presence of 9-Cl-TIBO at 0.0 (■–■), 1.56 (+–+), 3.1 (*–*) and 6.2 μM (■–■) give $K_m = 0.30$ μM for dGTP. The insert replot of slope (■–■) and intercept (+–+) vs. 9-Cl-TIBO gives $K_{is} = 2.20$ μM and $K_{ii} = 4.30$ μM.

3.4. Inhibition of HIV-1 RT by PFA and ddGTP

PFA caused a non-competitive inhibition of RT (Arg-138), as shown in Fig. 4, and a mixed type inhibition of RT (His-188) (not shown). The inhibition constants were higher for the mutants than for wt enzyme when $(rC)_n(dG)_{12-18}$ was used as template-primer and ^{3}H-dGTP was the varied substrate. The Michaelis-Menten kinetic constants are presented in Table 6. A reduced inhibitory effect by PFA was observed for the (Arg-138) mutant. The RT (His-188) had properties similar to those of RT (wt).

The guanosine triphosphate analogue ddGTP was a competitive inhibitor of all RT preparations with respect to ^{3}H-dGTP. The effect of ddGTP on RT activity is

Table 4
Inhibition of HIV-1 (wt) and mutant RT by 9-Cl-TIBO using $(rC)_n(dG)_{12-18}$ and ^{3}H-dGTP

Reverse transcriptase	K_m (μM)	K_{is} (μM)	K_{ii} (μM)	IC_{50} (μM)	Mode of inhibition
HIV-RT (wt)	0.20	0.12	0.30	0.20 ± 0.05[b]	non-competitive
HIV-RT (Ile-100)[c]	0.44	32[a]	176[a]	32.2 ± 2.80	mixed
HIV-RT (Arg-138)	0.30	2.20	4.30	3.25 ± 1.0	mixed
HIV-RT (Cys-181)[c]	0.24	7.4	40	13.0 ± 7.40	mixed
HIV-RT (His-188)	0.17	9.50	31.0	10.3 ± 3.20	mixed

[a]Extrapolated values.
[b]± S.D. of at least two independent experiments.
[c]Data from Zhang et al. (1993).

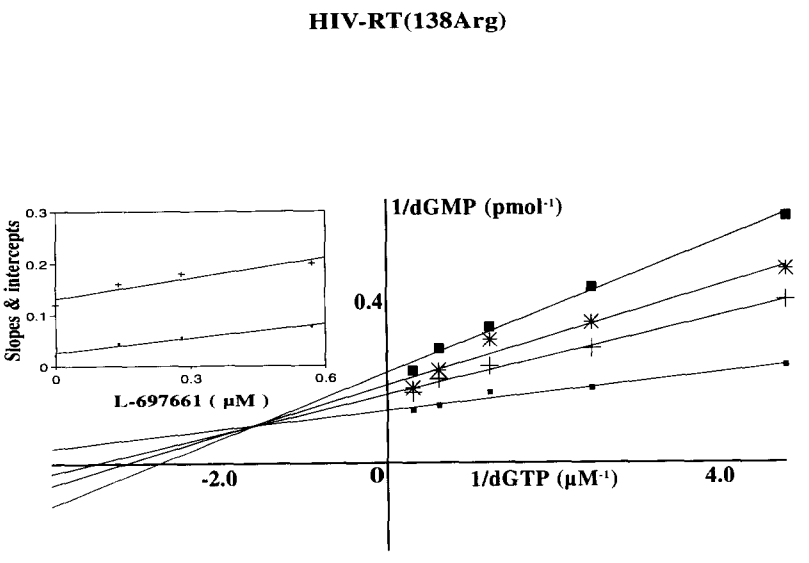

Fig. 3. Inhibition of HIV-1 RT (Arg-138) by L-697,661. Double-reciprocal plots of initial velocities of HIV-1 RT (Arg-138) using $(rC)_n(dG)_{12-18}$ as primer-template in the presence of L-697,661 at 0.0 (■–■), 0.14 (+–+), 0.28 (*–*) and 0.57 μM (■–■) give $K_m = 0.18$ μM for dGTP. The insert replot of slope (■–■) and intercept (+–+) vs. L-697,661 gives $K_{is} = 0.10$ μM and $K_{ii} = 0.35$ μM.

exemplified for RT (Arg-138) in Fig. 5. The kinetic constants are presented in Table 7. ddGTP was more inhibitory to RT (His-188) than to RT (wt).

For comparison the inhibition by PFA and ddGTP of the RT (Leu-100→Ile) and RT (Tyr-181→Cys) are given in Tables 6 and 7.

4. Discussion

Resistance development is a severe obstacle to the development of useful drugs

Table 5
Inhibition of HIV-1 (wt) and mutant RT by L-697,661 using $(rC)_n(dG)_{12-18}$ and ^{3}H-dGTP

Reverse transcriptase	K_m (μM)	K_{is} (μM)	K_{ii} (μM)	IC_{50} (μM)	Mode of inhibition
HIV-RT (wt)	0.16	0.04	0.043	0.064 ± 0.014[a]	non-competitive
HIV-RT (Ile-100)[b]	0.34	5.70	12.0	0.85 ± 0.68	mixed
HIV-RT (Arg-138)	0.18	0.10	0.35	0.24 ± 0.05	mixed
HIV-RT (Cys-181)[b]	0.28	12.0	24.0	7.70 ± 2.80	mixed
HIV-RT (His-188)	0.50	0.64	0.97	0.60 ± 0.24	mixed

[a] ± S.D. of at least two independent experiments.
[b] Data from Zhang et al. (1993).

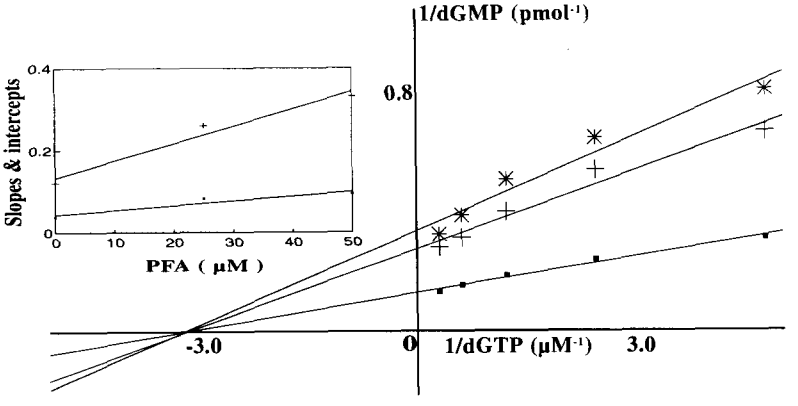

Fig. 4. Inhibition of HIV-1 RT (Arg-138) by PFA. Double-reciprocal plots of initial velocities of HIV-1 RT (Arg-138) using $(rC)_n(dG)_{12-18}$ as primer-template in the presence of PFA at 0.0 (■–■), 25 (+–+) and 50 (*–*) μM give $K_m = 0.32$ μM for dGTP. The insert replot of slope (■–■) and intercept (+–+) vs. PFA gives $K_{is} = K_{ii} = 39$ μM.

Table 6
Inhibition of HIV-1 (wt) and mutant RT by PFA using $(rC)_n(dG)_{12-18}$ and ^{3}H-dGTP

Reverse transcriptase	K_m (μM)	K_{is} (μM)	K_{ii} (μM)	IC_{50} (μM)	Mode of inhibition
HIV-RT (wt)	0.23	6.80	21.0	17.0 ± 14.0[a]	mixed
HIV-RT (Ile-100)[b]	0.65	25.0	52.0	29.0 ± 7.50	mixed
HIV-RT (Arg-138)	0.32	39.0	38.0	39.0 ± 9.0	non-competitive
HIV-RT (Cys-181)[b]	0.39	23.0	50.0	24.0 ± 5.0	mixed
HIV-RT (His-188)	0.22	11.7	45.0	15.0 ± 5.0	mixed

[a] ± S.D. of at least two independent experiments.
[b] Data from Zhang et al. (1993).

Table 7
Inhibition of HIV-1 (wt) and mutant RT by ddG-TP using $(rC)_n(dG)_{12-18}$ and ^{3}H-dGTP

Reverse transcriptase	K_m (μM)	K_i (μM)	K_{i/K_m}	IC_{50} (μM)	Mode of inhibition
HIV-RT (wt)	0.20	0.29	1.45	0.13 ± 0.11[a]	competitive
HIV-RT (Ile-100)[b]	0.48	0.44	0.92	0.27 ± 0.05	competitive
HIV-RT (Arg-138)	0.55	0.16	0.29	0.12 ± 0.03	competitive
HIV-RT (Cys-181)[b]	0.29	0.30	1.03	0.11 ± 0.09	competitive
HIV-RT (His-188)	0.23	0.03	0.13	0.02	competitive

[a] ± S.D. of at least two independent experiments.
[b] Data from Zhang et al. (1993).

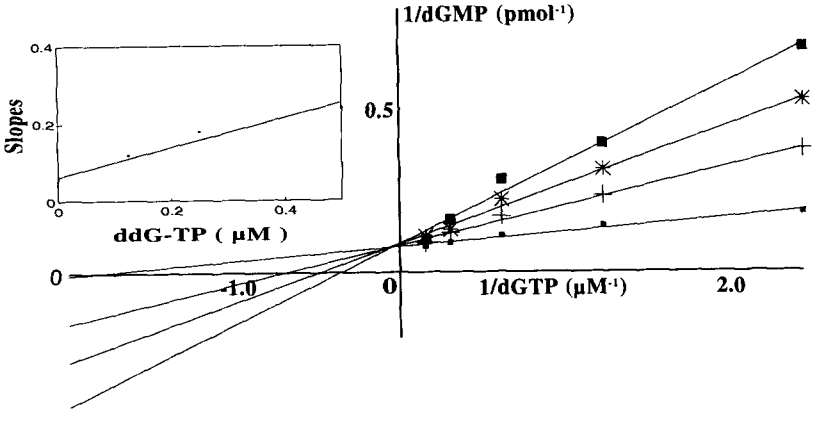

Fig. 5. Inhibition of HIV-1 RT (Arg-138) by ddGTP. Double-reciprocal plots of initial velocities of HIV-1 RT (Arg-138) using $(rC)_n(dG)_{12-18}$ as primer-template in the presence of ddGTP at 0.0 (■–■), 0.125 (+–+), 0.25 (*–*) and 0.5 μM (■–■) give $K_m = 0.55$ μM for dGTP. The insert replot of slope (■–■) vs. ddGTP gives $K_{is} = 0.16$ μM.

against HIV/AIDS. In the attempts to overcome the resistance problem, RT mutants are useful in the development of new inhibitors.

Several amino acid substitutions in the RT have been observed to cause resistance both in HIV-infected cell cultures and in patients treated with RT inhibitors. There is an obvious correlation between the selection of resistant mutants in cell cultures and in patients (Richman, 1993). The extensive cross-wise resistance to different non-nucleoside RT inhibitors observed in cell cultures for HIV mutants is also natural in view of the similar patterns of point mutations observed (Nanni et al., 1993).

HIV-1 RT's with mutations Tyr-188→His and Glu-138→Arg were constructed and purified to high purity (>90%). Attempts to purify RT (Lys-138) were not successful. RT (Lys-138) was expressed in *E. coli* to the same level as RT (Arg-138) but the material was degraded during purification. We do not yet know if the HIV-1 Glu-138→Lys mutation observed in cell cultures during resistance selection contains any mutation, in addition to the drastic change from Glu to Lys, which could compensate for the structural instability observed when this mutant RT was expressed in *E. coli*.

The wild type RT and the mutants were studied kinetically to compare the specific activities under first order conditions. Differences were seen as altered apparent K_m and k_{cat} values and in the altered catalytic efficiency (k_{cat}/K_m) as summarized in Tables 1 and 2. In general, K_m was lower for RT (wt) than for the RT mutants with

the exception of RT (His-188) which showed a lower K_m than the RT (wt) when $(dC)_n(dG)_{12-18}$ was used. The catalytic efficiency was influenced by the two point mutations as reflected by the highest k_{cat}/K_m values for RT (wt) and the lowest for RT (His-188). The k_{cat} values were considerably lower for RT (His-188) than for the other enzymes. The efficiency of catalysis (k_{cat}/K_m) differed slightly between homo- and heteropolymeric template-primers but the RT activity was dependent upon the templates used.

It is interesting to notice that RT (Arg-138) had the highest k_{cat} values of the RT mutants with both homo- and heteropolymeric templates, implying a minimal influence on the catalytic site on the p66 subunit. Scrutinizing the RT structure reported by Kohlstaedt et al. (1992) reveals a close proximity of Glu-138 in the p51 subunit to the nevirapine binding site on the p66 subunit in HIV-1 RT (B. Lindborg, unpublished data). Nanni et al. (1993) have also pointed out the location of Glu-138 in the p51 subunit close to the catalytic site, and to the non-nucleoside inhibitor binding pocket, of the p66 subunit.

The K_m values for RT (wt) (Table 2) are in close agreement with the values reported by Balzarini et al. (1992) using dTTP and dGTP and rRNA · DNA template primer. The kinetic properties of RT (Arg-138) and RT (His-188) have not been reported earlier.

Characterization of the sensitivity of wild type and mutant reverse transcriptases was done using nevirapine, 9-Cl-TIBO, L-697,661, PFA and ddGTP.

Merluzzi et al. (1990) found a K_i value of 200 nM for nevirapine which is close to our result of 300 nM (Table 3) for RT (wt). An IC_{50} value of 80 nM was reported by Tramontano and Cheng (1992) which is about half the value we observed for nevirapine. Debyser et al. (1991) reported that 9-Cl-TIBO inhibited HIV-1 RT activity with an IC_{50} of 600 nM when $(rC)_n(dG)_{12-18}$ and ^{3}H-dGTP were used. This value was higher than our observation ($IC_{50} = 200$ nM). We found the RT (wt) to be more sensitive to L-697,661, $IC_{50} = 0.069$ mM, than Dueweke et al. (1993) who reported an IC_{50} value of 0.67 μM, but it was in agreement with Sardana et al. (1992) who reported an IC_{50} of 0.052 μM when the assay included the homopolymeric template primer $(rC)_n(dG)_{12-18}$.

The sensitivity of RT (wt) to PFA when using $(rC)_n(dG)_{12-18}$ was somewhat higher, $IC_{50} = 17$ μM, than reported by Sardana et al. (1992), 8 μM, and by Tramontano and Cheng (1992), 3.9 μM. An IC_{50} value of 0.13 μM for ddG-TP on HIV-1 RT (wt) was the same as reported by Song et al. (1992).

In agreement with the reduced sensitivity in cell cultures of HIV-1 with a Glu-138→Lys mutation in RT (Balzarini et al., 1993a) we found the Glu-138→Arg mutation in RT to confer resistance of this mutant enzyme to TSAO compounds (Zhang, unpublished observation) as also been found with a crude bacterial lysate containing HIV-1 RT (Arg-138) (Balzarini et al., 1993b).

The Glu to Arg mutation of amino acid 138 in HIV-1 RT decreased the sensitivity to all the non-nucleoside RT inhibitors tested, but the difference to RT (wt) was not more than about 10-fold and only 2-fold for nevirapine. The sensitivity to ddGTP was not changed but RT (Arg-138) was less sensitive to PFA than RT (wt). Balzarini et al. (1993 a and b) have seen in cell cultures that the Lys-138 point

mutation in HIV-1 RT gives resistance to TSAO, 9-Cl-TIBO, L-697,661 but not to nevirapine and nucleoside analogs.

All the non-nucleoside RT inhibitors gave mixed types of inhibition of RT (Arg-138) (Figs. 2–6). PFA and ddGTP caused a non-competitive type and a competitive type of inhibition, respectively.

The His-188 mutation affects one of the aromatic amino acid groups involved in the binding of non-nucleoside RT inhibitors (Rice et al., 1993; Sardana et al., 1992; Nanni et al., 1993). This mutation resulted in a decreased sensitivity of the enzyme to non-nucleoside inhibitors, especially towards 9-Cl-TIBO. The sensivitity to PFA did not change. Interestingly, the sensitivity to ddGTP increased as compared to RT (wt). The possibility of increased sensitivities to other compounds is of considerable interest when resistance developes to one compound (St Clair et al., 1991; Chow et al., 1993).

Sardana et al. (1992) compared the relative inhibition (IC_{50} values) of RT (wt) and RT (His-188) by 9-Cl-TIBO, L-697,661, nevirapine and PFA and found that the sensitivity decreased by a factor of 20.9, 6.2, 1.1 and 0.038, respectively. The corresponding values observed in this study were 51.5, 9.4, 4.8 and 0.88 when $(rC)_n(dG)_{12-18}$ was used. The reason for the difference in sensitivity to PFA is not clear.

Access to several RT mutants increases the possibility to rapidly evaluate new compounds and to design inhibitors able to overcome the resistance problem as single drugs or as combinations.

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