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The role of T139 in the human immunodeficiency virus type 1 reverse transcriptase sensitivity to (+)-calanolide A

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Running title: Calanolide A sensitivity to HIV-1 T139 RT mutants

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List of non-standard abbreviations: NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs: non-nucleoside reverse transcriptase inhibitors; RT, reverse transcriptase; HIV, human immunodeficiency virus; ddGTP: 2',3'-dideoxyguanosine-5'-triphosphate; dGTP, 2'-deoxyguanosine-5'-triphosphate; dNTP, 2'-deoxynucleoside-5'-triphosphate; DTT, dithiothreitol; EDTA, ethylene diaminotetraacetate; BSA, bovine serum albumin; TSAO, *ter*butyldimethylsilyl aminooxathiole dioxide.

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ABSTRACT

The coumarins represent a unique class of non-nucleoside reverse transcriptase inhibitors (NNRTIs) that were isolated from tropical plants. (+)-Calanolide A, the most potent compound of this class, selects for the T139I resistance mutation in HIV-1 reverse transcriptase (RT). Seven RTs mutated at amino acid position 139 (A, K, Y, D, I, S and Q) were constructed by site-directed mutagenesis. The mutant T139Q enzyme retained full catalytic activity compared to wild-type RT, while the mutant T139I, T139S and T139A RTs retained only 85% to 50% of the activity. Mutant T139K, T139D and T139Y RTs had seriously impaired catalytic activities. The mutations in the T139I and T139D RTs were shown to destabilize the RT heterodimer. (+)-Calanolide A lost inhibitory activity (up to 20-fold) against the mutant T139Y, T139Q, T139K and T139I enzymes. All mutant enzymes retained marked susceptibility towards the other NNRTIs including nevirapine, delavirdine, efavirenz, the thiocarboxanilide UC-781, the quinoxaline GW867420X and the TSAO derivatives, and the nucleotide inhibitor ddGTP. The fact that the T139I RT (i) proved resistant to (+)-calanolide A, (ii) represents a catalytically efficient enzyme, and (iii) requires only a single transition point mutation (ACA →ATA) in codon 139 appears to explain why mutant T139I RT virus strains, but not virus strains containing other amino acid changes at this position, predominantly emerge in cell cultures under (+)-calanolide A pressure.

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INTRODUCTION

Polycyclic coumarins, originally isolated as natural products from several plants of the genus *Calophyllum*, have been demonstrated to be active against HIV-1 (for a review, see Yu et al., 2003). (+)-Calanolide A, the most potent compound of this class, has been evaluated in antiviral activity studies against non-nucleoside reverse transcriptase inhibitor (NNRTI)-resistant HIV-1 strains and related mutated RTs (Buckheit et al., 1999; Quan et al., 1999). Also, detailed enzyme kinetic studies on RT-inhibition by (+)-calanolide A have been performed. Unlike NNRTIs, which non-competitively inhibit RT with respect of the substrate and template/primer, (+)-calanolide A is at least partly competitive with respect to dNTP binding (Currens et al., 1996b). These findings suggest that (+)-calanolide A most likely interacts with RT in a manner that is mechanistically different from that of other NNRTIs described earlier. In this respect, (+)-calanolide A may represent a unique class of HIV-1-specific NNRTIs. Despite these kinetic differences with NNRTIs, (+)-calanolide A, like most NNRTIs, is inactive against HIV-2 strains or other (retro)viruses (Currens et al., 1996a; Kashman et al., 1992).

One of the major problems associated with the NNRTIs is the rapid emergence of drug resistant virus strains (Balzarini, 1999, 2004; De Clercq, 1999; Vandamme et al., 1998). Indeed, HIV-1 resistance to regular NNRTIs is primarily associated with mutations of amino acids that line the lipophilic NNRTI-specific binding pocket (Balzarini, 1999). (+)-Calanolide A selects in cell culture for the rather unusual T139I mutation in the HIV-1 RT (Buckheit et al., 1995). The mutant T139I HIV-1 strains are resistant to (+)-calanolide A but retain marked sensitivity to many other NNRTIs as well as several nucleoside RT inhibitors (NRTIs) (Buckheit et al., 1999). The T139 amino acid is part of the so-called β7-β8 loop, which comprises a six amino acid-

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motif denoted as SINNET. Whereas this loop is exposed to the solvent in the p66 subunit, the equivalent loop in p51 is snugly lodged into a cleft on the surface of the p66 subunit (Kohlstaedt et al., 1992). In fact, this loop, which is close to both the putative entrance to the NNRTI binding pocket and the active site (Fig. 1), is essential for the catalytic function of the p66 subunit as it is required to form a stable heterodimeric enzyme (Pandey et al., 2002; Pandey et al., 2001). It is also worth mentioning that resistance to the TSAO class of NNRTIs is achieved through mutation of E138 to lysine in the p51 β7–β8 loop (Balzarini et al., 1993a, 1993b). Due to the close proximity of T139 and E138 in HIV-1 RT, it is plausible that both (+)-calanolide A and TSAO derivatives share a similar site and/or mode of interaction with HIV-1 RT. Extending this analogy, (+)-calanolide A-resistance must arise from the T139I mutation taking place in the p51 subunit rather than in the p66 subunit. This is in sharp contrast with the vast majority of mutations in the NNRTI-binding pocket conferring resistance to other NNRTIs, which are due to substitutions occurring in the p66 subunit rather than in the p51 subunit. Recent reports on the mechanism of TSAO-resistance and structural modelling have suggested an influence of TSAO on RT dimerization, which places this compound in a unique position amongst the NNRTIs (Sluis-Cremer et al., 2000; Rodríguez-Barrios et al., 2001).

In this study we constructed seven different recombinant RT enzymes bearing a mutation at position 139 of RT and determined their catalytic activity as well as their resistance profiles against a variety of NNRTIs including (+)-calanolide A and TSAO. The data obtained provide a rationale for the finding that it is the T139I mutation and not any other substitution at position 139 of HIV-1 RT that emerges under (+)-calanolide A pressure in cell culture.

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MATERIALS AND METHODS

Compounds. [2',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide) derivatives of N^3-methylthymine (TSAO-m^3T) and thymine (TSAO-T) were synthesized as previous described (Péréz-Péréz et al., 1992). Nevirapine (BI-RG-587, dipyridodiazepinone) was obtained from Boehringer Ingelheim (Ridgefield, CT). Delavirdine [bis(heteroaryl)piperazine (BHAP)] U-90152) and efavirenz (DMP-266) were provided by Dr. R. Kirch (at that time at Hoechst AG, Frankfurt, Germany) and Dr. J.-P. Kleim (currently at GlaxoSmithKline, Stevenage, UK). The thiocarboxanilide derivative UC-781 was obtained from W.G. Brouwer (Middlebury, CT, and Guelph, Ontario, Canada). The quinoxaline GW420867X was provided by Dr. J.-P. Kleim (GlaxoSmithKline, Stevenage, UK). 2',3'-Dideoxyguanosine-5'-triphosphate (ddGTP) was obtained from Sigma Chemical Ltd. (St. Louis, MO). (+)-Calanolide A was delivered by Sarawak MediChem Pharmaceuticals Inc. (Sarawak, Malaysia).

Site-directed mutagenesis of HIV-1 reverse transcriptase. Mutant RT-enzymes containing the T139A, T139Q, T139Y, T139K, T139I, T139S or T139D mutations in both p66 and p51 subunits were derived from the RT sequence cloned in pKRT2His (D'Aquila and Summers, 1989). Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Westburg, Leusden, The Netherlands), as described previously (Pelemans et al., 1998). Two synthetic oligonucleotide primers (Sigma Chemical Ltd.) contained the desired mutation at amino acid position 139 of HIV-1 RT. The presence of the desired mutation was confirmed by sequencing of the RT gene on an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA), using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). For all

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mutants, the mutation was introduced in both p66 and p51 subunits. Only for the mutant T139I and T139D RT, the mutation was introduced solely in p66, solely in p51 or in both p66 and p51 subunits.

Construction of plasmids expressing mutant and wild-type recombinant HIV-1 RTs. Recombinant HIV-1 RTs were expressed from a two-plasmid co-expression system as previously described (Jonckheere et al., 1996). The p66 subunit of RT was expressed from pACYC66His and the p51 subunit from pKRT51. To construct wild-type and 139-mutated pACYC66His, wild-type and 139-mutated pKRT2His were digested with *Eco*RI and *Fsp*I and the RT-containing fragments were ligated into pACYC184 digested with *Eco*RI and *Sca*I. To construct wild-type and 139-mutated pKRT51, wild-type and 139-mutated pKRT2His were digested with *Nco*I and *Kpn*I and the RT-containing fragment was ligated into pKRT51 digested with *Nco*I and *Kpn*I.

Expression and purification of wild-type and mutant HIV-1 RT. Expression of recombinant HIV-1 RT was performed as described previously (Auwerx et al., 2004). LB medium (800 ml) containing 100 µg/ml ampicillin and 10 µg/ml tetracycline was inoculated with an overnight culture of *E. coli* JM109 transformed with both plasmids of the co-expression system and started at an OD_{600} of 0.1. The culture was grown at 37°C, induced with 1 mM final concentration of IPTG for expression of RT, and after centrifugation the pellet was stored at –20°C. Later, the bacterial cell pellet was resuspended in 15 ml lysis buffer (50 mM Na-phosphate buffer, 5 mM β-mercapto-ethanol, 0.9% glucose, 100 mM NaCl, 1mM PMSF, 10 µg/ml pepstatin, 10 µg/ml leupeptin and 10% glycerol) and passed through

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a SLM Aminco French Pressure Cell Press (Beun de Ronde, La Abcoude, The Netherlands). The lysate was centrifuged for 20 min at 17,000 g.

The purification of RT was performed as described previously (Auwerx et al., 2004). Briefly, the supernatant of the lysed bacterial cell culture was incubated with Ni-NTA resin (Qiagen). After sedimentation of the Ni-NTA resin with the bound $(His)_6$-tagged proteins, a column was formed and washed twice with a sodium phosphate buffer containing 10 mM imidazole. Then, the RT was eluted from the column with a sodium phosphate buffer containing 125 mM imidazole. The imidazole-containing buffer was exchanged by a Tris-HCl buffer and the eluate was concentrated to 2 ml using Ultrafree-15 centrifugal filtration devices (Millipore, Brussels, Belgium). The $(His)_6$-tagged RT was further purified to about 98% purity over a HiTrap Heparin column (Amersham Biosciences, Roosendaal, The Netherlands). All fractions containing heterodimer RT were pooled and stored in a 50% glycerol buffer at –20°C. Protein concentrations in these stock solutions were determined using the Bio-Rad Protein Assay (Bio-Rad, Nazareth, Belgium) with bovine serum albumin as standard.

Reverse transcriptase assay. For determination of the 50% inhibitory concentration (IC_{50}) of the test compounds against HIV-1 RT, the RNA-dependent DNA polymerase assay was performed as follows: the reaction mixture (50 µl) contained 50 mM Tris.HCl (pH 7.8), 5 mM DTT, 300 µM glutathione, 500 µM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 µg of bovine serum albumin, a fixed concentration of the labelled substrate [8-^{3}H]dGTP (1.6 µM, 1 µCi; specific activity, 12.6 Ci/mmol; Amersham Biosciences), a fixed concentration of the template/primer poly(rC)·oligo$(dG)_{12-18}$ (0.1 mM; Amersham Biosciences), 0.06% Triton X-100, 5 µl of inhibitor solution [containing various concentrations (10-fold dilutions) of the compounds], and 5 µl of the RT preparations that correspond to 8, 11, 6, 61, 575, 6, 9

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and 137 ng of enzyme (protein) for wild-type and the T139A, T139Q, T139Y, T139K, T139I, T139S and T139D mutant RTs, respectively. The reaction was initiated by addition of the enzyme, and the reaction mixtures were incubated at 37°C for 30 min, at which time 200 µl of yeast RNA (2 mg/ml) and 1 ml of trichloroacetic acid (5%, v/v) in 20 mM $Na_4P_2O_7$ were added. The solutions were incubated on ice for at least 30 min, after which the acid-insoluble material was filtered over Whatman GF/C glass-fiber filters and washed with 5% trichloroacetic acid in H_2O and ethanol. The filters were then analyzed for radioactivity in a liquid scintillation counter (Canberra Packard, Zellik, Belgium). The IC_{50} for each test compound was determined as the compound concentration that inhibited HIV-1 RT activity by 50%.

Steady-state kinetic assays were also performed as described previously (Balzarini et al., 1992), except that the reaction mixtures were incubated for 30 min instead of 60 min during the assays with variable substrate (dGTP) or template/primer [poly(rC)·oligo$(dG)_{12-18}$] concentrations. Under these experimental conditions the catalytic reaction of the different enzymes proceeded linearly and proportionally with time. The K_m and V_{max} (k_{cat}) values for poly(rC)·oligo$(dG)_{12-18}$ and dGTP were determined in the presence of fixed concentrations of [8-^{3}H]dGTP {specific radioactivity 14.1 Ci/mmol [1.25 µM (1 µCi)]} and poly(rC)·oligo$(dG)_{12-18}$ (0.1 mM), respectively. The K_m and V_{max} (k_{cat}) values were derived from the double reciprocal Lineweaver-Burk plots of the variable substrate (dGTP) or template/primer [poly(rC)·oligo$(dG)_{12-18}$] concentrations *versus* the velocities of dGTP incorporation at each substrate or template/primer concentration.

Stability of wild-type and mutant heterodimer HIV-1 RTs in the presence of urea. Denaturation curves were plotted by pre-incubation of RT with different concentrations of urea ranging from 0.0625 M up to 2.5 M for 10 min at 37°C in 50-

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µl reaction buffer [containing 50 mM Tris-HCl (pH 7.8), 0.06% Triton X100, 5 mM DTT, 150 mM, 0.3 mM glutathione, 1.25 mg/ml BSA, 0.5 mM EDTA, 5 mM $MgCl_2$ and 1.4 mM poly(rC)·oligo$(dG)_{12-18}$ (Amersham Biosciences)]. The polymerase reaction was initiated by adding [8-^{3}H]dGTP (0.1 mM, 1 mCi/ml) (Amersham Biosciences) substrate. After incubating for 10 min at 37 °C the reactions were terminated by addition of 1 ml of ice-cold TCA 5% in 200 mM $Na_4P_2O_7$ and 200 µl yeast RNA (2 mg/ml, pH 8.0). Reaction products were incubated on ice for 30 min and precipitated on a Whatman GF/C filter. The filters were washed with 20 ml TCA 5% and dried with 2 ml ethanol. The amount of incorporated radioactive substrate was analyzed in a TR-2500 liquid scintillation counter (Perkin Elmer NV Life Sciences, Zaventem, Belgium) by adding 4 ml of HiSafe2 (Perkin-Elmer). Polymerase activity was determined as the amount of nucleotide incorporated at each urea concentration relative to the amount of nucleotide incorporation in the absence of denaturant. The percentage polymerase activity was plotted *versus* the urea concentration and the data were fitted to a curve using the program SigmaPlot Version 8.0 (SPSS Inc.) to determine the concentration of urea at the midpoint of the denaturation curve.

Molecular modeling and structure visualization. The protein environment around T139 in HIV-1 RT was visualized and pictured using the PyMOL molecular graphics program (De Lano, 2004) and the x-ray coordinates of a covalently trapped catalytic complex between HIV-1 RT and a DNA template:primer deposited in the Protein Data Bank (PDB, http://www.rcsb.org/PDB/) with code 1RTD (Huang et al., 1998).

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RESULTS

RNA-dependent DNA polymerase activities of wild-type and mutant T139X HIV-1 RTs. To assess the influence of changes at the amino acid residue T139 on the catalytic activity of HIV-1 RT, seven recombinant RTs were constructed by site-directed mutagenesis: T139A, T139Q, T139Y, T139K, T139I, T139S and T139D. In this way, the different types of amino acid side chains were represented: an aliphatic side chain in alanine, an aromatic side chain in tyrosine, a protonated amino group in the positively charged lysine, a carboxylate group in the negatively charged aspartic acid, and a polar uncharged group in serine and glutamine. The T139I mutation was also introduced because it consistently appears in cell culture under (+)-calanolide A selective pressure. The mutations were introduced in both p66 and p51 subunits of the RT heterodimer, and all mutant recombinant RTs were purified to ≥ 98% homogeneity through Ni-NTA- and heparin-containing affinity columns.

Using poly(rC)·oligo$(dG)_{12\text{-}18}$ as the template/primer and [8-^{3}H]dGTP as the radiolabeled substrate, the RNA-dependent DNA polymerase (RDDP) activitiy was fully retained in the T139Q mutant and was reduced by only 15% in the mutant T139I RT compared to wild-type (Fig. 2). The polymerase activity was reduced by about 50% in the T139S and T139A RT mutants and was seriously impaired in the other mutants containing the negatively charged 139D, the positively charged 139K and the aromatic amino acid mutation 139Y.

To assess the role of the T139D and T139I mutations when separately located in the p66 and the p51 subunits of the RT heterodimer, 4 additional mutant RTs were constructed in which the T139D or T139I mutation were introduced in either solely the p66 or solely the p51 subunit of the heterodimeric RT enzyme. Whereas the mutant RT enzyme in which T139D was solely present in the p51 subunit had a

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catalytic activity that was 21 ± 3% of wild-type enzyme, the heterodimeric enzyme at which T139D was solely present in p66 had a catalytic activity of 91 ± 3% of the wild-type enzyme. For the T139I mutation solely present in the p51 subunit the catalytic activity was 59 ± 1% of wild-type while the presence of this mutation in the p66 alone was 89 ± 3% of wild-type. Thus, the exclusive presence of the T139D or T139I mutation in the p51 subunit of the RT heterodimer had a much more deleterious effect on the catalytic activity of the mutant enzyme than when these mutations were solely present in the p66 subunit of the RT heterodimer.

Inhibitory activities of NNRTIs and ddGTP against wild-type and mutant T139X HIV-1 RTs. The mutant T139Q/I/S/A/K/D/Y RT enzymes were evaluated for their sensitivity to the inhibitory activity of a variety of NNRTIs and the NRTI ddGTP (Table 1). Among all NNRTIs evaluated, (+)-calanolide A showed the most pronounced loss of inhibitory potential against the mutated enzymes. Indeed, the mutant T139K (20-fold), T139I (8-fold), T139Y (6-fold) and T139Q (6-fold) HIV-1 RT enzymes displayed marked resistance towards (+)-calanolide A compared with wild-type enzyme.

The RT enzyme bearing the T139K mutation was 4-fold less susceptible to the inhibitory activity of the TSAO derivatives TSAO-T and TSAO-m³T. In contrast, most mutant enzymes gained significant sensitivity toward the second-generation NNRTIs such as thiocarboxanilide UC-781 and efavirenz. This was most noticeable for the mutant T139A and T139D RTs (up to 5- to 10-fold and 3- to 4-fold increases in sensitivity for UC-781 and efavirenz, respectively). The greater susceptibility of most mutant T139 enzymes to UC781 and efavirenz was not a general property of second-generation NNRTIs since the quinoxaline GW867420X kept a virtually similar inhibitory potential against each of the mutant RTs. As already observed for

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UC-781 and efavirenz, the mutated T139D RT enzyme was 5-fold more sensitive toward the inhibitory activity of the first-generation NNRTI nevirapine (Table 1). Surprisingly, ddGTP showed a markedly decreased inhibitory activity (~7-fold) against several mutant RTs, in particular T139Y and T139D RT.

When (+)-calanolide A was evaluated for its inhibitory activity against the HIV-1 RT heterodimers that contained the T139I or T139D mutation solely in either the p66 or the p51 subunit, marked resistance of mutant T139I RT towards (+)-calanolide A is only evident when the T139I mutation is solely introduced in the p51 subunit (Fig. 3). Thus the resistance against (+)-calanolide A is clearly originating from the amino acid mutation in the p51 subunit and not in the p66 subunit of the heterodimer.

Kinetic analysis of wild-type and mutant T139I HIV-1 RTs. Kinetic analysis of the wild-type and mutant T139I RTs was performed with the substrate dGTP or the template/primer poly(rC)·oligo(dG) as variables. The kinetic parameters are summarized in Table 2. When dGTP or the template/primer poly(rC)·oligo(dG) was used as the variable substrate, no marked differences in K_m were noted between wild-type and mutant T139I enzyme. The catalytic efficiency of the mutant enzyme (k_{cat}/K_m) was very comparable between the mutant T139I and wild-type enzyme, indicating that the T139I mutation has no marked influence on the positioning of the template/primer or the substrate in an optimal position to allow efficient catalysis.

Effects of urea on wild-type and mutant T139I and T139D HIV-1 RT activity. Wild-type and mutant T139I and T139D RTs were exposed to a variety of urea concentrations and their catalytic activity was determined (Fig. 4). For the wild-type enzyme, the polymerase activity gradually decreased in the presence of increasing concentrations of urea. A urea concentration as high as 0.5 M decreased

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the catalytic activity of wild-type RT by 20% (residual activity ~80%), while 2.0 M urea decreased its catalytic activity to less than 10%. Half of the wild-type RT catalytic activity was retained at ~0.80 M urea (i.e., the urea-IC_{50}). When the mutant T139I and T139D RT enzymes were exposed to similar concentrations of urea, the enzymes showed increased sensitivity toward the denaturing effect of urea compared with wild-type RT. Indeed, whereas the urea-IC_{50} shifted from 0.80 M to 0.55 M for the mutant T139I RT, the urea IC_{50} was further decreased to 0.45 M for the mutant T139D RT enzyme (Fig. 4). A similar increased sensitivity to urea was observed for mutant T139D RT when the T139D mutation was solely introduced in the p51 subunit (urea-IC_{50}= 0.60 M), whereas the sensitivity to urea was not increased (urea-IC_{50}= 0.95 M) when the T139D mutation was solely introduced in the p66 subunit (data not shown). There was a close correlation between the catalytic activity of the mutant T139D RT with mutation in both subunits, p66 solely and p51 solely and the urea concentration required to decrease RT activity by 50%. The r-value of the regression line was as high as 0.995.

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DISCUSSION

(+)-Calanolide A possesses antiviral properties characteristically ascribed to NNRTIs (i.e., selectivity for HIV-1, but not HIV-2 strains and rapid selection of drug-resistant virus strains containing NNRTI-characteristic mutations in RT). Interestingly, (+)-calanolide A exhibits a 10-fold enhanced activity against certain drug-resistant viruses that bear the most prevalent NNRTI resistance mutations such as the Y181C mutation (Buckheit et al., 1999). (+)-Calanolide A-resistant virus strains may carry, besides T139I, also L100I, Y188H, L187F and N348K mutations in the RT (Buckheit et al., 1999; Currens et al., 1996b). Among these mutations, the single amino acid substitution T139I proved of major importance for the resistance against (+)-calanolide A and the effect of this mutation on drug resistance is engendered from its presence in the p51 subunit (Boyer et al., 1994). This p51 subunit dependence was also proven by the fact that resistance towards (+)-calanolide A was found in the mutant RT with a wild-type p66 and a mutant T139I p51 subunit and not *vice versa*. However, the occurrence of additional amino acid changes besides the T139I mutation in the RT of the selected virus strains must explain the pronounced resistance to (+)-calanolide A, since the single amino acid T139I mutation confers a relatively low level of resistance to this drug (up to 20-fold).

To the best of our knowledge, no detailed site-directed mutagenesis studies of the RT enzyme at this amino acid position have ever been performed to assess the impact of the different mutations on (+)-calanolide A sensitivity, RT catalytic activity and the structural dynamics of HIV-1 RT. Moreover, the proximity of T139 to E138, the amino acid that is important for binding of TSAO derivatives to HIV-1 RT (Camarasa et al., 2004), also suggests that the binding sites for (+)-calanolide A and TSAO in HIV-1 RT could be in close proximity to each other or even overlapping.

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E138 is one of the amino acid residues located near the putative entrance to the well-defined NNRTI binding pocket (Esnouf et al., 1997). Although (+)-calanolide A is structurally quite distinct from TSAO it is not unfeasible that the predominant interaction of (+)-calanolide A with HIV-1 RT occurs with the β7-β8 loop located in the p51 RT subunit of RT. However, attempts to dock this drug in this region failed to provide a unique and distinctive binding mode (data not shown). This β7-β8 loop is important for heterodimerization of the RT enzyme as shown earlier for the TSAO interaction with E138 of the β7-β8 loop (Pandey et al., 2002; Pandey et al., 2001; Rodriguez-Barrios et al., 2001). As found for TSAO-derivatives, (+)-calanolide A can perhaps also enhance RT heterodimer dissociation, a feature that has previously not been observed for other types of NNRTIs (Sluis-Cremer et al., 2000).

The T139I mutation that appears under (+)-calanolide A selection, is indeed located in the β7-β8 loop of p51 (Fig. 1) that is of crucial importance for efficient dimerization of both subunits. In agreement with the experimental data, molecular modelling of TSAO-m³T binding to this loop in wild-type RT suggests that the observed destabilization of the heterodimeric RT may result from structural and conformational perturbations at the RT subunit interface (Rodríguez-Barrios et al., 2001; Sluis-Cremer et al., 2000). Likewise, mutations at the 139 position of the HIV-1 RT may destabilize the p66/p51 heterodimer in a similar way thus diminishing the catalytic activity of the enzyme as previously described for some amino acid mutations at position 138 (Pelemans et al., 2001) and recently also at position 136 (Balzarini et al., 2005) and 137 (Auwerx et al., 2005) of HIV-1 RT. Indeed, a seriously compromised RT activity was observed for several amino acid mutations at position 139, especially for the lysine and aspartic acid residues, which resulted also in a higher susceptibility of the mutated RT to the inactivating (denaturation) action of

urea (Fig 4). It may therefore be hypothesized that certain mutations at position 139 such as K or D compromise the optimal conformation at the p66/p51 heterodimer interface, resulting in a decreased catalytic activity and easier separation of both subunits in the presence of urea. Low concentrations of urea probably have already a marked influence on RT activity because it can destabilize the heterodimer before a full dissociation into monomers occurs. Sluis *et al.* (2000) reported a higher urea-IC_{50} for wild-type RT (2.3 M) than found in our study. This could be explained by the fact that the reaction buffer and purification methods of the RT are quite different in our methods and it can also not be excluded that different salt concentrations have an influence on the urea-IC_{50} as well. Also, the different nature of the particular RT gene construct may account for the observed differences.

Although T139 is rather conserved in wild-type HIV-1 strains, Ceccherini-Silberstein *et al.* (2004) found that 3.7% of drug-treated HIV-1-infected patients were bearing mutations at position 139. These amino acid mutations appeared to be A, R, K, M, S, V, I and P. Absence of the aspartic acid and the tyrosine mutations at position 139 in NNRTI-treated patients is in agreement with our site-directed mutagenesis observations, since viruses with highly impaired enzymes, such as the mutant T139D RT (2.5% of wild-type activity) and the mutant T139Y RT (0.6% of wild-type activity), would likely not be viable in cell culture and/or patients. Since none of the patients were treated with (+)-calanolide A, it seems that other drugs may in some cases also select for mutations at T139 either to decrease the sensitivity of the virus for the particular drug, or to compensate for a potential decreased replication capacity of other mutations in RT. There was clearly a drug pressure on the virus since in drug-naïve individuals, six different mutations were spontaneously found in 10 out of 457 isolates (i.e. 1 V, 1 M, 1 A, 2 R, 2 I and 3 P), whereas in drug-treated

individuals nine different mutations were found in 72 out of 1556 isolates (i.e. 19 K, 18 R, 13 A, 9 Q, 6 M, 3 I, 2 S, 1 V and 1 P).

A mutation at position 139 to either Q, I, K or Y would result in 6- to 20-fold resistance to this compound at the enzymatic level while the influence of the mutations Q, I, K or Y on the resistance/sensitivity of RT to other NNRTIs or NRTIs are very minor (Table 1). Therefore, these amino acid mutations can be theoretically expected to appear under (+)-calanolide A pressure. However, a T to I conversion can emerge by a single transition point mutation (ACA→ATA), whereas double transversion point mutations (ACA→CAA) are required for the T to Q conversion, and even triple transversion point mutations (ACA→TAT) are needed for the T to Y conversion. Therefore, a T139I RT mutation would be more likely to appear than the T139Q or T139Y mutations in HIV-1 RT. Moreover, the (+)-calanolide A-resistant T139K RT (which can also arise from a single point mutation) and mutant T139Y RT have a much lower catalytic activity than the mutant T139I RT enzyme. Thus, our site-directed mutagenesis and kinetic analysis of mutated T139X RTs makes it obvious why the T139I mutation must preferentially occur under (+)-calanolide A pressure in cell culture.

In a previous study using the Y2H RT dimerization assay (+)-calanolide A had no detectable effect on RT dimerization (Tachedjian and Goff, 2003; Tachedjian et al., 2001). However, failure of an effect of (+)-calanolide A on RT dimerization can be explained by lack of entry of this compound in the yeast cells. Because the kinetic studies with (+)-calanolide A may suggest more than one site of interaction with the HIV-1 RT (Currens et al., 1996b) it would be very interesting to evaluate (+)-calanolide A in other p66-p51 RT dimerization assays. Given the fact that it has been shown that TSAO derivatives affect RT subunit dimerization, the appearance of

the T139I resistance mutation under (+)-calanolide A pressure, which is in close proximity to the TSAO binding amino acid E138, supports this view. In addition, the fact that the mutant T139D and T139I RT enzymes have increased sensitivity towards the denaturation activity of urea, is also supportive of a role for T139 in the stabilization of the HIV-1 RT heterodimer. In addition, the lower catalytic efficiency that was observed for the mutant T139I enzyme can also be explained by ensuing structural/conformational differences at the dimerization interface that critically affect proper positioning of the primer/template and/or the orientation of the incoming substrate molecules (i.e. dGTP). Additional support to this hypothesis is provided by the markedly decreased inhibitory activity of the NRTI ddGTP against some of the T139X mutants (Table 1).

The observed hypersensitivity (up to 20-fold) towards the thiocarboxanilide UC-781 (and efavirenz) for the majority of HIV-1 RT mutants except for T139I is intriguing. In particular, hypersensitivity of RT to UC-781 as a result of mutations at amino acid position 139 in RT is rather puzzling because UC-781 makes direct contacts with K101, V106, Y181 and F227 of p66, but not with the p51 subunit (Balzarini et al., 1998; Ren et al., 2004). One possible explanation is that the definite shape that the NNRTI binding pocket adopts when this inhibitor is lodged into it can be achieved more easily in the presence of this type of mutations.

The protein stretch ranging from I135 to T139 makes up the tip of the so-called β7–β8 loop that is present in both p66 and p51 subunits. An important difference, however, is that in p66 this loop is exposed to the solvent whereas in p51 it lies close to the dimerization interface (Fig. 1). The importance of the structural support imparted by the β7–β8 loop is demonstrated by the severe impairment of the polymerase function of the heterodimeric RT enzyme upon deletion or alanine

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substitution of amino acids 136-139 (Pandey et al, 2001) and, more specifically, by the dramatic changes in activity taking place upon mutation of T139 (Fig. 2). In the absence of direct experimental structural evidence, we would suggest that the presence of K, D or Y at this position is incompatible with the required loop conformation that is necessary for tight interaction with the p66 subunit and essential for catalytic activity. On the other hand, this conformation should virtually remain intact in the case of T139→Q and T139→I substitutions, and would be perturbed only slightly in the case of T139→A or T139→S mutations.

In conclusion, among the T139 RT mutants investigated, T139I RT has the highest resistance profile against (+)-calanolide A. It also retains a marked catalytic activity (85% of wild-type), which may result in a preferential replication of the mutated T139I RT virus in the presence of (+)-calanolide A, compared with the other mutant RT viruses. In addition, the single transition point mutation ACA→ATA that is necessary to convert the wild-type T139 into the mutant T139I can occur more readily than double or triple transversion base changes [that code for Q (102% of wild-type activity) or Y (0.6% of wild-type activity) respectively]. Thus, these kinetic properties of the mutant RTs (a pronounced catalytic activity of the T139I RT enzyme and a marked degree of resistance of T139I RT against (+)-calanolide A and the requirement of only one (transition) point mutation in the 139 codon to afford an amino acid substitution may likely explain why the T139I mutation in HIV-1 RT consistently and predominantly appears in cell culture under (+)-calanolide A pressure. Based on the location of the 139-mutation in the crucial β7-β8 loop of the p51 subunit in the p66/p51 dimer interface and the more pronounced denaturation effect of urea against mutant T139I and T139D *versus* wild-type RT enzymes, it can be well possible that (+)-calanolide A affects dimerization of the HIV-1 RT

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heterodimer and thus represents the prototype compound of a new class of RT dimerization inhibitors.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Left. Schematic representation of the dimeric structure of HIV-1 RT in complex with a DNA template:primer. The protein Cα trace of each subunit is shown as a ribbon, colored pink for p66 and cyan for p51, whereas the DNA molecule (C atoms in grey) and the incoming deoxythymidine triphosphate nucleotide (C atoms in green) are displayed as sticks. The Mg^{2+} ions at the active site are shown as yellow spheres. **Right.** Enlarged view of the framed area shown on the left providing detail of the location of the β7–β8 loop of p51 at the subunit interface which includes T139 (side chain as sticks with C atoms in cyan and the OH oxygen in red).

Fig. 2. Catalytic RNA dependent DNA polymerase activity of mutant T139Q, T139I, T139S, T139A, T139K, T139D and T139Y HIV-1 RT enzymes. 8 ng wild-type, 11 ng T139A, 6 ng T139Q, 61 ng T139Y, 575 ng T139K, 6 ng T139I, 9 ng T139S and 137 ng T139D mutant enzyme (protein) were used in the enzyme assays.
Data (± S.D.) are the mean of at least 2 to 3 independent experiments.

Fig. 3. Inhibitory activity of (+)-calanolide A against the catalytic activity of wild-type and mutant HIV-1 RT enzymes that contain the T139I or T139D mutation solely in the p66, solely in the p51 or both in the p66 and p51 subunits.

Fig. 4. Effect of urea on the catalytic activity of mutant T139I, T139D and wild-type HIV-1 RTs.

TABLE 1

Inhibitory activity of test compounds against mutant T139Q/I/S/A/K/D/Y HIV-1 RTs

	IC_{50} (μM)[a]								
RT mutation[b]	**nevirapine**	**delavirdine**	**efavirenz**	**UC-781**	**GW867420X**	**TSAO-m³T**	**TSAO-T**	**(+)-calanolideA**	**ddGTP**
WT	1.1 ± 0.1	0.5 ± 0.3	0.011 ± 0.003	0.11 ± 0.09	0.011 ± 0.00	1.6 ± 0.6	1.6 ± 1.0	0.03 ± 0.01	0.05 ± 0.02
T139Y	0.9 ± 0.5	0.9 ± 0.3	0.012 ± 0.006	0.04 ± 0.02	0.03 ± 0.01	0.4 ± 0.2	0.5 ± 0.2	0.19 ± 0.02	0.38 ± 0.11
T139K	1.2 ± 0.0	1.5 ± 0.8	0.016 ± 0.004	0.039 ± 0.004	0.010 ± 0.001	6.9 ± 1.4	6.2 ± 1.2	0.69 ± 0.25	0.11 ± 0.07
T139I	1.2 ± 0.1	0.6 ± 0.4	0.02 ± 0.01	0.10 ± 0.07	0.009 ± 0.001	1.4 ± 0.1	0.9 ± 0.3	0.24 ± 0.04	0.045 ± 0.004
T139A	0.9 ± 0.3	0.33 ± 0.05	0.0026 ± 0.0009	0.023 ± 0.009	0.008 ± 0.002	2.0 ± 0.1	2.0 ± 1.8	0.09 ± 0.07	0.024 ± 0.00
T139S	1.1 ± 0.0	0.6 ± 0.2	0.0065 ± 0.0002	0.030 ± 0.004	0.008 ± 0.002	1.4 ± 0.3	1.6 ± 0.6	0.09 ± 0.07	0.033 ± 0.002
T139D	0.29 ± 0.09	0.32 ± 0.07	0.004 ± 0.001	0.012 ± 0.004	0.006 ± 0.003	0.7 ± 0.1	1.0 ± 0.7	0.039 ± 0.006	0.30 ± 0.06
T139Q	1.4 ± 0.2	0.88 ± 0.07	0.022 ± 0.000	0.042 ± 0.003	0.02 ± 0.01	0.8 ± 0.2	0.7 ± 0.1	0.18 ± 0.01	0.10 ± 0.04

Template/primer: 1.4 mM poly(rC)·oligo$(dG)_{12\text{-}18}$; substrate: 1.6 μM [8-³H]dGTP

Data are the means of 2 to 3 independent experiments ± standard deviation

[a] 50% inhibitory concentration or compound concentration required to inhibit HIV-1 RT activity by 50%

[b] The amino acid mutations are concomitantly present in both p66 and p51 subunits of the HIV-1 RT heterodimer

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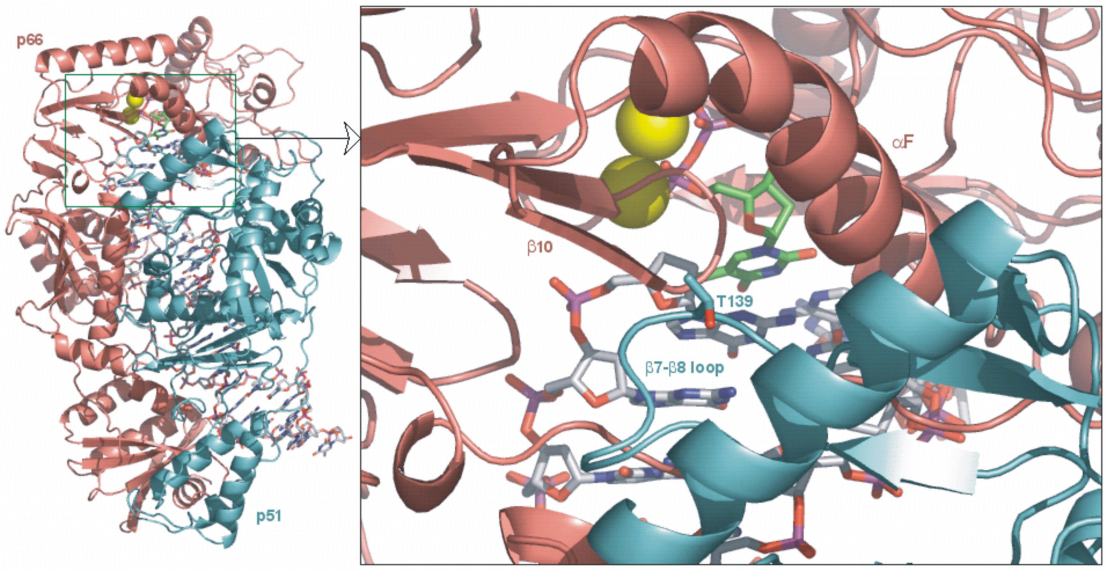
TABLE 2

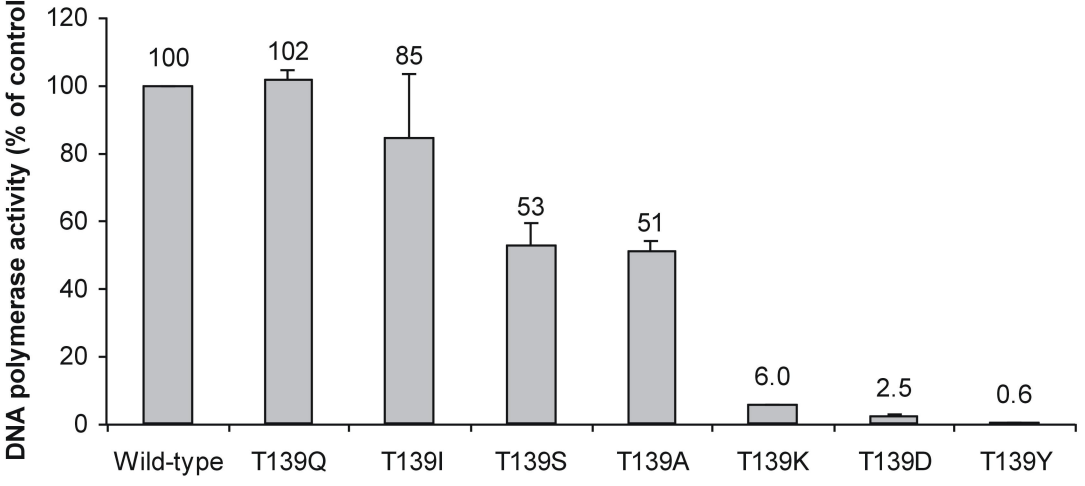
Kinetic analysis of wild-type and mutant T139I RT enzymes

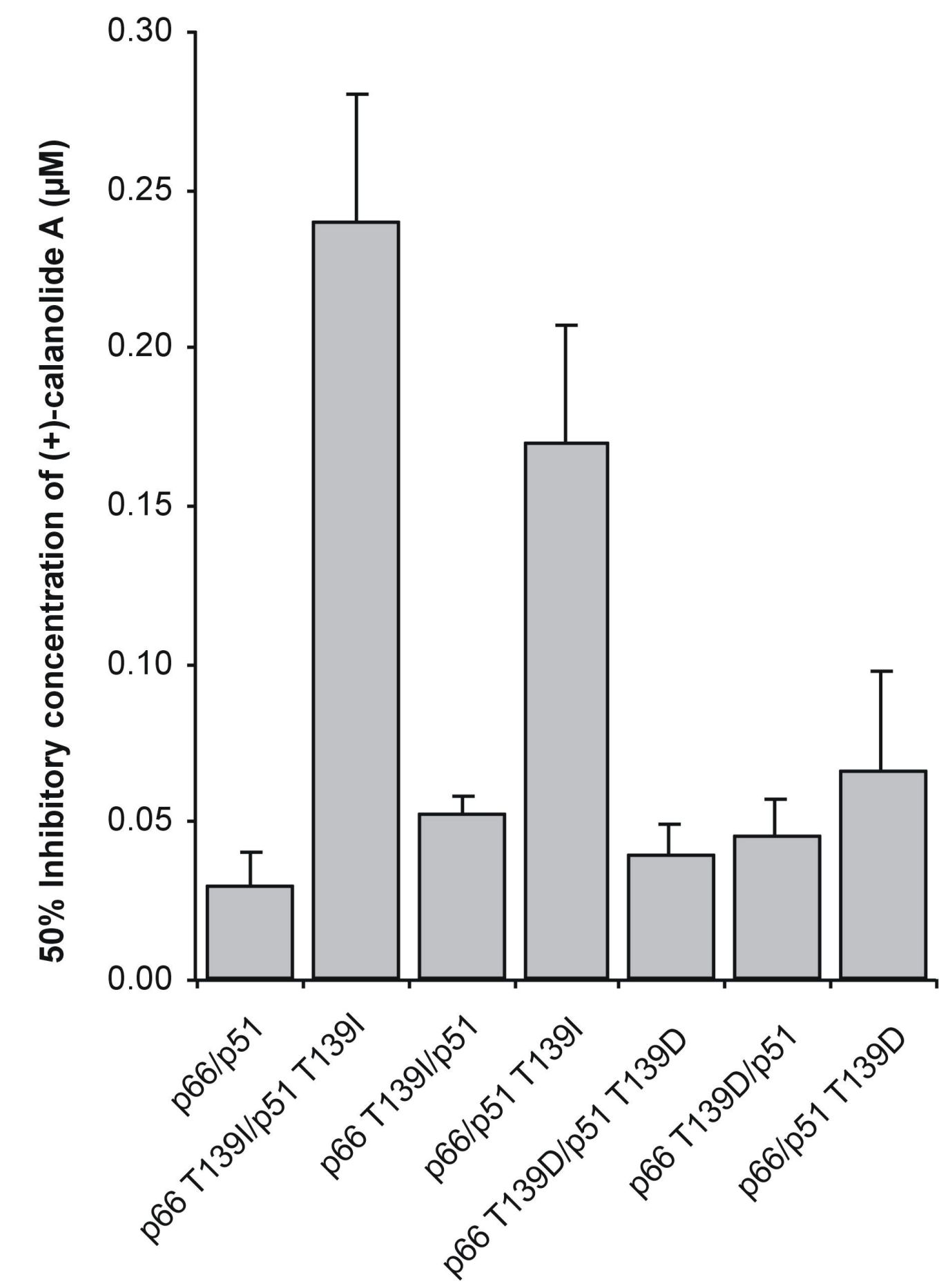
Variable substrate or template/primer	Kinetic parameters	Reverse transcriptase	
		Wild-type	T139I
dGTP	K_m [a]	0.50 ± 0.05	0.8 ± 0.2
	k_{cat} [b]	0.015 ± 0.004	0.014 ± 0.001
	k_{cat}/K_m [c]	0.03	0.018
poly(rC)·oligo(dG)$_{12-18}$	K_m	0.25 ± 0.08	0.5 ± 0.2
	k_{cat}	0.15 ± 0.01	0.17 ± 0.04
	k_{cat}/K_m	0.6	0.34

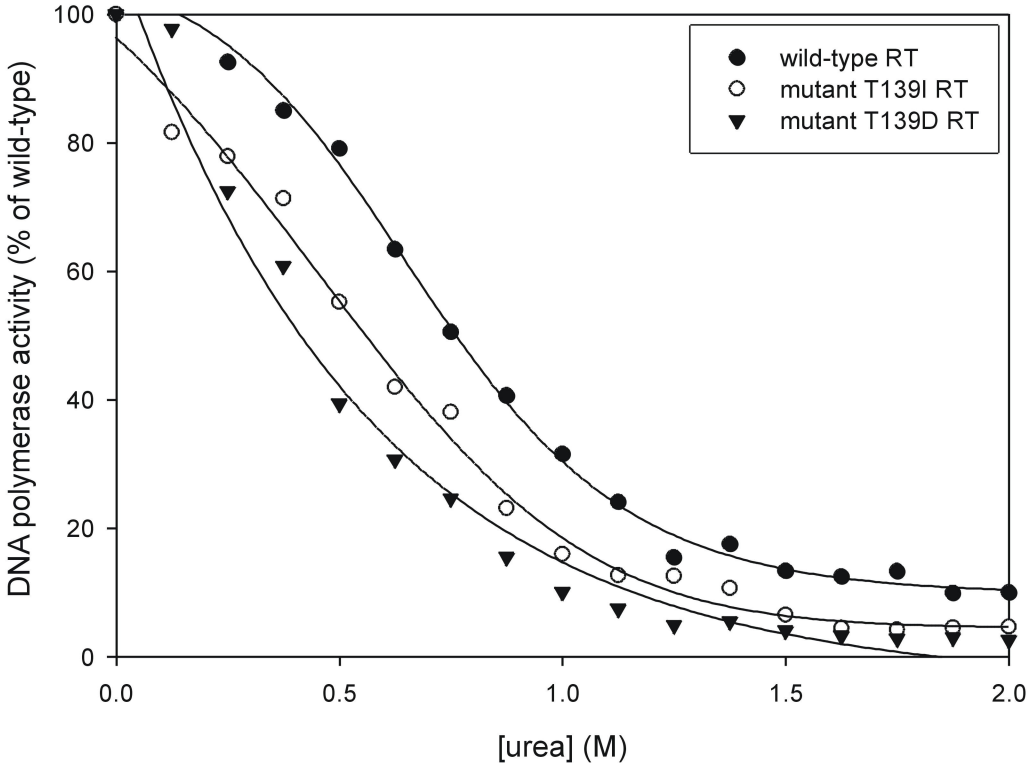
[a] K_m is in μM; [b] k_{cat} is in s^{-1}; [c] k_{cat}/K_m in $\mu M^{-1}s^{-1}$;

The data are means (± S.D.) of at least two to three independent experiments.


p66
p51
αF
β10
T139
β7-β8 loop


DNA polymerase activity (% of control)
120
100
80
60
40
20
0
100
102
85
53
51
6.0
2.5
0.6
Wild-type
T139Q
T139I
T139S
T139A
T139K
T139D
T139Y


50% Inhibitory concentration of (+)-calanolide A (μM)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
p66/p51
p66 T139I/p51 T139I
p66 T139I/p51
p66/p51 T139I
p66 T139D/p51 T139D
p66 T139D/p51
p66/p51 T139D


wild-type RT
mutant T139I RT
mutant T139D RT
DNA polymerase activity (% of wild-type)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
[urea] (M)