KATHOLIEKE UNIVERSITEIT LEUVEN
FACULTEIT WETENSCHAPPEN
FACULTEIT GENEESKUNDE
Departement Microbiologie en Immunologie - Rega Instituut
Afdeling Virologie en Chemotherapie

Molecular and kinetic studies of the interaction of lentiviral reverse transcriptases (RT) with non-nucleoside RT inhibitors of HIV-1

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Proefschrift voorgedragen tot
het behalen van de graad van
Doctor in de Wetenschappen

door
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JUNE 2005

DANKWOORD

Doctoreren doe je nooit alleen. Veel mensen hebben hier dan ook een aandeel in, zowel op wetenschappelijk als persoonlijk vlak. Nu het resultaat er eindelijk is, wil ik graag iedereen bedanken die op eender welk vlak heeft bijgedragen.

In eerste plaats wil ik Prof. Jan Balzarini danken omdat hij me een vijftal jaar geleden overhaalde om de voor mij toen nog onbekende wereld der virussen en chemotherapie te betreden. Hij heeft me met zijn onvoorwaardelijke steun, enthousiasme en mateloos dynamisme steeds opnieuw gemotiveerd verder te gaan op het juiste pad.
Prof. Erik De Clercq ben ik dankbaar voor het voorrecht dat hij me gaf deel uit te maken van het AIDS labo van het Rega Instituut.

De overige juryleden Prof. Roger Huybrechts, Prof. Lut Arckens, Prof. Luc Van Meervelt, Prof. Christophe Pannecouque, Prof. Paul Lewi en Prof. Herman Egberink wil ik eveneens bedanken voor hun kritische evaluatie van dit proefschrift.

Verder wil ik Lotte Bral, Dirk Daelemans, Lies De Dier, Kristien Erven, Katrien Francois, Annelies Hauspie, Cindy Heens, Heidi Patrouille, Miguel Stevens, Kris Uyttersprot bedanken voor opbouwende discussies en de toffe sfeer binnen het P3-groepje en in het bijzonder ook Prof. Christophe Pannecouque voor zijn hulp met vanalles en nog wat en voor zijn verwoede (maar ik vrees toch soms onvruchtbare) pogingen zijn liefde voor scheikundige structuren over te brengen op mij.

Ik kon ook rekenen op een aantal andere collega's van het AIDS labo en het Rega die ik dan ook wil danken voor het creëren van een leuke werkplek en hun hulp bij vanalles en nog wat. In het bijzonder wil ik nog Kristel Van Laethem en Yoeri Schrooten bedanken voor hun hulp en expertise bij het sequeneren van al mijn RT-mutanten en Heidi Pelemans voor de overdracht van al haar RT-kennis.

Een speciale dank gaat uit naar Joke Van Nieuwenhove en Kris Uyttersprot, die met hun thesisresultaten een belangrijke bijdrage leverden aan dit werk. Ook Kristien Minner, Lizette Van Berckelaer, Ann Absillis en Ria Van Berwaer ben ik erg dankbaar voor hun expertise en hulp bij de vele experimenten. Chris Callebaut, Inge Aerts, Dominique Brabants en Ria Swinnen voor hun hulp bij administratieve aangelegenheden.

Structural Biology Division, University of Oxford, United Kingdom

I'm grateful to Robert Esnouf for his fruitful discussion and ideas concerning the RT-chimeras and for his beautiful RT-figures. To Jingshan Ren, Louise Bird and Prof. Dave Stammers for their nice modelling studies of HIV-2 RT.

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A special thanks to Prof. Federico Gago and Fátima Rodríguez-Barrios, whose modelling studies and figures of the RT interface enhanced without any doubt the value of this work.

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Dankbaarheid gaat uit naar Prof. Herman Egberink, Nancy Schuurman en Arno Van Vliet voor mijn leerrijk verblijf in het gezellige Utrecht en hun hulp en expertise in FIV en kattencellen.

Natuurlijk is er, ondanks de geruchten, naast doctoreren ook plaats voor ontspanning. Voor de nodige babbels, feestjes, filmpjes, festivallekes, weekendjes, pintjes,... kon ik steeds bij mijn vrienden en vriendinnen terecht. Greg, Jessa, Lente, Karen, Mieke, Joost en alle anderen, wreed bedankt! Dank ook aan mijn duikbuddies die me toelieten af en toe de wereld eens vanuit een heel ander perspectief te zien in deze hectische tijden.

Natuurlijk wil ik ook mama, papa en mijn zus Nina bedanken voor hun steun, liefde en voor alles dat ze al voor mij gedaan hebben.

Joeri
Juni 2005

CONTENTS

ABBREVIATIONS

α-APA	alpha-anilinophenylacetamide
A	alanine
abacavir	ABC; (-)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopentene-1-methanol
adefovir	PMEA; 9-(2-phosphonylmethoxyethyl)adenine
AIDS	acquired immune deficiency syndrome
APOBEC-1	apolipoprotein B mRNA editing catalytic subunit 1
ARV	AIDS related virus
ATP	adenosine 5'-triphosphate
bp	basepair
BSA	bovine serum albumin
C	cysteine
CA	capsid protein
capravirine	AG1549; S-1153; 5-[(3,5-dichlorophenyl)thio]-4-isopropyl-1-(4-pyridinylmethyl)-1*H*-imidazole-2-methanol carbamate ester
$CCID_{50}$	cell culture infective dose 50

CCR5	CC-chemokine receptor 5
CD	circular dichroism
CD4	cluster of differentiation type 4
CPE	cytopathogen effect
cPPT	central polypurine tract
CRM1	chromosome region maintenance 1
CTS	central termination sequence
CXCR4	CXC-chemokine receptor 4
D	aspartic acid
dapivirine	R147681; TMC-120; *N*-[2-(4-cyanophenylamino)-4-pyrimidinyl]-2,4,6-trimethylaniline
DAPY	diarylpyrimidine
dCTP	2'-deoxycytidine-5'-triphosphate
ddG	2',3'-dideoxyguanosine
ddATP	2',3'-dideoxyadenosine-5'-triphosphate
ddCTP	2',3'-dideoxycytidine-5'-triphosphate
ddGTP	2',3'-dideoxyguanosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
delavirdine	BHAP U-90152; 1-[3-[(1-methylethyl)-amino]-2-pyridinyl] -4-[[5-[(methylsulphonyl)amino]-1*H*-indol-2yl]carbonyl]-piperazine
didanosine	ddI; 2',3'-dideoxyinosine
d4TTP	2',3'-didehydro-2',3'-dideoxythymidine-5'-triphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside-5'-triphosphate
ds	double stranded
DTT	dithiotreitol
dTTP	2'-deoxythymidine-5'-triphosphate
E	glutamic acid
EC_{50}	50% effective concentration
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetra-acetate
efavirenz	DMP 266; α-1,4-dihydro-2*H*-3,1-benzoxazin-2-one
emivirine	MKC-442; 6-benzyl-1-ethoxymethyl-5-isopropyluracil

env	envelope gene
etravirine	R165335; TMC-125; 4-[[6-amino-5-bromo-2-[(4-cyanophenyl)amino]-4-pyrimidinyl]oxy]-3,5-dimethylbenzonitrile
ER	endoplasmatic reticulum
F	phenylalanine
FIV	feline immunodeficiency virus
FPLC	fast protein liquid chromatography
G	glycine
gag	group-specific antigen
gp	glycoprotein
GW420867X	[S]-ethyl-7-fluoro-3-oxo-3,4-dihydro-2*H*-quinoxaline-1-carboxylic acid isopropyl ester
H	histidine
HAART	highly active antiretroviral therapy
HEPT	1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine
His	histidine
HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
I	isoleucine
IC_{50}	50% inhibitory concentration
IN	integrase
IPTG	isopropyl-β-D-thiogalactopyranoside
K	lysine
K_m	Michaelis-Menten constant
L	leucine
lamivudine	3TC; (-)-L-2',3'-dideoxy-3'-thiacytidine
LTR	long terminal repeat
M	methionine
MA	matrix protein
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MuLV	murine leukaemia virus
N	asparagine
NC	nucleocapsid protein

Nef	negative regulatory factor
nevirapine	dipyridodiazepinone BI-RG-587
Ni-NTA	nickel-nitrilotriacetic acid
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NtRTI	nucleotide reverse transcriptase inhibitor
NAM	nucleoside analogue mutation
OD_{600}	optical density at wavelength 600 nm
P	proline
p66	66 kDa subunit of HIV-1 RT
p51	51 kDa subunit of HIV-1 RT
PAGE	polyacrylamide gel electrophoresis
PBS	primer binding site
PCR	polymerase chain reaction
PETT	phenylethylthiazolylthiourea
PFA	phosphonoformic acid, foscarnet
PIC	preintegration complex
PMSF	phenylmethylsulfonylfluoride
pol	polymerase gene
PPT	polypurine tract
PR	protease
Q	glutamine
R	short repeat in both 5' and 3' LTR arginine
Rev	regulator of protein HIV expression
rilpivirine	R278474; TMC278; 4-[[4-[[4-[(1*E*)-2-cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzonitrile
RNA	ribonucleic acid
RNase H	ribonuclease H
Rpm	rotations per minute
RRE	Rev-response element
RT	reverse transcriptase
S	serine
SAR	structure activity relationship

SDS	sodium dodecylsulphate
SIV	simian immunodeficiency virus
ss	single strand
(-) sss	minus strand strong stop
(+) sss	plus strand strong stop
stavudine	d4T; 2',3'-didehydro-2',3'-dideoxythymidine
SU	surface protein
T	threonine
TAM	thymidine analogue mutation
Tat	transcriptional transactivator of HIV
Tar	transactivating response element
TCA	trichloroacetic acid
tenofovir	PMPA; (R)-9-(2-phosphonylmethoxypropyl)adenine
TIBO	4,5,6,7-tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepine-2(1*H*)-one
TM	transmembrane envelope protein
tRNA	transfer RNA
TSAO	[2',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-*β*-D-ribofuranosyl]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide)
TSAO-m^{3}T	[2',5'-Bis-*O*-(*tert*-butyldimethylsilyl)-*β*-D-ribofuranosyl]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide)-N^3-methyl-thymine
U3	unique region of the 3' LTR
U5	unique region of the 5' LTR
UC-781	*N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furancarbothioamide
V	valine
Vif	virion infectivity factor
Vpr	viral protein R
Vpu	viral protein U
W	tryptophan
WT	wild-type
Y	tyrosine
zalcitabine	ddC; 2',3'-dideoxycytidine
zidovudine	AZT; 3'-azido-2',3'-dideoxythymidine

SUMMARY

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) specifically interact with the reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1). NNRTIs are highly active inhibitors of HIV-1 with a generally low toxicity, which are as a rule completely inactive against other lentiviruses. NNRTIs bind to a specific site of the RT that is close to, but distinct from the polymerase active site. Widespread use of NNRTIs in anti-HIV chemotherapy has been hampered by the rapid emergence of NNRTI-resistant virus strains. In this study we focused on the NNRTI-interaction with the lentiviral RTs, encoded by HIV-1, HIV-2 and feline immunodeficiency virus (FIV). Our first goal was to investigate whether an FIV RT could be constructed that is susceptible to NNRTIs and that could therefore be ultimately used as a model for evaluation of new NNRTIs in the *in vivo* background of a relevant cat model. Second, we studied the molecular interactions and resistance pattern of MSK-076, a member of the PETT NNRTI series that is, besides targeting HIV-1, exceptionally also active against HIV-2 RT. These studies may have important implications for the development of novel NNRTIs with activity against a wider range of lentiviruses, including HIV-2. Third, we wanted to identify new interaction points in the NNRTI-specific binding pocket that can be reached by NNRTIs and may improve the binding and resilience of

NNRTIs to resistance development by HIV-1. The interaction points that we focused on are located at the bottom of the HIV-1 NNRTI-pocket and are also part of the p66/p51 heterodimer interface. In this way, we wanted to contribute to a better understanding of the NNRTI-binding pocket and interface interactions. These insights may lead to a more rational design of novel NNRTIs and/or a new type of RT dimerisation inhibitors that can lead to a better suppression of NNRTI resistance development of HIV-1.

We first investigated the molecular determinants that are responsible for the intrinsic resistance of FIV RT towards NNRTIs (Chapter 3). In parallel, an attempt was made to sensitize FIV RT to NNRTIs by construction of eight different HIV-1/FIV RT chimeras. Modified FIV RTs with high sensitivity towards NNRTIs could eventually be used in a recombinant virus model and later in animal model studies where cats are infected with a molecular FIV clone containing this NNRTI-sensitive RT. However, all constructed HIV-1/FIV chimeric RTs in the FIV RT background retained resistance towards NNRTIs, whereas chimeric HIV-1/FIV RTs in the HIV-1 background could be made entirely insensitive to NNRTIs. In addition, we found that the majority of the chimeric FIV/HIV-1 RTs had a markedly decreased catalytic efficacy while their affinity for the natural substrates was comparable with that of the wild-type HIV-1 and FIV RTs. Since the corresponding amino acids of the putative NNRTI-specific pocket in FIV RT only differ in a few amino acids, we introduced the mutations Q101K, D179V and Y227F in FIV RT. Surprisingly no susceptibility towards NNRTIs was noticed. Thus, exchanging amino acid segments or substituting relevant amino acids in FIV RT by their HIV-1 RT counterparts did not suffice to make FIV RT sensitive towards NNRTIs. Moreover, such exchanges were often accompanied by a decrease or even total loss of DNA polymerase activity. In addition we studied the influence of the p51 subunit of FIV RT on the sensitivity of HIV-1 RT towards NNRTIs (Chapter 2). Construction of enzymatic hybrids with exchanged subunits between HIV-1 and FIV RT revealed that replacing the p51 subunit of HIV-1 RT by that of FIV RT has no influence on the sensitivity of the hybrid HIV-1 RT to any of the analyzed NNRTIs. *Vice versa*, replacing the p51 subunit in FIV RT by the p51 subunit of HIV-1 RT did not alter the FIV RT resistance to NNRTIs. As a rule, the constructed hybrid RTs had a poor catalytic efficacy that may suggestive for a sub optimal interaction between the subunits of the different RTs.

Some derivatives of the NNRTI class of phenylethylthiazolylthiourea (PETT) compounds (i.e. MSK-076 and PETT-2) possess the unusual property to inhibit HIV-2 RT. In order to understand the mechanism of inhibition and the target of anti-HIV-2 RT action we

selected resistant virus strains under drug (MSK-076) pressure and we performed mechanistic studies to unravel the molecular interaction of the compound with RT (Chapter 4). We detected a mode/site of action in HIV-2 RT that was similar to the NNRTI interaction in HIV-1 RT. Resistance mutations in the equivalent presumable NNRTI-binding region of HIV-2 RT (i.e. A101P) but also distal to the NNRTI-pocket and in close proximity of the active site of HIV-2 RT (i.e. G112E) were found. Our studies revealed that, although HIV-2 RT is, as a rule, insensitive to NNRTIs, some compounds still could interact with HIV-2 RT and inhibit the catalytic activity and virus replication at a site in HIV-2 RT that is distinct from, but closely related to the NNRTI-pocket.

To contribute to the rational design of improved NNRTIs that can cope with, or circumvent the problem of, resistance development, we searched for 'unmutatable' amino acids in the NNRTI binding pocket (Chapter 5). In particular, we searched for interaction sites located in the β7-β8 loop of the p51 subunit of HIV-1 RT, which forms the bottom of the NNRTI binding pocket and is crucial for dimerisation interactions between p66 and p51. This approach makes it possible to develop novel classes of NNRTIs that can also interfere with the interface of the RT heterodimer, which might become a new target for anti-HIV therapy. We focused on two amino acids, N136 and N137, which are (i) part of the interface, (ii) at the bottom of the NNRTI binding pocket, (iii) highly conserved among lentiviruses and (iv) as yet unidentified as sites for resistance mutations. Using site-directed mutagenesis, eight N136 (i.e. A, Q, Y, K, T, S, L and D) and nine N137 (i.e. A, Q, Y, K, T, E, D, H and S) mutant RTs were constructed. Kinetic analysis of the catalytic activities of these mutant RTs revealed severely impaired RNA- and DNA-polymerase activities for all N136 and N137 mutant RTs with the exception of N137H and N137S (both are found in patients) and N137A and N137Q. We provided evidence that the severely compromised polymerase activities of most of the N136 and N137 mutant RTs suggest an important functional or structural role of these amino acids in maintaining the heterodimeric form of the RT. The disturbance of the interface by mutating these amino acids was confirmed by denaturation analysis by urea and/or acetonitrile of several mutant N136 and N137 RTs, size exclusion chromatography and circular dichroism spectra. When the mutant RTs were evaluated for their sensitivity/resistance towards NNRTIs, they retained in general a marked sensitivity to the NNRTIs. Only when NRTIs were evaluated, a marked resistance was observed that correlated well with the level of decreased catalytic activity. Our data identified both N136 and N137 in the HIV-1 RT as amino acids with a crucial functional role in maintaining the heterodimeric form of RT and a structural function in the formation of the bottom of the NNRTI-binding pocket. These amino acids

should be optimally targeted by novel or optimized NNRTIs. By achieving this goal, the affinity of the NNRTIs to HIV-1 RT would be strengthened and it would be expected that drug resistance development could be delayed due to the inability of these amino acids to mutate without seriously compromising the catalytic activity of HIV-1 RT. More importantly, such NNRTI derivatives may interact with a new potential target (i.e. dimerisation) on the RT, resulting in a different resistance and cross-sensitivity profile.

An existing drug that may potentially interact with the interface of the HIV-1 p66/p51 heterodimer is (+)-calanolide A, the most active and most studied compound among the anti-HIV-1 coumarines. The compound selects for a T139I mutation located in the p51 subunit at the interface of the p66/p51 heterodimer and has an unclear mode of action that includes at least the pyrophosphate binding site. Because of the unique character of (+)-calanolide A, we choose to investigate this peculiar resistance phenomenon and tried to resolve why a T139I mutation is consistently selected under (+)-calanolide A drug pressure (Chapter 6). We therefore constructed seven RTs mutated at amino acid position 139 (i.e. A, K, Y, D, I, S and Q). We evaluated the mutant T139 RTs for their DNA polymerase activities and determined their resistance profile against (+)-calanolide A and several other NNRTIs. Since mutant T139I RT was amongst the RTs that retained most of their catalytic activity and (+)-calanolide A lost part of its activity against mutant T139I RT and against a few other mutant T139 RTs, we could explain why the T139I RT mutation is the most likely mutation to emerge under (+)-calanolide A drug pressure. This mutation in HIV-1 RT is at the interface of p66 and p51, but we found that it is probably not a good target for the design of optimised NNRTIs.

SAMENVATTING

Niet-nucleoside reverse transcriptase inhibitoren (NNRTIs) interageren specifiek met het reverse transcriptase (RT) van het humaan immunodeficiëntie virus type 1 (HIV-1). NNRTIs zijn erg actieve inhibitoren van HIV-1 met in het algemeen een lage toxiciteit en ze vertonen geen activiteit tegen andere lentivirussen. NNRTIs binden aan een specifieke plaats in het RT die dicht bij de actieve substraat DNA polymerase site ligt. Uitgebreid gebruik van NNRTIs in anti-HIV chemotherapie veroorzaakt een snelle opkomst van NNRTI-resistente virusstammen. In deze studie concentreerden we ons op de NNRTI-interactie met lentivirale RTs die gecodeerd worden door HIV-1, HIV-2 en het katten (feline) immunodeficiëntie virus (FIV).

Onze eerste doelstelling was de constructie van een FIV RT met hoge gevoeligheid voor NNRTIs. Zulk RT kan later gebruikt worden voor de constructie van hybride RT-FHIVs ter evaluatie van nieuwe NNRTIs in katten. Vervolgens bestudeerden we de moleculaire interacties en resistentiepatronen van MSK-076, een fenylethylthiazolylthiourea (PETT) NNRTI derivaat welk, naast anti-HIV-1 activiteit, ook een uitzonderlijke activiteit tegenover HIV-2 vertoont. Deze studies hebben mogelijk belangrijke gevolgen voor de ontwikkeling van nieuwe NNRTIs met activiteit tegen een ruimere verscheidenheid aan lentivirussen, inclusief

HIV-2. Ten derde trachtten we aminozuren in de NNRTI-specifieke bindingsplaats op te sporen die kunnen interageren met NNRTIs en welke de binding van NNRTIs aan het RT en het profiel van NNRTIs ten opzichte van resistentie kunnen verbeteren. De interactiepunten waarop we ons toespitsten zijn gelegen aan de basis van de HIV-1 NNRTI-bindingsplaats en maken ook deel uit van de interfase van het p66/p51 heterodimeer. Op deze manier pogen we bij te dragen tot een beter begrip van de rol en de interacties van welbepaalde aminozuren in de NNRTI-bindingsplaats. Deze inzichten kunnen leiden tot een rationeler design van nieuwe NNRTIs en/of de ontwikkeling van nieuwe types van RT dimerisatie-inhibitoren die een betere remming van resistentie-ontwikkeling tegenover NNRTIs tot gevolg hebben.

Als eerste onderzochten we de moleculaire factoren die verantwoordelijk zijn voor de natuurlijke resistentie van FIV RT tegenover NNRTIs (Hoofdstuk 3). In parallel werd een poging ondernomen om FIV RT gevoelig te maken aan NNRTIs via de rationele constructie van acht verschillende chimere HIV-1/FIV RTs. Gemodificeerde FIV RTs met hoge gevoeligheid aan NNRTIs kunnen bovendien gebruikt worden in een recombinant virus en later in een diermodel waarbij katten geïnfecteerd worden met een moleculaire FIV kloon die een NNRTI-gevoelig RT bevat. Uit onze resultaten bleek echter dat alle geconstrueerde chimere FIV/HIV-1 RTs in de FIV RT genetische achtergrond hun natuurlijke resistentie tegen NNRTIs behielden, terwijl chimere HIV-1/FIV RTs in een HIV-1 genetische achtergrond volledig ongevoelig gemaakt konden worden voor NNRTIs. Bijkomend vonden we dat de meeste van de chimere FIV/HIV-1 RTs een sterk verlaagde katalytische efficiëntie vertoonden terwijl hun affiniteit voor het natuurlijk substraat vergelijkbaar was met dat van wildtype HIV-1 en FIV RTs.

Vermits de overeenkomstige aminozuren van de analoge ‘NNRTI-specifieke bindingsplaats’ in FIV RT slechts in enkele residuen verschillen, werden de mutaties Q101K, D179V en Y227F in de FIV RT genetische achtergrond geïntroduceerd. Er werd bij deze mutante RTs echter geen stijgende gevoeligheid tegenover NNRTIs waargenomen. We kunnen dus stellen dat uitwisseling van aminozuursegmenten of het vervangen van relevante aminozuren in FIV RT door hun HIV-1 RT equivalenten in FIV RT onvoldoende is om FIV RT gevoelig te maken voor NNRTIs. Zulke uitwisselingen bleken zelfs vaak samen te gaan met een daling of zelfs het totale verlies van DNA polymerase activiteit. Hierbij aansluitend hebben we ook onderzocht wat de invloed van de p51 subeenheid van FIV RT was op NNRTI-gevoeligheid van HIV-1 RT (Hoofdstuk 2). De constructie van enzymatische hybriden met uitgewisselde subeenheden tussen HIV-1 en FIV RT onthulden dat het vervangen van de p51 subeenheid van HIV-1 RT door de p51 subeenheid van FIV RT geen

enkele invloed had op de gevoeligheid van zulk hybride HIV-1/FIV RT voor de geanalyseerde NNRTIs. *Vice versa*, wanneer de p51 subeenheid in FIV RT vervangen werd door de p51 subeenheid van HIV-1 RT werd er geen verandering in het resistentieprofiel van FIV RT voor NNRTIs waargenomen. De geconstrueerde hybride RTs hadden een opmerkelijk lagere katalytische activiteit welke suggereert dat er in de hybride RTs suboptimale interacties plaatsvinden tussen de subeenheden van de verschillende RTs.

Sommige derivaten van de NNRTI klasse van de PETT analogen (nl. MSK-076 en PETT-2) bezitten de ongewone eigenschap om HIV-2 te inhiberen in celcultuur. Om het mechanisme te begrijpen dat hierachter schuil gaat en het doelwit van deze anti-HIV-2 activiteit te bepalen, selecteerden we resistente virusstammen onder druk van MSK-076 en voerden we studies uit om de moleculaire interacties van deze verbinding met HIV RT te onthullen (Hoofdstuk 4). Er werden resistentiemutaties gevonden in de overeenkomstige NNRTI bindingsplaats van HIV-2 RT (nl. A101P), maar ook verwijderd van deze bindingsplaats en in nabijheid van de actieve substraatsbindingsplaats (nl. G112E). Onze studies toonden aan dat sommige verbindingen met een NNRTI-structuur, ondanks de intrinsieke ongevoeligheid van HIV-2 voor NNRTIs, kunnen interageren met HIV-2 RT via een bindingsplaats die verschillend is van (maar erg gelijkend op) deze in HIV-1 RT waardoor de virus replicatie en de katalytische RT activiteit onderdrukt wordt.

Om bij te dragen tot de rationele ontwikkeling van nieuwe of gewijzigde NNRTIs met een beter resistentieprofiel zochten we naar aminozuren in de NNRTI bindingsplaats die na mutatie de katalytische activiteit van HIV-1 RT gevoelig verlagen of zelfs vernietigen (Hoofdstuk 5). Meer bepaald zochten we naar interactiepunten binnen RT die zich bevinden in de β7-β8 lus van de p51 subeenheid van HIV-1 RT welke de basis van de NNRTI-bindingsplaats vormt en cruciaal is voor dimerisatie-interacties tussen de p66 en p51 subeenheid. Deze benadering maakt het mogelijk om een nieuwe klasse NNRTIs te ontwikkelen die eveneens kan interageren met de interfase van het heterodimeer RT, wat een nieuw doelwit kan worden voor anti-HIV therapie. We concentreerden ons voornamelijk op twee aminozuren, N136 en N137, die (i) deel van de interfase uitmaken tussen p66 en p51, (ii) zich bevinden aan de basis van de NNRTI-bindingsplaats, (iii) sterk geconserveerd zijn in lentivirale RTs en (iv) nog niet beschreven zijn als resistentie-geassocieerde aminozuren in celcultuur of in behandelde patiënten. Met behulp van plaatsgerichte mutagenese werden acht mutante N136 (nl. A, Q, Y, K, T, S, L en D) en negen mutante N137 (nl. A, Q, Y, K, T, E, D, H en S) RTs geconstrueerd. Analyse van de katalytische activiteiten van deze mutante RTs onthulde sterk gereduceerde DNA polymerase (RT) activiteit voor alle N136 en N137 mutante

RTs behalve voor N137H en N137S (welke beiden gevonden worden in behandelde patiënten), en N137A en N137Q. We toonden aan dat de sterk gereduceerde DNA polymerase activiteit van de meeste N136 en N137 mutante RTs wijst op een belangrijke functionele of structurele rol van deze aminozuren in het behoud van de heterodimere vorm van het RT. De verstoring van de interfase door mutaties ter hoogte van deze aminozuren werd bevestigd door denaturatie studies van verschillende N136 en N137 mutante RTs met behulp van urea of acetonitrile, gelchromatografie en circulair dichroïsme spectra. Wanneer echter de mutante RTs geëvalueerd werden voor hun gevoeligheid/resistentie tegenover NNRTIs behielden ze over het algemeen een sterke gevoeligheid voor deze producten. Enkel wanneer NRTIs bestudeerd werden, werd een resistentiepatroon waargenomen dat sterk correleerde met het gereduceerde niveau van katalytische RT activiteit. Onze resultaten identificeerden zowel N136 als N137 in HIV-1 RT als aminozuren met een cruciale functionele rol in het behouden van de heterodimere vorm van het RT en met een structurele rol in de vorming van de basis van de NNRTI-bindingsplaats. Wanneer deze aminozuren dan als doelwit gebruikt worden door nieuwe of geoptimaliseerde NNRTIs wordt verwacht dat de affiniteit van de NNRTIs voor het RT zal vergroten en/of de drugresistentie ontwikkeling zal worden uitgesteld door het onvermogen van deze aminozuren om te muteren zonder een serieuze daling van de katalytische activiteit van HIV-1 RT te veroorzaken. Belangrijk is ook het feit dat zulke NNRTI derivaten kunnen interageren met een nieuw potentieel doelwit (nl. dimerisatie) van het RT dat mogelijk resulteert in een ander resistentie- en kruisgevoeligheidsprofiel dan de ‘klassieke’ NNRTIs.

Een gekende NNRTI die mogelijk interacties maakt met de interfase van het HIV-1 heterodimeer is (+)-calanolide A, de meest actieve en bestudeerde verbinding onder de anti-HIV coumarines. Dit product selecteert voor de T139I mutatie die zich bevindt in de p51 subeenheid van RT, meer bepaald aan de interfase van de p66/p51 heterodimeer. (+)-Calanolide A heeft een nog onduidelijk werkingsmechanisme en maakt waarschijnlijk gebruik van de pyrofosfaat-bindingsplaats. Door het unieke karakter van (+)-calanolide A en het feit dat dit product selecteert voor mutaties van een aminozuur dat in de p66/p51 interfase gelokaliseerd is, besloten we dit ongewoon resistentie fenomeen te onderzoeken en trachtten we te verklaren waarom de T139I mutatie geselecteerd wordt onder (+)-calanolide A druk (Hoofdstuk 6). Hiervoor construeerden we zeven HIV-1 RTs gemuteerd op aminozuurpositie 139 (i.e. A, K, Y, D, I, S en Q). We evalueerden de T139 mutante RTs voor hun DNA polymerase activiteit en onderzochten hun resistentieprofiel tegenover (+)-calanolide A en andere NNRTIs. Het mutante T139I RT bewaarde zijn katalytische activiteit goed en

vertoonde daarbij ook een uitgesproken resistentie voor (+)-calanolide A. Bovendien is er maar één puntmutatie nodig om T te muteren tot I, terwijl er minimum twee puntmutaties nodig zijn om T te muteren naar Q, S of A. Laatst genoemde aminozuren vertonen eveneens een goede katalytische activiteit. Onze resultaten verklaren dus waarom T139I de meest waarschijnlijke mutatie is die verschijnt onder (+)-calanolide A druk. Deze mutatie ligt op de interfase van de p66 en p51 subeenheden maar we vonden echter dat dit aminozuur waarschijnlijk geen geschikt doelwit is voor optimalisatie van NNRTIs.

CHAPTER I

GENERAL INTRODUCTION

1.1. HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

1.1.1. Discovery of HIV-1 as the causative agent of AIDS

The human immunodeficiency virus type 1 (HIV-1) was discovered in 1983 as the primary causative agent of the acquired immunodeficiency syndrome (AIDS). Three groups independently isolated this retrovirus from lymphoid tissue of a patient with AIDS. It was first called lymphadenopathy-associated virus (LAV) (Barré-Sinoussi *et al.*, 1983), later human T cell lymphotropic virus type III (HTLV-III) (Gallo *et al.*, 1983; Popovic *et al.*, 1984), and AIDS related virus (ARV) (Levy *et al.*, 1984). Although there was a rather high diversity in the envelope-coding gene (Starcich *et al.*, 1986), electron microscopy and comparison of the genome sequences of the three isolates showed very high homology and relationship with lentiviruses, genus of the retroviridae (Ratner *et al.*, 1985a). In 1986 they were all given the name HIV-1. In 1985, a second antigenic variant, designated HIV-2, was isolated from an AIDS patient in West Africa (Barin *et al.*, 1985; Clavel *et al.*, 1986). Both HIV-1 and HIV-2 are the result of an interspecies transmission of simian immunodeficiency virus (SIV) from chimpanzees (Gao *et al.*, 1999) and sooty mangabeys (Hirsch *et al.*, 1989) to humans, respectively. HIV-2 shares approximately 60% nucleotide sequence similarity with HIV-1 and is primarily found in West Africa, while HIV-1 has spread globally. Direct human contact with SIV-infected blood resulted probably in the zoonotic transmission of SIV to humans and its appearance as an epidemic at the end of the past century and the beginning of the present century is a result from socio-behavioural changes related to our time (Gao *et al.*, 1999; Hahn *et al.*, 2000; Korber *et al.*, 2000; Salemi *et al.*, 2001).

Another lentivirus that causes an immunodeficiency syndrome in cats that has a similar pathogenesis as AIDS was reported in 1987 (Pedersen *et al.*, 1987). This virus shares many physical and biochemical properties with HIV-1 and HIV-2 and is designated feline immunodeficiency virus (FIV).

1.1.2. The AIDS epidemic

The spread of AIDS has been dramatic since the early 1980s. At present, an estimated 40 million adults and children are infected with HIV and/or living with AIDS. During 2003, 5 million people became infected with HIV and 3 million people died from the consequences of the immune deficiency caused by HIV/AIDS. Half of the people infected with HIV, acquire

the disease before the age of 25 and die before they are 35. This has resulted in more than 11 million AIDS orphans. The majority of HIV-infected people (95%) live in the developing world, where the infection rates continue to increase due to socio-cultural factors and limited resources for prevention and health care. The impact of the infection in the developing countries is dramatic because it disrupts the complete social structure by affecting a large part of the working population and because it leaves behind a large number of orphans. It is clear that the epidemic in the developing world is more problematic than in the western world, where the disease is better controlled due to availability of antiretroviral chemotherapy and numerous prevention campaigns. Unfortunately, due to the overall access to different antiviral drugs in the western world, there is complacency about the risks of HIV and safe sex behaviour is being eroded.

Overall, early diagnosis and prevention should remain of highest priority to overcome new infections and development of new antiviral drugs and effective HIV vaccines are important to stop the expansion of the epidemic (UNAIDS, 2003).

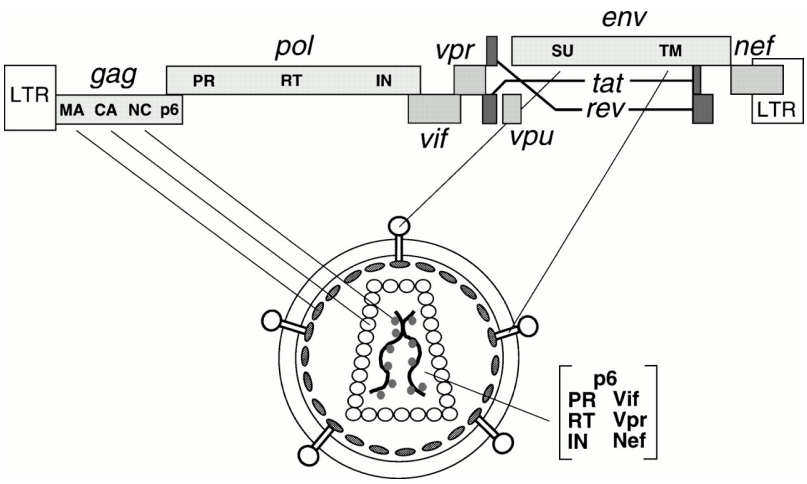

Figure 1.1 Organisation of the HIV-1 genome and virion (adapted from Frankel en Young (1998))

1.1.3. Molecular biology of HIV-1

1.1.3.1. Virion structure and genomic organisation

The HIV virions have a spherical shape of about 110 nm diameter and the conical capsid is enveloped by a lipid bilayer that is derived from the membrane of the host cell. The envelope contains exposed surface glycoproteins (i.e. gp120) that are anchored to the virus via

non-covalent interactions with the transmembrane protein gp41. A shell of matrix protein (MA, p17) lines the inner surface of the envelope membrane and surrounds the capsid (CA, p24). The capsid contains two identical copies of unspliced (+)-single stranded viral RNA genome of about 9.2 kb long, which is stabilized as a ribonucleoprotein complex with the nucleocapsid protein p7 (NC) and associated with two copies of reverse transcriptase (RT). It also contains the viral enzymes protease (PR) and integrase (IN), as well as the accessory proteins Vif, Vpr and Nef. Three additional proteins that are functional in the host cell, Tat, Rev and Vpu, do not appear to be packaged in the viral particle (Fig 1.1).

The HIV-1 genome is flanked by two long terminal repeats (LTRs) and contains nine open reading frames. Three of them encode for the Gag, Pol and Env polyproteins which are specific for retroviruses and are proteolyzed to yield the Gag structural proteins MA (matrix), CA (capsid), NC (nucleocapsid) and p6, the Env structural proteins SU (surface or gp120) and TM (transmembrane or gp41) as well as the Pol enzymes PR, RT and IN. The six additional open reading frames that flank the *env* gene encode for the regulatory proteins Tat and Rev and the accessory proteins Nef, Vif, Vpr and Vpu. The 5' LTR contains the promoter sequence that controls viral expression, while the 3' LTR is involved in polyadenylation.

1.1.3.2. Replication cycle

The $CD4^+$ T-lymphocytes, cells of the monocyte/macrophage lineage and dendritic cells are the target cells for HIV-1 infection because they express the human leukocyte antigen (HLA) class II receptor CD4 on their surface that is known as the primary target receptor of HIV (Dalgleish *et al.*, 1984; Schubert and Strebel, 1994). Viral entry is initiated by the interaction of viral gp120 with the cellular CD4 receptor. This interaction induces a conformational change in the gp120 molecule that allows binding with chemokine co-receptors. Depending on this co-receptor usage, HIV isolates can be divided into two major groups. T-cell- or T-tropic isolates that use the CXCR4 co-receptor were first identified and CXCR4 was earlier designated as fusin (Feng *et al.*, 1996; Oberlin *et al.*, 1996), whereas macrophage- or M-tropic isolates use the CCR5 co-receptor (Deng *et al.*, 1996; Dragic *et al.*, 1996) (Fig 1.2).

Membrane fusion of the viral envelope with the target cell is followed by a poorly understood uncoating event that results in the release of the viral nucleoprotein complex in the cytoplasm of the infected cell (Farnet and Haseltine, 1991). In this nucleoprotein complex - which contains parts of MA, IN, RT and Vpr - the single stranded RNA genome of HIV is

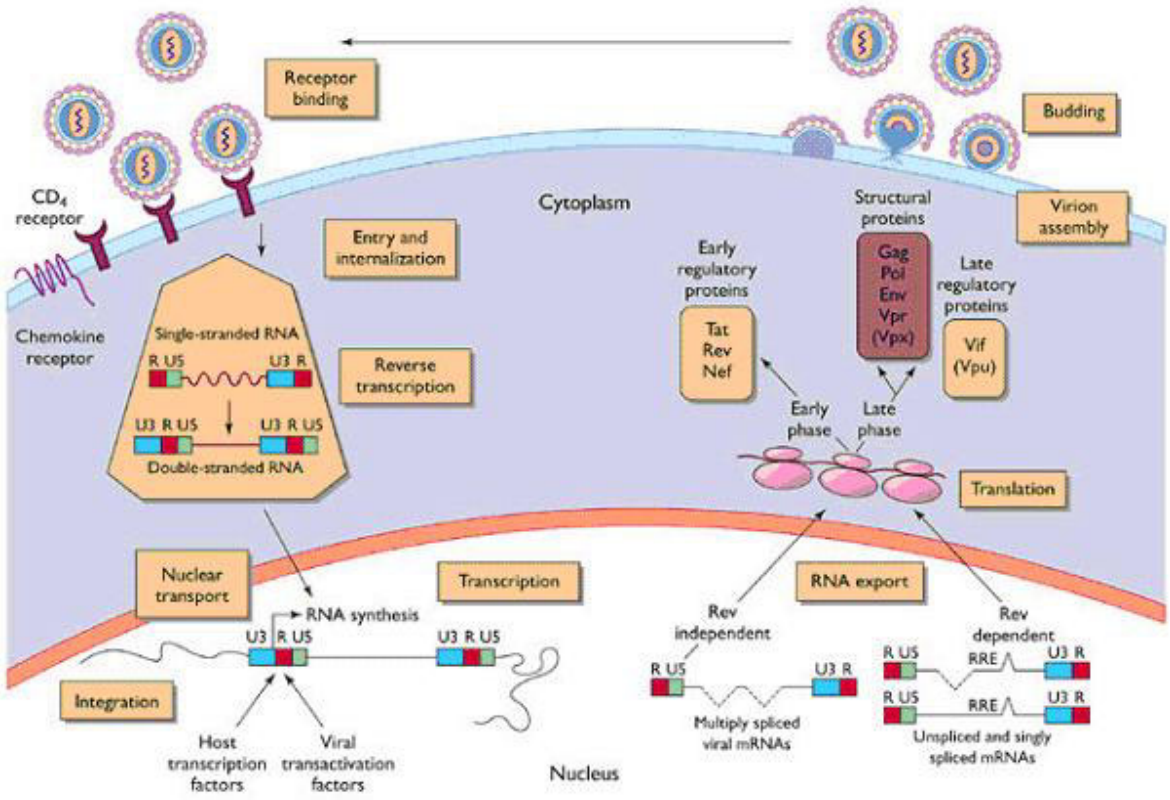

Figure 1.2 The HIV replication cycle (Taken from Perez *et al.*, 2001)

now reverse transcribed into a double stranded DNA molecule by a complex mechanism involving RNA- and DNA-templated polymerase and RNase H activities that are carried out by the reverse transcriptase (RT). The accessory protein Vpr appears to participate in the reverse transcription process by facilitating the initiation of reverse transcription by interacting with the lysine tRNA synthetase, since this interaction correlates with an inhibition of the aminoacylation of $tRNA^{lys}$, the primer that is necessary for reverse transcription (Stark and Hay, 1998). Also, the NC enhances reverse transcription by promoting the annealing of the $tRNA^{lys}$ primer to the primer-binding site (PBS) (Huang *et al.*, 1998b). The large nucleoprotein complex is now called the pre-integration complex (PIC) and transported to the nucleus (Miller *et al.*, 1997). In contrast to other retroviruses that require cell division and concomitant breakdown of the nuclear membrane to gain access to the nuclear compartment, the PIC is actively transported into the nucleus, which is directed by nuclear localization sequences (NLS) on the MA protein and IN and by the accessory protein Vpr (Vodicka, 2001). MA, an abundant PIC protein, was found to display nucleophilic properties and contains, besides an NLS, also a nuclear export signal (NES) that is necessary for packaging (Dupont *et al.*, 1999). Vpr does not contain an NLS but appears to connect the PIC to the

nuclear import machinery (Popov *et al.*, 1998a and 1998b). Nuclear localization is also dependent on the central DNA flap, a central, 99 nucleotides-long plus strand overlap in the linear DNA molecule (Zennou *et al.*, 2000). As part of the PIC, IN recognizes attachment sites (*att*) in the long terminal repeats (LTR) at the 5' and 3' ends of the viral DNA and subsequently removes a dinucleotide, adjacent to a highly conserved CA dinucleotide, from the 3' strand of the U3 and U5 viral LTR (3'-processing). After nuclear import, IN covalently joins the 3' and 5' ends of the cellular DNA of the host cell in the nucleus. The unpaired nucleotides at the viral 5' ends are now removed and the ends are joined to the target site 3' ends, generating an integrated provirus. Once integrated, the viral DNA remains permanently associated with the host cellular DNA as long as the lifetime of the cells.

The provirus serves as the template for the synthesis of unspliced and spliced mRNAs transcripts, which are transported out of the nucleus for translation in the cytoplasm. Initially, only multiply-spliced mRNAs encoding Tat, Rev and Nef are synthesized and translated (early phase). Tat is an essential transcriptional activator that interacts with a *cis*-acting RNA hairpin sequence at the 5' end of nascent RNA transcripts, the Tat responsive element (TAR). This Tat/TAR interaction results in assembling of transcriptional complexes with cellular proteins that stimulates transcriptional elongation by RNA polymerase II (Berkhout *et al.*, 1989) and increases initiation of the transcription. HIV produces only one primary transcript upon transcription from the integrated proviral DNA, which serves as template for the synthesis of all structural and regulatory proteins. Consequently, full length and singly-spliced viral mRNA transcripts are appearing in the cytoplasm. The nuclear export of the unspliced and singly-spliced mRNAs, needed for Gag, Pol and Env synthesis and packaging, is mediated through the chromosome region maintenance 1 (CRM1) export pathway and by the posttranscriptional transactivator Rev that contains an NLS (Pollard and Malim, 1998). Rev binds as an oligomer to a highly structured RNA located within the unspliced viral transcripts of the *env* gene, the Rev responsive element (RRE). As Rev accumulates in the nucleus, more unspliced and singly-spliced mRNAs associate with Rev and are shuttled out of the nucleus. In this manner, Rev functions as a switch between the early synthesis of multiply-spliced mRNAs (encoding Tat, Rev and Nef) and the later synthesis of unspliced (encoding the Gag and Gag-Pol proteins) and the singly-spliced (encoding Env, Vpu, Vif and Vpr) mRNAs.

The Env precursor protein gp160 is synthesized by the endoplasmatic reticulum (ER)-bound ribosomes and post-translationally modified and glycosylated in the ER and Golgi apparatus. The polyprotein is cleaved into gp120 and gp41 by cellular endoproteases while transported to the cell membrane. The accessory protein Vpu binds to the ER-synthesized

CD4 and promotes the degradation of CD4 by the ubiquitin-proteosome pathway (Bour *et al.*, 1995; Schubert *et al.*, 1998; Schubert and Strebel, 1994). In this way, the premature binding of CD4 to gp160 in the ER can be prevented, resulting in efficient transport and processing of gp160 (Crise *et al.*, 1990). Similarly, cell-surface major histocompatibility complex-I (MHC-I) molecules are downregulated by endosomal degradation upon internalisation by the accessory protein Nef. While downregulation of CD4 may increase virion release from the cell surface and may increase higher infectious viruses, the MHC-I downregulation protects HIV-1 infected cells from both cytotoxic T-lymphocytes and natural killer cells (Lama, 2003) and prevents reinfection by budding virions (Mangasarian and Trono, 1997).

The Gag and Gag-Pol polyproteins are synthesized on free ribosomes. The N-terminally myristoylated MA domain of the polyproteins directs binding to the cellular membrane (Bryant and Ratner, 1990). During or after transport to the cell membrane, the Gag precursor interacts with the Gag-Pol precursor and is responsible for the assembly of virion particles and the recruitment and encapsidation of two copies of unspliced viral RNA, the $tRNA^{lys}$ primer and Vpr into the budding virions. During or immediately after budding PR cleaves the Gag and Gag-Pol polyproteins to produce the viral enzymes and structural proteins. The structural proteins rearrange via a process called maturation to form an infectious virus particle.

1.2. REVERSE TRANSCRIPTASE

1.2.1. Biosynthesis of RT

The reverse transcriptase is encoded by the *pol* gene that is translated as part of the Gag-Pol polyprotein precursor during the late stage of virus replication. The Gag-Pol is synthesized by a -1 ribosomal frameshift during the translation of the *gag* gene to the *pol* gene. This ribosomal frameshifting occurs by an estimated frequency of 5 to 10%, which results in a Gag to Gag-Pol proteins ratio of 9/1. The Gag-Pol precursor is packaged into the virus particle and further RT maturation is mediated by the viral protease, which is released by autocatalytic cleavage. This cleavage occurs between amino acids F440 and Y441 of the 66 kDa subunit (denoted as p66) to yield the 51 kDa subunit (p51). Both subunits contain an identical amino-terminal sequence and form an asymmetric heterodimer. Interactions of the carboxyl group of E438 with neighbouring residues are critical to protect the F440-Y441 in

the p66 from cleavage and helps preventing eventual further processing of the heterodimer to a p51/p51 homodimer (Navarro *et al.*, 2001) (Fig 1.3).

Two models have been proposed for the generation of the mature p66/p51 heterodimer. In the first sequential model, initially a homodimer of two p66 subunits is formed in which one of the subunits resembles the tertiary structure of a p51 conformation. This forces the carboxyl terminus to partially unwind, becoming accessible for proteolytic cleavage by the protease (Davies *et al.*, 1991; Hostomska *et al.*, 1991). According to a second concerted model, the formation of the heterodimer occurs in a two-step dimerisation process, which involves the rapid binding of the p66 and p51 subunits into an inactive dimer (association phase), followed by slow conformational changes (activation phase), yielding an active heterodimeric enzyme (Divita *et al.*, 1995). Recent data support the first sequential model since the dimerisation defective mutant L234A RT resulted in the formation of p66 only without any p51-processing (Sluis-Cremer *et al.*, 2004). However, *in vivo*, a combination of both mechanisms might still occur (Morris *et al.*, 1999b).

1.2.2. Molecular structure of RT

1.2.2.1. Crystal structures

Elucidation of the molecular structure of HIV-1 RT by crystallographic studies gives opportunities to determine the structure-function relationship of the biochemical reactions catalyzed by HIV-1 RT, to evaluate structure-activity relationship (SAR) of existing drugs and to rationally design more specific and potent new drugs (Ren and Stammers, 2005). Today, several crystallographic HIV-1 RT structures are already available. The first reported RT structure was a complex of RT with the non-nucleoside inhibitor (NNRTI) nevirapine (Kohlstaedt *et al.*, 1992). Since then several other structures were crystallized and determined including: the apo-form of the enzyme (Hsiou *et al.*, 1996; Rodgers *et al.*, 1995), HIV-1 RT bound to a 18/19-mer DNA oligonucleotide duplex and the Fab fragment of a monoclonal antibody (Arnold *et al.*, 1992; Ding *et al.*, 1998; Jacobo-Molina *et al.*, 1993), HIV-1 RT complexed with a dideoxy-terminated DNA template/primer and dTTP (Huang *et al.*, 1998a), RT bound to the polypurine tract (PPT) (Sarafianos *et al.*, 2001), RT complexed with other inhibitors such as α-APA (loviride), TIBO, HEPT and thiocarboxanilide derivatives (Ding *et al.*, 1995b; Hopkins *et al.*, 1996; Ren *et al.*, 1995a; Ren *et al.*, 1995b; Ren *et al.*, 1998) as well as crystal structures of mutant drug-resistant RTs (Chamberlain *et al.*, 2002; Das *et al.*, 1996; Hsiou *et al.*, 1998).

Only recently the crystal structure of HIV-2 RT was elucidated. It has a similar overall fold to HIV-1 RT but has structural differences within the non-nucleoside RT inhibitor (NNRTI) pocket at both conserved and nonconserved residues (see below) (Bird *et al.*, 2003; Ren *et al.*, 2002).

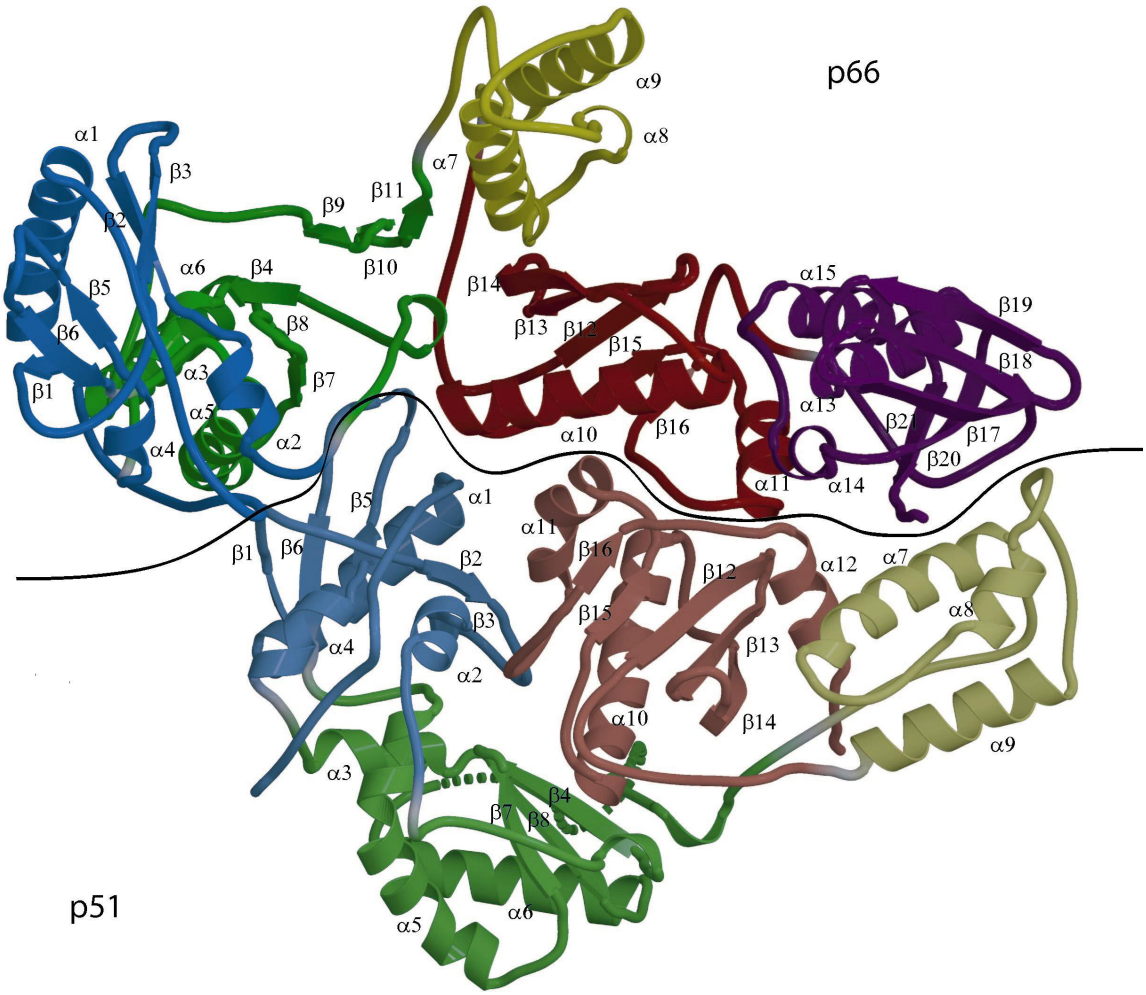

Figure 1.3 Three-dimensional structure of unliganded HIV-1 RT (by courtesy of Dr. R. Esnouf)

Fingers subdomains are blue, palm subdomains are green, thumb subdomains are yellow and connection subdomains are red. The RNase H domain in p66 is purple. p51 does not contain β9, β10, β11 or RNase H domain. p66 does not contain α12.
In literature two nomenclatures are used to describe the structural elements of HIV-1 RT. The HIV-1 structure and nomenclature in this figure is adapted from Ren *et al.*, 1995a. In the text the nomenclature of Kohlsteadt *et al.* (1992) is used unless otherwise stated. To convert the nomenclature of Kohlsteadt *et al.* (1992) (on the left side of the arrow) to the nomenclature of Ren *et al.* (1995a) (on the right side of the arrow), following conversions have to be made:

β3 → β2	β7 → β5	β12 → β9	αB → α2
β4 → β3	β8 → β6	β13 → β10	αE → α5
β5 → (1)	β9 → β7	β14 → β11	αH → α7
β6 → β4	β10 → β8	β15 → (2)	αI → α8

(1) In the structure of Ren *et al.* (1995a) an α2' is observed instead of a β5 strand
(2) In the structure of Ren *et al.* (1995a) there is no β strand at this position

1.2.2.2. Overall structure

The polymerase domain of the p66 subunit of HIV-1 RT resembles anatomically a right hand and consists of four subdomains named fingers, palm and thumb. The fourth subdomain lies between the polymerase site and the RNase H subdomain and is therefore called connection. Unlike the p66, the p51 misses the 15 kDa RNase H domain (Kohlstaedt *et al.*, 1992). The polymerase subdomains of the p66 subunit are folded into an open right-hand conformation, which creates a cleft between the thumb and fingers subdomain creating a binding-site for the template/primer duplex. The overall folding in the p51 subunit is similar to the folding in the p66 subunit but the spatial arrangement of subdomains is different and it lacks the RNase H subdomain. This results in an asymmetric heterodimer. Most notably, the connection domain of the p51 subunit is rotated to cover the palm, filling in the region that forms the nucleic acid binding cleft in p66 and displacing the thumb, which is now moved away from the palm and which makes contacts with the RNase H domain.

HIV-1 RT is a flexible enzyme, especially the p66 subunit has the opportunity of large-scale motions while the p51 subunit is essentially rigid. The global motion of the RT heterodimer is comprised of movements of the p66 fingers, accompanied by fluctuations of the RNase H domain and p51 thumb. The hinge-bending residues that control the highly concerted motion of the subdomains include the catalytic active site and the non-nucleoside inhibitor binding pocket of the p66 subunit (Bahar *et al.*, 1999). Binding of RT to dsDNA increases the flexibility and the ability of the p66 fingers to close down after binding of a dNTP molecule (Madrid *et al.*, 2001).

1.2.2.3. The polymerase active site

The catalytic site for polymerisation lies in the palm subdomain and three aspartic acids are identified to be essential for catalysis: D185 and D186 which are part of the highly conserved YMDD motif, and D110 (Larder *et al.*, 1987). Subunit-specific mutagenesis of this catalytic triad demonstrated its importance in the p66 subunit, since selective mutation of these residues in p51 did not result in loss of catalytic activity (Le Grice *et al.*, 1991). This was in agreement with the three-dimensional data, as discussed above, which showed that there is no nucleic acid binding cleft in the p51 and that the aspartic acid triad is buried (Kohlstaedt *et al.*, 1992). For the binding of dNTP during polymerisation, a metal coordination function is required and mediated by these aspartic acids. The phosphodiester bond involves two Mg^{2+} ions (Kaushik *et al.*, 1996; Pandey *et al.*, 1996; Patel *et al.*, 1995). A first mechanistic model proposed an initial binding of dNTP mediated via the coordination of

D186 and D110 with the β- and γ-phosphate of dNTP by the first Mg^{2+} ion, followed by an alignment of the 3'-OH of the primer terminus and the α-phosphate of dNTP by the second Mg^{2+} ion and the aspartic acid triad. The transition state of the reaction is then stabilised by D186 and D110. D186 is the residue that coordinates with the α-phosphate of dNTP and catalyses the phosphodiester bond formation with release of pyrophosphate (Kaushik *et al.*, 1996). A second model is based on the structure of HIV-1 RT complexes with template/primer and is similar to the first one, except that the second Mg^{2+} ion chelates only with D185 and the α-phosphate of dNTP to form an α-phosphate-Mg^{2+}-D185 complex that facilitates the nucleophilic attack by the oxygen atom of the 3'-OH of the primer terminus through enhancing the electropositive character of the phosphorus atom (Patel *et al.*, 1995). However, according to the crystal structure of RT complexed with a DNA template/primer and dTTP, the side chains from D110 and D185 and the phosphates of dTTP seem to be coordinated by the first Mg^{2+} ion (see Fig.1.4) (Huang *et al.*, 1998a). Thus, the exact mechanism has not been entirely clear yet.

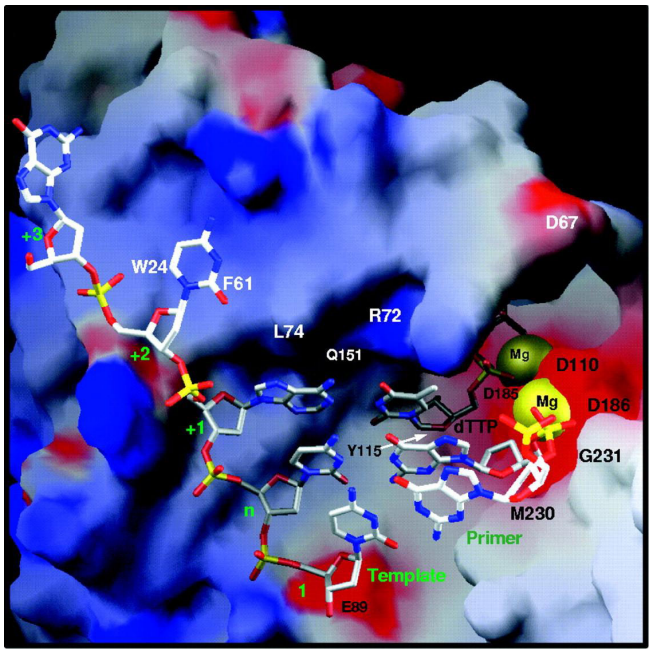

Figure 1.4 The active site (adapted from Huang *et al*. (1998)).

View into the dNTP pocket, formed by closure of the fingers domain (upper left, foreground) against the palm (center, background) and toward the thumb (lower right).

Template and primer strands and dTTP are in stick representation; the protein is a surface rendering (red and blue indicate negative and positive electrostatic potential, respectively). Mg^{2+} ions are yellow spheres.

1.2.2.4. The dNTP binding site

Prior to nucleotide incorporation, RT must select the appropriate dNTP. Besides the constraints imposed by Watson-Crick base pairing, also the structure of the polymerase active site is involved in the selection of the proper base. Besides the catalytic aspartic acid triad discussed above, modelling and mutational studies revealed that several other amino acids are in positions where they could affect the topology of the dNTP-binding site (Kaushik *et al.*, 1996 and 1997). Y183 and M184, two amino acids from the highly conserved YMDD are in close proximity to the 3'OH primer terminus and the bound dNTP, respectively and hence part of the dNTP-binding pocket (Ding *et al.*, 1998; Harris *et al.*, 1998a and 1998b; Wilson *et al.*, 1996). Other important amino acids lining the dNTP-pocket are K65, R72, D113, F116, F160 and especially Y115 and Q151 (Harris *et al.*, 1998a; Huang *et al.*, 1998a; Patel *et al.*, 1995; Tantillo *et al.*, 1994).

The V148 and Q151 residues differ from Y115 because their interaction with the substrate dNTP is important to provoke misincorporations rather than accurate DNA polymerisation. Mutations in residues 148 and 151 indeed affect the ability of the RT to complete mismatch extension. A decrease of this misinsertion fidelity influences overall viral mutagenesis (Weiss *et al.*, 2000 and 2004).

Overall, the dNTP pocket is formed by closure of the fingers domain against the palm and toward the thumb in a way that various residues in the fingers form part of the dNTP-binding site (Huang *et al.*, 1998a). The base of the dNTP is stacked on the terminus of the primer strand and the side chains of R72 and Q151 are packed against its outer surface. K65, R72, the main-chain-NH groups of D113 and A114, and two Mg^{2+} ions coordinate the triphosphate moiety. The 3'OH of the dNTP projects into a small pocket (3' pocket) lined by the side chains of D113, Y115, F116, Q151 and the peptide backbone between residue 113 and 115. The template nucleotide, which is paired with the incoming nucleotide, is blocked beneath the side chain of L74 and the backbone of G152 (Huang *et al.*, 1998a).

1.2.2.5. Template/primer binding site

RT has a large surface of interaction with the nucleic acid compared to other DNA binding proteins. The majority of the RT-template/primer contacts involve the residues of the fingers, palm and thumb subdomains that form a hand shaped clamp, which mediate the positioning of the template/primer relative to the polymerase active site (Jacobo-Molina *et al.*, 1993). The numerous interactions between the template/primer and RT are van der Waals contacts and hydrogen bonds that are not sequence-dependent and primarily involve the sugar-

phosphate backbone of the nucleic acid and structural elements of the palm, thumb and RNase H of p66 (Ding *et al.*, 1997). This is consistent with the fact that RT can copy a wide variety of different templates. In addition, hydrophobic interactions make a major contribution to the stability of the polymerase-DNA complex (Beard *et al.*, 1998).

In an RT/dsDNA complex, the bound DNA has a hybrid structure (Jacobo-Molina *et al.*, 1993). Also, in the crystal structure of RT in complex with the PPT RNA:DNA this hybrid form is observed as an A-form near the polymerase active site and a B-form towards the RNase H active site. This minor groove width dimensions of the B-form determines the susceptibility to RNase H cleavage (Fedoroff *et al.*, 1997; Han *et al.*, 1997). At the junction of the A-form and B-form regions, the DNA hybrid shows a 40 to 45° bend that is 5-9 bp away from the polymerase active site. In total, the nucleic acid binding cleft is ~60 Å in length and the distance in nucleotides between the polymerase and RNase H catalytic sites is 18 bp. While the contacts between RT and the DNA primer strands are very similar in the RT complexes with RNA:RNA and DNA:DNA, the contacts with the RNA template are different from those with the DNA template (Sarafianos *et al.*, 2001).

RT has numerous interactions with 2'-OH groups of the RNA template that include residues 280 and 284 of the p66 thumb, residues of the template grip including 89 and 91 of the p66 palm and RNase H residues (Sarafianos *et al.*, 2001). The β12-β13 hairpin, which includes residues 227 to 235 in the p66 palm subdomain, is located near the polymerase active site and nucleotides at the 3'-primer terminus (Fig 1.5). This element is the primer grip and is involved in positioning the 3'-OH end of the primer in the appropriate orientation for the nucleophilic attack on an incoming dNTP (Ghosh *et al.*, 1996; Jacobo-Molina *et al.*, 1993). Interactions of the primer grip with the 3' terminus of the primer strand involve the peptide main-chain of M230 and G231 and the primer terminal phosphate (Ding *et al.*, 1998). Alanine-scanning of the primer grip revealed that both DNA polymerase and RNase H activities are altered by substituting W229, M230, G231 and Y232 (Ghosh *et al.*, 1996; Ghosh *et al.*, 1997; Palaniappan *et al.*, 1997).

The helix clamp, which grasps the template/primer complex, consists of two anti-parallel α-helices (αH and αI) in the thumb subdomain of p66. Helix αI is directly adjacent to the sugar-phosphate backbone of the template strand, whereas αH is partially embedded in the minor groove of the dsDNA and interacts primarily with the primer strand (Hermann and Heumann, 1996; Jacobo-Molina *et al.*, 1993; Maier *et al.*, 1999). These two helices are also of major importance for proper RNaseH functioning, as they are required for the removal of the polypurine tract (PPT) (Powell *et al.*, 1999).

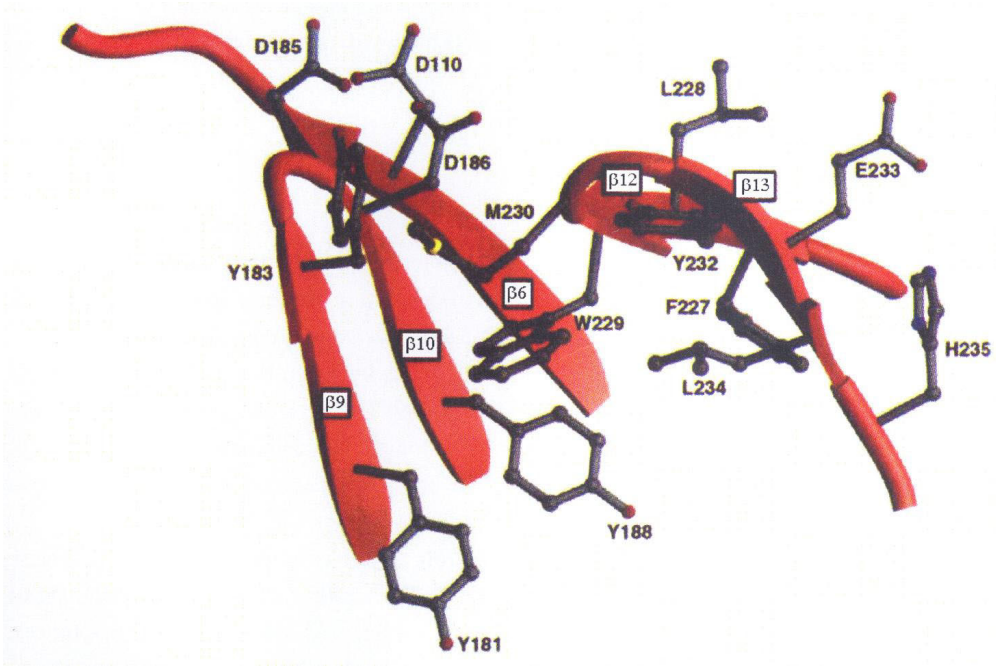

Figure 1.5 The primer grip (adapted from Ghosh *et al.* (1996))

Disposition of the side chains of F227-H235 within structural elements comprising the β12-β13 hairpin, or primer grip, of p66. Additionally, secondary structural elements from the DNA polymerase active site and relevant side-chain atoms have been highlighted.

1.2.2.5. RNase H

The active site of the RNase H domain comprises four important residues that are required to interact with the divalent cations in the catalytic reaction: D443, E478, D498 and D549 (Davies *et al.*, 1991; Mizrahi *et al.*, 1990). Mutagenesis of D443 and E478 completely abolished the RNase H activity but did not affect wild-type polymerase activity, however, substitutions of D498 resulted in an unstable enzyme and has probably a function in the folding of the heterodimeric RT (Mizrahi *et al.*, 1990 and 1994; Schatz *et al.*, 1989). Davies *et al*. (1991) identified two metal binding sites in the crystal structure of RNase H in close proximity to the active site. However, Ren *et al*. (1995) observed only one metal binding site interacting with D443, D498 and D549. Rodgers *et al*. (1995) also observed one binding site located between D443, E478 and D498. In a crystal structure of RT complexed with DNA template/primer only one Mg^{2+} ion was complexed with D443, D549 and the template phosphate, which was located between E478 and H539 (Huang *et al.*, 1998a). The conflicting observations from different RT structures probably reflect a flexibility of the RNase H active site to bind metal ions in a variety of ways. The functional role of such flexibility is still unclear. The RNase H active site is also capable of binding other bivalent cations than Mg^{2+},

like Mn^{2+} (Cirino *et al.*, 1995) or Fe^{2+} (Gotte *et al.*, 1998). The latter permits the enzyme even to cleave dsDNA by an oxidative mechanism instead of an enzymatic mechanism (Gotte *et al.*, 1998).

The RNase H domain makes significant contact with the thumb domain of the p51 subunit that affects the level of the RNase H activity of RT (Morris *et al.*, 1999a). The importance of the p51 thumb in obtaining the optimal RNase H structure is clearly shown when replacement of the p54 thumb in HIV-2 RT - which exhibits a ten-fold lower RNase activity - by the p51 thumb domain of HIV-1 RT was found to restore the RNase H activity level as high as that of HIV-1 RT (Sevilya *et al.*, 2001). A more detailed mutagenesis study revealed that the difference in RNase H activity between HIV-1 and HIV-2 RT is restricted to the Q294 residue in the p54 thumb of HIV-2 RT (Sevilya *et al.*, 2003a). Other residues that affect the *in vitro* RNase H function are the highly conserved C280 (Sevilya *et al.*, 2003b), W266 (Powell *et al.*, 1999), Y501 (Arion *et al.*, 2002), T473, E475 and K476, which make contact with the nascent DNA primer, and H539 and N474 that interact with the scissile phosphate of the RNA template (Arion *et al.*, 2002; Rausch *et al.*, 2002).

1.2.2.6. The role of p51 in the heterodimer

As stated above, RT is a heterodimer that consists of a p66 and p51 subunit with identical sequences but a different overall folding. In addition, the p51 lacks the p15 RNase H domain. Although p66/p66 and p51/p51 homodimers could possibly exist, the p66/p51 heterodimer is the most stable form and the dimeric form is necessary for catalytic activity (Bathurst *et al.*, 1990). Thus, the p51/p51 homodimer is catalytically inactive (Hizi *et al.*, 1988; Starnes *et al.*, 1988). However, other groups observed certain degrees of activity for p51 homodimers (Lori *et al.*, 1988; Restle *et al.*, 1990). Several studies have tried to elucidate the role of p51 in heterodimer RT. A chimeric HIV-1 RT containing the amino-terminal of HIV-1 RT and the carboxyl-terminal domain of murine leukaemia virus (MuLV) RT, which exists as a monomer, resulted in a monomeric enzyme with intact polymerase and RNase H functions (Misra *et al.*, 1998). This revealed that RT lacks two amino acid motifs located between the polymerase and the RNase H domain that are present in MuLV RT, which are responsible for the incompatibility of HIV-1 RT to be active as monomer. A later study demonstrated that the insertion of only a 26 amino acids-long peptide from MuLV RT into the connection domain of HIV-1 RT resulted already in a monomeric active HIV-1 RT (Pandey *et al.*, 2001b).

Study of dynamic characteristics of both subunits show that the p66 subunit is very flexible, while the p51 subunit is rigid, which implies a structure-supporting function of the

p51 subunit (Bahar *et al.*, 1999). Besides retaining the structural form of the heterodimer (Amacker and Hubscher, 1998), the p51 may also be involved in loading of the p66 subunit onto the template/primer. This is proven by the fact that, once bound to the template/primer, the heterodimer remains catalytically active after dissociation of the p51 subunit (Harris *et al.*, 1998b). In addition, an important interaction between the p51 thumb domain and the RNase H domain exists that plays, besides the role in RNase H functioning that is described above, also a role in proper folding of the template/primer and the tRNA-binding site during maturation of heterodimeric RT (Morris *et al.*, 1999a). Previous studies already suggested that p51 contributes to the binding site for the $tRNA^{Lys}$ primer as well as the binding site for template/primer (Jacques *et al.*, 1994; Zakharova *et al.*, 1995).

1.2.2.7. The p66/p51 interface

There are three distinct contacts between p51 and p66, namely the connection domain, the tip of the finger subdomain of p51 and the extended thumb region that supports the RNase H region of p66 (Becerra *et al.*, 1991). The connection subdomain, which lies between the polymerase and RNase H active sites in p66, plays a central role in the formation of the reverse transcriptase heterodimer. Extensive and very different intra- and intersubunit contacts are made by the connection subdomains of each of the subunits that constitute approximately one-third of the total contacts between subunits of the heterodimer. Conversion of an open p66 polymerase domain structure to a closed p51-like structure results in a reduction in solvent-accessible surface area and the burying of an extensive hydrophobic surface. Thus, the monomeric forms of both p66 and p51 are proposed to have the same closed structure as seen in the p51 subunit of the heterodimer. The free energy required to convert p66 from a closed p51-like structure to the observed open p66 polymerase domain structure is generated by the burying of a large, predominantly hydrophobic surface area upon formation of the heterodimer. It is likely that the only kind of dimer that can form is an asymmetric one like that seen in the heterodimer structure, since one dimer interaction surface exists only in p51 and the other only in p66 (Wang *et al.*, 1994).

One of the most studied interface regions is the hydrophobic tryptophan-rich cluster in the connection domain that consists of six W residues at codons 398, 401, 402, 406, 410 and 414. These amino acids are conserved among primate lentiviral RTs and mutagenesis studies revealed their importance in heterodimer formation (Tachedjian *et al.*, 2003). The effect of these residues is mainly directed from the p66 subunit. A second stretch suggested to be involved in dimerisation contains a leucine hepta-repeat motif (L282-L310) (Baillon, 1991).

This region in p66 has shown to be instrumental in protein-protein interactions required for p66/p51 RT dimerisation. Goel *et al.* (1993) demonstrated that the mutant L289K p66 subunit was unable to dimerise with either the mutant L289K or the wild-type p51 subunit, but not *vice versa* (i.e. dimerisation of wild-type p66 with mutant p51), which suggests a critical role for the leucine repeat motif in p66 (but not in p51) in heterodimer formation. A third important interface region exists as a minor groove at the floor of the polymerase binding cleft of the p66 subunit. The β7-β8-loop of the p51 subunit, formed by 6 amino acids noted as SINNET between two prolines at positions 133 and 140, seems to fit in this groove-like structure and stabilizes the polymerase domain of the p66 subunit (Pandey *et al.*, 2001 and 2002).

1.2.3. Enzymatic activities of RT

HIV-1 RT exhibits two distinct enzymatic activities: a DNA polymerase that can use either DNA or RNA as a template and a nuclease, termed ribonuclease H (RNase H), which specifically degrades the RNA in RNA/DNA hybrids. The polymerase domain is associated with the amino terminus of both the RT subunits, while the RNase H activity is associated with the carboxyl-terminal domain of the p66 subunit (Hansen *et al.*, 1987). However, the polymerase site is only active in the open RT conformation, which is present in the p66 subunit alone (Kohlstaedt and Steitz, 1992). The native p66/p51 heterodimer is the most stable form and the dimeric form of the enzyme possesses significant RT activity (Restle *et al.*, 1990; Restle *et al.*, 1992).

1.2.3.1. DNA polymerase activity

The initial step of DNA polymerisation by RT is template/primer binding. Moreover, the primer will bind first to the free enzyme followed by template binding to this complex (Majumdar *et al.*, 1989). Subsequently, the matching dNTP binds to the RT-template/primer complex. Next, a nucleophilic attack on the 3'OH of the primer will occur in this ternary complex to yield the phosphodiester bond and release of pyrophosphate. Both template/primer and nucleotide binding are two-step processes (Divita *et al.*, 1993; Hsieh *et al.*, 1993; Kati *et al.*, 1992): First, the complex undergoes a conformational change prior to the covalent linkage of the incoming nucleotide. Second, an isomerisation reaction takes place that results into a more tightly bound state. This reaction is rate limiting for nucleotide incorporation and also provides a second level of accuracy in nucleotide selection preceding phosphodiester bond

formation (Tong *et al.*, 1997). The initial binding of the template/primer merely involves electrostatic interactions between the primer grip and the template, whereas the subsequent conversion to a tightly bound complex involves conformational changes of both enzyme and template, leading to proper positioning and alignment (Patel *et al.*, 1995).

The lack of an intrinsic 3' to 5' exonuclease activity is one of the most important features of HIV-1 RT in terms of development of resistance because it does not proof-read replication errors (Roberts *et al.*, 1988). In addition, RT is able to efficiently extend mismatched primer termini (Perrino *et al.*, 1989; Yu and Goodman, 1992). These features explain the unusual high error rate of HIV-1 RT compared with other DNA polymerases. The average error rate of RT was determined *in vitro* at 1.7 x 10^{-3} per nucleotide incorporated (Roberts *et al.*, 1988). RT is unusually inaccurate for single base deletions, additions and substitutions in homopolymeric sequences (Bebenek *et al.*, 1989 and 1993). These errors involve template/primer slippage rather than direct base miscoding. The fidelity of DNA synthesis is higher with RNA than with DNA templates (Boyer *et al.*, 1992).

An extensive G to A hypermutation is characteristic for HIV-1, primarily at GpA and GpG sites (Vartanian *et al.*, 1991 and 1994). The transitions in GpG appear to be the consequence of base mispairing at the ends of runs of G residues, whereas G to A transitions within GpA may result from temporary dislocation of the primer and template strands by a single base (Vartanian *et al.*, 1994). The G to A hypermutation is likely an example of an induced mutation whereby the viral reverse transcriptase is forced in making errors by imbalances in the intracellular dCTP pools.

The *in vitro* processivity of HIV-1 RT is low (Abbotts *et al.*, 1993; Klarmann *et al.*, 1993). Processivity is defined as the average number of nucleotides incorporated before RT and template dissociate. Natural RNA and DNA templates contain 'pause sites', sequences where the processivity of the RT is very low. Homopolymeric nucleotide runs and regions with secondary structures exhibit strong pauses in DNA synthesis (Abbotts *et al.*, 1993; DeStefano *et al.*, 1992a; Klarmann *et al.*, 1993).

1.2.3.2. Ribonuclease H activity

The RNase H catalyzes the specific cleavage of the RNA moiety of an RNA/DNA hybrid. Degradation of the RNA in the nascent RNA/(-)DNA hybrid hereby enables the (+) strand DNA synthesis. Additionally, RNase H generates specific RNA primers for (+) strand initiation and also removes the RNA primers from the nascent (-) and (+) DNA strands to allow full length DNA synthesis. RNase H hydrolyzes, in contrast to other ribonucleases,

phosphodiester bonds to produce 3'-hydroxyls and 5'-phosphates to generate primers with a 3'-OH.

RNase H has a polymerase-dependent and a polymerase-independent mode of cleavage (Gopalakrishnan *et al.*, 1992; Peliska and Benkovic, 1992). During (-) strand synthesis, RNase H will introduce cuts in the U5 region of the genomic RNA, while the polymerase site is still bound to the 3' end of the growing DNA strand (i.e. the polymerase dependent activity). The polymerase-independent activity occurs after (-) strand synthesis when large uncleaved RNA fragments remain annealed to the newly synthesized (-) strand DNA. These fragments are removed by binding of the RNase active site to each fragment, which is then degraded without binding of the polymerase active site (DeStefano *et al.*, 1994; Palaniappan *et al.*, 1996). Both endo- and exonuclease activity have been reported for HIV-1 RNase H (Schatz *et al.*, 1990). However, only a partially processive 3' to 5' endonuclease was observed that further degrades the RNA primer (DeStefano *et al.*, 1991).

1.3. REVERSE TRANSCRIPTION

The reverse transcription of the viral ssRNA genome to a dsDNA provirus occurs mainly in the cytoplasm approximately 3 hours after viral entry, although there is evidence of limited reverse transcription in virions (Oude Essink *et al.*, 1996; Trono, 1992). Reverse transcription is probably initiated at the time of virus assembly but pauses after the virions are released from the host cell because of low dNTP pools in the virus particle. A large nucleoprotein complex is formed after viral entry where the DNA synthesis takes place (Bowerman *et al.*, 1989; Brown *et al.*, 1987). During reverse transcription, the internal RNA sequences unique to the 3'- (U3) and 5'- (U5) ends of the viral RNA are duplicated, generating the long terminal repeats (LTRs) that are composed of U3-R-U5 components, which are characteristic for the viral genome. Two template switches are required to generate the LTRs, known as the strand-transfers.

RT is capable of carrying out the total reverse transcription process by itself *in vitro* since it displays DNA polymerase activity on both RNA and DNA templates as well as RNase H activity. However, *in vivo*, it is stated that certain viral (NC, Tat, Vpr, Vif and Nef) and cellular proteins (such as topoisomerase I and cyclophilin A) increase the efficiency of reverse transcription.

1.3.1. Initiation of the reverse transcription

Reverse transcription is initiated by the annealing of the 3'-terminal 18 nucleotides of the partially unwound cellular $tRNA^{Lys}$ with the primer binding site (PBS), located in the 5' untranslated region of the genomic RNA (Ratner *et al.*, 1985b). RT itself is unable to discriminate between its cognate $tRNA^{Lys}$ primer and other tRNA forms (Thrall *et al.*, 1996), therefore the $tRNA^{Lys}$ primer is selectively incapsidated into the viral particles by specifically recruitment by the Gag-Pol precursor polyprotein (Mak *et al.*, 1994). The unwinding of the tRNA cloverleaf and subsequent PBS annealing is mediated by the nucleocapsid protein and the RT itself (Barat *et al.*, 1989 and 1993; Oude Essink *et al.*, 1995). The viral RNA and the $tRNA^{Lys}$ primer form several interactions in addition to annealing that are crucial for the efficiency and the specificity of the initiation of reverse transcription (Arts *et al.*, 1998; Isel *et al.*, 1993 and 1995) and facilitate the subsequent transition to a less specific elongation phase (Isel *et al.*, 1996 and 1998; Lanchy *et al.*, 1996). Due to the possible interactions with the tertiary structure of the RNA/$tRNA^{Lys}$ initiation complex, RT is allowed to interact with the template strand up to 20 nucleotides upstream from the polymerase site (Lanchy *et al.*, 2000). However, recent data suggested that the structural elements are already present in the viral RNA prior to $tRNA^{Lys}$ annealing, thus explaining that extensive interactions of RNA with the primer are not required to bind RT (Goldschmidt *et al.*, 2004).

1.3.2. Minus strand synthesis

The synthesis of the (-) strand, initiated from the $tRNA^{Lys}$ primer annealed to the PBS, results in the formation of a short (-) strong-stop DNA fragment (sssDNA) that consists of the U5 and repeat (R) regions of the LTR (Fig. 1.6). The 5' end of the genomic RNA is degraded by the RNase H and makes it possible for the sssDNA to hybridise with the complementary sequence in R at the 3' end of the viral genome, a process that is known as the first strand-transfer reaction. Efficient strand-transfer is dependent on the RNase H-mediated removal of the residual RNA (DeStefano *et al.*, 1992b; Peliska and Benkovic, 1992). Also, strand-transfer is facilitated by structural features in the R region (Berkhout *et al.*, 2001). However, transfer can be initiated at internal regions of the homologous R sequence without requiring total cleavage at the 5'-end. The sssDNA will invade at the gaps created by RNase H (Chen *et al.*, 2003). The positions that are more favourable for invasion are the so-called dimerisation initiation sequences (DIS) (Balakrishnan *et al.*, 2003). Since virus particles contain two

identical genomic RNA molecules, this strand-transfer can occur in an intermolecular (between the two RNA templates) or an intramolecular (from the 5' end to the 3' end of the same template RNA molecule) manner (van Wamel and Berkhout, 1998). After the first strand-transfer, the synthesis of the (-) strand continues to the 5' end of the template, which is now at the 5' of the PBS, since R and U5 have been removed earlier by RNase H. Concomitant with the elongation of the (-) DNA strand, the viral RNA template is degraded by the RNase H.

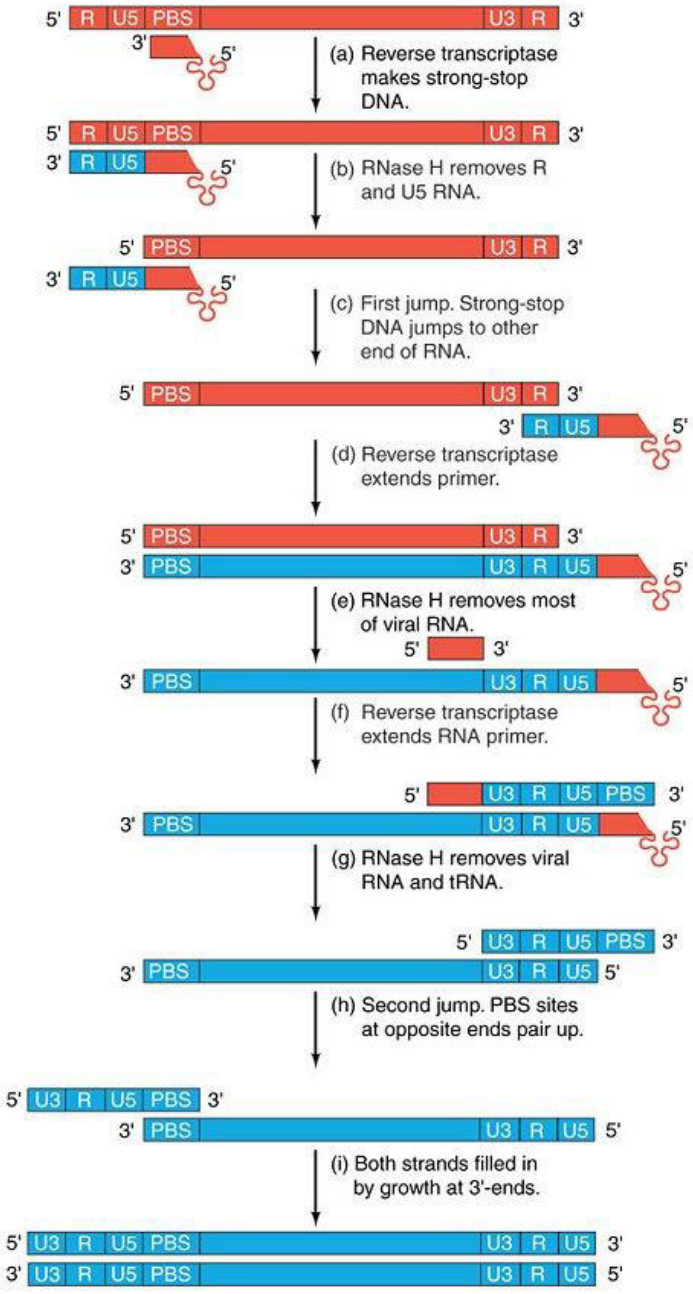

Figure 1.6 Mechanism of reverse transcription.

1.3.3. Plus strand synthesis

For the synthesis of the second (+) DNA strand RT utilizes two primers: a polypurine track (PPT) at the border of the U3 domain of the 3' LTR and a central PPT (cPPT) that is located in the center of the genome, at the end of the *pol* gene (Fig. 1.6) (Charneau *et al.*, 1994; Pullen and Champoux, 1990; Pullen *et al.*, 1992). After (-) strand synthesis approximately 15% of the RNA template remains as oligomers annealed to the newly formed DNA, but only the PPTs are used as primer for (+) strand synthesis (Fuentes *et al.*, 1995). Unlike other polymerases, RT can bind either to the 5' end of a primer for degradation or to the 3' end for synthesis. The competition between the two binding modes might determine whether a primer will be extended or degraded (Palaniappan *et al.*, 1998). However, Sarafianos *et al*. (2001) suggested that the selection of these particular polypurine RNA sequences is due to the resistance of the PPTs to RNase H digestion. More specific, the PPTs contain rA-stretches that have an unusual narrow minor groove that might prevent the RNA template from reaching the catalytic residues of RNase H, which results in unproductive cleavage (Sarafianos *et al.*, 2001). The rG stretch present in the PPT is also a critical requirement for primer function (Powell and Levin, 1996) and the bending at the rG:dC/rA:dT junction might be involved in proper binding of RT to the 3' end of the PPT (Powell and Levin, 1996; Sarafianos *et al.*, 2001 and 2003).

The synthesis of the (+) strand is initiated at the 3' PPT and generates the (+) strand strong-stop DNA ((+) sssDNA), which is terminated at the first methylated adenosine of the $tRNA^{Lys}$ primer that is still attached to the 5' terminus of the (-) DNA strand. In this way, a DNA copy is created of the PBS at the (+) sssDNA terminus. The short RNA/DNA hybrid, which is formed when a part of the $tRNA^{Lys}$ primer is used as a template, permits the removal of the primer tRNA by RNase H. This enables the $tRNA^{Lys}$ PBS in the (+) strand to anneal with the PBS at the 3' end of the (-) sssDNA. This event occurs only intramolecularly and is known as the second strand-transfer. After this second strand-transfer, the elongation of the (+) and (-) DNA strands continue. The (+) DNA strand is further elongated to the central termination sequence (CTS) located immediately at the 3' end of the cPPT. Additionally, the (+) DNA strand initiated at the cPPT is elongated to the end of the (-) DNA strand template. About 99 nucleotides of (+) DNA strand between the cPPT and CTS are displaced, resulting in a defined and stable overlap (Charneau *et al.*, 1994). This central DNA flap is necessary for nuclear import of the HIV-1 genome (Zennou *et al.*, 2000). After nuclear import, the cellular enzymes are presumed to remove the displayed sequences and to seal the (+) strand to yield

double-stranded linear DNA, which has an LTR sequence at each end (Charneau and Clavel, 1991).

1.3.4. Accessory proteins

1.3.4.1. Nucleocapsid protein

The nucleocapsid protein (NC) of HIV-1 is generated from the Gag and Gag-Pol polyprotein precursor by proteolytic cleavage of the HIV-1 protease (Henderson *et al.*, 1992). NC is a 55 amino acid long basic protein that is the major component of the virion nucleocapsid and essential for packaging the genomic RNA. NC molecules also have a high affinity for nucleic acids and coat the viral dimeric RNA at a ratio of one NC per every seven nucleotides depending on their secondary structure (Golinelli and Hughes, 2003), which offers an efficient protection against nucleases. Due to their close association with the viral genome, they play significant but also distinct roles in the reverse transcription process. NCs can promote (-) strand DNA synthesis by enhancing the annealing of the $tRNA^{Lys}$ primer to the PBS (Barat *et al.*, 1989), most likely by unwinding the tRNA structure (Khan and Giedroc, 1992). NC stimulates primer-specific proviral DNA synthesis while concurrently inhibiting non-specific reverse transcription of viral and cellular RNAs (Lapadat-Tapolsky *et al.*, 1997; Li *et al.*, 1996). During elongation of the viral DNA, NC increases the RT processivity by removing secondary RNA structures that obstruct RT progression (Ji *et al.*, 1996; Rong *et al.*, 1998). Furthermore, NC facilitates the first strand-transfer by promoting the annealing of the R region of the (-) sssDNA with the complementary region of the 3' end of the genomic RNA (Peliska *et al.*, 1994; Rodriguez-Rodriguez *et al.*, 1995). Finally, NC has been shown to enhance the incorporation of errors by the RT into the proviral DNA during (-) strand elongation by facilitating the annealing of mutated (-) cDNA during strand transfer (Lapadat-Tapolsky *et al.*, 1997). Since, besides the two strand transfers, also several additional strand transfers due to copy choice recombination occur during reverse transcription, NC could have a significant influence on the mutation rate of the viral genome. Overall, template switching of RT in reverse transcription occurs frequently and is facilitated by template proximity and the level of homology (Andersen *et al.*, 2003).

1.3.4.2. Vpr

Viral protein R (Vpr) is known to be essential for productive infection of macrophages and monocytes. Vpr is involved in nuclear import of the PIC in non-dividing cells (Heinzinger

et al., 1994). Additionally, Vpr interacts with the cellular Lys-tRNA synthetase and inhibits its enzymatic activity, blocking the aminoacylation of $tRNA^{Lys}$ and probably resulting in targeting the $tRNA^{Lys}$ primer to the assembling virion (Stark and Hay, 1998).

1.3.4.3. Vif

The viral infectivity factor (Vif) is necessary for the production of infectious virions from some cell lines (von Schwedler *et al.*, 1993). Viruses with a deletion in *Vif* were unable to complete the synthesis of proviral DNA, although they could enter the cell in a normal way (Camaur and Trono, 1996; Courcoul *et al.*, 1995). Recently it was shown that Vif interacts with a member of the cellular cytidine deaminase enzyme family (APOBEC) (Stopak *et al.*, 2003). In virions with deletions in Vif, APOBEC3G will be encapsidated and will deaminate the (-) strand of newly synthesized transcripts when a new infection and uncoating process starts, resulting in linear double-stranded viral DNA that contains uracil on the (-) strand and G to A transitions on the (+) strand. Most of the uracil-containing DNA will be degraded by cellular DNA repair enzymes before integration (Navarro and Landau, 2004). In normal virions, APOBEC3G will be specifically targeted and degraded by Vif (Marin *et al.*, 2003; Sheehy *et al.*, 2003).

1.3.4.4. Tat

HIV-1 lacking the transcriptional activator (Tat) is virtually defective in initiating reverse transcription upon infection of peripheral blood mononuclear cells (Harrich *et al.*, 1996). The TAR stem-loop RNA, which is located in the 5' LTR and serves as the binding site for Tat, is involved in the regulation of (-) sssDNA synthesis (Harrich *et al.*, 1997). Tat may facilitate the interactions between TAR RNA and other RNA elements, including the $tRNA^{Lys}$ primer and U5 RNA by a mechanism that is different from the mechanism used by Tat to modulate gene expression, because domain specific mutations of Tat can highly influence gene expression but has no effects on reverse transcription (Ulich *et al.*, 1999).

1.3.4.5. Nef

Nef also has been reported to influence efficient reverse transcription in infected cells (Schwartz *et al.*, 1995), which could account for the enhanced infectivity of wild-type virus compared to *nef* mutant virus (Chowers *et al.*, 1994). *Nef*-defective virions seem to be blocked at the stage of the HIV-1 life cycle between entry and reverse transcription, possibly virion

uncoating. A possible explanation is that Nef could enhance the affinity of RT for RNA *in vitro* (Fournier *et al.*, 2002).

1.4. INHIBITORS OF HIV RT

Since reverse transcriptase is virus-specific and essential for virus replication, and exerts its function before the irreversible integration of the viral DNA into the host genome, it is an excellent target enzyme to inhibit retroviral replication in an HIV infected individual. Over the last 15 years a variety of compounds, identified to interact with the RT, can be divided into four distinct groups of RT inhibitors (De Clercq, 2000; De Clercq, 2004): (i) 2',3'-dideoxynucleoside analogues, designated nucleoside RT inhibitors or NRTIs, (ii) acyclic nucleoside phosphonate analogues (ANPs), designated as nucleotide RT inhibitors (NtRTIs), (iii) pyrophosphate analogues and (iv) non-nucleoside RT inhibitors (NNRTIs).

1.4.1. NRTIs

The NRTIs have a typical 2',3'-dideoxynucleoside structure and principally act as competitive RT inhibitors with respect to the dNTP substrate after intracellular phosphorylation by cellular kinases (Fig. 1.7). More importantly, when NRTIs are incorporated in the growing template/primer they act as DNA chain terminators due to the lack of a 3' OH in the sugar part that prevents a nucleophilic attack on the α-phosphate and further phosphodiester bonds with other dNTPs. Because RT does not exhibit a 3'-5' exonuclease activity, the NRTIs once incorporated, prevent further viral DNA synthesis. The initial phosphorylation step of NRTIs to the 5'-monophosphate form is often the most crucial and rate-limiting step in the metabolic conversion of the NRTIs. Today, seven NRTIs are approved by the FDA: zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC) and emtricitabine ((-)FTC) (for an overview see Squires, 2001)

1.4.2. NtRTIs

The NtRTIs, such as adefovir (PMEA) and tenofovir ((R)-PMPA) can be considered as NRTIs that have an intact nucleobase part but an acyclic sugar (aliphatic) part equipped with a phosphonate (P-C) bond (Fig. 1.7). This circumvents the problem of the initial, limiting

phosphorylation step that NRTIs have to encounter. NtRTIs are also further phosphorylated by cellular kinases to the corresponding mono- and diphosphates and act, like NRTIs, as DNA chain terminators of nascent viral DNA synthesis.

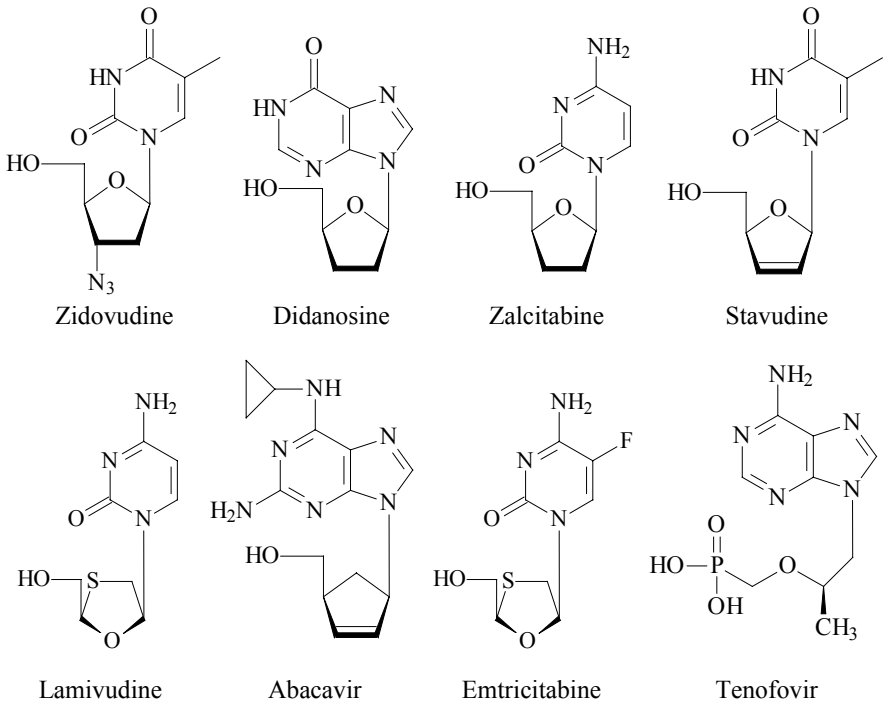

Figure 1.7 Structural formulae of NRTIs and the NtRTI tenofovir

1.4.3. Pyrophosphate analogues

Phosphonoformic acid (PFA) or foscarnet is a pyrophosphate analogue that inhibits reverse transcription by interacting with the pyrophosphate site close to the dNTP binding site of the RT enzyme (Fig. 1.8). PFA is a non-competitive inhibitor of RT with respect to the natural substrates and an uncompetitive inhibitor of RT against the template/primer.

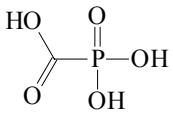

Figure 1.8 Structural formula of the pyrophosphate analogue foscarnet

1.4.4. NNRTIs

A structurally diverse group of compounds, which comprises at least 50 distinct structural classes different from the nucleoside structure, are designated as non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Fig. 1.9). All these NNRTIs bind to the NNRTI-binding pocket: an allosteric hydrophobic site close to, but distinct from, the catalytic active site of the RT (Kohlstaedt *et al.*, 1992). As they are inhibitory against RT as such, NNRTIs do not require intracellular metabolic activation. NNRTIs are non-competitive (or mixed-type) inhibitors with respect to the substrates and non-competitive or uncompetitive inhibitors with respect to the template/primer. Whereas NRTIs and NtRTIs do not markedly discriminate between RTs from different lentiviruses, the NNRTIs are highly specific for HIV-1 RT and do not recognize other lentiviral RTs or any other DNA or RNA polymerases from viral or cellular origin. This explains the highly selective anti-HIV-1 activity of the NNRTIs (for an overview, see Balzarini, 2004). NNRTIs that are approved for clinical use by the FDA are nevirapine, delavirdine and efavirenz, whereas etravirine, dapivirine and rilpivirine are currently subject of clinical phase I/II studies, either for potential microbicide use (dapivirine) or for systemic (oral) therapy of HIV-1.

The general structure of an NNRTI resembles that of a butterfly (Ding *et al.*, 1995a). One wing contains a substituted aromatic ring and can form a hydrogen bridge to the peptide main chain of K101. The other wing contains often a π-electron system that interacts with the aromatic residues of Y181, Y188 and W229. The body of the butterfly is polar and close to Y181 and results in a movement of the hydroxyphenyl group of this amino acid upon binding. Although most NNRTIs adapt such a shape, several NNRTIs do have impaired wings like delavirdine. Dapivirine, etravirine and rilpivirine are diarylpyrimidine (DAPY) derivatives and resemble a horseshoe rather than a butterfly shape. Structural and molecular modeling studies of these inhibitors with HIV-1 RT revealed a binding to RT in different conformations. The torsional flexibility of the inhibitors can generate numerous conformational variants and the compactness of the inhibitors permits significant repositioning and reorientation (translation and rotation) within the pocket. Such adaptations appear to be critical for the ability of the DAPY compounds to retain their potency against a wide range of drug-resistant HIV-1 RTs (Das *et al.*, 2004 and 2005; Janssen *et al.*, 2005)

TSAO NNRTI molecules, which have a totally different structure comparing to other NNRTIs, seem to interact with a binding site on the interface between p66 and p51 of HIV-1 RT, which is distinct from the binding site of the other NNRTIs (Arion *et al.*, 1996;

Rodriguez-Barrios *et al.*, 2001). These unusual properties of the TSAO NNRTIs were found by modelling of the TSAO molecules in the RT, and proved to be in agreement with biochemical, enzyme kinetic and structural data and are also in agreement with the observation that TSAO molecules enhance urea- and acetonitrile-mediated destabilisation of the dimer (Harris *et al.*, 1998b; Sluis-Cremer *et al.*, 2000b). These findings point to an interaction of TSAO with the earlier described β7-β8-loop of the p51 subunit. Therefore, this area in RT can be potentially used as a novel target site on RT for inhibitor design either by small molecules like TSAO derivatives or by oligopeptides or peptidomimetics. Previous studies have shown that the tryptophan-rich area at the p66/p51 interface can be targeted by oligopeptides resulting in destabilisation of RT and even a pronounced anti-HIV activity in cell culture (de Soultrait *et al.*, 2003; Divita *et al.*, 1994; Morris *et al.*, 1999b)

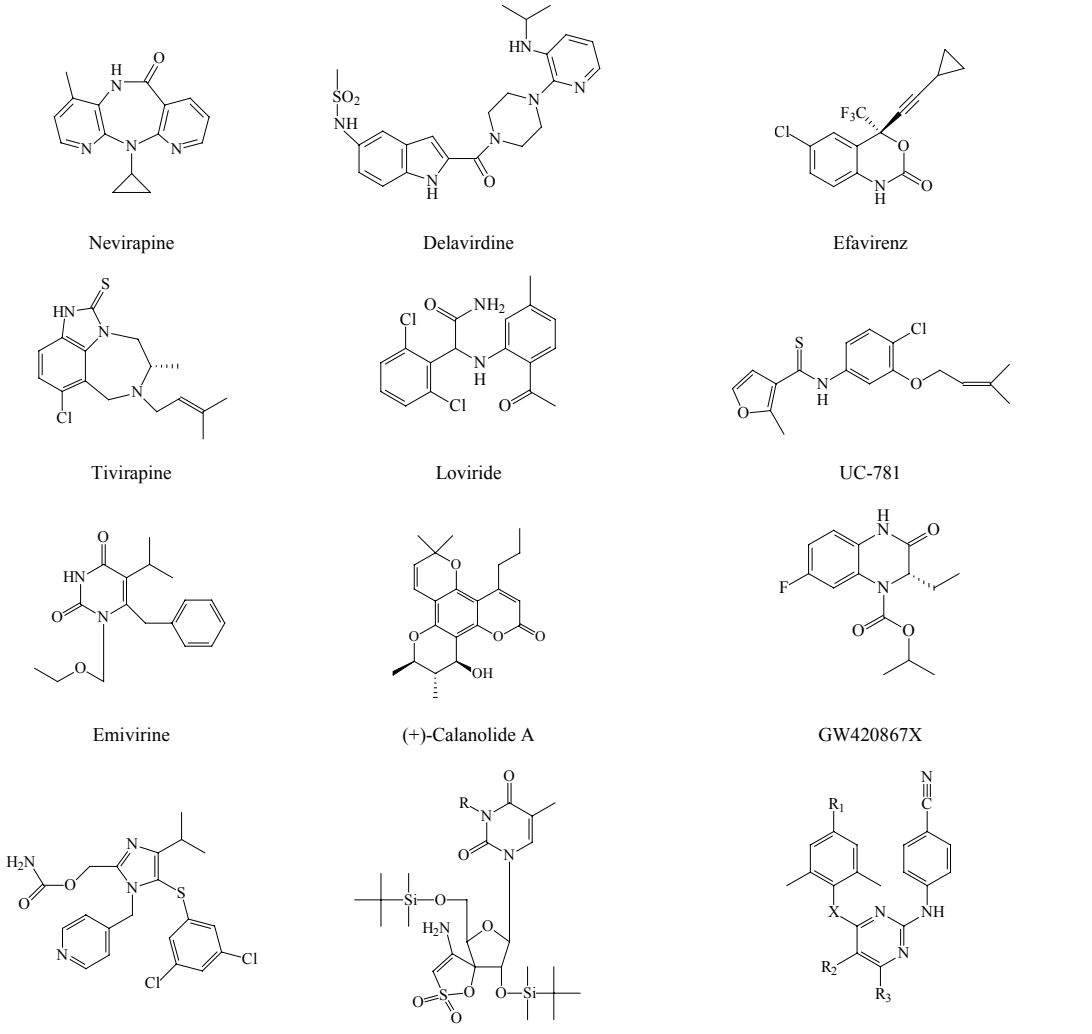

Figure 1.9 Structural formulae of NNRTIs

1.5. RESISTANCE

1.5.1. Genetic diversity of HIV

The most important step in virus replication to generate mutations in the viral genome is the reverse transcription process: (i) RT itself is highly error-prone due to the lack of a proofreading function, (ii) retroviral recombination occurs between the two different RNA genomes co-packaged in the virions (Hu and Temin, 1990a), (iii) two strand-transfers implicate template switching and (iv) the enormous viral replication rate provides a huge generation of errors. Retroviral recombination allows HIV-1 to repair breaks in the RNA and to exchange nucleic acid sequences (Temin, 1993). Recombination occurs when the growing DNA chain switches from one RNA template to another during minus-strand DNA synthesis (copy-choice model). Alternatively, the RT can also be forced to switch to the second RNA because of a break in the first RNA template (forced copy choice model). Finally, the new DNA that is displaced during the discontinuous plus-strand synthesis can hybridise to the minus-strand DNA synthesized with the second molecule of viral RNA as template. If this displaced plus-strand fragment is incorporated into the viral genome, a recombinant virus is generated (displacement-assimilation model) (Hu and Temin, 1990b).

The huge diversity of virions observed in patients infected with HIV-1 is a direct result of the high rate of virus replication and the large viral load (Coffin, 1995). Even during periods of clinical latency of the virus as many as 10^8 to 10^{10} virions are produced every day. In fact, clinical latency is the result of a dynamic equilibrium between vigorous HIV replication and HIV clearance on the one hand and $CD4^+$ cell depletion and renewal on the other hand (Coffin, 1995; Ho *et al.*, 1995; Wei *et al.*, 1995). Due to this high replication rate, the high prevalence of mutations and the total body viral burden in infected patients, every single point mutation is probably generated multiple times each day (Coffin, 1995). As a consequence, HIV does not have a fixed genome, but rather exists as a swarm of closely related but non-identical quasispecies (Wain-Hobson, 1993). Thus, pre-existence of viruses carrying mutations related to resistance to RT inhibitors is a major problem, because under selective pressure of retroviral drugs these mutant viruses will rapidly emerge and ultimately dominate the virus population.

1.5.2. Resistance mutations and their mechanism of action against NRTIs and NtRTIs

High-level resistance to zidovudine, the first drug that was administered to patients with HIV infection, requires an accumulation of mutations at positions 41, 67, 70, 210, 215 and 219 (Boucher *et al.*, 1992; Kellam *et al.*, 1992; Larder and Kemp, 1989). These mutations seem to occur in a particular ordering that progressively increases the level of drug resistance (Boucher *et al.*, 1992). The K70R mutation is often found as the initial mutation, typically followed by the appearance of a T215Y mutation, the disappearance of K70R and the subsequent emerging of M41L, D67N and K219Q/E/N mutations. Under further treatment, an additional L210W mutation can arise and the K70R may reappear (Harrigan *et al.*, 1996; Hooker *et al.*, 1996; Larder and Kemp, 1989). Because these mutations are selected under thymidine analogues like zidovudine and stavudine, they are known as thymidine analogue mutations (TAMs) that confer resistance by enhancing primer unblocking and do not alter nucleoside incorporation rates. TAMs decrease the *in vivo* activity of all N(t)RTIs, and are also referred to as nucleoside analogue mutations (NAMs). However, the molecular explanations for the mechanism of TAMs are rather difficult because it was shown *in vitro* that RT bearing TAMs could still be susceptible to zidovudine (Larder *et al.*, 1989). It has been proposed that the primary mechanism of zidovudine resistance is the phosphorolytic removal of this chain-terminator (Arion *et al.*, 1998; Arion *et al.*, 2000). The K70R and D67N mutations were shown to confer increased sensitivity (and thus selectivity of RT) to pyrophosphate. Also, an increased DNA synthesis was observed for the mutant RT bearing mutations at positions 67, 70, 215 and 219, which would compensate for the increase in phosphorolysis and should facilitate HIV-1 replication in the presence of zidovudine (Arion *et al.*, 1998; Arion *et al.*, 2000).

The appearance of K65R and T69D in the β3-β4 loop of the finger domain were associated with tenofovir, abacavir, stavudine, zalcitabine or didanosine treatment (Fitzgibbon *et al.*, 1992; Gu *et al.*, 1994; Miller, 2004). K65 interacts with the γ-phosphate of the incoming dNTP (Huang *et al.*, 1998a) and has importance in the nucleotide-binding specificity since a K65R mutation resulted in a 10-fold decreased binding of ddCTP and ddATP to the mutated enzyme (Sluis-Cremer *et al.*, 2000a). K65R confers (partial) resistance to zalcitabine, didanosine, abacavir, tenofovir, lamivudine and possibly stavudine (Garcia-Lerma *et al.*, 2003; Ruane and Luber, 2004; Wainberg *et al.*, 1999). K65R reduces TAM-associated resistance probably by reducing TAM-mediated primer unblocking (Sluis-Cremer *et al.*, 2000a).

Table 1.1 Resistance mutations associated with the officially approved nucleoside RT inhibitors (NRTIs), nucleotide RT inhibitors (NtRTIs) and non-nucleoside RT inhibitors (NNRTIs)

Compound	Amino acid mutation
NRTI	
Zidovudine (AZT)	*M41L*, D67N, K70R, L210W, T215*F*/Y, K219*Q*/**E**
Abacavir (ABC)	K65R, L74V, Y115F, M184V
Zalcitabine (ddC)	K65R, *T69D*, *L74V*, V75T, M184V, *T215C*
Didanosine (ddI)	*M41L*, K65R, *D67N*, *T69A/S*, *K70R*, *L74V*, V75T, M184V, *L210W*, *T215Y*, *K219Q*
Stavudine (d4T)	*M41L*, *D67N*, V75T, *T215Y*, *K219Q*
Lamivudine (3TC)	*P157S*, M184I/**T**/V
Emtricitabine [(-)FTC]	M184I/**T**/V
NtRTI	
Tenofovir DF (TDF)	K65R
NNRTIs	
Nevirapine	*A98G*, *L100I*, *K103N*, V106A, *V108I*, Y181C/*I*, *Y188C*, *G190A*
Delavirdine	K103*N*/T, *Y181C*, P236L
Efavirenz	*A98G*, L100I, **K101E**, K103N, V108I, **V179D**, **Y181C**, **Y188L**, *G190S*, *P225H*

Adapted from Schinazi *et al*., 2000. Mutations in bold represent mutations only observed *in vitro*, mutations in italics represent mutations only observed *in vivo*.

Viruses that contain a K65R mutation have a diminished replicative capacity, similar to viruses with M184V mutations (White *et al.*, 2002). T69D probably exerts its influence by inducing a conformational change in the fingertips that makes contacts with the dNTP (Huang *et al.*, 1998a).

Insertions at fingers regions between codons 67 and 70, most commonly 2 amino acids (S-S or S-A) at position 69, can occur in patients receiving nucleoside analogues and are typically found in addition to TAMs (Balotta *et al.*, 2000; de Jong *et al.*, 1999). Biochemical analyses have demonstrated that resistance conferred by these amino acid inserts is ATP-dependent, which suggests an enhanced primer unblocking (Goldschmidt and Marquet, 2004; Larder *et al.*, 1999). Computer modeling also suggests that the increased mobility of the β3-β4 loop caused by the T69 insertion mutation could result in an increased rate of ATP-mediated excision and thus contribute to the high-level and broad N(t)RTI resistance (White *et al.*, 2004). The inserts themselves confer minor degree of resistance but this can markedly increase when present in addition with TAMs (Larder *et al.*, 1999; Winters *et al.*, 1998). Besides 6 bp nucleotide inserts, also 3 bp deletions were found in HIV-1 RT from patients

with high level of multinucleoside resistance. This probably results in a loss of hydrogen bonds between the RT and an incoming dNTP (Winters *et al.*, 2000).

L74 directly interacts with the template/primer and the incorporating dNTP and with the side chains of R72 and Q151 (Huang *et al.*, 1998a). The L74V mutation, which is most frequently observed in patients receiving didanosine monotherapy (St Clair *et al.*, 1991) or abacavir therapy (Harrigan *et al.*, 2000; Miller *et al.*, 2000; St Clair *et al.*, 1991), confers resistance by selectively reducing incorporation rates of specific nucleoside analogues. A valine at position 74 also permits more fluctuation of the templating base, which makes it more difficult for a ddNTP to form a base pair (Huang *et al.*, 1998a).

M184 is a residue of the catalytic polymerase site that directly interacts with the incoming dNTP (Huang *et al.*, 1998a). The M184I/V causes steric hindrance between the oxathiolane ring of 3TC-TP and the side chain of β-branched amino acids (like V, I and T), leading to a perturbation of the inhibitor binding and selectively reduces the incorporation of nucleosides like lamivudine, emtricitabine, didanosine, zalcitabine and abacavir (Gao *et al.*, 2000; Sarafianos *et al.*, 1999). Lamivudine rapidly selects for the M184I mutation that is soon replaced by the M184V mutation due to a fitness advantage (Keulen *et al.*, 1997; Schuurman *et al.*, 1995). In patients under lamivudine treatment (Schuurman *et al.*, 1995), M184V is also selected upon abacavir monotherapy (Harrigan *et al.*, 2000; Miller *et al.*, 2000), emtricitabine therapy and occasionally on didanosine monotherapy and abacavir/zidovudine therapy (Winters *et al.*, 1997). M184V confers high-level resistance to emtricitabine and lamivudine (Schinazi *et al.*, 1993; Tisdale *et al.*, 1993) but, on itself, only minor resistance to abacavir or didanosine. In combination with the additional K65R, L74V and Y115F mutations, the rate of resistance to abacavir markedly increases (Tisdale *et al.*, 1997). M184V actually reverses TAM-associated resistance to zidovudine, stavudine and tenofovir (Larder *et al.*, 1995; Miller *et al.*, 2001; Whitcomb *et al.*, 2003) by decreasing the rate of primer unblocking (Boyer *et al.*, 2002; Gotte *et al.*, 2000).

The Q151M mutation develops under treatment with thymidine analogues and didanosine combinations and causes resistance to all NRTIs (Coakley *et al.*, 2000; Picard *et al.*, 2001; Van Laethem *et al.*, 2000). Because Q151 is located in a conserved region that interacts with the incoming dNTP (Huang *et al.*, 1998a; Sarafianos *et al.*, 1995), the mutation can interfere with nucleoside binding and incorporation (Deval *et al.*, 2002). Q151M is generally associated with A62V, V75I/F, F77L and F116Y. Each of these mutations substantially increases the degree of resistance and/or the fitness of the isolates containing Q151M (Maeda *et al.*, 1998; Shafer *et al.*, 1996). Mutations at positions 62, 75 and 77 modify

the hydrophobic core of the fingers to adjust the orientation of the methionine and the F116Y restores a hydrogen bond that is lost in the Q151M mutation (Huang *et al.*, 1998a). Tenofovir remains active against viruses with this multidrug complex (Miller *et al.*, 2001), and lamivudine may also retain some activity (Schmit *et al.*, 1998).

1.5.3. Resistance mutations against PFA

Several RT mutations are associated with resistance to foscarnet (PFA), including W88G/S, E89G/K, L92I, S156A, Q161L and H208Y (Fig. 1.10). The mutations at amino acid positions 88, 161 and 208 have also been observed in HIV strains of patients under PFA treatment (Mellors *et al.*, 1995). The PFA resistance mutations W88G/S, E89G/K and L92I are located close to the template/primer binding site and rather distant from the putative pyrophosphate-binding site. This suggests that PFA resistance is likely to be mediated through an altered interaction of the mutant enzyme with the template strand of the template/primer, which distorts the formation of the active site and leads to inefficient binding of PFA (Tachedjian *et al.*, 1995). In contrast, the Q161L mutation is located at the active site of HIV-1 RT and can have a more direct effect on PFA binding by inducing conformational changes in the dNTP binding site and the putative pyrophosphate-binding site (Mellors *et al.*, 1995).

1.5.4. Resistance against NNRTIs

1.5.4.1. The NNRTI-binding pocket in HIV-1 RT

Crystallographic structures of RT enzyme complexed with an NNRTI revealed that they all bind in a specific site within the p66 palm domain. This NNRTI-binding site is represented by a lipophilic pocket at the basis of a three-stranded β-sheet (β4, β7 and β8). At the top of this sheet, the three catalytic D110, D185 and D186 residues are approximately 10-15 Å distant from the bound NNRTIs. The pocket is further formed by the β4, β7, β8 sheet (amino acid residues 105-110 and 179-191), the β9, β10, β11 sheet (amino acid residues 224-241) and the structure preceding β4 (amino acid residues 98-104). The entrance to the pocket contains the P225 and P236 residues that are located on flexible peptide chains, and thought to be able to close the pocket following the entrance of an NNRTI. The aromatic residues Y181, Y188 and W229 form the roof of the pocket. The pocket walls are lined with the hydrophobic residues L100, V106 and L234. The pocket floor consists of main-chain atoms of several

lysines in the 101-K-K-K-103 domain preceding β4. Two aromatic residues (F227 and Y318) are located toward the pocket entrance. Amino acid residues E138 and T139 derived from the p51 subunit are positioned left of the pocket, beyond residues L100 and Y181 (Ding *et al.*, 1995b; Kohlstaedt *et al.*, 1992; Smerdon *et al.*, 1994; Stammers *et al.*, 1994).

1.5.4.2. The 'NNRTI-binding pocket' in other lentiviral RTs

It is still intriguing why NNRTIs are not inhibitory to RT enzymes other than HIV-1 RT. Several chimeric enzymes involving HIV-1 RT and other lentiviral RTs have been constructed, contributing to a further understanding of the function of the individual subunits within HIV-1 RT heterodimer, the mapping of the catalytic sites and the NNRTI-binding pocket. It was found that exchanging one or few amino acids from HIV-1 RT by the corresponding amino acids in HIV-2 RT is sufficient to markedly reduce sensitivity to NNRTIs and, *vice versa*, exchange of one or a few amino acids or several amino stretches from HIV-2 RT by the corresponding amino acids of HIV-1 RT is sufficient for HIV-2 RT to gain sensitivity to NNRTIs (Hizi *et al.*, 1993; Isaka *et al.*, 2001; Yang *et al.*, 1996). Resolution of the crystal structure of HIV-2 RT at 2-3.5 Å resolution showed that it has a similar overall folding to HIV-1 RT but there were structural differences within the putative NNRTI-pocket at both conserved and non-conserved residues. There are sequence differences that result in unfavourable NNRTI contacts at amino acid positions 101, 106, 138, 181, 188 and 190 (Bird *et al.*, 2003; Ren *et al.*, 2002). The crystal structure also confirms that the I181 in HIV-2 RT could be a significant contributory factor to the inherent drug resistance of HIV-2 RT against NNRTIs. This is also in agreement with the fact that mutant Y181I HIV-1 RT markedly lost sensitivity to NNRTIs (Balzarini *et al.*, 1994).

When an alignment was made for a wide variety of primary amino acid sequences that line the NNRTI-pocket of HIV-1 RT with other lentiviral RTs, a higher sequence similarity was found for FIV than for HIV-2 and SIV RT. Even, the majority of amino acids in the NNRTI pocket of HIV-1 RT that are instrumental in obtaining sensitivity to NNRTIs are identical in FIV RT, except for Q101, A138, D179 and Y227. However, no inhibitory effect of NNRTIs on FIV RT could been found, even at very high drug concentrations (Auwerx *et al.*, 2004; Auwerx *et al.*, 2002). In addition, none of the hybrid RTs in which well-defined parts of the p66 FIV RT subunit are exchanged by their HIV-1 RT conterpart could restore the sensitivity of FIV RT to NNRTIs (Auwerx *et al.*, 2004). As long as no crystal structure is available of FIV RT, the structural basis of this NNRTI-resistance remains unsolved.

1.5.4.3. NNRTIs: Mechanism of action

The NNRTI-binding pocket does not physically exist in the unliganded RT because the side chains of Y181, Y188 and W229 occupy this region (Hsiou *et al.*, 1996; Rodgers *et al.*, 1995). However, upon entry of an NNRTI, a conformational shift creates a pocket by moving the Y181 and Y188 towards the aspartic acid triad of the active site (Hopkins *et al.*, 1996). The β12-β13-β14 sheet that contains the primer grip is repositioned and the functional groups of Y181 and Y188 are rotated towards the polymerase active site (Ding *et al.*, 1995b). Repositioning of the primer grip probably alters the position and the 3' OH group of the template/primer relative to the active site, resulting in inhibition of polymerase activity. In NNRTI-bound structures, the positions of Y181 and Y188 closely mimic the conformations of the equivalent side chains in the (inactive) p51 subunit of the enzyme (Esnouf *et al.*, 1995). Concomitantly, the whole β-sheet comprising β4, β7 and β8 and the aspartic acid triad shift by ~2 Å. This shift upon NNRTI-binding is presumed to result in a dramatic slowing down of the catalytic activity of the enzyme (Esnouf *et al.*, 1995). These structural explanations of NNRTI inhibition are consistent with kinetic mechanistic studies which showed that the major effect of NNRTIs is on the chemical step (nucleotide transfer) of DNA polymerisation (Rittinger *et al.*, 1995; Spence *et al.*, 1995). A third possible hypothesis for the mechanism of action of NNRTIs is that the position of the p66 thumb domain, which is of major importance in DNA binding, is in hyper-extension when RT is bound to an NNRTI (Hsiou *et al.*, 1996). It is possible that interference with the mobility of the p66 thumb domain, which is normally in a folded down position in unliganded RT and in an upright position in DNA-bound RT (Hsiou *et al.*, 1996; Jacobo-Molina *et al.*, 1993; Rodgers *et al.*, 1995), can lead to inhibition of polymerisation. In more recent studies it was also suggested that binding of NNRTIs to RT decreases the flexibility of the fingers and thumb domain of the p66 subunit, which is necessary for binding and translocation of the nucleic acid substrate after nucleotide incorporation (Madrid *et al.*, 2001; Shen *et al.*, 2003). However, according to Esnouf *et al.* (1995) the orientation of the thumb domain depends on the hydration state of the crystal lattice rather than on binding of an NNRTI.

1.5.4.4. Resistance mutations

Whereas NRTI-specific drug mutations are scattered around several domains of the RT (fingers, palm and thumb), the NNRTI-specific mutations are clustered in the lipophylic NNRTI-binding pocket (for an overview, see Balzarini, 2004 and Schinazi *et al.*, 2001). In this pocket, several clusters of mutations can be identified, namely A98G, L100I, K101E/Q/I,

K103N/T/Q/R, V106A/I/L, V108I in the β-sheet comprising β5b and β6; V179D/E, Y181C/I, Y188C/H/L, V189I and G190E/T/E/A/S, located in the β-sheet, comprising β9 and β10 and I135M/T/L, E138K, T139I and G141E mutations located in the interface between the p66 and p51 subunit. Furthermore, P225H, F227L, E233V, P236L and K238T are also related to resistance to NNRTIs (Fig. 1.10).

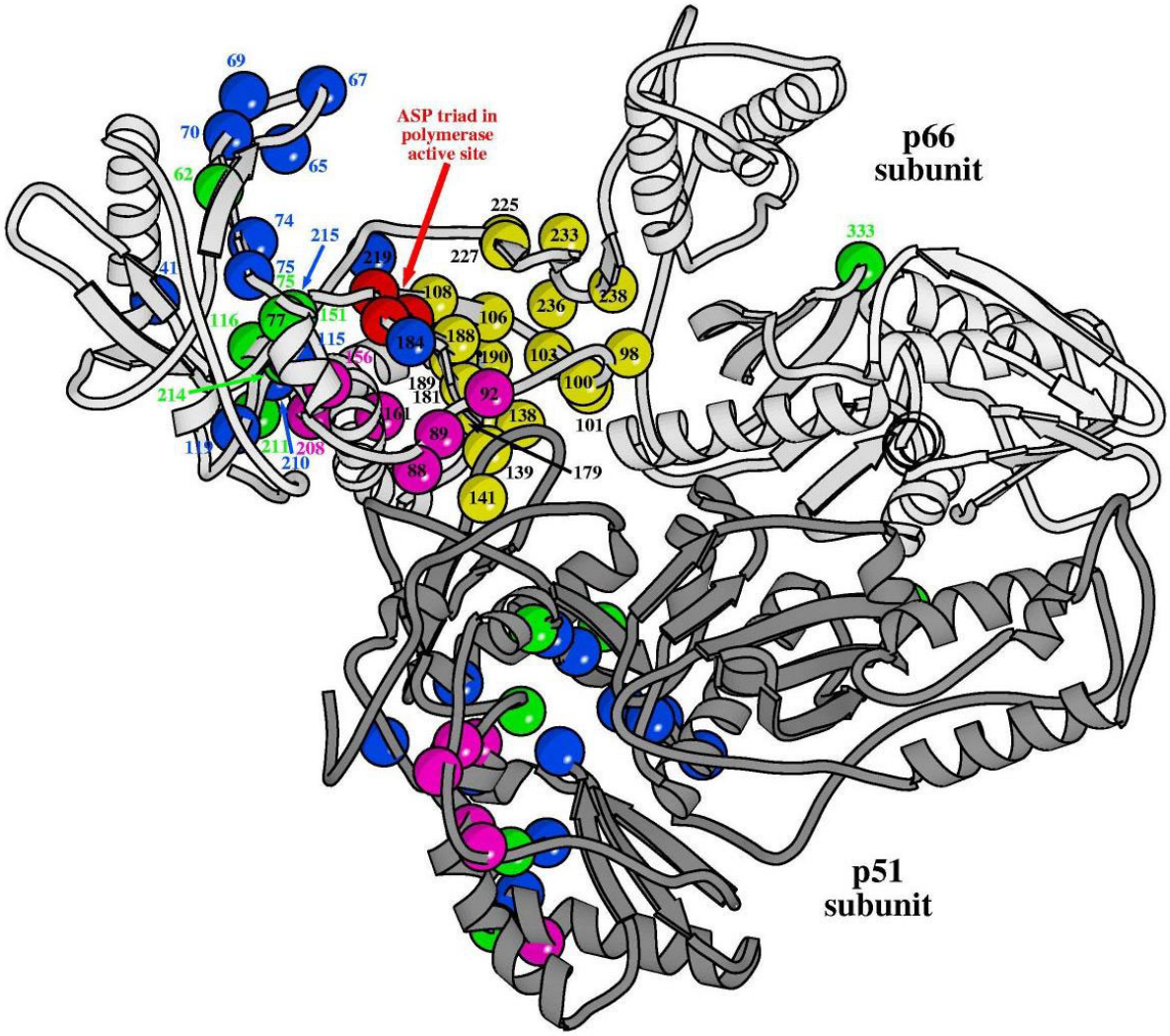

Figure 1.10 Overview of the location in the p66/p51 heterodimer of the resistance mutations associated with the different classes of RT inhibitors.

NRTI and NtRTI resistance mutations are in blue, foscarnet resistance mutations are in magenta and NNRTI resistance mutations are in yellow. The multidrug NRTI-resistance mutations are shown in green (taken from J. Balzarini (1999)).

Only a single amino acid mutation in the NNRTI-pocket can result in a marked resistance to first-generation NNRTIs like nevirapine, delavirdine, loviride and TIBO

(Balzarini *et al.*, 1993). Such a single amino acid mutation can even result in a pronounced cross-resistance to other first-generation NNRTIs because they all bind in the same pocket, which results in a very different resistance pattern than that observed for NRTIs. This has been shown in structural studies with Y181C and Y188L mutations, where the loss of the tyrosine side chain can lead to a reduction in favourable contacts between the enzyme and different kinds of NNRTIs like tivirapine (8-chloro-TIBO) and quinoxaline HBY 097 (Das *et al.*, 1996; Hsiou *et al.*, 1998). The major importance of Y181 and Y188 is also proven in a study where I188 and L181 of the NNRTI-insensitive HIV-2 RT are replaced by tyrosines, resulting in an RT with NNRTI-susceptibility (Isaka *et al.*, 2001; Yang *et al.*, 1996). Furthermore, other NNRTI specific mutations like V108A/I and L100I may also act indirectly by a shift of the distally-positioned tyrosine side-chains and a perturbation in ring stacking interactions with the NNRTI (Ren *et al.*, 2004). In addition, the K103N mutation, which is located near the putative entrance of the NNRTI-pocket and which confers resistance against a large number of NNRTIs, may also act *via* an indirect mechanism by forming an unfavourable hydrogen bond with Y188, thereby closing the pocket due to numerous interactions with water molecules in and nearby the pocket (Hsiou *et al.*, 2001; Rodriguez-Barrios and Gago, 2004). Different amino acid changes at one single location, like G190A confers resistance to nevirapine, but not to quinoxalines, while the G190E confers resistance to nevirapine and quinoxalines by introducing a substantial steric bulky and a hydrophilic, potentially charged group in the NNRTI-pocket (Hsiou *et al.*, 1998; Kleim *et al.*, 1997).

In contrast to first-generation NNRTIs, the currently most active second-generation NNRTIs like efavirenz, the thiocarboxanilide UC-781, quinoxaline GW420867X, PETT-5 (MIV-150), CP-94,707, capravirine (AG1549) and etravirine (TMC-125) can usually deal very well with single mutations in the NNRTI-pocket and need several NNRTI-specific mutations in the RT to achieve high-level resistance against these NNRTIs (Andries *et al.*, 2004; Das *et al.*, 2004; Pata *et al.*, 2004). Additional NNRTI-specific mutations do not necessarily result in an additive effect when appearing in the same RT. V108I and P225H mutations do not confer resistance as high as its single mutations, but in combination with K103N drug resistance increases up till 100-fold. The K103N mutation in combination with L100I results in substantial resistance to efavirenz while the single mutations have a much lower impact.

1.5.5. Hypersensitivity

Some NNRTI-specific mutations can confer an increase in susceptibility to some other well-defined NNRTIs, even when they are combined with NRTI-specific mutations. Mutations that are located at the pocket entrance increase the inhibitory effect of delavirdine (i.e. P225H) and other NNRTIs (i.e. P236L), including nevirapine, pyridinone and 9-chloro-TIBO. The Y181C mutation is also able to reverse the phenotypic resistance of virus strains that contain typical AZT resistance mutations (Larder, 1992). The introduction of the Y181C substitution suppresses the increased repair (or unblocking) of the AZT-MP-terminated primer provided by the AZT resistance substitutions in RT (Chong *et al.*, 2003). The L100I mutation is able to reverse phenotypic AZT resistance when added onto an AZT-resistance background (Byrnes *et al.*, 1994). Foscarnet resistance mutations W88G, E89K, L92I and Q161L are hypersensitive to zidovudine, nevirapine, tivirapine and 9-chloro TIBO (Mellors *et al.*, 1995; Tachedjian *et al.*, 1995; Tachedjian *et al.*, 1996). Thus, there exists a complicated interplay and communication between several amino acids that belong to clearly different locations but it is difficult to understand and/or predict these effects based on the structural data from the crystal structure of the RT.

1.5.6. Compensatory mutations

Resistance mutations often decrease the catalytic efficacy of the RT enzyme and additional mutations are required to restore the fitness of the resistant viruses and to compensate for the (partial) loss of catalytic activity. In this context, a G190E mutated RT that emerges under quinoxaline pressure displays only ~5% of the catalytic activity of wild-type RT (Huang *et al.*, 2003; Kleim *et al.*, 1997). Prolonged exposure to quinoxalines resulted in the appearance of additional mutations (L74V/I and V75L/I) that restore the replication rate and polymerase activity by returning the distorted template/primer back in its original position. These mutations also confer resistance to the NRTIs stavudine and didanosine (Boyer *et al.*, 1998; Kleim *et al.*, 1996). A mutagenesis study revealed that mutation of the W229 residue to other amino acids generally leads to mutant enzymes with severely compromised catalytic activity. Only W229Y and W229F mutant RTs retained RT activity but also retained their sensitivity to most NNRTIs (Pelemans *et al.*, 2000). Additional mutations in the RT (i.e. I63M, V189I and E396G) were found to compensate for the loss in polymerase activity due to W229Y mutant (Pelemans *et al.*, 2001).

REFERENCES

Abbotts J, Bebenek K, Kunkel TA and Wilson SH (1993) Mechanism of HIV-1 reverse transcriptase. Termination of processive synthesis on a natural DNA template is influenced by the sequence of the template-primer stem. *J Biol Chem* **268**:10312-23.

Amacker M and Hubscher U (1998) Chimeric HIV-1 and feline immunodeficiency virus reverse transcriptases: critical role of the p51 subunit in the structural integrity of heterodimeric lentiviral DNA polymerases. *J Mol Biol* **278**:757-65.

Andersen ES, Jeeninga RE, Damgaard CK, Berkhout B and Kjems J (2003) Dimerization and template switching in the 5' untranslated region between various subtypes of human immunodeficiency virus type 1. *J Virol* **77**:3020-30.

Andries K, Azijn H, Thielemans T, Ludovici D, Kukla M, Heeres J, Janssen P, De Corte B, Vingerhoets J, Pauwels R and de Bethune MP (2004) TMC125, a novel next-generation nonnucleoside reverse transcriptase inhibitor active against nonnucleoside reverse transcriptase inhibitor-resistant human immunodeficiency virus type 1. *Antimicrob Agents Chemother* **48**:4680-6.

Arion D, Fletcher RS, Borkow G, Camarasa MJ, Balzarini J, Dmitrienko GI and Parniak MA (1996) Differences in the inhibition of human immunodeficiency virus type 1 reverse transcriptase DNA polymerase activity by analogs of nevirapine and [2',5'-bis-O-(tert-butyldimethylsilyl)-3'-spiro-5"-(4"-amino-1", 2"-oxathiole-2",2"-dioxide] (TSAO). *Mol Pharmacol* **50**:1057-64.

Arion D, Kaushik N, McCormick S, Borkow G and Parniak MA (1998) Phenotypic mechanism of HIV-1 resistance to 3'-azido-3'-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. *Biochemistry* **37**:15908-17.

Arion D, Sluis-Cremer N, Min KL, Abram ME, Fletcher RS and Parniak MA (2002) Mutational analysis of Tyr-501 of HIV-1 reverse transcriptase. Effects on ribonuclease H activity and inhibition of this activity by N-acylhydrazones. *J Biol Chem* **277**:1370-4.

Arion D, Sluis-Cremer N and Parniak MA (2000) Mechanism by which phosphonoformic acid resistance mutations restore 3'-azido-3'-deoxythymidine (AZT) sensitivity to AZT-resistant HIV-1 reverse transcriptase. *J Biol Chem* **275**:9251-5.

Arnold E, Jacobo-Molina A, Nanni RG, Williams RL, Lu X, Ding J, Clark AD, Jr., Zhang A, Ferris AL, Clark P and et al. (1992) Structure of HIV-1 reverse transcriptase/DNA complex at 7 A resolution showing active site locations. *Nature* **357**:85-9.

Arts EJ, Miller JT, Ehresmann B and Le Grice SF (1998) Mutating a region of HIV-1 reverse transcriptase implicated in tRNA(Lys-3) binding and the consequences for (-)-strand DNA synthesis. *J Biol Chem* **273**:14523-32.

Auwerx J, Esnouf R, De Clercq E and Balzarini J (2004) Susceptibility of feline immunodeficiency virus/human immunodeficiency virus type 1 reverse transcriptase chimeras to non-nucleoside RT inhibitors. *Mol Pharmacol* **65**:244-51.

Auwerx J, North TW, Preston BD, Klarmann GJ, De Clercq E and Balzarini J (2002) Chimeric human immunodeficiency virus type 1 and feline immunodeficiency virus reverse transcriptases: role of the subunits in resistance/sensitivity to non-nucleoside reverse transcriptase inhibitors. *Mol Pharmacol* **61**:400-6.

Bahar I, Erman B, Jernigan RL, Atilgan AR and Covell DG (1999) Collective motions in HIV-1 reverse transcriptase: examination of flexibility and enzyme function. *J Mol Biol* **285**:1023-37.

Baillon JG, Nashed NT, Kumar A, Wilson SH and Jerina DM (1991) A leucine zipper-like motif may mediate reverse transcriptase subunit binding. *The New Biologist* **3**:1015-1019.

Balakrishnan M, Roques BP, Fay PJ and Bambara RA (2003) Template dimerization promotes an acceptor invasion-induced transfer mechanism during human immunodeficiency virus type 1 minus-strand synthesis. *J Virol* **77**:4710-21.

Balotta C, Violin M, Monno L, Bagnarelli P, Riva C, Facchi G, Berlusconi A, Lippi M, Rusconi S, Clementi M, Galli M, Angarano G and Moroni M (2000) Prevalence of multiple dideoxynucleoside analogue resistance (MddNR) in a multicenter cohort of HIV-1-infected Italian patients with virologic failure. *J Acquir Immune Defic Syndr* **24**:232-40.

Balzarini J (2004) Current status of the non-nucleoside reverse transcriptase inhibitors of human immunodeficiency virus type 1. *Curr Top Med Chem* **4**:921-44.

Balzarini J, Karlsson A, Perez-Perez MJ, Vrang L, Walbers J, Zhang H, Oberg B, Vandamme AM, Camarasa MJ and De Clercq E (1993) HIV-1-specific reverse transcriptase inhibitors show differential activity against HIV-1 mutant strains containing different amino acid substitutions in the reverse transcriptase. *Virology* **192**:246-53.

Balzarini J, Karlsson A, Sardana VV, Emini EA, Camarasa MJ and De Clercq E (1994) Human immunodeficiency virus 1 (HIV-1)-specific reverse transcriptase (RT) inhibitors may suppress the replication of specific drug-resistant (E138K)RT HIV-1 mutants or select for highly resistant (Y181C-->C181I)RT HIV-1 mutants. *Proc Natl Acad Sci U S A* **91**:6599-603.

Barat C, Lullien V, Schatz O, Keith G, Nugeyre MT, Gruninger-Leitch F, Barre-Sinoussi F, LeGrice SF and Darlix JL (1989) HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA. *Embo J* **8**:3279-85.

Barat C, Schatz O, Le Grice S and Darlix JL (1993) Analysis of the interactions of HIV1 replication primer tRNA(Lys,3) with nucleocapsid protein and reverse transcriptase. *J Mol Biol* **231**:185-90.

Barin F, M'Boup S, Denis F, Kanki P, Allan JS, Lee TH and Essex M (1985) Serological evidence for virus related to simian T-lymphotropic retrovirus III in residents of west Africa. *Lancet* **2**:1387-9.

Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W and Montagnier L (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**:868-71.

Bathurst IC, Moen LK, Lujan MA, Gibson HL, Feucht PH, Pichuantes S, Craik CS, Santi DV and Barr PJ (1990) Characterization of the human immunodeficiency virus type-1 reverse transcriptase enzyme produced in yeast. *Biochem Biophys Res Commun* **171**:589-95.

Beard WA, Bebenek K, Darden TA, Li L, Prasad R, Kunkel TA and Wilson SH (1998) Vertical-scanning mutagenesis of a critical tryptophan in the minor groove binding track of HIV-1 reverse transcriptase. Molecular nature of polymerase-nucleic acid interactions. *J Biol Chem* **273**:30435-42.

Bebenek K, Abbotts J, Roberts JD, Wilson SH and Kunkel TA (1989) Specificity and mechanism of error-prone replication by human immunodeficiency virus-1 reverse transcriptase
Recent studies of the fidelity of DNA synthesis. *J Biol Chem* **264**:16948-56.

Bebenek K, Abbotts J, Wilson SH and Kunkel TA (1993) Error-prone polymerization by HIV-1 reverse transcriptase. Contribution of template-primer misalignment, miscoding, and termination probability to mutational hot spots. *J Biol Chem* **268**:10324-34.

Becerra SP, Kumar A, Lewis MS, Widen SG, Abbotts J, Karawya EM, Hughes SH, Shiloach J and Wilson SH (1991) Protein-protein interactions of HIV-1 reverse transcriptase: implication of central and C-terminal regions in subunit binding. *Biochemistry* **30**:11707-19.

Berkhout B, Silverman RH and Jeang KT (1989) Tat trans-activates the human immunodeficiency virus through a nascent RNA target. *Cell* **59**:273-82.

Berkhout B, Vastenhouw NL, Klasens BI and Huthoff H (2001) Structural features in the HIV-1 repeat region facilitate strand transfer during reverse transcription. *Rna* **7**:1097-114.

Bird LE, Chamberlain PP, Stewart-Jones GB, Ren J, Stuart DI and Stammers DK (2003) Cloning, expression, purification, and crystallisation of HIV-2 reverse transcriptase. *Protein Expr Purif* **27**:12-8.

Boucher CA, O'Sullivan E, Mulder JW, Ramautarsing C, Kellam P, Darby G, Lange JM, Goudsmit J and Larder BA (1992) Ordered appearance of zidovudine resistance mutations during treatment of 18 human immunodeficiency virus-positive subjects. *J Infect Dis* **165**:105-10.

Bour S, Schubert U and Strebel K (1995) The human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: implications for the mechanism of degradation. *J Virol* **69**:1510-20.

Bowerman B, Brown PO, Bishop JM and Varmus HE (1989) A nucleoprotein complex mediates the integration of retroviral DNA.Correct integration of retroviral DNA in vitro. *Genes Dev* **3**:469-78.

Boyer JC, Bebenek K, Kunkel TA, Abbotts J, Roberts JD and Wilson SH (1992) Unequal human immunodeficiency virus type 1 reverse transcriptase error rates with RNA and DNA templates. *Proc Natl Acad Sci U S A* **89**:6919-23.

Boyer PL, Gao HQ and Hughes SH (1998) A mutation at position 190 of human immunodeficiency virus type 1 reverse transcriptase interacts with mutations at positions 74 and 75 via the template primer. *Antimicrob Agents Chemother* **42**:447-52.

Boyer PL, Sarafianos SG, Arnold E and Hughes SH (2002) The M184V mutation reduces the selective excision of zidovudine 5'-monophosphate (AZTMP) by the reverse transcriptase of human immunodeficiency virus type 1. *J Virol* **76**:3248-56.

Brown PO, Bowerman B, Varmus HE and Bishop JM (1987) Correct integration of retroviral DNA in vitro. *Cell* **49**:347-56.

Bryant M and Ratner L (1990) Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc Natl Acad Sci U S A* **87**:523-7.

Byrnes VW, Emini EA, Schleif WA, Condra JH, Schneider CL, Long WJ, Wolfgang JA, Graham DJ, Gotlib L, Schlabach AJ and et al. (1994) Susceptibilities of human immunodeficiency virus type 1 enzyme and viral variants expressing multiple resistance-engendering amino acid substitutions to reserve transcriptase inhibitors. *Antimicrob Agents Chemother* **38**:1404-7.

Camaur D and Trono D (1996) Characterization of human immunodeficiency virus type 1 Vif particle incorporation. *J Virol* **70**:6106-11.

Chamberlain PP, Ren J, Nichols CE, Douglas L, Lennerstrand J, Larder BA, Stuart DI and Stammers DK (2002) Crystal structures of Zidovudine- or Lamivudine-resistant human immunodeficiency virus type 1 reverse transcriptases containing mutations at codons 41, 184, and 215. *J Virol* **76**:10015-9.

Charneau P and Clavel F (1991) A single-stranded gap in human immunodeficiency virus unintegrated linear DNA defined by a central copy of the polypurine tract. *J Virol* **65**:2415-21.

Charneau P, Mirambeau G, Roux P, Paulous S, Buc H and Clavel F (1994) HIV-1 reverse transcription. A termination step at the center of the genome. *J Mol Biol* **241**:651-62.

Chen Y, Balakrishnan M, Roques BP, Fay PJ and Bambara RA (2003) Mechanism of minus strand strong stop transfer in HIV-1 reverse transcription. *J Biol Chem* **278**:8006-17.

Chong Y, Akula N, Chu CK, Selmi B, Deval J, Alvarez K, Boretto J, Sarfati S, Guerreiro C, Canard B, Rigourd M, Goldschmidt V, Brule F, Morrow CD, Ehresmann B, Ehresmann C and Marquet R (2003) Studies of molecular mechanism of tenofovir against 3TC- and AZT-resistance mutant HIV-1 reverse transcriptase. *Bioorg Med Chem Lett* **13**:4019-22.

Chowers MY, Spina CA, Kwoh TJ, Fitch NJ, Richman DD and Guatelli JC (1994) Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene. *J Virol* **68**:2906-14.

Cirino NM, Cameron CE, Smith JS, Rausch JW, Roth MJ, Benkovic SJ and Le Grice SF (1995) Divalent cation modulation of the ribonuclease functions of human immunodeficiency virus reverse transcriptase. *Biochemistry* **34**:9936-43.

Clavel F, Guetard D, Brun-Vezinet F, Chamaret S, Rey MA, Santos-Ferreira MO, Laurent AG, Dauguet C, Katlama C, Rouzioux C and et al. (1986) Isolation of a new human retrovirus from West African patients with AIDS. *Science* **233**:343-6.

Coakley EP, Gillis JM and Hammer SM (2000) Phenotypic and genotypic resistance patterns of HIV-1 isolates derived from individuals treated with didanosine and stavudine. *Aids* **14**:F9-15.

Coffin JM (1995) HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science* **267**:483-9.

Courcoul M, Patience C, Rey F, Blanc D, Harmache A, Sire J, Vigne R and Spire B (1995) Peripheral blood mononuclear cells produce normal amounts of defective Vif- human immunodeficiency virus type 1 particles which are restricted for the preretrotranscription steps. *J Virol* **69**:2068-74.

Crise B, Buonocore L and Rose JK (1990) CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus type 1 glycoprotein precursor. *J Virol* **64**:5585-93.

Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF and Weiss RA (1984) The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**:763-7.

Das K, Clark AD, Jr., Lewi PJ, Heeres J, De Jonge MR, Koymans LM, Vinkers HM, Daeyaert F, Ludovici DW, Kukla MJ, De Corte B, Kavash RW, Ho CY, Ye H, Lichtenstein MA, Andries K, Pauwels R, De Bethune MP, Boyer PL, Clark P, Hughes SH, Janssen PA and Arnold E (2004) Roles of conformational and positional adaptability in structure-based design of TMC125-R165335 (etravirine) and related non-nucleoside reverse transcriptase inhibitors that are highly potent and effective against wild-type and drug-resistant HIV-1 variants. *J Med Chem* **47**:2550-60.

Das K, Ding J, Hsiou Y, Clark AD, Jr., Moereels H, Koymans L, Andries K, Pauwels R, Janssen PA, Boyer PL, Clark P, Smith RH, Jr., Kroeger Smith MB, Michejda CJ, Hughes SH and Arnold E (1996) Crystal structures of 8-Cl and 9-Cl TIBO complexed with wild-type HIV-1 RT and 8-Cl TIBO complexed with the Tyr181Cys HIV-1 RT drug-resistant mutant. *J Mol Biol* **264**:1085-100.

Das K, Clark AD, Jr., Lewi PJ, Heeres J, De Jonge MR, Koymans LM, Vinkers HM, Daeyaert F, Ludovici DW, Kukla MJ, De Corte B, Kavash RW, Ho CY, Ye H, Lichtenstein MA, Andries K, Pauwels R, De Bethune MP, Boyer PL, Clark P, Hughes SH, Janssen PA and Arnold E (2004) Roles of conformational and positional adaptability in structure-based design of TMC125-R165335 (etravirine) and related non-nucleoside reverse transcriptase inhibitors that are highly potent and effective against wild-type and drug-resistant HIV-1 variants. *J Med Chem* **47**:2550-60.

Das K, Lewi PJ, Hughes SH and Arnold E (2005) Crystallography and the design of anti-AIDS drugs: conformational flexibility and positional adaptability are important in the design of non-nucleoside HIV-1 reverse transcriptase inhibitors. *Prog Biophys Mol Biol* **88**:209-31

Davies JF, 2nd, Hostomska Z, Hostomsky Z, Jordan SR and Matthews DA (1991) Crystal structure of the ribonuclease H domain of HIV-1 reverse transcriptase. *Science* **252**:88-95.

De Clercq E (2000) Reverse trancriptase inhibitors as anti-HIV drugs, in *Antivirals against AIDS* (Unger RE, Kreuter, J., and Rübsamen-Waigmann, H. ed) pp 107-150, Marcel Dekker, Inc., New York.

De Clercq E (2004) Antiviral drugs in current clinical use. *J Clin Virol* **30**:115-33.

de Jong JJ, Goudsmit J, Lukashov VV, Hillebrand ME, Baan E, Huismans R, Danner SA, ten Veen JH, de Wolf F and Jurriaans S (1999) Insertion of two amino acids combined with changes in reverse transcriptase containing tyrosine-215 of HIV-1 resistant to multiple nucleoside analogs. *Aids* **13**:75-80.

de Soultrait VR, Desjobert C and Tarrago-Litvak L (2003) Peptides as new inhibitors of HIV-1 reverse transcriptase and integrase. *Curr Med Chem* **10**:1765-78.

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR and Landau NR (1996) Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**:661-6.

DeStefano JJ, Buiser RG, Mallaber LM, Bambara RA and Fay PJ (1991) Human immunodeficiency virus reverse transcriptase displays a partially processive 3' to 5' endonuclease activity. *J Biol Chem* **266**:24295-301.

DeStefano JJ, Buiser RG, Mallaber LM, Fay PJ and Bambara RA (1992a) Parameters that influence processive synthesis and site-specific termination by human immunodeficiency virus reverse transcriptase on RNA and DNA templates. *Biochim Biophys Acta* **1131**:270-80.

DeStefano JJ, Mallaber LM, Fay PJ and Bambara RA (1994) Quantitative analysis of RNA cleavage during RNA-directed DNA synthesis by human immunodeficiency and avian myeloblastosis virus reverse transcriptases. *Nucleic Acids Res* **22**:3793-800.

DeStefano JJ, Mallaber LM, Rodriguez-Rodriguez L, Fay PJ and Bambara RA (1992b) Requirements for strand transfer between internal regions of heteropolymer templates by human immunodeficiency virus reverse transcriptase. *J Virol* **66**:6370-8.

Deval J, Selmi B, Boretto J, Egloff MP, Guerreiro C, Sarfati S and Canard B (2002) The molecular mechanism of multidrug resistance by the Q151M human immunodeficiency virus type 1 reverse transcriptase and its suppression using alpha-boranophosphate nucleotide analogues. *J Biol Chem* **277**:42097-104. Epub 2002 Aug 22.

Ding J, Das K, Hsiou Y, Sarafianos SG, Clark AD, Jr., Jacobo-Molina A, Tantillo C, Hughes SH and Arnold E (1998) Structure and functional implications of the polymerase active site region in a complex of HIV-1 RT with a double-stranded DNA template-primer and an antibody Fab fragment at 2.8 A resolution. *J Mol Biol* **284**:1095-111.

Ding J, Das K, Moereels H, Koymans L, Andries K, Janssen PA, Hughes SH and Arnold E (1995a) Structure of HIV-1 RT/TIBO R 86183 complex reveals similarity in the binding of diverse nonnucleoside inhibitors. *Nat Struct Biol* **2**:407-15.

Ding J, Das K, Tantillo C, Zhang W, Clark AD, Jr., Jessen S, Lu X, Hsiou Y, Jacobo-Molina A, Andries K and et al. (1995b) Structure of HIV-1 reverse transcriptase in a complex with the non-nucleoside inhibitor alpha-APA R 95845 at 2.8 A resolution. *Structure* **3**:365-79.

Ding J, Hughes SH and Arnold E (1997) Protein-nucleic acid interactions and DNA conformation in a complex of human immunodeficiency virus type 1 reverse transcriptase with a double-stranded DNA template-primer. *Biopolymers* **44**:125-38.

Divita G, Muller B, Immendorfer U, Gautel M, Rittinger K, Restle T and Goody RS (1993) Kinetics of interaction of HIV reverse transcriptase with primer/template. *Biochemistry* **32**:7966-71.

Divita G, Restle T, Goody RS, Chermann JC and Baillon JG (1994) Inhibition of human immunodeficiency virus type 1 reverse transcriptase dimerization using synthetic peptides derived from the connection domain. *J Biol Chem* **269**:13080-3.

Divita G, Rittinger K, Geourjon C, Deleage G and Goody RS (1995) Dimerization kinetics of HIV-1 and HIV-2 reverse transcriptase: a two step process. *J Mol Biol* **245**:508-21.

Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP and Paxton WA (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**:667-73.

Dupont S, Sharova N, DeHoratius C, Virbasius CM, Zhu X, Bukrinskaya AG, Stevenson M and Green MR (1999) A novel nuclear export activity in HIV-1 matrix protein required for viral replication. *Nature* **402**:681-5.

Esnouf R, Ren J, Ross C, Jones Y, Stammers D and Stuart D (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. *Nat Struct Biol* **2**:303-8.

Farnet CM and Haseltine WA (1991) Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. *J Virol* **65**:1910-5.

Fedoroff OY, Ge Y and Reid BR (1997) Solution structure of r(gaggacug):d(CAGTCCTC) hybrid: implications for the initiation of HIV-1 (+)-strand synthesis. *J Mol Biol* **269**:225-39.

Feng Y, Broder CC, Kennedy PE and Berger EA (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**:872-7.

Fitzgibbon JE, Howell RM, Haberzettl CA, Sperber SJ, Gocke DJ and Dubin DT (1992) Human immunodeficiency virus type 1 pol gene mutations which cause decreased susceptibility to 2',3'-dideoxycytidine. *Antimicrob Agents Chemother* **36**:153-7.

Fournier C, Cortay JC, Carbonnelle C, Ehresmann C, Marquet R and Boulanger P (2002) The HIV-1 Nef protein enhances the affinity of reverse transcriptase for RNA in vitro. *Virus Genes* **25**:255-69.

Frankel AD and Young JA (1998) HIV-1: fifteen proteins and an RNA. *Annu Rev Biochem* **67**:1-25.

Fuentes GM, Rodriguez-Rodriguez L, Fay PJ and Bambara RA (1995) Use of an oligoribonucleotide containing the polypurine tract sequence as a primer by HIV reverse transcriptase. *J Biol Chem* **270**:28169-76.

Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D, Sidhu GD, Stahl RE, Zolla-Pazner S, Leibowitch J and Popovic M (1983) Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**:865-7.

Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, Cummins LB, Arthur LO, Peeters M, Shaw GM, Sharp PM and Hahn BH (1999) Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**:436-41.

Gao HQ, Boyer PL, Sarafianos SG, Arnold E and Hughes SH (2000) The role of steric hindrance in 3TC resistance of human immunodeficiency virus type-1 reverse transcriptase. *J Mol Biol* **300**:403-18.

Garcia-Lerma JG, MacInnes H, Bennett D, Reid P, Nidtha S, Weinstock H, Kaplan JE and Heneine W (2003) A novel genetic pathway of human immunodeficiency virus type 1 resistance to stavudine mediated by the K65R mutation. *J Virol* **77**:5685-93.

Ghosh M, Jacques PS, Rodgers DW, Ottman M, Darlix JL and Le Grice SF (1996) Alterations to the primer grip of p66 HIV-1 reverse transcriptase and their consequences for template-primer utilization. *Biochemistry* **35**:8553-62.

Ghosh M, Williams J, Powell MD, Levin JG and Le Grice SF (1997) Mutating a conserved motif of the HIV-1 reverse transcriptase palm subdomain alters primer utilization. *Biochemistry* **36**:5758-68.

Goel R, Beard WA, Kumar A, Casas-Finet JR, Strub MP, Stahl SJ, Lewis MS, Bebenek K, Becerra SP, Kunkel TA and et al. (1993) Structure/function studies of HIV-1(1) reverse transcriptase: dimerization-defective mutant L289K. *Biochemistry* **32**:13012-8.

Goldschmidt V and Marquet R (2004) Primer unblocking by HIV-1 reverse transcriptase and resistance to nucleoside RT inhibitors (NRTIs). *Int J Biochem Cell Biol* **36**:1687-705.

Goldschmidt V, Paillart JC, Rigourd M, Ehresmann B, Aubertin AM, Ehresmann C and Marquet R (2004) Structural variability of the initiation complex of HIV-1 reverse transcription. *J Biol Chem* **279**:35923-31. Epub 2004 Jun 11.

Golinelli MP and Hughes SH (2003) Secondary structure in the nucleic acid affects the rate of HIV-1 nucleocapsid-mediated strand annealing. *Biochemistry* **42**:8153-62.

Gopalakrishnan V, Peliska JA and Benkovic SJ (1992) Human immunodeficiency virus type 1 reverse transcriptase: spatial and temporal relationship between the polymerase and RNase H activities. *Proc Natl Acad Sci U S A* **89**:10763-7.

Gotte M, Arion D, Parniak MA and Wainberg MA (2000) The M184V mutation in the reverse transcriptase of human immunodeficiency virus type 1 impairs rescue of chain-terminated DNA synthesis. *J Virol* **74**:3579-85.

Gotte M, Maier G, Gross HJ and Heumann H (1998) Localization of the active site of HIV-1 reverse transcriptase-associated RNase H domain on a DNA template using site-specific generated hydroxyl radicals. *J Biol Chem* **273**:10139-46.

Gu Z, Gao Q, Fang H, Salomon H, Parniak MA, Goldberg E, Cameron J and Wainberg MA (1994) Identification of a mutation at codon 65 in the IKKK motif of reverse transcriptase that encodes human immunodeficiency virus resistance to 2',3'-dideoxycytidine and 2',3'-dideoxy-3'-thiacytidine. *Antimicrob Agents Chemother* **38**:275-81.

Hahn BH, Shaw GM, De Cock KM and Sharp PM (2000) AIDS as a zoonosis: scientific and public health implications. *Science* **287**:607-14.

Han GW, Kopka ML, Cascio D, Grzeskowiak K and Dickerson RE (1997) Structure of a DNA analog of the primer for HIV-1 RT second strand synthesis. *J Mol Biol* **269**:811-26.

Hansen J, Schulze T and Moelling K (1987) RNase H activity associated with bacterially expressed reverse transcriptase of human T-cell lymphotropic virus III/lymphadenopathy-associated virus. *J Biol Chem* **262**:12393-6.

Harrich D, Ulich C, Garcia-Martinez LF and Gaynor RB (1997) Tat is required for efficient HIV-1 reverse transcription. *Embo J* **16**:1224-35.

Harrich D, Ulich C and Gaynor RB (1996) A critical role for the TAR element in promoting efficient human immunodeficiency virus type 1 reverse transcription. *J Virol* **70**:4017-27.

Harrigan PR, Kinghorn I, Bloor S, Kemp SD, Najera I, Kohli A and Larder BA (1996) Significance of amino acid variation at human immunodeficiency virus type 1 reverse transcriptase residue 210 for zidovudine susceptibility. *J Virol* **70**:5930-4.

Harrigan PR, Stone C, Griffin P, Najera I, Bloor S, Kemp S, Tisdale M and Larder B (2000) Resistance profile of the human immunodeficiency virus type 1 reverse transcriptase inhibitor abacavir (1592U89) after monotherapy and combination therapy. CNA2001 Investigative Group. *J Infect Dis* **181**:912-20.

Harris D, Kaushik N, Pandey PK, Yadav PN and Pandey VN (1998a) Functional analysis of amino acid residues constituting the dNTP binding pocket of HIV-1 reverse transcriptase. *J Biol Chem* **273**:33624-34.

Harris D, Lee R, Misra HS, Pandey PK and Pandey VN (1998b) The p51 subunit of human immunodeficiency virus type 1 reverse transcriptase is essential in loading the p66 subunit on the template primer. *Biochemistry* **37**:5903-8.

Heinzinger NK, Bukinsky MI, Haggerty SA, Ragland AM, Kewalramani V, Lee MA, Gendelman HE, Ratner L, Stevenson M and Emerman M (1994) The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc Natl Acad Sci U S A* **91**:7311-5.

Henderson LE, Bowers MA, Sowder RC, 2nd, Serabyn SA, Johnson DG, Bess JW, Jr., Arthur LO, Bryant DK and Fenselau C (1992) Gag proteins of the highly replicative MN strain of human immunodeficiency virus type 1: posttranslational modifications, proteolytic processings, and complete amino acid sequences. *J Virol* **66**:1856-65.

Hermann T and Heumann H (1996) Strained template under the thumbs. How reverse transcriptase of human immunodeficiency virus type 1 moves along its template. *Eur J Biochem* **242**:98-103.

Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH and Johnson PR (1989) An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**:389-92.

Hizi A, McGill C and Hughes SH (1988) Expression of soluble, enzymatically active, human immunodeficiency virus reverse transcriptase in Escherichia coli and analysis of mutants. *Proc Natl Acad Sci U S A* **85**:1218-22.

Hizi A, Tal R, Shaharabany M, Currens MJ, Boyd MR, Hughes SH and McMahon JB (1993) Specific inhibition of the reverse transcriptase of human immunodeficiency virus type 1 and the chimeric enzymes of human immunodeficiency virus type 1 and type 2 by nonnucleoside inhibitors. *Antimicrob Agents Chemother* **37**:1037-42.

Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM and Markowitz M (1995) Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**:123-6.

Hooker DJ, Tachedjian G, Solomon AE, Gurusinghe AD, Land S, Birch C, Anderson JL, Roy BM, Arnold E and Deacon NJ (1996) An in vivo mutation from leucine to tryptophan at position 210 in human immunodeficiency virus type 1 reverse transcriptase contributes to high-level resistance to 3'-azido-3'-deoxythymidine. *J Virol* **70**:8010-8.

Hopkins AL, Ren J, Esnouf RM, Willcox BE, Jones EY, Ross C, Miyasaka T, Walker RT, Tanaka H, Stammers DK and Stuart DI (1996) Complexes of HIV-1 reverse transcriptase with inhibitors of the HEPT series reveal conformational changes relevant to the design of potent non-nucleoside inhibitors. *J Med Chem* **39**:1589-600.

Hostomska Z, Matthews DA, Davies JF, 2nd, Nodes BR and Hostomsky Z (1991) Proteolytic release and crystallization of the RNase H domain of human immunodeficiency virus type 1 reverse transcriptase. *J Biol Chem* **266**:14697-702.

Hsieh JC, Zinnen S and Modrich P (1993) Kinetic mechanism of the DNA-dependent DNA polymerase activity of human immunodeficiency virus reverse transcriptase. *J Biol Chem* **268**:24607-13.

Hsiou Y, Das K, Ding J, Clark AD, Jr., Kleim JP, Rosner M, Winkler I, Riess G, Hughes SH and Arnold E (1998) Structures of Tyr188Leu mutant and wild-type HIV-1 reverse transcriptase complexed with the non-nucleoside inhibitor HBY 097: inhibitor flexibility is a useful design feature for reducing drug resistance. *J Mol Biol* **284**:313-23.

Hsiou Y, Ding J, Das K, Clark AD, Jr., Boyer PL, Lewi P, Janssen PA, Kleim JP, Rosner M, Hughes SH and Arnold E (2001) The Lys103Asn mutation of HIV-1 RT: a novel mechanism of drug resistance. *J Mol Biol* **309**:437-45.

Hsiou Y, Ding J, Das K, Clark AD, Jr., Hughes SH and Arnold E (1996) Structure of unliganded HIV-1 reverse transcriptase at 2.7 A resolution: implications of conformational changes for polymerization and inhibition mechanisms. *Structure* **4**:853-60.

Hu WS and Temin HM (1990a) Genetic consequences of packaging two RNA genomes in one retroviral particle: pseudodiploidy and high rate of genetic recombination. *Proc Natl Acad Sci U S A* **87**:1556-60.

Hu WS and Temin HM (1990b) Retroviral recombination and reverse transcription. *Science* **250**:1227-33.

Huang H, Chopra R, Verdine GL and Harrison SC (1998a) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **282**:1669-75.

Huang W, Gamarnik A, Limoli K, Petropoulos CJ and Whitcomb JM (2003) Amino acid substitutions at position 190 of human immunodeficiency virus type 1 reverse transcriptase increase susceptibility to delavirdine and impair virus replication. *J Virol* **77**:1512-23.

Huang Y, Khorchid A, Gabor J, Wang J, Li X, Darlix JL, Wainberg MA and Kleiman L (1998b) The role of nucleocapsid and U5 stem/A-rich loop sequences in tRNA(3Lys) genomic placement and initiation of reverse transcription in human immunodeficiency virus type 1. *J Virol* **72**:3907-15.

Isaka Y, Miki S, Kawauchi S, Suyama A, Sugimoto H, Adachi A, Miura T, Hayami M, Yoshie O, Fujiwara T and Sato A (2001) A single amino acid change at Leu-188 in the reverse transcriptase of HIV-2 and SIV renders them sensitive to non-nucleoside reverse transcriptase inhibitors. *Arch Virol* **146**:743-55.

Isel C, Ehresmann C, Keith G, Ehresmann B and Marquet R (1995) Initiation of reverse transcription of HIV-1: secondary structure of the HIV-1 RNA/tRNA(3Lys) (template/primer). *J Mol Biol* **247**:236-50.

Isel C, Keith G, Ehresmann B, Ehresmann C and Marquet R (1998) Mutational analysis of the tRNA3Lys/HIV-1 RNA (primer/template) complex. *Nucleic Acids Res* **26**:1198-204.

Isel C, Lanchy JM, Le Grice SF, Ehresmann C, Ehresmann B and Marquet R (1996) Specific initiation and switch to elongation of human immunodeficiency virus type 1 reverse transcription require the post-transcriptional modifications of primer tRNA3Lys. *Embo J* **15**:917-24.

Isel C, Marquet R, Keith G, Ehresmann C and Ehresmann B (1993) Modified nucleotides of tRNA(3Lys) modulate primer/template loop-loop interaction in the initiation complex of HIV-1 reverse transcription. *J Biol Chem* **268**:25269-72.

Janssen PA, Lewi PJ, Arnold E, Daeyaert F, de Jonge M, Heeres J, Koymans L, Vinkers M, Guillemont J, Pasquier E, Kukla M, Ludovici D, Andries K, de Bethune MP, Pauwels R, Das K, Clark AD, Jr., Frenkel YV, Hughes SH, Medaer B, De Knaep F, Bohets H, De Clerck F, Lampo A, Williams P, Stoffels P, Van Herrewege Y, Vanham G, Michiels J, Fransen K, Kestens L, Janssen P, Lewi P, De Jonge MR, Koymans LM, Vinkers HM, Ludovici DW, Kukla MJ, De Corte B, Kavash RW, Ho CY, Ye H, Lichtenstein MA, Boyer PL and Clark P (2005) In search of a novel anti-HIV drug: multidisciplinary coordination in the discovery of 4-[[4-[[4-[(1E)-2-cyanoethenyl]-2,6-dimethylphenyl]amino]-2-pyrimidinyl]amino]benzonitrile (R278474, rilpivirine). *J Med Chem* **48**:1901-9.

Jacobo-Molina A, Ding J, Nanni RG, Clark AD, Jr., Lu X, Tantillo C, Williams RL, Kamer G, Ferris AL, Clark P and et al. (1993) Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. *Proc Natl Acad Sci U S A* **90**:6320-4.

Jacques PS, Wohrl BM, Howard KJ and Le Grice SF (1994) Modulation of HIV-1 reverse transcriptase function in "selectively deleted" p66/p51 heterodimers. *J Biol Chem* **269**:1388-93.

Ji X, Klarmann GJ and Preston BD (1996) Effect of human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein on HIV-1 reverse transcriptase activity in vitro. *Biochemistry* **35**:132-43.

Kati WM, Johnson KA, Jerva LF and Anderson KS (1992) Mechanism and fidelity of HIV reverse transcriptase. *J Biol Chem* **267**:25988-97.

Kaushik N, Harris D, Rege N, Modak MJ, Yadav PN and Pandey VN (1997) Role of glutamine-151 of human immunodeficiency virus type-1 reverse transcriptase in RNA-directed DNA synthesis. *Biochemistry* **36**:14430-8.

Kaushik N, Rege N, Yadav PN, Sarafianos SG, Modak MJ and Pandey VN (1996) Biochemical analysis of catalytically crucial aspartate mutants of human immunodeficiency virus type 1 reverse transcriptase. *Biochemistry* **35**:11536-46.

Kellam P, Boucher CA and Larder BA (1992) Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. *Proc Natl Acad Sci U S A* **89**:1934-8.

Keulen W, Back NK, van Wijk A, Boucher CA and Berkhout B (1997) Initial appearance of the 184Ile variant in lamivudine-treated patients is caused by the mutational bias of human immunodeficiency virus type 1 reverse transcriptase. *J Virol* **71**:3346-50.

Khan R and Giedroc DP (1992) Recombinant human immunodeficiency virus type 1 nucleocapsid (NCp7) protein unwinds tRNA. *J Biol Chem* **267**:6689-95.

Klarmann GJ, Schauber CA and Preston BD (1993) Template-directed pausing of DNA synthesis by HIV-1 reverse transcriptase during polymerization of HIV-1 sequences in vitro. *J Biol Chem* **268**:9793-802.

Kleim JP, Rosner M, Winkler I, Paessens A, Kirsch R, Hsiou Y, Arnold E and Riess G (1996) Selective pressure of a quinoxaline nonnucleoside inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on HIV-1 replication results in the emergence of nucleoside RT-inhibitor-specific (RT Leu-74-->Val or Ile and Val-75-->Leu or Ile) HIV-1 mutants. *Proc Natl Acad Sci U S A* **93**:34-8.

Kleim JP, Winkler I, Rosner M, Kirsch R, Rubsamen-Waigmann H, Paessens A and Riess G (1997) In vitro selection for different mutational patterns in the HIV-1 reverse transcriptase using high and low selective pressure of the nonnucleoside reverse transcriptase inhibitor HBY 097. *Virology* **231**:112-8.

Kohlstaedt LA and Steitz TA (1992) Reverse transcriptase of human immunodeficiency virus can use either human tRNA(3Lys) or Escherichia coli tRNA(2Gln) as a primer in an in vitro primer-utilization assay. *Proc Natl Acad Sci U S A* **89**:9652-6.

Kohlstaedt LA, Wang J, Friedman JM, Rice PA and Steitz TA (1992) Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**:1783-90.

Korber B, Muldoon M, Theiler J, Gao F, Gupta R, Lapedes A, Hahn BH, Wolinsky S and Bhattacharya T (2000) Timing the ancestor of the HIV-1 pandemic strains. *Science* **288**:1789-96.

Lama J (2003) The physiological relevance of CD4 receptor down-modulation during HIV infection. *Curr HIV Res* **1**:167-84.

Lanchy JM, Ehresmann C, Le Grice SF, Ehresmann B and Marquet R (1996) Binding and kinetic properties of HIV-1 reverse transcriptase markedly differ during initiation and elongation of reverse transcription. *Embo J* **15**:7178-87.

Lanchy JM, Isel C, Keith G, Le Grice SF, Ehresmann C, Ehresmann B and Marquet R (2000) Dynamics of the HIV-1 reverse transcription complex during initiation of DNA synthesis. *J Biol Chem* **275**:12306-12.

Lapadat-Tapolsky M, Gabus C, Rau M and Darlix JL (1997) Possible roles of HIV-1 nucleocapsid protein in the specificity of proviral DNA synthesis and in its variability. *J Mol Biol* **268**:250-60.

Larder BA (1992) 3'-Azido-3'-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. *Antimicrob Agents Chemother* **36**:2664-9.

Larder BA, Bloor S, Kemp SD, Hertogs K, Desmet RL, Miller V, Sturmer M, Staszewski S, Ren J, Stammers DK, Stuart DI and Pauwels R (1999) A family of insertion mutations between codons 67 and 70 of human immunodeficiency virus type 1 reverse transcriptase confer multinucleoside analog resistance. *Antimicrob Agents Chemother* **43**:1961-7.

Larder BA, Darby G and Richman DD (1989) HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* **243**:1731-4.

Larder BA and Kemp SD (1989) Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**:1155-8.

Larder BA, Kemp SD and Harrigan PR (1995) Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. *Science* **269**:696-9.

Larder BA, Purifoy DJ, Powell KL and Darby G (1987) Site-specific mutagenesis of AIDS virus reverse transcriptase. *Nature* **327**:716-7.

Le Grice SF, Naas T, Wohlgensinger B and Schatz O (1991) Subunit-selective mutagenesis indicates minimal polymerase activity in heterodimer-associated p51 HIV-1 reverse transcriptase. *Embo J* **10**:3905-11.

Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM and Oshiro LS (1984) Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225**:840-2.

Li X, Quan Y, Arts EJ, Li Z, Preston BD, de Rocquigny H, Roques BP, Darlix JL, Kleiman L, Parniak MA and Wainberg MA (1996) Human immunodeficiency virus Type 1 nucleocapsid protein (NCp7) directs specific initiation of minus-strand DNA synthesis primed by human tRNA(Lys3) in vitro: studies of viral RNA molecules mutated in regions that flank the primer binding site. *J Virol* **70**:4996-5004.

Lori F, Scovassi AI, Zella D, Achilli G, Cattaneo E, Casoli C and Bertazzoni U (1988) Enzymatically active forms of reverse transcriptase of the human immunodeficiency virus. *AIDS Res Hum Retroviruses* **4**:393-8.

Luciw PA (1996) Human immunodeficiency viruses and their replication., in *Fundamental viologyr* (Fields BN, Knipe, D.M. and Howley, P.M. ed) pp 1912.

Madrid M, Lukin JA, Madura JD, Ding J and Arnold E (2001) Molecular dynamics of HIV-1 reverse transcriptase indicates increased flexibility upon DNA binding. *Proteins* **45**:176-82.

Maeda Y, Venzon DJ and Mitsuya H (1998) Altered drug sensitivity, fitness, and evolution of human immunodeficiency virus type 1 with pol gene mutations conferring multi-dideoxynucleoside resistance. *J Infect Dis* **177**:1207-13.

Maier G, Dietrich U, Panhans B, Schroder B, Rubsamen-Waigmann H, Cellai L, Hermann T and Heumann H (1999) Mixed reconstitution of mutated subunits of HIV-1 reverse transcriptase coexpressed in Escherichia coli - two tags tie it up. *Eur J Biochem* **261**:10-8.

Majumdar C, Stein CA, Cohen JS, Broder S and Wilson SH (1989) Stepwise mechanism of HIV reverse transcriptase: primer function of phosphorothioate oligodeoxynucleotide. *Biochemistry* **28**:1340-6.

Mak J, Jiang M, Wainberg MA, Hammarskjold ML, Rekosh D and Kleiman L (1994) Role of Pr160gag-pol in mediating the selective incorporation of tRNA(Lys) into human immunodeficiency virus type 1 particles. *J Virol* **68**:2065-72.

Mangasarian A and Trono D (1997) The multifaceted role of HIV Nef. *Res Virol* **148**:30-3.

Marin M, Rose KM, Kozak SL and Kabat D (2003) HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. *Nat Med* **9**:1398-403. Epub 2003 Oct 05.

Mellors JW, Bazmi HZ, Schinazi RF, Roy BM, Hsiou Y, Arnold E, Weir J and Mayers DL (1995) Novel mutations in reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. *Antimicrob Agents Chemother* **39**:1087-92.

Miller MD (2004) K65R, TAMs and tenofovir. *AIDS Rev* **6**:22-33.

Miller MD, Farnet CM and Bushman FD (1997) Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. *J Virol* **71**:5382-90.

Miller MD, Margot NA, Hertogs K, Larder B and Miller V (2001) Antiviral activity of tenofovir (PMPA) against nucleoside-resistant clinical HIV samples. *Nucleosides Nucleotides Nucleic Acids* **20**:1025-8.

Miller V, Ait-Khaled M, Stone C, Griffin P, Mesogiti D, Cutrell A, Harrigan R, Staszewski S, Katlama C, Pearce G and Tisdale M (2000) HIV-1 reverse transcriptase (RT) genotype and susceptibility to RT inhibitors during abacavir monotherapy and combination therapy. *Aids* **14**:163-71.

Misra HS, Pandey PK and Pandey VN (1998) An enzymatically active chimeric HIV-1 reverse transcriptase (RT) with the RNase-H domain of murine leukemia virus RT exists as a monomer. *J Biol Chem* **273**:9785-9.

Mizrahi V, Brooksbank RL and Nkabinde NC (1994) Mutagenesis of the conserved aspartic acid 443, glutamic acid 478, asparagine 494, and aspartic acid 498 residues in the ribonuclease H domain of p66/p51 human immunodeficiency virus type I reverse transcriptase. Expression and biochemical analysis. *J Biol Chem* **269**:19245-9.

Mizrahi V, Usdin MT, Harington A and Dudding LR (1990) Site-directed mutagenesis of the conserved Asp-443 and Asp-498 carboxy-terminal residues of HIV-1 reverse transcriptase. *Nucleic Acids Res* **18**:5359-63.

Morris MC, Berducou C, Mery J, Heitz F and Divita G (1999a) The thumb domain of the P51-subunit is essential for activation of HIV reverse transcriptase. *Biochemistry* **38**:15097-103.

Morris MC, Robert-Hebmann V, Chaloin L, Mery J, Heitz F, Devaux C, Goody RS and Divita G (1999b) A new potent HIV-1 reverse transcriptase inhibitor. A synthetic peptide derived from the interface subunit domains. *J Biol Chem* **274**:24941-6.

Navarro F and Landau NR (2004) Recent insights into HIV-1 Vif. *Curr Opin Immunol* **16**:477-82.

Navarro JM, Damier L, Boretto J, Priet S, Canard B, Querat G and Sire J (2001) Glutamic residue 438 within the protease-sensitive subdomain of HIV-1 reverse transcriptase is critical for heterodimer processing in viral particles. *Virology* **290**:300-8.

Oberlin E, Amara A, Bachelerie F, Bessia C, Virelizier JL, Arenzana-Seisdedos F, Schwartz O, Heard JM, Clark-Lewis I, Legler DF, Loetscher M, Baggiolini M and Moser B (1996) The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**:833-5.

Oude Essink BB, Das AT and Berkhout B (1995) Structural requirements for the binding of tRNA Lys3 to reverse transcriptase of the human immunodeficiency virus type 1. *J Biol Chem* **270**:23867-74.

Oude Essink BB, Das AT and Berkhout B (1996) HIV-1 reverse transcriptase discriminates against non-self tRNA primers. *J Mol Biol* **264**:243-54.

Palaniappan C, Fuentes GM, Rodriguez-Rodriguez L, Fay PJ and Bambara RA (1996) Helix structure and ends of RNA/DNA hybrids direct the cleavage specificity of HIV-1 reverse transcriptase RNase H. *J Biol Chem* **271**:2063-70.

Palaniappan C, Kim JK, Wisniewski M, Fay PJ and Bambara RA (1998) Control of initiation of viral plus strand DNA synthesis by HIV reverse transcriptase. *J Biol Chem* **273**:3808-16.

Palaniappan C, Wisniewski M, Jacques PS, Le Grice SF, Fay PJ and Bambara RA (1997) Mutations within the primer grip region of HIV-1 reverse transcriptase result in loss of RNase H function. *J Biol Chem* **272**:11157-64.

Pandey PK, Kaushik N, Singh K, Sharma B, Upadhyay AK, Kumar S, Harris D and Pandey VN (2002) Insertion of a small peptide of six amino acids into the beta7-beta8 loop of the p51 subunit of HIV-1 reverse transcriptase perturbs the heterodimer and affects its activities. *BMC Biochem* **3**:18.

Pandey PK, Kaushik N, Talele TT, Yadav PN and Pandey VN (2001a) The beta7-beta8 loop of the p51 subunit in the heterodimeric (p66/p51) human immunodeficiency virus type 1 reverse transcriptase is essential for the catalytic function of the p66 subunit. *Biochemistry* **40**:9505-12.

Pandey PK, Kaushik N, Talele TT, Yadav PN and Pandey VN (2001b) Insertion of a peptide from MuLV RT into the connection subdomain of HIV-1 RT results in a functionally active chimeric enzyme in monomeric conformation. *Mol Cell Biochem* **225**:135-44.

Pandey VN, Kaushik N, Rege N, Sarafianos SG, Yadav PN and Modak MJ (1996) Role of methionine 184 of human immunodeficiency virus type-1 reverse transcriptase in the polymerase function and fidelity of DNA synthesis. *Biochemistry* **35**:2168-79.

Pata JD, Stirtan WG, Goldstein SW and Steitz TA (2004) Structure of HIV-1 reverse transcriptase bound to an inhibitor active against mutant reverse transcriptases resistant to other nonnucleoside inhibitors. *Proc Natl Acad Sci U S A* **101**:10548-53. Epub 2004 Jul 12.

Patel PH, Jacobo-Molina A, Ding J, Tantillo C, Clark AD, Jr., Raag R, Nanni RG, Hughes SH and Arnold E (1995) Insights into DNA polymerization mechanisms from structure and function analysis of HIV-1 reverse transcriptase. *Biochemistry* **34**:5351-63.

Pedersen NC, Ho EW, Brown ML and Yamamoto JK (1987) Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* **235**:790-3.

Pelemans H, Esnouf R, De Clercq E and Balzarini J (2000) Mutational analysis of trp-229 of human immunodeficiency virus type 1 reverse transcriptase (RT) identifies this amino acid residue as a prime target for the rational design of new non-nucleoside RT inhibitors. *Mol Pharmacol* **57**:954-60.

Pelemans H, Esnouf R, Min KL, Parniak M, De Clercq E and Balzarini J (2001) Mutations at amino acid positions 63, 189, and 396 of human immunodeficiency virus type 1 reverse transcriptase (rt) partially restore the dna polymerase activity of a trp229tyr mutant rt. *Virology* **287**:143-50.

Peliska JA, Balasubramanian S, Giedroc DP and Benkovic SJ (1994) Recombinant HIV-1 nucleocapsid protein accelerates HIV-1 reverse transcriptase catalyzed DNA strand transfer reactions and modulates RNase H activity. *Biochemistry* **33**:13817-23.

Peliska JA and Benkovic SJ (1992) Mechanism of DNA strand transfer reactions catalyzed by HIV-1 reverse transcriptase. *Science* **258**:1112-8.

Perez KA, Saag MS and Kilby JM (2001). Human immunodeficiency virus. In: Atlas of AIDS. Edited by Mandell GL, Donna Mildvan. Current Medicine Inc.

Perrino FW, Preston BD, Sandell LL and Loeb LA (1989) Extension of mismatched 3' termini of DNA is a major determinant of the infidelity of human immunodeficiency virus type 1 reverse transcriptase. *Proc Natl Acad Sci U S A* **86**:8343-7.

Picard V, Angelini E, Maillard A, Race E, Clavel F, Chene G, Ferchal F and Molina JM (2001) Comparison of genotypic and phenotypic resistance patterns of human immunodeficiency virus type 1 isolates from patients treated with stavudine and didanosine or zidovudine and lamivudine. *J Infect Dis* **184**:781-4. Epub 2001 Aug 07.

Pollard VW and Malim MH (1998) The HIV-1 Rev protein. *Annu Rev Microbiol* **52**:491-532.

Popov S, Rexach M, Ratner L, Blobel G, Bukrinsky M, Zybarth G, Reiling N, Lee MA, Lane CM and Moore MS (1998a) Viral protein R regulates docking of the HIV-1 preintegration complex to the nuclear pore complex. *J Biol Chem* **273**:13347-52.

Popov S, Rexach M, Zybarth G, Reiling N, Lee MA, Ratner L, Lane CM, Moore MS, Blobel G and Bukrinsky M (1998b) Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. *Embo J* **17**:909-17.

Popovic M, Sarngadharan MG, Read E and Gallo RC (1984) Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**:497-500.

Powell MD, Beard WA, Bebenek K, Howard KJ, Le Grice SF, Darden TA, Kunkel TA, Wilson SH and Levin JG (1999) Residues in the alphaH and alphaI helices of the HIV-1 reverse transcriptase thumb subdomain required for the specificity of RNase H-catalyzed removal of the polypurine tract primer. *J Biol Chem* **274**:19885-93.

Powell MD and Levin JG (1996) Sequence and structural determinants required for priming of plus-strand DNA synthesis by the human immunodeficiency virus type 1 polypurine tract. *J Virol* **70**:5288-96.

Pullen KA and Champoux JJ (1990) Plus-strand origin for human immunodeficiency virus type 1: implications for integration. *J Virol* **64**:6274-7.

Pullen KA, Ishimoto LK and Champoux JJ (1992) Incomplete removal of the RNA primer for minus-strand DNA synthesis by human immunodeficiency virus type 1 reverse transcriptase. *J Virol* **66**:367-73.

Ratner L, Gallo RC and Wong-Staal F (1985a) HTLV-III, LAV, ARV are variants of same AIDS virus. *Nature* **313**:636-7.

Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, Doran ER, Rafalski JA, Whitehorn EA, Baumeister K and et al. (1985b) Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* **313**:277-84.

Rausch JW, Lener D, Miller JT, Julias JG, Hughes SH and Le Grice SF (2002) Altering the RNase H primer grip of human immunodeficiency virus reverse transcriptase modifies cleavage specificity. *Biochemistry* **41**:4856-65.

Ren J, Bird LE, Chamberlain PP, Stewart-Jones GB, Stuart DI and Stammers DK (2002) Structure of HIV-2 reverse transcriptase at 2.35-A resolution and the mechanism of resistance to non-nucleoside inhibitors. *Proc Natl Acad Sci U S A* **17**:17.

Ren J, Esnouf R, Garman E, Somers D, Ross C, Kirby I, Keeling J, Darby G, Jones Y, Stuart D and et al. (1995a) High resolution structures of HIV-1 RT from four RT-inhibitor complexes. *Nat Struct Biol* **2**:293-302.

Ren J, Esnouf R, Hopkins A, Ross C, Jones Y, Stammers D and Stuart D (1995b) The structure of HIV-1 reverse transcriptase complexed with 9-chloro-TIBO: lessons for inhibitor design. *Structure* **3**:915-26.

Ren J, Esnouf RM, Hopkins AL, Warren J, Balzarini J, Stuart DI and Stammers DK (1998) Crystal structures of HIV-1 reverse transcriptase in complex with carboxanilide derivatives. *Biochemistry* **37**:14394-403.

Ren J, Nichols CE, Chamberlain PP, Weaver KL, Short SA and Stammers DK (2004) Crystal structures of HIV-1 reverse transcriptases mutated at codons 100, 106 and 108 and mechanisms of resistance to non-nucleoside inhibitors. *J Mol Biol* **336**:569-78.

Ren J and Stammers DK (2005) HIV reverse transcriptase structures: designing new inhibitors and understanding mechanisms of drug resistance. *Trends Pharmacol Sci* **26**:4-7.

Restle T, Muller B and Goody RS (1990) Dimerization of human immunodeficiency virus type 1 reverse transcriptase. A target for chemotherapeutic intervention. *J Biol Chem* **265**:8986-8.

Restle T, Muller B and Goody RS (1992) RNase H activity of HIV reverse transcriptases is confined exclusively to the dimeric forms. *FEBS Lett* **300**:97-100.

Rittinger K, Divita G and Goody RS (1995) Human immunodeficiency virus reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside inhibitors. *Proc Natl Acad Sci U S A* **92**:8046-9.

Roberts JD, Bebenek K and Kunkel TA (1988) The accuracy of reverse transcriptase from HIV-1. *Science* **242**:1171-3.

Rodgers DW, Gamblin SJ, Harris BA, Ray S, Culp JS, Hellmig B, Woolf DJ, Debouck C and Harrison SC (1995) The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **92**:1222-6.

Rodriguez-Barrios F and Gago F (2004) Understanding the basis of resistance in the irksome Lys103Asn HIV-1 reverse transcriptase mutant through targeted molecular dynamics simulations. *J Am Chem Soc* **126**:15386-7.

Rodriguez-Barrios F, Perez C, Lobaton E, Velazquez S, Chamorro C, San-Felix A, Perez-Perez MJ, Camarasa MJ, Pelemans H, Balzarini J and Gago F (2001) Identification of a putative binding site for [2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3'-spiro-5"- (4"-amino-1",2"-oxathiole-2",2"-dioxide)thymine (TSAO) derivatives at the p51-p55 interface of HIV-1 reverse transcriptase. *J Med Chem* **44**:1853-65.

Rodriguez-Rodriguez L, Tsuchihashi Z, Fuentes GM, Bambara RA and Fay PJ (1995) Influence of human immunodeficiency virus nucleocapsid protein on synthesis and strand transfer by the reverse transcriptase in vitro. *J Biol Chem* **270**:15005-11.

Rong L, Liang C, Hsu M, Kleiman L, Petitjean P, de Rocquigny H, Roques BP and Wainberg MA (1998) Roles of the human immunodeficiency virus type 1 nucleocapsid protein in annealing and initiation versus elongation in reverse transcription of viral negative-strand strong-stop DNA. *J Virol* **72**:9353-8.

Ruane PJ and Luber AD (2004) K65R-associated virologic failure in HIV-infected patients receiving tenofovir-containing triple nucleoside/nucleotide reverse transcriptase inhibitor regimens. *MedGenMed* **6**:31.

Salemi M, Strimmer K, Hall WW, Duffy M, Delaporte E, Mboup S, Peeters M and Vandamme AM (2001) Dating the common ancestor of SIVcpz and HIV-1 group M and the origin of HIV-1 subtypes using a new method to uncover clock-like molecular evolution. *Faseb J* **15**:276-8. Epub 2000 Dec 08.

Sarafianos SG, Clark AD, Jr., Tuske S, Squire CJ, Das K, Sheng D, Ilankumaran P, Ramesha AR, Kroth H, Sayer JM, Jerina DM, Boyer PL, Hughes SH and Arnold E (2003) Trapping HIV-1 reverse transcriptase before and after translocation on DNA. *J Biol Chem* **278**:16280-8.

Sarafianos SG, Das K, Clark AD, Jr., Ding J, Boyer PL, Hughes SH and Arnold E (1999) Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. *Proc Natl Acad Sci U S A* **96**:10027-32.

Sarafianos SG, Das K, Tantillo C, Clark AD, Ding J, Whitcomb JM, Boyer PL, Hughes SH and Arnold E (2001) Crystal structure of HIV-1 reverse transcriptase in complex with a polypurine tract RNA:DNA. *Embo J* **20**:1449-61.

Sarafianos SG, Pandey VN, Kaushik N and Modak MJ (1995) Glutamine 151 participates in the substrate dNTP binding function of HIV-1 reverse transcriptase. *Biochemistry* **34**:7207-16.

Schatz O, Cromme FV, Gruninger-Leitch F and Le Grice SF (1989) Point mutations in conserved amino acid residues within the C-terminal domain of HIV-1 reverse transcriptase specifically repress RNase H function. *FEBS Lett* **257**:311-4.

Schatz O, Mous J and Le Grice SF (1990) HIV-1 RT-associated ribonuclease H displays both endonuclease and 3'----5' exonuclease activity. *Embo J* **9**:1171-6.

Schinazi RF, Larder BA and Mellors JW (2001) Mutations in retroviral genes associated with drug resistance: 2000-2001 update. *International Antiviral News* **8**:65-91.

Schinazi RF, Lloyd RM, Jr., Nguyen MH, Cannon DL, McMillan A, Ilksoy N, Chu CK, Liotta DC, Bazmi HZ and Mellors JW (1993) Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. *Antimicrob Agents Chemother* **37**:875-81.

Schmit JC, Van Laethem K, Ruiz L, Hermans P, Sprecher S, Sonnerborg A, Leal M, Harrer T, Clotet B, Arendt V, Lissen E, Witvrouw M, Desmyter J, De Clercq E and Vandamme AM (1998) Multiple dideoxynucleoside analogue-resistant (MddNR) HIV-1 strains isolated from patients from different European countries. *Aids* **12**:2007-15.

Schubert U, Anton LC, Bacik I, Cox JH, Bour S, Bennink JR, Orlowski M, Strebel K and Yewdell JW (1998) CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. *J Virol* **72**:2280-8.

Schubert U and Strebel K (1994) Differential activities of the human immunodeficiency virus type 1-encoded Vpu protein are regulated by phosphorylation and occur in different cellular compartments. *J Virol* **68**:2260-71.

Schuurman R, Nijhuis M, van Leeuwen R, Schipper P, de Jong D, Collis P, Danner SA, Mulder J, Loveday C, Christopherson C and et al. (1995) Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). *J Infect Dis* **171**:1411-9.

Schwartz O, Marechal V, Danos O and Heard JM (1995) Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. *J Virol* **69**:4053-9.

Sevilya Z, Loya S, Adir N and Hizi A (2003a) The ribonuclease H activity of the reverse transcriptases of human immunodeficiency viruses type 1 and type 2 is modulated by residue 294 of the small subunit. *Nucleic Acids Res* **31**:1481-7.

Sevilya Z, Loya S, Duvshani A, Adir N and Hizi A (2003b) Mutagenesis of cysteine 280 of the reverse transcriptase of human immunodeficiency virus type-1: the effects on the ribonuclease h activity. *J Mol Biol* **327**:19-30.

Sevilya Z, Loya S, Hughes SH and Hizi A (2001) The ribonuclease H activity of the reverse transcriptases of human immunodeficiency viruses type 1 and type 2 is affected by the thumb subdomain of the small protein subunits. *J Mol Biol* **311**:957-71.

Shafer RW, Winters MA, Iversen AK and Merigan TC (1996) Genotypic and phenotypic changes during culture of a multinucleoside-resistant human immunodeficiency virus type 1 strain in the presence and absence of additional reverse transcriptase inhibitors. *Antimicrob Agents Chemother* **40**:2887-90.

Sheehy AM, Gaddis NC and Malim MH (2003) The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat Med* **9**:1404-7. Epub 2003 Oct 05.

Shen L, Shen J, Luo X, Cheng F, Xu Y, Chen K, Arnold E, Ding J and Jiang H (2003) Steered Molecular Dynamics Simulation on the Binding of NNRTI to HIV-1 RT. *Biophys J* **84**:3547-63.

Sluis-Cremer N, Arion D, Abram ME and Parniak MA (2004) Proteolytic processing of an HIV-1 pol polyprotein precursor: insights into the mechanism of reverse transcriptase p66/p51 heterodimer formation. *Int J Biochem Cell Biol* **36**:1836-47.

Sluis-Cremer N, Arion D, Kaushik N, Lim H and Parniak MA (2000a) Mutational analysis of Lys65 of HIV-1 reverse transcriptase. *Biochem J* **348**:77-82.

Sluis-Cremer N, Dmitrienko GI, Balzarini J, Camarasa MJ and Parniak MA (2000b) Human immunodeficiency virus type 1 reverse transcriptase dimer destabilization by 1-[Spiro[4"-amino-2",2" -dioxo-1",2" -oxathiole-5",3'-[2', 5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]]]-3-ethylthy mine. *Biochemistry* **39**:1427-33.

Smerdon SJ, Jager J, Wang J, Kohlstaedt LA, Chirino AJ, Friedman JM, Rice PA and Steitz TA (1994) Structure of the binding site for nonnucleoside inhibitors of the reverse transcriptase of human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **91**:3911-5.

Spence RA, Kati WM, Anderson KS and Johnson KA (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* **267**:988-93.

Squires KE (2001) An introduction to nucleoside and nucleotide analogues. *Antivir Ther* **6 Suppl 3**:1-14.

St Clair MH, Martin JL, Tudor-Williams G, Bach MC, Vavro CL, King DM, Kellam P, Kemp SD and Larder BA (1991) Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. *Science* **253**:1557-9.

Stammers DK, Somers DO, Ross CK, Kirby I, Ray PH, Wilson JE, Norman M, Ren JS, Esnouf RM, Garman EF and et al. (1994) Crystals of HIV-1 reverse transcriptase diffracting to 2.2 A resolution. *J Mol Biol* **242**:586-8.

Starcich BR, Hahn BH, Shaw GM, McNeely PD, Modrow S, Wolf H, Parks ES, Parks WP, Josephs SF, Gallo RC and et al. (1986) Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. *Cell* **45**:637-48.

Stark LA and Hay RT (1998) Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) interacts with Lys-tRNA synthetase: implications for priming of HIV-1 reverse transcription. *J Virol* **72**:3037-44.

Starnes MC, Gao WY, Ting RY and Cheng YC (1988) Enzyme activity gel analysis of human immunodeficiency virus reverse transcriptase. *J Biol Chem* **263**:5132-4.

Stopak K, de Noronha C, Yonemoto W and Greene WC (2003) HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. *Mol Cell* **12**:591-601.

Tachedjian G, Aronson HE, de los Santos M, Seehra J, McCoy JM and Goff SP (2003) Role of residues in the tryptophan repeat motif for HIV-1 reverse transcriptase dimerization. *J Mol Biol* **326**:381-96.

Tachedjian G, Hooker DJ, Gurusinghe AD, Bazmi H, Deacon NJ, Mellors J, Birch C and Mills J (1995) Characterisation of foscarnet-resistant strains of human immunodeficiency virus type 1. *Virology* **212**:58-68.

Tachedjian G, Mellors J, Bazmi H, Birch C and Mills J (1996) Zidovudine resistance is suppressed by mutations conferring resistance of human immunodeficiency virus type 1 to foscarnet. *J Virol* **70**:7171-81.

Tantillo C, Ding J, Jacobo-Molina A, Nanni RG, Boyer PL, Hughes SH, Pauwels R, Andries K, Janssen PA and Arnold E (1994) Locations of anti-AIDS drug binding sites and resistance mutations in the three-

dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. *J Mol Biol* **243**:369-87.

Temin HM (1993) Retrovirus variation and reverse transcription: abnormal strand transfers result in retrovirus genetic variation. *Proc Natl Acad Sci U S A* **90**:6900-3.

Thrall SH, Reinstein J, Wohrl BM and Goody RS (1996) Evaluation of human immunodeficiency virus type 1 reverse transcriptase primer tRNA binding by fluorescence spectroscopy: specificity and comparison to primer/template binding. *Biochemistry* **35**:4609-18.

Tisdale M, Alnadaf T and Cousens D (1997) Combination of mutations in human immunodeficiency virus type 1 reverse transcriptase required for resistance to the carbocyclic nucleoside 1592U89. *Antimicrob Agents Chemother* **41**:1094-8.

Tisdale M, Kemp SD, Parry NR and Larder BA (1993) Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3'-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc Natl Acad Sci U S A* **90**:5653-6.

Tong W, Lu CD, Sharma SK, Matsuura S, So AG and Scott WA (1997) Nucleotide-induced stable complex formation by HIV-1 reverse transcriptase. *Biochemistry* **36**:5749-57.

Trono D (1992) Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses. *J Virol* **66**:4893-900.

Ulich C, Dunne A, Parry E, Hooker CW, Gaynor RB and Harrich D (1999) Functional domains of Tat required for efficient human immunodeficiency virus type 1 reverse transcription. *J Virol* **73**:2499-508.

UNAIDS 2004 Report on the Global AIDS epedemic 2004

Van Laethem K, Witvrouw M, Balzarini J, Schmit JC, Sprecher S, Hermans P, Leal M, Harrer T, Ruiz L, Clotet B, Van Ranst M, Desmyter J, De Clercq E and Vandamme AM (2000) Patient HIV-1 strains carrying the multiple nucleoside resistance mutations are cross-resistant to abacavir. *Aids* **14**:469-71.

van Wamel JL and Berkhout B (1998) The first strand transfer during HIV-1 reverse transcription can occur either intramolecularly or intermolecularly. *Virology* **244**:245-51.

Vartanian JP, Meyerhans A, Asjo B and Wain-Hobson S (1991) Selection, recombination, and G----A hypermutation of human immunodeficiency virus type 1 genomes. *J Virol* **65**:1779-88.

Vartanian JP, Meyerhans A, Sala M and Wain-Hobson S (1994) G-->A hypermutation of the human immunodeficiency virus type 1 genome: evidence for dCTP pool imbalance during reverse transcription. *Proc Natl Acad Sci U S A* **91**:3092-6.

Vodicka MA (2001) Determinants for lentiviral infection of non-dividing cells. *Somat Cell Mol Genet* **26**:35-49.

von Schwedler U, Song J, Aiken C and Trono D (1993) Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *J Virol* **67**:4945-55.

Wainberg MA, Miller MD, Quan Y, Salomon H, Mulato AS, Lamy PD, Margot NA, Anton KE and Cherrington JM (1999) In vitro selection and characterization of HIV-1 with reduced susceptibility to PMPA. *Antivir Ther* **4**:87-94.

Wain-Hobson S (1993) The fastest genome evolution ever described: HIV variation in situ. *Curr Opin Genet Dev* **3**:878-83.

Wang J, Smerdon SJ, Jager J, Kohlstaedt LA, Rice PA, Friedman JM and Steitz TA (1994) Structural basis of asymmetry in the human immunodeficiency virus type 1 reverse transcriptase heterodimer. *Proc Natl Acad Sci U S A* **91**:7242-6.

Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH and et al. (1995) Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**:117-22.

Weiss KK, Chen R, Skasko M, Reynolds HM, Lee K, Bambara RA, Mansky LM and Kim B (2004) A role for dNTP binding of human immunodeficiency virus type 1 reverse transcriptase in viral mutagenesis. *Biochemistry* **43**:4490-500.

Weiss KK, Isaacs SJ, Tran NH, Adman ET and Kim B (2000) Molecular architecture of the mutagenic active site of human immunodeficiency virus type 1 reverse transcriptase: roles of the beta 8-alpha E loop in fidelity, processivity, and substrate interactions. *Biochemistry* **39**:10684-94.

Whitcomb JM, Parkin NT, Chappey C, Hellmann NS and Petropoulos CJ (2003) Broad nucleoside reverse-transcriptase inhibitor cross-resistance in human immunodeficiency virus type 1 clinical isolates. *J Infect Dis* **188**:992-1000. Epub 2003 Sep 16.

White KL, Chen JM, Margot NA, Wrin T, Petropoulos CJ, Naeger LK, Swaminathan S and Miller MD (2004) Molecular mechanisms of tenofovir resistance conferred by human immunodeficiency virus type 1 reverse transcriptase containing a diserine insertion after residue 69 and multiple thymidine analog-associated mutations. *Antimicrob Agents Chemother* **48**:992-1003.

White KL, Margot NA, Wrin T, Petropoulos CJ, Miller MD and Naeger LK (2002) Molecular mechanisms of resistance to human immunodeficiency virus type 1 with reverse transcriptase mutations K65R and K65R+M184V and their effects on enzyme function and viral replication capacity. *Antimicrob Agents Chemother* **46**:3437-46.

Wilson JE, Aulabaugh A, Caligan B, McPherson S, Wakefield JK, Jablonski S, Morrow CD, Reardon JE and Furman PA (1996) Human immunodeficiency virus type-1 reverse transcriptase. Contribution of Met-184 to binding of nucleoside 5'-triphosphate. *J Biol Chem* **271**:13656-62.

Winters MA, Coolley KL, Cheng P, Girard YA, Hamdan H, Kovari LC and Merigan TC (2000) Genotypic, phenotypic, and modeling studies of a deletion in the beta3-beta4 region of the human immunodeficiency virus type 1 reverse transcriptase gene that is associated with resistance to nucleoside reverse transcriptase inhibitors. *J Virol* **74**:10707-13.

Winters MA, Coolley KL, Girard YA, Levee DJ, Hamdan H, Shafer RW, Katzenstein DA and Merigan TC (1998) A 6-basepair insert in the reverse transcriptase gene of human immunodeficiency virus type 1 confers resistance to multiple nucleoside inhibitors. *J Clin Invest* **102**:1769-75.

Winters MA, Shafer RW, Jellinger RA, Mamtora G, Gingeras T and Merigan TC (1997) Human immunodeficiency virus type 1 reverse transcriptase genotype and drug susceptibility changes in infected individuals receiving dideoxyinosine monotherapy for 1 to 2 years. *Antimicrob Agents Chemother* **41**:757-62.

Yang G, Song Q, Charles M, Drosopoulos WC, Arnold E and Prasad VR (1996) Use of chimeric human immunodeficiency virus types 1 and 2 reverse transcriptases for structure-function analysis and for mapping susceptibility to nonnucleoside inhibitors. *J Acquir Immune Defic Syndr Hum Retrovirol* **11**:326-33.

Yu H and Goodman MF (1992) Comparison of HIV-1 and avian myeloblastosis virus reverse transcriptase fidelity on RNA and DNA templates. *J Biol Chem* **267**:10888-96.

Zakharova OD, Tarrago-Litvak L, Fournier M, Litvak S and Nevinsky GA (1995) DNA synthesis primed by mononucleotides (de novo synthesis) catalyzed by HIV-1 reverse transcriptase: tRNA(Lys,3) activation. *FEBS Lett* **373**:255-8.

Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L and Charneau P (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101**:173-85.

CHAPTER II

THE FUNCTIONAL ROLE OF THE p51 SUBUNIT IN RECOMBINANT HYBRID HIV-1 AND FIV REVERSE TRANSCRIPTASES

The results presented in this chapter have been published in the following article:

Auwerx, J., North, T. W., Preston, B. D., Klarmann, G. J., De Clercq, E. and Balzarini, J. (2002) Chimeric human immunodeficiency virus type 1 and feline immunodeficiency virus reverse transcriptases: Role of the subunits in resistance/sensitivity to non-nucleosides reverse transcriptase inhibitors. *Mol. Pharmacol.* **61,** 400-406.

SUMMARY

NNRTIs are specific for HIV-1 RT and do not inhibit HIV-2. Given the higher similarity of amino acids lining the NNRTI-specific pocket of HIV-1 RT with the corresponding FIV RT amino acids than with those of HIV-2 RT, the susceptibility of FIV RT and hybrid HIV-1/FIV RTs to NNRTIs and the role of the p51 subunit in the inhibitory action of NNRTIs were investigated. We found that the wild-type FIV RT and the FIVp66/HIVp51 hybrid enzyme showed no susceptibility for NNRTIs. On the other hand, the hybrid HIVp66/FIVp51 RT retained a similar sensitivity spectrum for NNRTIs as the wild-type HIV-1 RT. The non-competitive nature of inhibition of HIV-1 RT by nevirapine was also observed with the HIVp66/FIVp51 hybrid enzyme. Inhibition of the hybrid RTs by NRTIs and PFA was in the same range as observed for the corresponding HIVp66/HIVp51 and FIVp66/FIVp51 wild-type enzymes. The hybrid RTs had a comparable affinity (K_m) for their dNTP substrate and template/primer as the wild-type HIV-1 and FIV RTs, but their catalytic efficacy (k_{cat}) was markedly decreased whereas the decreased catalytic efficacy of the RT hybrids may suggest suboptimal interactions between p66 and p51 in the hybrid enzymes and thus point to an instrumental role of p51 in the functional integrity of RT. But the p51 subunit seems to play a minor role in the (in)sensitivity of HIV (and FIV) RT.

INTRODUCTION

The heterodimeric form is the most stable and active form of the RT enzyme (Lightfoote *et al.*, 1986; Lowe *et al.*, 1988). Sequence comparisons between HIV-1 RT and FIV RT revealed 63% identity at the nucleotide level and 48% identity and 67% similarity at the amino acid level (Amacker *et al.*, 1995). The p66 subunit forms a DNA binding cleft with the active site residues and encodes both the polymerase and RNase H activity of the enzyme, whereas the p51 subunit is catalytically inactive (Boyer *et al.*, 1992; Cheng *et al.*, 1991; Hostomsky *et al.*, 1992; Le Grice *et al.*, 1991). The role of p51 is still uncertain and several possible functions have been suggested: (i) a role in processivity of the p66 subunit (Huang *et al.*, 1992), (ii) involvement in tRNA primer binding (Dufour *et al.*, 1998; Mishima and Steitz, 1995), (iii) loading of the p66 subunit onto the template primer (Harris *et al.*, 1998), (iv) enhancement of the strand displacement DNA synthesis (Amacker *et al.*, 1995; Hottiger *et al.*, 1994) and (v) a role in induction and maintenance of an optimal structural conformation (Tasara *et al.*, 1999).

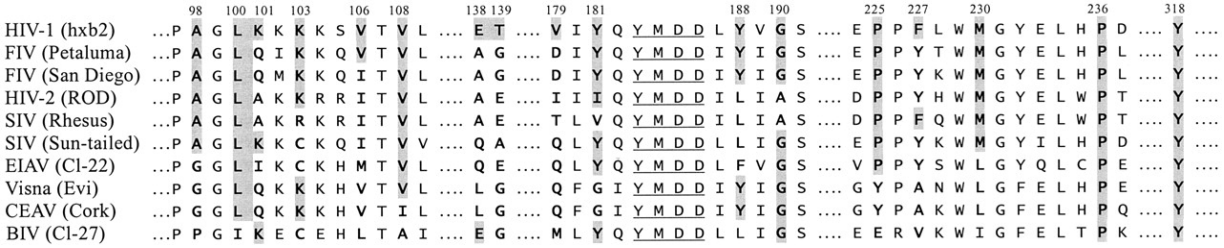

Figure 2.1 Alignment of important amino acid stretches in the NNRTI-binding pocket of HIV-1 compared with the corresponding amino acids in other lentiviral RTs.

The amino acids that are instrumental for susceptibility of the RT to NNRTIs are indicated in bold and shaded. The underlined sequence is conserved between the lentiviral RTs and includes the D185 and D186 amino acid residues critical for polymerase activity.

Although very similar to HIV-1 RT, HIV-2 RT shows no susceptibility to NNRTIs. However, when an alignment was made for the primary amino acid sequences of the NNRTI-specific pocket of HIV-1 RT with the corresponding amino acids in HIV-2 RT and FIV RT, a much higher sequence similarity was found for FIV RT than for HIV-2 RT (Fig. 2.1). Hybrid enzymes involving HIV-1 RT and other lentiviral RTs have been constructed by other groups contributing to a further understanding of the function of the individual subunits within the HIV-1 RT heterodimer, the mapping of the catalytic sites and the NNRTI binding pocket. In this perspective, the construction of HIV-1/HIV-2 (Howard *et al.*, 1991; Shih *et al.*, 1991; Yang *et al.*, 1996), SIV/HIV-1 (Isaka *et al.*, 1998), HIV-1/MuLV RT hybrids (Hizi *et al.*, 1993; Misra *et al.*, 1998) have already been reported. The hybrid FIV/HIV-1 made by Amacker and Hubscher (1998) had the purpose to investigate the role of the p51 subunit in the heterodimer. The HIVp66/FIVp51 hybrid in their study was found to be resistant to AZTTP and nevirapine. FIVp66/HIVp51 RT and even the wild-type FIV RT were reported to be susceptible to the inhibitory activity of nevirapine. In order to investigate the NNRTI-sensitivity to FIV RT in more detail and to gain further insights in the potential role of the p51 subunit in the sensitivity to and/or inhibition by NNRTIs we constructed, besides wild-type FIVp66/FIVp51 RT, also stable and functionally active hybrid HIVp66/FIVp51 and FIVp66/HIVp51 RTs and investigated their sensitivity spectrum in the presence of a variety of different classes of NNRTIs.

We found in our kinetic studies that the hybrid RT enzymes had a comparable affinity towards their dNTP substrate and template as wild-type HIV-1 and FIV RTs, but their catalytic efficacy was markedly decreased. Inhibition by nevirapine or any other NNRTI was neither observed for wild-type FIV RT nor for the hybrid FIVp66/HIVp51 RT. Instead, the

hybrid HIVp66/FIVp51 RT retained marked sensitivity to the inhibitory effects of all NNRTIs investigated. Inhibition of the hybrid RTs by NRTIs was in the same range as observed for the HIV-1 RTs and FIV RTs.

MATERIALS AND METHODS

Test 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) was obtained from Dr. M.-J. Camarasa (Consejo Superior de Investigaciones Científicas, Madrid, Spain). Nevirapine (BI-RG-587; dipyridodiazepinone) was kindly provided by Boehringer (Ingelheim, Ridgefield, CT). Delavirdine (BHAP [bis(heteroaryl)piperazine] U-90152), efavirenz (DMP 266) and capravirine were provided by Dr. R. Kirch (Hoechst AG, Frankfurt, Germany) and Dr. J.-P. Kleim (GlaxoSmithKline, Stevenage, UK). Emivirine (MKC-442) was kindly provided by Dr. Ph. A. Furman (Triangle Pharmaceuticals, NC). The thiocarboxanilide derivative UC-781 was obtained from Uniroyal Chemical Ltd. (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) and foscarnet (PFA) were obtained from Sigma Chemical Ltd. (St. Louis, MO).

Cloning of p66 and p51 subunits.

The complete FIV RT coding sequence of the Petaluma isolate was ligated into the *Eco*RI-*Pst*I digested expression vector pKK223-3 with inducible *tac* promoter (Amersham Biosciences, Roosendaal, the Netherlands) creating the pFIV66-WT. An analogous construct, pFIV51-WT, was created for expression of the p51 FIV RT subunit. HIV-1 RT was expressed by the pKRT2 expression vector (D'Aquila and Summers, 1989) under the control of the *trc* promoter. The p51 subunit of HIV-1 RT was expressed by pKRT51, like pKRT2, based on pKK233-2 (Amersham Biosciences). For further purification of the HIV-1 and FIV RT enzymes we used the glutathione-S-transferase (GST) fusion system. To make sure that the p51 subunit of the hybrid RTs was not derived from eventual bacterial proteolysis we purified the enzymes by a GST-tag on the p51 amino terminus. Therefore, we cloned the FIV p51 and HIV-1 p51 RT sequences into pGEX 4T-1 (Amersham Biosciences). The p51 RT sequences were amplified from pFIV51-WT and pKRT51, respectively, by means of PCR with *Pfu*

polymerase. The primers used for PCR contained add-on sequences for restriction endonuclease sites. The 5' primer contained an *Eco*RI restriction site and the 3' primer a *Not*I restriction site. The desired fragment was digested with *Eco*RI and *Not*I and then purified using QIAquick gel purification kit (Qiagen, Westburg, Leusden, the Netherlands). This fragment was ligated into the *Eco*RI–*Not*I digested pGEX 4T-1 vector creating pGEX51F and pGEX51H, which express respectively the FIV p51 subunit and the HIV-1 p51 subunit together with the GST fusion protein at the amino terminus.

Recombinant FIV RT enzymes were expressed from a two-plasmid co-expression system. The *Sal*I–*Dra*I portion of pFIV66-WT was subcloned into the *Sal*I–*Sma*I digested pREP4 (Qiagen) for the construction of pREP66. This plasmid is compatible with pGEX51H and pGEX51F in *Escherichia coli* and contains the kanamycine resistance gene. HIV-1 RT was expressed from the two-plasmid co-expression system as described by (Jonckheere *et al.*, 1996). The p66 subunit was subcloned into pACYC184 containing the p15A *ori* (Chang and Cohen, 1978) and a tetracycline resistance gene (pACYC66). The hybrids were formed by different plasmid combinations. In this way, we constructed, besides the wild-type expression systems, a pREP66-pGEX51H and pACYC66-pGEX51F system expressing, respectively, FIVp66/HIVp51 and HIVp66/FIVp51.

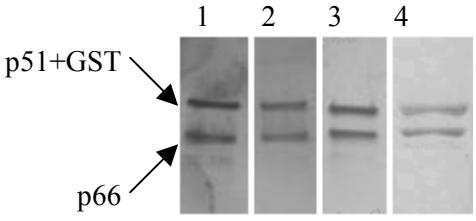

Figure 2.2 Purification of hybrid and wild-type RT heterodimers. A silverstained 12% SDS-polyacrylamide gel of the pooled RT fraction is shown.

lane 1: HIVp66/FIVp51; lane 2, FIVp66/HIVp51; lane 3, FIVp66/FIVp51; lane 4, HIVp66/HIVp51 RT heterodimer.

Expression and purification of reverse transcriptase enzymes.

Luria Broth medium (800 ml) containing the appropriate antibiotics were inoculated with an overnight culture of *E. coli* JM109 transformed with both plasmids of the expression system. The culture was started at an A_{600} of 0.1 and incubated at 37 °C with vigorous shaking. Expression of recombinant RT was induced by adding isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 4 h, the cells were harvested, washed, and kept frozen overnight at –20 °C. Cell lysis was accomplished by

mechanical lysis in the French Pressure® Cell Press (Sim Aminco). The cell paste was resuspended in 15 ml of lysis buffer (50 mM sodium phosphate buffer pH 7.8, 100 mM NaCl, 5 mM β-mercaptoethanol, 0,9% glucose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml pepstatin A, 10 μg/ml leupeptin and 10% glycerol) and subsequently placed in the French Press unit, which was kept at 4 °C. After lysis, the cell lysate was centrifuged for 25 min at 13,000 g. The supernatant was incubated with 1 ml of pre-equilibrated glutathione-S-sepharose beads (Amersham Biosciences) at 4 °C while rotating for at least 1 h. After incubation the beads were washed three times with 20 ml buffer (50 mM sodium phosphate buffer pH 7.8, 0.5 mM NaCl, 5 mM β-mercaptoethanol and 10% glycerol). The RT was eluted from the beads by bulk incubation with elution buffer [containing 20 mM reduced glutathione (Sigma Chemical Ltd.)] at 4 °C, while rotating for 15 min. The beads were recovered by centrifugation at 500 g and the supernatant was collected. This elution procedure was repeated at least 4 times. The elution fractions were pooled and afterwards analysed by SDS PAGE. The pooled sample was concentrated to ~2 ml volume and the elution buffer was exchanged by Hep A buffer (20 mM Tris-HCl, pH 7.8, 0.05 M NaCl, 1 mM EDTA, 1 mM dithiotreitol (DTT) and 10% glycerol) to remove high concentrations of reduced glutathione using the Vivaspin 15 centrifugal filtration devices (Vivascience, Van der Heyden, Brussels, Belgium). The protein was further FPLC-purified to about 98% purity over a Hitrap Heparin column (Amersham Biosciences). After the binding of the RT to the heparin column, elution was accomplished by a linear salt gradient of 0.05–1 M NaCl. Heterodimer RT eluted at approximately 0.3 M NaCl, as determined by SDS PAGE. All fractions with the same relative amounts of the p51 and p66 subunits were pooled (Fig. 2.2) and stored in buffer containing 0.3 M NaCl and 25% glycerol at –20 °C. Protein concentrations in the stock solutions were determined with the Pierce Protein Assay (Polylab, Antwerp, Belgium), using bovine serum albumin as a standard.

Preparation of *E. coli* lysates

Luria Broth medium (25 ml) containing the appropriate antibiotics were inoculated with an overnight culture of *E. coli* JM109 transformed with both plasmids of the expression system at an A_{600} of 0.1. The culture was grown at 37 °C, induced with IPTG and stored as described in the previous section. The cell pellet was resuspended in 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 7.8, 2 mM EDTA, 5 mM DTT, 1 mM PMSF, 0.1% Triton X-100, 1 mg/ml lysozyme and 10% glycerol) and sonicated for 5 to 10 min. The lysate was centrifuged (13,000 g, 20 min) and the supernatant was stored at –80 °C in aliquots of 80 μl.

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), 0.06% Triton X100, 5 mM DTT, 0.3 mM glutathione, 150 mM KCl, 5 mM $MgCl_2$, 1.25 mg/ml BSA, 0.5 mM EDTA, 0.1 mM template/primer poly(rC).oligo$(dG)_{12-18}$ (Amersham Biosciences), a fixed concentration of the labelled substrate [8-^{3}H]dGTP (1.6 µM, 1 µCi; specific activity, 12.6 Ci/mmol; Amersham Biosciences), 5 µl of inhibitor solution [containing various concentrations (10-fold dilutions) of the compounds], and 5 µl of the RT preparations. The reaction mixtures were incubated at 37°C for 30 min, and the reaction was terminated by adding 200 µl of yeast RNA (2 mg/ml) and 1 ml of trichloroacetic acid (TCA) (5% in 20 mM $Na_4P_2O_7$). The solutions were kept on ice for at least 30 min, after which the acid-insoluble material was precipitated on Whatman GF/C glass-fibre filters and washed with 20 ml TCA (5% in water) and 2 ml ethanol. The amount of incorporated radioactive substrate was analyzed 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%.

To assess the processivity of hybrid HIVp66/FIVp51 RT compared to wild-type HIVp66/HIVp51 RT the enzyme activity was determined at different time points. The amount of dGTP incorporated by a fixed concentration of RT enzyme (8 ng HIVp66/HIVp51 and 670 ng HIVp66/FIVp51 RT) was examined at a variety of different incubation periods ranging from 15 min to 150 min.

Determination of the relative specific activity of hybrid HIVp66/FIVp51 RT and wild-type HIVp66/HIVp51 RT was also performed by measuring the RT activity in a 30 min RT assay as described above, using various enzyme concentrations ranging from 3.2 ng to 10.7 µg in the enzyme assays (50 µl).

Steady-state kinetic assays were 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 dTTP) or template/primer [poly(rC).oligo(dG_{12-18}) or poly(rA).oligo(dT_{12-18})]. Under these experimental conditions the catalytic reaction of the different enzymes proceeded linearly and proportionally with time. The K_m and k_{cat} values for poly(rC).oligo(dG_{12-18}) and dGTP were determined in the presence of 1.4 µM (1 µCi) [8-^{3}H] dGTP (specific radioactivity 14.1 Ci/mmol) and 0.1 mM poly(rC).oligo(dG_{12-18}), respectively. The K_m and V_{max} (k_{cat}) values were derived from the double reciprocal Lineweaver-Burk plots of the concentrations of the variable substrate (dGTP) or template/primer

[poly(rC).oligo(dG_{12-18})] versus the velocities of dGTP incorporation at each substrate or template/primer concentration. In the assays using [^{3}H]dTTP as the labeled substrate and poly(rA).oligo(dT_{12-18}) as the template/primer, the K_m and k_{cat} values for dTTP were determined in the presence of 0.15 mM poly(rA).oligo(dT_{12-18}).

To determine the K_i value of nevirapine ($K_{i,nev}$) and the kinetic mechanism (competitive/non-competitive) of wild-type HIV-1, FIV and the hybrid RTs, the assays (using [8-^{3}H]dGTP and poly(rC).oligo(dG_{12-18})) were performed in the presence of different concentrations of nevirapine ranging from 1.5 to 7.5 μM, respectively.

RESULTS

2.1. Kinetic analysis of the wild-type (HIVp66/HIVp51 and FIVp66/FIVp51) and hybrid (HIVp66/FIVp51 and FIVp66/HIVp51) RTs

Kinetic analysis of the reverse transcriptases was performed with both the substrates (i.e. dGTP or dTTP) and the template/primer (i.e. poly(rC).oligo(dC)) as variables. The kinetic parameters for the different RT enzymes with dGTP and dTTP as the variable substrate are summarized in Table 2.1. The K_m value of the HIVp66/FIVp51 hybrid for dGTP was 3.2-fold higher than the K_m value for wild-type HIV-1 RT. The K_m values of wild-type FIV RT, and the hybrid FIVp66/HIVp51 RT were similar as observed for wild-type HIV-1 RT. The k_{cat} values of the wild-type HIV-1 and FIV RT enzymes were about 1 pmol/μg protein/s. In contrast, the k_{cat} values for the two hybrids were substantially lower (23- to 30-fold) than for the wild-type RT enzymes, indicating that the hybrids allow fewer substrate molecule incorporations per unit time than the wild-type RTs. The lower k_{cat} values of FIVp66/HIVp51 and HIVp66/FIVp51 combined with the slightly higher K_m values resulted in a catalytic efficiency (k_{cat}/K_m) that was only 2.3% and 1.2%, respectively of the catalytic efficiency of the wild-type RTs.

To confirm these results found for dGTP as the variable substrate we determined also the K_m values of wild-type HIV-1 RT and hybrid HIVp66/FIVp51 RT with dTTP as variable substrate, finding K_m values of 8.5 μM and 7.9 μM, respectively. These values were comparable to those found for hybrid HIVp66/FIVp51 (8.6 μM) with dGTP as the variable substrate. In this assay we found a k_{cat} value for the hybrid HIVp66/FIVp51 that was, like in the assay with dGTP as the variable substrate, also considerably lower than the k_{cat} value found for wild-type HIV-1 RT. This results in a catalytic efficiency of HIVp66/FIVp51 RT that is approximately 100 fold less than the wild-type catalytic RT activity.

The kinetic parameters of the different RT enzymes with poly(rC).oligo(dG) as the variable template/primer are summarized in Table 2.1. Wild-type HIV-1 RT had a K_m value of 2.9 µM for poly(rC).oligo(dG). The K_m value of the hybrid HIVp66/FIVp51 RT containing the HIV-1 p66 subunit was ~3.5 times higher. The K_m value of FIVp66/HIVp51 RT subunit was 4.2 µM, and, thus, ~2-fold lower than that of wild-type FIV RT. Thus, the k_{cat} values of the RT hybrids are both in the same range and are 10 to 60 times lower than the k_{cat} values of the corresponding wild-type RTs. This might indicate that the bound template/primer is not in an optimal position to allow efficient catalysis probably due to subtle differences in dimerisation of both subunits.

Table 2.1 Kinetic analysis of HIV-1 and FIV wild-type and hybrid RT enzymes

Variable substrate or template/primer	**Kinetic parameter**	**Reverse transcriptase**			
		HIVp66/HIVp51	**HIVp66/FIVp51**	**FIVp66/FIVp51**	**FIVp66/HIVp51**
dGTP[c]	K_m [a]	2.7 ± 0.7	8.6 ± 1.2	3.1 ± 0.6	4.2 ± 0.2
	k_{cat} [b]	1.0 ± 0.2	0.043 ± 0.009	1.1 ± 0.1	0.036 ± 0.001
	k_{cat}/K_m	0.40	0.005	0.36	0.008
	$K_{i,nev}$	0.52	0.47	>50	>50
poly(rC).oligo(dG)[d]	K_m	2.9 ± 0.5	10 ± 1.9	8.8 ± 1.7	4.2 ± 0.4
	k_{cat}	3.4 ± 0.7	0.05 ± 0.02	0.76 ± 0.03	0.06 ± 0.002
	k_{cat}/K_m	1.2	0.005	0.086	0.015
	$K_{i,nev}$	0.55	1.2	>50	>50
dTTP[e]	K_m	8.5	7.9	-	-
	k_{cat}	1.1	0.01	-	-
	k_{cat}/K_m	0.14	0.01	-	-

[a] K_m and $K_{i,,nev}$ are in µM; [b] k_{cat} is in pmol/µg protein/s; [c] poly(rC).oligo(dG) as template/primer ; [d] dGTP (1 µCi) as substrate; [e] poly(rA).oligo(dT) as template/primer. The data are means of at least two to three independent experiments (means ± standard deviations).

2.2. Processivity and relative activity of wild-type HIVp66/HIVp51 RT and hybrid HIVp66/FIVp51 RT

In order to understand the low catalytic efficiency of the hybrid enzymes, in particular the NNRTI-sensitive hybrid HIVp66/FIVp51 RT, we investigated the processivity of these enzymes over a broad incubation time period (Fig. 2.3). We observed a linear progression of

the reaction for wild-type HIV-1 RT up to 2 hours after initiation of the reaction. In contrast the hybrid HIVp66/FIVp51 RT did not proceed linearly anymore after ±45 min incubation. To obtain a comparable incorporation of [^{3}H]dGTP in these reactions, 80-fold more hybrid HIVp66/FIVp51 RT than wild-type HIVp66/HIVp51 RT was required.

We also determined the relative specific enzyme activity over a broad range of RT concentrations to assess the linearity of the reaction in the presence of these enzyme concentrations (Fig. 2.4). We observed for HIVp66/HIVp51 RT a linear [^{3}H]dGTP incorporation up to ~1000 ng enzyme. Higher enzyme concentrations likely caused an extensive consumption of template/primer or substrate in the reaction mixture leading to staggering of the enzyme reaction. For hybrid HIVp66/FIVp51 RT the enzyme activity is much lower than in the case of HIVp66/HIVp51 RT, and resulted in a linear incorporation of [^{3}H]dGTP in function of all enzyme concentrations (as low as 3.2 ng) tested. Thus, there was no indication that there occurred reduced subunit association at the lowest enzyme concentrations that may have resulted in substantial amounts of potential p66/p66 homodimer formation (and concomitantly lower enzyme activity).

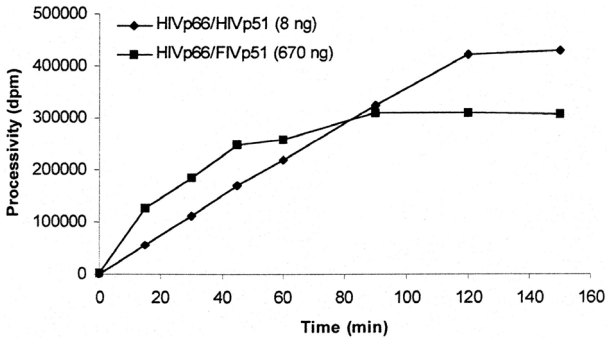

Figure 2.3 Relative processivity of HIVp66/HIVp51 RT and HIVp66/FIVp51 RT in function of incubation time

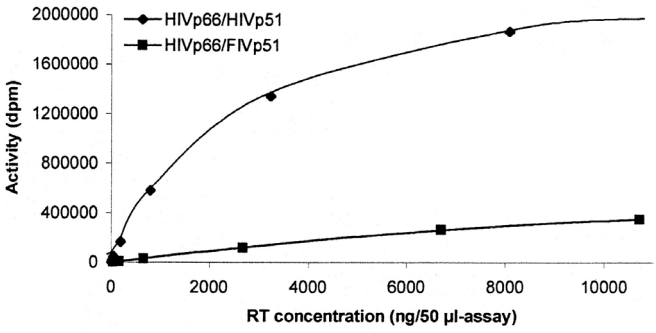

Figure 2.4 Relative specific activities of HIVp66/FIVp51 and HIVp66/FIVp51 RTs in function of different enzyme concentrations

2.3. Inhibitory activities of NNRTIs, ddGTP and PFA against wild-type (HIVp66/HIVp51 and FIVp66/FIVp51) and hybrid (HIVp66/FIVp51 and FIVp66/HIVp51) RTs

The wild-type HIV-1 and FIV RTs and the two RT hybrids were evaluated for their sensitivities to the inhibitory activity of a variety of NNRTIs, ddGTP and PFA (Table 2.2).

HIV-1 RT showed a pronounced sensitivity to the inhibitory effect of a variety of NNRTIs. The extent of inhibition was dependent on the nature of the NNRTIs, the quinoxaline GW867420 and thiocarboxanilide UC-781 representing the most potent inhibitors of HIV-1 RT. Whereas HIVp66/FIVp51 RT showed a marginal decrease in sensitivity to capravirine (4.6-fold) and TSAO-m^3T (3.6-fold) compared to wild-type HIV-1 RT, all the other NNRTIs including nevirapine, delavirdine, efavirenz, emivirine, the quinoxaline GW420867X and the thiocarboxanilide UC-781 showed similar inhibitory activity against this hybrid enzyme, as compared to wild-type RT. Also, the NRTI ddGTP and PFA had comparable inhibitory effects on both enzymes. Thus, overall, there was a close correlation between the inhibitory effects of NNRTIs, ddGTP and PFA on the HIVp66/HIVp51 and HIVp66/FIVp51 RT enzymes, containing the HIV-1 p66 subunit in common. This was shown in a linear regression plot with the IC_{50} values of the different NNRTIs, ddGTP and PFA for the wild-type HIVp66/HIVp51 RT and the hybrid HIVp66/FIVp51 RT (Fig. 2.5). The correlation coefficient (r) was 0.96, which points to a remarkable similarity between the two enzyme constructs with regard to their ddGTP and NNRTI binding sites.

In contrast, the wild-type FIVp66/FIVp51 RT showed full resistance to all NNRTIs that were included in the study that is, at drug concentrations that are at least more than 100 to 10,000-fold higher than reported to efficiently inhibit HIV-1 p66 containing wild-type and hybrid RT enzymes. ddGTP had ~8-fold less inhibitory effect on FIV RT compared to HIV-1 RT. Also FIV RT was ~5-fold less sensitive to PFA, as compared to HIV-1 RT. The hybrid FIVp66/HIVp51 RT also showed full resistance to all NNRTIs studied, as did wild-type FIV RT. The inhibitory values of ddGTP and PFA against FIVp66/HIVp51 were also in the same range as observed for wild-type FIV RT.

2.4. Kinetic analysis of the nature of inhibition of wild-type HIVp66/HIVp51 and hybrid HIVp66/FIVp51 RTs by nevirapine

The K_i value for the NNRTI nevirapine ($K_{i,nev}$) against dGTP or the template/primer is shown in Table 2.1. The $K_{i,nev}$ value with dGTP as the variable substrate was 0.52 μM for wild-type HIV-1 RT and 0.47 μM for hybrid HIVp66/FIVp51 RT. These nearly identical $K_{i,nev}$

values confirm the results found for the determination of the IC_{50} values of nevirapine which were very similar for wild- type HIV-1 and hybrid HIVp66/FIVp51 RT (see above and Table 2.1). If poly(rC).oligo(dG) was used as the variable template, the $K_{i,nev}$ value for wild-type HIV-1 RT was 0.55 µM, and 1.2 µM for HIVp66/FIVp51

Table 2.2 Sensitivity of wild-type HIV-1, FIV and hybrid HIVp66/FIVp51 and FIVp66/HIVp51 RTs to the inhibitory effects of NNRTIs, ddGTP and PFA

	IC_{50} (µM) [a]			
	HIVp66/HIVp51	HIVp66/FIVp51	FIVp66/FIVp51	FIVp66/HIVp51
NNRTIs				
Nevirapine	1.15	1.58	>100	>100
Delavirdine	0.34	0.21	>100	>100
Efavirenz	0.025	0.001	>100	>100
Emivirine	0.13	0.16	>100	>100
Capravirine	0.011	0.051	>100	>100
GW420867X	0.007	0.021	>100	>100
UC-781	0.024	0.018	>100	>100
TSAO-m³T	0.69	2.49	>80	>80
NRTI				
ddGTP	0.073	0.107	0.52	0.56
PFA	28	21	147	147

[a] IC_{50}, or 50% inhibitory concentration required to inhibit the enzyme activity by 50%, using poly(rC).oligo(dG_{12-18}) as the template/primer and [8-^{3}H]dGTP as the radiolabelled substrate.

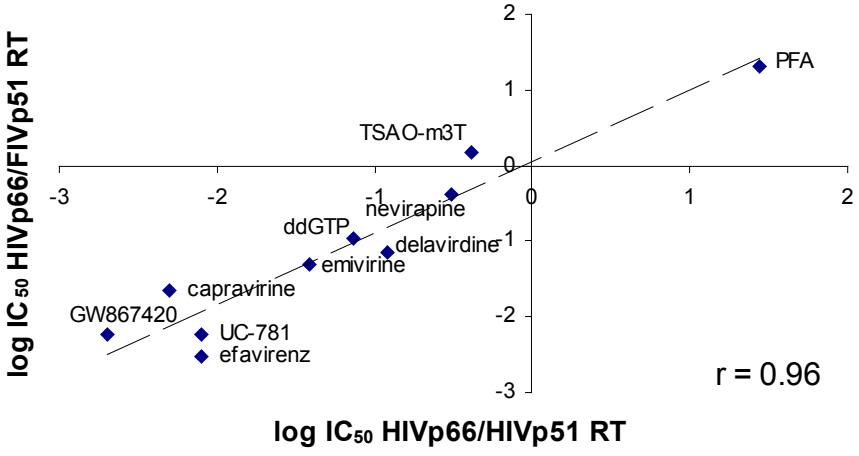

Figure 2.5 Regression analysis of IC_{50} values obtained for wild-type HIVp66/HIVp51 and hybrid HIVp66/FIVp51 RTs.

RT. The $K_{i,nev}$ value for wild-type FIV and FIVp66/HIVp51 RT could not be determined because of full resistance of these enzymes to NNRTIs, including nevirapine (IC_{50}>100 µM).

To investigate the kinetic inhibition mechanism of nevirapine, we analysed the mode of inhibition of both enzymes in the presence of various concentrations of the NNRTI (Fig. 2.6). Double-reciprocal Lineweaver-Burk plots for the inhibition of RT by nevirapine with respect to dGTP as variable substrate, or poly(rC).oligo(dG) as variable template/primer, revealed non-competitive inhibition in all cases indicating a binding of the drug that was independent from the binding of the substrate or template/primer to the RT.

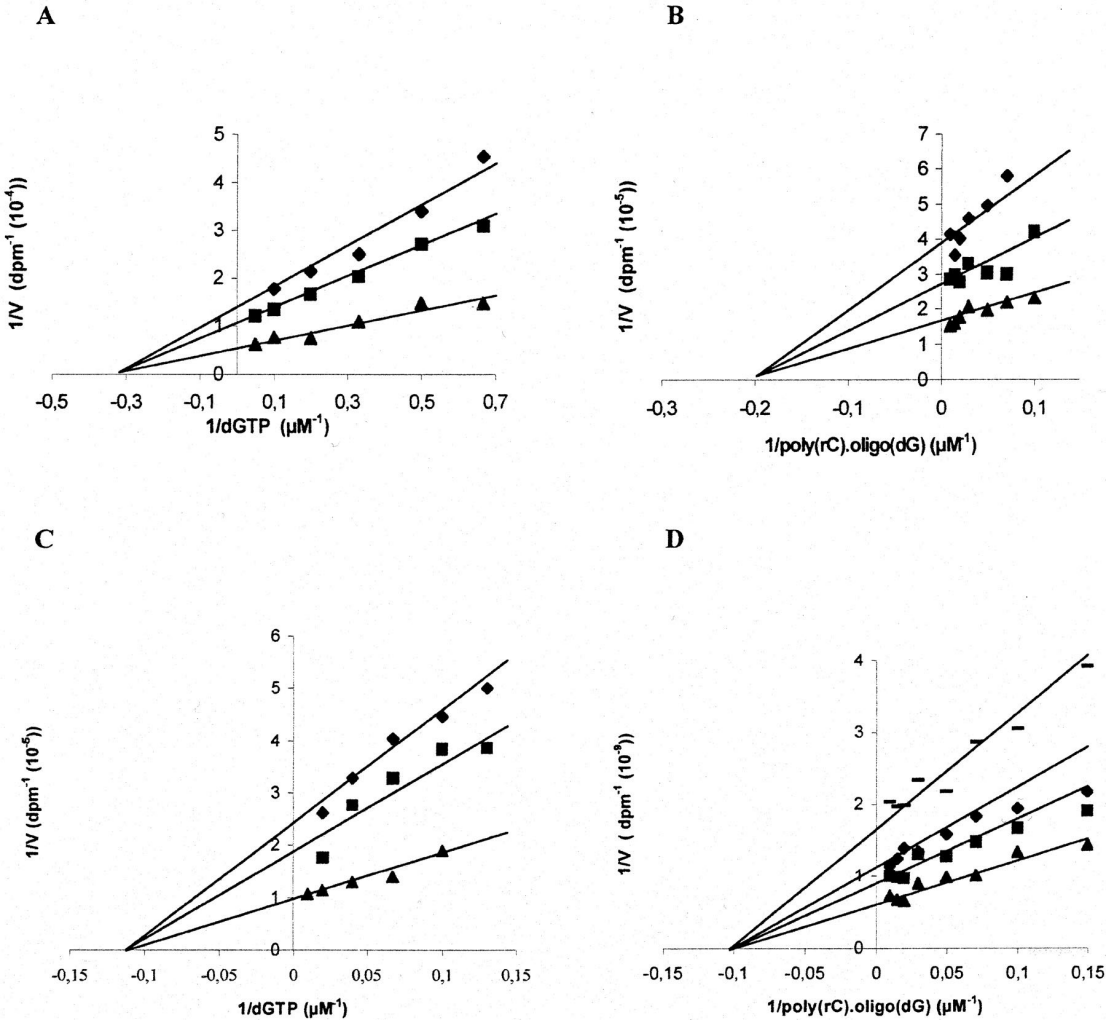

Figure 2.6 Double-reciprocal plots for inhibition of wild-type HIV-1 (A and B) and hybrid HIVp66/FIVp51 (C and D) RT by nevirapine. Nevirapine 7.5 µM [-], 3 µM [♦], 1.5 µM [■] and 0 µM (control) [▲].

(A and C) 0.1 mM of template/primer [poly(rC).oligo(dG)] and variable concentrations of [^{3}H]dGTP were used. (B and D) 1.4 µM of [^{3}H]dGTP and variable concentrations of template/primer [poly(rC).oligo(dG)] were used. (C and D) are performed on RT lysate.

DISCUSSION

In our study we have examined the separate roles of the p66 and p51 subunits in the susceptibility of FIV RT to NNRTIs by constructing hybrid HIV-1/FIV RTs (HIVp66/FIVp51 and FIVp66/HIVp51 RTs), which could be expressed in a co-expression system and purified as stable heterodimers as shown by SDS PAGE (Fig. 2.2). Although we used a GST-tag in our RT purification assays, the presence of this GST-tag did not influence the kinetic properties of the enzymes, and we found similar IC_{50} values for the NNRTIs as found against wild-type HIV-1 RT in the literature.

Since diverse mutations and even single mutations may change the enzymatic activity of RT and may show different local conformational structures (Tantillo *et al.*, 1994), we presume that a replacement of one of both subunits by a homologous subunit of another lentiviral RT can induce important conformational changes. Moreover, it has been suggested that HIV-1 p51 plays a role in the processivity of the p66 subunit (Huang *et al.*, 1992), in loading the HIV-1 p66 subunit onto the template/primer (Amacker and Hubscher, 1998) and a role in the maintenance of an optimal structural enzyme conformation (Tasara *et al.*, 1999). In this perspective, the relatively low catalytic activity and processivity of the hybrids of HIV-1 and FIV RTs can be ascribed to conformational changes and/or suboptimal interaction of p66 and p51 in the hybrid enzymes. Amacker and Hubscher (1998) showed a 2.5-fold increase in RNase H activity compared to the native HIV-1 RT heterodimer. Since significant portions of the p51 helical structure interact with the p66 RNase H domain, these observations suggest a significant alteration of the p51/p66 interactions in the hybrid enzymes. It would therefore be interesting to reveal whether the HIVp66/FIVp51 heterodimeric hybrid enzymes described in our study, have a decreased RNase H activity.

Interestingly, the majority of amino acids in the NNRTI pocket of HIV-1 RT that are instrumental to keep sensitivity to NNRTIs (Schinazi *et al.*, 2001) are identical in FIV RT except (the corresponding amino acids in FIV RT are in parentheses) K101 (Q101), E138 (A138), V179 (D179) and F227 (Y227) (see also Fig. 2.1). However, we could not find any inhibitory effect of NNRTIs on FIV RT even at drug concentrations that are several orders of magnitude higher than required to fully suppress HIV-1 RT activity. The relatively minor differences in amino acid composition in FIV RT can probably not fully explain the complete resistance of FIV RT against NNRTIs. According to our data no major influence of the p51 FIV RT subunit of the hybrid HIVp66/FIVp51 RT on the sensitivity to NNRTIs was observed except for a marginal decrease of the inhibitory potential of capravirine and TSAO-m^3T.

These observations are in agreement with previous observations that the p66 subunit, but not the p51 subunit, predominantly determines the sensitivity of HIV-1 RT to the NNRTIs (Boyer *et al.*, 1994; Jonckheere *et al.*, 1994). Also, the sensitivity (IC_{50}) of HIVp66/FIVp51 RT to the NRTI ddGTP and to PFA was in the same range as that of the wild-type HIV-1 RT.

We have also shown that inhibition of both the wild-type HIV-1 and the hybrid HIVp66/FIVp51 RTs by nevirapine is non-competitive with respect to the substrate and also non-competitive with respect to template/primer (Fig. 2.6), which suggests a similar interaction of this drug with the p66 subunit of wild-type HIV-1 and hybrid HIVp66/FIVp51 RT.

The conclusions of our findings differ from those reported by Amacker *et al.* (1998). These investigators found that nevirapine was inhibitory towards FIV RT and the hybrid FIVp66/HIVp51 RT whereas our results for nevirapine and all other NNRTIs point to a complete inactivity against FIV p66 containing wild-type or hybrid heterodimer RTs, at least at drug concentrations that are 100- to 10,000-fold higher than the inhibitory values for HIV-1 RT. The K_i values of nevirapine for the wild-type and hybrid enzymes with dGTP as substrate or poly(rC).oligo(dG) as template/primer was comparable with the value (0.45 μM) these investigators (Amacker *et al.*, 1998) found with poly(rA).oligo(dT) as the template/primer. It should be mentioned that none of the NNRTIs included in our studies, proved to be inhibitors of the replication of FIV in Crandell feline kidney cells (data not shown) and these observations are in full agreement with our enzyme data.

In conclusion, the role of the RT p51 subunit is limited to the maintenance of the optimal conformation of the p66 subunit in the heterodimeric RT enzyme. Replacement of the p51 subunit in wild-type HIV-1 or FIV RTs by the FIV or HIV-1 p51 counterpart has a profound effect on the catalytic activity of the RT heterodimer, but does not to markedly change the sensitivity/resistance profile of RT towards NNRTIs.

REFERENCES

Amacker M, Hottiger M and Hubscher U (1995) Feline immunodeficiency virus reverse transcriptase: expression, functional characterization, and reconstitution of the 66- and 51-kilodalton subunits. *J Virol* **69**:6273-9.

Amacker M and Hubscher U (1998) Chimeric HIV-1 and feline immunodeficiency virus reverse transcriptases: critical role of the p51 subunit in the structural integrity of heterodimeric lentiviral DNA polymerases. *J Mol Biol* **278**:757-65.

Balzarini J, Perez-Perez MJ, San-Felix A, Camarasa MJ, Bathurst IC, Barr PJ and De Clercq E (1992) Kinetics of inhibition of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase by the novel HIV-1-specific nucleoside analogue [2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3'-spiro-5 "-(4"-amino-1",2"-oxathiole-2",2"-dioxide)thymine (TSAO-T). *J Biol Chem* **267**:11831-8.

Boyer PL, Ding J, Arnold E and Hughes SH (1994) Subunit specificity of mutations that confer resistance to nonnucleoside inhibitors in human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* **38**:1909-14.

Boyer PL, Ferris AL and Hughes SH (1992) Mutational analysis of the fingers domain of human immunodeficiency virus type 1 reverse transcriptase. *J Virol* **66**:7533-7.

Chang AC and Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* **134**:1141-56.

Cheng N, Painter GR and Furman PA (1991) Crosslinking of substrates occurs exclusively to the p66 subunit of heterodimeric HIV-1 reverse transcriptase. *Biochem Biophys Res Commun* **174**:785-9.

D'Aquila RT and Summers WC (1989) HIV-1 reverse transcriptase/ribonuclease H: high level expression in Escherichia coli from a plasmid constructed using the polymerase chain reaction. *J Acquir Immune Defic Syndr* **2**:579-87.

Dufour E, El Dirani-Diab R, Boulme F, Fournier M, Nevinsky G, Tarrago-Litvak L, Litvak S and Andreola ML (1998) p66/p51 and p51/p51 recombinant forms of reverse transcriptase from human immunodeficiency virus type 1--interactions with primer tRNA(Lys3), initiation of cDNA synthesis, and effect of inhibitors. *Eur J Biochem* **251**:487-95.

Harris D, Lee R, Misra HS, Pandey PK and Pandey VN (1998) The p51 subunit of human immunodeficiency virus type 1 reverse transcriptase is essential in loading the p66 subunit on the template primer. *Biochemistry* **37**:5903-8.

Hizi A, Tal R, Shaharabany M, Currens MJ, Boyd MR, Hughes SH and McMahon JB (1993) Specific inhibition of the reverse transcriptase of human immunodeficiency virus type 1 and the chimeric enzymes of human immunodeficiency virus type 1 and type 2 by nonnucleoside inhibitors. *Antimicrob Agents Chemother* **37**:1037-42.

Hostomsky Z, Hostomska Z, Fu TB and Taylor J (1992) Reverse transcriptase of human immunodeficiency virus type 1: functionality of subunits of the heterodimer in DNA synthesis. *J Virol* **66**:3179-82.

Hottiger M, Podust VN, Thimmig RL, McHenry C and Hubscher U (1994) Strand displacement activity of the human immunodeficiency virus type 1 reverse transcriptase heterodimer and its individual subunits. *J Biol Chem* **269**:986-91.

Howard KJ, Frank KB, Sim IS and Le Grice SF (1991) Reconstitution and properties of homologous and chimeric HIV-1.HIV-2 p66.p51 reverse transcriptase. *J Biol Chem* **266**:23003-9.

Huang SC, Smith JR and Moen LK (1992) Contribution of the p51 subunit of HIV-1 reverse transcriptase to enzyme processivity. *Biochem Biophys Res Commun* **184**:986-92.

Isaka Y, Sato A, Kawauchi S, Suyama A, Miki S, Hayami M and Fujiwara T (1998) Construction of the chimeric reverse transcriptase of simian immunodeficiency virus sensitive to nonnucleoside reverse transcriptase inhibitor. *Microbiol Immunol* **42**:195-202.

Jonckheere H, De Vreese K, Debyser Z, Vandekerckhove J, Balzarini J, Desmyter J, De Clercq E and Anne J (1996) A two plasmid co-expression system in Escherichia coli for the production of virion-like reverse transcriptase of the human immunodeficiency virus type 1. *J Virol Methods* **61**:113-25.

Jonckheere H, Taymans JM, Balzarini J, Velazquez S, Camarasa MJ, Desmyter J, De Clercq E and Anne J (1994) Resistance of HIV-1 reverse transcriptase against [2',5'-bis-O-(tert-butyldimethylsilyl)-3'-spiro-5"-(4"-amino-1",2"- oxathiole-2",2"-dioxide)] (TSAO) derivatives is determined by the mutation Glu138-->Lys on the p51 subunit. *J Biol Chem* **269**:25255-8.

Le Grice SF, Naas T, Wohlgensinger B and Schatz O (1991) Subunit-selective mutagenesis indicates minimal polymerase activity in heterodimer-associated p51 HIV-1 reverse transcriptase. *Embo J* **10**:3905-11.

Lightfoote MM, Coligan JE, Folks TM, Fauci AS, Martin MA and Venkatesan S (1986) Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. *J Virol* **60**:771-5.

Lowe DM, Aitken A, Bradley C, Darby GK, Larder BA, Powell KL, Purifoy DJ, Tisdale M and Stammers DK (1988) HIV-1 reverse transcriptase: crystallization and analysis of domain structure by limited proteolysis. *Biochemistry* **27**:8884-9.

Mishima Y and Steitz JA (1995) Site-specific crosslinking of 4-thiouridine-modified human tRNA(3Lys) to reverse transcriptase from human immunodeficiency virus type I. *Embo J* **14**:2679-87.

Misra HS, Pandey PK and Pandey VN (1998) An enzymatically active chimeric HIV-1 reverse transcriptase (RT) with the RNase-H domain of murine leukemia virus RT exists as a monomer. *J Biol Chem* **273**:9785-9.

Schinazi RF, Larder BA and Mellors JW (2001) Mutations in retroviral genes associated with drug resistance: 2000-2001 update. *International Antiviral News* **8**:65-91.

Shih CK, Rose JM, Hansen GL, Wu JC, Bacolla A and Griffin JA (1991) Chimeric human immunodeficiency virus type 1/type 2 reverse transcriptases display reversed sensitivity to nonnucleoside analog inhibitors. *Proc Natl Acad Sci U S A* **88**:9878-82.

Tantillo C, Ding J, Jacobo-Molina A, Nanni RG, Boyer PL, Hughes SH, Pauwels R, Andries K, Janssen PA and Arnold E (1994) Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. *J Mol Biol* **243**:369-87.

Tasara T, Amacker M and Hubscher U (1999) Intramolecular chimeras of the p51 subunit between HIV-1 and FIV reverse transcriptases suggest a stabilizing function for the p66 subunit in the heterodimeric enzyme. *Biochemistry* **38**:1633-42.

Yang G, Song Q, Charles M, Drosopoulos WC, Arnold E and Prasad VR (1996) Use of chimeric human immunodeficiency virus types 1 and 2 reverse transcriptases for structure-function analysis and for mapping susceptibility to nonnucleoside inhibitors. *J Acquir Immune Defic Syndr Hum Retrovirol* **11**:326-33.

CHAPTER III

SUSCEPTIBILITY OF RECOMBINANT FIV/HIV-1 REVERSE TRANSCRIPTASE CHIMERAS TO NNRTIs

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The results presented in this chapter have been published in the following article:

Auwerx, J., Esnouf, R., De Clercq, E. and Balzarini, J. (2004) Susceptibility of feline immunodeficiency virus type 1 reverse transcriptase chimeras to non-nucleoside RT inhibitors. *Mol. Pharmacol.* **65,** 244-251.

SUMMARY

To map the determinants of the lack of susceptibility of FIV RT to HIV-1-specific NNRTIs, a variety of chimeric HIV-1/FIV RTs were constructed. The majority of chimeric RTs had an affinity (K_m) for their natural substrates comparable with that of the wild-type HIV-1 and FIV RTs, but their catalytic efficacy was decreased. Whereas HIV-1 RT could be made entirely insensitive to NNRTIs by exchanging the amino acid sequence 97 through 205 of FIV RT, none of the reverse FIV/HIV-1 RT chimeras gained susceptibility to NNRTIs. The amino acids which are thought to be involved in NNRTI susceptibility and that are different from those in HIV-1 RT have also been introduced in FIV RT. These mutant RTs gained only minor susceptibility to efavirenz or capravirine. *Vice versa*, when these HIV-1-specific amino acids were replaced by their FIV RT counterparts in HIV-1 RT, susceptibility to the NNRTIs was lost. Thus, replacing segments or substituting relevant amino acids in FIV RT by their HIV-1 RT counterparts did not suffice to make FIV RT sensitive towards NNRTIs and was often accompanied by a substantial decrease or even total loss of polymerase activity. It is postulated that, in contrast to the results found for HIV-1/HIV-2 RT chimeras, there exist significant differences in the structure and/or flexibility of FIV RTs that may prevent NNRTIs from interacting with the FIV RT.

INTRODUCTION

NNRTIs are highly specific for HIV-1 and they are not active against HIV-2 or any other retrovirus, including simian immunodeficiency virus (SIV) and FIV. This is in contrast to the NRTIs, which are not specific inhibitors of HIV-1 but broadly effective to other lentiviruses as well, including FIV (North *et al.*, 1990).

Interestingly, the majority of amino acids in the NNRTI pocket of HIV-1 RT that are instrumental in displaying marked sensitivity to NNRTIs (Balzarini, 1999; Schinazi *et al.*, 2001) are also present in FIV RT except for (the corresponding amino acids in FIV RT are in parentheses) K101 (Q101), E138 (A138), V179 (D179) and F227 (Y227) (Fig 2.1). In this respect, FIV RT is surprisingly more similar to HIV-1 RT than HIV-2 RT, which differs in at least 8 amino acids including: A101, I106, A138, I179, I181, L188, A190 and Y227. The specific parts containing amino acids lining the NNRTI pocket and important for NNRTI sensitivity are residues 98 to 110, 179 to 190, and 225 to 236. In fact, exchanging these amino

acid chains between HIV-1 and HIV-2 RT rendered HIV-2 RT susceptible to NNRTIs such as nevirapine, whereas, *vice versa*, HIV-1 RT containing the amino acid present in HIV-2 RT, lost their susceptibility to these NNRTIs (Isaka *et al.*, 2001; Shih *et al.*, 1991; Yang *et al.*, 1996). These observations clearly suggest that these amino acids stretches in the NNRTI-specific pocket are of crucial importance for recognition of NNRTIs by HIV-1 RT. In an attempt to allow NNRTI-testing in the *in vivo* (i.e. monkey) setting, hybrid SIV strains in which the entire RT gene has been replaced by the HIV-1 RT gene (designated RT-SHIV) have been constructed (Uberla *et al.*, 1995). Construction of an RT-FHIV strain in which the FIV RT gene has been replaced by the HIV-1 RT gene, has not been reported.

Despite the high similarity of the NNRTI pocket between HIV-1 RT and FIV RT, no inhibitory effect of NNRTIs against FIV RT has ever been reported, even at drug concentrations that are by several orders of magnitude higher than those required to fully suppress HIV-1 RT activity (Auwerx *et al.*, 2002) (see also Chapter 2). Therefore, single, double and triple amino acid substitutions were introduced into FIV RT and HIV-1 RT to study the influence of these amino acids on the NNRTI susceptibility of the RTs. In addition, we constructed a variety of FIV/HIV-1 chimeras in which relevant well-defined parts of the p66 subunit of FIV RT were substituted by the corresponding HIV-1 p66 amino acid stretches. These amino acid stretches in FIV RT were also introduced into the p66 subunit of HIV-1 RT. Recombinant wild-type, chimeric and mutant enzymes were expressed and purified using a $(His)_6$-tag and their sensitivity spectrum to RT inhibitors and their kinetic properties were analysed.

These studies revealed that FIV RT must be profoundly different from HIV-1 and HIV-2 RT in terms of structure and/or flexibility, so as to prevent interaction of FIV RT with NNRTIs even after this enzyme has been equipped with those amino acids that are proven to engender susceptibility of HIV-1 RT towards NNRTIs.

MATERIALS AND METHODS

Test compounds

Test compounds used in this study were obtained as described in Chapter 2.

Construction of plasmids for the expression of intramolecular chimeras

HIV-1 RT and FIV RT were expressed as previously described by in Chapter 2. The *E. coli* strain JM109 was used for enzyme expression. Exchanges of amino acid stretches

between HIV-1 and FIV RTs were made by the use of six restriction sites, *Nco*I, *Sst*I, *Sst*II, *Nsi*I, *Nhe*I and *Pac*I. Of these sites, *Nco*I, *Nhe*I and *Pac*I were present only in wild-type FIV RT and *Nsi*I was present in both RTs. The *Sst*I and *Sst*II restriction sites were absent from both RT sequences. Each of these sites and combinations of the sites were created by site-directed mutagenesis, if necessary. Creation of the restriction sites did not lead to undesirable amino acid substitutions because the nucleotide replacements resulted in silent mutations. The restriction sites allowed exchanges between or within the amino acid subdomains in RT. The locations of the sites with respect to codon positions are shown in Fig. 3.1.

The chimeras were constructed with pKRT2 (D'Aquila and Summers, 1989) and pFIV66 (North *et al.*, 1994) as the parental plasmids which contain both a $(His)_6$-tag sequence at the ATG start codon of the RT gene which is translated into an N-terminal His-tag used in the protein purification procedure.

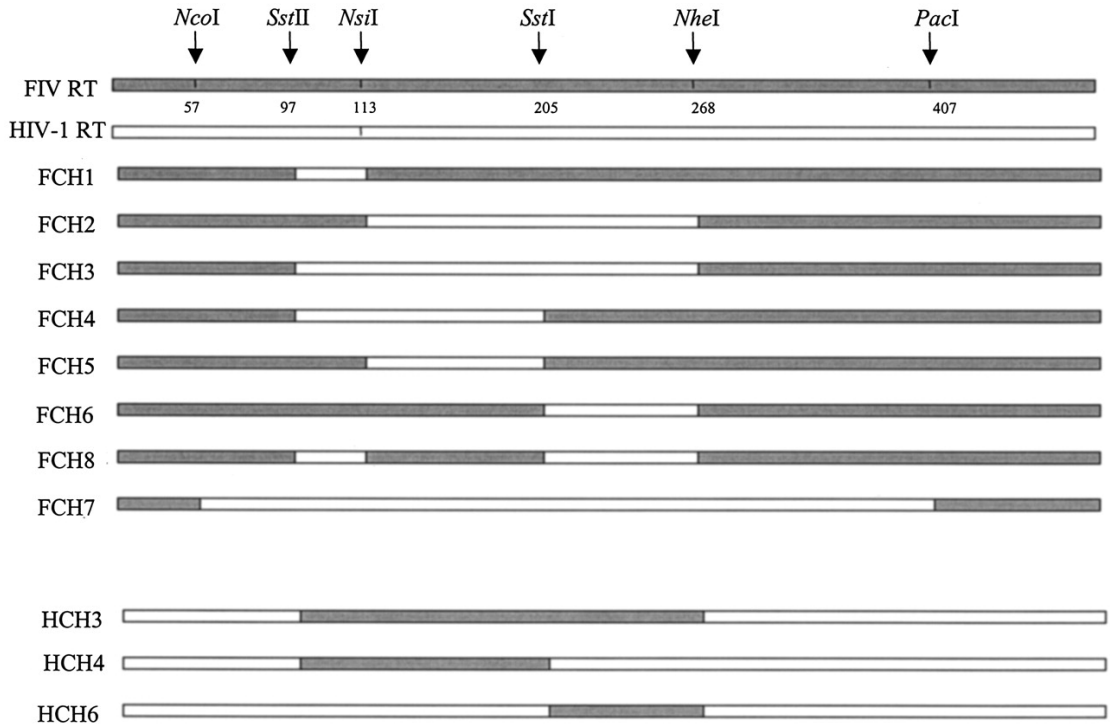

Figure 3.1 Overview of the different chimeras.

The numbers under the upper bar indicate the position of the amino acids where each restriction enzyme recognizes their site. The open bar represents HIV-1 RT and the grey bar FIV RT p66. The grey or open segments in each chimeric construct represent the parental [FIV (grey) or HIV-1 (open)] RT part of which the sequences were derived. A restriction site that was already present in the genome of the RT gene is marked with a vertical line. If the site was not present, it was introduced by site-directed mutagenesis.

Introduction of point mutations in FIV and HIV-1 RT

Besides the construction of chimeras, we also introduced single, double and triple amino acid substitutions in the FIV and HIV-1 RTs. Because we focused on the NNRTI pocket (Fig 2.1) only those amino acids that are known to be of major importance in recognizing the NNRTIs by HIV-1 RT were changed. Therefore, in HIV-1 RT the K101Q, V179D and F227Y substitutions were made, whereas, *vice versa*, in FIV RT Q101K, D179V and Y227F mutations were introduced. The mutations were introduced using the site-directed mutagenesis method (Stratagene, Westburg, Leusden, the Netherlands), as described previously by (Pelemans *et al.*, 1998).

Purification of RT

Expression and cell lysis of recombinant RT was performed as described in Chapter 2. The supernatant was then incubated with 2 ml of pre-equilibrated NiNTA beads (Qiagen) at 4 °C, while rotating for at least 1 h. After incubation and sedimentation of the NiNTA resin with the bound $(His)_6$-tagged proteins, the column was formed and washed three times with 20 ml phosphate buffer (50 mM sodium phosphate buffer pH 7.8, 0.5 mM NaCl, 5 mM β-mercaptoethanol, 25 mM imidazole and 10% glycerol). The HIV-1 and FIV RTs were eluted from the column with the same phosphate buffer containing 125 mM imidazole. The imidazole-containing elution buffer was exchanged by a Tris buffer (20 mM Tris-HCl, pH 7.8, 0.05 M NaCl, 1 mM EDTA, 1 mM DTT and 10% glycerol) and the eluate was concentrated to 2 ml using Vivaspin 15 centrifugal filtration devices (Vivascience). The protein was further purified by means of FPLC as described in Chapter 2.

Preparation of *E. coli* lysates

RT lysates were prepared as described in Chapter 2.

Reverse transcriptase assay

For determination of the 50% inhibitory concentration (IC_{50}) values of the test compounds against the wild-type and chimeric HIV-1 and FIV RTs and for studying steady-state kinetic parameters, the enzyme assays were performed as described previously (Balzarini *et al.*, 1992) and in Chapter 2.

RESULTS

3.1. Catalytic activity of the chimeric RT enzymes

The expression plasmids were examined for their ability to encode stable chimeric RT proteins. The bacterial lysates and the purified enzymes were analysed by SDS PAGE to verify the accumulation of stable protein. Only FCH1 and HCH6 of the chimeras showed significant activity that was almost comparable with their corresponding wild-type enzymes. FCH5 and FCH6 had a markedly reduced activity but were still sufficiently active to perform RT assays for determination of the IC_{50} values (Table 3.1). The chimeras in which larger amino acid parts were exchanged (i.e. FCH7 and FCH8 and HCH4) were only active in a crude lysate. No catalytic activity in the other chimeric RT constructs (i.e. FCH2, FCH3, FCH4 and HCH3) could be detected, in either the crude bacterial lysates or with the purified enzymes.

3.2. Kinetic properties of the chimeric RT enzymes

To further study the characteristics of the chimeric enzymes, we determined their K_m and k_{cat} values with dGTP as substrate (Table 3.1). The K_m values of the wild-type FIV66 and HIV66 homodimers were 2.5 and 5.5 μM, respectively. The RT chimera FCH1 had a K_m value of 7.6 μM which was about 3 times higher than that observed for the K_m of wild-type FIV RT. The other active RT chimeras had K_m values that were in the same range as the K_m value of the respective wild-type enzymes, which reflects an affinity of the individual enzymes for their substrate that is comparable with their corresponding wild-type RTs. Instead, the k_{cat} value of the different chimeric enzymes was significantly lower than the values found for the wild-type RT enzymes, which reflects a low catalytic efficacy of the chimeric enzymes. Indeed, for the FCH5 RT a k_{cat} of only 0.5% of the k_{cat} of wild-type FIV RT was observed, while the FCH6 chimeric RT enzyme had a catalytic efficiency that was only 0.01% of that of wild-type FIV RT. HCH6 and FCH1 had k_{cat} values that were about 2% of that of their respective wild-type enzymes (Table 3.1).

Table 3.1 Inhibitory activity of NNRTIs, PFA and ddGTP against a variety of purified FIV/HIV-1 RT chimeric enzymes

				IC_{50} (µM)[a,b]				
Chimeric RT	**RT-activity[c] (%)**	K_m	k_{cat}[d]	**nevirapine**	**efavirenz**	**GW420867X**	**ddGTP**	**PFA**
FIV66	100	2.5	0.50	>1000	>1000	>1000	0.74 ± 0.11	71 ± 32
FCH1	28	7.6	0.013	>1000	>1000	>1000	1.51 ± 0.06	>500
FCH2	-							
FCH3	-							
FCH4	-							
FCH5	18.7	3.8	0.0025	>1000	>1000	>1000	0.69 ± 0.45	136 ± 27
FCH6	3.3	5.9	0.0005	>1000	>1000	>1000	1.60 ± 0.57	>500
FCH7[e]	1.3	6.1	-	>1000	>1000	>1000	-	-
FCH8[e]	1.9	4.5	-	>1000	>1000	>1000	-	-
HIV66	100	5.5	0.57	1.61± 0.6	0.034 ± 0.008	0.010 ± 0.002	0.08 ± 0.04	8.5 ± 5.5
HCH3	-							
HCH4[e]	0.2	8.0	-	>1000	>1000	>1000	1.43 ± 0.27	>500
HCH6	3.2	6.7	0.018	3.0 ± 2.3	0.016 ± 0.006	0.014 ± 0.007	0.051 ± 0.007	7 ± 3

[a] 50% inhibitory concentration or compound concentration required to inhibit the RT reaction by 50%. Poly(rC).oligo(dG) and [^{3}H]dGTP were used as the template/primer and radiolabelled substrate, respectively

[b] Data are the mean ± S.D. for at least 2 to 3 independent experiments

[c] RT-activity is expressed as dpm/ng protein. 100% FIV RT activity represents 222,250 dpm/ng protein and 100% HIV-1 RT activity represents 2,246,934 dpm/ng protein (100%)

[d] k_{cat} is in pmol/µg protein/s

[e] The RT was not purified. The experiments were performed on 10-fold diluted bacterial RT lysates. RT activity values were expressed as a percentage of the activity found in the wild-type (purified) RT sample

3.3. Sensitivity of the chimeric RT enzymes to NNRTIs, ddGTP and PFA

In an attempt to identify the regions in FIV RT that are responsible for the lack of susceptibility to NNRTIs, the enzymatically active FIV/HIV-1 and HIV-1/FIV chimeric RT enzymes and the two parental FIV and HIV-1 RTs were evaluated for their sensitivities to inhibition by the NNRTIs nevirapine, efavirenz and GW420867X. Nevirapine represents a first-generation clinically used NNRTI, whereas efavirenz and the quinoxaline derivative represent both second-generation NNRTIs. The IC_{50} values of the NNRTIs for the chimeric RTs are presented in Table 3.1. As expected, FIV RT was not inhibited by any NNRTI even at concentrations as high as 1000 μM. In contrast, HIV-1 RT proved exquisitely sensitive to efavirenz and GW420867X (IC_{50}: 0.034 and 0.01 μM, respectively) and, to a lesser extent, nevirapine (1.61 μM), that is at compound concentrations that is 1000 to more than 100,000 fold lower than the highest concentration of drug that proved inactive against FIV RT. The FIV RT-derived chimeras which contained several amino acid fragments from HIV-1 RT (Fig 2) and which showed sufficient catalytic activity (i.e. FCH1, FCH5, FCH6, FCH7 and FCH8) did not gain any susceptibility to the NNRTIs (Table 3.1). Interestingly, the chimeric RT enzyme HCH4, which was derived from the HIV-1 RT and contained the homologous amino acid stretch of FIV RT from amino acid 97 to 205, fully lost susceptibility (IC_{50}>1000 μM) to the three NNRTIs tested.

Besides NNRTIs, ddGTP and PFA were also evaluated for their inhibitory activities against the chimeric RTs (Table 3.1). The values obtained for inhibition of the chimeric enzymes by ddGTP were found to be comparable with the parental RTs, except the chimeric RT enzyme HCH4 which had an IC_{50} value of 1.43 μM, that is at a 18-fold higher concentration than required to inhibit the parental HIV-1 RT, but close to the IC_{50} value found for wild-type FIV RT (0.74 μM). This observation may suggest that the substrate specificity of RT is predominantly determined by the conformation of the amino acid stretch between 97 and 205 (containing the catalytic aspartic acid triad at amino acid positions 110, 185 and 186). When examining PFA, varying IC_{50} values were found for the FIV RT derived chimeras: FCH1 and FCH6 became fully resistant to this drug while FCH5 remained susceptible at PFA concentrations that were also required to inhibit the parental enzyme. In contrast to the HIV-1 and HCH6 RTs, the HCH4 RT chimeric enzyme was resistant to PFA. Thus, the HCH4 chimeric RT enzyme seemed to have gained properties that were more comparable to FIV RT than HIV-1 RT.

3.4. Introduction of point mutations into FIV RT and HIV-1 RT and sensitivity of the mutated enzymes to NNRTIs, ddGTP and PFA

Besides the construction of the chimeric enzymes, in which relatively large internal segments were exchanged, introduction of single, double and triple amino acid changes were also made in both HIV-1 and FIV RTs. These mutant enzymes had catalytic activities that were comparable with those of their wild-type enzymes (data not shown). The amino acids of choice represented those that are instrumental for the HIV-1 RT enzyme to keep full sensitivity to NNRTIs but that are different in FIV RT. We determined whether the complete loss of sensitivity of FIV RT to NNRTIs is due to the inability of these compounds to bind to the putative pocket in FIV RT in the presence of these amino acids. The introduced amino acid mutations in FIV RT were Q101K, D179V and Y227F and all possible combinations derived thereof.

Poor susceptibility to the inhibitory effect of efavirenz (IC_{50} value of 114 μM for the single D179V FIV RT mutant and 143 μM for the triple Q101K+D179V+Y227F mutant) was found (Table 3.2). Also capravirine very slightly inhibited the mutant FIV RT enzymes with IC_{50} values ranging from 329 to 844 μM, but wild-type FIV RT was also slightly inhibited by capravirine at a concentration of 988 μM. Therefore, it should be concluded that the amino acid mutations introduced in the FIV RT did not influence the inhibitory values for capravirine. All the other NNRTIs did not show any inhibitory activity against any FIV RT mutant. Besides the different classes of NNRTIs, ddGTP and PFA were also included in the enzyme assays and their inhibitory values found were highly comparable with those recorded for wild-type FIV RT (i.e. 0.74 μM for ddGTP and 71 μM for PFA) (Table 3.2).

To assess the importance of the role of the amino acids mentioned above in NNRTI resistance/sensitivity, we introduced the same homologous residues of FIV RT into HIV-1 RT by site-directed mutagenesis, and the results are shown in Table 3.3. By introducing the K101Q mutation in HIV-1 RT we found a slight decrease of sensitivity of the enzyme to nevirapine (3-fold) and TSAO-m^3T (10- to 15-fold). Mutating the amino acid position 179 decreased the sensitivity towards the second-generation thiocarboxanilide NNRTI UC781 by 50-fold. The F227Y mutant did not lead to any marked change in the sensitivity of the HIV-1 RT enzyme to most NNRTIs.

Table 3.2 Inhibitory activity of NNRTIs, ddGTP and PFA against mutant FIV RTs

	IC_{50} [a,b] (μM)								
	Q101K	D179V	Y227F	D179V+Y227F	Q101K+Y227F	Q101K+D179V	Q101K+D179V+Y227F	FIV RT	HIV-1 RT
Nevirapine	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	1.6 ± 0.6
Delavirdine	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.52 ± 0.23
Efavirenz	994 ± 63	114 ± 3	>1000	>1000	>1000	>1000	143 ± 22	>1000	0.034 ± 0.008
Emivirine	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.16 ± 0.05
UC-781	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.016 ± 0.002
GW420867X	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.010 ± 0.002
Capravirine	679 ± 184	507 ± 78	677 ± 2	844 ± 9	537 ± 69	755 ± 2	329 ± 62	988 ± 171	0.004 ± 0.001
TSAO-m^3T	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.6 ± 0.2
ddGTP	0.63 ± 0.02	0.75 ± 0.16	0.78 ± 0.0	0.81 ± 0.14	0.74 ± 0.13	0.59 ± 0.12	0.74 ± 0.16	0.74 ± 0.11	0.08 ± 0.04
PFA	106 ± 47	118 ± 30	153 ± 29	174 ± 22	129 ± 47	116 ± 20	262 ± 184	71 ± 32	8.5 ± 5.5

Table 3.3 Inhibitory activity of NNRTIs, ddGTP and PFA against mutant HIV-1 RTs

	IC_{50} [a,b] (μM)								
	K101Q	V179D	F227Y	V179D +F227Y	K101Q +F227Y	K101Q +V179D	K101Q +V179D +F227Y	HIV-1 RT	FIV RT
Nevirapine	3.0 ± 0.1	4.4 ± 1	2.05 ± 0.03	1.39 ± 0.00	4.8 ± 3.7	6.1 ± 0.3	5.0 ± 1.3	1.6 ± 0.6	>1000
Delavirdine	0.9 ± 0.5	2.63 ± 0.55	2.4 ± 1.4	5.8 ± 1.5	2.9 ± 1.7	2.4 ± 1.0	0.10 ± 0.02	0.52 ± 0.23	>1000
Efavirenz	0.06 ± 0.02	0.044 ± 0.009	0.033 ± 0.002	0.06 ± 0.02	0.28 ± 0.06	0.136 ± 0.009	0.10 ± 0.01	0.034 ± 0.008	>1000
Emivirine	0.14 ± 0.01	0.84 ± 0.07	0.19 ± 0.02	0.23 ± 0.04	0.91 ± 0.12	0.62 ± 0.23	0.84 ± 0.12	0.16 ± 0.05	>1000
UC-781	0.16 ± 0.09	0.8 ± 0.2	0.07 ± 0.03	0.06 ± 0.01	0.16 ± 0.08	0.1 ± 0.1	0.8 ± 0.3	0.016 ± 0.002	>1000
GW420867X	0.07 ± 0.03	0.10 ± 0.06	0.015 ± 0.008	0.03 ± 0.01	0.10 ± 0.03	0.08 ± 0.03	0.07 ± 0.05	0.010 ± 0.002	>1000
Capravirine	0.079 ± 0.005	0.08 ± 0.05	0.017 ± 0.004	0.09 ± 0.05	0.08 ± 0.06	0.15 ± 0.02	0.0071 ± 0.0004	0.004 ± 0.001	988 ± 171
TSAO-m^3T	6.1 ± 5.4	6.1 ± 1.2	0.8 ± 0.6	0.395 ± 0.007	6.7 ± 1.0	9 ± 1	6.5 ± 2.0	0.6 ± 0.2	>1000
ddGTP	0.04 ± 0.03	0.03 ± 0.01	0.046 ± 0.001	0.054 ± 0.005	0.018 ± 0.004	0.07 ± 0.02	0.069 ± 0.008	0.08 ± 0.04	0.74 ± 0.11
PFA	3.0 ± 0.7	20 ± 15	9.0 ± 4.6	6.4 ± 2.1	18.3 ± 2.5	8.2 ± 1.8	27 ± 5	8.5 ± 5.5	71 ± 32

[a] 50% inhibitory concentration or compound concentration required to inhibit the RT reaction by 50%
[b] Data are the mean ± S.D. for at least 2 to 3 independent experiments

The decreased inhibitory activity against the mutated HIV-1 RT enzymes afforded by single amino acid changes were not always additive when multiple mutations derived thereof were combined. For example, we found for delavirdine a 5-fold reduction of drug sensitivity when mutating positions 101 and 179, and a 11-fold reduction when changing residues 179 and 227. However, when the triple RT mutant was compared with wild-type HIV-1 RT, a 5-fold increase of susceptibility to delavirdine occurred. These observations point to a complicated interplay between several amino acids on the conformation of the NNRTI pocket and/or on the affinity to the individual NNRTIs.

DISCUSSION

Out of 20 relevant amino acids lining the NNRTI pocket in HIV-1 RT, only the NNRTI-characteristic K101, E138, V179 and F227 amino acids in HIV-1 RT are not present in FIV RT (Fig 2.1). According to the crystal structure of the binding site for NNRTIs in HIV-1 RT, Y181 and Y188 are extremely important for sensitivity of HIV-1 RT to NNRTIs, in particular to first-generation NNRTIs (Esnouf *et al.*, 1995; Hsiou *et al.*, 1998; Kohlstaedt *et al.*, 1992). As shown in Fig 2.1, these two tyrosine residues at positions 181 and 188 are also present in FIV RT whereas they are absent in the RTs of SIV and HIV-2, which are, like FIV RT, also insensitive to NNRTIs. Previously, it has been shown that replacing the I181 and L188 (or even the L188 residue alone) in HIV-2 RT by the tyrosine residues present in HIV-1 RT results in a mutated HIV-2 RT enzyme that is highly susceptible to NNRTIs (i.e. nevirapine) (Isaka *et al.*, 2001; Shih *et al.*, 1991).

In this study, we aimed to make FIV RT susceptible to NNRTIs by replacing one or several relevant amino acids in the enzyme or by exchanging important amino acid stretches in the FIV RT enzyme to determine the minimum requirements to restore NNRTI sensitivity in FIV RT. Therefore, to map the regions of FIV RT that determine the lack of susceptibility to NNRTIs, enzymatically active FIV/HIV-1 chimeric enzymes were constructed and evaluated for sensitivity towards inhibition by a variety of relevant NNRTIs. In contrast to the HIV-1/HIV-2 chimeric RTs, none of our constructed chimeric RT enzymes gained any significant susceptibility and most chimeras even lost their major enzymatic activities. We interpret the decreased (or lack of) catalytic activity of the FIV/HIV-1 chimeric enzymes as due to either a change in the overall structure of the chimeric RTs, or perturbed dimerisation of the subunits of the enzyme and/or an altered structure of the substrate binding site of the RT enzyme. In fact, we found already that hybrid heterodimers of HIV-1 and FIV RT (i.e. p66 of

HIV-1 RT and p51 of FIV RT or *vice versa*, p66 of FIV RT and p51 of HIV-1 RT) had a compromised catalytic activity, which was not observed for HIV-1/HIV-2 heterodimeric RT enzymes (see Chapter 2). Therefore, we believe that, in contrast with HIV-2, exchanges of amino acid stretches between FIV RT and HIV-1 RT may somewhat compromise the overall folding or conformation of the chimeric subunits, resulting in lowering or even abolition of the enzymatic activity.

Our observations that none of the FIV RT chimeras showed sensitivity to NNRTIs, including the FCH4 chimeric enzyme that contained the amino acid stretch of HIV-1 RT important for the sensitivity of the HIV-1 RT to NNRTIs, seem to imply that there must exist structural restrictions on the ability to change amino acid parts between both enzymes. In this respect it is important to note that the HCH4 chimeric HIV-1 RT enzyme fully lost NNRTI susceptibility, pointing to the crucial importance of this part of the enzyme for NNRTI sensitivity. The majority of mutations that are reported to affect NNRTI sensitivity to HIV-1 RT are indeed located within this amino acid stretch (Table 3.4).

Although the level of sequence identity between FIV and HIV-1 RTs (48%) is somewhat lower than that between HIV-1 and HIV-2 RTs (62%), it is still sufficiently high for suggesting an overall similar structure. Indeed, the recent crystal structure of HIV-2 RT confirms its high level of structured similarity with HIV-1 RT (Bird *et al.*, 2003; Ren *et al.*, 2002). The fact that all the chimeric RTs could bind to their substrates with K_m values that are comparable to those of the wild-type enzymes also supports this view. However, the nature of polymerase activity is that the binding site is fairly tolerant to small changes as the requirement for translocation of the template/primer without dissociation precludes unnecessarily specific interactions. Also, no marked preference of HIV-1 RT for a template/primer containing a polymerizable end over one containing a non-polymerizable end was observed showing that the specificity of HIV-1 RT is rather low (Huang *et al.*, 2000). There is a large reduction in k_{cat} for all the chimeric RTs that were constructed, implying that within the overall polymerase binding site there are distortions that affect the catalytic residues. Some of these distortions may arise from the nature of the chimeras constructed since they are based on existing restriction sites (or ones introduced that result in silent mutations) and are not aligned on structural domain boundaries. Chimeras constructed based on structural considerations may lead to fewer distortions, at the expense of point mutations, and thereby greater activity. The chimeras with residual activity do not involve significant changes to the residues implicated in NNRTI susceptibility, and so the lack of NNRTI susceptibility is not surprising.

Although structural distortion may affect NNRTI binding in the chimeric RTs with residual activity, this is unlikely to be the case for the FIV RTs bearing point mutations where the activity is close to wild-type. Given that the sequence conservation between HIV-1 and FIV RT suggests similarity of structure, the lack of sensitivity of (mutant) FIV RTs to NNRTIs may be ascribed to any of the following factors: (i) Some structural feature of FIV RT makes it impossible for the NNRTI binding pocket to be created. In unliganded HIV-1 RT, the NNRTI binding pocket does not exist, but opens up on binding of the inhibitor (Esnouf *et al.*, 1995; Esnouf *et al.*, 1997; Hsiou *et al.*, 1996; Rodgers *et al.*, 1995). Binding of an NNRTI (such as nevirapine) to RT requires conformational changes of the overall structure as the thumb rotates away from the fingers and also induces a small shift in the fingers-palm unit. In the catalytic complex, the fingers domain closes in toward the palm and the palm itself shifts slightly with respect to the core of the unliganded RT (Huang *et al.*, 2000). Were FIV RT more rigid than its HIV-1 counterpart, then this opening may be at too great an energy cost for NNRTIs to penetrate into the pocket. (ii) The NNRTIs simply cannot penetrate the (mutant) FIV RT binding pocket, perhaps due to changes in the flexibility of FIV RT or differences in the subunit interactions. (iii) The NNRTIs may enter but cannot bind in the enzyme pocket due to conformational hindrance. While this may well be the case for wild-type FIV RT, in the mutant FIV RTs the amino acids lining the NNRTI pocket are those of HIV-1 RT and so lack of binding seems unlikely. (iv) The NNRTIs may efficiently bind in the enzyme's putative pocket but fail to inhibit the catalytic activity. While this possibility cannot be ruled out, the fact that HIV-2 RT can be made sensitive to NNRTIs by point mutations suggests a similar conformation between HIV-1 RT and HIV-2 RT.

This analysis leads us to the suggestion that structural flexibility may play an important role in NNRTI binding and that FIV RT may be somewhat more rigid that HIV-1 RT, whilst still having the same overall structure. Were this the case, then one might see significant sequence differences between the two RTs for amino acids expected to form domain and subunit interfaces. In several places this seems to be so. The lack of enzymatic activity of HIV-1/FIV RT heterodimers, unlike HIV-1/HIV-2 RT heterodimers, also points to substantial differences in the subunit interactions (see Chapter 2).

For the HIV-1 RTs bearing point mutations that represent the analogue amino acids present in FIV RT, there is a complex interplay between the resistance mutations with both drug resistance and hypersensitivity being observed. Similar observations have been found in numerous NNRTI resistance studies (for an overview, see Bacheler, 1999; Schinazi *et al.*,

2001) and the complexities have been ascribed to the varying abilities of NNRTIs to adapt to the changing shape and interaction possibilities afforded by the mutant NNRTI pocket.

In conclusion, we demonstrated in this study that FIV RT behaves differently from HIV-1 and HIV-2 RT in terms of exchange of individual amino acids and amino acid stretches that determine the NNRTI susceptibility of HIV-1 RT. In contrast with HIV-2 RT, FIV RT cannot be mutated or engineered to acquire susceptibility to the inhibitory effects of the NNRTIs. A crystal structure of FIV RT may resolve the molecular/structural basis of these observations and provide deeper insights in the similarities and differences of this family of RT enzymes that are very closely related to each other.

Table 3.4 Overview of amino acids in HIV-1 RT reported to play a role in sensitivity/resistance of HIV-1 RT against NNRTIs

Amino acid position In HIV-1 RT	HIV-1 wild-type amino acid	NNRTI-mutated amino acid	Corresponding FIV RT amino acid
98	Ala	Gly	Ala
100	Leu	Ile	Leu
101	Lys	Glu	Gln
103	Lys	Asn, Thr	Lys
106	Val	Ala	Val, Ile
108	Val	Ile	Val
135	Ile	Met, Thr, Leu	Lys
138	Glu	Lys	Ala
139	Thr	Ile	Gly
179	Val	Asp, Glu	Asp
181	Tyr	Cys, Ile	Tyr
184	Met	Ile, Val	Met
188	Tyr	Cys, His, Leu	Tyr
190	Gly	Ala, Glu, Ser	Gly
225	Pro	His	Pro
227	Phe	Leu	Tyr
229	Trp	-	Trp
233	Glu	Val	Glu
236	Pro	Leu	Pro
238	Lys	Thr	Thr
318	Tyr	-	Tyr

Data taken from Balzarini (1999) and Schinazi *et al.* (2000)

REFERENCES

Auwerx J, North TW, Preston BD, Klarmann GJ, De Clercq E and Balzarini J (2002) Chimeric human immunodeficiency virus type 1 and feline immunodeficiency virus reverse transcriptases: role of the subunits in resistance/sensitivity to non-nucleoside reverse transcriptase inhibitors. *Mol Pharmacol* **61**:400-6.

Bacheler LT (1999) Resistance to non-nucleoside inhibitors of HIV-1 reverse transcriptase. *Drug Resist Updat* **2**:56-67.

Balzarini J (1999) Suppression of resistance to drugs targeted to human immunodeficiency virus reverse transcriptase by combination therapy. *Biochem Pharmacol* **58**:1-27.

Balzarini J, Perez-Perez MJ, San-Felix A, Camarasa MJ, Bathurst IC, Barr PJ and De Clercq E (1992) Kinetics of inhibition of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase by the novel HIV-1-specific nucleoside analogue [2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3'-spiro-5 "-(4"-amino-1",2"-oxathiole-2",2"-dioxide)thymine (TSAO-T). *J Biol Chem* **267**:11831-8.

Bird LE, Chamberlain PP, Stewart-Jones GB, Ren J, Stuart DI and Stammers DK (2003) Cloning, expression, purification, and crystallisation of HIV-2 reverse transcriptase. *Protein Expr Purif* **27**:12-8.

D'Aquila RT and Summers WC (1989) HIV-1 reverse transcriptase/ribonuclease H: high level expression in Escherichia coli from a plasmid constructed using the polymerase chain reaction. *J Acquir Immune Defic Syndr* **2**:579-87.

Esnouf R, Ren J, Ross C, Jones Y, Stammers D and Stuart D (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. *Nat Struct Biol* **2**:303-8.

Esnouf RM, Ren J, Hopkins AL, Ross CK, Jones EY, Stammers DK and Stuart DI (1997) Unique features in the structure of the complex between HIV-1 reverse transcriptase and the bis(heteroaryl)piperazine (BHAP) U-90152 explain resistance mutations for this nonnucleoside inhibitor. *Proc Natl Acad Sci U S A* **94**:3984-9.

Hsiou Y, Das K, Ding J, Clark AD, Jr., Kleim JP, Rosner M, Winkler I, Riess G, Hughes SH and Arnold E (1998) Structures of Tyr188Leu mutant and wild-type HIV-1 reverse transcriptase complexed with the non-nucleoside inhibitor HBY 097: inhibitor flexibility is a useful design feature for reducing drug resistance. *J Mol Biol* **284**:313-23.

Hsiou Y, Ding J, Das K, Clark AD, Jr., Hughes SH and Arnold E (1996) Structure of unliganded HIV-1 reverse transcriptase at 2.7 A resolution: implications of conformational changes for polymerization and inhibition mechanisms. *Structure* **4**:853-60.

Huang H, Harrison SC and Verdine GL (2000) Trapping of a catalytic HIV reverse transcriptase*template:primer complex through a disulfide bond. *Chem Biol* **7**:355-64.

Isaka Y, Miki S, Kawauchi S, Suyama A, Sugimoto H, Adachi A, Miura T, Hayami M, Yoshie O, Fujiwara T and Sato A (2001) A single amino acid change at Leu-188 in the reverse transcriptase of HIV-2 and SIV renders them sensitive to non-nucleoside reverse transcriptase inhibitors. *Arch Virol* **146**:743-55.

Kohlstaedt LA, Wang J, Friedman JM, Rice PA and Steitz TA (1992) Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**:1783-90.

North TW, Cronn RC, Remington KM and Tandberg RT (1990) Direct comparisons of inhibitor sensitivities of reverse transcriptases from feline and human immunodeficiency viruses. *Antimicrob Agents Chemother* **34**:1505-7.

North TW, Hansen GL, Zhu Y, Griffin JA and Shih CK (1994) Expression of reverse transcriptase from feline immunodeficiency virus in Escherichia coli. *Antimicrob Agents Chemother* **38**:388-91.

Pelemans H, Esnouf RM, Jonckheere H, De Clercq E and Balzarini J (1998) Mutational analysis of Tyr-318 within the non-nucleoside reverse transcriptase inhibitor binding pocket of human immunodeficiency virus type I reverse transcriptase. *J Biol Chem* **273**:34234-9.

Ren J, Bird LE, Chamberlain PP, Stewart-Jones GB, Stuart DI and Stammers DK (2002) Structure of HIV-2 reverse transcriptase at 2.35-A resolution and the mechanism of resistance to non-nucleoside inhibitors. *Proc Natl Acad Sci U S A* **17**:14410-5.

Rodgers DW, Gamblin SJ, Harris BA, Ray S, Culp JS, Hellmig B, Woolf DJ, Debouck C and Harrison SC (1995) The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **92**:1222-6.

Schinazi RF, Larder BA and Mellors JW (2001) Mutations in retroviral genes associated with drug resistance: 2000-2001 update. *International Antiviral News* **8**:65-91.

Shih CK, Rose JM, Hansen GL, Wu JC, Bacolla A and Griffin JA (1991) Chimeric human immunodeficiency virus type 1/type 2 reverse transcriptases display reversed sensitivity to nonnucleoside analog inhibitors. *Proc Natl Acad Sci U S A* **88**:9878-82.

Uberla K, Stahl-Hennig C, Bottiger D, Matz-Rensing K, Kaup FJ, Li J, Haseltine WA, Fleckenstein B, Hunsmann G, Oberg B and et al. (1995) Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors. *Proc Natl Acad Sci U S A* **92**:8210-4.

Yang G, Song Q, Charles M, Drosopoulos WC, Arnold E and Prasad VR (1996) Use of chimeric human immunodeficiency virus types 1 and 2 reverse transcriptases for structure-function analysis and for mapping susceptibility to nonnucleoside inhibitors. *J Acquir Immune Defic Syndr Hum Retrovirol* **11**:326-33.

CHAPTER IV

THE PHENYLETHYLTHIAZOLYLTHIOUREA NNRTI MSK-076 SELECTS FOR A RESISTANCE MUTATION IN THE SUBSTRATE ACTIVE SITE OF HIV-2 RT

The results presented in this chapter have been published in the following article:

Auwerx, J., Stevens, M., Van Rompay, A. R., Bird, L. E., Ren, J., Öberg, B., Stammers, D. K., De Clercq, E., Karlsson, A. and Balzarini, J. (2004) The phenylethylthiazolylthiourea non-nucleoside reverse transcriptase (RT) inhibitor MSK-076 selects for a resistance mutation in the active site of HIV-2 RT. *J. Virol.* **78**, 7427-7437.

SUMMARY

The phenylethylthiazolylthiourea (PETT) derivative MSK-076 shows, besides high potency against HIV-1, marked activity against HIV-2 (EC_{50} of 0.63 μM) in cell culture. Time-of-addition experiments pointed to HIV-2 RT as the target of action of MSK-076. Recombinant HIV-2 RT was inhibited by MSK-076 at 23 μM. As also found for HIV-1 RT, MSK-076 inhibited HIV-2 RT in a non-competitive manner with respect to dGTP and poly(rC).oligo(dG) as substrate and template/primer, respectively. MSK-076 selected for A101P and G112E mutations in HIV-2 RT and for K101E, Y181C and G190R mutations in HIV-1 RT when exposed to HIV-2 and HIV-1, respectively in cell culture. The selected mutated strains of HIV-2 were fully resistant to MSK-076 and the mutant recombinant HIV-2 RT enzymes in which the A101P and/or G112E were introduced by site-directed mutagenesis, showed more than 50-fold resistance to MSK-076. Mapping the resistance mutations to the HIV-2 RT structure ascertained that A101P is located at what is equivalent to the NNRTI-binding site of HIV-1 RT. G112E, however, is distal to the putative NNRTI-binding site in HIV-2 RT, but close to the active site, implying a novel molecular mode of action and mechanism of resistance. Our findings may have important implications for the development of new NNRTIs with pronounced activity against a wider range of lentiviruses.

INTRODUCTION

Given the highly conserved nature of the antiretroviral target (reverse transcriptase) (Fig 2.1) the specificity of NNRTIs for HIV-1 strains is remarkable. The HIV-2 serotype was shown to be closely related to SIV_{mac} and has approximately 60% overall amino acid identity with HIV-1 RT and a comparable catalytic polymerase activity (Hizi *et al.*, 1991). Despite this amino acid similarity with HIV-1 RT the NNRTIs are inactive against HIV-2 and SIV. It became clear that a relatively small number of amino acids in the HIV-2 RT were responsible for the lack of the inhibitory activity of the NNRTIs. Chimeric HIV-1/HIV-2 RT constructs (Bacolla *et al.*, 1993; Yang *et al.*, 1996) and site-directed mutagenesis of HIV-1 and HIV-2 RT (Condra *et al.*, 1992; Hizi *et al.*, 1993; Isaka *et al.*, 2001; Shih *et al.*, 1991) revealed that the nature of the amino acids at positions 181 and 188 in RT play a major role in the recognition of the first-generation NNRTIs (i.e. HEPT, TIBO, nevirapine, pyridinone, delavirdine, TSAO, etc.). Also, some mutant HIV-1 strains that were selected in cell culture for high-level resistance against delavirdine contain the 181I or 188L mutations in the RT

(Balzarini *et al.*, 1994; Ren *et al.*, 2001). Such single-mutated virus strains proved highly resistant to all first-generation NNRTIs and contain those aliphatic amino acids (I, L) at locations 181 or 188 that are present in wildcritical role of these and other amino acids in the recognition of NNRTIs has been visualized in the crystal structures of RT/NNRTI drug complexes (Ren *et al.*, 1995). These studies also revealed that a variety of different NNRTI structures bind to a well-defined lipophilic pocket in HIV-1 RT, and usually subtle differences in their interactions with the protein could be found between the different NNRTIs. The recently published crystal structure of HIV-2 RT (Bird *et al.*, 2003; Ren *et al.*, 2002) revealed that HIV-2 RT has a similar overall fold to HIV-1 RT but has structural differences in a putative NNRTI pocket at both conserved and non-conserved residues. The crystal structure points to a role of sequence differences that can give rise to unfavorable inhibitor contacts or cause destabilisation of parts of the binding pocket at amino acid positions 101, 106, 138, 181, 188 and 190. There is also confirmation that the HIV-2 RT Ile-181 amino acid compared with the HIV-1 RT Tyr-181 could be a significant contributing factor to the inherent drug resistance of HIV-2 to NNRTIs. However, there have been a few reports on a modest inhibitory activity of some NNRTIs against other lentiviruses. TIBO has found to be inhibitory to several SIV strains in MT-4 cell cultures (Debyser *et al.*, 1992), and delavirdine and a few other NNRTIs were reported to be inhibitory to the HIV-2 EHO and SIV agm3 strains (but not to the HIV-2 ROD and SIV mac251 and SIV mndGB1 strains) in MT-4 cell cultures (Witvrouw *et al.*, 1999).

One defined series of NNRTIs previously described are the phenylethylthiazoylthiourea (PETT) derivatives that were reported to have potent activity against HIV-1 RT (Ahgren *et al.*, 1995; Bell *et al.*, 1995; Cantrell *et al.*, 1996; Hogberg *et al.*, 1999). Further PETT analogues have been designed and synthesized based on structure-activity relationships and molecular modeling (Hu *et al.*, 2002; Ludovici *et al.*, 2001; Mao *et al.*, 1998; Ranise *et al.*, 2003; Sahlberg *et al.*, 1998; Vig *et al.*, 1998) and several of them showed activity against HIV-1 in the low nanomolar concentration range. Interestingly, some members among the NNRTI PETT derivatives inhibit HIV-2 RT at ~2 μM (Ren *et al.*, 2000). Kinetic analysis of a member of the PETT series (PETT-2) with both HIV-1 and HIV-2 RT indicated non-competitive inhibition modes with respect to dNTP. PETT-2 also showed non-competitive inhibition of HIV-2 RT with respect to either template/primer or substrate. Such kinetic results are consistent with PETT-2 binding to HIV-2 RT at a site equivalent to the HIV-1 RT NNRTI-binding site but do not eliminate the possibility that the inhibitor binds at a distinctive allosteric site.

In the present study we investigated the anti-HIV-1 and -HIV-2 activity of MSK-076 (Fig 4.1) and selected for PETT-resistant HIV-1 and HIV-2 strains in cell culture. The nature of the amino acid mutations that appeared in the HIV-2 RT were found to be the novel mutations at amino acid positions 112 and 101, resulting in full resistance of mutant HIV-2 in cell culture and recombinant mutant HIV-2 RT to MSK-076. The significance of these findings will be discussed from a structural viewpoint as based on the available coordinates of the HIV-2 RT structure.

MATERIALS AND METHODS

Viruses

HIV-1 (III_B) and HIV-2 (ROD) were kindly provided by Dr. R.C. Gallo (at that time at the National Cancer Institute, NIH, Bethesda, MD, USA) and Dr. L. Montagnier (at that time at the Pasteur Institute, Paris, France), respectively.

Cells

CEM cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% foetal bovine serum (Integro, Leuvenheim, The Netherlands), 2mM L-Glutamine (Invitrogen) and 0.075% $NaHCO_3$ (Invitrogen).

Test compounds

The PETT derivative MSK-076 was kindly provided by Medivir AB, Huddinge, Sweden. Delavirdine and lamivudine were provided by Dr. Jörg-Peter Kleim (GlaxoSmithKline, Stevenage, UK). Nevirapine BI-RG587 was obtained from Boehringer Ingelheim (Ridgefield, CT). UC-781 was obtained from Uniroyal Chemical Ltd. (Middlebury, CT & Guelph, ON, Canada). Efavirenz was obtained from Dr. L. Bacheler (DuPont Pharmaceuticals, Wilmington, DE). MSK-076 was provided by Medivir (Lunastigen 7, Huddinge, Sweden). Foscarnet (PFA) and zidovudine were purchased from Sigma Chemicals (St. Louis, MO). Stavudine, didanosine and zalcitabine were provided by Dr. D. G. Johns (NCI, NIH, Bethesda, MD). Tenofovir was obtained from Gilead Sciences (Foster City, CA). The bicyclam, AMD3100, a CXCR4 antagonist, was provided by Dr. G. Henson, AnorMed (Langley, Canada). Ritonavir was obtained from Abbott Laboratories (Abbott Park, IL). The

quinolone-derivative K-37, an inhibitor of HIV mRNA synthesis (Baba *et al.*, 1998), was kindly provided by Prof. M. Baba (Kagoshima, Japan).

MSK-076 PETT-2

Figure 4.1 Structural formula of the PETT derivatives MSK-076 and PETT-2

Drug activity assay against wild-type and mutant HIV-1 and HIV-2 strains

CEM cells were suspended at 250,000 cells/ml of RPMI 1640 cell culture medium (supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 0.075% $NaHCO_3$) and infected with wild-type HIV-1(III_B) and HIV-2(ROD) or mutant HIV-1 or HIV-2 strains at ~100 $CCID_{50}$ (50% cell culture infected doses) per ml. Then, 100 µl of the infected cell suspension was added to 200 µl-microtiter plate wells containing 100 µl of an appropriate concentration of the test compounds. After 4 days of incubation at 37°C, the cell cultures were examined for HIV-induced syncytium formation. The 50% effective concentration (EC_{50}) was determined as the compound concentration required to inhibit HIV-induced syncytium formation by 50%.

Selection of PETT-and lamivudine-resistant HIV-1 and HIV-2 strains in CEM cell cultures

MSK-076 or lamivudine were exposed at fixed concentrations (indicated in the Tables 4.1 and 4.3) to 1 ml HIV-1- or HIV-2-infected CEM cell cultures in 48-well microtiter plates. For each subcultivation (every 3 or 4 days), 0.1 ml of the drug-treated HIV-infected cell cultures was added to 900 µl of fresh CEM cells (at ~300,000 cells/well). The drug concentrations were not increased during ~14 subsequent subcultivations and the drug-exposed HIV-infected CEM cell culture supernatants were frozen in aliquots at –70°C after abundant syncytium formation became evident. Those cell cultures that did not show visible giant cell formation after 12 subcultivations were further passaged for at least an additional 3 subcultivations in the absence of the test compounds. Then, p24-determinations were performed on the culture supernatant fluids by a p24 enzyme-linked immunosorbent assay

(ELISA) (DuPont, Brussels, Belgium) according to the manufacturer's instructions, to confirm lack of virus production in the cell cultures.

Time-of-drugaddition experiments in CEM cells

CEM cell cultures (5 x 10^5 cells/ml) were infected with HIV-2(ROD) at approximately 100 times the 50% cell culture infectious dose ($CCID_{50}$) per ml. Following a 2 h adsorption period, cells were washed 3 times and incubated at 37 °C. The following test compounds were added at ≥100-fold their EC_{50} values at different time points (0, 1, 3, 5, 7, 9, 12, 18, 24, 36 h) after virus infection: the CXCR4 antagonist AMD3100 at 10 μM, the NRTI lamivudine at 90 μM, the NtRTI tenofovir at 700 μM, MSK-076 at 50 μM, the HIV protease inhibitor ritonavir at 15 μM and the HIV transcription inhibitor K-37 at 5 μM. Viral p24 antigen production was determined at 72 hours post infection by a HIV-2 p24 ELISA (Innogenetics, Ghent, Belgium).

Inhibitory effect of test compounds against recombinant HIV-1 and HIV-2 reverse transcriptase and kinetic assays

The assay procedure for measuring the inhibitory effect of the test compounds against HIV RT and steady-state kinetic assays have been described previously (Balzarini *et al.*, 1992) and were performed as described in chapter 2. To determine the K_i value of PETT (MSK-076) ($K_{i,\ PETT}$) and its kinetic mechanism of RT inhibition (competitive/non-competitive/uncompetitive), the assays (using [8-^{3}H]dGTP and poly(rC).oligo(dG_{12-18})) were performed in the presence of different concentrations of MSK-076 [26 and 52 μM for HIV-2 RT and 0.013, 0.026 and 0.052 μM for HIV-1 RT].

Site-directed mutagenesis of amino acids A101P and G112E in HIV-2(ROD) RT

Mutant enzymes used in this study were translated from the HIV-2(ROD) RT sequence which was cloned into pET21RT2p68. The *Nco*I-*Hind*III fragment from this plasmid, which contains the HIV-2 RT gene, was ligated into *Nco*I-*Hind*III-digested pKRT2His (Pelemans *et al.*, 1998) to create pKRT68His which contains a $(His)_6$ tag with the purpose of easy purification. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Briefly, supercoiled double-stranded pKRT68His DNA and two synthetic oligonucleotide primers containing the desired mutation were used. For the mutation of Ala to Pro at position 101 primer JA7 (5'CAGTTAGGAATACCACACCCAGCAGG-ATTGCCCAAG-AAGAG) and the complement primer JA8 were used that contained the desired mutation at position 101 and a silent mutation that alters an *Eco*RI site (underlined).

For the G112E mutation primer JA9 (5'GAATTACTGTTCTAGATGTAGAGGATGCT-TACTTTTCCATAC) and the complement primer JA10 were used that contained the desired mutation and a silent mutation creating a unique *Xba*I restriction site (underlined). The procedure is further performed as described by Pelemans *et al.* (1998). Final confirmation of the presence of the mutation was done by sequencing the complete RT gene on an ABI Prism 3100 sequencer (Perkin Elmer) using the dRhodamine terminator cycle sequencing reaction kit (Perkin Elmer).

Preparation of HIV-2 RT-containing *Escherichia coli* extracts

Expression of recombinant RT and lysis of the bacterial pellet was performed as described in Chapter 3.

Location of resistance mutations in HIV reverse transcriptase structures

The position of the resistance mutations selected by MSK-076 in relation to ligand binding sites on RT was assessed in the program O (Jones *et al.*, 1991) using structures of HIV-1 and HIV-2 RTs. PETT-2, an analogue of MSK-076 which differs by a substituted phenyl ring instead of a substituted pyridine ring, and a cyclopropyl bridge instead of an ethylene bridge (see Fig 4.1), was modeled with the program SHP (Stuart *et al.*, 1979) into HIV-2 RT using an overlap of the 110 residues around the NNRTI site of the HIV-1 RT-PETT-2 structure (Ren *et al.*, 2000) with the corresponding residues of HIV-2 RT (Ren *et al.*, 2002). The structure of the catalytic complex of HIV-1 RT (Huang *et al.*, 1998) was used to map the relationship of G112 to the RT active site including the template-primer and dTTP ligands.

RESULTS

4.1. Antiviral activity of MSK-076

MSK-076 was evaluated for its inhibitory activity against HIV-1(III_B) and HIV-2(ROD) replication in CEM cell cultures. The compound inhibited HIV-1-induced cytopathicity at a 50% effective concentration (EC_{50}) of 0.0018 μM. The HIV-2-induced cytopathicity was inhibited at an EC_{50} of 0.63 μM (Table 4.6), that is at a ~300- fold higher concentration than the concentration required to inhibit HIV-l. The compound was not markedly cytotoxic to the cell cultures at 50 μM.

Table 4.1 Appearance of HIV-2(ROD)-induced cytopathicity in CEM cell cultures in the presence of fixed concentrations of MSK-076 and lamivudine

Compound	Conc (μM)	Passage number									
		1	2	3	4	5	6	7	8	//	12
MSK-076	1.0	12	100	100	100	100	100	100*	-		-
MSK-076	2.1	3	25	75	100	100	100	100*	-		-
MSK-076	4.2	0	0	3	12	100	100	100*	-		-
MSK-076	8.3	0	0	0	0	0	6	25	100		100*
Lamivudine	1.7	0	0	12	100	100	100	100*	-		-
Lamivudine	3.5	0	0	6	100	100	100	100*	-		-
Lamivudine	4.2	0	0	25	100	100	100	100*	-		-
Lamivudine	14	0	0	50	100	100	100	100*	-		-
Lamivudine	28	0	0	25	100	100	100	100*	-		-

*Asterisk denotes the supernatants of the virus passage that has been prepared for PCR and sequence analysis of proviral DNA.

Table 4.2 Mutations in HIV-2(ROD) RT that appeared in HIV-2-infected CEM cell cultures under MSK-076 drug pressure

Concentration (μM)	Mutation in the HIV-2 RT	Codon change
First experiment		
1.0	Wild-type	-
2.1	Wild-type	-
4.2	G112E	GGG → GAG
8.3	G112E	GGG → GAG
Second experiment		
8.3	A101A/P	GCC → CCC/GCC

4.2. Time (site) of intervention

Time-of-drugaddition experiments were performed to pinpoint the possible step(s) of the replication cycle of HIV-2 that could be inhibited by MSK-076 (Fig. 4.2). The CEM cell cultures were infected at a high multiplicity of infection by HIV-2(ROD). The p24 antigen production was measured as the parameter of viral replication. The test compounds were added at different time points after infection. The experiments revealed that the antiviral potential of MSK-076 in the virus-infected cell cultures could be preserved as long as drug addition was delayed for no longer than 5 hours post infection. Longer delay of drug administration resulted in diminished suppression of virus production by MSK-076. This is comparable with earlier findings for NNRTIs in HIV-1-infected cell cultures (Pauwels *et al.*,

1990). NRTIs and NtRTIs, such as lamivudine and tenofovir, loose their antiviral activity when added later than 4 hours after infection, thus approximately 0.5 to 1 hour, before the NNRTIs (including MSK-076) loose their antiviral potential. The time-of-addition studies performed in the same experiment clearly confirmed that the bicyclam entry-inhibitor AMD3100 interacted with an early replication step (virus entry) and the protease inhibitor ritonavir with a late stage of the HIV replication cycle. Also, administration of K-37, a compound that inhibits mRNA transcription, could be markedly delayed before its antiviral activity was lost (Fig 4.2). Our findings indicate that MSK-076 most likely interacts at the reverse transcription process during HIV-2 infection, and thus might act as an "NNRTI" against HIV-2 in cell culture.

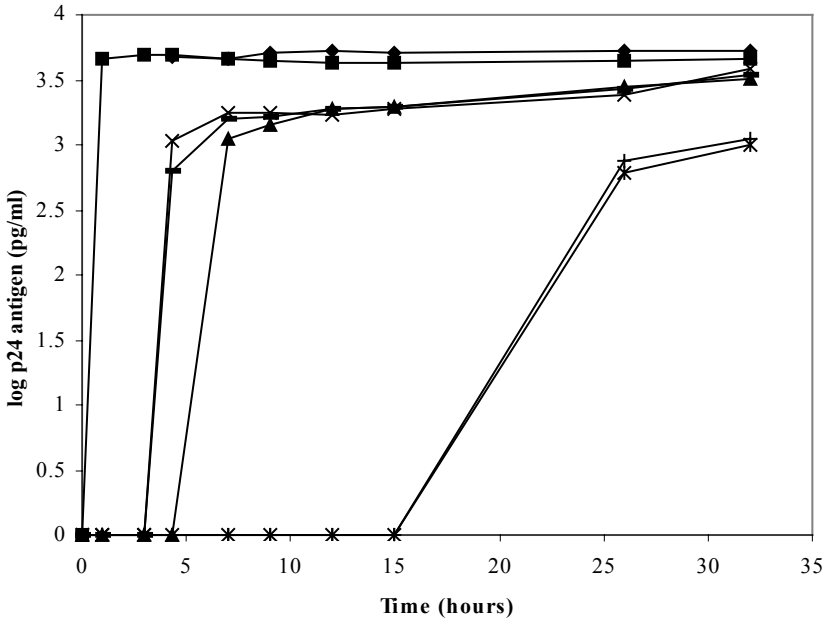

Figure 4.2 Time-of-drugaddition experiment. CEM cells were infected with HIV-2(ROD) at approximately 100 times the 50% cell culture infectious dose ($CCID_{50}$) per ml.

Test compounds were added at different times post infection. Viral p24 antigen production was determined at 72 h post infection. The drug concentrations were as follows: ■, AMD3100 at 10 µM; ▬,lamivudine at 90 µM; x, tenofovir at 700 µM; ▲,MSK-076 at 50 µM; *, ritonavir at 15 µM; +, K-37 at 5 µM; ♦, nontreated control.

Table 4.3 Breakthrough of HIV-1(III_B) in CEM cell cultures treated with different concentrations of MSK-076 or lamivudine

Compound	Drug concentration (μM)	Passage number[a]													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
MSK-076	0.002	75	100	100	100	100	100	100	100	100	100*	-	-	-	-
MSK-076	0.002	50	100	100	100	100	100	100*	-	-	-	-	-	-	-
MSK-076	0.01	0	6	3	12	50	100	100	100	100	100*	-	-	-	-
MSK-076	0.01	0	0	1	6	50	100	100	100*	-	-	-	-	-	-
MSK-076	0.05	0	0	0	0	0	0	0	0	0	0*	-	-	-	-
MSK-076	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0*
MSK-076	0.26	0	0	0	0	0	0	0	0	0	0	0	-	-	-
MSK-076	1.3	0	0	0	0	0	0	0	0	0	0	0	-	-	-
Lamivudine	0.04	100	100	100	100	100	100	100	100*	-	-	-	-	-	-
Lamivudine	0.09	75	100	100	100	100	100	100	100*	-	-	-	-	-	-
Lamivudine	0.22	18	50	82	100	100	100	100	100*	-	-	-	-	-	-
Lamivudine	0.44	0	12	12	12	100	100	100	100*	-	-	-	-	-	-
Lamivudine	0.88	0	3	0	6	50	100	100	100*	-	-	-	-	-	-
Lamivudine	1.7	0	0	0	0	25	100	100	100*	-	-	-	-	-	-
Lamivudine	3.5	0	0	0	0	25	100	100	100	100*	-	-	-	-	-
Lamivudine	7	0	0	0	0	3	0	6	6	25	75	100	100*	-	-
Lamivudine	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0*

*Asterisk denotes the supernatants of the virus passage that has been prepared for PCR and sequence analysis of proviral DNA.

[a] Virus breakthrough was monitored by scoring the appearance of virus-induced cytopathicity (CPE; syncytium formation) as percentage of the CPE observed in virus-infected control cultures in the absence of drug.

4.3. Selection of mutant HIV-2(ROD) and HIV-1(III_B) strains in the presence of MSK-076

Mutant HIV-2 strains resistant to MSK-076 were selected in CEM cell cultures by passaging the virus in the presence of a variety of fixed concentrations of MSK-076 (Table 4.1). HIV-2(ROD) was able to fully replicate in the continuous presence of 4.2 μM MSK-076 after 5 passages (designated HIV-2/MSK-076a) or after 8 passages in the presence of 8.2 μM MSK-076 (designated HIV-2/MSK-076b). Proviral DNA of these virus isolates was harvested at passage 7 and 12, respectively, and sequenced. Genotypic analysis of the RT-coding gene of the MSK-076-resistant viruses HIV-2/MSK-076a and HIV-2/MSK-076b revealed the amino acid mutation G112E (Table 4.2). In a second independent experiment, another MSK-076-exposed HIV-2 resistant strain selected in presence of 8.2 μM MSK-076 was isolated and shown to contain the amino acid mutation A101A/P in its RT.

Under similar experimental conditions the selection of HIV-2(ROD) strains was carried out in the presence of fixed concentrations of lamivudine (Table 4.1). Virus was able to afford full cytopathogenicity grow after 4 passages in the presence of lamivudine concentrations between 1.7 and 28 μM. Analyzing the genotype of the virus strains selected in the presence of lamivudine, showed that the Met184Ile was invariably present in the RTs of all virus isolates recovered at passage 7.

Table 4.4 Mutations in HIV-1(III_B) RT that appeared in HIV-1-infected CEM cell cultures under MSK-076 drug escalating concentrations

Concentration (μM)[a]	Mutation in the HIV-1 RT	Codon change
0.002	wild-type	-
		-
0.01	K101E	AAA→GAA
	Y181C/Y	TAT→TGT/TAT
↓		
0.05	K101E	AAA→GAA
	Y181C	TAT→TGT
↓		
0.26	K101E	AAA→GAA
	Y181C	TAT→TGT
	G190R	GGA→AGA

[a] The virus isolate that appeared in the presence of a fixed concentration of 0.01 μM was exposed to 0.05 μM MSK-076 for 5 subcultivations. The resulting virus isolate was then further exposed to 0.26 μM MSK-076 for an additional 10 subcultivations.

HIV-1(III_B) was also exposed to a variety of fixed concentrations MSK-076 (in duplicate) (Table 4.3). Full breakthrough of virus-induced cytopathicity replication occurred in the presence of a fixed MSK-076-concentration of 0.01 μM after 6 passages. Higher fixed concentrations of MSK-076 (i.e. 0.05, 0.26 and 1.3 μM) prevented breakthrough of virus. Genotypic analysis of the proviral DNA of the virus isolates grown in the presence of 0.01 μM revealed the K101E mutation and a mixture of Y181C/Y in HIV-1 RT (Table 4.4), which have already been described earlier as HIV-1 RT resistance mutations appearing in the presence of NNRTIs (for a review of NNRTI-specific mutations in RT, see Balzarini (1999)). When the mutant HIV-1 strain that emerged in the presence of 0.01 μM MSK-076 was exposed to escalating MSK-076 concentrations, additional mutations appeared. At a concentration of 0.05 μM MSK-076, a pure Y181C mutation was observed in combination with K101E, whereas, further increasing of the drug concentration to 0.26 μM resulted in the appearance of an additional (third) mutation (G190R) in the RT of the HIV-1 isolate (Table 4.4).

Selection of HIV-1 virus strains under lamivudine pressure showed that mutant virus strains emerged at different (fixed) drug concentrations that ranged between 0.44 and 7 μM. At 14 μM lamivudine, no virus breakthrough was observed. Analysis of the HIV-1 proviral DNA of the several isolates showed that the virus strains contained the Met184Ile mutation in their RT.

4.4. Inhibitory activities of NNRTIs, ddGTP and PFA against wild-type and mutant HIV-2 RT

By site-directed mutagenesis, we constructed mutant HIV-2 RTs with single amino acid replacements (A101P and G112E) and a double mutant containing both amino acid mutations in the same RT. The recombinant wild-type, mutant A101P, mutant G112E and double mutant A101P+G112E RTs were evaluated for their sensitivities to a variety of NNRTIs, ddGTP and PFA (with [^{3}H]dGTP as the radiolabeled substrate and poly(rC).oligo(dG) as the template/primer) (Table 4.5). The single and double mutant HIV-2 RTs retained their insensitivity to the NNRTIs efavirenz, delavirdine and UC-781, as being also the case for wild-type HIV-2 RT. However, in contrast to wild-type HIV-2 RT, which showed pronounced sensitivity to the inhibitory activity of MSK-076 (IC_{50}= 22.9 μM), none of the mutant HIV-2 RT enzymes showed sensitivity towards MSK-076 at a drug concentration as high as 300 μM. Thus, the single mutant A101P and G112E RTs and the double mutant RT bearing the two mutations together in one enzyme molecule showed a >15-

fold degree of resistance to MSK-076. The inhibition values obtained for ddGTP and PFA for the wild-type and mutant HIV-2 RTs were very similar.

Table 4.5 Inhibitory activity of test compounds against wild-type and mutant recombinant HIV-2 reverse transcriptase

Compound	IC_{50}[a] (μM)			
	wild-type	A101P	G112E	A101P+G112E
MSK-076	22.9 ± 0.1	> 300	> 300	> 300
Delavirdine	> 300	> 300	> 300	> 300
Efavirenz	> 300	> 300	> 300	> 300
UC-781	> 300	> 300	> 300	> 300
DdGTP	0.25 ± 0.20	0.23 ± 0.19	0.28 ± 0.01	0.22 ± 0.01
PFA	3.36 ± 1.25	2.58 ± 0.60	1.77 ± 0.13	0.56 ± 0.14

[a] 50% inhibitory concentration or compound concentration required to inhibit the HIV-2 RT activity by 50%. Poly(rC).oligo(dG) was used as template/primer and 1.4 μM [8-^{3}H]dGTP was used as the radiolabeled substrate.

Table 4.6 Sensitivity of mutant HIV-2 strains containing the mutations G112E and Met184Ile in their RT to the inhibitory effect of NRTIs, NtRTIs and NNRTIs

Compound	EC_{50}[a] (μM)				
	HIV-1 wild-type	HIV-2 wild-type	HIV-2/MSK-076a[b]	HIV-2/MSK-076b[c]	HIV-2/Met184Ile[d]
Zidovudine	0.0036 ± 0.0021	0.0071 ± 0.0037	0.01 ± 0.0	0.01 ± 0.002	0.005 ± 0.002
Stavudine	0.41 ± 0.32	0.42 ± 0.27	0.65 ± 0.21	0.48 ± 0.45	0.40 ± 0.14
Zalcitabine	0.030 ± 0.023	0.024 ± 0.014	0.06 ± 0.03	0.04 ± 0.02	0.08 ± 0.0
Didanosine	6.33 ± 3.21	13.8 ± 6.3	9.5 ± 3.5	10 ± 4.9	16 ± 5.7
Lamivudine	0.057 ± 0.026	0.11 ± 0.03	0.22 ± 0.13	0.31 ± 0.22	> 400
Adefovir	8.0 ± 3.6	7.9 ± 3.9	21 ± 13	26 ± 20	12 ± 0.0
PFA	44.4 ± 11.0	32.3 ± 13.0	20.8 ± 14.6	10.4 ± 7.3	78.1 ± 0.0
Nevirapine	0.029 ± 0.016	> 70	> 70	> 70	> 70
Delavirdine	0.019 ± 0.012	> 40	> 40	> 40	≥ 40
UC-781	0.0045 ± 0.0012	> 10	> 10	> 10	> 10
MSK-076	0.0018 ± 0.0003	0.63 ± 0.21	> 50	> 50	1.69 ± 0.18

[a]50% Effective concentration or compound concentration required to inhibit virus-induced cytopathicity by 50%.

[b] selected in the presence of 4.2 μM MSK-076

[c] selected in the presence of 8.3 μM MSK-076

[d] selected in the presence of 28 μM lamivudine

4.5. Inhibitory activity of NNRTIs, N(t)RTIs and PFA against wild-type and mutant HIV-2/MSK-076a and HIV-2/MSK-076b strains in CEM cell cultures

To reveal to what extent the novel mutation (G112E) found in two PETT-treated HIV-2 strains affected the sensitivity of these virus strains to MSK-076 and a variety of NNRTIs, NRTIs and the NtRTI adefovir, the two virus isolates together with a lamivudine resistant HIV-2 strain were evaluated for their sensitivity to these drugs (Table 4.6). Interestingly, the NtRTI and the NRTIs listed in Table 4.6 showed similar suppressive activity against the wild-type and the mutant HIV-2 strains except for lamivudine that had completely lost antiviral activity against the Met184Ile RT-mutated virus strain. However, MSK-076 completely lost (>80-fold) antiviral activity against the G112E RT mutated virus isolates, keeping full activity against the wild-type (EC_{50}= 0.63 μM) and Met184Ile RT mutated virus (EC_{50}= 1.69 μM).

4.6. Kinetic analysis of the nature of inhibition of HIV-2 and HIV-1 RT by MSK-076

The K_i values for MSK-076 ($K_{i,\ PETT}$) against HIV-1 and HIV-2 RT using substrate (dGTP) and template/primer poly(rC).oligo(dG) are shown in Table 4.7. The $K_{i,\ PETT}$ value for HIV-2 RT was 14.6 μM (against dGTP) and 26.1 μM [against template/primer poly(rC).oligo(dG)]. These values are in agreement with the corresponding IC_{50} values for drug-exposed RT (IC_{50}= 22.9 μM) in the presence of dGTP (2.5 μM). For HIV-1 RT we found $K_{i,\ PETT}$ values as low as 0.0052 μM when using dGTP as variable substrate and 0.0044 μM using poly(rC).oligo(dG) at variable template/primer concentrations. When the $K_{i,\ PETT}/K_M$ ratio for HIV-1 RT [0.0003 for poly(rC).oligo(dG) and 0.0023 for dGTP] was compared with that of HIV-2 RT [0.73 for poly(rC).oligo(dG) and 8.1 for dGTP], could be concluded that MSK-076 binds with a much higher affinity to HIV-1 RT than to HIV-2 RT. This is in line with the markedly higher (> 100-fold) inhibitory activity of MSK-076 against HIV-1 than HIV-2 replication in CEM cell cultures.

Table 4.7 Kinetic analysis of HIV-1 and HIV-2 RT enzymes with poly(rC).oligo(dG) as template/primer and dGTP as variable substrate

	HIV-1 RT		HIV-2 RT	
	poly(rC).oligo(dG)	dGTP	poly(rC).oligo(dG)	dGTP
$K_{i,\ PETT}$ (μM)	0.0044	0.0052	26.1	14.6
$K_{i,\ PETT}/K_M$	0.0003	0.0023	0.73	8.1

To investigate the kinetic mechanism of inhibition of HIV-2 RT by MSK-076, we analyzed the mode of inhibition of HIV-2 RT, in comparison with that of HIV-1 RT in the presence of various concentrations of MSK-076 (Fig 4.3). Double-reciprocal Lineweaver-Burk plots for the inhibition of the HIV-2 and HIV-1 RTs by MSK-076 with respect to dGTP as variable substrate, or poly(rC).oligo(dG) as variable template/primer, revealed in both cases a noncompetitive inhibition, indicating binding of the drug to the enzyme being independent from prior binding of the substrate or template/primer to the HIV-1 or HIV-2 RTs.

4.7. Structural context for the role of amino acid mutations G112E and A101P in HIV-2 RT

The position of PETT-2, an analogue of MSK-076 determined from the crystal structure of the complex with HIV-1 RT is shown in relation to the HIV-2 RT active site and putative NNRTI-binding pocket (Fig 4.4a). It is clear that the PETT compound is in close juxtaposition to the A101P mutation, whilst the separation between the NNRTI and G112E is much larger (>12 Å). The location of Gly112 in relation to the active site in the HIV-1 RT catalytic complex is shown in Fig. 4.4b. Gly112 is two residues away from one of the key catalytic aspartates, Asp110. The introduction of a bulky negatively charged side-chain adjacent to the polymerase active site, however, does not appear to compromise HIV-2 RT enzyme activity.

DISCUSSION

We have shown that MSK-076 is a potent inhibitor of HIV-1 in cell culture. This is in line with reports for other members of the PETT-series (Ahgren *et al.*, 1995; Cantrell *et al.*, 1996). However, the PETT compound reported here has also the ability to markedly inhibit HIV-2, albeit at a much higher concentration than required for HIV-1. Only recently the inhibitory activity of other related members of the PETT series against HIV-2 have been reported (Ren *et al.*, 2000). Also, a modest activity of the NNRTIs delavirdine and emivirine (MKC442) against the HIV-2(EHO) strain, but not the HIV-2(ROD) strain, has been reported (Witvrouw *et al.*, 1999). The possibility that the remarkable activity of MSK-076 against HIV-2 compared to the inactivity of other NNRTIs is due to interaction with a different target or, alternatively, that it inhibits RT but binds to an entirely different site of the enzyme than the "classical" NNRTIs, was considered. First, time-of-addition experiments reported here show that the

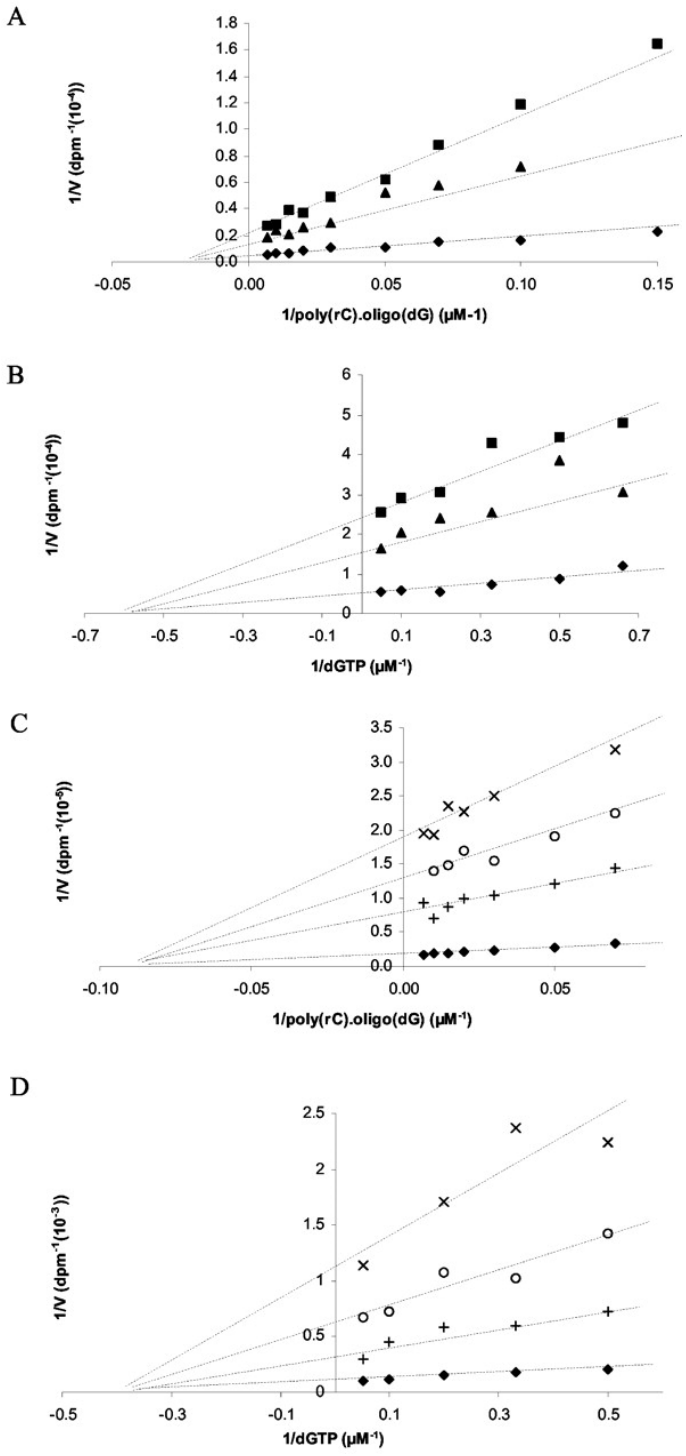

Figure 4.3 Double-reciprocal plots for inhibition of wild-type HIV-2 RT (A and B) and HIV-1 RT (C and D) by MSK-076.

MSK-076 concentrations were: ■, 52 µM; ▲, 26 µM; X, 0.052 µM, ○, 0.026 µM; +, 0.013 µM and ♦, 0 µM (control). In panels A and C, template/primer [0.1 mM poly(rC).oligo(dG)] and variable concentrations of [^{3}H]dGTP were used. In panels B and D, 1.4 µM [8-^{3}H] dGTP and variable concentrations of template/primer poly(rC).oligo(dG) were used.

molecular site of action of MSK-076 is most likely the HIV-2 RT. Compared to the NRTIs, which loose approximately one hour earlier their antiviral activity than MSK-076 when drug administration is delayed after the time of virus infection, the time of interaction with its target after infection is in agreement with that found for all other NNRTIs against HIV-1. Second, the MSK-076 has been found inhibitory to HIV-2 RT in contrast with the other "classical" NNRTIs such as efavirenz, UC-781, delavirdine and nevirapine. Moreover, the decreased affinity of HIV-2 RT *versus* HIV-1 RT for the MSK-076 compound correlated well with the decreased antiviral activity of MSK-076 against HIV-2 *versus* HIV-1 in cell culture. This observation is in agreement with the view that the HIV-2 target for MSK-076 inhibition is the virus-encoded RT. Third, kinetic studies, performed in an attempt to elucidate the mode of HIV-2 RT inhibition by MSK-076 in comparison with HIV-1 RT inhibition, showed that MSK-076 behaves noncompetitively with respect to both the substrate dGTP and the template/primer poly(rC).oligo(dG). This indicates that MSK-076 does not interfere with HIV-2 RT by binding at the dNTP site, or at the DNA-binding site, but binds at another allosteric site of the enzyme. These data also reveal that MSK-076 neither competes with the dNTP substrate nor with the template/primer, making it likely that MSK-076 interacts with a presumable NNRTI-pocket in HIV-2 RT similar or closely resembling the NNRTI-binding site in HIV-1 RT. Fourth, another important observation that points to RT as the target for the inhibitory effect of MSK-076 is the selection of a 101-A (GCC) to P (CCC) mutation and a 112-G (GGG) to E (GAG) mutation in the RT gene of HIV-2(ROD) under MSK-076 pressure. In fact, in HIV-1-infected cell cultures, MSK-076 also selects for an amino acid mutation at position 101 (K to E), but also at position 181 (Y to C) and position 190 (G to R). The latter amino acid mutations are already described to appear in the presence of NNRTIs (Balzarini, 1999; Schinazi *et al.*, 2001). Interestingly, in HIV-1 RT, the K101 plays an important role in binding of PETT derivatives through a hydrogen bond between the peptide main chain in the NNRTI-pocket and the thiourea moiety of PETT (Ren *et al.*, 2000). Emergence of this homologous amino acid mutation in HIV-2 RT may indicate that this amino acid may affect the binding of MSK-076 in HIV-2 RT as well. However, it should be noticed that the amino acid at position 101 in HIV-2 RT (A) is different from the corresponding amino acid in HIV-1 RT (K) and that mutation to a proline in HIV-2 RT has never been observed in HIV-1 RT at this amino acid site. Proline at residue 101 in HIV-2 RT may give rise to a more profound structural effect on the putative NNRTI pocket than the more classical mutations at residue 101 found in HIV-1 RT (i.e. E, I or Q), due to lower conformational flexibility and the replacement of the main-chain NH by an NC link. Indeed it is likely that P101 can distort the

key hydrogen bonding interaction from the inhibitor to the main-chain carbonyl observed for many NNRTIs in HIV-1 RT including PETT-2. The G112E mutation, on the other hand, also found to appear under MSK-076 pressure in HIV-2 RT, has never previously been described in relation to NNRTI resistance in HIV-1 RT (including PETT analogues). The location of this mutation is adjacent to the dNTP binding site in the RT (D110, D185, D186). Indeed it is interesting that the introduction of a bulky negatively charged side-chain can be tolerated at a position that is relatively close to the key catalytic machinery of the polymerase active site. Kinetic data indicate that MSK-076 is not competing at the dNTP site and thus the occurrence of G112E as a resistance mutation is somewhat surprising given that this mutation is distal to the putative NNRTI site. Generally mutations giving resistance to NNRTIs are in direct contact with the inhibitor in HIV-1 RT. There are exceptions, however, such as K103N and V108I, which do not necessarily interact directly with the NNRTIs but nevertheless are still situated close to the inhibitor binding site.

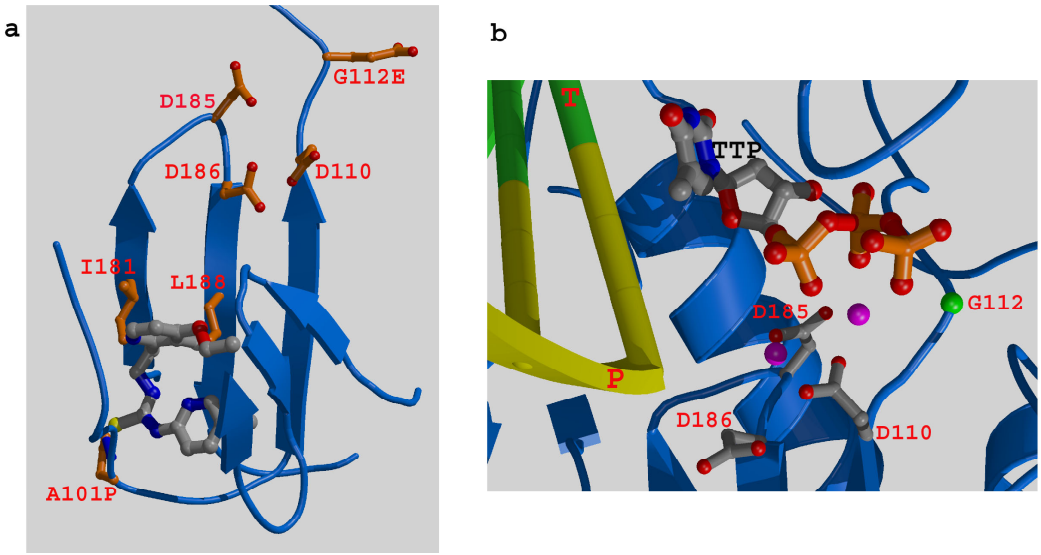

Figure 4.4 Positions of MSK-076 resistance mutations A101P and G112E in HIV RT **a)** A101P and G112E mutations relative to the polymerase active site and the putative NNRTI site in HIV-2 RT. The protein backbone is shown as blue ribbons and coils, and protein side-chains are shown as ball-and-sticks with carbon, oxygen and nitrogen atoms coloured in orange, red and blue, respectively. The thicker ball-and-sticks with carbon atoms coloured grey show the PETT-2 molecule to mark the putative NNRTI site. The possible orientations of 101P and 112E were shown.

b) Position of residue G112 (green sphere) relative to the polymerase active site of HIV-1 RT. The blue ribbons and coils represent the protein backbones. The protein side-chains and the substrate dTTP are shown as thinner and thicker ball-and-sticks, respectively. The two purple spheres represent manganese ions, and the yellow and green coloured ladder shows the bound oligo-nucleotide with the template and primer strands labeled with letters T and P, respectively. Figure generated with the programs O and SHP.

The kinetic data and the presence of the A101P mutation are consistent with MSK-076 binding to HIV-2 RT at the equivalent site as in the HIV-1 RT NNRTI-binding site, however the possibility of a different additional binding site for this inhibitor cannot be discounted. However, although RNase H binding, or both DNA polymerase and RNase H binding like the compounds described by (Borkow *et al.*, 1997) cannot be excluded, such a site of drug interaction may be unlikely due to the fact that resistance mutations in the RT of MSK-076-resistant virus strains are on a marked distance from the RNase H binding site. The structural basis for the mechanism of resistance to MSK-076 induced by G112E is not clear. It is known that the mechanism of inhibition of NNRTIs in HIV-1 RT is via a distortion of the active site aspartates (Esnouf *et al.*, 1995). It is conceivable that displacement of the active site aspartates presumed to be caused by the NNRTI-binding to HIV-2 RT can be prevented by the G112E mutation. However, another role of G112E in the resistance to MSK-076 cannot be excluded.

If we compared the speed of selection of resistant viruses between HIV-1 and HIV-2 in the presence of MSK-076, we observed a faster emergence of MSK-076-resistant virus in HIV-2, compared to the much slower breakthrough of drug-resistant virus for HIV-1 under MSK-076 pressure. Therefore, MSK-076 seems to behave more as a second-generation NNRTI against HIV-1 and rather as a first-generation NNRTI against HIV-2, probably due to its lesser potency as an anti-HIV-2 RT agent.

In this respect, it should also be noticed that the K_i value of MSK-076 and its EC_{50} value for HIV-1 in cell culture are close (0.0052 μM and 0.0018 μM, respectively) while the K_i value of MSK-076 for HIV-2 RT and its EC_{50} value for HIV-2 in cell culture differ by ~20 fold (14.6 μM and 0.63 μM, respectively). It has previously been observed for the first-generation NNRTIs that RT enzyme inhibition values (IC_{50}) can be considerably higher than the corresponding EC_{50} values in cell culture. This phenomenon has been ascribed to the rather artificial testing conditions in the enzyme assays (i.e. use of homopolymeric template/primer). Such a difference between K_i and EC_{50} values is usually less pronounced for second generation NNRTIs.

In conclusion, despite its inhibitory activity against HIV-2 RT, MSK-076 represents another member of the NNRTI class of compounds that act non-competitively at a specific site in both HIV-1 and HIV-2 RT. We could now show that the specificity of NNRTIs (including most PETT compounds) to solely inhibit HIV-1 RT could be broadened to a significant inhibition of HIV-2 (and HIV-2 RT) as well. Our kinetic, mutational and structural analysis revealed that the mode of binding of MSK-076 to HIV-2 RT might occur in a comparable manner as that for HIV-1 RT. These observations may have important implications for the

further development of novel NNRTIs with activity against HIV-2 because of the increasing prevalence and incidence of HIV-2 infections in developing countries. Furthermore, the rational design of potent drugs with a broad activity spectrum against a wider range of lentiviruses can be important for the treatment of (drug-resistant) HIV strains. The availability of the crystallographic HIV-2 RT structure coordinates may become instrumental in the design of more potent NNRTI (i.e. PETT) inhibitors with a broader antiretroviral spectrum.

REFERENCES

Ahgren C, Backro K, Bell FW, Cantrell AS, Clemens M, Colacino JM, Deeter JB, Engelhardt JA, Hogberg M, Jaskunas SR and et al. (1995) The PETT series, a new class of potent nonnucleoside inhibitors of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* **39**:1329-35.

Baba M, Okamoto M, Kawamura M, Makino M, Higashida T, Takashi T, Kimura Y, Ikeuchi T, Tetsuka T and Okamoto T (1998) Inhibition of human immunodeficiency virus type 1 replication and cytokine production by fluoroquinoline derivatives. *Mol Pharmacol* **53**:1097-103.

Bacolla A, Shih CK, Rose JM, Piras G, Warren TC, Grygon CA, Ingraham RH, Cousins RC, Greenwood DJ, Richman D and et al. (1993) Amino acid substitutions in HIV-1 reverse transcriptase with corresponding residues from HIV-2. Effect on kinetic constants and inhibition by non-nucleoside analogs. *J Biol Chem* **268**:16571-7.

Balzarini J (1999) Suppression of resistance to drugs targeted to human immunodeficiency virus reverse transcriptase by combination therapy. *Biochem Pharmacol* **58**:1-27.

Balzarini J, Karlsson A, Sardana VV, Emini EA, Camarasa MJ and De Clercq E (1994) Human immunodeficiency virus 1 (HIV-1)-specific reverse transcriptase (RT) inhibitors may suppress the replication of specific drug-resistant (E138K)RT HIV-1 mutants or select for highly resistant (Y181C-->C181I)RT HIV-1 mutants. *Proc Natl Acad Sci U S A* **91**:6599-603.

Balzarini J, Perez-Perez MJ, San-Felix A, Camarasa MJ, Bathurst IC, Barr PJ and De Clercq E (1992) Kinetics of inhibition of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase by the novel HIV-1-specific nucleoside analogue [2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3'-spiro-5 "-(4"-amino-1",2"-oxathiole-2",2"-dioxide)thymine (TSAO-T). *J Biol Chem* **267**:11831-8.

Bell FW, Cantrell AS, Hogberg M, Jaskunas SR, Johansson NG, Jordan CL, Kinnick MD, Lind P, Morin JM, Jr., Noreen R and et al. (1995) Phenethylthiazolethiourea (PETT) compounds, a new class of HIV-1 reverse transcriptase inhibitors. 1. Synthesis and basic structure-activity relationship studies of PETT analogs. *J Med Chem* **38**:4929-36.

Bird LE, Chamberlain PP, Stewart-Jones GB, Ren J, Stuart DI and Stammers DK (2003) Cloning, expression, purification, and crystallisation of HIV-2 reverse transcriptase. *Protein Expr Purif* **27**:12-8.

Borkow G, Fletcher RS, Barnard J, Arion D, Motakis D, Dmitrienko GI and Parniak MA (1997) Inhibition of the ribonuclease H and DNA polymerase activities of HIV-1 reverse transcriptase by N-(4-tert-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone. *Biochemistry* **36**:3179-85.

Cantrell AS, Engelhardt P, Hogberg M, Jaskunas SR, Johansson NG, Jordan CL, Kangasmetsa J, Kinnick MD, Lind P, Morin JM, Jr., Muesing MA, Noreen R, Oberg B, Pranc P, Sahlberg C, Ternansky RJ, Vasileff RT, Vrang L, West SJ and Zhang H (1996) Phenethylthiazolylthiourea (PETT) compounds as a new class of HIV-1 reverse transcriptase inhibitors. 2. Synthesis and further structure-activity relationship studies of PETT analogs. *J Med Chem* **39**:4261-74.

Condra JH, Emini EA, Gotlib L, Graham DJ, Schlabach AJ, Wolfgang JA, Colonno RJ and Sardana VV (1992) Identification of the human immunodeficiency virus reverse transcriptase residues that contribute to the activity of diverse nonnucleoside inhibitors. *Antimicrob Agents Chemother* **36**:1441-6.

Debyser Z, De Vreese K, Pauwels R, Yamamoto N, Anne J, De Clercq E and Desmyter J (1992) Differential inhibitory effects of TIBO derivatives on different strains of simian immunodeficiency virus. *J Gen Virol* **73**:1799-804.

Esnouf R, Ren J, Ross C, Jones Y, Stammers D and Stuart D (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. *Nat Struct Biol* **2**:303-8.

Hizi A, Tal R and Hughes SH (1991) Mutational analysis of the DNA polymerase and ribonuclease H activities of human immunodeficiency virus type 2 reverse transcriptase expressed in Escherichia coli. *Virology* **180**:339-46.

Hizi A, Tal R, Shaharabany M, Currens MJ, Boyd MR, Hughes SH and McMahon JB (1993) Specific inhibition of the reverse transcriptase of human immunodeficiency virus type 1 and the chimeric enzymes of human immunodeficiency virus type 1 and type 2 by nonnucleoside inhibitors. *Antimicrob Agents Chemother* **37**:1037-42.

Hogberg M, Sahlberg C, Engelhardt P, Noreen R, Kangasmetsa J, Johansson NG, Oberg B, Vrang L, Zhang H, Sahlberg BL, Unge T, Lovgren S, Fridborg K and Backbro K (1999) Urea-PETT compounds as a new class of HIV-1 reverse transcriptase inhibitors. 3. Synthesis and further structure-activity relationship studies of PETT analogues. *J Med Chem* **42**:4150-60.

Hu W, Timmons DJ and Doyle MP (2002) In search of high stereocontrol for the construction of cis-disubstituted cyclopropane compounds. Total synthesis of a cyclopropane-configured urea-PETT analogue that is a HIV-1 reverse transcriptase inhibitor. *Org Lett* **4**:901-4.

Huang H, Chopra R, Verdine GL and Harrison SC (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **282**:1669-75.

Isaka Y, Miki S, Kawauchi S, Suyama A, Sugimoto H, Adachi A, Miura T, Hayami M, Yoshie O, Fujiwara T and Sato A (2001) A single amino acid change at Leu-188 in the reverse transcriptase of HIV-2 and SIV renders them sensitive to non-nucleoside reverse transcriptase inhibitors. *Arch Virol* **146**:743-55.

Jones TA, Zou JY, Cowan SW, Kjeldgaard M (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr* **A47**:110-9.

Ludovici DW, Kukla MJ, Grous PG, Krishnan S, Andries K, de Bethune M, Azijn H, Pauwels R, De Clercq E, Arnold E and Janssen PA (2001) Evolution of anti-HIV drug candidates. Part 1: From alpha-Anilinophenylacetamide (alpha-APA) to imidoyl thiourea (ITU). *Bioorg Med Chem Lett* **11**:2225-8.

Mao C, Vig R, Venkatachalam TK, Sudbeck EA and Uckun FM (1998) Structure-based design of N-[2-(1-piperidinylethyl)]-N'-[2-(5-bromopyridyl)]-thiourea and N-[2-(1-piperazinylethyl)]-N'-[2-(5-bromopyridyl)]-thiourea as potent non-nucleoside inhibitors of HIV-1 reverse transcriptase. *Bioorg Med Chem Lett* **8**:2213-8.

Pauwels R, Andries K, Desmyter J, Schols D, Kukla MJ, Breslin HJ, Raeymaeckers A, Van Gelder J, Woestenborghs R, Heykants J and et al. (1990) Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. *Nature* **343**:470-4.

Pelemans H, Esnouf RM, Jonckheere H, De Clercq E and Balzarini J (1998) Mutational analysis of Tyr-318 within the non-nucleoside reverse transcriptase inhibitor binding pocket of human immunodeficiency virus type I reverse transcriptase. *J Biol Chem* **273**:34234-9.

Ranise A, Spallarossa A, Schenone S, Bruno O, Bondavalli F, Vargiu L, Marceddu T, Mura M, La Colla P and Pani A (2003) Design, synthesis, SAR, and molecular modeling studies of acylthiocarbamates: a novel series of potent non-nucleoside HIV-1 reverse transcriptase inhibitors structurally related to phenethylthiazolylthiourea derivatives. *J Med Chem* **46**:768-81.

Ren J, Bird LE, Chamberlain PP, Stewart-Jones GB, Stuart DI and Stammers DK (2002) Structure of HIV-2 reverse transcriptase at 2.35-A resolution and the mechanism of resistance to non-nucleoside inhibitors. *Proc Natl Acad Sci U S A* **17**:14410-5.

Ren J, Diprose J, Warren J, Esnouf RM, Bird LE, Ikemizu S, Slater M, Milton J, Balzarini J, Stuart DI and Stammers DK (2000) Phenylethylthiazolylthiourea (PETT) non-nucleoside inhibitors of HIV-1 and HIV-2 reverse transcriptases. Structural and biochemical analyses. *J Biol Chem* **275**:5633-9.

Ren J, Esnouf R, Garman E, Somers D, Ross C, Kirby I, Keeling J, Darby G, Jones Y, Stuart D and et al. (1995) High resolution structures of HIV-1 RT from four RT-inhibitor complexes. *Nat Struct Biol* **2**:293-302.

Ren J, Nichols C, Bird L, Chamberlain P, Weaver K, Short S, Stuart DI and Stammers DK (2001) Structural mechanisms of drug resistance for mutations at codons 181 and 188 in HIV-1 reverse transcriptase and the improved resilience of second generation non-nucleoside inhibitors. *J Mol Biol* **312**:795-805.

Sahlberg C, Noreen R, Engelhardt P, Hogberg M, Kangasmetsa J, Vrang L and Zhang H (1998) Synthesis and anti-HIV activities of urea-PETT analogs belonging to a new class of potent non-nucleoside HIV-1 reverse transcriptase inhibitors. *Bioorg Med Chem Lett* **8**:1511-6.

Schinazi RF, Larder BA and Mellors JW (2001) Mutations in retroviral genes associated with drug resistance: 2000-2001 update. *International Antiviral News* **8**:65-91.

Shih CK, Rose JM, Hansen GL, Wu JC, Bacolla A and Griffin JA (1991) Chimeric human immunodeficiency virus type 1/type 2 reverse transcriptases display reversed sensitivity to nonnucleoside analog inhibitors. *Proc Natl Acad Sci U S A* **88**:9878-82.

Stuart DI, Levine M, Muirhaed H, Stammers DK (1979). The crystal structure of cat pyruvate kinase at a resolution of 2.6 Å. *J Mol Biol* **134**:109-42.

Vig R, Mao C, Venkatachalam TK, Tuel-Ahlgren L, Sudbeck EA and Uckun FM (1998) Rational design and synthesis of phenethyl-5-bromopyridyl thiourea derivatives as potent non-nucleoside inhibitors of HIV reserve transcriptase. *Bioorg Med Chem* **6**:1789-97.

Witvrouw M, Pannecouque C, Van Laethem K, Desmyter J, De Clercq E and Vandamme AM (1999) Activity of non-nucleoside reverse transcriptase inhibitors against HIV-2 and SIV. *Aids* **13**:1477-83.

Yang G, Song Q, Charles M, Drosopoulos WC, Arnold E and Prasad VR (1996) Use of chimeric human immunodeficiency virus types 1 and 2 reverse transcriptases for structure-function analysis and for mapping susceptibility to nonnucleoside inhibitors. *J Acquir Immune Defic Syndr Hum Retrovirol* **11**:326-33.

CHAPTER V

ROLE OF THE ASPARAGINES AT POSITION 136 AND 137 IN THE p51 β7-β8 LOOP OF HIV-1 RT ON STABILIZATION OF THE HETERODIMER

The results presented in this chapter have been published in the following articles:

Auwerx, J., Van Nieuwenhove, J., Rodríguez-Barrios, F., de Castro, S., Velázquez, S., Ceccherini-Silberstein, F., De Clercq, E., Camarasa, M.-J., Perno, C.-F., Gago, F. and Balzarini, J. (2005) The N137 and P140 amino acids in the p51 and the P95 amino acid in the p66 subunit of human immunodeficiency virus type 1 reverse transcriptase are instrumental to maintain catalytic activity and to design new classes of anti-HIV-1 drugs. *FEBS Letters* **579**, 2294-2300.

Balzarini, J., Auwerx, J., Rodríguez-Barrios, F., Chedad, A., Farkas, V., Ceccherini-Silberstein, F., García-Aparicio, C., Velázquez, S., De Clercq, E., Perno, C.-F., Camarasa, M.-J. and Gago, F. (2005) The amino acid N136 in HIV-1 reverse transcriptase (RT) maintains efficient association of both RT subunits and enables the rational design of novel RT dimerisation inhibitors. *Mol. Pharmacol.* **68**, *In press*

SUMMARY

The highly conserved N136, N137 and P140 in the p51 subunit of HIV-1 RT are part of the β7-β8-loop that contributes to the formation of the base of the NNRTI-specific pocket and makes up a substantial part of the dimerisation interface. Amino acid P95 in p66 also markedly contributes to the dimerisation binding energy. Site-directed mutagenesis has revealed that the RNA-dependent DNA polymerase activity of RT mutated at positions N136 or N137 (and also mutant P95A or P140A RT) is heavily compromised. The detrimental effect of the N136 mutations occurred when the mutated amino acid was present in the p51 subunit, but not in the p66 subunit of the p66/p51 RT heterodimer. None of the mutant N136 and N137 RT enzymes showed marked resistance against any of the clinically used NNRTIs. Mutant N137 RTs surprisingly lost significant sensitivity for NRTIs such as ddGTP and d4TTP. Most mutant enzymes were also markedly more sensitive to the inactivating (denaturing) effect of urea (and acetonitrile) than wild-type RT, and the degree of increased urea sensitivity was highly correlated with the degree of (lower) catalytic activity of the mutant enzymes. Replacing wild-type N136 in HIV-1 RT by other amino acids resulted in notably increased amounts of free p51 and p66 monomers in the RT preparation. Our findings identify a structural/functional role for N136 (and N137) in stabilization of the RT p66/p51 heterodimer and provide clues for the rational design of novel NNRTIs or drugs targeting either N136 or N137 in the β7-β8 loop of p51 or its anchoring points on p66 (the peptide backbone of H96 or the stretch comprising L92-G93-I94). Such drugs would disturb the RT dimerisation process and/or the structural support that the p51 subunit provides to the p66 subunit and which is essential for catalytic activity of the enzyme.

INTRODUCTION

The NNRTI-specific pocket mainly consists of amino acids that belong to the p66 subunit, but the bottom of the pocket is closely located to a peptide stretch that is contributed by the p51 subunit and better known as the β7-β8 loop (Ding *et al.*, 1995b; Esnouf *et al.*, 1995; Hopkins *et al.*, 1996; Kohlstaedt *et al.*, 1992; Ren *et al.*, 1995). Indeed, E138 and T139, but also S134, I135, N136 and N137 from the p51 subunit line the outer part of the NNRTI binding pocket in the p66 subunit, and also provide a portion of the dimerisation interface (Fig. 5.1).

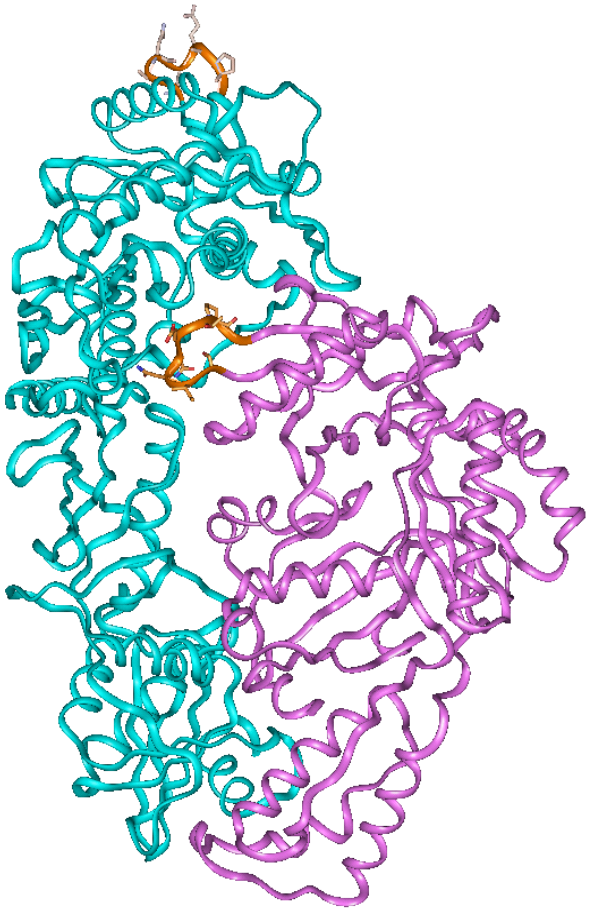

Figure 5.1 Location of the β7-β8 loop in a ribbon representation of the HIV-1 RT.
p66 and p51 are colored in cyan and pink, respectively. The 133-140 stretch (PSINNETP) in both subunits has been colored orange, and side chains are displayed as sticks. Figure generated using ViewerLite 5.0 software (Accelrys Inc.; http://www.accelrys.com)

To date, two NNRTIs are known to select for amino acid mutations outside the NNRTI pocket that are located at the p66/p51 interface. One is TSAO-m^{3}T, which selects for E138K (Balzarini *et al.*, 1993; Balzarini *et al.*, 1994; Boyer *et al.*, 1994; Jonckheere *et al.*, 1994), and the other one is (+)-calanolide A, which selects for the T139I mutation in HIV-1 RT (Buckheit *et al.*, 1995). For TSAO-m^{3}T and its derivatives, modelling studies, as well as experimental results, have provided evidence that these drugs may interact with the association of p66 and p51 subunits, thereby destabilizing the HIV-1 RT heterodimeric enzyme (Arion *et al.*, 1996; Rodriguez-Barrios *et al.*, 2001; Sluis-Cremer *et al.*, 2000). The fact that both N136 and N137 are highly conserved among the RTs of all HIV-1 strains, but also of HIV-2, SIV, FIV and several other lentiviruses that have been characterized so far (Table 5.1) points to a defined, but as yet unidentified, functional and/or structural role for these residues. Both N136 and N137 are exposed to the solvent in the p66 subunit, but the side-chain carboxamide group of

N136 on the p51 subunit is engaged in two hydrogen bonds with the peptide backbone of H96 in the p66 subunit.

Table 5.1 Alignment of amino acids S134 to G141 in reverse transcriptase enzymes from different lentivirus strains

Lentivirus	Amino acid position in the RT							
	134	135	136	137	138	139	140	141
HIV-1 (HXB2)	S	I	N	N	E	T	P	G
HIV-2 (ROD)	S	V	N	N	A	E	P	G
SIV(Rhesusmac)	S	V	N	N	A	E	P	G
SIV (Sun tailed)	S	V	N	N	Q	A	P	G
FIV (Petaluma)	R	K	N	N	A	G	P	G
BIV (Cl 127)	P	V	N	R	E	G	P	I
EIAV (Cl 22)	S	I	N	H	Q	E	P	D
Visna (Evi)	S	P	N	N	L	G	P	C
CAEV (CORK)	S	P	N	N	L	G	P	C

Interestingly, no mutations at amino acid position 136 or 137 of RT have ever been detected in NNRTI-exposed HIV-1-infected cell cultures. In HIV-1-infected individuals, naïve for antiretroviral drugs, the presence of mutations at these positions was completely absent (0 out of 457 patients) and less than 1% in HIV-1-infected individuals that were HAART-treated with NRTIs/NNRTIs (Table 5.2). Due to their highly conserved nature, N136 and N137 seem to play a crucial role in the integrity of HIV RT and/or its catalytic function. Therefore, we decided to perform an in-depth site-directed mutagenesis study on the N136 and N137 positions of HIV-1 RT to gain detailed information on their effect in the catalytic activity of HIV-1 RT, their role in the potential interaction of RT with existing NNRTIs, and their role in the stabilization of the dimer interface of the p66/p51 RT heterodimer.

Our studies revealed that N136 and N137 are essential to preserve the catalytic activity of HIV RT and play a crucial role in the stabilization of the enzyme heterodimer. We also provide evidence that the area in the NNRTI pocket of p66 where these amino acids from p51 are located can be a potential target in the design of novel NNRTIs endowed with higher antiviral potency and/or a more favourable resistance profile than the current NNRTIs in clinical use.

Table 5.2 Mutation profile of the amino acids in the β7-β8 loop of the p51 subunit in HIV-1 RT for drug-naïve patients (***n*** = 457) and NRTI/NNRTI-treated patients (***n*** = 1779).

Naïve				Treated		
n	**%**	**Mutation**	**Wild-type**	**Mutation**	**%**	***n***
1	0.22	S_1	**P133**		0.00	0
3	0.66	R_1C_2	**S134**	T_1R_1	0.13	2
200	43.76	$P_1Q_2K_3M_5L_5R_{14}V_{27}T_{143}$	**I135**	$T_{572}V_{108}L_{92}M_{38}R_{14}K_{10}A_2$	52.89	823
0	0.0		**N136**	$T_{12}I_2K_1$	0.96	15
0	0.0		**N137**	S_4H_2	0.39	6
21	4.6	$R_1K_2G_2A_{16}$	**E138**	$A_{79}G_{22}Q_{17}K_{14}T_3S_2D_2R_2$	8.42	131
10	2.2	$V_1M_1A_1R_2I_2P_3$	**T139**	$K_{19}R_{18}A_{13}Q_9M_6I_3S_2V_1P_1$	4.37	68
1	0.22	L_1	**P140**	Q_2T_2	0.26	4

n represents the number of patients with a mutation at a particular amino acid position. The individual number of patients with a specific amino acid mutation is indicated in subscript. The wild-type consensus amino acid is represented in bold.

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 (Perez-Perez *et al.*, 1992). (+)-Calanolide A was delivered by Sarawak MediChem Pharmaceuticals Inc. (Sarawak, Malaysia). 2',3'-didehydro-2',3'-dideoxythymidine-5'-triphosphate (d4TTP) was obtained from Sigma Chemical Ltd. (St. Louis, MO). Other compounds were obtained as described in Chapter 2 and 3.

Site-directed mutagenesis of HIV-1 RT

Mutant RT-enzymes containing the N136X, N137X, P95A or P140A mutations were derived from the RT sequence cloned in pKRT2His (D'Aquila and Summers, 1989; Pelemans *et al.*, 1998). Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and as described in Chapter 3. 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).

Construction of mutant recombinant HIV-1 reverse transcriptases

Recombinant HIV-1 RT enzymes were expressed from a two-plasmid coexpression system previously described by (Jonckheere *et al.*, 1996). The p66 subunit of RT was expressed from pACYC66His and the p51 subunit from pKRT51. To construct wild-type and 136-mutated pACYC66His, wild-type and 136-mutated pKRT2His were digested with *Eco*RI and *Avi*II and the RT-containing fragments were ligated into pACYC184 digested with *Eco*RI and *Sca*I. To construct wild-type and 136-mutated pKRT51, wild-type and 136-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. For all mutant enzymes, the mutation was introduced in both p66 and p51 subunits. Only for the mutant N136T RT, the mutation was introduced solely in p66, solely in p51 or in both p66 and p51 subunits.

Expression and purification of wild-type and mutant recombinant HIV-1 RT

Expression and purification of recombinant RT was performed as described in Chapter 3.

Construction and expression of wild-type and mutant HIV-1 RT fusion plasmids in bacterial expression vectors

Glutathione *S*-transferase-tagged p51 mutant subunits (GST-p51) containing either the N136Q, N136T, N136Y or N136K substitutions were constructed by subcloning a *Nsi*I–*Kpn*I restricted fragment from the p51-encoding portion of the above described pKRT51 containing the desired mutation (Q, T, Y or K) at position 136 into the *Nsi*I–*Kpn*I digested pGEX51H (see Chapter 2). Since the N136 amino acid in the p66 subunit is located in the finger domain of the RT and, therefore, has no influence on dimerisation of the RT, we used wild-type p66-encoding pKRT2His, described above. Expression and purification of the wild-type and mutant N-terminal GST-tagged p51 subunits and N-terminal $(His)_6$-tagged p66 (His-p66) were performed as described in Chapter 2.

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 described in Chapter 2. In case where d4TTP (or PFA) has been evaluated for its inhibitory activity, 0.15 mM poly(rA).oligo dT_{12-18} had been used as the template/primer, and 1.6 μM [^{3}H]dTTP as the radiolabeled substrate.

For determination of the K_m value of the template/primer against HIV-1 RT in the presence of different concentrations of urea, the RT activity was measured as described above in the presence of variable concentrations poly rC.oligo dG. The amounts of urea varied between 0.3 and 1.5 M.

Catalytic activity of wild-type and mutant heterodimer HIV-1 RTs in the presence of urea and acetonitrile

Denaturation curves were plotted by preincubation of RT with different concentrations of urea ranging from 0.0625 M up to 2.0 M or acetonitrile ranging from 2% up to 14% for 30 min at 37°C in 50-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) (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, precipitated, washed and analyzed as described in Chapter 2. 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.

FPLC Size exclusion chromatography

Size exclusion chromatography was performed using a 10 x 300 mm Superdex 200 HR 10/30 column (Amersham Biosciences). Freshly prepared RT samples or RT samples that were left in elution buffer for 24 hrs, 72 hrs or 336 hrs after elution from a Ni NTA column, and that contain 5-10 µg protein were applied on the size exclusion column and eluted with 200 mM potassium phosphate pH 7.0 at the flow rate of 0.5 ml as previously described (Restle *et al.*, 1990).

Circular dichroism (CD) spectroscopy

CD spectra of wild-type and mutant N136T and N136L RTs in the far-UV region (190-260 nm) were acquired at 25°C on a Jasco J-600A spectropolarimeter using a cuvette of 1 ml. Protein solutions were 0.17 and 0.25 µM in 0.75 mM EDTA, 0.75 mM DDT, 2%

glycerol and 7.5 mM Tris at pH 7.8. The data were expressed as residual ellipticity [Θ] (deg cm^2 $dmol^{-1}$), using 114.75 as the mean residue weight for HIV-1 RT. The spectra were obtained with a 1 nm bandwidth, a 1 s time constant and a data density of 10 points/nm. In order to estimate the fractions of the different types of secondary structure, analysis of CD data was performed with the CDNN-program (Bohm *et al.*, 1992).

Three-dimensional structure visualization

The RT structure was visualized using the ViewerLite 5.0 software (Accelrys Inc.; http://www.accelrys.com) and the X-ray coordinates of a covalently-trapped catalytic complex with a DNA template:primer deposited in the Protein Data Bank (PDB, http://www.rcsb.org/PDB/) with code 1RTD (Huang *et al.*, 1998).

RESULTS

5.1. Catalytic DNA polymerase activity of mutant and wild-type HIV-1 RT

To investigate the influence of changes at the amino acid residue N136 or N137 of HIV-1 RT activity, we constructed different recombinant mutant RTs by site-directed mutagenesis. Different types of amino acid side chains were represented: an aliphatic side chain in alanine and leucine, an aromatic side chain in tyrosine, a protonated amino group in the positively charged lysine and histidine, a carboxylate in the negatively charged aspartic and glutamic acid, and a polar uncharged group in serine, threonine and glutamine. 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 the Ni-NTA- and heparin-containing affinity columns.

As a rule, the RNA-dependent DNA polymerase (RdDp) activities of all mutant enzymes were severely impaired (Fig. 5.2). The catalytic activity ranged between 0.07 and 36 percent of wild-type. The catalytically most active mutant N136 RT contained the N136T mutation (2.1% activity of wild-type) and the presence of the N136Y, N136L and N136D amino acid mutations in HIV-1 RT resulted in mutant enzymes endowed with the poorest catalytic activity (≤ 0.1%). The mutant N136T RT had the highest catalytic activity among all mutant N136 RTs. In this enzyme, the N136T mutation was present in both p66 and p51 subunits. To assess the role of the subunit location of the N136T mutation in the decreased catalytic activity of the mutant RT, two additional mutant RTs were constructed in which the

N136T mutation was introduced in either solely the p66 or solely the p51 subunit of the heterodimeric RT enzyme. Whereas the mutant RT enzyme in which N136T was solely present in the p51 subunit had a catalytic activity that was 8.4 ± 3.7% of wild-type enzyme, the heterodimeric enzyme at which N136T was solely present in p66 had a catalytic activity of 71 ± 23% of wild-type enzyme. Thus, the exclusive presence of the N136T mutation in p51 had a much more deleterious effect on the catalytic activity of the mutant enzyme than when it was solely present in p66.

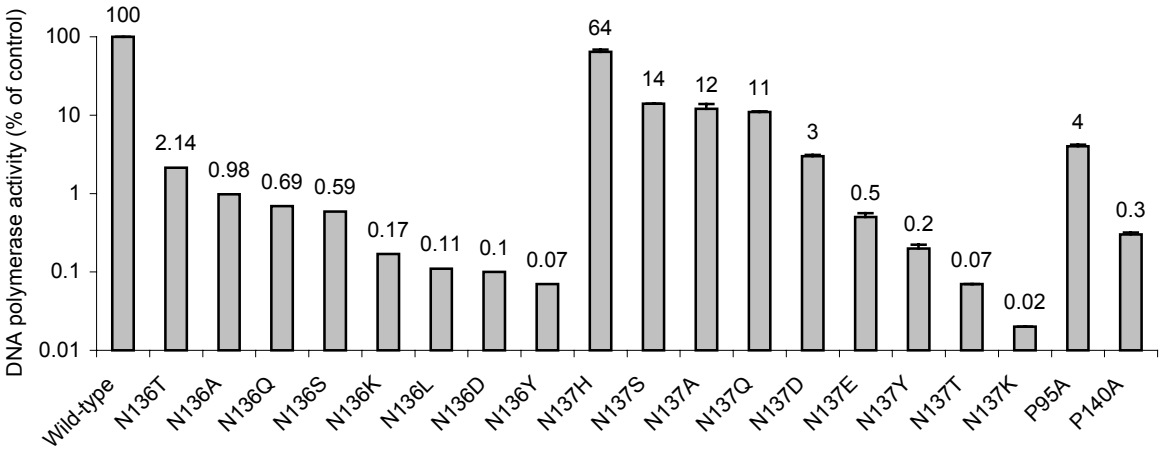

Figure 5.2 Catalytic RNA-dependent DNA polymerase activity of mutant N136 HIV-1 RT enzymes. Poly(rC).oligo(dG) was used as the template/primer and [^{3}H]dGTP as the radiolabeled substrate.

The mutant N137H RT was the most active N137 mutant RT and retained 64% of the catalytic activity. The N137S, N137A and N137Q RTs showed markedly compromised catalytic activity (11-14% of that of wild-type RT) among the mutated N137X RTs. Seriously impaired catalytic activities were noted for the other five mutant N137X RTs. They only retained 3% (for N137D) to less than 0.1% (for N137T and N137K) catalytic activity. The proline to alanine mutations at position 95 and 140 of HIV-1 RT also resulted in markedly decreased DNA polymerase activity (4% for mutant P95A RT enzyme and 0.3% for mutant P140A RT enzyme) (Fig. 5.2).

5.2. Inhibitory activity of NNRTIs, PFA, d4TTP and ddGTP against wild-type and mutant N136, N137, P95A and P140A recombinant HIV-1 RTs

The mutant enzymes were evaluated for their sensitivity to the inhibitory activity of a variety of NNRTIs, PFA and the NRTIs d4TTP and ddGTP (Table 5.3 and Table 5.4). The

N136T RT mutant that was endowed with the highest catalytic activity among the N136 mutant RTs virtually kept pronounced sensitivity to all NNRTIs and ddGTP. The inhibitory activity of both NNRTIs and ddGTP against wild-type RT ranged between an IC_{50} of 0.022 and 3.8 μM depending on the nature of the compound. Also, the mutant N136D RT reasonably kept its sensitivity to the drugs (decrease of the inhibitory activity of the NNRTIs ranged between 1.4- to 5-fold). In contrast to their susceptibility to the inhibitory effect of the NNRTIs, the other mutant N136 RT enzymes substantially lost between 10- to >25-fold sensitivity to some of the evaluated drugs depending on the nature of the drug and the introduced amino acid mutation. Interestingly, efavirenz, which was among the most potent inhibitors of the wild-type RT enzyme, only lost its inhibitory potential by 8-fold at most. It is remarkable that the NRTI derivative ddGTP in most cases lost at least 10-fold and in some cases even 50- to 100-fold its inhibitory potential against one or several of the mutant RT enzymes (Table 5.3).

Table 5.3 Inhibitory activity of test compounds against mutant N136X and wild-type HIV-1 RTs

	IC_{50}[a]				
RT mutation	**nevirapine**	**delavirdine**	**efavirenz**	**GW420867X**	**ddGTP**
WT	1.1 ± 0.1	0.5 ± 0.3	0.011 ± 0.003	0.011 ± 0.000	0.05 ± 0.02
N136Y	> 10	> 10	0.12 ± 0.01	≥ 1	1.6 ± 0.32
N136K	> 10	≥ 10	0.16 ± 0.021	≥ 1	0.93 ± 0.64
N136T	1.0 ± 0.29	0.90 ± 0.16	0.035 ± 0.006	0.033 ± 0.004	0.065 ± 0.007
N136D	2.7 ± 2.3	1.3 ± 1.2	0.036 ± 0.000	0.037 ± 0.002	0.19 ± 0.021
N136A	> 10	6.6 ± 2.4	0.21 ± 0.20	0.28 ± 0.01	0.28 ± 0.48
N136Q	≥ 10	4.5 ± 1.7	0.051 ± 0.018	0.39 ± 0.21	1.4 ± 0.20
N136S	5.1 ± 2.6	3.1 ± 1.7	0.052 ± 0.044	0.35 ± 0.32	0.58 ± 0.51
N136L	> 10	9.1 ± 1.3	0.13 ± 0.014	0.80 ± 0.021	6.1 ± 1.0

[a] 50% inhibitory concentration, or compound concentration required to inhibit RT activity with 50%.

Template/primer: 0.1 mM poly(rC).oligo(dG); substrate: 1.6 μM [^{3}H]dGTP.

Data are the means of 2 to 3 independent experiments ± S.D.

Most of the mutant N137 RTs kept full sensitivity to the NNRTIs tested. Only the mutant N137K RT showed moderate resistance to (+)-calanolide A (5-fold), but other NNRTIs retained their inhibitory activity against this enzyme. Interestingly, all mutant N137 RTs significantly gained sensitivity against the thiocarboxanilide UC-781 (Table 5.4). Also,

Table 5.4 Inhibitory activity of test compounds against mutant N137X, P95A and P140A and wild-type HIV-1 RTs

	IC_{50} [b] (μM)[a]										
RT Mutation	**nevirapine**	**delavirdine**	**efavirenz**	**UC-781**	**GW420867X**	**TSAO-m³T**	**TSAO-T**	**(+)-calanolideA**	**ddGTP**	**d4TTP**	**PFA**
WT	1.1 ± 0.1	0.5 ± 0.3	0.011 ± 0.003	0.11 ± 0.09	0.011 ± 0.000	1.6 ± 0.6	1.6 ± 1.0	0.03 ± 0.01	0.05 ± 0.02	0.19 ± 0.02	3.4 ± 0.4
N137Y	0.61 ± 0.04	0.6 ± 0.2	0.008 ± 0.000	0.020 ± 0.005	0.008 ± 0.003	0.3 ± 0.1	0.41 ± 0.03	0.02 ± 0.01	1.2 ± 0.3	2.6 ± 0.3	39 ± 15
N137K	1.3 ± 0.3	0.8 ± 0.2	0.019 ± 0.004	0.04 ± 0.01	0.02 ± 0.01	3.3 ± 0.0	2.1 ± 0.8	0.155 ± 0.007	6.4 ± 1.1	3.3 ± 1.8	50 ± 0
N137T	1.1 ± 0.3	0.8 ± 0.3	0.011 ± 0.005	0.03 ± 0.01	0.03 ± 0.02	0.3 ± 0.2	0.7 ± 0.1	0.08 ± 0.01	2.2 ± 0.6	1.7 ± 0.0	15 ± 8
N137D	0.92 ± 0.02	0.3 ± 0.1	0.007 ± 0.001	0.020 ± 0.003	0.011 ± 0.003	1.2 ± 0.2	1.1 ± 0.7	0.07 ± 0.03	0.13 ± 0.07	1.6 ± 0.3	33 ± 7
N137A	1.0 ± 0.0	0.6 ± 0.5	0.009 ± 0.002	0.030 ± 0.001	0.010 ± 0.002	0.9 ± 0.4	1.8 ± 0.4	0.04 ± 0.03	0.2 ± 0.1	0.21 ± 0.05	28 ± 3
N137E	1.0 ± 0.1	0.7 ± 0.3	0.02 ± 0.01	0.032 ± 0.002	0.013 ± 0.001	1.7 ± 0.6	2 ± 1	0.09 ± 0.05	0.083 ± 0.002	0.04 ± 0.02	1.7 ± 0.5
N137Q	0.6 ± 0.2	0.4 ± 0.2	0.005 ± 0.001	0.027 ± 0.004	0.007 ± 0.000	0.8 ± 0.3	0.66 ± 0.00	0.07 ± 0.03	0.5 ± 0.1	0.24 ± 0.07	39 ± 15
N137H	0.49 ± 0.26	0.18 ± 0.04	0.015 ± 0.009	0.048 ± 0.001	0.011 ± 0.003	1.0 ± 0.42	1.1 ± 0.21	0.034 ± 0.0	0.70 ± 0.04	-	-
N137S	0.36 ± 0.02	0.27 ± 0.01	0.005 ± 0.0	0.050 ± 0.001	0.021 ± 0.001	1.4 ± 0.4	0.95 ± 0.22	0.055 ± 0.007	0.39 ± 0.01	-	-
P140A	0.8 ± 0.2	0.9 ± 0.1	0.009 ± 0.002	0.045 ± 0.009	0.010 ± 0.002	0.6 ± 0.4	1.07 ± 0.00	0.04 ± 0.01	1.3 ± 0.3	-	-
P95A	1.0 ± 0.2	0.39 ± 0.00	0.008 ± 0.001	0.014 ± 0.001	0.012 ± 0.000	0.9 ± 0.7	0.9 ± 0.8	0.9 ± 0.1	1.4 ± 0.1	-	-

[a] 50% inhibitory concentration, or compound concentration required to inhibit RT activity with 50%.

Template/primer: 0.1 mM poly(rC).oligo(dG); substrate: 1.6 μM [³H]dGTP for all inhibitors except for d4TTP and PFA where 0.15 mM poly(rA).oligo(dT) was used as the template/primer and 1.6 μM [³H]dTTP as the radiolabeled substrate. Data are the means of 2 to 3 independent experiments ± S.D.

the P95A and P140A RTs were 3- to 10-fold more sensitive to the inhibitory effect of UC-781. In contrast, substantial resistance to (+)-calanolide A was observed for mutant P95A RT enzyme (30-fold). Surprisingly, all mutant RT enzymes showed less susceptibility and often even relatively high resistance towards the nucleotide RT inhibitor ddGTP (up to 130-fold for the mutant N137K RT), and, to a lesser extent, also to d4TTP (Table 5.4). Also, the mutant enzymes showed several degrees of resistance (up to 15-fold) against the pyrophosphate analogue PFA depending on the nature of the mutation in RT.

5.3. Effects of exposure of urea and acetonitrile on mutant N136X and N137X RT activity

Wild-type and four mutant N136 HIV-1 RTs (i.e. N136T, N136K, N136Y, N136L) were exposed to a wide variety of urea (or acetonitrile for N136T and N136L) concentrations, and the catalytic activity of the urea- (and acetonitrile)-exposed enzymes was measured (Fig. 5.3 panel A, C). For the wild-type enzyme, the RT activity gradually decreased in the presence of increasing urea concentrations (Fig. 5.3, panel A). A urea concentration as low as 0.20 M slightly decreased the catalytic activity of wild-type RT, and 2.0 M urea abolished its catalytic activity almost completely. Half of the RT catalytic activity was retained at around 1.0 M urea. However, when the mutant N136 RT enzymes were exposed to the different concentrations of urea, the enzymes invariably had gained a markedly higher sensitivity to the denaturing activity of urea. Whereas the urea IC_{50} shifted from 1 M for wild-type enzyme to 0.6 M for the N136T mutant RT, the urea IC_{50} was further decreased to 0.25 M and 0.20 M for the mutant N136Y and N136K RT, respectively. The N136Q RT mutant showed an intermediate sensitivity to urea (IC_{50} ~ 0.4 M). An equally increased sensitivity to urea was observed for mutant N136T RT when the N136T mutation was solely introduced in p51 but not in p66 (the open-square curve compared with the closed-square curve in Fig. 5.3, panel A).

When mutant N137A, N137D and N137E RTs were exposed to different concentrations of urea, the enzymes had gained increased sensitivity towards the denaturing effect of urea (Fig. 5.3, panel B). Whereas the urea-IC_{50} shifted from 0.75 M to 0.55 M for the mutant N137A and N137D RTs, the urea-IC_{50} was further decreased to 0.35 M for the mutant N137E RT enzyme.

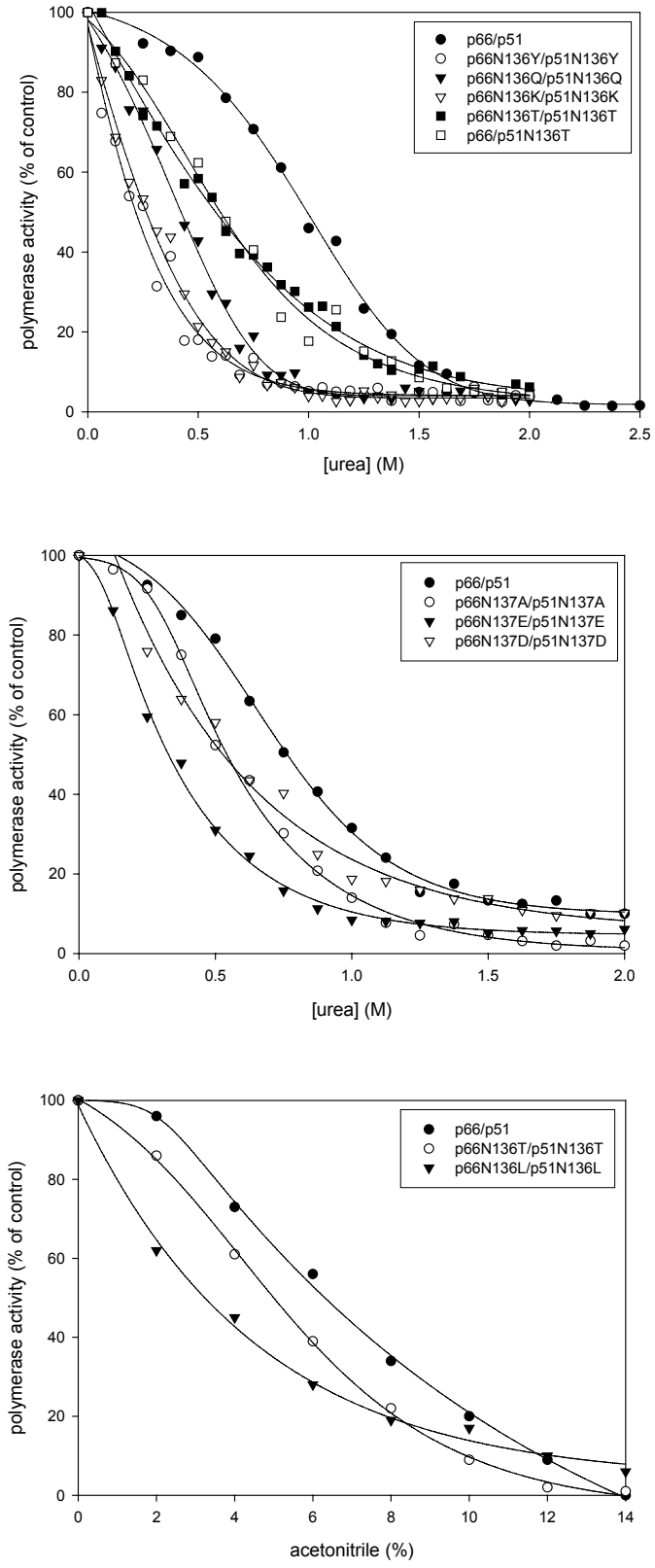

Figure 5.3 Effect of urea on the catalytic activity of mutant N136 (panel A) and N137 HIV-1 RTs (panel B). In panel C the effect of acetonitrile on the catalytic activity of N136 mutant HIV-1 RTs is presented.

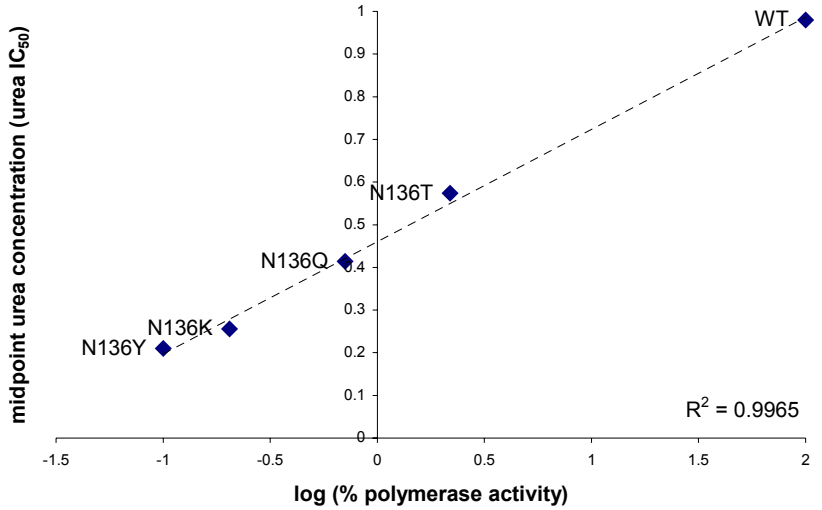

Figure 5.4 Correlation between the catalytic activity of mutant N136 HIV-1 RT enzymes and the 50% inhibitory concentration of urea against these mutated enzymes.

The log percent RT activity was taken from Fig. 5.2 and the midpoint of the urea concentration at which urea resulted in 50% inhibition of enzyme activity was calculated from the curves depicted in Fig. 5.3.

Table 5.5 Affinity constant (K_m) of wild-type and mutant N136RT enzymes for poly(rC).oligo(dG) in the presence of varying concentrations of urea

RT Enzyme	Concentration of urea (M)	K_m of template/primer[a] (μM) poly(rC).oligo(dG)
Wild-type	0	10
	1.0	15
	1.5	47
N136T	0	15
	0.6	22
	0.9	38
N136L	0	33
	0.3	68
	0.45	98

[a]Data are the mean of 3 to 4 independent experiments.

5.4. Determination of the affinity constant (K_m) values of template/primer for wild-type and mutant N136X HIV-1 RTs in the presence or absence of urea

The K_m values of the template/primer poly(rC).oligo(dG) for wild-type and mutant N136T and N136L HIV-1 RTs were determined in the presence or absence of different urea concentrations (Table 5.5). The K_m of template/primer was lowest for wild-type RT (10 μM) and increased for the mutant enzymes as a function of their more pronounced compromised

catalytic activity; thus, the K_m was 1.5-fold higher than wild-type for mutant N136T RT (15 μM) and highest for mutant N136L RT (33 μM). Interestingly, in all cases (wild-type, N136T RT and N136L RT), the presence of urea further dose-dependently increased the K_m of the enzymes for the template/primer (4.7-, 2.5- and 3-fold, respectively) at the highest urea concentrations tested (1.5 M for WT RT, 0.9 M for N136T RT and 0.45 M for N136L RT, respectively). The different urea concentrations chosen for the individual (wild-type and mutant) enzymes corresponded to comparably inactivating effects of these urea concentrations on the wild-type and (mutant) enzymes.

5.5. Analysis of monomer content of wild-type and mutant N136X heterodimer RTs at different time points after isolation

Wild-type and mutant N136T and N136Y HIV-1 RT heterodimers (consisting of p66-His and p51-GST) were isolated using Glutathione Sepharose 4B beads and a HiTrap heparin column as described in Chaper 2. The fraction that corresponded to the p66/p51 heterodimer (as ascertained by subsequent gel electrophoresis) was then left in the elution solution at 4°C and analysed on a size exclusion FPLC column after 24 hrs, 72 hrs and 336 hrs (14 days). Whereas >99% wild-type RT enzyme still existed as a p66/p51 heterodimer at all time points measured, the mutant N136T RT heterodimer consisted of a mixture of p66/p51 heterodimer plus p66 and p51 monomers after 24 hrs. After 72 hrs, the formation of monomers in the mixture had proceeded still further (Fig. 5.5). The ratio of monomer/heterodimer content of the mutant N136Y RT was even higher than that observed for the mutant N136T RT after 24 and 72 hrs (Fig. 5.5). Thus, the lower the catalytic activity of the enzyme, the higher the ratio of free monomer/heterodimer in the enzyme preparation at ≥24 hrs after isolation of the enzyme.

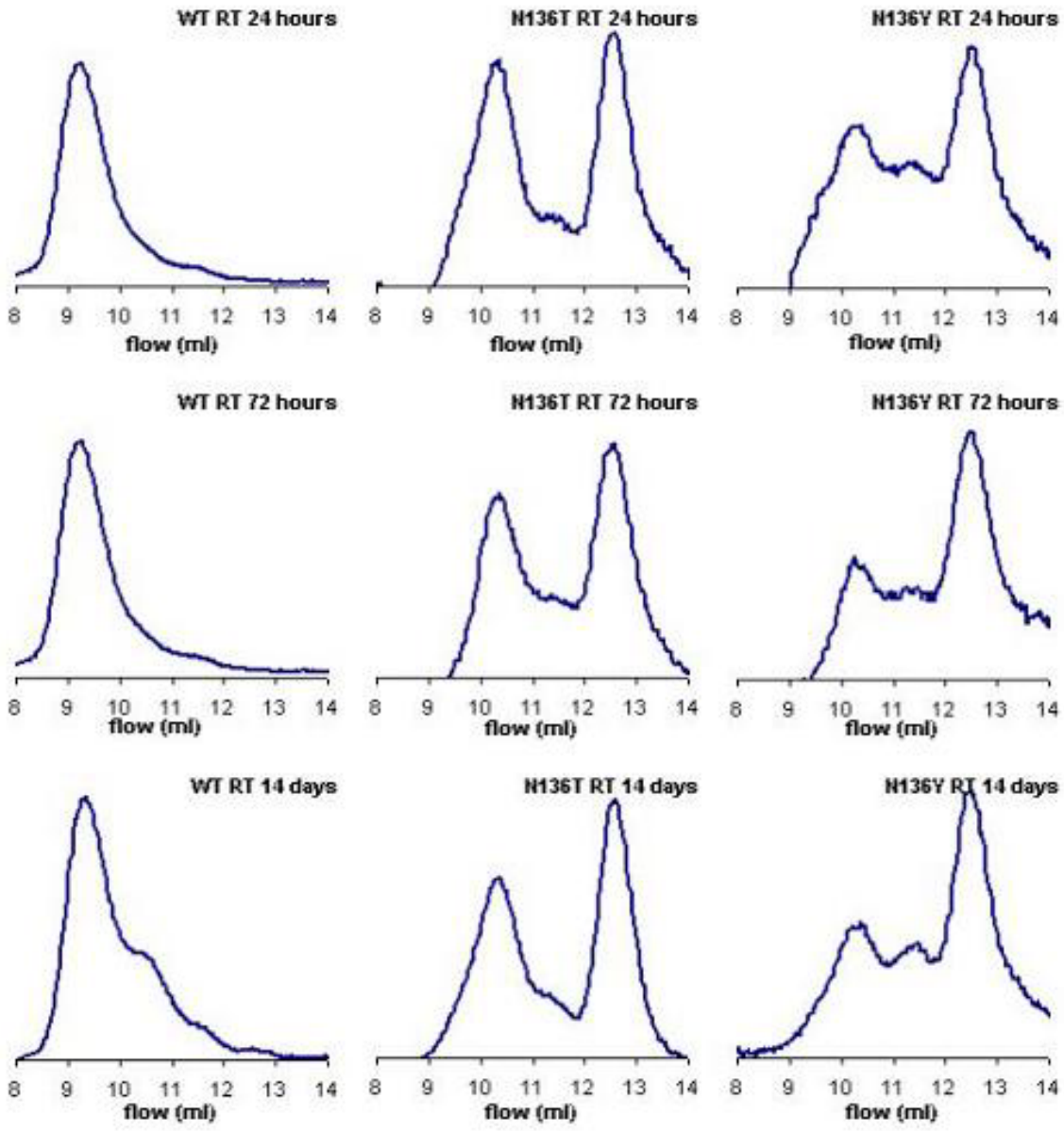

Figure 5.5 Determination of p66/p51 heterodimer and free p66 and p51 monomers in wild-type and mutant N136T and N136Y RT heterodimer enzyme preparations by size exclusion chromatography as a function of incubation time and based on size exclusion chromotography.

Freshly prepared RT samples were left in elution buffer for 24 hrs (top), 72 hrs (middle) or 336 hrs (14 days) (bottom) after elution from a HiTrap heparin column. Left peaks eluting between 8 to 11 ml represent heterodimer; right peaks eluting between 12 and 14 ml represent a mixture of monomers.

5.6. Circular dichroism (CD) spectra of wild-type and mutant N136X HIV-1 RTs

To reveal whether the mutations at amino acid position 136 had an effect on the general structure and conformation of the HIV reverse transcriptase, the circular dichroism spectra of wild-type and the mutant N136T and N136L RTs were determined and compared (Fig. 5.6).

The CD spectrum of wild-type HIV-1 RT exhibits a broad and prominent band of negative ellipticity between 260 nm and 202 nm with a peak at 212 nm and a shoulder at 226 nm. The ellipticity becomes positive in the region 202-190 nm. The CD spectrum of the mutant N136T RT is also dominated by a negative ellipticity band but compared with the WT HIV-1 RT it is considerably blue-shifted with a peak at 204 nm, a shoulder at 220 nm and a crossover at 196 nm. The overall shape of the spectrum of the mutant N136L RT is similar to that of the mutant N136T RT but with a slightly increased negative and positive ellipticity and a crossover at 198 nm (Fig 5.6). Both mutant RTs display CD spectra typical for a considerable amount of α-helical structures with a positive band at 190 nm and a double minimum in the region of 205-220 nm. These spectra, therefore, show a similar secondary structure that, however, is clearly different from that of the wild-type RT. In order to quantify the observed spectral changes, the contribution of the various secondary structure elements to the measured CD spectra was determined using the CDNN program of Böhm *et al.* The results obtained with this deconvolution program are listed in Table 5.6 and confirm the increased α-helical content in the mutants. Thus, the substitution of N136 by T or L induces structural effects in HIV-1 RT that are observed as an increase in the amount of α-helices (11-13%) and a slight decrease of both random coil (8%) and β-sheet (3%).

Table 5.6 Secondary structure analysis of the circular dichroism of wild-type and mutant N136 L/T RTs

	Helix (%)	Anti-parallel (%)	Parallel (%)	Beta-turn (%)	Random Coil (%)	Total (%)
HIV1-RT	50.4	5.9	5.3	14.2	24.2	100
N136L	62.7	2.3	4.7	14	16.3	100
N136T	63.6	3.6	3.6	12.8	16.6	100.1

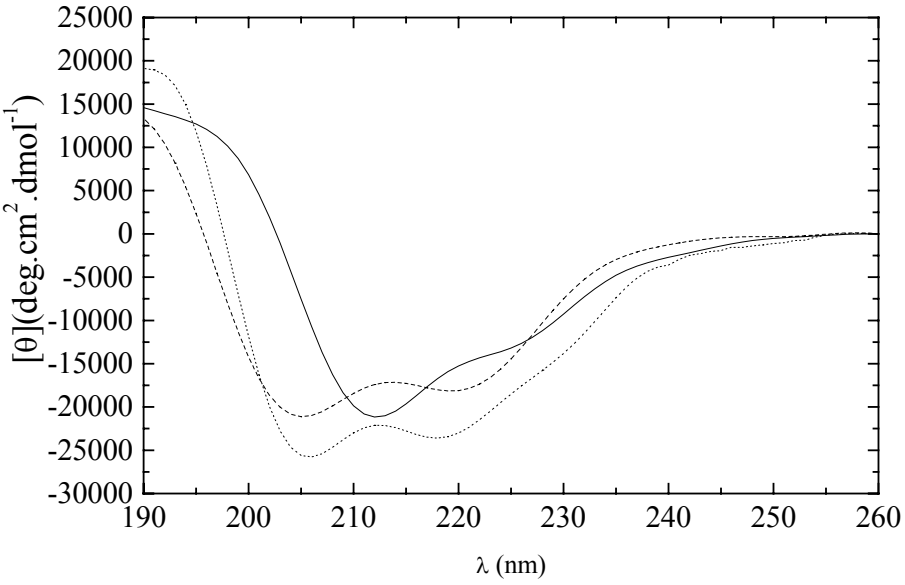

Figure 5.6 Circular dichroism spectra of wild-type and mutant N136T and N136L RTs.
Solid line: wild-type HIV-1 RT; dotted line: N136L HIV-1 RT; broken line: N136T HIV-1 RT.

5.7. Structural role of amino acids N137, P140 and P95 in HIV-1 RT

Accordingly, whereas the side chain of N137 in the p66 subunit is facing the solvent it is found lying on the peptide backbone of p66 in a stretch comprising L92-G93-I94. It is worth mentioning that the region encompassing amino acids 83-99 displays a different conformation in both subunits despite having identical composition (Fig. 5.10). Thus, a short α-helix from P97 to G99 is apparent in the larger (p66) subunit but it is absent in the smaller (p51) subunit. Furthermore, the backbone of these two segments can be superimposed only on a relatively short stretch made up of G93, I94, P95, H96, and P97, which presents a highly invariant amino acid region even in the presence of NNRTI (i.e. nevirapine, delavirdine and efavirenz) drug pressure (Ceccherini-Silberstein *et al.*, 2004).

DISCUSSION

X-ray crystallographic analyses have revealed subtle structural differences in a large number of complexes of HIV-1 RT with different NNRTIs including details of amino acid arrangements in and around the hydrophobic NNRTI-binding pocket as well as the amino acid interactions at the interface between the p66 and p51 subunits (Ding *et al.*, 1995a; Esnouf *et al.*, 1995; Hopkins *et al.*, 1996; Kohlstaedt *et al.*, 1992; Ren *et al.*, 1995). The energetics of dimerisation have also been computationally studied and the contributions of individual

residues to the surface area that is buried upon dimer association have been dissected (Rodriguez-Barrios *et al.*, 2001). The segment from I135 to P140 in p51, which is part of the β7−β8 loop and essential for the catalytic activity of the p66 subunit (Pandey *et al.*, 2002; Pandey *et al.*, 2001), has been identified as a "hot spot" of binding energy (Rodriguez-Barrios *et al.*, 2001) (Fig. 5.7). Of these amino acids, N136 is the most buried (Rodriguez-Barrios *et al.*, 2001) and engages its side-chain carboxamide group in two hydrogen bonds to the backbone of H96 in the p66 subunit (Fig. 5.8). Interestingly, N136 and also N137 are highly conserved among all known lentiviral RTs (Table 5.1), and our site-directed mutagenesis studies now reveal that none of the mutant N136 and N137 RT enzymes shows significant catalytic RT activity except for the N137H. The marginal RNA-dependent DNA polymerase activity of HIV-1 RT ranged between 0.08 and 64% of control (wild-type) depending on the nature of the amino acid replacing the asparagine at position 136 or 137. It is noteworthy that the N136T and N136S and N137S and N137H RT mutants, which were endowed with the highest catalytic activity among all HIV-1 RT mutants tested in this study, are the most prevalent mutant enzymes found in HIV-1-infected patients treated with NNRTIs, with a prevalence of <1% (Table 5.2). However, it should be mentioned that these mutations at amino acid position 136 or 137 are not the sole amino acid mutations present in the RT of these drug-treated HIV-1-infected individuals, but are accompanied by several other amino acid changes that may affect not only drug resistance but also fitness (by increasing replication competence) of these mutant virus strains. N136 and N137 in the p66 subunit are located in the solvent-exposed β7-β8 loop structure markedly distant from both the substrate active site and the NNRTI-binding pocket. Instead, N136 and N137 in the p51 subunit are located right at the interface between p66 and p51, and contribute to the formation of the base of the NNRTI pocket (Fig. 5.1). Therefore, it is obvious that the heavily compromised catalytic activity of the N136- and N137-mutated HIV-1 RT is predominantly due to the effect of the mutated amino acid in the p51 subunit rather than in the p66 unit. Indeed, when the N136T mutation was solely introduced in the p66 subunit, the RdDp activity was substantially restored (71% of wild-type) whereas the mutation solely in p51 resulted in only 8% of wild-type RT activity. It is unclear, however, why the catalytic competence of the mutant p66 N136T/p51 enzyme remained lower than that of wild-type RT.

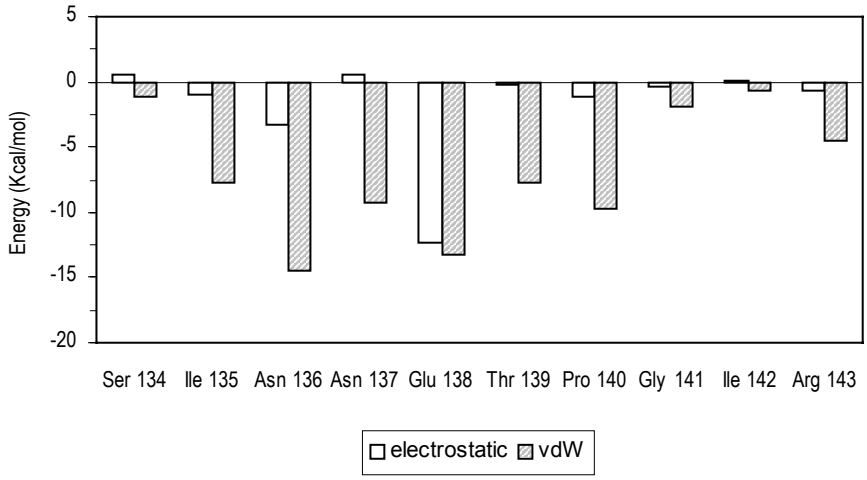

Figure 5.7 Van der Waals (hatched) and electrostatic (blanc) contributions of the residues making up the tip of the p51 β7-β8 loop to the dimerisation energy of HIV-1 RT (Rodriguez-Barrios *et al.*, 2001).

The observed effect of the mutated amino acid at position 136 and 137 in p51 of the HIV-1 RT on the catalytic activity is in full agreement with the findings of Pandey *et al.*, (2001) who demonstrated that the β7-β8 loop of p51 is a key structural element for RT dimerisation, and also that duplication of the six amino acids from the β7-β8 loop in p51, but not in p66, results in a substantial loss of both DNA binding and catalytic efficiency (Pandey *et al.*, 2002). It is thus clear that N136 and N137 in HIV-1 RT must fulfil a crucial structural function in the p51 β7-β8 loop to preserve the catalytic activity of the heterodimer. The observed decreased affinity of the (mutant) enzymes for the template/primer (the lower the catalytic activity of the mutant RT, the higher the K_m for the template/primer), and the further decreased apparent affinity for the template/primer in the presence of urea is in full agreement with the requirement of an intact β7-β8 loop of p51 for loading the p66 subunit on the template primer (Harris *et al.*, 1998).

There are three major contact areas between the p66 and the p51 subunits of RT (Becerra *et al.*, 1991; Wang *et al.*, 1994). The most intensively studied interface area between p66 and p51 is the tryptophan-rich amino acid stretch in the connection subdomain of both RT subunits consisting of six tryptophan residues at codons 398, 401, 402, 406, 410, and 414. These amino acids are also highly conserved amongst all primate lentiviral RTs. Mutagenesis studies at these amino acid locations revealed an important role in RT dimerisation (Tachedjian *et al.*, 2003). Interestingly, the effect of these amino acids on dimerisation proved to be mediated mainly through the p66 subunit. Thus, mutations at W401 and W414 in p66

resulted in a complete lack of RT activity. Such mutations impaired RT subunit dimerisation by altering the proper positioning of these structural elements against those residues in p51, which make important contacts with p66. A second stretch suggested to be involved in dimerisation contains a leucine hepta-repeat motif (L282-L310) (Baillon *et al.*, 1991). Goel *et al.* (1993) demonstrated that mutant L289K p66 was unable to dimerise with (either mutant L289K or wild-type) p51 but not *vice versa* (dimerisation of wild-type p66 with mutant p51) suggesting a critical role for the leucine repeat motif of p66 (but not of p51) in heterodimeric formation. From the crystallographic analyses of HIV-1 RT both in the apo form and in complex with inhibitors, it is apparent that N136 and N137 are located in the middle of the third major contact area at the interface between p66 and p51. Likewise, we believe that N136 and N137 in the β7-β8 loop of HIV-1 RT p51 may also fulfil a function in p66/p51 dimerisation comparable to that of the tryptophans in the 398-414 amino acid stretch of p66 and that of L289 in the leucine hepta-repeat stretch of p66. Indeed, it was found by FPLC size exclusion chromatography that, after mixing equal amounts of p66 and p51 subunits, mutant N136T and N136Y RT consisted of markedly less intact p66/p51 heterodimer and much more free p66 and p51 subunits than wild-type after 24 hrs (Fig. 5.5). In this respect, the mutant N136Y RT enzyme, which also showed the lowest catalytic activity (0.08% of wild-type), contained the highest amounts of monomeric p66 and p51. The N136T RT enzyme (catalytic activity: 2.1% of wild-type) contained lower amounts of monomeric p66 and p51 than the N136Y RT whereas the wild-type RT contained virtually no free monomer. These observations are strongly suggestive for a less optimal binding of the p66 and p51 subunits in the mutated enzymes than in the wild-type RT. This disturbed binding efficiency between p66 and p51 can be related to the structural changes the mutations at N136 afford on RT (increased amount of α helices and less random coil according to CD analysis of the mutant RT enzymes) (Fig. 5.6 and Table 5.5). The pronounced effect of the N136 mutation on the RT p66/p51 interface also has a clear effect on the efficiency of DNA binding, since the K_m of the mutant enzymes for template/primer is higher than the K_m for wild-type enzyme. This observation is in full agreement with the instrumental role of the p51 subunit in the loading of DNA onto the heterodimeric enzyme.

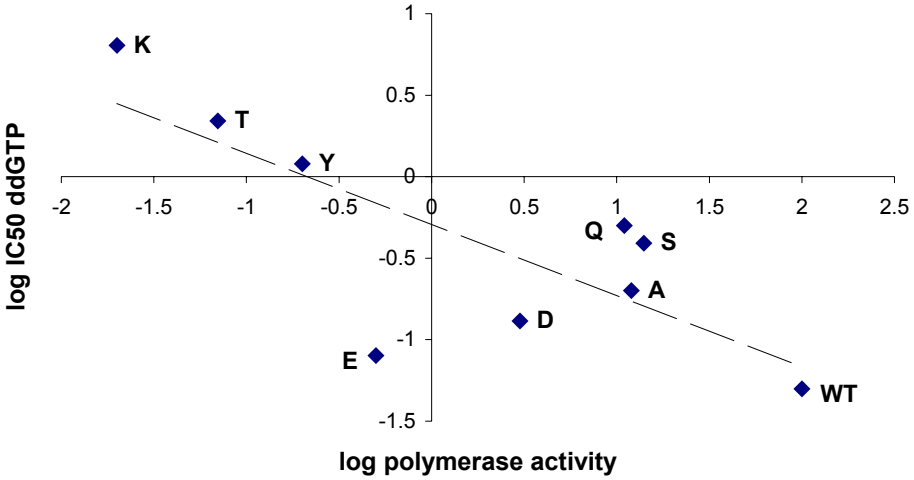

Figure 5.8 Inverse correlation between the catalytic DNA polymerase activity of the mutant N137X RT enzymes and their resistance to the inhibitory activity of the NRTI ddGTP.
Poly(rC).oligo(dG) was used as the template primer and [^{3}H]dGTP as the radiolabeled substrate.

When mutant N136 and N137 RT enzymes were exposed to a variety of urea concentrations, we found that the lower the catalytic activity of the mutant enzymes, the more easily they were denatured by urea. In fact, a very close correlation was found between the catalytic activity of the mutant RT enzymes and the mid-point concentration of urea at which the enzyme activity was reduced by 50% (Fig. 5.4). Such a phenomenon was not observed when HIV-1 RT enzymes mutated at other subdomain areas [i.e. at Y318 that is located near the thumb domain of RT and also results in compromised catalytic activity when mutated (Pelemans *et al.*, 1998)] were exposed to urea. These findings may also point to a less tight association between both RT subunits at the dimerisation interface in the mutant N136 or N137 enzymes and, again, to the crucial role of this highly conserved N136 at this location in p51 for maintaining two hydrogen bonds with the main chain of H96 of p66 thus ensuring tight subunit association (Fig. 5.9). Indeed, Sluis-Cremer and co-workers reported that in the presence of relatively low urea concentrations (<2 M), loss of RT activity is a direct result of dissociation of the heterodimeric RT and not a result of a conformational change in protein secondary structure (Sluis-Cremer *et al.*, 2002). Their studies also implied that no significant structural changes (or unfolding events) are associated with the dissociation of the RT heterodimer subunits at the urea concentrations used in our studies. Similar findings on the effect of low concentrations of urea on heterodimerisation of RT were reported earlier (Menendez-Arias *et al.*, 2001).

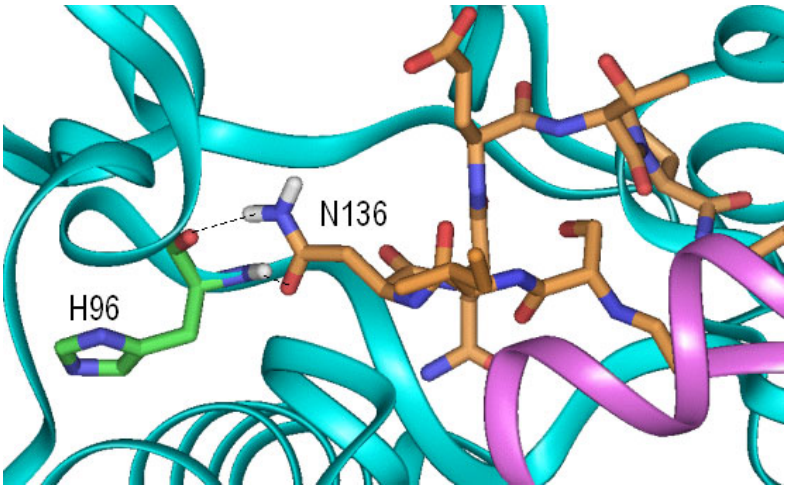

Figure 5.9 Detail of the HIV-1 RT subunit interface showing the hydrogen bonding interaction of the side chain of N136 in p51 (pink) with the backbone of His96 in p66 (dark cyan). Generated using ViewerLite 5.0 software (Accelrys Inc.; http://www.accelrys.com)

When several NNRTIs and the NRTI ddGTP were evaluated for their inhibitory activity against the mutant N136 RT enzymes, the first-generation NNRTI compounds (i.e. nevirapine, delavirdine) lost in the majority of cases their inhibitory activity to a variable extent depending on the nature of both the amino acid mutation and the evaluated drug. However, the second generation NNRTIs (i.e. efavirenz, quinoxaline) kept a marked inhibitory potential against the mutant enzymes irrespective of the nature of the mutation at amino acid 136. Since the p51 subunit has been shown to be instrumental in loading the p66 subunit onto the template/primer (Harris *et al.*, 1998), disturbance of the p66/p51 dimerisation interface by the N136 RT mutations may also indirectly affect both the substrate active site and the NNRTI-specific binding pocket. This may explain the varying degrees of sensitivity/resistance of the variety of NNRTIs. Interestingly, as already mentioned earlier, the very potent efavirenz, which is known to enhance RT dimerisation (Tachedjian and Goff, 2003; Tachedjian *et al.*, 2001), and also the quinoxaline derivative GW420867X, retain pronounced inhibitory activity against all N136-mutated RT enzymes. Therefore, the impact of a mutation at N136 in the p51 of the HIV-1 p66/p51 RT heteroduplex on the sensitivity of the mutant enzymes to second-generation NNRTIs seems to be relatively minor. Also various degrees of resistance towards ddGTP were observed for the mutant N136 RTs but especially for the mutant N137 RT enzymes, which also revealed resistance, although to a lesser extent, towards d4TTP and PFA. Such dramatic change in the sensitivity of HIV-1 RT to NRTIs and PFA upon mutations in the neighbourhood of the distant NNRTI pocket has, to the best of our

knowledge, never been observed before. In fact, we observed a close inverse correlation between the catalytic efficiency of the mutant enzymes on the one hand, and their degree of resistance to the inhibitory activity of the substrate analogue ddGTP (Fig. 5.8) and d4TTP on the other (r = 0.77 and 0.58, respectively). Indeed, if the substrate active site has been compromised by the N137 (or N136) mutations in p51, it is not unlikely that also the efficiency of binding of ddGTP (and other NRTIs such as d4TTP) to the active site is affected.

Thus, by identifying the importance of N136 and N137 for enzyme structure and functionality, our study now suggests that designing N136 and N137 mimetics, possibly possessing a similar carboxamide group, might become a novel strategy to develop drugs that interfere with the β7-β8 dimerisation interface. Another unexplored possibility, given the relative proximity of N136 and N137 to the NNRTI binding pocket, would be to rationally design modifications of existing NNRTIs to additionally target this highly conserved amino acid in the p51 subunit to improve their resistance profile and suppressive effect on wild-type HIV-1 strains.

In conclusion, N136 and N137 represent highly conserved amino acids among all known lentivirus RTs whose role in HIV-1 RT are shown here to be severely compromised upon mutation to other amino acids. Natural mutations at this amino acid site of RT will lead to a virtually inactive enzyme (and thus poorly replicating virus, if viable at all) that nevertheless will still be sensitive to several second-generation NNRTIs including the clinically used efavirenz. Therefore, it is very unlikely that mutations at position 136 or 137 in HIV RT will appear under selective pressure of these drugs, because they will markedly suppress viability of the mutant virus by heavily compromising subunit dimerisation due to the structurally changed interface. N136 and N137 could therefore become attractive targets for the design of novel NNRTIs with improved potency and increased ability to suppress virus drug resistance development. Such drugs would represent a completely new family of compounds aimed at disrupting RT subunit dimerisation by targeting a previously unexplored interface region. In addition, short peptides or peptidomimetics should be designed to interact with the β7-β8 loop in p51 to compromise efficient RT dimerisation of the enzyme.

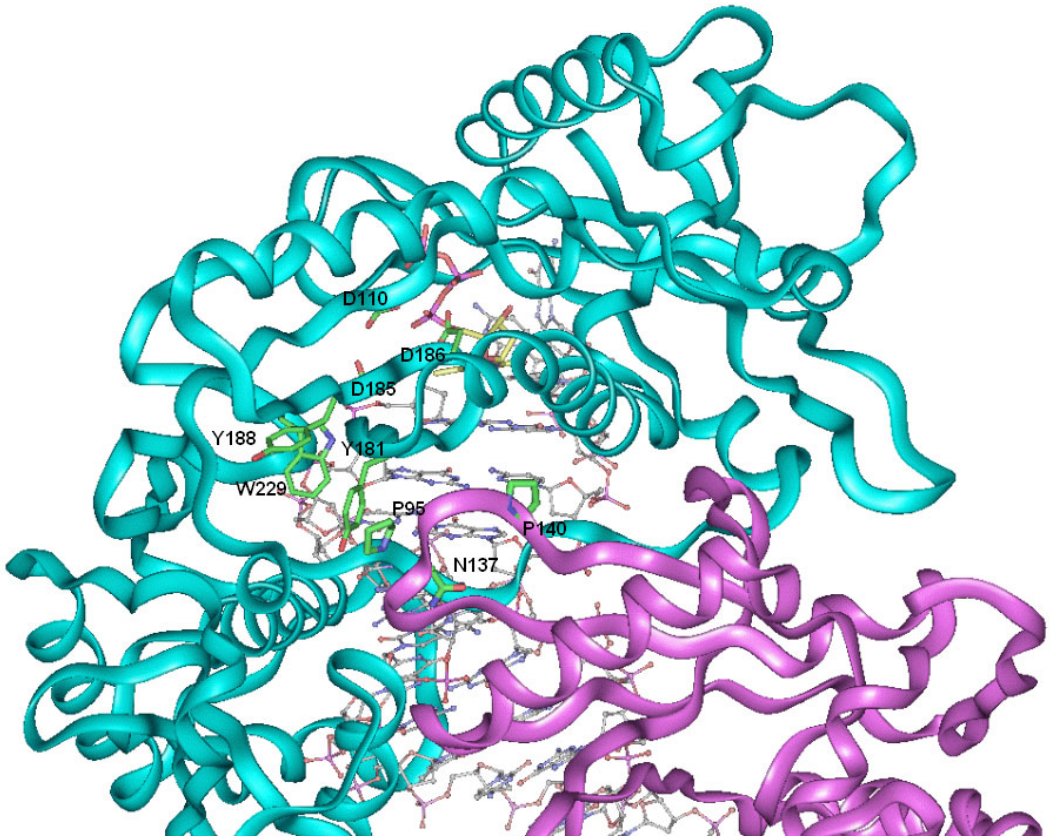

Figure 5.10 Detail of the HIV-1 RT subunit interface showing as sticks the side chains of N137 and P140 in the β7-β8 loop of p51 (pink ribbon) and of P95 in the p66 subunit (cyan ribbon). Generated using ViewerLite 5.0 software (Accelrys Inc.; http://www.accelrys.com)

Carbon atoms of the DNA template:primer are colored grey whereas those of the incoming nucleotide triphosphate are colored yellow. Residues Y181, Y188 and W229 making up the NNRTI binding pocket and the catalytic D110, D185 and D186 are shown as thick and thin sticks, respectively.

REFERENCES

Arion D, Fletcher RS, Borkow G, Camarasa MJ, Balzarini J, Dmitrienko GI and Parniak MA (1996) Differences in the inhibition of human immunodeficiency virus type 1 reverse transcriptase DNA polymerase activity by analogs of nevirapine and [2',5'-bis-O-(tert-butyldimethylsilyl)-3'-spiro-5"-(4"-amino-1", 2"-oxathiole-2",2"-dioxide] (TSAO). *Mol Pharmacol* **50**:1057-64.

Baillon JG, Nashed NT, Kumar A, Wilson SH and Jerina DM (1991) A leucine zipper-like motif may mediate reverse transcriptase subunit binding. *The New Biologist* **3**:1015-1019.

Balzarini J, Karlsson A, Vandamme AM, Perez-Perez MJ, Zhang H, Vrang L, Oberg B, Backbro K, Unge T, San-Felix A and et al. (1993) Human immunodeficiency virus type 1 (HIV-1) strains selected for resistance against the HIV-1-specific [2',5'-bis-O-(tert-butyldimethylsilyl)-3'-spiro- 5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide)]-beta-D-pentofurano syl (TSAO) nucleoside analogues retain sensitivity to HIV-1-specific nonnucleoside inhibitors. *Proc Natl Acad Sci U S A* **90**:6952-6.

Balzarini J, Kleim JP, Riess G, Camarasa MJ, De Clercq E and Karlsson A (1994) Sensitivity of (138 Glu-->Lys) mutated human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) to HIV-1-specific RT inhibitors. *Biochem Biophys Res Commun* **201**:1305-12.

Becerra SP, Kumar A, Lewis MS, Widen SG, Abbotts J, Karawya EM, Hughes SH, Shiloach J and Wilson SH (1991) Protein-protein interactions of HIV-1 reverse transcriptase: implication of central and C-terminal regions in subunit binding. *Biochemistry* **30**:11707-19.

Bohm G, Muhr R and Jaenicke R (1992) Quantitative analysis of protein far UV circular dichroism spectra by neural networks. *Protein Eng* **5**:191-5.

Boyer PL, Ding J, Arnold E and Hughes SH (1994) Subunit specificity of mutations that confer resistance to nonnucleoside inhibitors in human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* **38**:1909-14.

Buckheit RW, Jr., Fliakas-Boltz V, Decker WD, Roberson JL, Stup TL, Pyle CA, White EL, McMahon JB, Currens MJ, Boyd MR and et al. (1995) Comparative anti-HIV evaluation of diverse HIV-1-specific reverse transcriptase inhibitor-resistant virus isolates demonstrates the existence of distinct phenotypic subgroups. *Antiviral Res* **26**:117-32.

D'Aquila RT and Summers WC (1989) HIV-1 reverse transcriptase/ribonuclease H: high level expression in Escherichia coli from a plasmid constructed using the polymerase chain reaction. *J Acquir Immune Defic Syndr* **2**:579-87.

Ding J, Das K, Moereels H, Koymans L, Andries K, Janssen PA, Hughes SH and Arnold E (1995a) Structure of HIV-1 RT/TIBO R 86183 complex reveals similarity in the binding of diverse nonnucleoside inhibitors. *Nat Struct Biol* **2**:407-15.

Ding J, Das K, Tantillo C, Zhang W, Clark AD, Jr., Jessen S, Lu X, Hsiou Y, Jacobo-Molina A, Andries K and et al. (1995b) Structure of HIV-1 reverse transcriptase in a complex with the non-nucleoside inhibitor alpha-APA R 95845 at 2.8 A resolution. *Structure* **3**:365-79.

Esnouf R, Ren J, Ross C, Jones Y, Stammers D and Stuart D (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. *Nat Struct Biol* **2**:303-8.

Goel R, Beard WA, Kumar A, Casas-Finet JR, Strub MP, Stahl SJ, Lewis MS, Bebenek K, Becerra SP, Kunkel TA and et al. (1993) Structure/function studies of HIV-1(1) reverse transcriptase: dimerization-defective mutant L289K. *Biochemistry* **32**:13012-8.

Harris D, Lee R, Misra HS, Pandey PK and Pandey VN (1998) The p51 subunit of human immunodeficiency virus type 1 reverse transcriptase is essential in loading the p66 subunit on the template primer. *Biochemistry* **37**:5903-8.

Hopkins AL, Ren J, Esnouf RM, Willcox BE, Jones EY, Ross C, Miyasaka T, Walker RT, Tanaka H, Stammers DK and Stuart DI (1996) Complexes of HIV-1 reverse transcriptase with inhibitors of the HEPT series reveal conformational changes relevant to the design of potent non-nucleoside inhibitors. *J Med Chem* **39**:1589-600.

Huang H, Chopra R, Verdine GL and Harrison SC (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **282**:1669-75.

Jonckheere H, De Vreese K, Debyser Z, Vandekerckhove J, Balzarini J, Desmyter J, De Clercq E and Anne J (1996) A two plasmid co-expression system in Escherichia coli for the production of virion-like reverse transcriptase of the human immunodeficiency virus type 1. *J Virol Methods* **61**:113-25.

Jonckheere H, Taymans JM, Balzarini J, Velazquez S, Camarasa MJ, Desmyter J, De Clercq E and Anne J (1994) Resistance of HIV-1 reverse transcriptase against [2',5'-bis-O-(tert-butyldimethylsilyl)-3'-spiro-5"-(4"-amino-1",2"- oxathiole-2",2"-dioxide)] (TSAO) derivatives is determined by the mutation Glu138-->Lys on the p51 subunit. *J Biol Chem* **269**:25255-8.

Kohlstaedt LA, Wang J, Friedman JM, Rice PA and Steitz TA (1992) Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**:1783-90.

Menendez-Arias L, Abraha A, Quinones-Mateu ME, Mas A, Camarasa MJ and Arts EJ (2001) Functional characterization of chimeric reverse transcriptases with polypeptide subunits of highly divergent HIV-1 group M and O strains. *J Biol Chem* **276**:27470-9.

Pandey PK, Kaushik N, Singh K, Sharma B, Upadhyay AK, Kumar S, Harris D and Pandey VN (2002) Insertion of a small peptide of six amino acids into the beta7-beta8 loop of the p51 subunit of HIV-1 reverse transcriptase perturbs the heterodimer and affects its activities. *BMC Biochem* **3**:18.

Pandey PK, Kaushik N, Talele TT, Yadav PN and Pandey VN (2001) The beta7-beta8 loop of the p51 subunit in the heterodimeric (p66/p51) human immunodeficiency virus type 1 reverse transcriptase is essential for the catalytic function of the p66 subunit. *Biochemistry* **40**:9505-12.

Pelemans H, Esnouf RM, Jonckheere H, De Clercq E and Balzarini J (1998) Mutational analysis of Tyr-318 within the non-nucleoside reverse transcriptase inhibitor binding pocket of human immunodeficiency virus type I reverse transcriptase. *J Biol Chem* **273**:34234-9.

Perez-Perez MJ, San-Felix A, Balzarini J, De Clercq E and Camarasa MJ (1992) TSAO analogues. Stereospecific synthesis and anti-HIV-1 activity of 1-[2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3'-spiro -5"- (4"-amino-1",2"-oxathiole 2",2"-dioxide) pyrimidine and pyrimidine-modified nucleosides. *J Med Chem* **35**:2988-95.

Ren J, Esnouf R, Garman E, Somers D, Ross C, Kirby I, Keeling J, Darby G, Jones Y, Stuart D and et al. (1995) High resolution structures of HIV-1 RT from four RT-inhibitor complexes. *Nat Struct Biol* **2**:293-302.

Restle T, Muller B and Goody RS (1990) Dimerization of human immunodeficiency virus type 1 reverse transcriptase. A target for chemotherapeutic intervention. *J Biol Chem* **265**:8986-8.

Rodriguez-Barrios F, Perez C, Lobaton E, Velazquez S, Chamorro C, San-Felix A, Perez-Perez MJ, Camarasa MJ, Pelemans H, Balzarini J and Gago F (2001) Identification of a putative binding site for [2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3'-spiro-5"- (4"-amino-1",2"-oxathiole-2",2"-dioxide)thymine (TSAO) derivatives at the p51-p55 interface of HIV-1 reverse transcriptase. *J Med Chem* **44**:1853-65.

Sluis-Cremer N, Arion D and Parniak MA (2002) Destabilization of the HIV-1 reverse transcriptase dimer upon interaction with N-acyl hydrazone inhibitors. *Mol Pharmacol* **62**:398-405.

Sluis-Cremer N, Dmitrienko GI, Balzarini J, Camarasa MJ and Parniak MA (2000) Human immunodeficiency virus type 1 reverse transcriptase dimer destabilization by 1-[Spiro[4"-amino-2",2" -dioxo-1",2" -oxathiole-5",3'-[2', 5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]]]-3-ethylthy mine. *Biochemistry* **39**:1427-33.

Tachedjian G, Aronson HE, de los Santos M, Seehra J, McCoy JM and Goff SP (2003) Role of residues in the tryptophan repeat motif for HIV-1 reverse transcriptase dimerization. *J Mol Biol* **326**:381-96.

Tachedjian G and Goff SP (2003) The effect of NNRTIs on HIV reverse transcriptase dimerization. *Curr Opin Investig Drugs* **4**:966-73.

Tachedjian G, Orlova M, Sarafianos SG, Arnold E and Goff SP (2001) Nonnucleoside reverse transcriptase inhibitors are chemical enhancers of dimerization of the HIV type 1 reverse transcriptase. *Proc Natl Acad Sci U S A* **98**:7188-93.

Wang J, Smerdon SJ, Jager J, Kohlstaedt LA, Rice PA, Friedman JM and Steitz TA (1994) Structural basis of asymmetry in the human immunodeficiency virus type 1 reverse transcriptase heterodimer. *Proc Natl Acad Sci U S A* **91**:7242-6.

CHAPTER VI

ANALYSIS OF MUTATIONS AT T139 IN HIV-1 RT AND THEIR EFFECT ON SENSITIVITY TO (+)-CALANOLIDE A

The results presented in this chapter will be published in the following article:

Auwerx, J., Rodríguez-Barrios, F., de Castro, S., San-Félix, A., Velázquez, S., Ceccherini-Silberstein, F., De Clercq, E., Camarasa, M.-J., Perno, C.-F., Gago, F. and Balzarini, J. (2005) Role of T139 in the human immunodeficiency virus type 1 reverse transcriptase sensitivity to (+)-calanolide A. *Mol. Pharmacol. In press*

SUMMARY

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. (+)-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. Our studies also revealed that the T139 amino acid in HIV-1 RT would not be an appropriate target for novel or modified NNRTIs to increase the potency or to attenuate the drug resistance profile.

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 series has been evaluated in antiviral activity studies against 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 and 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.*, 1999). 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-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 6.4), is essential for the catalytic function of the p66 subunit as it is required to form a stable heterodimeric enzyme (Pandey *et al.*, 2001 and 2002). 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 and 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 dimerisation, which places this compound in a unique position amongst the NNRTIs (Rodriguez-Barrios *et al.*, 2001; Sluis-Cremer *et al.*, 2000).

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.

MATERIALS AND METHODS

Compounds

All compounds were obtained as previously mentioned in Chapter 5.

Site-directed mutagenesis of HIV-1 reverse transcriptase

Mutant RT-enzymes containing the T139A, T139Q, T139Y, T139K, T139I, T139S or T139D mutations were derived from the RT sequence cloned in pKRT2His (D'Aquila and Summers, 1989). Site-directed mutagenesis and confimation by sequencing was performed as described in Chapter 2. For all 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 reverse transcriptases

Recombinant HIV-1 RTs were expressed from a two-plasmid co-expression, constructed as described in Chapter 5.

Expression and purification of wild-type and mutant HIV-1 RT

Expression and purification of recombinant RT were performed as described in Chapter 3.

Reverse transcriptase assay

The assay procedure for measuring the inhibitory effect of the test compounds against HIV RT and steady-state kinetic assays were performed as described in Chapter 2.

Stability of wild-type and mutant heterodimer HIV-1 RTs in the presence of urea

Denaturation curves were determined as described in Chapter 5.

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).

RESULTS

6.1. 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) 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. 6.1). 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 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.

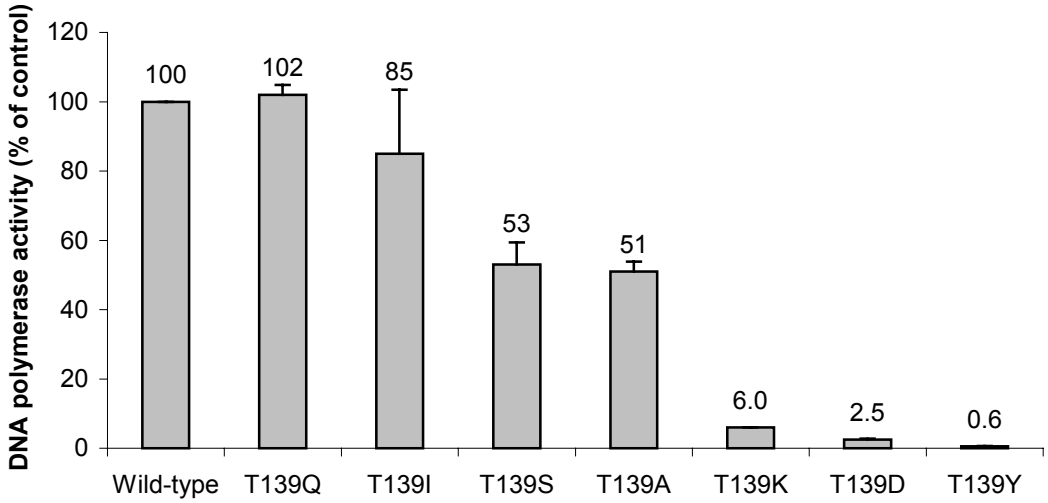

Figure 6.1 Catalytic RNA dependent DNA polymerase activity of mutant T139X HIV-1 RT enzymes.

6.2. 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 6.1). 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-m^{3}T. 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 GW420867X kept a virtually similar inhibitory potential against each of the mutant RTs. As already observed for 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 6.2). 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. 6.3). 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.

6.3. 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 6.1. 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.

Table 6.1 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)	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 is in $\mu M^{-1}s^{-1}$; The data are means (± S.D.) of at least two to three independent experiments.

Table 6.2 Inhibitory activity of test compounds against mutant T139X HIV-1 RTs

	IC_{50} (µM)[a]								
RT mutation	**nevirapine**	**Delavirdine**	**Efavirenz**	**UC-781**	**GW420867X**	**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); substrate: 1.6 µM [8-³H]dGTP

Data are the means of 2 to 3 independent experiments ± S.D.

[a] 50% inhibitory concentration or compound concentration required to inhibit RT activity with 50%

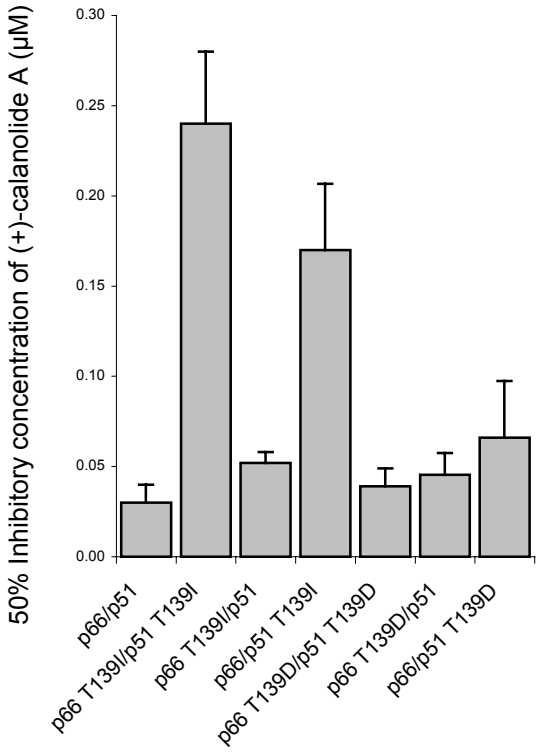

Figure 6.2 Inhibitory activity of (+)-calanolide A against the catalytic activity of wild-type and mutant HIV-1 RT enzymes that contain the T139I or T139D mutation solely in the p66, solely in the p51 or both in the p66 and p51 subunits.

6.4. 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. 6.3). 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 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 ~0.75 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 0.75 M to 0.55 M for the mutant T139I RT, the urea IC_{50} was further decreased to 0.40 M for the mutant T139D RT enzyme (Fig. 6.3). 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 (data not shown).

6.5. Structural context of amino acid 139 in HIV-1 RT

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 dimerisation interface (Fig. 6.4). 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 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. 6.1). 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 only slightly be perturbed in the case of T139→A or T139→S mutations.

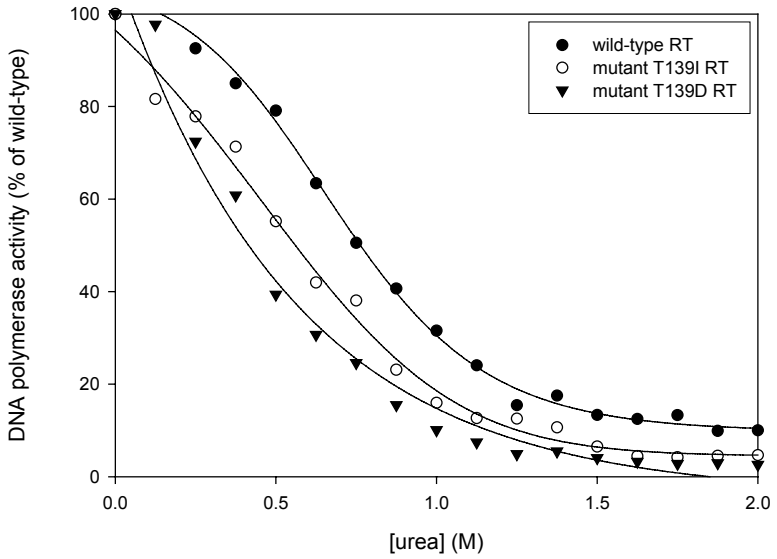

Figure 6.3 Effect of urea on the catalytic activity of mutant T139I, T139D and wild-type HIV-1 RTs.

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.*, 1996a). 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. 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 heterodimerisation of the RT enzyme as shown earlier for the TSAO interaction with E138 of the β7-β8 loop (Pandey *et al.*, 2001 and 2002; 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. 6.4) that is of crucial importance for efficient dimerisation 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 destabilisation of the heterodimeric RT may result from structural and conformational perturbations at the RT subunit interface (Rodriguez-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 and 137 (see Chapter 5) 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 (denaturating) action of urea (Fig. 6.3). 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.

Although T139 is rather conserved in wild-type HIV-1 strains, Ceccherini-Silberstein and coworkers (2004) found that 3.7% of drug-treated HIV-1-infected patients were bearing mutations at position 139 (see also Table 5.2). 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.

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 6.2). 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)

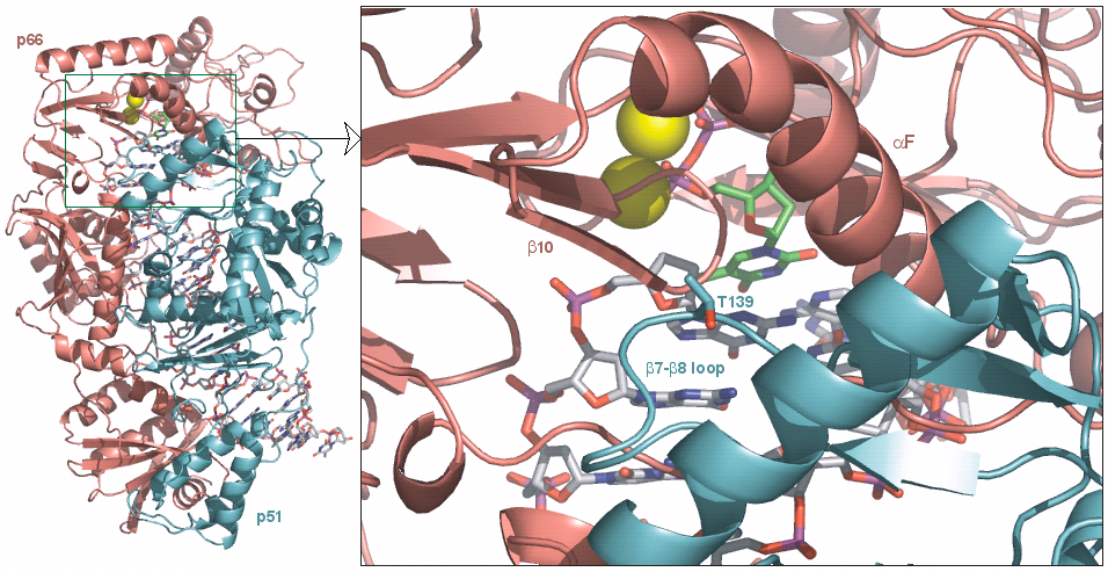

Figure 6.4 Left. Schematic representation of the dimeric structure of HIV-1 RT in complex with a DNA template/primer generated by PyMOL Molecular Graphics System.

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).

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 dimerisation assay (+)-calanolide A had no detectable effect on RT dimerisation (Tachedjian and Goff, 2003; Tachedjian *et al.*, 2001). However, failure of an effect of (+)-calanolide A on RT dimerisation 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 dimerisation assays. Given the fact that it has been shown that TSAO derivatives affect RT subunit dimerisation, 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 dimerisation 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 6.2).

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.

In conclusion, among the T139 RT mutants investigated, the mutant 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 three kinetic properties of the mutant RTs (a pronounced catalytic activity of the T139I RT enzyme, 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 denaturating effect of urea against mutant T139I and T139D *versus* wild-type RT enzymes, it can be well possible that (+)-calanolide A affects dimerisation of the HIV-1 RT heterodimer and thus represents the prototype compound of a new class of RT dimerisation inhibitors.

REFERENCES

Balzarini, J. (1999). Suppression of resistance to drugs targeted to human immunodeficiency virus reverse transcriptase by combination therapy. *Biochem Pharmacol* **58**(1)**,** 1-27.

Balzarini, J. (2004). Current status of the non-nucleoside reverse transcriptase inhibitors of human immunodeficiency virus type 1. *Curr Top Med Chem* **4**(9)**,** 921-44.

Balzarini, J., Karlsson, A., Vandamme, A. M., Perez-Perez, M. J., Zhang, H., Vrang, L., Oberg, B., Backbro, K., Unge, T., San-Felix, A., and et al. (1993a). Human immunodeficiency virus type 1 (HIV-1) strains selected for resistance against the HIV-1-specific [2',5'-bis-O-(tert-butyldimethylsilyl)-3'-spiro- 5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide)]-beta-D-pentofurano syl (TSAO) nucleoside analogues retain sensitivity to HIV-1-specific nonnucleoside inhibitors. *Proc Natl Acad Sci U S A* **90**(15)**,** 6952-6.

Balzarini, J., Pelemans, H., Esnouf, R., and De Clercq, E. (1998). A novel mutation (F227L) arises in the reverse transcriptase of human immunodeficiency virus type 1 on dose-escalating treatment of HIV type 1-infected cell cultures with the nonnucleoside reverse transcriptase inhibitor thiocarboxanilide UC-781. *AIDS Res Hum Retroviruses* **14**(3)**,** 255-60.

Balzarini, J., Velazquez, S., San-Felix, A., Karlsson, A., Perez-Perez, M. J., Camarasa, M. J., and De Clercq, E. (1993b). Human immunodeficiency virus type 1-specific [2',5'-bis-O-(tert- butyldimethylsilyl)-beta-D-ribofuranosyl]-3'-spiro-5"-(4"-amino-1",2"- oxathiole-2",2"-dioxide)-purine analogues show a resistance spectrum that is different from that of the human immunodeficiency virus type 1-specific non-nucleoside analogues. *Mol Pharmacol* **43**(1)**,** 109-14.

Boyer, P. L., Ding, J., Arnold, E., and Hughes, S. H. (1994). Subunit specificity of mutations that confer resistance to nonnucleoside inhibitors in human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* **38**(9)**,** 1909-14.

Buckheit, R. W., Jr., White, E. L., Fliakas-Boltz, V., Russell, J., Stup, T. L., Kinjerski, T. L., Osterling, M. C., Weigand, A., and Bader, J. P. (1999). Unique anti-human immunodeficiency virus activities of the nonnucleoside reverse transcriptase inhibitors calanolide A, costatolide, and dihydrocostatolide. *Antimicrob Agents Chemother* **43**(8)**,** 1827-34.

Camarasa, M. J., San-Felix, A., Velazquez, S., Perez-Perez, M. J., Gago, F., and Balzarini, J. (2004). TSAO compounds: the comprehensive story of a unique family of HIV-1 specific inhibitors of reverse transcriptase. *Curr Top Med Chem* **4**(9)**,** 945-63.

Currens, M. J., Gulakowski, R. J., Mariner, J. M., Moran, R. A., Buckheit, R. W., Jr., Gustafson, K. R., McMahon, J. B., and Boyd, M. R. (1996a). Antiviral activity and mechanism of action of calanolide A against the human immunodeficiency virus type-1. *J Pharmacol Exp Ther* **279**(2)**,** 645-51.

Currens, M. J., Mariner, J. M., McMahon, J. B., Boyd, M. R., Shulman, N., and Winters, M. (1996b). Kinetic analysis of inhibition of human immunodeficiency virus type-1 reverse transcriptase by calanolide A. *J Pharmacol Exp Ther* **279**(2)**,** 652-61.

D'Aquila, R. T., and Summers, W. C. (1989). HIV-1 reverse transcriptase/ribonuclease H: high level expression in Escherichia coli from a plasmid constructed using the polymerase chain reaction. *J Acquir Immune Defic Syndr* **2**(6)**,** 579-87.

De Clercq, E. (1999). Perspectives of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. *Farmaco* **54**(1-2)**,** 26-45.

DeLano WL (2004) The PyMOL Molecular Graphics System. DeLano Scientific LLC, San Carlos, CA, USA (http://www.pymol.org).

Esnouf, R. M., Ren, J., Hopkins, A. L., Ross, C. K., Jones, E. Y., Stammers, D. K., and Stuart, D. I. (1997). Unique features in the structure of the complex between HIV-1 reverse transcriptase and the bis(heteroaryl)piperazine (BHAP) U-90152 explain resistance mutations for this nonnucleoside inhibitor. *Proc Natl Acad Sci U S A* **94**(8)**,** 3984-9.

Huang, H., Chopra, R., Verdine, G. L., and Harrison, S. C. (1998). Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **282**(5394)**,** 1669-75.

Kashman, Y., Gustafson, K. R., Fuller, R. W., Cardellina, J. H., 2nd, McMahon, J. B., Currens, M. J., Buckheit, R. W., Jr., Hughes, S. H., Cragg, G. M., and Boyd, M. R. (1992). The calanolides, a novel HIV-inhibitory class of coumarin derivatives from the tropical rainforest tree, Calophyllum lanigerum. *J Med Chem* **35**(15)**,** 2735-43.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992). Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**(5065)**,** 1783-90.

Pandey, P. K., Kaushik, N., Singh, K., Sharma, B., Upadhyay, A. K., Kumar, S., Harris, D., and Pandey, V. N. (2002). Insertion of a small peptide of six amino acids into the beta7-beta8 loop of the p51 subunit of HIV-1 reverse transcriptase perturbs the heterodimer and affects its activities. *BMC Biochem* **3**(1)**,** 18.

Pandey, P. K., Kaushik, N., Talele, T. T., Yadav, P. N., and Pandey, V. N. (2001). The beta7-beta8 loop of the p51 subunit in the heterodimeric (p66/p51) human immunodeficiency virus type 1 reverse transcriptase is essential for the catalytic function of the p66 subunit. *Biochemistry* **40**(32)**,** 9505-12.

Pelemans, H., Aertsen, A., Van Laethem, K., Vandamme, A. M., De Clercq, E., Perez-Perez, M. J., San-Felix, A., Velazquez, S., Camarasa, M. J., and Balzarini, J. (2001). Site-directed mutagenesis of human immunodeficiency virus type 1 reverse transcriptase at amino acid position 138. *Virology* **280**(1)**,** 97-106.

Quan, Y., Motakis, D., Buckheit, R., Jr., Xu, Z. Q., Flavin, M. T., Parniak, M. A., and Wainberg, M. A. (1999). Sensitivity and resistance to (+)-calanolide A of wild-type and mutated forms of HIV-1 reverse transcriptase. *Antivir Ther* **4**(4)**,** 203-9.

Ren, J., Nichols, C. E., Chamberlain, P. P., Weaver, K. L., Short, S. A., and Stammers, D. K. (2004). Crystal structures of HIV-1 reverse transcriptases mutated at codons 100, 106 and 108 and mechanisms of resistance to non-nucleoside inhibitors. *J Mol Biol* **336**(3)**,** 569-78.

Rodriguez-Barrios, F., Perez, C., Lobaton, E., Velazquez, S., Chamorro, C., San-Felix, A., Perez-Perez, M. J., Camarasa, M. J., Pelemans, H., Balzarini, J., and Gago, F. (2001). Identification of a putative binding site for [2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3'-spiro-5"- (4"-amino-1",2"-oxathiole-2",2"-dioxide)thymine (TSAO) derivatives at the p51-p55 interface of HIV-1 reverse transcriptase. *J Med Chem* **44**(12)**,** 1853-65.

Sluis-Cremer, N., Dmitrienko, G. I., Balzarini, J., Camarasa, M. J., and Parniak, M. A. (2000). Human immunodeficiency virus type 1 reverse transcriptase dimer destabilization by 1-[Spiro[4"-amino-2",2" -dioxo-1",2" -oxathiole-5",3'-[2', 5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]]]-3-ethylthy mine. *Biochemistry* **39**(6)**,** 1427-33.

Tachedjian, G., and Goff, S. P. (2003). The effect of NNRTIs on HIV reverse transcriptase dimerization. *Curr Opin Investig Drugs* **4**(8)**,** 966-73.

Tachedjian, G., Orlova, M., Sarafianos, S. G., Arnold, E., and Goff, S. P. (2001). Nonnucleoside reverse transcriptase inhibitors are chemical enhancers of dimerization of the HIV type 1 reverse transcriptase. *Proc Natl Acad Sci U S A* **98**(13)**,** 7188-93.

Vandamme, A.-M., Van Laethem, K., Van Vaerenbergh, K., and De Clercq, E. (1998). Anti-HIV virus combination therapy and resistance management. *International Antiviral News* **6**(10)**,** 182-187.

Yu, D., Suzuki, M., Xie, L., Morris-Natschke, S. L., and Lee, K. H. (2003). Recent progress in the development of coumarin derivatives as potent anti-HIV agents. *Med Res Rev* **23**(3)**,** 322-45.

CHAPTER VII

GENERAL DISCUSSION AND PERSPECTIVES

The current treatment for HIV-infected patients is based on combination therapies of RT and protease (and fusion) inhibitors better known as highly active antiretroviral therapy (HAART) (Vandamme *et al.*, 1998b). However, HAART is not always able to completely suppress HIV-1 replication, resulting in the emergence and selection of (multi)drug-resistant viral variants, with therapy failure a consequence (Vandamme *et al.*, 1998a; De Clercq, 2004a). Also, toxicity and inconvenience of the present drug regimens force many patients to stop or change treatment or to inappropriately take their drugs. Therefore, new and better drugs targeted at RT or other steps in the viral life cycle are still necessary to ensure sufficient options for both first-line and second-line or salvage therapy.

RT inhibitors play a crucial role in the efficient treatment of HIV-infected individuals, such as the NRTIs, the NtRTI tenofovir and the NNRTIs (De Clercq, 2000). Among these different drug classes, NNRTIs are very potent and highly selective inhibitors as they interact with a molecular site of the HIV-1 RT that does not exist in cellular DNA polymerases and that is also absent in the RT of other lentiviruses including HIV-2, FIV and SIV. However, their clinical use for anti-AIDS therapy has been limited by the relatively rapid emergence of drug-resistant viruses. Currently the FDA has already approved the NNRTIs nevirapine, delavirdine and efavirenz, but several other classes of NNRTIs have been discovered and are currently under investigation and/or in clinical trials (Balzarini, 2004; De Clercq, 2004b).

Due to the high specificity of the NNRTIs for HIV-1 RT there is a lack of a useful animal model that could account for the numerous clinical trials that are performed today with HIV-infected patients. The feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus that is associated with immunodeficiency and opportunistic infections in FIV-infected cats. FIV is very similar to HIV-1 regarding genomic organization, biologic properties and the tendency to evolve as a persistent infection in the natural host. The immunodeficiency and (neuro)pathogenesis in FIV-infected cats are remarkably similar to AIDS in humans (Bendinelli *et al.*, 1995). Thus, FIV would be a strong candidate to function as a relatively small animal model that is not hazardous to humans and that has already been used for evaluating NRTIs (such as AZT and PMEA) in the past (North *et al.*, 1989). However, FIV is not susceptible for NNRTIs. Therefore, part of our study was aimed to investigate the molecular determinants that cause resistance of FIV RT towards NNRTIs. In parallel an attempt was made to sensitize FIV RT to NNRTIs by construction of RT chimeras to eventually obtain a useful RT enzyme that shows pronounced NNRTI sensitivity and which could be used in infections with molecular FIV clones. However, whereas HIV-1 RT could be made entirely insensitive to NNRTIs by exchanging one or several amino acid stretches of

HIV-1 RT by those of FIV RT, none of the FIV RT chimeras in which one or several amino acid stretches of FIV RT were exchanged by those of HIV-1 RT gained susceptibility to NNRTIs. Moreover, the majority of chimeric RTs had a strong decreased catalytic efficacy while their affinity for their natural substrates was comparable with that of the wild-type HIV-1 and FIV RTs. Thus, replacing segments or substituting relevant amino acids in FIV RT by their HIV-1 RT counterparts did not suffice to make FIV RT sensitive towards NNRTIs and was often accompanied by a decrease or even total loss of polymerase activity. This was in sharp contrast with the results found for HIV-2 and SIV RT where only a L188Y replacement renders them sensitive towards the NNRTIs delavirdine and efavirenz (Isaka *et al.*, 2001) and with the results of chimeric HIV-1/HIV-2 RTs that show pronounced sensitivity towards several NNRTIs (Hizi *et al.*, 1993; Shih *et al.*, 1991). In an attempt to allow NNRTI-testing in the *in vivo* (i.e., monkey) setting, hybrid SIV strains in which the entire RT gene was replaced by the HIV-1 RT gene (designated RT-SHIV) have already been constructed (Balzarini *et al.*, 1995; Uberla *et al.*, 1995). Later, the importance of mutations at residues Y188 and Y181 in SIV and HIV-2 RT in resistance towards NNRTIs was also confirmed with structural data of HIV-2 RT (Ren *et al.*, 2002). Intriguingly, FIV RT is an enzyme that possesses the corresponding Y181 and Y188 amino acids of HIV-1 RT, but is still completely insensitive to NNRTIs. There probably exist significant differences in the structure and/or flexibility in FIV RT that may prevent NNRTIs from interacting with the FIV RT and that lead to fatal perturbations when amino acid parts are eventually replaced. It can also not be excluded whether the entry of NNRTIs to reach their binding pocket in FIV RT is sterically prevented, or alternatively whether NNRTIs may efficiently reach their binding pocket but that binding, in contrast with HIV-1 RT, does not lead to significant enzyme inhibition. Biacore experiments to detect binding of NNRTIs to FIV RT should be conducted to reveal this issue. Also, the elucidation of the crystal structure of FIV RT at a high resolution is still highly required to gain insight in (the lack of) potential interactions of FIV RT with different NNRTIs.

When we studied the influence of the p51 subunit of FIV RT on the sensitivity of HIV-1 RT in FIV/HIV-1 RT hybrids towards NNRTIs, construction of enzymatic hybrids with exchanged subunits revealed that replacing the p51 subunit has no influence on the pronounced sensitivity of HIV-1 RT to any of the analyzed NNRTIs and, *vice versa*, replacing the p51 subunit in FIV RT did not alter the resistance to NNRTIs. In contrast, the constructed chimeras had a poor catalytic efficacy that suggested suboptimal interaction between the subunits of the different RTs. It would be worthwhile to study the interactions of HIV-1 p66

with FIV p51 and FIV p66 with HIV-1 p51 at their interfaces in more detail. We may learn on the crucial amino acid areas that are instrumental at the interface of both subunits to keep both the structural and functional integrity of the heterodimers. Such insights may help to design novel types of dimerisation inhibitors.

Among the NNRTIs, some members of the PETT derivatives (i.e. PETT-2) have the unusual property to show activity towards HIV-2 (Ren *et al.*, 2000). Our studies on resistance development of HIV-2 and HIV-1 against another PETT derivative (i.e. MSK-076) revealed that MSK-076 inhibited HIV-1 RT in the nanomolar range and HIV-2 RT in the micromolar range. Our kinetic analyses of MSK-076 together with data obtained with the PETT-2 compound (Ren *et al.*, 2000) showed non-competitive inhibition of HIV-2 RT similar to all other NNRTI compounds that are exclusively active against HIV-1 RT. This NNRTI-like mode of action was also confirmed by our time of drug addition experiments in which it was shown that the PETT derivative lost antiviral activity when added to virus-infected cell cultures at a time point after the reverse transcription process. However, to distinguish between the presence of an equivalent NNRTI-binding site and another allosteric site for PETT binding present in the HIV-2 RT, we selected for amino acid mutations in HIV-1 and HIV-2 RT under MSK-076 drug pressure. Data from molecular modeling suggested that MSK-076 resistance mutations were found in the equivalent NNRTI-binding region of HIV-2 RT (i.e. A101P) but also distal to the NNRTI-pocket and in close proximity of the active site of HIV-2 RT (i.e. G112E). The latter mutation could imply a novel mechanism of drug interaction and enzyme inhibition. Our studies together with data from the crystal structure of HIV-2 RT revealed that although HIV-2 RT is insensitive to NNRTIs, some compounds still could interact with the enzyme and inhibit the catalytic RT activity and virus replication at a site that is distinct from, but closely related with the NNRTI-pocket. However, similar to HIV-1 where treatment with NNRTIs in monotherapy rapidly selects for highly resistant HIV strains, treatment of HIV-2-infected cells with the NNRTI MSK-076 also leads rapidly to selection of mutations in HIV-2 RT that confer resistance towards the NNRTIs. It would be of particular interest to search for additional NNRTIs that inhibit HIV-2 RT. Resistance selection and in depth enzyme kinetics allows insights in other binding mechanisms that may lead to the development of new classes of NNRTI-like compounds with an altered resistance profile and broader virus-specificity than the currently available NNRTIs.

To circumvent the problem of rapid selection of resistant HIV strains, a combination therapy is mostly applied where several inhibitors are administered at the same time and that preferentially act at different targets of the viral life cycle. Consequently, the viral load will

become reduced to low levels making it more difficult for the virus to produce a variety of mutant strains and consequently select for drug-resistant variants. Therefore, a drug for which resistance can only be achieved by multiple mutations will be of particular interest. If these mutations affect important residues that, upon change, create replication compromised virus strains, we believe it might be possible to avoid or at least markedly delay drug-resistance development when novel NNRTIs can be directed to such crucial amino acids in the NNRTI-pocket. An additional advantage might occur when these novel NNRTIs target also, besides the known NNRTI-pocket, amino acids that are crucial to maintain the quaternary structure, i.e. the heterodimer, of the enzyme. In this respect, TSAO derivatives are one of the compound classes that exhibit the property to interact with amino acids on the interface of the heterodimer RT (i.e. E138) and disturb the interaction of the p66 and p51 subunits resulting in significant loss of enzymatic activity (Rodriguez-Barrios *et al.*, 2001; Sluis-Cremer *et al.*, 2000). In fact, two such amino acids (i.e. W229 and Y318) lining the NNRTI binding pocket and compromising the RT catalytic activity once mutated have already been revealed (Pelemans *et al.*, 2000; Pelemans *et al.*, 1998). Bearing this in mind, we searched for additional amino acids in the NNRTI binding pocket and/or on the interface of HIV-1 RT that are 'unmutatable'. Several residues, i.e. P95, N136, N137, T139 and P140, were identified which are part of, or directly interact with the β7-β8 loop of the p66/p51 interface and also form the bottom of the NNRTI-pocket. We mainly focused on the asparagines that are highly conserved among the lentiviral RTs and which, to date, have not been observed to mutate in NNRTI-exposed HIV-1-infected cell cultures (Ceccherini *et al.*, unpublished). We could demonstrate that mutating N136 or N137 to other amino acids results in a severe decrease of catalytic activity of the mutated enzyme. Only the N137H, N137S, N137Q and N137A mutant enzymes retained substantial DNA polymerase activity. We hypothesized that the decreased catalytic activity of most mutant enzymes could be a result of the disturbance of the dimer interface. This has been proven by the fact that the mutant enzymes are markedly more sensitive to the denaturing effect of urea than wild-type RT. Also the degree of increased urea sensitivity was highly correlated with the degree of (lower) catalytic activity of the mutant enzymes. Replacing wild-type N136 in HIV-1 RT by other amino acids resulted in notably increased amounts of free p51 and p66 monomers in the RT preparation as found by FPLC size exclusion chromatography. The dramatic effect on enzyme activity of most N136 and N137 mutations suggest an important function and/or structural role of the carboxamide function in maintaining interactions with the p66 subunit through p66 H96 in the amino acid stretch where also P95 is located. Moreover, data from circular dichroism spectra suggested

that some mutations on position 136 in p51 could even have a pronounced effect on the tertiary structure of the RT. Interesting is the fact that almost all mutated enzymes kept pronounced sensitivity towards second-generation NNRTIs. Therefore, the N136 and N137 mutations did not significantly increase the drug resistance profile of the virus. It would be important to further search for 'unmutatable' interaction points of potential drugs with HIV-1 RT to enable the rational design of new and more efficient anti-HIV drugs. Having the information available for W229, Y318 and the β7-β8 loop N136, N137 and surrounding amino acids, computer-assisted modelling studies should now be initiated to investigate whether existing NNRTIs can be modified in such a way that they efficiently interact with the functional groups of the above described amino acids. In addition, short peptides (and in a later stage also peptidomimetics) should be designed to interact with the β7-β8 loop in p51 to affect efficient dimerisation of the enzyme. Our studies, together with studies on TSAO and other compounds such as the *N*-acyl hydrazones (Sluis-Cremer *et al.*, 2002), revealed that the dimer interface might form a novel target site for anti-HIV drugs.

The coumarines also form a special class of NNRTIs because they are the only anti-HIV-1 RT drugs that are derived from a natural origin (Kashman *et al.*, 1992) and they select for an amino acid mutation also located in the β7-β8 loop structure of p51. (+)-Calanolide A is the most active and most studied compound of the coumarines. Inhibition by (+)-calanolide A has a mode of action that is still not fully understood but it involves at least two binding sites including the pyrophosphate binding site (Currens *et al.*, 1996a and 1996b). As said, they select for the rather unusual T139I mutation (Buckheit *et al.*, 2000), which is on the interface of the p66/p51 heterodimer and thus (+)-calanolide A may interact with both subunits, a mechanism that is similar to the TSAO compounds that have also a binding site at the interface in such a way that it makes only partial use of the NNRTI binding pocket (Rodriguez-Barrios *et al.*, 2001). Our studies on the T139 amino acid demonstrated that T139I recombinant RT was among the most catalytically active mutant recombinant RTs and proved also to be one with the highest degree of enzyme resistance against (+)-calanolide A. The T139I mutation in HIV-1 RT will eventually result in a fitter replicating virus than other T139 mutated HIV-1 strains. Although our studies suggested that mutations at position 139 could have an influence on dimerisation, these effects are not as pronounced as found for those mutations at positions 136 and 137. We revealed that targeting T139 in p51 of HIV-1 RT should not necessarily lead to new drugs with better drug-resistance profile or a compromised catalytic activity.

In conclusion, much has still to be learned with regard to drug interaction on the one hand and the role of several amino acids to keep the structural and functional integrity of lentiviral RTs on the other hand. Design of new drugs based on the increased molecular insights of drug interaction and enzyme structure more and more becomes successful and helpful to optimize drug therapy of HIV-1-infected individuals. Our studies have contributed to gaining better insights in the interplay between drugs and the RT enzyme.

REFERENCES

Balzarini J (2004) Current status of the non-nucleoside reverse transcriptase inhibitors of human immunodeficiency virus type 1. *Curr Top Med Chem* 4:921-44.

Balzarini J, Weeger M, Camarasa MJ, De Clercq E and Uberla K (1995) Sensitivity/resistance profile of a simian immunodeficiency virus containing the reverse transcriptase gene of human immunodeficiency virus type 1 (HIV-1) toward the HIV-1-specific non-nucleoside reverse transcriptase inhibitors. *Biochem Biophys Res Commun* 211:850-6.

Bendinelli M, Pistello M, Lombardi S, Poli A, Garzelli C, Matteucci D, Ceccherini-Nelli L, Malvaldi G and Tozzini F (1995) Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin Microbiol Rev* 8:87-112.

Buckheit RW, Jr., Russell JD, Xu ZQ and Flavin M (2000) Anti-HIV-1 activity of calanolides used in combination with other mechanistically diverse inhibitors of HIV-1 replication. *Antivir Chem Chemother* 11:321-7.

Currens MJ, Gulakowski RJ, Mariner JM, Moran RA, Buckheit RW, Jr., Gustafson KR, McMahon JB and Boyd MR (1996a) Antiviral activity and mechanism of action of calanolide A against the human immunodeficiency virus type-1. *J Pharmacol Exp Ther* **279**:645-51.

Currens MJ, Mariner JM, McMahon JB, Boyd MR, Shulman N and Winters M (1996b) Kinetic analysis of inhibition of human immunodeficiency virus type-1 reverse transcriptase by calanolide A. *J Pharmacol Exp Ther* **279**:652-61.

De Clercq E and Vandamme A.-M. (2004a) Milestones in drug therapy: Combination therapy of AIDS, Birkhäuser Verlag, Basel, Switzerland.

De Clercq E (2004b) Non-nucleoside reverse transcriptase inhibitors (NNRTIs): past, present and future. *Chem. & Biodiv.* **1**: 44-64.

De Clercq E (2000) Reverse trancriptase inhibitors as anti-HIV drugs, in *Antivirals against AIDS* (Unger RE, Kreuter, J., and Rübsamen-Waigmann, H. ed) pp 107-150, Marcel Dekker, Inc., New York.

Hizi A, Tal R, Shaharabany M, Currens MJ, Boyd MR, Hughes SH and McMahon JB (1993) Specific inhibition of the reverse transcriptase of human immunodeficiency virus type 1 and the chimeric enzymes of human immunodeficiency virus type 1 and type 2 by nonnucleoside inhibitors. *Antimicrob Agents Chemother* **37**:1037-42.

Isaka Y, Miki S, Kawauchi S, Suyama A, Sugimoto H, Adachi A, Miura T, Hayami M, Yoshie O, Fujiwara T and Sato A (2001) A single amino acid change at Leu-188 in the reverse transcriptase of HIV-2 and SIV renders them sensitive to non-nucleoside reverse transcriptase inhibitors. *Arch Virol* **146**:743-55.

Kashman Y, Gustafson KR, Fuller RW, Cardellina JH, 2nd, McMahon JB, Currens MJ, Buckheit RW, Jr., Hughes SH, Cragg GM and Boyd MR (1992) The calanolides, a novel HIV-inhibitory class of coumarin derivatives from the tropical rainforest tree, Calophyllum lanigerum. *J Med Chem* **35**:2735-43.

North TW, North GL and Pedersen NC (1989) Feline immunodeficiency virus, a model for reverse transcriptase-targeted chemotherapy for acquired immune deficiency syndrome. *Antimicrob Agents Chemother* **33**:915-9.

Pelemans H, Esnouf R, De Clercq E and Balzarini J (2000) Mutational analysis of trp-229 of human immunodeficiency virus type 1 reverse transcriptase (RT) identifies this amino acid residue as a prime target for the rational design of new non-nucleoside RT inhibitors. *Mol Pharmacol* **57**:954-60.

Pelemans H, Esnouf RM, Jonckheere H, De Clercq E and Balzarini J (1998) Mutational analysis of Tyr-318 within the non-nucleoside reverse transcriptase inhibitor binding pocket of human immunodeficiency virus type I reverse transcriptase. *J Biol Chem* **273**:34234-9.

Ren J, Bird LE, Chamberlain PP, Stewart-Jones GB, Stuart DI and Stammers DK (2002) Structure of HIV-2 reverse transcriptase at 2.35-A resolution and the mechanism of resistance to non-nucleoside inhibitors. *Proc Natl Acad Sci U S A* **17**:17.

Ren J, Diprose J, Warren J, Esnouf RM, Bird LE, Ikemizu S, Slater M, Milton J, Balzarini J, Stuart DI and Stammers DK (2000) Phenylethylthiazolylthiourea (PETT) non-nucleoside inhibitors of HIV-1 and HIV-2 reverse transcriptases. Structural and biochemical analyses. *J Biol Chem* **275**:5633-9.

Rodriguez-Barrios F, Perez C, Lobaton E, Velazquez S, Chamorro C, San-Felix A, Perez-Perez MJ, Camarasa MJ, Pelemans H, Balzarini J and Gago F (2001) Identification of a putative binding site for [2',5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]-3'-spiro-5"- (4"-amino-1",2"-oxathiole-2",2"-dioxide)thymine (TSAO) derivatives at the p51-p55 interface of HIV-1 reverse transcriptase. *J Med Chem* **44**:1853-65.

Shih CK, Rose JM, Hansen GL, Wu JC, Bacolla A and Griffin JA (1991) Chimeric human immunodeficiency virus type 1/type 2 reverse transcriptases display reversed sensitivity to nonnucleoside analog inhibitors. *Proc Natl Acad Sci U S A* **88**:9878-82.

Sluis-Cremer N, Arion D and Parniak MA (2002) Destabilization of the HIV-1 reverse transcriptase dimer upon interaction with N-acyl hydrazone inhibitors. *Mol Pharmacol* **62**:398-405.

Sluis-Cremer N, Dmitrienko GI, Balzarini J, Camarasa MJ and Parniak MA (2000) Human immunodeficiency virus type 1 reverse transcriptase dimer destabilization by 1-[Spiro[4"-amino-2",2" -dioxo-1",2" -oxathiole-5",3'-[2', 5'-bis-O-(tert-butyldimethylsilyl)-beta-D-ribofuranosyl]]]-3-ethylthy mine. *Biochemistry* **39**:1427-33.

Uberla K, Stahl-Hennig C, Bottiger D, Matz-Rensing K, Kaup FJ, Li J, Haseltine WA, Fleckenstein B, Hunsmann G, Oberg B and et al. (1995) Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors. *Proc Natl Acad Sci U S A* **92**:8210-4.

Vandamme A-M, Van Laethem K, Van Vaerenbergh K and De Clercq E (1998a) Anti-HIV virus combination therapy and resistance management. *International Antiviral News* **6**:182-187.

Vandamme AM, Van Vaerenbergh K and De Clercq E (1998b) Anti-human immunodeficiency virus drug combination strategies. *Antivir Chem Chemother* **9**:187-203.