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The Role of Second Generation Antiretroviral Drugs in HIV-1 Subtype B and non-B Variants Harboring Natural Polymorphisms and Drug Resistance Mutations.

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Résumé

Cette thèse traite de la résistance du VIH-1 aux antirétroviraux, en particulier de l'activité antivirale de plusieurs inhibiteurs non nucléosidiques de la transcriptase inverse (INNTI) ainsi que des inhibiteurs de protéase (IP). Nous avons exploré l'émergence et la spécificité des voies de mutations qui confèrent la résistance contre plusieurs nouveaux INNTI (étravirine (ETR) et rilpivirine (RPV)) (chapitres 2 et 3). En outre, le profil de résistance et le potentiel antirétroviral d'un nouvel IP, PL-100, est présenté dans les chapitres 4 et 5.

Pour le premier projet, nous avons utilisé des sous-types B et non-B du VIH-1 pour sélectionner des virus résistants à ETR, et ainsi montré que ETR favorise l'émergence des mutations V90I, K101Q, E138K, V179D/E/F, Y181C, V189I, G190E, H221H/Y et M230L, et ce, en 18 semaines. Fait intéressant, E138K a été la première mutation à émerger dans la plupart des cas. Les clones viraux contenant E138K ont montré un faible niveau de résistance phénotypique à ETR (3,8 fois) et une diminution modeste de la capacité de réplication (2 fois) par rapport au virus de type sauvage. Nous avons également examiné les profils de résistance à ETR et RPV dans les virus contenant des mutations de résistance aux INNTI au début de la sélection. Dans le cas du virus de type sauvage et du virus contenant la mutation unique K103N, les premières mutations à apparaître en présence d'ETR ou de RPV ont été E138K ou E138G suivies d'autres mutations de résistance aux INNTI. À l'inverse, dans les mêmes conditions, le virus avec la mutation Y181C a évolué pour produire les mutations V179I/F ou A62V/A, mais pas E138K/G. L'ajout de mutations à la position 138 en présence de Y181C n'augmente pas

les niveaux de résistance à ETR ou RPV. Nous avons également observé que la combinaison de Y181C et E138K peut conduire à un virus moins adapté par rapport au virus contenant uniquement Y181C. Sur la base de ces résultats, nous suggérons que les mutations Y181C et E138K peuvent être antagonistes.

L'analyse de la résistance au PL-100 des virus de sous-type C et CRF01_AE dans les cellules en culture est décrite dans le chapitre 4. Le PL-100 sélectionne pour des mutations de résistance utilisant deux voies distinctes, l'une avec les mutations V82A et L90M et l'autre avec T80I, suivi de l'addition des mutations M46I/L, I54M, K55R, L76F, P81S et I85V. Une accumulation d'au moins trois mutations dans le rabat protéique et dans le site actif est requise dans chaque cas pour qu'un haut niveau de résistance soit atteint, ce qui démontre que le PL-100 dispose d'une barrière génétique élevée contre le développement de la résistance. Dans le chapitre 5, nous avons évalué le potentiel du PL-100 en tant qu'inhibiteur de protéase de deuxième génération. Les virus résistants au PL-100 émergent en 8-48 semaines alors qu'aucune mutation n'apparaît avec le darunavir (DRV) sur une période de 40 semaines. La modélisation moléculaire montre que la haute barrière génétique du DRV est due à de multiples interactions avec la protéase dont des liaison hydrogènes entre les groupes di-tétrahydrofuranne (THF) et les atomes d'oxygène des acides aminés A28, D29 et D30, tandis que la liaison de PL-100 est principalement basée sur des interactions polaires et hydrophobes délocalisées à travers ses groupes diphényle. Nos données suggèrent que les contacts de liaison hydrogène et le groupe di-THF dans le DRV, ainsi que le caractère hydrophobe du PL-100, contribuent à la liaison à la protéase ainsi qu'à la haute barrière génétique contre la résistance et que la refonte de

la structure de PL-100 pour inclure un groupe di-THF pourrait améliorer l'activité antivirale et le profil de résistance.

Mot-clés: VIH-1 sous-type, le traitement antirétroviral, la résistance aux médicaments, l'activité antivirale, la résistance croisée, la deuxième génération, la capacité de réplication, barrière génétique, l'étravirine, rilpivirine, PL-100.

Abstract

This thesis focuses on HIV-1 drug resistance and on the antiviral activity of several non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). We have explored the mutational pathways and resistance patterns of several new NNRTIs (etravirine (ETR) and rilpivirine (RPV)) (Chapters 2 and 3). In addition, the drug resistance profile and potential of a novel protease inhibitor (PI) PL-100 is presented in Chapters 4 and 5. In the first project, we used both B and non-B subtypes of HIV-1 to select for ETR resistance and showed that ETR selected for mutations at positions V90I, K101Q, E138K, V179D/E/F, Y181C, V189I, G190E, H221H/Y and M230L within 18 weeks of commencing drug pressure. Interestingly, E138K was the first mutation to emerge in most instances. Viral clones containing E138K displayed low-level phenotypic resistance to ETR (3.8-fold) and modestly impaired replication capacity (2-fold) compared to wild-type virus. We also examined resistance patterns to ETR and RPV in viruses containing NNRTI mutations at baseline. In wild-type (wt) viruses and viruses containing K103N alone, E138K or E138G mutations were observed in the presence of either ETR or RPV drug pressure followed by the appearance of other NNRTI resistance mutations. Alternatively, subtype B viruses containing Y181C generated V179I/F or A62V/A on exposure to ETR or RPV drug pressure, respectively, but not E138K. The addition of mutations at position 138 to Y181C did not significantly enhance levels of resistance to ETR or RPV. We also observed that the combination of Y181C and E138K may lead to a less fit virus compared to virus containing Y181C alone. Based on these findings, we suggest that Y181C may be antagonistic to E138K.

The tissue culture drug resistance analysis of PL-100 in subtype C and CRF01_AE viruses is described in Chapter 4. PL-100 selected for PI resistance mutations along either of two distinct pathways, one of which involved resistance mutations at positions V82A and L90M while the other involved a mutation at position T80I, with other mutations being observed at positions M46I/L, I54M, K55R, L76F, P81S and I85V. An accumulation of at least three mutations in the protease flap and enzyme active sites were required in each case for high-level resistance to occur, demonstrating that PL-100 has a high genetic barrier against the development of drug resistance. In Chapter 5, we evaluated the potential of PL-100 as a second generation HIV-1 protease inhibitor. PL-100 resistant variants emerged within 8-48 weeks while darunavir (DRV) did not select for resistance mutations over a period of 40 weeks. Structural modeling demonstrated that the high genetic barrier of DRV is due to numerous interactions with protease that include hydrogen-bonding to PR backbone oxygens at amino acid positions A28, D29 and D30 via di-tetrahydrofuran (THF) groups, while binding of PL-100 was predominantly based on polar interactions and delocalized hydrophobic interactions through its diphenyl groups. Our data suggest that hydrogen bonding contacts and the di-THF group in DRV, as well as the hydrophobic nature of PL-100, contribute to PI binding and a high genetic barrier for resistance and that redesigning the structure of PL-100 to include a di-THF group might improve it antiviral potency and drug resistance profile.

Key Words: HIV-1 subtype, antiretroviral treatment, drug resistance, antiviral activity, cross-resistance, second generation, replication capacity, genetic barrier, etravirine, rilpivirine, PL-100.

Preface

Chapter 1 of this thesis includes literature review and a general introduction. Parts of Chapter 1 were published as a review in Antimicrobial Agents and Chemotherapy. Original publications presented in Chapters 2-5 inclusive are either published or submitted for publication. A general discussion is presented in Chapter 6. Four original research papers described in Chapter 2-5 are presented as followed:

Chapter 2: **Asahchop EL**, Maureen Oliveira, Mark A Wainberg, Bluma G. Brenner, Daniela Moisi, Thomas d'Aquin Toni, Cecile L. Tremblay. Characterization of the E138K Resistance Mutation in HIV-1 Reverse Transcriptase on Susceptibility to Etravirine in B and non-B HIV-1 Subypes. Antimicrobial Agents and Chemotherapy, Feb 2011, 55(2):600-7.

Chapter 3: Asahchop EL, Wainberg MA, Oliveira M, Xu HT, Brenner BG, Moisi D, Ibanescu IR, and Tremblay CL. Distinct Resistance Patterns to Etravirine and Rilpivirine in Viruses Containing NNRTI Mutations at Baseline. Submitted to AIDS.

Chater 4: Asahchop EL, Oliveira M, Brenner BG, Martinez-Cajas JL, Toni TA, Ntemgwa M, Moisi D, Tremblay CL and Wainberg MA. Tissue Culture Drug Resistance Analysis of a Novel HIV-1 Protease Inhibitor Termed PL-100. Antiviral Research, Sept. 2010, 87(3):367-72.

Chapter 5: Asahchop EL, Oliveira M, Quashie PK, Moisi D, Martinez-Cajas JL, Brenner BG, Tremblay CL and Wainberg MA. *In Vitro* and Structural Evaluation of PL-100 as a Potential Second Generation HIV-1 Protease Inhibitor. J. Antimicrobial Chemotherapy July 2012. Accepted.

Other manuscripts not included in this thesis, but to which a significant contribution was made by the Candidate include;

Martinez-Cajas JL, Wainberg MA, Oliveira M, **Asahchop EL**, Doualla-Bell F, Lisovsky I, Moisi D, Mendelson E, Grossman Z, and Brenner BG. The Role of Polymorphisms at Position 89 in the HIV-1 Protease Gene on the Development of Drug Resistance to HIV-1 Protease Inhibitor. J. Antimicrobial Chemotherapy, 2012 Feb 7.

Xu HT**, Asahchop EL**, Oliveira M, Quashie PK, Quan Y, Brenner BG, Wainberg MA. Compensation by the E138K Mutation in HIV-1 Reverse Transcriptase of Deficits in Viral Replication Capacity and Enzyme Processivity Associated with the M184I/V Mutations. J Virol. 2011 Aug, 85(21): 11300-11308.

Xu HT, Quan Y, **Asahchop EL**, Maureen Oliveira, Mark A Wainberg. Comparative biochemical analysis of recombinant reverse transcriptase enzymes of HIV-1 subtype B and subtype C. Retrovirology, Oct 2010, 7:80.

Toni TA, Brenner BG **Asahchop EL**, Ntemgwa M, Moisi D and Wainberg MA. Development of an allele-specific polymerase chain reaction for detection of K65R resistance mutation in patients infected by subtype C Human Immunodeficiency Virus type 1. Antimicrobial Agent and Chemotherapy. Feb. 2010, 54(2):907-11.

Toni TA, **Asahchop EL**, Moisi D, Ntemgwa M, Oliveira M, Masquelier B, Brenner BG, Wainberg MA. Detection of HIV-1 M184V and K103N Minority Variants in Primary HIV Infected Patients. Antimicrobial Agents Chemother. 2009 Apr, 53(4):1670-2.

Ntemgwa M, Toni TA, Brenner BG, Oliveira M, **Asahchop EL**, Moisi D, and Wainberg MA. Nucleoside and nucleotide analogs select in culture for different patterns of drug resistance in human immunodeficiency virus types 1 and 2. Antimicrobial Agents Chemother. 2009 Feb;53(2):708-15.

Ondoa P, Vereecken C, **Asahchop EL**, Litzroth A, Diallo A, Fransen K, Dieye T, Ryder R, Mboup S and Kestens L. Proof of principle: An HIV p24 microsphere immunoassay with potential application to HIV clinical diagnosis. Cytometry B Clin Cytom. 2009 May;76(3):231-6.

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List of Abbreviations

3TC:	Lamivudine
ABC:	Abacavir
AIDS:	Acquired immune deficiency syndrome
APOBEC:	apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1-like protein
APV:	Aprenavir
ATC :	Apricitabine
bp :	base pair
C :	cysteine
CBMC :	Cord blood mononuclear cell
CCR5:	C Chemokine receptor 5
cDNA:	complementary deoxyribonucleic acid
CXCR4:	CXC-chemokine receptor 4
d4T:	stavudine
ddC:	zalcitabine
DNA:	deoxyribonucleic acid

dNTP: 2’-deoxyribonucleoside-5’-triphosphate

DRM: drug resistant mutations

DUET: Demonstrate Undetectable viral load in patients Experienced with ARV Therapy

EC_{50} : 50% effective concentrations

Env: envelope gene

ER: endoplamic reticulum

ESCRT: endosomal sorting complexes required for transport

F: phenylalanine

FC: fold change

FDA: Food and Drug Administrator, USA

FTC: Emtricitabine

G: glycine

gag: group antigen gene

gp: glycoprotein

H: histidine

HIV-1: human immunodeficiency virus type 1

HIV-2:	human immunodeficiency virus type 2
I:	isoleucine
IDV:	indinavir
IN:	integrase
K:	lysine
Kb:	kilobase
LPV:	lopinavir, ABT-378
LTR:	long terminal repeat
M:	methionine
MA:	matrix protein
MDR:	multidrug resistance
mRNA:	messenger ribonucleic acid
N:	asparagines
NAM:	nucleoside analogue mutation
NC:	nucleocapsid protein
Nef:	negative regulatory factor

NFV:	nelfinavir, AG1343
NIH:	National Institute of Health
NNRTI:	non-nucleoside reverse transcriptase inhibitor
NRTI:	nucleoside reverse transcriptase inhibitor
Nt:	nucleotide
NVP:	nevirapine, BI-RG-587, dipyridodiazepinone
P:	proline
p51:	51 KDA subunit of HIV-1 RT
p66:	66 KDA subunit of HIV-1 RT
PBMC:	peripheral blood mononuclear cell
PCR:	polymerase chain reaction
PDB:	protein databank
PI:	Protease inhibitor
PR:	protease
RNA:	ribonucleic acid
RT:	reverse transcriptase

TAM:	Thymidine analogue mutation
Tat:	tansactivator of transcription
TDF:	Tenofovir
THF:	tetrahydrofuran
TM:	transmembrane envelope protein
TPV:	tipranavir
U3:	unique region of the 3'LTR
U5:	unique region of the 5'LTR
V:	valine
Vif:	virion infectivity factor
VPr:	viral protein R
Vpu:	viral protein U
W:	tryptophan
wt:	wild type
Y:	tyrosine
ZDV:	zidovudine

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Dedication

This Ph.D. thesis is dedicated to my dear wife Asongaze Nadege and our two kids Bryant and Kayla. Thank you all for your patience during this work.

CHAPTER 1

1.0 INTRODUCTION

This chapter intends to give a brief overview of the life cycle, structure and epidemiology of Human Immunodeficiency virus (HIV) and to summarize those aspects of HIV research that are relevant to the original work presented in subsequent chapters. The main focus is placed on antiretroviral (ARV) therapy and mechanisms of resistance. Aspects of replication capacity and fitness in the presence of mutations are also discussed.

Sections of this chapter were published as a review entitled "Antiviral Drug Resistance and the Need for Development of New HIV-1 Reverse Transcriptase Inhibitors", authored by **Asahchop EL**, Wainberg MA, Sloan RD, Tremblay CL. **Antimicrobial Agents and Chemotherapy. June 2012.**

1.1 Historical Background of HIV/AIDS

In 1981, the Acquired Immunodeficiency Syndrome (AIDS) was first reported among the male homosexual community of San Francisco with immunodeficiency associated with conditions such as Kaposi's sarcoma, pneumocystis pneumonia, and mucosal candidiasis [1,2,3]. Later on, other groups like haemophiliacs [4], intravenous drug users [3], immigrants or residents from Haiti and Africa [5] and infants born from infected mothers were also found affected by the same syndrome [6]. It is now well established through epidemiological and public health studies that transmission of human immunodeficiency virus (HIV) occurs by sexual contact, blood or blood product transfusion, intravenous drug use by needle sharing and from infected mother to her child through breast feeding or pre- or perinatal contact [2,3,7,8,9]. Human immunodeficiency virus type 1 (HIV-1) was later identified as the pathogenic cause of AIDS in 1983 [10]. AIDS is a disease characterized by a decreased T-helper (CD4+) cell subset leading to impaired immune function [11]. Clinical manifestations of immunodeficiency syndrome are weight loss, adenopathy, malignancies, neural syndrome and development of opportunistic infections [12]. In 1986, another type of human immunodeficiency virus (HIV-2), was identified in Portugal from AIDS patients from West Africa [13]. In HIV-2, disease progression and eventual progression to AIDS is much slower [14].

The most plausible origins of HIV-1 and HIV-2 transmission to humans are from Simian Immunodeficiency Virus (SIV) infected Chimpanzees (SIV_{cpz}) and SIV sooty mangabeys ($SIV_{sm)}$ respectively [15,16]. Simian Immunodeficiency Virus (SIV) is a retrovirus that infects non-human primates. Phylogenetic analysis has shown that SIV clusters closely

with HIV. HIV is a retrovirus and belongs to the genus Lentivirus and family Retroviridae: these are viruses, which have single-stranded RNA as their genetic material, but replicate via a double stranded DNA intermediate. To date no vaccine has been developed that can protect against HIV. This is largely due to the variability of the HIV virus which changes or mutates over time.

1.2 Epidemic in 2011

At the end of 2011, a total of 34.2 million people were living with HIV worldwide according to the joint UNAIDS/WHO report [17]. Amongst these, 30.8 million are adults and 3.4 are children less than 15 years old. In 2011, 1.7 million deaths due to AIDS and 2.5 million new HIV infections were registered, with 7000 new HIV infections per day. The highest burden is within Sub-Saharan Africa where the mortality rate due to AIDS is high. This region alone harbored about 23.5 million (68% of all cases worldwide) HIV infected people in 2011, followed by South and Southeast Asia with 4.2 million cases. There are an estimated 3.4 million orphans as a result of AIDS mortality in this region. Worldwide, an increase from 1.5 million in 2001 to 3.4 million in 2011 has been observed in children (under 15 years) living with HIV. The burden falls most heavily on poor countries and on the poorest individuals within those countries. Regional summaries of the HIV epidemic in 2011 are shown in Figure 1.

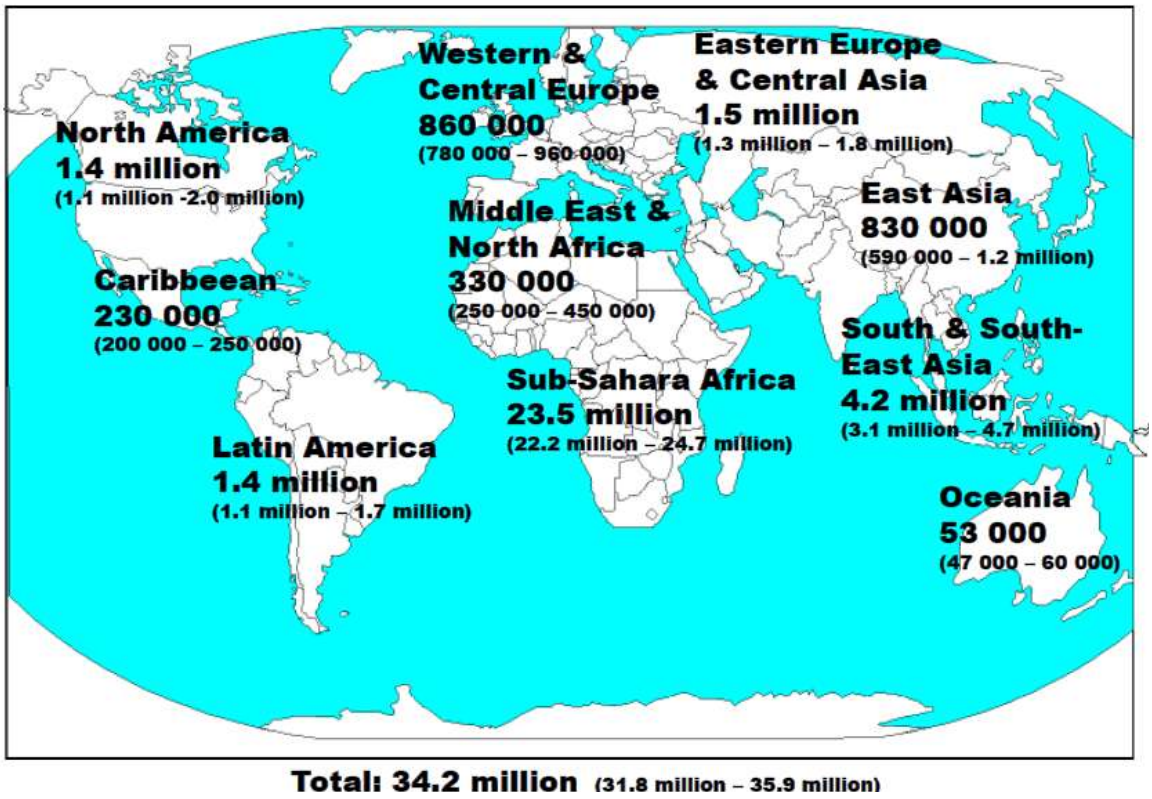


Figure 1: Adults and Children Estimated to be living with HIV in 2011. Regional summaries (Adapted from the joint UNAIDS/WHO 2012 HIV/AIDS report).

1.3 Structure of HIV-1 Virion

All lentiviruses have an envelope. The envelope contains a lipid bilayer that originates from the host cell membrane. HIV-1 has an icosahedral structure containing approximately 10 spikes per virion and an overall diameter of 100 nm (Figure 2) [18,19,20]. The surface glycoprotein (SU, gp120) and the transmembrane glycoprotein (TM, gp41) form the two major proteins on the viral envelope. The gp120 protein is the external (exposed) region of viral envelope while the gp41 portion attaches the *env* gene

to the surface of the cell membrane. An infectious virus contains three structural Gag proteins: the matrix (MA, p17) protein forms the inner shell in the virus particle next to the lipid envelope, capsid (CA, p24) protein is the cone shaped structure that surrounds the viral genomic RNA and nucleocapsid (NC, p7) encodes viral RNA within the capsid. The PTAPP and YSPTL motifs in p6 of Gag each interact with an endosomal sorting complex required for transport i.e. (ESCRT) I and III respectively [21,22,23]. The cellular proteins TSG 101 and ALIX are found in ESCRT I and ESCRT III respectively. Specifically the PTAPP motif in the p6 of Gag binds with TSG 101 while the YSPTL motif binds to ALIX [23]. These interactions facilitate the recruitment and assembly of host proteins on the cell membrane and thus the budding of viral particles. It has been shown that when the TSG 101 pathway for budding is defective (HIV-1 PTAPP mutant), cellular expression of ALIX can rescue this virus release defect and this rescue depends on an unaltered ALIX binding site on p6 [21]. The capsid contains two copies of viral genomic RNA, the viral enzymes reverse transcriptase (RT), protease and integrase, and approximately 2000 molecules of the processed nucleocapsid (NC) p7 [24,25]. The proteolytic cleavage of the 55 kD Gag precursor by HIV-1 protease yields p17, p24 and p7 [26]. The two copies of single-stranded HIV-1 RNA molecules located inside the core are about 9.2 kilobases each. In general, a single HIV-1 virion is estimated to contain 2400 Gag molecules, roughly 80 RT molecules, and up to three molecules each of gp120 and gp41 that form one heterotremiric glycoprotein spike [19,27,28,29].

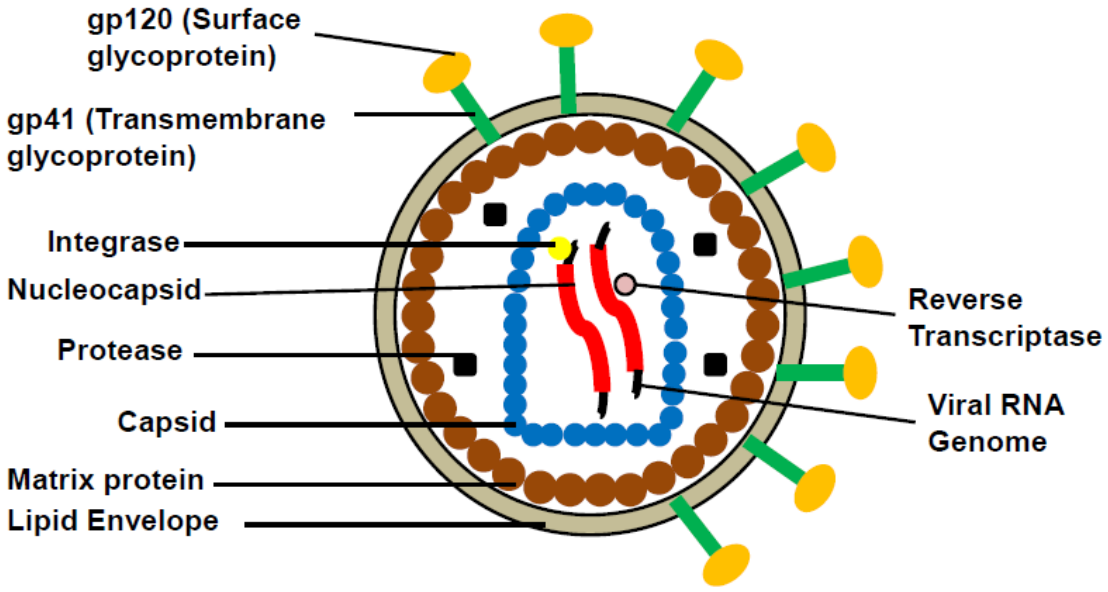


Figure 2: Structure of HIV Virus.

1.4 Genome Organization

The genomic organization of HIV-1 is illustrated in Figure 3. During the process of reverse transcription of the viral RNA, two identical elements, the long terminal repeats (LTRs), are generated at each end of the provirus. Promoters and transcriptional enhancer sequences of the LTR include; a TATA promoter, polyadenylation signal sequences, cis-acting elements, negative regulatory elements and NF-kB binding regions, and have been shown to be necessary for efficient viral replication [30,31]. The two LTRs are flanked by genes encoding structural proteins and viral enzymes, and genes coding for a series of regulatory and accessory proteins. HIV-1 exhibits three essential regions located between the 5'-and the 3'LTRs. The *gag* gene is situated at the 5' end of the genome and codes for

a Pr55 polyprotein. The proteolytic cleavage of the Gag precursor p55 results in smaller proteins, including p24, p17, p9, p6, p2 and p1. The *Pol* gene is cleaved into three enzymes including; reverse transcriptase (RT) for conversion of RNA to DNA, integrase (IN) that is responsible for the integration of viral DNA into host cell and protease (PR) for processing of polyprotein precursors. The proteolytic processing site of Gag and Gag-pol polyprotein is unique to the protease that is encoded by HIV-l [32]. The precursor of *env* gene gp160 is cleaved into surface protein gp120 and transmembrane protein gp41. The endoprotease furin, is responsible for the cleavage of gp160 [33]. Extracellular gp120 is involved in recognition and binding, while transmembrane protein gp41 in membrane fusion. Specific conserved regions within the gp120 of Env are responsible for binding to the CD4 receptor [19,34]. The gp120 layers that are involved in binding also regulate the interaction of gp41 [35]. The binding of gp120 to the CD4 receptor exposes the co-receptor binding site. Macrophage (M)-tropic HIV-1 viruses use CCR5 as a co-receptor while T-cell tropic HIV-1 uses CXCR4 [19]. It has been shown that the V3 loop of Env plays a role in fusion and is co-receptor specific [36,37]. The complete binding of gp120 to the CD4 receptor and co-receptor results in a change in the conformation of gp41, leading to the release of the fusion peptide that penetrates into the cell membrane and forms a fusion pore [19].

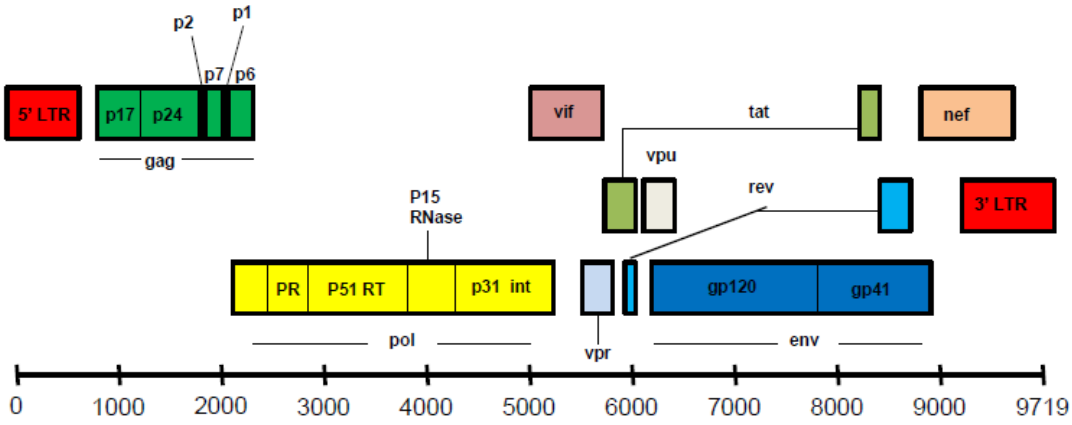


Figure 3: Genomic organization of HIV-1. (Adapted from the Los Alamos Laboratory HIV Sequence Book).

In addition to the prototypic *gag, pol and env* coding sequences, HIV-1 possesses at least six other genes coding for auxiliary proteins namely: Tat, Rev, Vif, Vpr, Vpu and Nef (reviewed in [38,39,40,41]). The classification of these proteins into two groups depends on the time of their regulatory expression during HIV replication. For example, early in the viral life cycle, Tat, Rev, and Nef are synthesized from Rev-independent multiply spliced mRNA while at late stages of HIV-1 replication, Vif, Vpr, and Vpu are expressed from a Rev dependent singly spliced mRNAs (reviewed in [38,39]). The function of the Tat protein is to enhance transcriptional elongation by binding to the TAR (*trans* - activating response element) and is a major protein involved in up-regulating HIV replication. Another viral regulatory protein, Rev (regulator of viral protein expression) plays a role in the export of unspliced and singly spliced viral mRNAs into the cytoplasm [42,43]. The functions of the viral protein Nef (negative factor) include cell activation

and enhanced infectivity. Nef plays a role in signal transduction and cell activation by interacting with cellular proteins (serine kinases) [44]. Another function of Nef is the down regulation of CD4 surface expression by mediating endocytosis and lysosomal degradation of CD4 [45]. The Vif protein is also called viral infectivity factor and is produced from a singly spliced mRNA that accumulates late in HIV life cycle [46]. Vif acts during assembly by allowing the generation of particles (viral proteins) that allow a new cycle of infection to be initiated [47]. APOBEC3G/3F (apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1-like protein 3G or 3F) has been described as a host restriction factor. HIV containing Vif counteracts the antiviral effect of APOBEC leading to infection while HIV delta Vif has been shown to be non-infectious [48,49]. CD4 is down regulated by *vpu* at the surface of the endoplasmic reticulum (ER), thus preventing any premature binding of viral gp160 to CD4. In addition, Vpu facilitates the release of HIV-1 virons by counteracting the host protein tetherin [50,51]. Vpr is responsible for the transport of the preintegration complex, after reverse transcription, from the cytoplasm into the nucleus in non-dividing target cells [52]. The proteins Vpr and Matrix independently permit import of the preintegration complex to the nucleus via distinct nuclear localization signal (NLS) sequences [52]. Vpr has also been reported to play an important role in G2 cell cycle arrest by formation of foci and recruitment of the DNA Damage-Binding protein 1-cullin 4A (DDB1-CUL4A (VPRBP)). The presence of E3 ubiquitin ligase in these nuclear foci can activate the host cell response leading to cell cycle arrest [53,54]. Vpx is a viral protein expressed in HIV-2 and not HIV-1 [55]. In HIV-2, Vpx counteracts the effect of the restriction factor SAMHD1 for proteasomal degradation in the nucleus and thus induces an antiviral innate immune response [55,56].

1.5. HIV-1 Life Cycle

The life cycle of HIV begins when the viral particle binds to host cell receptors on the cell membrane (Figure 4). Specifically, the viral gp120 envelop protein recognizes host cell surface receptor proteins.

1.5.1. Attachment, Entry and Uncoating

Entry of HIV-1 into the host cell involves binding of glycoprotein gp120 with the cellular receptor CD4 on helper T lymphocytes [57]. A conformational change occurs on the surface of gp120, exposing new epitopes that can interact with a co-receptor (either CXCR4 or CCR5 or both) (Figure 4) [58,59,60]. The co-receptors for HIV are members of seven-transmembrane domain proteins, α (CXCR4) and β (CCR5) chemokines. Macrophage tropic (M-tropic) are non-syncytium inducing viruses that uses CCR5 for replication in primary infection while T-cell-line tropic (T-tropic) are syncytium inducing viruses that replicates with CXCR4 during the late stage of the disease. The significant role of CCR5 in HIV entry is shown by individuals who are homozygous for mutations (32 bp deletion) within CCR5 develop resistance to infection by HIV-1 [61,62]. Recently, Hutter *et al* were able to show HIV remission in a patient with acute myeloid leukemia and HIV-1 infection after transplantation of stem cells from a donor who was homozygous for CCR5 delta32 [63]. This patient discontinued antiretroviral therapy without any viral rebound for several years. Entry of HIV-1 into the host cell occurs via two mechanisms which can either be a fusion process by a clathrin-mediated endocytosis

at a low PH or a PH-independent fusion of virus with the cell membrane [19,64,65]; as a result, the viral core and associated RNA are engulfed.

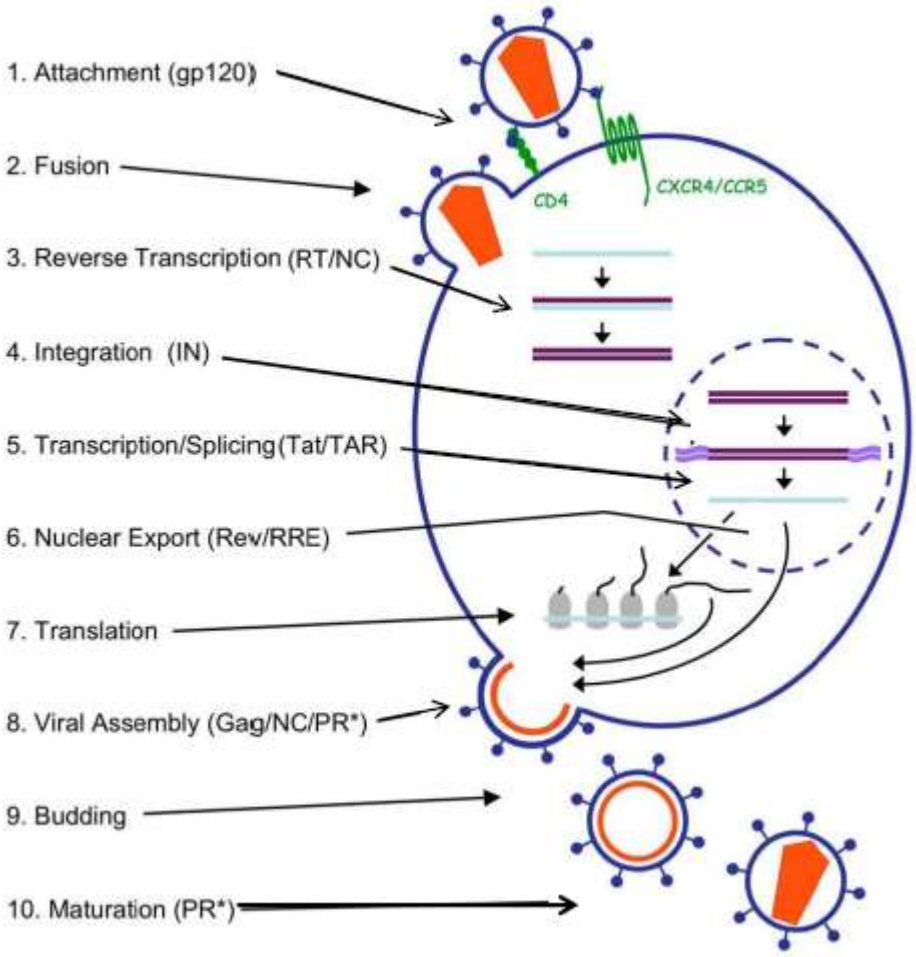


Figure 4: HIV-1 Replication cycle [66]. (Reproduced from Held DM *et al*, 2006 with permission).

1.5.2. Reverse transcription and Integration

Once inside the cell, viral RT transcribes the viral genome into cDNA, using a cellular lysine tRNA molecule as a primer [67]. The process involves the synthesis of double stranded DNA from single stranded RNA by the enzyme DNA polymerase, and the degradation of RNA from the RNA:DNA duplexes by RNase H. The synthesized viral DNA moves to the host cell nucleus (a process facilitated by the HIV proteins Vpr and Vif as well as Vpr and p17) as a pre-integration complex (PIC). The movement of the PIC into the cell nucleus is facilitated by viral and cellular factors. Once inside the nucleus, the viral integrase enzyme integrates proviral cDNA into the host genome, using the 3' processing and strand transfer mechanism [68]. This integrated form of HIV called provirus has two identical long terminal repeats (LTR) flanking the coding regions. Eradication of HIV-1 from infected patients has not been achieved so far. This is in part due to the fact that HIV-1, like all retroviruses, integrates into the host genome.

1.5.3. Virus Expression

After cDNA integration, viral gene expression commence in the presence of cellular transcription machinery. HIV-1 gene expression is initiated by two regulatory protein Tat and Rev. Cellular transcription factors are recruited to the promoter, leading to increased viral gene transcription by host RNA polymerase II by the HIV regulatory protein Tat (reviewed in [69,70]). Specifically, Tat induces transcriptional elongation through interaction with HIV-1 transactivation response element (TAR) RNA [69]. P-TEFb is a factor that binds Tat and has two components involved in Tat binding; Cyclin T 1 and

CDK9 kinase. CycT1 has been shown to form a stable complex with CDK9, Tat and TAR RNA [71]. When Tat is formed, it binds to CycT1 and CDK9 components of the P-TEFb, and the resulting complex then binds to TAR RNA [69,71]. The formation of an active preinitiation complex (PIC) involves the recruitment of TATA-binding protein (TBP) and TFIIB by the Tat-CycT1-CDK9 bound TAR. Here phosphorylation of the C-terminal domain of the RNA polymerase II (RNAPII) by CDK9 occurs [69]. The activity of Tat on transcriptional elongation is evidence by the formation of a highly competent PIC that recruit TFIIH and it kinase CDK7 to hypophorylates the RNAPII. This complex of activities brought on the PIC by Tat triggers polymerase departure [69].

Another regulatory protein Rev and the Rev response element (RRE) play a role in export of mRNA from the nucleus to the cytoplasm [23]. HIV-1 has developed a mechanism to export unspliced and partially spliced RNA from the nucleus to the cytoplasm. In this mechanism, Rev bind to RRE and interacts with chromosome maintenance region 1 (CRM-1) protein and exportin 1 protein which in turn interacts with phenylalanine-glycine (FG) repeats of nucleoporins [23,72]. This pathway of export is energy-dependent requiring Ran GTPase [73,74]. This interaction mediates the export of unspliced and partially spliced RNA. A number factor have also been described to play a role directly or indirectly in Rev-mediated transport/expression [72]. The Rev/RRE mediated export of unspliced and partially spliced RNA from nucleus to cytoplasm occurs when the nuclear matrix protein matrin 3 binds to Rev/RRE containing viral RNA [75,76].

1.5.4. Virus Assembly and Budding

The transcribed viral cDNA can serve as either a viral genome copy or an unspliced, singly-spliced or multi-spliced mRNA used for viral protein translation. The unspliced viral mRNA expresses the Gag and Gag-Pol precursor polyproteins that are subsequently directed to the cell plasma membrane for virion assembly ([77,78] reviewed in [79]). Genomic RNA associates with Gag precursor polyproteins through the packaging sequence present in NC [80] while the interactions of CA and Gag transport the Gag-Pol into assembling virions [81]. The MA protein of Gag interacts with Env and directs Env incorporation into virions [82,83]. The p6 protein of Gag interacts with endosomal sorting complexes required for transport (ESCRT) and initiates release of viral particles from the plasma membrane [23,84] The viral protease is derived from the Gag-Pol precursor through self-cleavage. This viral protease functions in the proteolytic cleavage of other viral polyproteins that result in viral maturation, to eventually generate an infectious viral particle ([85,86] reviewed in [82]).

1.6. Natural History of HIV Infection.

The course of HIV infection in the absence of treatment follows the trends shown in Figure 5. This involves three stages of infection. The acute infection occurs within the first 12 weeks of infection, when viral load (red line) rapidly increases in the plasma, peaks, and declines to a level known as the viral set point. HIV-specific CD8+ T-cells (not shown) peak within a few weeks, decline and remain at low levels throughout the infection. Early intervention with therapy has shown a significant increase in CD8+ T-

cells [87]. At the peak of viremia there is a drastic decrease in CD4+ T-cell counts (blue line) [88,89]. The acute phase ends when HIV specific antibodies emerge [90,91,92]. This is known as seroconversion. An asymptomatic period of chronic infection develops in the presence of continued antibody production, fluctuating viral loads, persistent CTL responses and a slow decline of CD4+ T cell counts (reviewed in [93,94]).

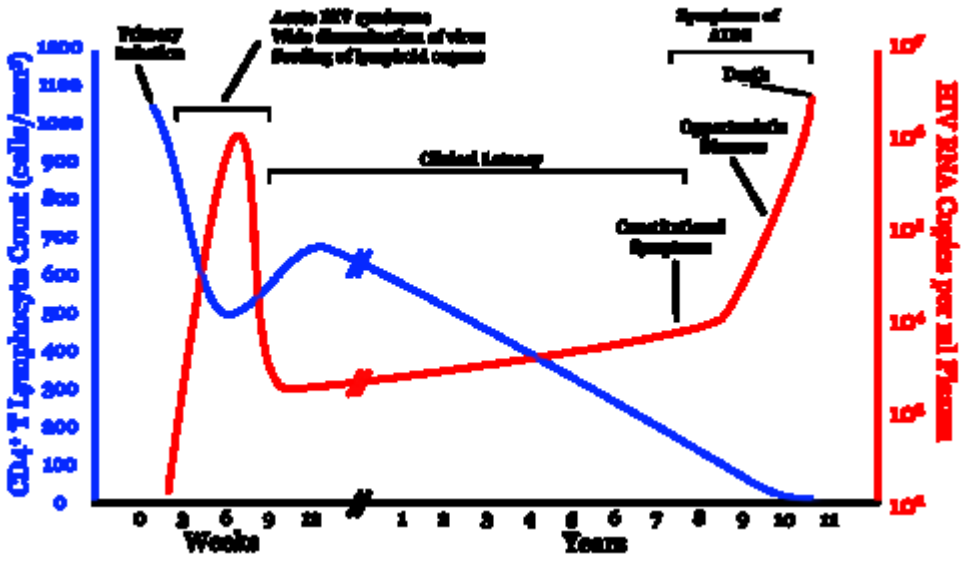


Figure 5: The Relationship between HIV copies (viral RNA) and CD4 counts during the course of infection. (Source: http://www.thenakedscientists.com/HTML/uploads/tx_naksciimages/Hiv-timecourse.png).

Depending on the individual, the chronic period can lasts on average 7-9 years. This ranges from one to 15 years in rapidly progressing and slowly progressing patients, respectively. The length of the chronic phase correlates inversely with the viral set point

[95,96]. A decreased CD4+ counts lead to failure of the adaptive immune response, and individuals tend to acquire opportunistic infections and thus enter the third phase of infection i.e. the acquired immunodeficiency syndrome (AIDS). *Pneumocystis carinii* pneumonia, cytomegalovirus disease, systemic fungal infections and atypical *Mycobacterium* infections are the most common opportunistic infections seen in AIDS; and if untreated, these HIV infected individuals will die of an AIDS-related illness after a median of 10 years (reviewed in [97]). In some individuals, the asymptomatic stage does not progress to AIDS [98,99,100]. These individuals, known as elite controllers or long-term non-progressors (LTNP), are able to sufficiently maintain high CD4+ T cell levels and low level or undetectable viral loads [101].

1.7 Structure of HIV-1 RT

HIV-1 RT is encoded by the *pol* gene and expressed from the unspliced mRNA in the context of the Gag-Pol precursor by a frameshift event [102]. The HIV-1 RT consists of two subunits; the p66 and the p51-KD subunits. The p66 includes functions for reverse transcription, the DNA polymerase and RNase H domains while p51 is a carboxy-terminal end of p66 without the RNase H domain. Using subunit site directed mutagenesis, it has been confirmed that both the DNA polymerase and RNase H activities of RT reside within the p66 subunit [103]. The p51 subunit of RT plays a role in maintaining the conformation of the p66 subunit and in tRNA Lys3 binding [104,105,106]. Crytallographic data show that HIV-1 RT resembles a right hand [107,108]. The subdomains have been described as the fingers, palm, thumb, and

connection domains (Figure 6) [108]. Within the p66 subunit, the polymerase and RNase H domains are linked by the connection domain that may play a structural role [109].

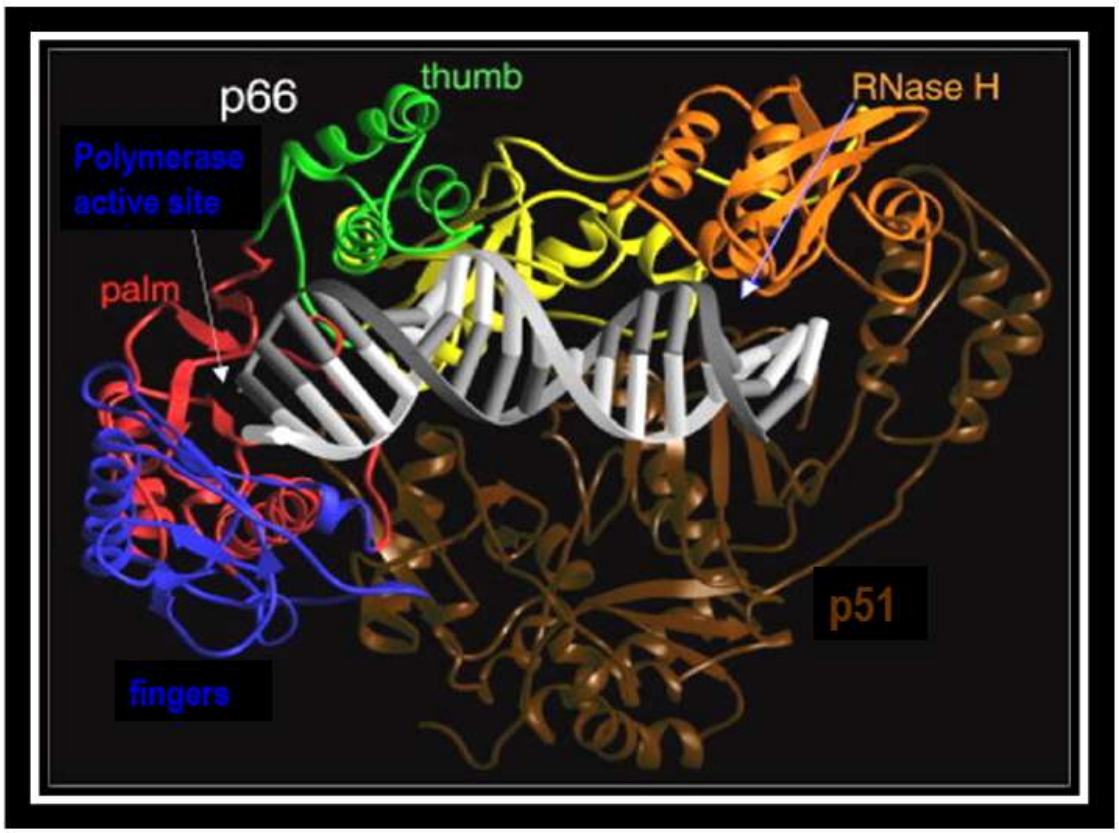


Figure 6: Structure of HIV-1 RT (Reproduced with permission from Sarafianos SG, 2009 [110]). Ribbon representation of HIV-1 RT in a complex with nucleic acid. The fingers, palm, thumb, connection, and RNase H subdomains of the p66 subunit are shown. The p51 subunit is shown, as well as the template and primer DNA strands.

1.8. Protease

The HIV-1 protease (PR) is a 99-amino acid, symmetrical and obligate homodimer. It is structurally similar to other aspartyl proteases [111,112], as well as those from other retroviruses including: HIV-2 [113] and simian immunodeficiency virus (SIV) [114]. The HIV-1 PR consists of three domains: the flaps, active site and dimerization domain (Figure 7). The dimerization process leads to a mechanism that regulates and controls activation of the enzyme (reviewed in [115]). In a concentrated environment (e.g. in the budded virion), the protease is activated, and in a highly dilute environment (e.g. in the host cell), the protease is inactivated. The substrate-binding cleft forms the centre of the enzyme. The centre of the enzyme then interplays with a variety of substrate cleavage site sequences in the Gag and Gag-Pol proteins. Cleavage of the Gag and Gag-Pol polyproteins at specific sites by HIV-1 protease results in mature viral proteins. This cleavage is for specific HIV-1 protease at these sites *in vivo*.[116].

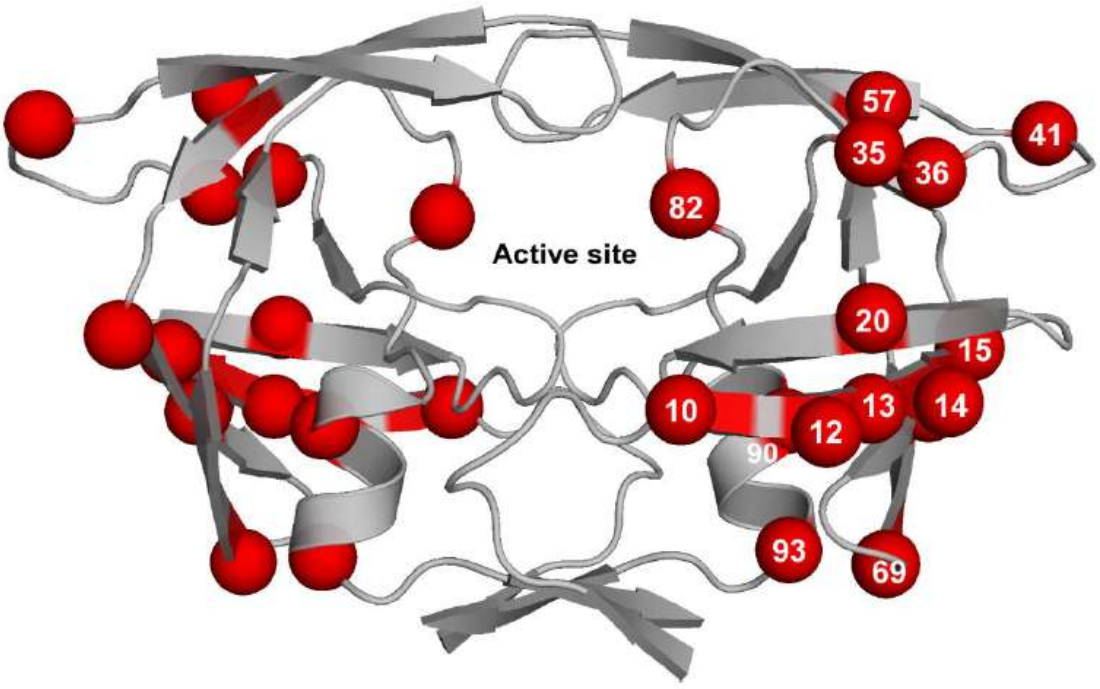


Figure 7: Structure of HIV-1 PR (Reproduced with permissionfrom Ali A 2010 [117]). The catalytic active site formed at the dimeric interface. Red spheres represent amino acid positions.

1.9. Recombination

Retroviral recombination is the combination of two regions of the viral genome during reverse transcription. Although recombination can occur within all regions of the viral genome, it is more specific in the gag and envelope regions [118,119,120]. For recombination to occur, two non-identical viruses need to successfully infect the same cell and the two diverse genomic RNA should have the capacity to form heterodimers [121]. This phenomenon could take place early in the infection process, with a latently infected cell or a cell in which virus is suppressed [122,123]. Some studies have shown that HIV-1 recombines about two to three times per genome per replication cycle,

indicating that it has a higher recombination rate than other retroviruses [124,125]. During recombination, the RT uses the plus-strand genomic RNA variants in the cell as a template to start the synthesis of minus strand DNA. As synthesis proceeds, homopolymeric nucleotide tracks cause pausing of the reverse transcriptase and thereby promoting template switch and recombination and the nascent minus-strand DNA copied from the first variant to transfer to the genomic RNA of second variant [126]. Once copied, the genomic RNA is degraded by the RNase H enzyme during reverse transcription. Efficient degradation of the template by the RNase H enzyme (which can occur during transcription pausing) promotes recombination [121]. The process of homologous recombination could allow two defective viruses to become cytopathic [127] and could introduce new strains into the population, thereby challenging therapy (via drug resistance) and vaccine approaches [118,128,129].

1.10 Variability and Global Distribution of HIV

HIV possesses an extremely high genetic variability. This extensive heterogeneity is due to several factors. First, genetic diversity is the result of the high error rate of reverse transcriptase that lacks proofreading capacity [130,131,132] and the fast turnover of virions (approximately 10 billion copies) in HIV infected individuals [133]. Secondly, the genetically diverse simian viruses that have been introduced into humans [15] could cause this heterogeneity. Lastly, the highly recombinogenic property of the virus results to intra and interpatients recombination [124,134]. The variability of HIV is of particular importance since it may affect a protein's structure, function and

antigenicity/immunogenicity with respect to therapy, vaccine development and diagnostic tests. Point mutations occur frequently during reverse transcription leading to quasi-species [135]. Thus, a patient is generally infected with a homogenous population which gradually changes over time to produce a heterogeneous virus population (qausi-species) [136,137,138]. The homogenous population is known as transmitted/founder virus. As the transmitted/founder (T/F) virus replicates, the virus selects for mutations due to immune pressure. This eventually results to a diverse viral population with impaired replication [139]. It has also been shown that the transmitted/founder virus Env uses the CCR5 co-receptor [140]. In a recent study Parrish *et al* showed that the ability with which the envelope gene of T/F and chronic controls use CD4 and CCR5 were similar [141]. In addition the authors demonstrated that in both the T/F and chronic controls the CD4+ T cell subset tropism and sensitivity to neutralization by CD4 binding site were the same [141]. Two recent studies showed that the ability of both T/F virus and chronic controls to replicate in T-lymphocytes was the same, whereas T/F virus replicated less efficiently in monocytes derived macrophages than chronic controls [142,143]. It has also been demonstrated that the glycosylation pattern of envelope of T/F virus is markedly different from that of chronic in the degree of occupancy and levels of complex gycans [144]. Recently it was shown that African Green Monkeys can acquire SIV infection through the mucosal routes (intrarectal and intravaginal) by one or two T/F virus and that susceptibility to infection was proportional to the number of CD4+ expressing CCR5 in the mucosal [145]. Another study has identified neutralizing antibodies in SIVmac-infected with T/F virus after 5 to 8 months of infection [146]. Although their emergence

was slow and titer was low, these neutralizing antibodies induced escape mutants harboring mutations and deletions in the variable region of the envelope.

The AIDS epidemic is caused by HIV-1 and HIV-2. HIV-1 can be classified into four groups: group M (major), O (outlier), N (new, non-M, non-O) and group P. The new HIV (group P) was recently isolated and found to be closely related to gorilla SIV [147,148]. Group M is the most diverse that is further classified into nine genetic subtypes; A, B, C, D, F, G, H, J and K and four sub-subtypes; A1, A2, F1 and F2 [149,150,151]. The heterogeneity of HIV-1 group M subtypes in amino acid substitutions in the *env* and *gag* genes is 20% and 15%, respectively [149,152]. In addition, 51 circulating recombinant forms (CRFs) have been reported to date [153]. The identification of a new subtype, sub-subtype or CRFs requires the identification of three complete identical genome sequences from individuals not known to be linked in a direct chain of transmission that results in the identified sequences (epidemiological linkage) [154].

HIV-1 subtype distribution and prevalence vary in the different regions of the globe (Figure 8). The world-wide distribution of HIV subtypes and recombinants is relatively stable, although CRFs are playing an increasing role in the HIV pandemic [155]. The most prevalent HIV-1 genetic subtypes worldwide are subtype C (48%), subtype A (12%), subtype B (11%), CRF02_AG (8%), CRF01_AE (5%), subtype G (5%) [155]. Central Africa has the greatest diversity, where all major subtypes and groups M, O, N and P are circulating [147,148,155]. In West Africa, the dominant variants are

CRF02_AG and subtype G although all subtypes have been identified, while subtype B is the dominant subtype in North America, the Caribbean, Latin America, western and central Europe and Australia [155]. In southern Africa, Ethiopia and India, subtype C is the major cause of the epidemic. Additional subtypes and CRFs are regularly identified, and migrating populations have been observed to play a greater role in the patterns of new subtype identification and distribution [156,157,158]. There is particular concern in regard to HIV-1 subtype C, A, and the AG recombinant forms that are predominant in Africa where HIV is rapidly spreading.

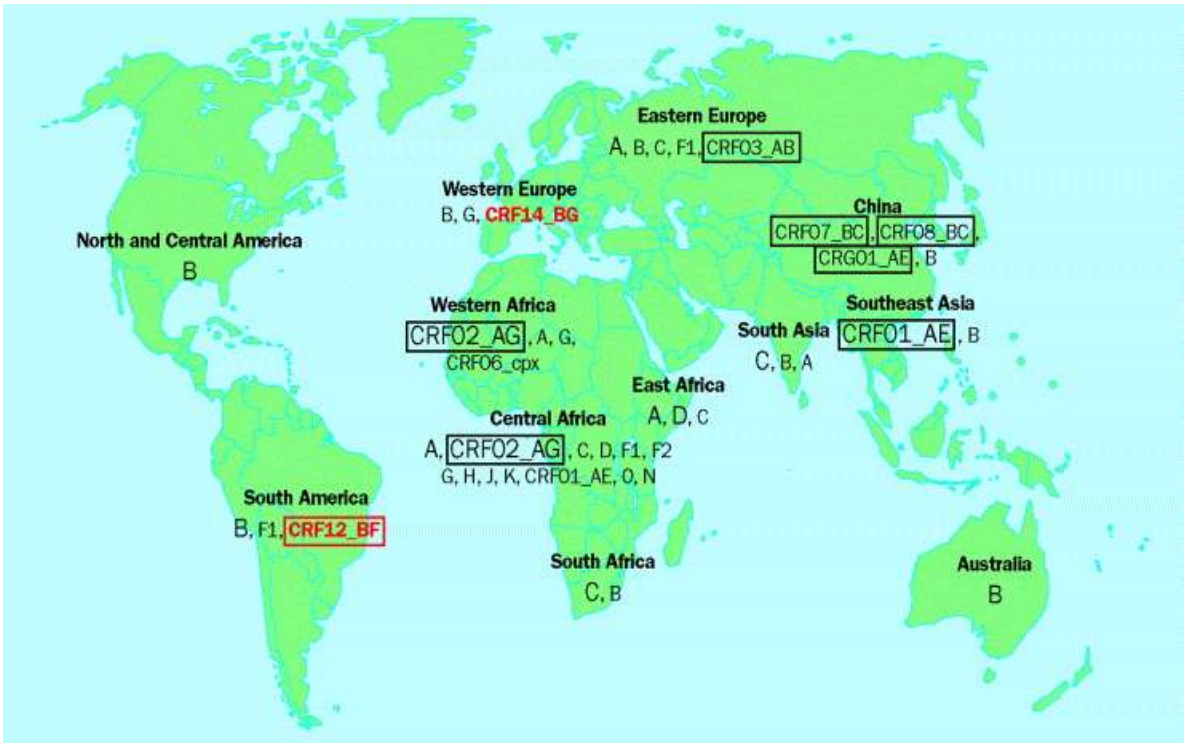


Figure 8: Geographic Distribution of HIV subtypes [159]. (Reproduced with permission from Thomson MM et al 2002).

1.11. HIV Drug Classes

The success of HIV research today has been the availability of new classes of drugs, allowing the introduction of combination antiretroviral (ARV) therapy [highly active antiretroviral therapy (HAART)] and the gradual evolution of HIV infection into a chronic, usually non-fatal condition [160]. Antiretroviral drugs are classified according to the step they inhibit in the viral life-cycle. Most of these drugs target viral enzymes (RT, PR and IN inhibitors) or important steps in the viral life cycle (entry and maturation inhibitors). Entry, RT, PR and IN inhibitors are currently approved for treatment of HIV patients while maturation inhibitors are under pre-clinical and clinical development.

The five different classes of approved anti-HIV drugs for treatment of HIV infected patients include; nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), Entry Inhibitors (EIs), and integrase inhibitors (INI). Each antiretroviral drug class targets the HIV life cycle differently. These drug classes differ at the stage of HIV replication at which each acts.

1.11.1. Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTIs)

NRTIs block the reverse transcription process of HIV RT. These chemically modified nucleosides and nucleotides (analogs) compete with the natural building blocks (dNTPs). This results to binding of the compound to the viral DNA in the place of the dNTPs. The structure of these compounds are different from the natural dNTPs and do not allow any further replication of HIV, thus helping to stop the replication of the virus (competitive

inhibitors of natural dNTPs). NRTIs are dideoxy compounds that lack a 3'-OH group, stopping DNA synthesis when they are bound into the growing DNA strand [161,162,163]. They act as chain terminators, causing premature termination of the proviral DNA chain. Nucleoside analogues undergo three phosphorylation steps for HIV inhibition to occur. Nucleotide analogues do not need the first phosphorylation step because they are already phosphorylated. Six nucleosides are currently approved by the US Food and Drug Administration (FDA) and Health Canada (Table 1). They include; abacavir (ABC), didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T) and zidovudine (ZDV) while tenofovir (TDF) is the only approved nucleotide analogue. Second (next) generation nucleosides inhibitors in clinical development include; elvucitabine (ELV) and Aprecitabine (ATC) (Table 2).

Table 1: Represents first generation NRTIs that have been approved for treatment of HIV patients.

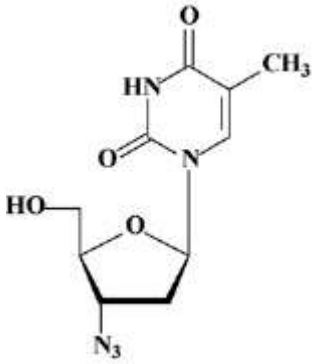
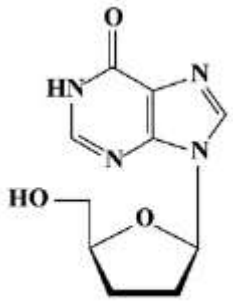
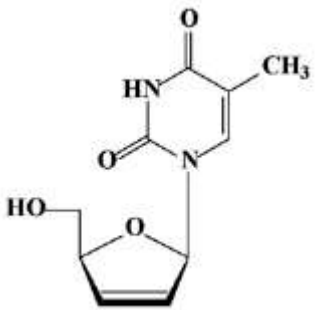
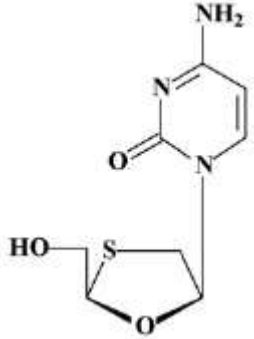
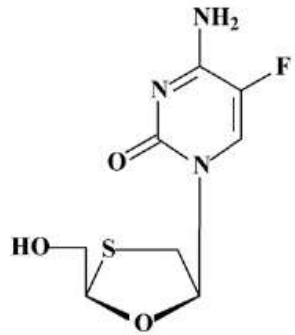
Drug Candidate	ZDV	ddI	d4T	3TC	FTC
Chemical Structure					
Date of FDA/Health Canada approval	1987/1990	1991/2002	1994/1996	1995/1995	2003/2006

Table 1: Represents first generation NRTIs that have been approved for treatment of HIV patients (cont.).

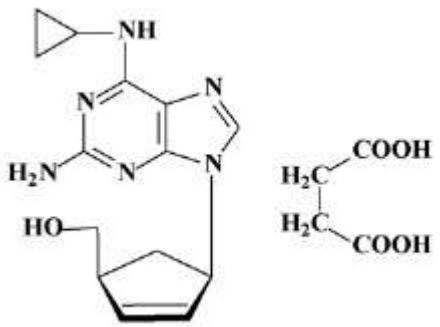
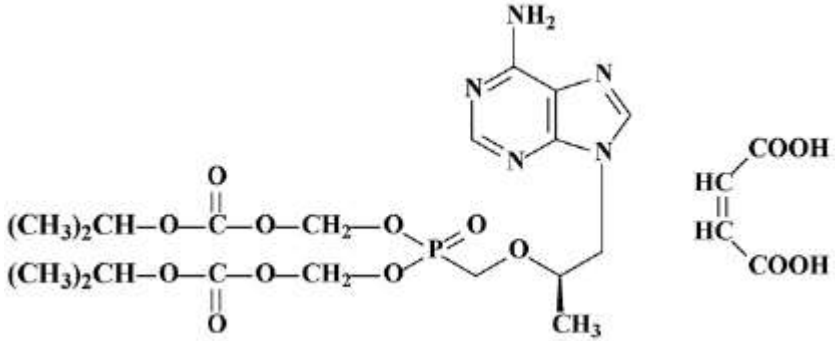
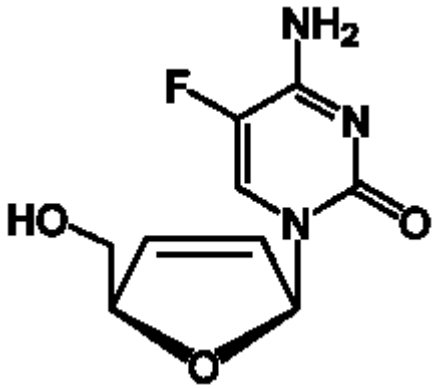
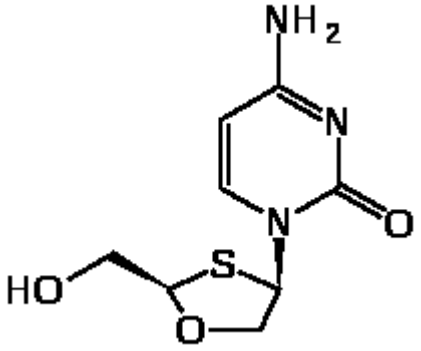
Drug Candidate	**ABC**	**TDF**
Chemical Structure		
Date of FDA/Health Canada approval	1998/1999	2001/2004

Table 2: Represents second generation NRTIs undergoing clinical development.

Drug Candidate	ELV	ATC
Chemical Structure		
Phase of Development	IIb	IIb

1.11.2. Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

NNRTIs also target the reverse transcriptase enzyme by a mechanism of action that is different from that used by NRTIs. NNRTIs are not derivatives of natural dNTPs. They are non-competitive inhibitors of HIV-1 RT and do not require metabolic activation. Moreover they show extremely diverse molecular structures that play an important role in binding. The structure of NNRTIs allows them to bind to the hydrophobic pocket within the p66 subunit of RT enzyme which is close to the polymerase active site [108,164,165], inhibiting HIV-1 replication by preventing the conversion of viral RNA into DNA [108,166,167,168]. Crystallographic and biochemical studies have revealed that NNRTIs may interrupt with the binding between the 3' end of the primer and the incoming dNTP, thus decreasing the efficiency of the chemical step [169]. Four NNRTIs are currently approved by the US FDA and Health Canada. They include first generation drugs efavirenz (EFV) and nevirapine (NVP) (Table 3), while etravirine (ETR) and rilpivirine (RPV) are second generation drugs (Table 4). Dapivirine (TMC120) is a second generation NNRTI license for microbicide development. Novel second generation NNRTIs under clinical development include; Lersivirine and RDEA 806 (Table 4).

Table 3: Represents first generation NRTIs that have been approved for treatment of HIV patients.

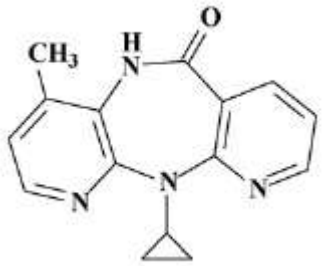
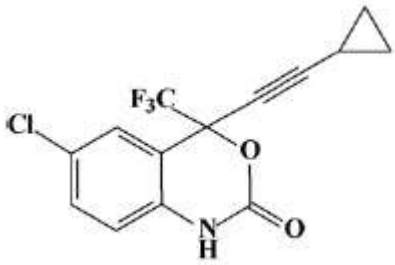
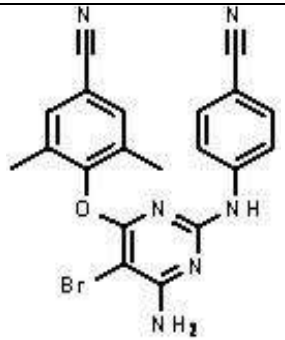
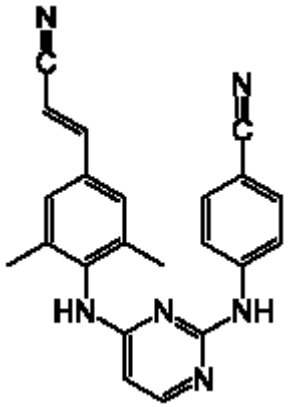
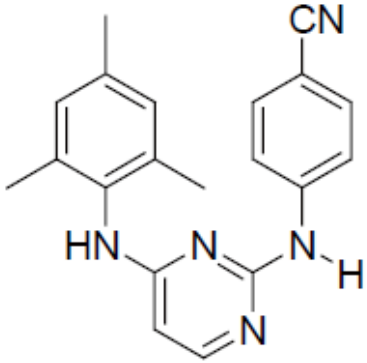
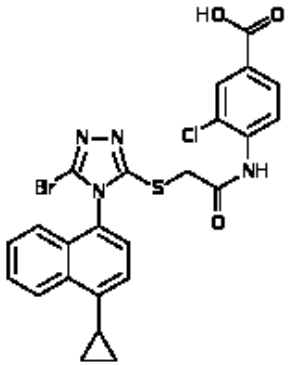
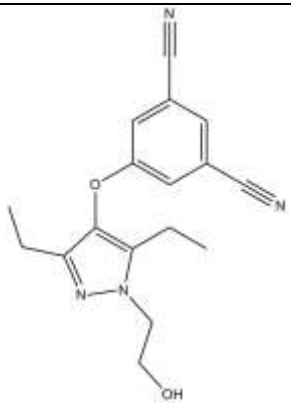
Drug Candidate	NVP	EFV
Chemical Structure		
Date of FDA/Health Canada approval	1996/1998	1998/1999

Table 4**:** Represents second generation NNRTIs that have been approved or are undergoing clinical development

Drug candidate	ETR	RPV	TMC 120	RDEA806	Lersivirine (UK-453061)
Chemical structure					
Phase of development	FDA (2008), Health Canada (2008)	FDA (2011), Health Canada (2011)	License for HIV-1 microbicide development	IIb	IIb

1.11.3. Protease Inhibitors (PIs)

The role of the HIV protease enzyme is to cleave immature viral proteins to produce mature infectious virus particles [32]. PIs inhibit the cleavage of immature Gag and Gag-Pol precursor protein (prevent maturation) and thus render the virus non-infectious. The US Food and Drug Administration (FDA) and Health Canada has approved eight PIs for treatment of HIV patients [170] (Table 5 and 6). They include; saquinavir (SQV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), lopinavir (LPV), atazanavir (ATV), tipranavir (TPV) and darunavir (DRV). PI treatment is accompanied by a low dose of ritonavir (RTV) as a boosting agent (a compound that inhibits the host enzyme that metabolizes other PIs and thus leads to higher concentrations of PIs in blood). All PIs are known as competitive peptidomimetic inhibitors, except tipranavir. These competitive inhibitors mimic the natural substrate of the viral protease. A hydroxyethylene core present in the peptidomimetic inhibitors prohibits cleavage of the inhibitor by the HIV-1 protease. In tipranavir a dihydropyrone ring replaces the peptidomimetic hydroxyethylene core (reviewed in [171]).

Table 5: Represents first generation PIs that have been approved for treatment of HIV patients.

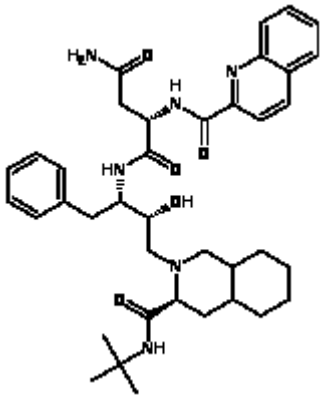
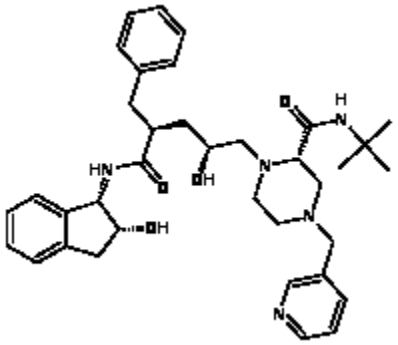
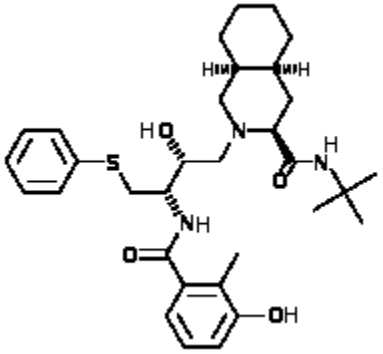
Drug Candidate	SQV	IDV	NFV
Chemical Structure			
Date of FDA/Health Canada approval	1995/1996	1996/1996	1997/1998

Table 5: Represents first generation PIs that have been approved for treatment of HIV patients (cont.).

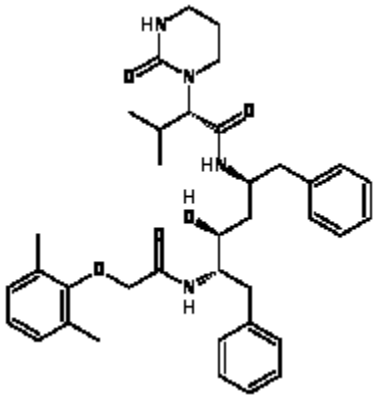
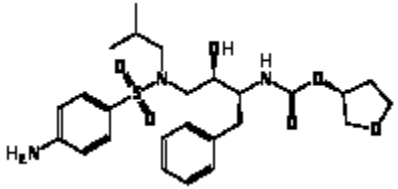
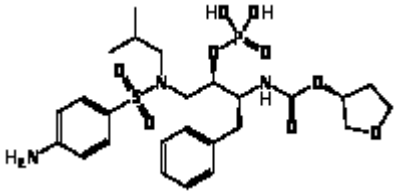
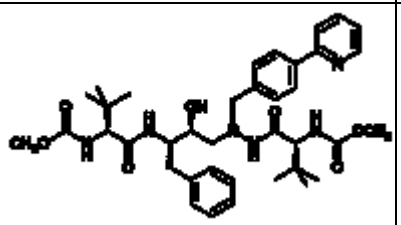
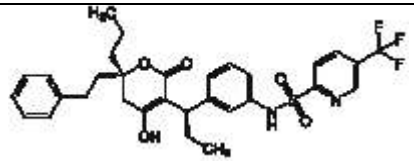
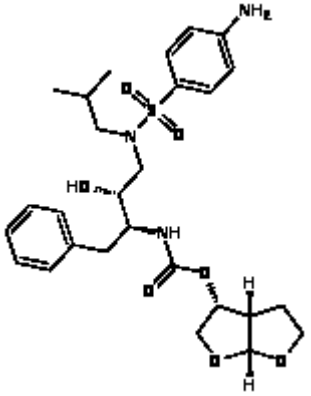
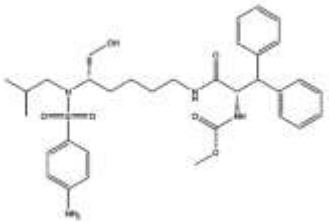
Drug Candidate	LPV	APV	Fosamprenavir
Chemical Structure			
Date of FDA/Health Canada approval	2000/2001	1999/none	2003/2004

Table 6**:** Represents second generation PIs that have been approved or are undergoing pre-clinical development

Drug candidate	ATV	TPV	DRV	PL-100
Chemical structure				
Phase of development	2003/2004	2005/2005	2006/2009	Pre-clinical development

1.11.4. Integrase Inhibitors (INIs)

Integrase inhibitors are a novel class of ARV drugs recently included in HIV management [172]. The role of the integrase enzyme is to integrate viral genetic material into the host cell genome. This serves as an important drug target. The HIV integrase has essentially two important catalytic functions which include; 3'-processing and strand transfer. Raltegravir is an INI approved by FDA in 2007 (reviewed in [173,174]) and elvitegravir recently approved (August 2012) are targeted at strand transfer reaction while dolutegravir, a second generation INI in clinical development also target the strand transfer reaction.

1.11.5. Entry Inhibitors (EIs) and Fusion Inhibitors (FIs)

EIs prevent HIV entry into the cell. FIs inhibit HIV by targeting and blocking HIV entry into CD4 cells. FIs block fusion by binding to gp41, which is involved in the fusion of the viral particle and the membrane of the CD4 cell [175]. Blocking the fusion process by FIs prevents the HIV genetic material from entering the CD4 cells. The only FI that is FDA approved to date is enfuvirtide (T-20) [176,177]. T-20 is a C-peptide of the HIV-1 gp41 C-terminal heptad repeat (CHR) region. It also contains partial N-terminal heptad repeat (NHR) and lipid binding domains. The N- and C- terminal sequences of T-20 bind with the NHR of gp41 and cell membrane respectively, and thus inhibit the fusion process [178].

A new entry inhibitor, maraviroc was recently approved. It is the only antiretroviral that targets the host cell rather than the virus. It binds to the CCR5 co-receptor (CCR5

antagonist) and thus prevents HIV from entering the cell. However, the activity of maraviroc is specific to patients with R5-tropic viruses. CXCR4-CCR5 mixed tropic viruses and X4-using viruses are resistant to maraviroc treatment [179].

1.12. Antiretroviral (ARV) Therapy

HAART is the standard treatment for HIV patients. It involves the use of three anti-HIV drugs e.g. two NRTIs in combination with either a non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor or, most recently, integrase inhibitor [180]. The goal of HAART is the maximal and long term inhibition of HIV replication with an improved immune function [181]. Rational drug selection is necessary for optimal therapy response, reduced toxicity and cross resistance, in order to preserve options for future treatment and increase overall duration of viral suppression [reviewed in [182]]. Although numerous antiretroviral combinations may provide potent suppression of viral replication, therapeutic choices necessitate careful consideration of the potential impact of viral resistance on subsequent treatment options.

Potential causes of therapy failure during antiretroviral therapy include insufficient viral suppression (virological failure), a continuous decrease in CD4 cell counts (immunological failure), adverse drug effects (toxicity) and progression of clinical disease [reviewed in [182]]. Virological failure results from pre-existing drug resistance or suboptimal drug levels and may lead to further accumulation of resistance mutations [183].

1.12.1. HIV Drug Resistance during Antiretroviral Therapy

Despite the successes in antiretroviral therapy that have improved the lives of HIV-infected patients, the selection of drug resistance mutations is inevitable [160]. The emergence of resistance variants is facilitated by the error-prone reverse transcriptase that has an impaired ability to proofread [132] together with an increased number of replication cycles occurring in an infected individual [184]. Sub-optimal drug levels within certain compartments allows viral replication to occur and can also lead to selection of resistance mutations [185]. In a review by Asahchop *et al* 2012, it was reported that "these selected mutations are located on the genes that encode antiretroviral targets such as RT, PR, IN and gp41 resulting in the production of viral proteins that are subtly different than their wild-type counterparts in structure and function. Although these proteins are still able to play their role in HIV replication, they are not targeted as effectively as wild-type proteins by the ARV drugs. The number of mutations required for resistance to occur varies from drug to drug. Many factors determine the relative rate of resistance selection with different drugs and drug combinations, and this is reflected in the `genetic barrier' to resistance. Interactions between mutations and the effects of individual resistance mutations on viral replication and fitness influence mutational pathways and the overall impact of resistance mutations on viral phenotype". Certain mutations such as K65R and thymidine analog mutations (TAMs) may be antagonistic [186], whereas others may confer resistance to some drugs while conferring hypersusceptibility to others. An example is the K103N mutation that confers high level resistance to both NVP and EFV but is susceptible to ETR [187].

Different mechanisms through which HIV-1 escapes from drug pressure have been described. These mechanisms differ from one drug class to another and sometimes differ even between drugs of the same class.

1.12.2. Changes in Viral Population Dynamics during ARV Therapy

Due to the high error rate of HIV RT, the rate of mutations within the viral genome is high during HIV infection. Although most of these mutations do not have an effect on viral function, some at specific sites may be lethal to the virus or affect drug susceptibility. Genetically diverse HIV populations harbor drug resistance mutations. However, in the absence of ARV therapy, these diverse viral variants containing mutations possess a decreased replication ability compared to wild-type viruses (i.e. they are less fit). They are only present at low levels in the viral population (i.e. minority populations) [188,189]. The administration of ARV therapy results in strong selective pressure on the HIV population, stopping replication of susceptible variants [184,188]. In the case of sub-optimal therapy, residual viral replication will occur resulting in mutant virus strains that will replicate and dominate the viral population. If drug therapy is withdrawn, resistant variants will continue to dominate. However, the wild-type virus will eventually predominate the viral population due the absence of therapy [184,190].

1.12.3. Mechanism of NRTI Resistance

HIV can become resistant to NRTIs via two distinct mechanisms as described by Asahchop *et al* 2012. The first is discrimination, whereby the mutated viral RT can selectively avoid incorporating NRTIs in favor of natural dNTPS; this mechanism is typified by such mutations as K65R, L74V, Q151M and M184V [191]. The second mechanism of resistance allows a mutated RT to enact the phosphorolytic excision of NRTIs from the 3' end of the viral DNA chain that extends from the primer, a process referred to as 'primer unblocking'. Examples of mutations involved in this process are those selected by zidovudine (ZDV) and stavudine (d4T) that are termed thymidine analogue mutations (TAMs), e.g. M41L, D67N, K70R, L210W, T215Y/F and K219Q/E [186,192]. TAMs confer resistance to all NRTIs except lamivudine (3TC) and emtricitabine (FTC). Although, there is a degree of cross-resistance associated with TAMs, ultimate levels of resistance depend on the specific NRTI and the number of TAM mutations found in the viral RT (reviewed in [192]). The two NRTI resistance mechanisms of discrimination and excision can also influence each other. For example, the M184V/I mutation that is selected by 3TC and FTC is a discrimination mutation but viruses that contain M184V/I are less likely to quickly develop TAMs under selective pressure with such drugs as ZDV. Viruses containing M184V/I are also more susceptible to ZDV and d4T than wild-type viruses (reviewed in [192]).

1.12.3.1. Resistance to First Generation NRTIs

The NRTI ZDV was the first HIV drug approved for treatment of HIV infected patients. Monotherapy with ZDV lead to the selection of single amino acid substitution with high

level resistance. Additional drugs in this class have also been approved and possess low genetic barrier to resistance as well as cross-resistance.

1.12.3.1.1. Resistance to ZDV and d4T

Among the recognized NRTI-associated mutations are the amino acid substitutions at codons 41, 67, 70, 210, 215 and 219, which are selected by ZDV or d4T [193,194]. TAMs are selected by and cause resistance to the thymidine analogues (ZDV and d4T). The timing and rates of selection of these particular mutations may differ between ZDV and d4T, although both agents show reduced susceptibility in their presence. Decreased activity of ZDV increases with accumulation of mutations. A mutation at codon 70 (K70R) results to ZDV failure, the emergence of mutations such as T215Y/F, D67N, M41L and K219Q/E follow later. TAMs confer cross-resistance to ZDV and d4T, and other NRTIs at varying degrees. Two distinct TAM pathways have been identified: the first consist of mutations M41L, L210W and T215Y and, the second involves D67N, K70R, T215F and K219Q/E [195,196]. The second pathway is more frequently observed than the first one and even result to higher level resistance to ZDV. However, several mutations from one group can co-exist with those from the other. Furthermore, these pathways may in some cases overlap, especially for viruses with five or six TAMs.

1.12.3.1.2. Resistance to 3TC and FTC

The M184V/I mutation is selected in all patients receiving therapy containing 3TC or FTC [197]. The M184V mutation results to approximately 100-fold resistance to 3TC and FTC, low-level resistance to ABC *in vitro*, but seem clinically not important except in the

presence of mutations such as TAMs, K65R or L74V. M184V acts antagonistically with TAMs mutations to improve susceptibility to ZDV. Its presence has been observed to delay the accumulation of TAMs. M184V results to reduced viral fitness. A decreased viral load with 3TC monotherapy was observed in HIV-1 infected patients compared to pre-treatment level despite the presence of M184V [198]. This data provide evidence for maintaining 3TC in a failing regimen.

1.12.3.1.3. Resistance to ddI

Antiretroviral therapy with ddI leads to the emergence of L74V mutation and rarely selects for K65R mutation [199]. *In vivo*, isolates containing the L74V alone has been observed to impact on the susceptibility to ddI. The L74V can confer cross-resistance to ABC and TDF if present together with TAMs. *In vitro*, the L74V mutation is associated with an increased susceptibility to ZDV [200].

1.12.3.1.4. Resistance to ABC

Treatment failure with ABC leads to the emergence of the following mutations: K65R, L74V, Y115F and M184V [201]. L74V and M184V are the most frequently observed mutations. It has been demonstrated that the combination of L74V and M184V shows increased susceptibility to ZDV and d4T. ABC seems to be able to select K65R only rarely.

1.12.3.1.5. Resistance to TDF

TDF selects the K65R mutation *in vitro*, which reduces susceptibility to TDF by 3- to 6-fold [202,203]. *In vitro*, this mutation shows decreased susceptibility to ddI, 3TC and ABC, but not to ZDV [204]. The selection of K65R in patients failing TDF has been

influenced by the presence or absence of TAMs at baseline and the use of NRTIs (TDF, ABC, d4T and ddI) [205,206,207,208]. Both *in vitro* and biochemical studies have shown that the selection K65R is facilitated in subtype C than in subtype B [202,209]. Recent studies revealed that the increased selection of K65R in subtype C depends on the nature of the subtype C RNA template rather the than subtype origin of the viral reverse transcriptase [209,210,211]. This is due to the pausing events that occur at codon 65 of subtype C viruses resulting in a high rate of K65R selection during reverse transcription [209,210]. In contrast, subtype B template pausing is observed at codon 67, resulting in generation of D67N and TAMs, and not K65R [209,210,212].

Clinical studies revealed that patients previously treated with ABC or ddI- containing regimen were not at higher risk to select K65R [213]. The existence of an antagonistic interaction between K65R and TAMs limit the coexistence of K65R and TAMs. The strongest antagonism is observed between K65R and T215Y [203]. This antagonism is the result of a K65R-mediated reduction of the excision resistance mechanism induced by TAMs [186]. In addition, biochemical studies have also shown that there is an antagonism between K65R and L74V. The presence of both mutations induces a deep loss of viral replicative capacity that is correlated with a low propensity of K65R + L74V double mutants to incorporate natural nucleotides relative to wild-type RT [214]. These results are in accordance with most clonal analyses performed on plasma samples [215,216]. More rarely, the K70E mutation can be selected by TDF *in vivo*.

1.12.3.2. Resistance to Second Generation NRTIs

Currently approved NRTIs have safety precautions. Limitations are specific to certain population such as children infected with HIV, HIV-infected pregnant women or those with NRTI resistance [217]. There is thus the need to develop new NRTIs with better pharmacological profiles. Several NRTIs are in various stages of clinical development.

1.12.3.2.1. Resistance to Elvucitabine

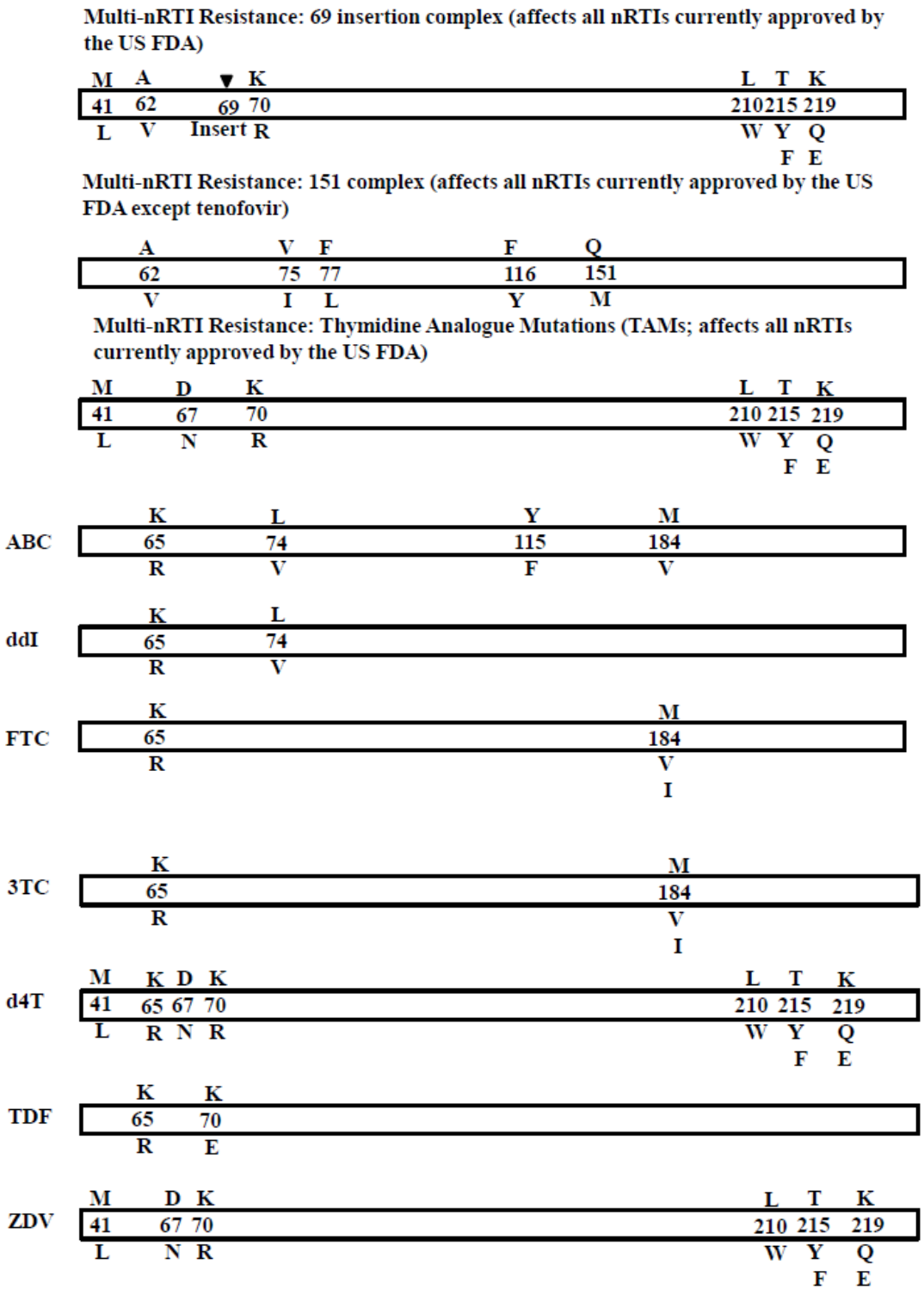
Elvucitabine (Table 2) is a novel NRTI currently in the late phase II study and developed by Achillion Pharmaceuticals. In a review by Asahchop *et al* in 2012, it was reported that only two amino acid substitutions, M184I and D237E, were identified in the resultant variant in an *in vitro* selection study [218,219]. The double mutation conferred moderate resistance to elvucitabine (about 10 fold) and cross-resistance to lamivudine but not to other nucleoside inhibitors tested. Elvucitabine shows more potent anti-HIV activity than 3TC, in part because of high intracellular levels of its triphosphate metabolite [220]. Elvucitabine has also demonstrated potent antiviral activity in HIV-infected patients with resistance to 3TC and other NRTIs. The drug has good oral bioavailability and an intracellular half life of >24 hours [221].

1.12.3.2.2. Resistance to Apricitabine

Apricitabine (ATC) (Table 2) is a novel deoxycytidine NRTI currently in clinical development for the treatment of HIV infection. In a review by Asahchop *et al* 2012, it was reported that, *in vitro* selection for resistance with ATC selected for M184V, V75I and K65R [219,222]. The resulting mutants from this selection conferred low-level

resistance of less than 4 fold. Others showed that continuous passage of HIV-1 already containing M184V, K65R or combinations of M41L, M184V and T215Y did not result in any additional mutations [223]. *In vitro* ATC has shown favorable antiviral activity against HIV-1 wt strains and clinical isolates containing NRTI mutations including M184V, L74V, and TAMs (TAMs) [224]. ATC yielded virological response in treatment-naïve and treatment-experienced HIV-1 infected patients whose viruses contained M184V and up to 5 TAMs. Resistance to ATC was reported to be slow to develop *in vitro,* and there is little evidence of the development of resistance to this drug in patients [225,226].

Table 7: Clinically Approved NRTIs and corresponding resistance –conferring mutations. (Adapted from Johnson VA et al 2011 [227]).



1.12.4. Mechanism of NNRTI Resistance

Mutations that are selected by NNRTIs are all located in the enzyme binding pocket and result in decreased interaction between the inhibitor and the enzyme [228,229,230]. These mutations decrease the ability of the inhibitors to bind to the enzyme. High level resistance resulting from a single mutation in the NNRTI-binding pocket may affect one or more NNRTIs. Amino acid residues that form the NNRTI binding pocket within the p66 subunit include; 95, 100, 101, 103, 106, 108, 179, 181, 188, 190, 227, 229, 234, 236 and 318 [107,108,168]. Some residues from p51, such as 138, also contribute to the NNRTI binding. Resistance to NNRTIs is a direct result of conformational change in the NNRTI binding pocket that prevents the binding of inhibitors (steric hindrance) [228,231]. All NNRTI mutations confer resistance by disrupting the interactions between the inhibitor and the enzyme. Structural studies have revealed three mechanisms of NNRTI resistance including; steric hindrance at the NNRTI binding pocket, abolishing key contacts between inhibitor and the NNRTI binding pocket, and overall change in architecture of the binding pocket [232]. The NNRTI mutation K103N can block the entry of inhibitor into the NNRTI binding pocket through steric hindrance [231]. Mutations such as Y181C can affect contacts (abolish key contacts) between the inhibitor and residues that line the NNRTI binding pocket. The Y188L and G190A mutations can alter the conformation or size (change in architecture) of the NNRTI binding pocket so that it becomes less specific for the inhibitor (reviewed in [232,233]). Some resistance mutations can affect the binding of NNRTIs through more than one mechanism.

1.12.4.1. Resistance to First Generation NNRTIs

First generation NNRTIs include NVP and EFV. The administration of NNRTIs as monotherapy or in the presence of sub-optimal concentrations of drug results in the rapid emergence of resistance [234,235,236]. Studies in which a single dose of NVP is used to prevent mother-to-child transmission of HIV-1 have shown the selection for NNRTI-resistant mutants [234,237]. The presence of a single amino acid substitution usually results to high level resistance. Additionally, a high degree of cross-resistance is seen among first generation NNRTIs [238,239].

1.12.4.1.1. Resistance to Nevirapine

The mutations K103N and Y181C remain the most common NNRTI mutations and most commonly selected in patients containing NVP regimen ([240] and reviewed in [241]). These mutations are responsible for high level phenotypic resistance to NVP [242]. The selection of K103N alone is often enough for treatment failure with first generation NNRTIs to occur [240] due to high level resistance and the fact that it is as fit as the wild-type [243,244]. Other amino acid substitutions conferring resistance to NVP occur at positions 106, 108, 188 and 190 [245]. In monotherapy of NVP to prevent mother to child transmission of HIV-1, these mutations have been selected in both mothers and in children postpartum [246,247,248,249]. The use of ZDV together with NVP results in a different pattern of mutations that does not involve the amino acid substitutions at position 181, demonstrating an interaction between NRTI and NNRTI mutations [245].

1.12.4.1.2. Resistance to Efavirenz

The most frequently selected mutation in EFV treatment failure and in *in vitro* studies is K103N [250,251,252,253]. The NNRTI resistance mutations V108I, P225H, L100I, K101E, K101Q, Y188H, Y188L, G190S, G190A and G190E were also selected in phase II trials [252,253]. In addition the V106A mutation has been observed to confer low level resistance to EFV [254]. This mutation is not frequently selected due to its negative impact on fitness [255]. The M230L is a rare NNRTI mutation because it decreases fitness compared to wild-type as observed in patients with EFV and NVP therapy [256,257] and confers a 15-20 fold resistance to EFV [258]. The selection of V106M mutation is common in subtype C viruses after exposure to NNRTIs such NVP or EFV. This subtype specific mutation is facilitated by a subtype C specific polymorphism within the RT at codon 106 [259,260]. Clinical relevance has been observed in regions where subtype C is endemic [261,262].

1.12.4.2. Resistance to Second Generation NNRTIs

The low genetic barrier to resistance and cross-resistance among first generation NNRTIs serves as a major limitation for prolong antiretroviral therapy and sequential use of inhibitors of this class [239,259,263]. Notably, a single point mutation in the RT enzyme is often associated with the development of high-level resistance and therapy failure. This has driven NNRTI drug development research towards the development of a new set of compounds known as second (next) generation NNRTIs. These compounds possess a high genetic barrier to resistance and improved pharmacological properties. This section

on resistance to approve second generation NNRTIs and those in preclinical development was reported in a review by Asahchop *at al* 2012.

1.12.4.2.1. Resistance to Etravirine

Etravirine (ETR) (Table 4), formerly known as TMC125, is a diarylpyrimidine (DAPY)-based NNRTI that possesses potent antiviral activity against both wild-type (wt) HIV-1 of multiple subtypes as well as against some viruses containing NNRTIs resistance mutations [187,264]. Specifically, ETR retains full activity against viruses containing the most prevalent NNRTI mutation, K103N [187]. *In vitro*, it is more difficult to develop resistance to ETR compared to first generation NNRTIs [264]. Clinical studies of ETR in combination with potent background regimens that included NRTIs, integrase and protease inhibitors, showed significant decrease in viral load in patients with resistance to older NNRTIs and some PIs [265,266,267].

In vitro and clinical studies have identified 20 ETR resistance-associated mutations (RAMs) (V90I, A98G, L100I, K101E/H/P, V106I, E138A/K/G/Q, V179D/F/T, Y181C/I/V, G190A/S, and M230L) and have allowed a weighted score to be assigned to each mutation [268,269,270]. A second score by Monogram has identified 30 ETR RAMs based on a correlation of phenotype and genotype results of 4,923 samples containing at least one NNRTI mutation [271]. Of these ETR RAMs, three or more are required for high level resistance to occur, thus demonstrating a high genetic barrier to resistance compared to older NNRTIs. The structure of ETR allows it to bind to the RT enzyme, such that mutations in the NNRTI-binding pocket do not compromise binding

and, thus, activity is maintained. ETR can rotate within the pocket allowing multiple interactions despite the presence of mutations in the binding pocket [272]. Because of its unique characteristic, ETR is the only NNRTI approved for treatment-experienced patients. Only a few studies have prospectively studied the efficacy of ETR in combination with other background regimen in NNRTI-experience patients [273,274]. In the phase 3 DUET-1 and DUET-2 studies, 57% of patients in the ETR arm versus 36% in the placebo had a viral load <50 copies/ml after 96 weeks of treatment [273]. In these DUET-1 and DUET-2 clinical studies, it was found that patients who experienced virologic failure had greater numbers of ETR resistance mutations at baseline than treatment successes. Furthermore, patients who experienced virologic failure were often found to have received less efficient background regimens compared to those who did not fail therapy [275]. In these studies, the V179F, V179I and Y181C mutations in RT were commonly associated with treatment failure alongside changes at positions K101 and E138 [275]. The authors concluded that these mutations usually emerge in a background of other multiple NNRTI mutations and were, in most cases, associated with a decrease in phenotypic sensitivity to ETR. Another sub-analysis of the DUET trial studied the impact of background regimen on virologic response to ETR, and the authors further confirmed that a higher virologic response rate was observed in patients who demonstrated an increased activity of the background regimen, with the highest responses being achieved in patients who used more than two active agents in addition to ETR [276]. In the TMC125-C227 (ETR) trial, ETR was inferior to a protease inhibitor (PI) in PI-naïve patients with a history of previous NNRTI failure [277]. In a post-hoc analysis of baseline resistance data for the TMC125-C227 trial, a diminished virological response was

observed in patients who possessed the following characteristics at baseline; the presence of Y181C, a baseline ETR fold change of ≥ 10, and a higher number of ETR resistance mutations [277].

1.12.4.2.2. Resistance to Rilpivirine

Rilpivirine (RPV) (Table 4), also known as TMC278, is another DAPY compound that was recently approved for treatment of NNRTI-naïve patients. The structure and binding of RPV in the NNRTI binding pocket is similar to that of ETR, which allows reorientation of both compounds within the pocket. *In vitro* RPV possess subnanomolar activity against wild-type HIV-1 of multiple subtypes and shows antiviral activity against viruses containing many NNRTI resistance-associated mutations [278]. NNRTI RAMs emerging in culture under RPV selective pressure included combinations of V90I, L100I, K101E, V106A/I, V108I, E138G/K/Q/R, V179F/I, Y181C/I, V189I, G190E, H221Y, F227C, and M230I/L. The resistance profile and genetic barrier to the development of resistance to RPV are comparable to those of ETR. In the ECHO and THRIVE phase 3 trials [279,280], resistance analysis showed a slightly higher proportion of treatment failures in the RPV arm compared to the EFV arm. The most frequent NNRTI mutation in the RPV arm was E138K in addition to mutations such as Y181C, K101E, H221Y, V901, E138Q, and V189I. Also the proportion of NRTI mutations that emerge in the study was higher in the RPV arm than EFV arm. The NRTI mutations selected includes M184I/V, K65R, K219E and Y115F.

The IAS-USA has published a total of 15 mutations (K101E/P, E138A/G/K/Q/R, V179L, Y181C/I/V, H221Y, F227C, and M230I/L) associated with decreased susceptibility to RPV [227]. These mutations have been described based on *in vitro* studies and in patients in who RPV was failing. The quantitative impact of each of these mutations on RPV resistance differs.

1.12.4.2.3. Resistance to Dapivirine (TMC 120)

Dapivirine (TMC 120) (Table 4) is another DAPY compound that can accommodate some mutations within the NNRTI binding site without significant loss of activity [263,281]. TMC 120 has shown potent antiviral activity against both wt and NNRTI-resistant HIV-1 strains [282,283]. In 2004, Janssen officially licensed the further development of TMC 120 for use as a vaginal microbicide to the International Partnership for Microbicides (IPM) to help prevent sexual transmission of HIV-1. The results of both phase I and II studies have shown that TMC 120 was widely distributed through the lower genital tract with low systemic absorption when administered as a vaginal gel formulation for up to 42 days [284,285]. The gel was safe and well tolerated. *In vitro* selection studies have identified drug resistance mutations in the presence of TMC 120, notably L100I, K101E, V108I, E138K/Q, V179M/E, Y181C and F227Y [286,287]. Most of these TMC 120 resistance-associated mutations occur at exactly the same position as many of the mutations associated with ETR and RPV resistance [268,269,278]. However, in one of these studies, it was shown that sub-optimal concentrations of TMC 120 alone facilitated the emergence of common NNRTI resistance mutations while sub-optimal concentrations of TMC 120 plus tenofovir (TFV)

gave rise to fewer mutations [287]. Due to the likelihood of transmitted resistant strains in HIV-1 infected individuals, resistance mutations might impact the ability of a single drug in preventing HIV-1 as a microbicide. Using a combination of antiviral drugs of different classes may be useful. Another study showed in an *in vitro* model that using TMC 120 in combination with TFV as a microbicide was more potent and exhibited synergy in comparison with either drug alone [283].

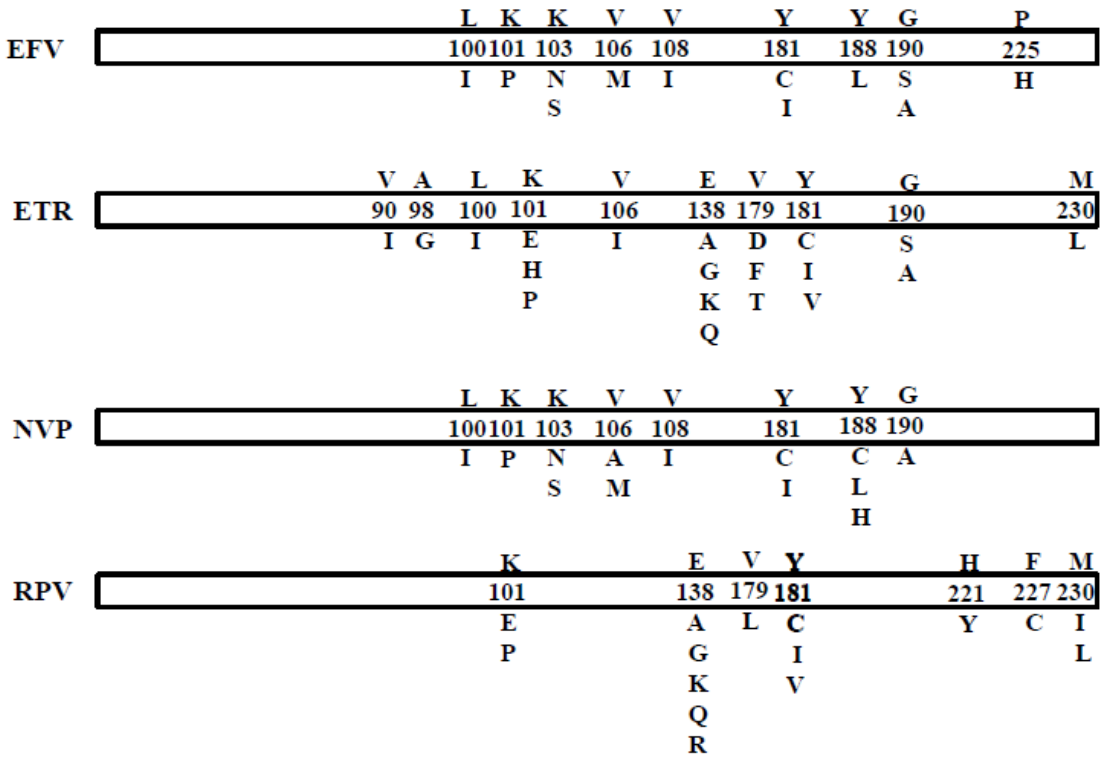
1.12.4.2.4. Resistance to Lersivirine

Lersivirine (Table 4) is a new NNRTI belonging to the pyrazole family and is being developed by Pfizer. In an *in vitro* resistance study, lersivirine selected for the amino acid substitutions; V108I, E138K, V179D, F227L and M230I [288]. In a phase II b trial, Pozniak and colleagues reported better responses in patients with EFV compared to lersivirine, i.e. 86% versus 79% respectively [289]. Amongst patients who failed lersivirine, the mutations identified included K101E, V106M, V108I, H221Y, Y188H, F227C/L and L234I.

1.12.4.2.4. Resistance to RDEA806

RDEA806 (Table 4) is a novel NNRTI developed by Ardea Biosciences. In a genotypic and phenotypic analysis of mutant viruses selected by RDEA806; the K104E, E138K, T240I, V179D and F227L substitutions were identified [290]. Phenotypic analysis of these mutations demonstrated that RDEA806 requires at least 3 mutations for greater than 10 fold loss of susceptibility. In a phase 2a trial, RDEA806 was well tolerated and exhibited robust antiviral activity with no genotypic or phenotypic changes [291].

Table 8: Clinically Approved NNRTIs and corresponding major resistance–conferring mutations. (Adapted from Johnson VA et al 2011 [227]).



1.12.5. Mechanism of resistance of connection domain and RNase H mutations

A number of studies continue to identify new mutations aside from those currently known to be involved in the development of drug resistance to RT inhibitors. Mutations at the C-terminal RT domains i.e. connection domain (amino acids 293-426) and RNase H domain (amino acids 427-560) have recently been shown to be involved in HIV drug resistance. Amino acid substitutions at position A371V and Q509L in the connection and RNase H domains respectively are co-selected on the same genome as TAMs and increase ZDV

resistance [292,293]. The connection domain mutations (E312Q, G335C/D, N348I, A360I/V, V365I, and A376S) together with the presence of TAMs have been shown to increase resistance to ZDV [294]. However, the clinical importance of connection domain mutations is still unknown and there is currently a debate whether these regions should be included in routine HIV genotyping for drug resistance ([294] and reviewed in [295]). A recent study demonstrated that patients who fail more than one NRTI-containing regimen with different TAM and NAM profiles confer varying levels of resistance to ZDV. In this study, the level of phenotypic resistance to ZDV was not affected by mutations in the RNase H domain. Interestingly, the co-presence of L74V and M184V contributed significantly to the phenotypic resistance of ZDV in patients with TAMs even in the presence of connection domain mutations [226]. RNase H mutations confer resistance to NRTIs through a mechanism that involves decreased degradation of RNA either in the presence or absence of TAMs by increasing the time required for excision of incorporated NRTIs [296]. Similarly, one of the most studied connection domain mutation, N348I, was shown to confer ZDV resistance by increasing nucleotide excision [297].

Connection domain (CN) mutations and RNase H mutations including; N348I, T369V/I, A376S, D549N have also been shown to play a role in NVP and EFV resistance [298,299,300]. Furthermore, CN mutations (G333D, N348I, A360V, T369I, and A376S) either decrease susceptibility to ETR or increase in the level of resistance in the presence of both combination of CN mutations and ETR RAMs [299,301,302]. To date, it is well established that CN and RNase H domain mutations exhibit dual resistance to both NRTIs and NNRTIs. The CN mutation N348I confers resistance to NNRTIs (NVP) by

more than one mechanism. These mechanisms include decreased affinity for inhibitor to the NNRTI binding pocket, decreased RT/RNase H activity, and affecting the orientation of the NNRTI binding site [303]. The mechanism of resistance of RNase H mutations is decreased activity of RNase H and thus a reduced degradation of RNA:DNA substrate [232]. However, this mechanism of resistance leads to low levels of resistance to NNRTIs compared to NRTIs. Recently, Nikolenko *et al* proposed that the mechanism for dual resistance to both NRTIs and NNRTIs is the interaction that exists between RNase H cleavage and NRTI excision/NNRTI dissociation [299]. The authors confirmed the proposed model by analysis of patient samples and showed that the ability of each NNRTI to bind to the RT is important in determining the reduction in RNase H cleavage that results in NNRTI resistance.

1.12.6. Mechanism of Protease Inhibitor (PI) Resistance

HIV-1 protease inhibitors play an important role in the management of both HIV-1 infected and AIDS patients. However, their therapeutic efficacy is affected by the selection of drug-resistant mutants, cross-resistance and by issues of patient compliance. Selection of resistance to PIs is considered to be a stepwise process during which mutations accumulate [304,305,306]. Clinical and biochemical studies have demonstrated that the initial PI selected mutations affect the binding of the PI to the viral PR, and the accumulation of additional mutations results in PI resistance [307,308,309]. The selected mutations are found in the substrate-binding cleft of the PR. Subsequently, the levels of resistance conferred by the initially selected resistance mutations are enhanced by the

selection of additional resistance mutations in PR [309]. These additional resistance mutations, when compared to the initially selected resistance mutations, are often located at a more distant position from the substrate-binding cleft.

Since mutations in the active site directly affect the drug/target interface, their mode of action has been interpreted in terms of loss of interactions or steric effects created by altered site geometries (reviewed in [310]). The role of non-active site mutations in inhibitor binding has remained largely elusive, but a few recent studies have suggested that when mutations are selected by PIs, accumulation of these mutations within the core of the protease molecule affects binding by altering the geometry of the binding site cavity [311,312]. PI resistance mutations are often designated as major (mutations which on their own can cause resistance) or minor (mutations that further increase the level of resistance induced by major mutations) resistance mutations.

1.12.6.1. Resistance to First Generation PIs

The initial resistance mutations that are selected can differ between the PIs. First generation PIs generally have a low genetic barrier to resistance. The selection and eventual accumulation of major PI resistance mutations in the binding cavity often result in loss of hydrophobic contacts with these PIs, thus leading to resistance [313,314]. Because most PIs differ only slightly in their chemical structure, cross-resistance is a common phenomenon amongst PIs [315].

1.12.6.1.1. Resistance to Saquinavir

Saquinavir (SQV) developed by Roche, was the first approved PI for treatment of HIV infected patients. *In vitro*, the most frequent mutations or variants selected for by SQV are G48V and L90M and the less frequent G48V/L90M double mutant [316,317,318]. The amino acid residues 48 and 90 are found in the flexible flap of the protease and outside the binding pocket of the enzyme, respectively. A substitution at position 48 may affect the flexibility of the flap while the L90M mutation could induce conformational changes in the enzyme, impacting on inhibitor binding at the active site. The L90M mutation further reduces the catalytic activity and structural stability of the protease [319]. Other PI mutations that have been observed *in vitro,* although at a lower frequency, include M36I, A71V/T and G73S.

1.12.6.1.2. Resistance to Indinavir

The third HIV PI licensed in the United States, indinavir (IDV), was developed by Merck & Co. A report of phase I/II clinical studies with IDV by Condra *et al* demonstrated that the rapid development of resistance occurs at suboptimal doses of the drug [306]. Phenotypic resistance was observed to correlate with multiple substitutions in the protease: L10I/V/R, K20M/R, L24I, M46I/L, I54V/A, L63P, I64V, A71V/T, V82A/F/T, I84V and L90M [320]. The emergence of mutations was random. i.e., resistance was the combined effect of multiple and variable combinations of amino acid substitutions. Most of the viral isolates displaying resistance to IDV also showed SQV and APV resistance.

1.12.6.1.3. Resistance to Nelfinavir

Nelfinavir (NFV), developed by Agouron Pharmaceuticals, demonstrated antiviral activity with EC_{50} of 9 nM and 60 nM against HIV-1 and HIV-2 strains, respectively [321]. Genotyping identified the D30N mutation in resistant variants from patients receiving NFV therapy [322,323]. Mutations that occurred at a low frequency included: E35D, M36I, M46I, A71T/V, V77I, I84V, N88D and L90M [321,322].

1.12.6.1.4. Resistance to Amprenavir

Amprenavir (APV) is a drug from GlaxoSmithKline, which belongs to a class of sulfonamide PIs and possesses antiviral activity against HIV-1 and HIV-2. The EC_{50} values for HIV-1 and HIV-2 are 48 nM and 420 nM, respectively [324]. Genotyping of HIV-1 protease of resistant variants showed a sequential selection of mutations at position L10F, M46I, I47V and I50V. For APV, the predominant resistant mutation in the binding site appears to be the I50V [325]. Furthermore, amino acid changes at position 54 and 84 were associated with the I50V mutation [325,326].

1.12.6.1.5. Resistance to Lopinavir

Lopinavir (LPV) was developed by Abbott Laboratories. It has a very high antiviral activity against HIV-1 and HIV-2 [327]. Decreased sensitivity to LPV results from the mutations M46I, I54V, A71V, V82A and I84V [327,328]. Other mutations involved in decreased susceptibility to LPV include L10F/I/R/V and L90M, as well as amino acid substitutions at codons 20, 24, 32, 33, 46, 47, 50, 53, 58, 63 and 90 [329,330]. These

mutations have shown decreased susceptibility or a combination is associated with complete resistance to LPV [331,332]. V47A also causes resistance to LPV in patients infected with HIV-2 [333].

1.12.6.2. Resistance to Second Generation PIs

Resistance to all the protease inhibitors is inevitable as shown in both *in vitro* and *in vivo* studies. In the absence of antiviral therapy or sub-optimal therapy in infected individuals, the high replication rate increases the diversity of the viral population, eventually leading to viral quasi-species [334]. As a result of resistance mutations, the development of novel PIs is focused on structural designing of HIV PIs that can form molecular bonds with the enzyme backbone atoms and possess a high genetic barrier to the development of resistance [335]. DRV possess a high genetic barrier to resistance and activity against multi-PI-resistant HIV-1 variants, because its forms bond with the main chain of the PR active-site amino acid (Asp-29 and Asp-30) [336]. An additional advantage is its molecular flexibility that can allow it to adapt to the changing shape of a mutant PR [337,338,339].

1.12.6.2.1. Resistance to Atazanavir

Atazanavir (ATV), a second generation HIV-1 protease inhibitor, was developed by Bristol-Myers Squibb as an alternative option for initial PI-based therapy. In a study by Collono *et al*, the authors showed that the resistance profile of ATV is different from that of other PIs based on phenotypic susceptibility results of resistant isolates [340]. Genotypic analysis of viral variants from patients failing ATV therapy showed

that patients selected for mutations at specific residues (10I/V/F, 20R/M/I, 24I, 33I/F/V, 36I/L/V, 46I/L, 48V, 54V/L, 63P, 71V/T/I, 73C/S/T/A, 82A/F/S/T, 84V, and 90M) and displayed decreased susceptibility to ATV. No single mutation or a combination of mutations led to high level resistance to ATV, while five of these mutations were required for loss of ATV susceptibility [340]. The I50L mutation was also observed in treatment naïve patients who experienced virological failure during unboosted atazanavir-based regimens. This mutation confers phenotypic resistance to ATV and impaired viral replication, but increases susceptibility to other PIs [341]. Viruses containing I50L confer resistance specifically to ATV while those with I50V confer resistance to APV, without evidence of cross-resistance [341].

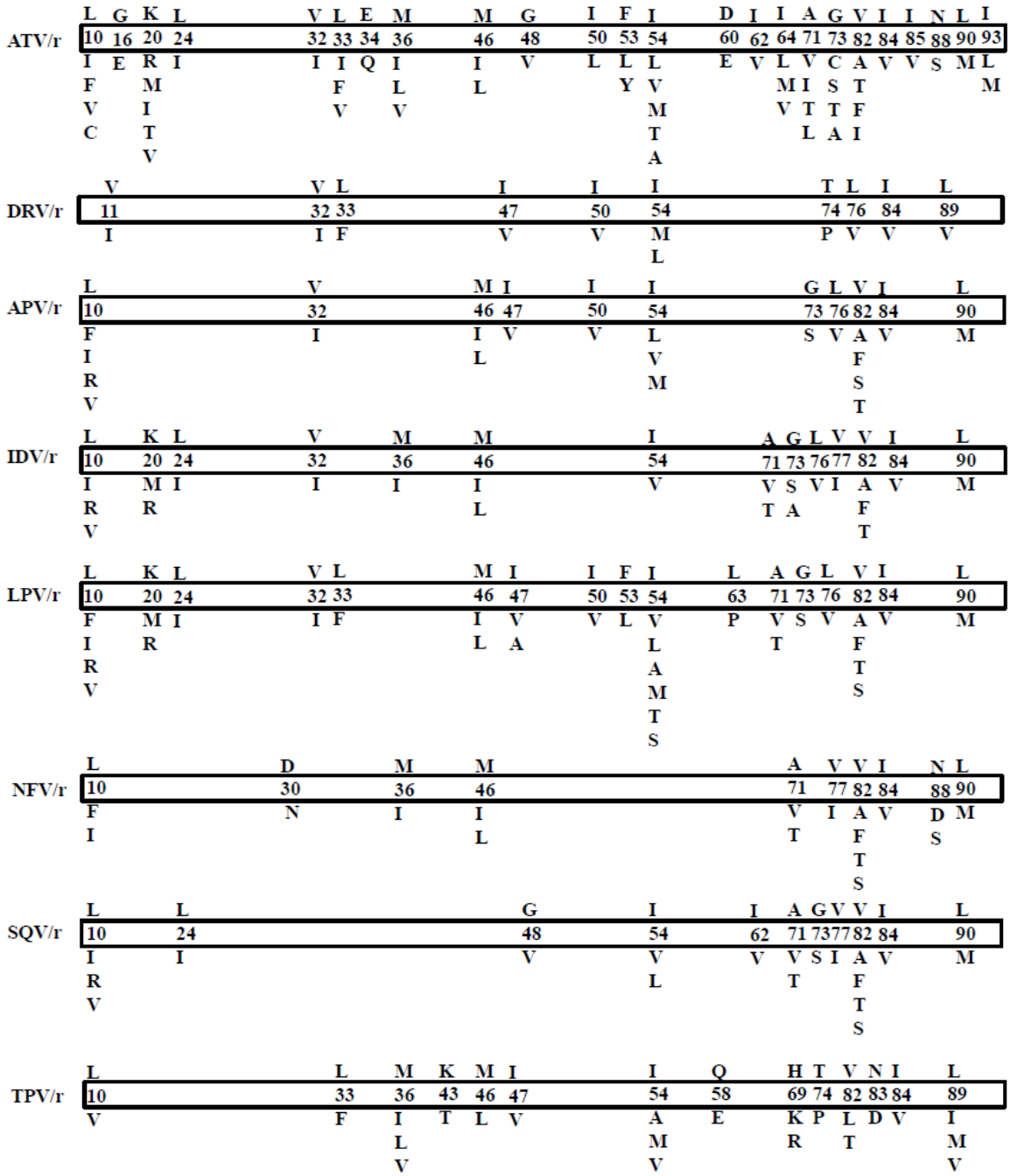
1.12.6.2.2. Resistance to Tipranavir

Tipranavir (TPV) was developed as a second generation HIV-1 protease inhibitor by Boehringer Ingelheim. It is a dihydropyrone that is structurally similar to coumadin that shows activity against HIV protease (reviewed in [342]. It is effective against HIV-1 isolates that are resistant to other PIs [343]. However, resistance to TPV can still occur and requires the accumulation of up to ten mutations for a high level of resistance to develop. In an *in vitro* selection experiment, accumulation of 10 mutations in the protease (L10F, I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V) together with mutations in the CA/SP1 gag cleavage site were identified and showed 87-fold impact on resistance to TPV. With the exception of SQV, Tipranavir resistant variants have been shown to display cross-resistance to other PIs [344]. The L33F and I84V are the dominant mutations that lead to the development of TPV resistance.

1.12.6.2.3. Resistance to Darunavir

Darunavir developed by Tibotec Inc. is a broad-spectrum potent protease inhibitor against HIV-1 isolates including multi-drug resistant clinical strains with minimal cyto-toxicity [336]. It also has higher genetic barrier for resistance development compared to existing PIs. Genotypic analysis of patients failing therapy identified amino acid substitutions including: V11I, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V and L89V. The presence of three or more of these mutations leads to a DRV fold change of greater than 10 and a decreased virological response [345].

Table 9: Clinically Approved PIs and corresponding major resistance–conferring mutations. (Adapted from Johnson VA et al 2011 [227]).



1.12.7. Mechanism of resistance of Gag cleavage site mutations

In HIV naïve patient treated with a boosted PI, mutations in the protease might be uncommon immediately after therapy failure [346]. It has been hypothesized that virological failure in isolates lacking protease mutations may be due to the fact that mutations are selected not in the protease region but within the *gag* gene [347].

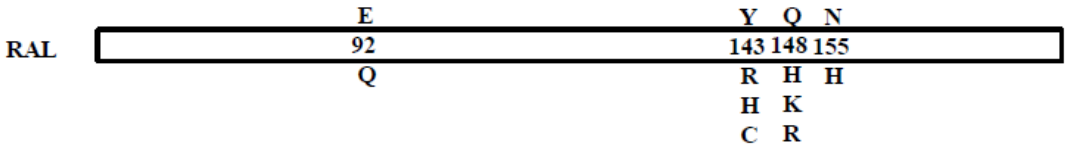
Gag cleavage site mutations (V128T, E428D, A431V, I437V, L449P/F, S451N, P453L) have been reported in virologic failure of HIV patients containing PI regimen [346,348]. The mutations at the p1/p6 cleavage site (L449F or P453L), individually do not confer PI resistance. However, in the presence of the background PI mutation I50V, decreased drug sensitivity is observed, demonstrating that the level of resistance is enhanced when mutations in the viral protease and Gag protein interact [349]. Two mechanisms leading to PI resistance have been proposed, first the drug selects for specific resistance mutations in Gag, aside from those in the protease gene resulting in an increased processing efficiency of the altered substrate by wt protease [347,350]. Secondly, the emergence of amino acid substitutions within the protease gene may exert selective pressure on the *gag* gene through compensatory mutations.

1.12.8. Resistance to INIs

Raltegravir (RAL) and elvitegravir (EVG) are integrase strand-transfer inhibitors (INSTIs) approved for clinical use. Though effective in first line and HIV experienced patients, resistance mutations can impact on the susceptibility of virus to INSTIs. The selection of single point mutations in the presence of RAL pressure can confer high level

resistance (fold change FC >5). Thus, the genetic barrier for resistance development to RAL is low. Three resistance pathways for resistance development to RAL have been identified; E92QV/N155H, T97A/Y143CHR and G140CS/Q148HKR [351]. These resistance pathways can either emerge separately or may be linked. The G140S/C and E92Q/V single mutations confer 5-10 fold resistance to RAL [352], but usually emerge as secondary mutations after the N155H and Q148HKR mutations are selected [353], leading to FC>100 for these combination of mutations. Some polymorphic and non-polymorphic residues such as T66I/L have been observed to decrease the susceptibility of RAL [354]. All major INSTIs resistance mutations play a role in IN activity and viral replicative capacity [355]. All mutations that confer resistance to RAL have also been observed to decreased the susceptibility to EVG with the exception of amino acid substitutions at position 143 [356]. Thus, there is a potential for cross-resistance for RAL and EVG. Dolutegravir is a next generation INSTIs in clinical development.

Table 10: Clinically Approved INIs and corresponding major resistance–conferring mutations. (Adapted from Johnson VA et al 2011) [227].



1.12.9. Resistance to EIs and FIs

Viral entry is an important drug target for the development of new HIV drugs. Molecular mechanisms have identified several steps that block the entry process. First, the viral

glycoprotein gp120 attaches to the membrane of CD4 cell. Secondly, binding of the gp120 to co-receptors CCR5 or CXCR4 occurs. Lastly, the gp41 mediates the fusion of the viral and cellular membranes. Compounds (attachment inhibitors) such as BMS-488043 and NBD-556 have been identified to inhibit binding of gp120 to the membrane of the CD4 cell [357,358,359]. These compounds are in preclinical development. It has been shown that amino acid substitutions in gp120 can decrease the sensitivity of these compounds [358]. Enfuvirtide was the first fusion inhibitor to be approved for treatment of AIDS and HIV infected patients [175,360]. In patients failing enfuvirtide containing regimens, the emergence of mutations in a larger fragment of the first heptad repeat (HR1), from codons 36 to 45 of gp41, was observed [361,362]. The decreased susceptibility to enfuvirtide was caused by a variety of mutations, with changes in simple amino acids resulting in high-level resistance [363]. The importance of the surface expression level of the co-receptor on target cells and fusion kinetics is seen when high levels of CCR5 on the cellular surface results in more rapid membrane fusion, thus decreasing the time required for gp41 to be targeted by enfuvirtide [364,365].

Table 11: Clinically Approved EIs and corresponding major resistance–conferring mutations. (Adapted from Johnson VA et al 2011) [227].

	36	37	38	39	40	42	43
Enfuvitide	G	I	V	Q	Q	N	N
	D	V	A	R	H	T	D
	S		M				
			E				

The CCR5 antagonist maraviroc is another entry inhibitor approved for clinical use. Maraviroc inhibits HIV entry by binding to the co-receptor CCR5 on the membrane of CD4 cells thus preventing the binding of gp120 and CCR5 [366]. Two main resistance pathways have been described. The first and most important is when there is a shift in co-receptor usage from R5 to X4. The second results from amino acid substitutions within the viral envelope that permit the interaction of gp120 and the co-receptor even in the presence of the inhibitor. A high affinity of gp120 for CCR5 allows HIV-1 and CCR5 inhibitors to compete for binding to the CCR5 co-receptor (reviewed in [367]). It has been shown that CCR5 antagonist-resistant variants allows the binding of gp120 and CCR5 co-receptor to occur in the presence of the inhibitor, and that the amino acids substitutions in the gp120 [V3, V2 and constant regions 2 and 4 (C2 and C4)] are responsible for the observed resistance [368,369]. Another compound that inhibits binding of another co-receptor (CXCR4) is AMD3100 [370]. This compound is not yet approved for treatment of AIDS and HIV infected patients.

1.12.10. Replication Capacity and Fitness of HIV-1

Fitness is a measure of the ability of an organism to replicate within a specific environment (reviewed in [188]). HIV fitness is the continuous production of infectious viral particles in a given environment. Replication capacity is a comparison of the replication ability of two or more viral variants in an experimentally defined *in vitro* host cell system (reviewed in [371]). The selection of resistance to antiretroviral drugs leads

to decreased fitness and the subsequent emergence of compensatory mutations in the targeted gene that will eventually improve fitness. A number of methods have been employed to study viral fitness including replication kinetics, single-cycle replication assays and growth competition assays. In replication kinetic assays, the rate of HIV-1 replication is quantified after infection of either cell lines (e.g. MT-2 and MT-4) or primary cells [372]. Cultures of these variants are grown in separate wells. Measurements of p24 antigen or the RT activity in culture supernatants at chosen time points are then used to determine the replication capacity of each virus [373]. A single-cycle replication assay is a modified assay used to measure antiretroviral phenotypic susceptibility that uses luciferase as a reporter gene [374]. Here, replication capacity can be measured in the absence or the presence of different drug concentrations [375]. In growth competition culture assays, two viral variants (e.g. wild-type and mutant) are co-cultured in the same well. Genotypic or phenotypic analysis is used to measure the proportions of the competing viruses [376,377].

Viral fitness and virulence are determined by the features present in individual HIV-1 variants. It might be possible to conclude that viral fitness determines the pathogenicity of a given variant (reviewed in [371]. A majority of resistance mutations have deleterious effects due to poor replication capacity and decreased fitness [378,379,380]. For example, the combination of K65R and M184V or L74V has been shown to decrease viral fitness due to the low processivity of the double mutant RT K65R/M184V [381]. The impact of viral fitness on HIV disease progression has not been clearly defined. However, drug selected mutant viruses that possesses a decreased *pol* replication capacity might

contribute to decreased plasma HIV-1 RNA level in patients with selected resistance mutations during HAART [378,382]. Nevertheless, data demonstrating a correlation between replicative capacity and plasma HIV-1 RNA levels in patients with viremia has been either weak [383] or limited to small pilot cohorts [377]. Viral replication fitness could be involved in the transmission of drug-resistant HIV. It has been suggested that a less fit virus has a low probability to be transmitted compared to a highly fit virus that is more easily transmitted (reviewed in [371]).

1.12.11. The Significance of minority drug-resistant Variants.

Routine genotyping for the detection of drug-resistant HIV-1 is based on direct population sequencing. Using this method, only viral variants that represent at least 20–25% of the viral population can be detected [384]. More sensitive methods such as allele specific real time PCR and pyrosequencing have been employed with sensitivity of 0.2 to 1% [385,386]. Clinical studies in antiretroviral experienced individuals have shown that drug-resistant HIV-variants exist at frequencies undetectable by routine population sequencing for prolonged durations not only after therapy failure [387,388,389,390], but also after interruption of therapy in patients initially infected with wt virus [391]. Using two different methods, (single genome sequencing and allele specific real time PCR) Halvas *et al*, identified higher percentages of these minority NNRTI-resistant variants in NNRTI-experienced patients compared to NNRTI-naïve patients [392]. At a frequency of 0.5%–1% of K103N variants, the efficacy of therapy containing efavirenz was significantly decreased while Y181C minority variants were not associated with virologic failure. In a recent study of 13 NNRTI treatment-naïve patients and 20 NNRTI-

experienced patients who developed a K103N mutation by routine genotyping, Varghese *et al* showed that the use of ultra deep pyrosequencing (UDPS) led to additional mutations being detected at low frequencies and this was associated with decreased resistance to the new-generation NNRTI ETR amongst NNRTI-experience patients [393]. In contrast, UDPS could not detect additional major NNRTI mutations as minorities in treatment naïve patients. A dose dependent increased risk of virologic failure after first-line therapy is significantly associated with low-frequency HIV-1 NNRTI resistance mutations (reviewed in [394]).

It has been observed that routine genotyping may underestimate the rates of transmitted drug resistance. In a study to investigate the prevalence of selected drug-resistance mutations as minority quasi-species by allele-specific real-time PCR, the protease L90M mutation and the RT K103N mutation were detected as minority quasi-species in two patients, whereas the RT M184V mutation represented a minority quasi-species in five patients [395]. These mutations were not detected by routine genotyping.

1.12.12. Transmitted Resistance

HIV resistance can either be transmitted (primary) or acquired (secondary). Transmitted resistance is the presence of drug resistant mutations (DRMs) in treatment naïve HIV infected patients while acquired drug resistance is the emergence of drug resistance in a background of transmitted virus in the presence of drugs [396,397]. The ability to transmit a virus containing resistance mutation is impaired compared to a wt virus. This is

probably due to decreased fitness of mutant variants in the absence of therapy [398]. In the viral population, the drug-resistant virus has impaired replication and is thus not transmitted at high frequency [396,398,399]. Transmitted resistance mutations are unstable in the absence of drug pressure [400], as seen in the transmitted mutation M184V that is less fit and reverts back to wild-type [401]. In patients with transmitted drug-resistant virus, the response to therapy is suboptimal [402,403].

1.12.13. Antiretroviral Drug Resistance to different HIV subtypes

The dominant HIV-1 subtype in the developed world is subtype B and differs from subtypes and recombinants that exist in Africa and Asia that harbor the greatest burden of infection. HIV subtypes contain nucleotide changes (silent mutations), polymorphisms and secondary mutations within RT and PR. Some of these silent mutations and polymorphisms have been shown to impact on resistance to NRTIs, NNRTIs and PIs. Many of these changes are known to facilitate the development of resistance but will not confer resistance to drugs on its own (reviewed in[404]).

Two clinical studies from Botswana and Malawi reported 30% and 23% of K65R in subtype C patients after failure of first line therapy containing d4T/ddI plus NVP or EFV and d4T/3TC/NVP, respectively [205,206]. The presence of higher rates of the K65R mutation in subtype C suggests that these viruses possess some factors that facilitate acquisition of this mutation, as has been described *in vitro* [202]. Importantly, biochemical data has shown that a subtype C RNA template mechanism is involved. There is a higher frequency of K65R mutagenesis in subtype C viruses than in other

subtypes [209,211]. Tissue culture selection and clinical studies have shown that in the presence of NVP or EFV drug pressure, a V106M mutation is commonly selected in subtype C viruses whereas the V106A mutation develops in subtype B. The basis for this difference is a nucleotide polymorphism at codon 106 in RT [259,260,261]. Natural resistance of HIV-2 to NNRTIs is determined by the presence of the Y188L natural polymorphism in HIV-2 isolates. HIV-2 has been shown to be sensitive after this amino acid position is reversed [405]. With respect to PR, D30N is preferentially selected by NFV in subtype B than non-B subtypes. As explained by molecular dynamic simulations, this predominance of D30N in subtype B appears to increase the flexibility of the PR flap region and destabilizes the PR-inhibitor complex, thus conferring resistance to NFV [406]. In addition, competition assays have shown that subtype C viruses bearing the D30N mutation replicate poorly and thus confirm the above findings [407]. A recent paper suggests that the selection of specific patterns of protease resistance mutations in different HIV-1 subtypes is influenced by polymorphisms at position 36 in PR [331].

1.13. Hypothesis

As exposed in the introduction to this thesis, PIs and NNRTIs are an important component in the treatment of both naïve and experienced patients and have shown a profound impact on disease progression and transmission. Our studies focused on next generation NNRTIs and PIs. Long term therapy and drug toxicity in HIV-positive individual's lead to adherence problems during treatment. Non-adherence to therapy and the error prone RT enzyme results in the selection of resistance mutations. Both B and non-B HIV-1 subtypes can develop resistance to all currently approved drugs. New anti-

retroviral should be active against multi-resistant viral strains and possess unique resistance pathways in both B and non-B subtypes. It is therefore necessary to determine the potential benefit of new drugs against viruses harboring known drug resistance mutations. The second generation NNRTIs (ETR and RPV) and a novel PI (PL-100) based lysine sulphonamide possess high activity for both resistant and wild-type viruses. Because these drugs have been designed based on structural data from subtype B viruses we wanted to explore how HIV genetic diversity (represented by different non-B subtypes) and viruses harboring known drug resistance mutations impact on the emergence of resistance under drug selective pressure.

We therefore hypothesize that the mutational pathways and resistant patterns to ETR, RPV and PL-100 in both B and non-B HIV-1 subtypes might be driven by the natural HIV-1 genetic diversity and/or baseline resistance mutations.

The aims of this project were to determine: 1) what mutations emerge under drug pressure in different subtypes and in the presence of baseline resistant mutations, 2) how rapidly they emerge upon drug pressure; and 3) what cross-resistance they confer (how could the efficacy of other NNRTIs or PIs be affected). Additionally, we also studied the replicative capacity of resistant variants. The first objective was to identify mutations selected under ETR, RPV and PL-100 drug pressure in B and non-B HIV-1 subtypes using wt and resistant clones, compared resistance profile and time to development of resistance. The second was to compare phenotypic susceptibility of the drug selected viruses to ETR, RPV and NNRTIs or to PL-100 and other PIs. Lastly, we wanted to study

the impact of novel mutations on the replication capacity and susceptibility of ETR, RPV or PL-100 by site directed mutagenesis.

Chapter 2

Characterization of the E138K Resistance Mutation in HIV-1 Reverse Transcriptase Conferring Susceptibility to Etravirine in B and non-B HIV-1 Subypes.

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Abstract

We have selected for resistance to etravirine (ETR) and efavirenz (EFV) in tissue culture using three subtype B, three subtype C and two CRF02_AG clinical isolates, grown in cord blood mononuclear cells. Genotypic analysis was performed at baseline and at various weeks of selection. Phenotypic resistance in regard to ETR, EFV and nevirapine (NVP) was evaluated at weeks 25 to 30 for all ETR selected viruses and in viral clones that contained specific resistance mutations that were inserted by site-directed mutagenesis into pNL-4.3 and AG plasmids. The results show that ETR selected mutations at positions V90I, K101Q, E138K, V179D/E/F, Y181C, V189I, G190E, H221H/Y and M230L and that E138K was the first of these to emerge in most instances. Time to emergence of resistance was longer in the case of ETR (18 weeks) compared to EFV (11 weeks) and no differences in the patterns of emergent mutations could be documented between the B and non-B subtypes. Viral clones containing E138K displayed low-level phenotypic resistance to ETR (3.8 fold) and modestly impaired replication capacity (2 fold) compared to wild-type virus. ETR-selected virus showed a high degree of cross-resistance to NVP but not to EFV. We identified K101Q, E138K, V179E, V189I, G190E and H221Y as mutations not included among the 17 currently recognized resistance-associated mutations (RAMs) for ETR.

Introduction

HIV-1 reverse transcriptase (RT) is responsible for the conversion of the single-stranded RNA genome into double-stranded DNA and is therefore an important target of anti-HIV-1 therapy (16). Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are noncompetitive inhibitors of HIV-1 RT that bind to the polymerase active site and disrupt enzyme function. NNRTIs represent an important component of standard antiretroviral (ARV) therapy in HIV-1 infected patients. NNRTIs can also exert additive effects when combined with nucleoside reverse transcriptase inhibitors (NRTIs) (5) and have potency comparable to that of protease inhibitors (PIs) (39, 41). However, the efficacy of first-generation NNRTIs such as nevirapine (NVP) and efavirenz (EFV) is often hindered by their low genetic barrier for resistance, since only a single amino acid substitution in the NNRTI binding pocket can often lead to high-level resistance against these drugs (3, 25). Furthermore, mutations associated with NNRTI resistance can be transmitted to others, and this has been documented in both developed and developing countries as occurring in between 10 and 20% of newly-infected individuals (1, 19).

Etravirine (ETR) is an expanded-spectrum NNRTI with potent antiviral activity against both wild-type (wt) HIV-1 subtypes and against some viruses resistant to narrow-spectrum NNRTIs (2, 47). Clinical studies have shown that ETR plus optimized background regimens consisting of NRTIs, as well as integrase and protease inhibitors, significantly decreased viral loads in patients with resistance against narrow-spectrum NNRTIs and some PIs (9, 27, 33).

The structure of ETR allows it to bind to the RT enzyme in more than one distinct mode through conformational adaptations based on changes in the NNRTI-binding pocket. This allows ETR to reorient itself and provides alternative binding conformation when mutations in the binding pocket occur (12). *In vitro* selection studies and both the DUET-1 and DUET-2 (Demonstrate Undetectable viral load in patients Experienced with ARV Therapy) clinical trials identified 17 ETR resistance-associated mutations (RAMs) (V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190A/S and M230L) and have permitted the assignment of a weighted score for each mutation (48). In general, three or more ETR RAMs are required for a diminished responsiveness to ETR to occur.

Non-subtype B infections represent >90% of the global HIV problem. Non-B viruses predominate in sub-Saharan Africa and are becoming more prevalent even in areas dominated by subtype B, such as North America and Western Europe (18). Although some studies have established that subtype polymorphisms can play a role in the development of drug resistance, based on specific mutations that are selected by antiviral pressure (6, 7, 11, 21), most have focused on narrow-spectrum antiretrovirals. It is known, however, that naturally occurring polymorphisms at RT sites that correspond to ETR RAMs can vary among subtypes (24, 25, 28, 36). We sought to investigate the possible importance of such polymorphisms in the development of ETR resistance, even though ETR seems to possess similar antiviral activity against various HIV subtypes (A to H) and some circulating recombinant forms (CRFs).

Materials and Methods

Virus isolates, cells, drugs and plasmids:

Three subtype B (BK132, 5326 and 5331), three subtype C (Mole 03, BG 05 and 10680) and two CRF02_AG (6383 and 6399) wild-type clinical isolates were studied (Fig. 1). BK132 was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. The 5326, 5331, 10680, 6383 and 6399 isolates were obtained with informed consent from drug-naive individuals at our clinics in Montreal, Canada. The two subtype C clinical samples Mole 03 and BG 05 originated in Botswana and were obtained courtesy of Dr. Max Essex of Harvard University, Boston MA.

ETR was a gift from Tibotec Inc., while lopinavir (LPV), NVP and EFV were obtained from Abbott Laboratories (North Chicago, IL), Boehringer Ingelheim Inc. and the NIH AIDS Research and Reference Reagent Program, respectively.

Cord blood mononuclear cells (CBMCs) were obtained through the Department of Obstetrics, Jewish General Hospital, Montreal, Canada. The HEK293T cell line was obtained from the American Type Culture Collection. The AG plasmid (p97GH-AG2) was kindly provided by Dr. Masashi Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan. The infectious molecular clone pNL4-3 and TZM-bl cells were obtained through the NIH AIDS Research and Reference Reagent Program, courtesy of Malcolm Martin and John C. Kappes, respectively.

Selection of resistance mutations in CBMCs:

Phytohemagglutinin (PHA)-stimulated CBMCs were infected with viruses (multiplicity of infection [MOI] of 0.1) for 2 hours, incubated at 37ºC, and subsequently washed with RPMI 1640 medIUM (Invitrogen) supplemented with 10% fetal bovine serum and seeded into a 24-well plate at a density of 2.5 x 10^6 cells per well (14). Selection for resistance in CBMCs was performed using increasing concentrations of drugs (ETR and EFV) at starting concentrations below the 50% effective concentration (EC50) of the drugs (34). As controls, all viruses were simultaneously passaged without drugs. RT assays were performed weekly as described to monitor viral replication (26, 35). Based on the ratio of RT value in culture fluids of control wells/wells with drug at the previous round of replication, drug concentrations were increased at subsequent passages. Selection at a particular drug concentration was considered to be complete when repeated passage revealed that RT levels in culture fluids had peaked at the same time as that of a control well that did not contain drugs. Virus-containing culture fluids were harvested and kept at -80ºC for subsequent genotypic analysis at the same time that drug concentrations were increased. Selections for resistance were performed over 25 to 30 weeks.

Nucleic acid extraction, amplification and sequencing analysis:

Viral RNA was extracted from culture supernatants using the Qiagen QIAamp viral extraction kit (Mississauga, Ontario, Canada). Viral RNA amplification was performed by reverse transcription-PCR and nested PCR using a previously published protocol (Virco BVBA, Mechelen, Belgium). The resulting PCR-amplified DNA fragment was

purified by using a QIAquick PCR purification kit (Mississauga, Ontario, Canada), as specified by the manufacturer. The presence of the 1.5kb PR and RT PCR product was confirmed by running 5 μL of each product on a 1% agarose gel with sybersafe (invitrogen).

Genotyping was performed by a published protocol (Virco, BVBA, Mechelen, Belgium), based on sequencing of a 1,200-bp fragment of the HIV-1 pol gene encompassing up to 400 amino acids in the RT region, using Virco primers with a BigDye Terminator sequencing kit (Version 1.1; Applied Biosystems, Forter City, CA) and automated sequencer (ABI Prism 3130 genetic analyzer; Applied Biosystems). Data were analyzed using SeqScape software version 2.5.

Site-directed mutagenesis and virus production:

The E138K mutation was introduced into pNL4-3 and the p97GH-AG2 plasmid by site-directed mutagenesis (SDM) using a QuikChange II XL site-directed mutagenesis kit (Stratagene, LaJolla, CA). For SDM of E138K in pNL4-3, the forward primer 5'-GCATTTACCATACCTAGTATAAACAATAAGACACCAGGGATTA-3' and reverse primer 5'-TAATCCCTGGTGTCTTATTGTTTATACTAGGTATGGTAAATGC-3' were used, while for E138K in p97GH-AG2 the forward primer was 5'-GCATTCACTATACCTAGTGTAAACAATAAGACACCAGGGATT-3' and the reverse primer was 5'-AATCCCTGGTGTCTTATTGTTTACACTAGGTATAGTGAATGC-3'. The underlined codons denote sites at which single nucleotide substitutions were introduced into the

plasmids. This was confirmed by sequencing and DNA was ultimately transformed into DH5α cells (Invitrogen) for high-yield of plasmid. Viruses were produced by transfection of 16μg of pNL4-3 wt (pNL4-3wt), pNL4-3E138K, p97GH-AG2 wt (AGwt) and AGE138K plasmids into HEK 293T cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. Two days after transfection, supernatants of transfected cells were clarified by centrifugation at 1500 rpm for 5 min to remove cellular debris, filtered through a 0.45μm-pore-size filter, and stored in aliquots at -80°C. Virus production was confirmed by measurement of RT activity and p24 production.

Phenotypic drug susceptibility:

Drug susceptibility to NNRTIs (i.e., ETR, EFV and NVP) was measured in cell-culture based phenotypic assays as previously described (26, 40). In brief, CBMCs were infected for 2 hours with either wt virus, drug selected variants, or recombinant viruses, washed to remove unbound virus; and plated into 96 well plates containing drugs or not. After 3 or 4 days of incubation at 37ºC, the cells were fed with fresh media containing appropriate drug dilutions, and RT assays were performed at day 7. The data were analyzed using Prism software (version 5.0; GraphPad, Inc.) to determine the EC50 for each drug tested. Resistance to a given drug was determined based on the published lower clinical cutoff (CCO), which determines the cutoff fold change value for the sensitivity of NNRTIs (46, 48). These values are 3.0 for ETR, 3.4 for EFV and 5.5 for NVP (46, 48).

Determination of relative replication capacity in TZM-bl cells:

The replicative capacities of competent clonal wt and E138K-containing HIV-1 were evaluated in a noncompetitive infectivity assay using TZM-bl cells (51). Twenty thousand cells per well were added into a 96-well culture plate in 100µl of Dulbecco modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin, and 1% L-glutamine (Invitrogen). Viral stocks for both wild-type and mutant viruses were normalized by p24 and recombinant viruses from AG and pNL4-3 plasmids were serially diluted 2 and 4-fold respectively from viral stock suspensions. After 4 hours, 50µl of DMEM was removed from the wells and replaced by 50µl of virus dilution; a control well did not contain virus. Virus and cells were co-cultured for 48 hours, after which 100µl of Bright-GloTM reagent was added and luciferase activity measured in a luminometer as described (Promega). The viral replication level was expressed as a percentage of relative light units (RLU) with reference to wild-type virus for each virus studied.

Viral Growth Kinetics in CBMCs:

PHA-stimulated CBMCs were infected with viruses at an MOI of 0.1 as described above for selection of resistance. Culture fluids were collected at various times to determine levels of RT activity.

Statistical Analysis:

A two-way analysis of variance (ANOVA) was performed on group means using Prism software (version 5.0; GraphPad, Inc.). A P value <0.05 was considered to represent a significant difference.

Results

Baseline genotyping of clinical isolates.

The first 240 amino acids in RT of all the isolates (BK132, 5326, 5331, 6383, 6399, Mole 03, BG 05 and 10680) were sequenced at baseline and compared with the HIV-1 subtype B reference strain HXB2 (Fig. 1). Amino acid changes were identified in 37 of 240 positions in RT and were more frequent in non subtype-B than B isolates. Only one of these substitutions, i.e. V90I, present at baseline in the 6383 isolate, is known to be implicated in HIV-1 resistance to NNRTIs with a weighted factor of 1.0 for ETR resistance (48).

Time to development of resistance to ETR

ETR-resistance was selected in culture using CBMCs as described above (Table 1). Despite attainment of high ETR concentrations at weeks 11-13, only the BK132 isolate selected the E138E/K mutation, consistent with previous results on the difficulty of selecting resistance to this compound (47). Subsequently, all isolates showed the

accumulation of other mutations at week 18-20 (V90I, K101Q, E138K, V179D/E/F, Y181C, V189I, G190E, H221Y, M230L). E138K emerged as the first mutation in all cases except for isolate 5331, in which it was detected between weeks 25 and 30. All selected mutations were maintained in culture under drug pressure as other mutations accumulated. A strong association was observed between the presence of E138K and Y181C and between Y181C and substitutions at position 179 for all isolates. Isolate 6399 selected for E138K alone. No differences in patterns of mutations for ETR were observed between subtype B and non-B viruses. Novel amino acid substitutions not included in lists of published RAMs for ETR (48) were K101Q, E138K, V179E, V189I, G190E and H221Y.

Time to development of resistance to EFV

As expected, all isolates had selected for mutations K103N/Q, V106I/M, V179D/V, Y188C/Y, and/or M230L/M that contribute to high level EFV resistance by weeks 11-13 (Table 2). Additional mutations (L100I, V108I/V, K101E/K, N348N/T) were observed at subsequent passages. This demonstrates the low genetic barrier of EFV. V106I and V106M were selected in subtypes B and C, respectively, due to a well-described polymorphism at this position (6). Other mutations selected by EFV included; L100I, K103N/Q, V108V/I, V179D/V, Y188C/L, G190A, H221Y, and N348I.

Resistance profile of ETR-selected variants.

Table 3 shows the phenotypic susceptibility of viruses containing ETR RAMs. At baseline, mean EC50s for ETR ranged between 1 to 4.5 nM while those for EFV ranged between 1.2 - 3.5 nM. Isolate 6383 that harbored V90I at baseline had the highest EC50 value of 4.5 nM for ETR. Isolate 6399 that selected for E138K alone after 25-30 weeks in culture displayed low-level resistance to ETR, EFV and NVP, with fold resistance of 5.1, 3.7 and 6.7 respectively. Isolate BK132 (subtype B) variants selected at weeks 25 to 30 possessed E138K, G190E, and H221H/Y and displayed moderate and low-level resistance to ETR and EFV, i.e., 8.7- and 3.8-fold, respectively (Table 3). ETR-resistant variants selected by three other non-B isolates at weeks 25-30 contained mutations as follows: 6383 (V90I, E138K, V179E Y181C, M230L), Mole 03 (V90I, E138K, V179D, Y181Y/C) and BG 05 (E138K, V179F, Y181C, M230L). These viruses all displayed very high level resistance to ETR and NVP (> 95-fold) and low-moderate resistance (< 11-fold) to EFV. The presence of Y181C in combination with other RAMs may have contributed to high-level ETR resistance in these situations. As a control, we used the isolate BG05 (E138K, V179F, Y181C, M230L) and the protease inhibitor LPV. The EC50 values of BG05 wt and the BG05 selected variant (E138K, V179F, Y181C, M230L) were 5.5 ± 2.1 and 6.0 ± 2.0 nM, respectively (fold change [FC] =1.1).

Impact of E138K in the pNL4-3**and AG plasmids**

We introduced E138K by site-directed mutagenesis into the pNL4-3 and AG plasmids to determine its impact on resistance to ETR, EFV and NVP in CBMCs. The EC50s of

pNL4-3wt and pNL4-3E138K were 1.6 ± 0.25 and 6.2 ± 1.2 for ETR [fold change (FC) =3.8], 1.73 ± 0.25 and 4.8 ± 0.69 for EFV (FC= 2.7) and 26 ± 6 and 38 ± 10 for NVP (FC=1.4) (nM), respectively. Similarly, the EC50s of AGwt and AGE138K were 1.3 ± 0.15 and 5.1 ± 0.17 for ETR (FC=3.9), 1.46 ± 0.15 and 3.2 ± 0.42 for EFV (FC=2.2), and 40.0 ± 7 and 75.0 ± 2 for NVP (FC=1.8) (nM), respectively. Decreases in susceptibility were modest for ETR, i.e., 3.8 and 3.9-fold for pNL4-3E138K and AGE138K, respectively (Fig 2). EFV and NVP both retained relative sensitivity to the pNL4-3E138K and AGE138K viruses containing E138K, i.e., FCs of 2.7 and 2.2 for EFV and 1.4 and 1.8 for NVP, respectively. The EC50s of a control drug, LPV, for pNL4-3wt and pNL4-3E138K were 9.5 ± 0.7 and 10.0 ± 0.02 (FC=1.05) (nM).

Viral replication capacity.

We also studied the relative replication capacity of wt and viruses carrying the E138K mutation by infecting TZM-bl cells with serially diluted viral stocks normalized for p24 production. Infectiousness of the wt and E138K viruses was determined by measuring luciferase activity at 48 hour postinfection. At a p24 input of 10,000 pg/ml, the relative replication of E138K compared to wt virus was diminished after 48 hr by 2-and 3-fold for pNL4-3E138K and AGE138K, respectively ($p < 0.01$ and $p < 0.001$) (Fig 3A and 3B). In a multiple round infection in CBMCs, viral growth experiments in the absence of drug confirmed that the E138K mutation conferred a 2-fold drop in replication rates for pNL4-3E138K compared to pNL4-3wt by both 6 and 8 days after infection (Fig 3C) ($p < 0.001$).

Discussion

Although ETR is currently approved for NNRTI-experienced patients, a number of studies have suggested that its use in patients with NNRTI mutations at baseline might lead to poor virological response (VR) (22, 29, 30, 37). In most of these studies, the presence in the background regimen of newer drugs such as enfuvirtide, darunavir or raltegravir was positively associated with VR while previous exposure to NVP and the presence of baseline mutations such as E138A, V179I and Y181C were negatively associated with VR. We provide here a comprehensive in vitro analysis of the resistance pattern of ETR in wild-type clinical isolates of subtype B, subtype C and CRF02_AG grown over 25-30 weeks in CBMCs in increasing concentrations of ETR compared with EFV. Our data show that ETR RAMs emerged after 18 weeks of drug pressure except for one isolate (BK132) that selected for E138E/K at weeks 11-13. The E138K mutation was selected in all isolates and was almost always the first mutation to emerge.

Although E138K has been reported in a few cases of ETR treatment failure (44, 49), its clinical significance has not yet been elucidated. Site-directed mutagenesis of E138K revealed low-level resistance to ETR in both pNL4-3 and AG plasmids, with a fold-change slightly above the clinical cutoff (CCO). Others have reported a median ETR FC of 2.6-fold for E138K with first and third-quartile values of 2.0 and 4.1-fold respectively, data that are consistent with site-directed mutagenesis results reported here (4). Although E138K cannot on its own exclude ETR as a potentially useful drug, its presence might

facilitate the accumulation of other resistance mutations or may result in an increased FC for resistance for ETR. In an analysis of the DUET studies, two different groups showed that mutations at position 138 were among the most frequent with E138K emerging in three patients (44, 49). Furthermore, all patients with E138 mutations demonstrated an increased FC for ETR over the upper CCO for resistance for ETR (>13), documenting the role of mutations at position 138 in ETR resistance. Of course, the emergence of resistance in the treatment-experienced DUET patients may not be comparable to in vitro selections beginning with wild-type viruses, especially because several of the NRTI mutations that patients possessed in the DUET studies are known to hyper-sensitize to ETR. Clinical studies with ETR in NNRTI-naïve patients will be required to validate the present results.

In contrast, a FC for EFV below the CCO was observed with the site-directed mutants of E138K. This is consistent with the fact that E138K is not selected in vitro by EFV and was not observed in EFV-treated patients. One study reported the emergence of E138K after in vitro passage of viruses containing amino acid substitutions at position 135 at baseline (15). These authors demonstrated a FC for EFV of 2 and 7 for E138K and I135T/E138K, respectively.

The ETR RAMs observed here include V90I, K101Q, E138K, V179D/E/F, Y181C, V189I, G190E, H221Y, and M230L, a pattern similar to that observed by others who also observed a high frequency of L100I if viruses contained some NNRTI mutations at

baseline (47). These authors also selected E138G if they began with a virus containing the K103N substitution.

We did not observe any difference in the pathway of ETR resistance in B and non-B subtypes. The Y181C mutation was associated with either E138K or V179D/E/F. Both Y181C and V179F were also observed to co-emerge in the DUET studies (44, 48), a finding that is possibly due to the fact that the combination of either E138K and Y181C or V179D/E/F and Y181C improve viral replication capacity compared to the presence of only a single mutation. In a recent analysis of genotypic and phenotypic changes of rebounders in the DUET studies, it was shown that V179 (n=35), E138 (n=17), Y181 (n=15) and K101 (n=13)(44) were the positions at which new mutations were most frequently observed. In contrast, EFV selected for classic mutations including V106I and V106M in subtypes B and C, respectively, at weeks 11-13, as previously described (6). The specific selection in this study of G190A and G190E by EFV and ETR, respectively, confirms the non-association of G190A with ETR (50). However, G190E has been shown to be selected by both ETR and another novel NNRTI, rilpivirine (RIL) (4, 47).

Our phenotypic data show that accumulation of several ETR RAMs especially Y181C and M230L resulted in high level ETR resistance. Viruses possessing high-level resistance to ETR, also displayed moderate and high-level resistance to EFV and NVP, respectively. This confirms cross-resistance between ETR and NVP and is also consistent with studies showing that the prior use of NVP is associated with resistance to ETR (23,

31, 42). The low and moderate level resistance to ETR displayed by isolates 6399 (E138K) and BK132 (E138K, G190E, H221H/Y) are probably due to the low weight of these mutations on resistance to ETR.

In general, NNRTI mutations that emerge early in treatment-experienced patients e.g. K103N, do not impair viral replication capacity to significant extend (10). We found that E138K in both pNL4-3and AG exerted only modest impact on replication capacity, a finding that helps to explain the frequency of this mutation in our selection as well as the fact that E138K was usually the first mutation to emerge. In contrast, M230L impairs viral replication capacity by ≈8-fold (51). This is consistent with the selection of E138K in all 8 isolates while M230L was selected in only 2 of 8 isolates in this study.

E138K has previously been selected in vitro by other broad-spectrum NNRTIs (4, 8, 13, 17, 20). However, it has low prevalence (0.5%) in clinical samples (43) and only little impact on susceptibility to narrow-spectrum NNRTIs. E138K (GAG→AAG) is found in the p51 subunit of RT as part of the NNRTI binding pocket. The K101 side-chain can normally form a salt bridge with E138, but the presence of E138K causes residues to move away from the NNRTI pocket due to the juxtaposition of like charges that result from the mutation (38), potentially preventing the specific binding of drug. In addition, interactions of Y318 and E138 in the presence of ETR have been described as unusual for NNRTI-RT complexes (45). The emergence of mutations at residues 138 and 318 could disrupt drug binding ability (4, 47). The identification of a new E138R mutation selected by RIL reinforces the importance of this position (4). E138K and other mutations (K101Q, V179E, V189I, G190E and H221Y) selected in our study are not yet included

among the list of ETR RAMs. Although G190E was previously selected by ETR and RIL (4, 47), its role, alone or in combination with other mutations, remains unknown. Both V179E and H221Y have also been previously shown to confer a degree of resistance to ETR (28, 31, 32). Additional studies in our laboratory will evaluate whether the mutations K101Q, V179E, V189I, G190E and H221Y either alone or in combination with E138K might affect susceptibility of ETR and other NNRTIs.

A recent study analyzed the weights of 17 ETR RAMs, based on the DUET 1 and 2 studies (48). Others have also assigned weighted scores for ETR mutations based on different work (32); the assigned scores were 2, 3, 1, and 1 for E138K, V179E, G190E, and H221Y, respectively. As new results of ETR resistance are released, there will be a need to improve algorithms to also take into account the partial resistance that is associated with certain mutations and mutational patterns.

Our in vitro results confirm that ETR is a potent antiviral drug with a higher genetic barrier for resistance than narrow spectrum NNRTIs. Although the data also suggest that virological response to ETR in NNRTI-naïve patients might be superior than in experienced individuals, clinical studies with patient samples will be needed to provide further information on this topic, as well as on the clinical significance of the E138K substitution in cases of ETR treatment failure.

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Table 1. Evolution of viral variants selected with ETR in tissue culture.

Variant(s) in:								
Week	**Subtype B**			**CRF02_A/G**		**Subtype C**		
	BK132	5326	5331	6383	6399	Mole03	BG05	10680
11-13	E138E/K	ND[a]	ND	V90I[b]	Wt	Wt	Wt	ND
18-20	E138K G190G/E H221H/Y	E138E/K V179D Y181C	K101Q V189I	V90I E138E/K V179V/E Y181C M230M/L	E138E/K	V90I E138K V179D Y181Y/C	E138K Y181C M230L	E138K K238K/N
25-30	E138K G190E H221H/Y	E138K V179D Y181C	V90I/V K101Q E138K V189I	V90I E138K V179E Y181C M230L	E138K	V90I E138K V179D Y181Y/C	E138K V179F Y181C M230L	E138K G190E/G K238K/N

[a] ND, not determined.

[b] V90I is a baseline polymorphism in isolate 6383.

Table 2. Evolution of viral variants selected with EFV in tissue culture.

Variant(s) in:								
Week	**Subtype B**			**CRF02_A/G**		**Subtype C**		
	BK132	5326	5331	6383	6399	Mole03	BG05	10680
11-13	K103Q V106I Y188C	ND[a]	ND	V90I[b] K103N	K103N	V106M Y188C/Y M230L/M	V106M V179D/V Y188C	Y188C
18-20	K103Q V106I Y188C	K101E/K V108I/V Y188C/Y	L100I N348I/N	V90I L100I K103N	N348N/T K103N	V106M Y188C	V106M V179D/V Y188C	V106M Y188C
25-30	K103Q V106I Y188C	L100I Y188L	L100I V108V/I H221Y N348I	V90I L100I K103N G190A	K103N	V106M Y188C	V106M V179D/V Y188C	V106M Y188C

[a] ND, not determined.

[b] V90I is a baseline polymorphism in isolate 6383.

Table 3: *In vitro* antiviral activity of NNRTIs against ETR-selected variants.

Isolate[a]	Subtype	NNRTI Mutation	Mean EC_{50} ± SD[b] (fold Change)		
			ETR (nM)	EFV (nM)	NVP (μM)
BK132	B	Wt	3.7 ± 0.9	3.2 ± 0.4	0.04 ± 0.02
BK132*	B	E138K, G190E, H221H/Y	32 ± 3 **(8.7)**	12 ± 4 **(3.8)**	ND[c]
6383	A/G	Wt	4.5 ± 0.7	3.5 ± 0.7	0.041 ± 0.012
6383*	A/G	V90I, E138K, V179E, Y181C, M230L.	430 ± 20 **(95.6)**	40 ± 10 **(11.4)**	>5.0 **(>121.9)**
6399	A/G	Wt	1 ± 0.2	1.2 ± 0.4	0.031 ± 0.001
6399*	A/G	E138K	5.1 ± 0.2 **(5.1)**	4.4 ± 0.1 **(3.7)**	0.21 ± 0.01 **(6.7)**
Mole 03	C	Wt	1.3 ± 0.4	1.5 ± 0.1	0.038 ± 0.01
Mole 03*	C	V90I, E138K, V179D, Y181Y/C.	140 ± 20 **(107.7)**	4.7 ± 2 **(3.1)**	>10.0 **(>263)**
BG 05	C	wt	3.5 ± 0.1	1.5 ± 0.2	0.012 ± 0.002
BG 05*	C	E138K, V179F, Y181C, M230L	490 ± 40 **(140)**	16 ± 1 **(10.7)**	>5.0 **(>416)**

[a] Asterisks (*) indicates an ETR-selected variant at weeks 25 to 30. As stated in the text, isolate 6383 contained the V90I mutation at baseline.

[b] The values represent means of two or three independent experiments, each performed in duplicate. Drug susceptibility was expressed as fold-change in EC_{50}, determined by calculating the ratio of EC_{50}s for selected variants and wt viruses (values in parentheses).

[c] ND, not determined.

Figure 1.

Baseline polymorphisms of HIV-1, as shown in an amino acid alignment (residues 1-240) of RT for all clinical isolates used in this study. Subtype B: BK132; 5326 and 5331; CRF02_AG, 6383 and 6399; Subtype C, Mole03, BG05, and 10680. HXB2 was used as a reference sequence. All isolates were sequenced at baseline and passaged simultaneously in the presence or absence of drugs. The sequence of the wild-type RT at baseline was the same as that of isolates passaged without drugs. The dots represent identical positions, while letters indicates variations in amino acids relative to HXB2. Isolate 6383 at baseline harbored the NNRTI resistance mutation V90I that is known to decrease susceptibility to ETR.

```
                10         20         30         40         50         60         70         80
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HXB2       PISPIETVPV KLKPGMDGPK VKQWPLTEEK IKALVEICTE MEKEGKISKI GPENPYNTPV FAIKKKDSTK WRKLVDFREL
BK132 WT   .......... .......... .......... .......... .......... .......... .......... ..........
5326 WT    .......... .......... .......... ........P. .......... .......... .......... ..........
5331 WT    .......... .......... .......... .......... .......... .......... .......... ..........
6383 WT    .......... .......... .......... ....T...I. .......... .........I ........G.. ..........
6399 WT    .......... .......... .......... ....T..... .........R. .........I .......... ..........
Mole03 WT  .......... .......... .......A.. ....T...E. .......T.. .......... .......... ..........
BG05 WT    .......... ...A.....N .......... ....T...E. .......T.. .......... .......... ..........
10680 WT   .....X.... .......... .......D.. ....TA..EX ......X... .....X.... .......... .........X

                90        100        110        120        130        140        150        160
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HXB2       NKRTQDFWEV QLGIPHPAGL KKKKSVTVLD VGDAYFSVPL DEDFRKYTAF TIPSINNETP GIRYQYNVLP QGWKGSPAIF
BK132 WT   .......... .......... .......... .......... .K........ ....T..... .......... ..........
5326 WT    ....X..... P...X..... .......... .......... .K........ HL..T.H... ......H... ..........
5331 WT    .....E.... .......... .......... .......... .K........ .......... .......... ..........
6383 WT    .........I .......... .......... .......... .......... ....V..... .......... ..........
6399 WT    .......... .......... .......... .......... ..S....... .......... .V........ ..........
Mole03 WT  .......... .......... .......... .......... .......... .......... .......... ..........
BG05 WT    .......... .......... .......... .......... ..G....... .......... .......... ..........
10680 WT   .....X.... .......... .....X.... ........XX ..X....... ....T...V. X...X..... X........X

               170        180        190        200        210        220        230        240
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
HXB2       QSSMTKILEP FRKQNPDIVI YQYMDDLYVG SDLEIGQHRT KIEELRQHLL RWGLTTPDKK HQKEPPFLWM GYELHPDKWT
BK132 WT   .C........ .......... .......... .......... .......... ...F...... .......... ..........
5326 WT    .......... ...R..E... .......... .........I .......... .......G.. .R........ ....P.....
5331 WT    .......... .......... .......... .......... ......X... K..F...... .......... ..........
6383 WT    .A........ ..TK..EM.. .......... .....E...A ......G... ...F...... .......... ..........
6399 WT    ....I..... Y.TK..EM.. .......... .....E...A ...Q..E... ...F...... .......... ..........
Mole03 WT  .A..I..... ..T...EM.. .......... .........A ......E... ...F...... .......... ..........
BG05 WT    .....I.... ..A...E... .......... .........A ......E... K..F...... .......... ..........
10680 WT   .......... ..A...X... .......... X......X.A ....X.N... ...X...... .......... .......X..
```

Figure 2.

Comparative phenotypes of recombinant viruses derived from pNL-4.3$_{E138K}$ and AG$_{E138K}$ and tested for susceptibilities to ETR, EFV and NVP in CBMCs. Recombinant clones containing E138K were tested for their susceptibilities to ETR, EFV and NVP in cell culture assays. The mean EC_{50}s of mutated variants were compared to those of wt pNL-4.3 or AG plasmids. The results shown are means of two or three independent experiments. Bars in the figure represent mean fold-change (FC) while error bars represent ± standard deviation (SD). Bars with dots, fold change (FC) to ETR; bars with slashes, fold change to EFV and open bars, fold change to NVP.

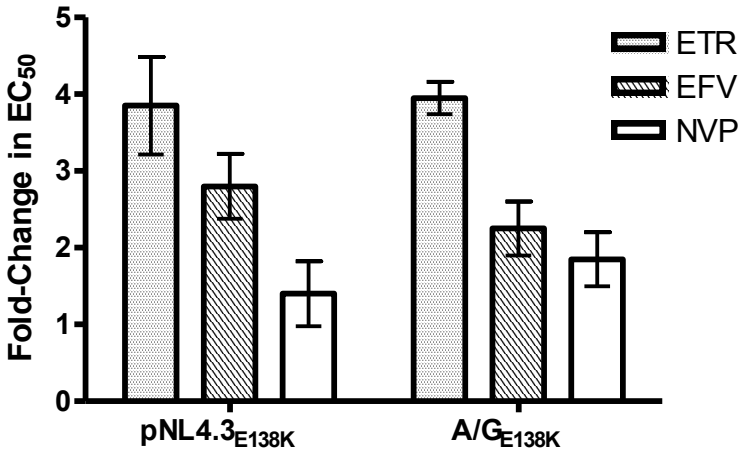
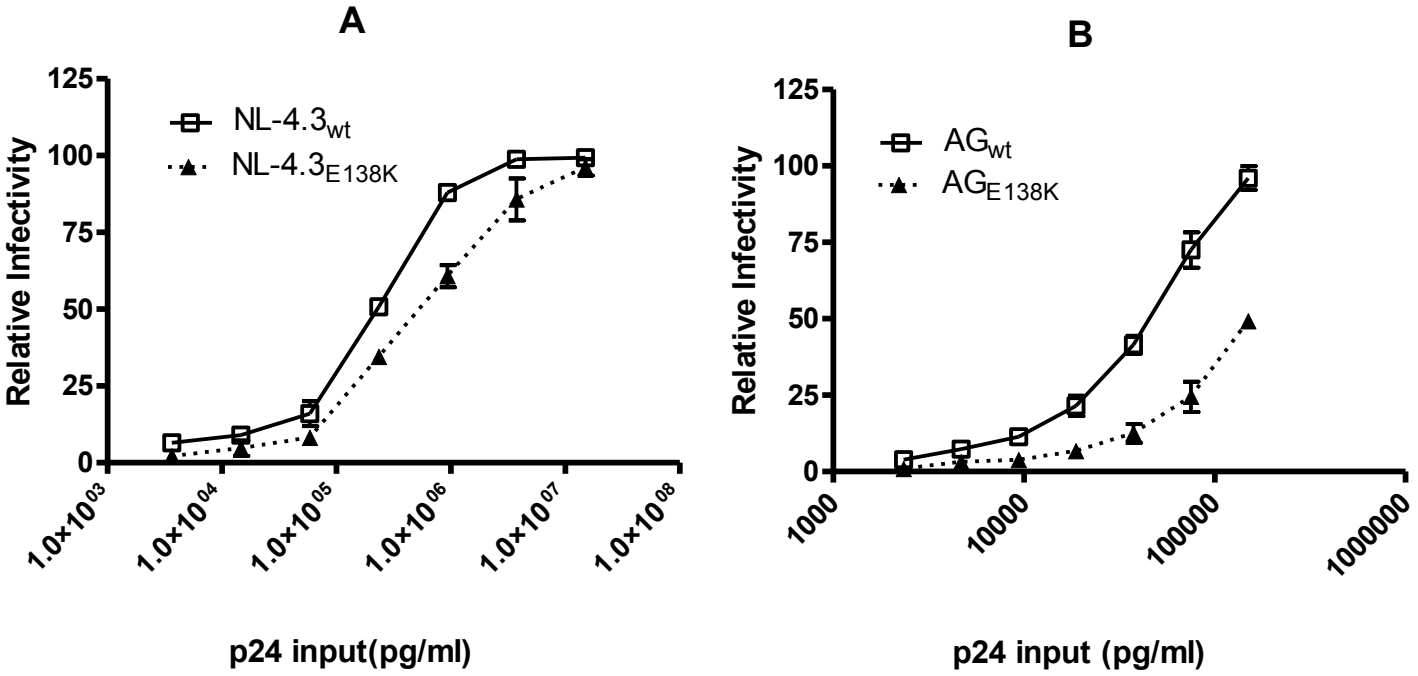
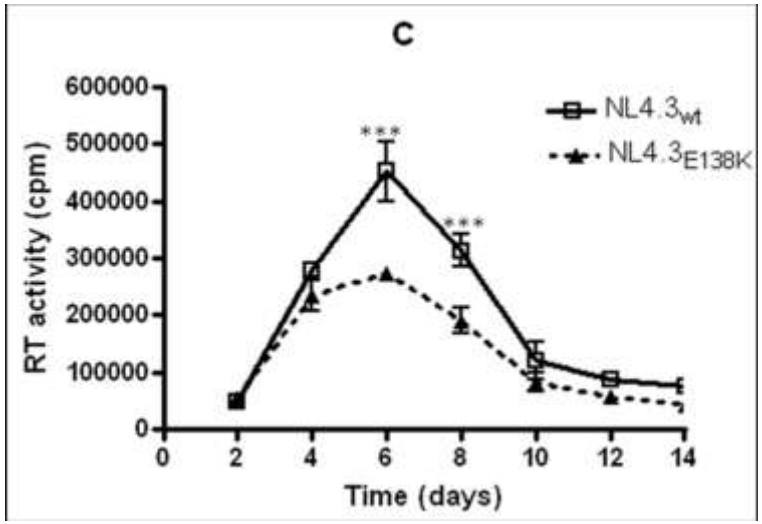

Fold-Change in EC50
5
4
3
2
1
0
ETR
EFV
NVP
pNL4.3E138K
A/GE138K

Figure 3.

Effect of E138K on viral replication capacity. Viral stocks of both wt and E138K-containing virus were normalized for p24 and used to infect TZM-bl cells. (A and B) The Luciferase activity was measured at 48 h postinfection as an indication of viral replication. The relative infectivity of wt compared to E138K-containing virus is shown on the *y* axis while the *x* axis denotes the input of p24. Statistical analysis using a two-way ANOVA shows that replication capacity was decreased by 2-and 3-fold compared to wt for pNL4-3_{E138K} and AG_{E138K}, respectively, after 48 hours. A significant difference was observed after a p24 input greater than 10,000 pg/ml. The points with asterisks (***) indicates a significant difference. CBMCs were also infected as described in Materials and Methods and viral growth was measured by determining RT activity in culture supernatants at different times (C). A significant difference was observed at the peak of infection (days 6 and 8) are indicated with asterisks (***). In the Figure, a *P* value of < 0.01 is represented by "**" while a p value of < 0.001 is represented by "***". Values are means of at least two independent experiments ± SD. Error bars represent the SD.


A
Relative Infectivity
125
100
75
50
25
0
NL-4.3wt
NL-4.3E138K
1.0×10^03
1.0×10^04
1.0×10^05
1.0×10^06
1.0×10^07
1.0×10^08
p24 input(pg/ml)
B
Relative Infectivity
125
100
75
50
25
0
AGwt
AGE138K
1000
10000
100000
1000000
p24 input (pg/ml)


C
600000
500000
400000
300000
200000
100000
0
RT activity (cpm)
0
2
4
6
8
10
12
14
Time (days)
***
***
NL4.3wt
NL4.3E138K

Chapter 3

Distinct Resistance Patterns to Etravirine and Rilpivirine in Viruses Containing NNRTI Mutations at Baseline.

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Running Title: Resistance Patterns to Etravirine and Rilpivirine

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Abstract

Objective: The current *in vitro* study examined HIV-1 drug resistance patterns following etravirine (ETR) and rilpivirine (RPV) drug pressure in viruses containing baseline NNRTI resistance mutations.

Design and Method: Several baseline mutations were introduced into pNL4-3by site-directed mutagenesis together with two subtype C clinical isolates containing baseline mutations, were passaged in increasing drug pressure of NNRTIs in cord blood mononuclear cells (CBMCs). Genotypic analysis was performed at different weeks. Phenotypic resistance for ETR, RPV and efavirenz (EFV) and the replication capacity (RC) of several mutations and combinations was tested.

Results: In wild-type (wt) viruses and viruses containing K103N alone at baseline, E138K or E138G mutations were observed following pressure with either ETR or RPV prior to the appearance of other NNRTI resistance mutations and these changes were observed regardless of viral subtype. However, subtype B viruses containingY181C generated V179I/F or A62V/A but not E138K following exposure to ETR or RPV, while subtype C viruses containing Y181C developed E138V together with Y188H and V179I under ETR pressure. The addition of mutations at position 138 to Y181C did not significantly enhance levels of resistance to ETR or RPV and the replicative capacity of viruses containing Y181C plus either E138K or E138A was similar to that of viruses containing either E138K or E138A alone.

Conclusion: These results demonstrate that each of ETR and RPV are likely to select for E138K as a major resistance mutation if no or very few other resistance mutations are present and that Y181C may be antagonistic to E138K.

Key words: etravirine, rilpivirine, cross-resistance, second generation NNRTI, replication capacity, antiviral activity.

Introduction

Antiretroviral therapy (ART) has significantly decreased HIV-associated morbidity and mortality [1]. Two classes of drugs that are used clinically are nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) [2]; the latter are noncompetitive inhibitors of HIV-1 reverse transcriptase (RT) that bind to a hydrophobic pocket in the p66 subunit near the polymerase active site, allosterically inhibiting the activity of the enzyme [2]. NNRTIs are routinely prescribed for both treatment-naïve and experienced patients [3-5]. However, non-adherence to therapy can result in the selection of drug-resistance mutations [6, 7], and all HIV-1 subtypes can develop resistance to all currently approved drugs.

Multiple studies have reported an increasing prevalence of NNRTI drug resistance in acute HIV-1 infection [8, 9], and individuals to whom at least one NNRTI resistance mutation has been transmitted are less likely to respond positively to treatment regimens that include first-generation NNRTIs such as nevirapine (NVP) and efavirenz (EFV) [10, 11]. The widespread use of single dose NVP to prevent mother-to-child transmission of HIV-1 has increased the prevalence of NNRTI resistance mutations (reviewed in [12]). In addition, cross-resistance among NNRTIs is common [13], such that the sequential use of first generation NNRTIs is excluded in treatment-experienced patients. Etravirine (ETR) is a second generation NNRTI that possesses high activity against both wild-type (wt) and drug-resistant viruses at nM concentrations and was approved for use in treatment-experienced patients with NNRTI-resistant viruses [14, 15]. ETR can bind to RT by rotating within the NNRTI binding pocket to accommodate resistance mutations and

retain activity [16] and has been shown to be effective in treatment-experienced, NNRTI-resistant patients in the DUET-1 and DUET-2 (Demonstrate Undetectable viral load in patients Experienced with ARV Therapy) studies [5, 17]. These same studies identified 20 ETR resistance-associated mutations (RAMs) that can diminish virological response: V90I, A98G, L100I, K101E/H/P, V106I, E138A/K/G/Q, V179D/F/T, Y181C/I/V, G190A/S and M230L [18, 19]. Each of these mutations has been assigned a weighted genotypic score and three or more of these mutations are required to significantly reduce virologic response to ETR [18].

Rilpivirine (RPV) is another second generation NNRTI whose mode of binding within the NNRTI binding pocket is similar to that of ETR [20]. In recent phase III studies (ECHO and THRIVE), HIV-1 drug-naïve patients who failed combination therapy containing RPV and emtricitabine (FTC) or lamivudine (3TC) were frequently observed to have the resistance mutations E138K/M184I and E138K/M184V [21, 22]. The same mutations are also selected in culture by RPV and ETR, resulting in high-level cross-resistance [23, 24].

The most prevalent NNRTI mutations for EFV and NVP include K103N, Y181C and G190A [25], of which K103N does not compromise susceptibility to either ETR or RPV [14, 23]. However, both Y181C and G190A can affect responsiveness to ETR [26] while only Y181C decreases susceptibility to RPV [23].

In contrast to the foregoing, the application of ETR pressure against wt viruses commonly selects for the E138K mutation that has also been identified in patients who have failed first-line regimens that have included RPV. The current study was performed to determine the basis for the preferential selection of E138K by ETR in wt viruses compared to the selection of other NNRTI mutations in the DUET clinical studies. In addition, we wanted to investigate whether HIV-1 that already contained E138K or M184I/V or E138K plus M184I/V could develop additional resistance mutations in the presence of ETR or RPV. Another NNRTI of the same family and structural similarity Dapivirine (TMC120) was also included in the study. TMC120 is licensed for microbicide development.

Materials and Methods

Viral isolates, Cells, drugs, and plasmids

The subtype C viral isolates 10680 and 4743 were obtained with informed consent from HIV-1 infected patients at our clinics in Montreal, Canada. Cord blood mononuclear cells (CBMCs) were obtained through the Department of Obstetrics, Jewish General Hospital, Montreal, Canada. The HEK293T cell line was obtained from the American Type Culture Collection. The infectious molecular clone pNL4-3and TZM-bl cells were obtained through the NIH AIDS Research and Reference Reagent Program, courtesy of Malcolm Martin and John C. Kappes, respectively. ETR and Dapivirine (TMC120) were gifts of Janssen Inc., while RPV and EFV were obtained from the NIH AIDS Research and

Reference Reagent Program. Tenofovir (TFV), also used in selections, was a gift of Gilead Sciences, Inc, Foster city, California.

Site-directed mutagenesis and virus production:

Amino acid changes in RT were introduced into the pNL4-3 plasmid by site-directed mutagenesis (SDM) using a QuikChange II XL site-directed mutagenesis kit (Stratagene, LaJolla, CA). Mutations were introduced individually by PCR in the case of double mutants. The list of mutations and primers employed are shown in a table (supplemental digital content 1). After SDM, the presence of mutations was confirmed by sequencing, and DNA was ultimately transformed into DH5α cells (Invitrogen) for high-yield of plasmid. Viruses were produced by transfection of 16μg of wt or mutant plasmid into HEK 293T cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. Two days after transfection, supernatants of transfected cells were clarified by centrifugation at 1500 rpm for 5 min to remove cellular debris, filtered through a 0.45μm-pore-size filter, and stored in aliquots at -80°C. Virus production was confirmed by measurement of RT activity and p24 production.

Selection of resistance mutations in CBMCs:

PHA-stimulated CBMCs were infected with viruses (multiplicity of infection of 0.1) for 2 hours, incubated at 37ºC, and subsequently washed with RPMI 1640 media (Invitrogen), supplemented with 10% fetal bovine serum, and seeded into a 24-well plate at a density

of 2.5 x 10^6 cells per well [27]. Selection for resistance in CBMCs was performed using increasing concentrations of drugs (ETR and EFV) at concentrations starting below the 50% effective concentration (EC_{50}) of the drugs [28]. As controls, all viruses were simultaneously passaged in the absence of drugs. RT assays were performed weekly as described to monitor viral replication [29, 30]. Based on the ratio of RT values in culture fluids of control wells/wells with drug at the previous round of replication, drug concentrations were increased at subsequent passages. Selection at a particular drug concentration was considered to be complete when repeated passage revealed that RT levels in culture fluids had peaked at the same time as that of a control well that did not contain drugs. Virus-containing culture fluids were harvested and kept at -80ºC for subsequent genotypic analysis at the same time that drug concentrations were increased. Selections for resistance were performed over 25-30 weeks.

Nucleic acid extraction, amplification and sequencing analysis:

Viral RNA was extracted from culture supernatants using the Qiagen QIAamp viral extraction kit (Mississauga, Ontario, Canada). Viral RNA amplification was performed by RT-PCR and nested PCR using a previously published protocol (Virco BVBA, Mechelen, Belgium). The resulting PCR-amplified DNA fragment was purified using the QIAquick PCR purification kit (Mississauga, Ontario, Canada), as specified by the manufacturer. The presence of the 1.5kb protease (PR) and RT PCR products was confirmed by running 5 μL of each product on a 1% agarose gel with Sybersafe (Invitrogen).

Genotyping was performed by a published protocol (Virco, BVBA, Mechelen, Belgium), based on sequencing of a 1,200-bp fragment of the HIV-1 *pol* gene encompassing up to 400 amino acids in the RT region, using Virco primers with a BigDye Terminator sequencing kit (Version 1.1; Applied Biosystems, Forter City, CA) and automated sequencer (ABI Prism 3130 genetic analyzer; Applied Biosystems). Data were analyzed using SeqScape software version 2.5.

Phenotypic drug susceptibility in TZM-bl. cells

Drug susceptibility to NNRTIs (ETR, RPV, TMC120 and EFV) was measured in a single cycle cell-culture based phenotypic assay in TZM-bl cells. In brief, 20.000 cells per well were added into a 96-well culture plate in 50µl of Dulbecco modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin, and 1% L-glutamine (Invitrogen). Cells were infected with either wt or mutant HIV and serially diluted drugs were added. Standardization of virus infections was determined by adding virus at a p24 concentration that would yield ~160.000 ±20.000 relative light units (RFU) as detected by luminescence. All cultures were maintained at 37°C under 5% CO2 for 48 h. After 48 h, 100µl of Bright-Glo™ reagent (Promega) was added to 100µl of infected TZM-bl cells. Drug efficacy was determined by quantifying luciferase activity as a measure of viral replication. RLU were detected with a 1450 MicroBeta TriLux microplate scintillation and luminescence counter (Perkin-Elmer). The 50% effective concentrations (EC50s) were determined by nonlinear regression with GraphPad Prism (version 5.01) software. Resistance to a given drug was

determined based on the previously published lower clinical cutoff (CCO) of a drug which determines the cutoff fold change value for drug sensitivity. These values are 3.0 for ETR, 3.4 for EFV and 2.0 for RPV [18, 31, 32].

Determination of relative replication capacity in TZM-bl cells.

The replicative capacities of competent clonal wt and mutant HIV-1 were evaluated in a noncompetitive infectivity assay using TZM-bl cells [33]. Twenty thousand cells per well were added into a 96-well culture plate in 100µl of Dulbecco modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin, and 1% L-glutamine (Invitrogen). Viral stocks for both wt and mutant viruses were normalized by p24 and recombinant viruses were serially diluted 2-fold from viral stock suspensions. After 4 hours, 50µl of DMEM were removed from the wells and replaced by 50µl of virus dilution; a control well did not contain virus. Virus and cells were co-cultured for 48 hours, after which 100µl of Bright-Glo^{TM} reagent was added and luciferase activity was measured in a luminometer as described (Promega). Viral replication levels were expressed as a percentage of RLU with reference to wt virus for each viral variant studied.

Results

Mutations selected in the presence of RPV drug pressure

To determine the impact of baseline NNRTI mutations on the pattern of RPV resistance the following NNRTI mutations and combinations were introduced into wt pNL4-3 by site-directed mutagenesis: K103N, G190A, Y181C, E138K, and M184I/V (Table 1). All viral clones were passaged in the absence of drugs and genotyped at the end of each passage. Genotyping results revealed that all baseline mutations were maintained at the end of the selections, except for viral clones containing the mutations M184I, M184V and E138K/M184I, in which case the M184I/V mutations reverted to wt.

In the wt virus, a combination of E138K and L100I was selected after 8 weeks in the presence of RPV (Fig 1). Continuous RPV drug pressure resulted in the emergence of K101E as a predominant viral variant while viruses containing the E138K and L100I mutations, previously selected in the dominant viral population, reverted to wt (Fig 1). At week 21, the E138K and L100I mutations re-emerged and predominated at week 26, while K101E reverted to wt. In the virus containing Y181C at baseline, only the NRTI multidrug resistant mutation A62A/V was selected, even when a RPV concentration of 300 nM was attained. In viruses containing both K103N and G190A at baseline, E138K was the first mutation to emerge at week 12 while L100I was identified in the virus containing E138K at baseline (Table 1). In the viral clones containing E138K/M184I and M184V, additional mutations did not develop. In contrast, clonal viruses containing

E138K/M184V or M184I at baseline yielded H221H/Y or M41L and I135M, respectively, under RPV pressure.

Mutations selected in the presence of ETR and ETR/TFV drug pressure

The viral clones containing the mutations K103N, E138K, Y181C, M184I/V, G190A, K103N/Y181C, K103N/G190A, E138K/M184I and E138K/M184V as well as two subtype C clinical isolates (isolates 4743 and 10680) containing baseline NNRTI mutations were exposed to ETR or combination of ETR plus TFV for 20-30 weeks (Table 2). Beginning with suboptimal concentrations of ETR for 20-30 weeks in selection experiments, we observed distinct mutational pathways. In the subtype B (pNL4-3) wt clone and the subtype C wt clinical isolate, E138K/G was observed in all selections followed by other ETR RAMs. In contrast, subtype B viruses containing either K103N or G190A at baseline generated E138K/L100I and V106I, respectively, while subtype C viruses containing G190A developed E138K (Table 2). The clones containing either M184I or M184V yielded E138K while the E138K/M184I and E138K/M184V clones developed the V189I and V118I mutations respectively. Subtype B viruses containing Y181C at baseline commonly developed V179I/F but not E138 substitutions.

The subtype C clinical isolate containing Y181C as a baseline mutation developed E138V under ETR pressure together with Y188H and V179I; K238K/N and E399G were also frequently observed. When the combination of K103N/G190A was present in pNL4-

3 (subtype B) at baseline, no mutation was selected, probably because the virus containing these mutations was not fit and was difficult to replicate in the presence of suboptimal drug concentration. Subtype C clinical isolates subjected to ETR/TFV drug pressure developed E138 mutations but not the TFV-associated K65R mutation (Table 2).

Mutations selected in the presence of EFV and EFV/TFV drug pressure

In the case of the subtype B (pNL4-3) wt and Y181C-containing viruses, the mutations selected under EFV pressure were K101E/Q, K103N or Y188H. Viruses containing K103N generated the minor NNRTI mutations Y318F and N348I/N (see table, supplemental digital content 2). The subtype B pNL4-3virus containing G190A at baseline mostly developed V106A. However, viruses containing the combinations of K103N/Y181C and K103N/G190A did not select additional substitutions. In the presence of M184I and M184V at baseline, amino acid substitutions at position 188 were observed whereas viruses containing E138K/M184I or E138K/M184V developed G190A or E399G, respectively, under EFV drug pressure. In the clone containing E138K alone at baseline, the additional K103N and L100I mutations were observed (see table, supplemental digital content 2).

Subtype C viruses that were wt or that contained G190A or Y181C at baseline selected V106M, followed by the accumulation of several additional mutations (see table,

supplemental digital content 2). The combination of EFV/TFV drug pressure did not select for K65R.

Mutations selected in the presence of dapivirine (TMC120) drug pressure

We also wanted to determine whether any additional mutations would develop in the presence of baseline mutations E138K, M184I, M184V, E138K/M184I and E138K/M184V under dapivirine (TMC120) drug pressure. The latter is an NNRTI that is structurally related to RPV and ETR and that is now undergoing evaluation as an anti-HIV microbicide. After 20 weeks wt viruses and viruses containing M184I and M184V yielded E138K, while viruses containing E138K/M184I or E138K/M184V developed L100I and V108I respectively (see table, supplemental digital content 3).

***In vitro* antiviral activity of NNRTIs against mutated viruses.**

The activity of the NNRTIs ETR, RPV, TMC120 and EFV were tested against pNL4-3 clones engineered to contain E138 and Y181C mutational combinations. Table 3 shows that the E138A and E138K amino acid substitutions conferred low-level resistance to ETR, RPV and TMC120, while E138V, that was co-selected with Y181C in tissue culture, did not. Y181C alone conferred a fold-change in susceptibility of 6.0, 2.3, 5.3, and 2.5 for ETR, RPV, TMC120 and EFV, respectively. The addition of mutations at position 138 to Y181C did not significantly enhance levels of resistance to ETR, RPV or TMC120 (Table 3). All of the combinations tested remained susceptible to EFV.

Impact of mutations at position 138 alone or in combination with Y181C on viral replication capacity (RC).

Since E138K was not selected by ETR and RPV, when using virus that contained Y181C at baseline, we next wished to determine the viral replication capacity of viruses containing E138K and Y181C or E138 together with other mutations. For this purpose, TZM-bl cells were infected with serially diluted viral stocks (normalized for p24 levels and relative light units) of wt virus or viruses containing the E138K, E138A, Y181C, E138K/Y181C or E138A/Y181C mutations. The infectiousness of viral clones was determined by measuring luciferase activity at 48 h postinfection. The results show that the replication capacity of both the E138K and E138A viruses were decreased by ≈3-fold compared to wt, while that of Y181C virus was only slightly decreased by ≈ 1.5 fold. The addition of E138K or E138A to Y181C further decreased replication capacity to about the same level (≈3-fold) as that of viruses containing either E138K or E138A alone (Figure 2).

Discussion

The current *in vitro* study examined HIV resistance patterns following selection with ETR in both wt viruses as well as in viruses containing a variety of NNRTI mutations at baseline. We showed that E138K or E138G was selected by each of a subtype B wt or K103N virus, a subtype C wt clinical isolate, and a subtype C virus containing G190A at baseline. However, subtype B viruses containing Y181C at baseline selected for V179I or V179F, but not E138K. These findings are consistent with previous *in vitro* data that

showed selection of E138K by ETR in wt viruses of multiple subtypes over 18 weeks [24]. In the same study, it was shown that E138K emerged first and that Y181C, together with V179I/F, was selected subsequently due to increased ETR pressure [24]. Possibly, the use of ETR in drug-naïve patients might also select for E138K, if such patients were to experience virologic failure. Others have also observed differences in mutational patterns among viral subtypes in SUPT1 cells exposed to NNRTI pressure [34].

E138K was observed following RPV drug pressure in wt subtype B viruses and viruses containing either K103N or G190A at baseline, while subtype B viruses containing Y181C at baseline did not yield E138K. This observation is in agreement with the recent phase III trials (ECHO and THRIVE) in which RPV was shown to preferentially select for E138K in patients undergoing first-line treatment failure [21, 22]. Under RPV drug pressure, wt viruses developed a mixture E138K, K101, and E101, that was dominated by E138K at the end of the selection experiment. This could be due to the presence of quasispecies that existed in the viral population and suggests that K101E and E138K cannot exist in the same viral clone possibly due to a salt bridge that exists between these two amino acids. Viral clones containing M184I/V at baseline did not select for NNRTI mutations after exposure to RPV, as observed with ETR in a recent *in vitro* study [35]. This could be due to decreased viral fitness of both clones and difficulties in increasing RPV drug concentration.

In the DUET studies that led to the approval of ETR for use in NNRTI-experienced patients, only three subtype B patients who previously harbored K103N developed E138K and failed ETR therapy, while one patient who harbored Y181C developed E138V [19]. However, most ETR related mutations were at positions 138, 179 and 181 [19]. A different study showed that 12 of 42 ETR failures who harbored mutations at position 181 at baseline contained at least one new NNRTI mutation but not E138K [36]. In an *in vitro* selection study, using a drug of the same family (TMC120), E138K was selected in wt virus but not in viruses containing Y181C at baseline [37]. The present study, together with evidence from other studies, suggests that Y181C may be antagonistic to E138K. As shown here, the combination of Y181C and E138K may lead to a less fit virus. In our study, amino acid substitutions at position 179 were frequently observed in viruses containing Y181C at baseline. This pathway has also been observed [36, 38].

The complex formed between RPV and wt RT is stable and comparable to the complex between RPV and K103N mutant RT. In the presence of RPV and mutant RT containing Y181C, there is a loss of aromatic interactions, resulting in a displacement of RPV further into the NNRTI pocket [39]. We believe that differences in binding interactions that exist between RPV or ETR and the Y181C RT mutant maybe the basis for potential antagonistism.

Although the prevalence of ≥3 ETR RAMs among viral isolates from patients experiencing treatment failure under EFV and NVP therapy was low, ranging from 4.6% to 10%, the prevalence of single ETR RAMs was high (17.4% to 35.9%) [11, 26, 40]. These studies concluded that there is a low prevalence of ETR resistance at baseline and that patients with prior failure to EFV and NVP could potentially benefit from ETR therapy. However, these data were obtained in developed countries in which full access to potent antiretroviral drugs is the norm. Now, studies in resource-limited settings have shown a high prevalence of many NNRTI resistance mutations associated with ETR resistance among patients failing EFV or NVP, putting into doubt the potential effectiveness of ETR and RPV in such settings [41-43]. There is concern that patients, who fail RPV, because of the E138K mutation, will be unlikely to derive future benefit from ETR.

In summary, we have shown that each of ETR and RPV are likely to select for E138K as a major resistance mutation in tissue culture if no or very few other resistance mutations are present. In contrast, viruses containing an array of other common NNRTI mutations associated with resistance to EFV and NVP, such as Y181C, may be less likely to select E138K following exposure to ETR and RPV, and may develop other substitutions instead. In part, this may be due to diminished viral replicative capacity. These tissue culture observations are consistent with the clinical experience and help to explain why the resistance profile of ETR as a second-line drug is different from what it might have been if this drug were to be more commonly employed in first-line therapy.

Chapter 3 References

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Table 1: Mutations Selected by Serial Passage Experiments in CBMCs in the Presence of RPV Drug Pressure

Virus	Baseline Genotype	Week 12		Week 20		Week 26	
		RPV (nM)	Acquired Mutations	RPV (nM)	Acquired Mutations	RPV (nM)	Acquired Mutations
NL-4.3	wt	60	K101E/K, E138E/K	60	L100I/L, K101K/E, E138E/K	40	L100I, E138K
NL-4.3	E138K	60	V108I/V, E138K	70	L100I/L, E138K	100	L100I/L, E138K
NL-4.3	E138K/M184I	60	E138K	40	E138K	90	E138K
NL-4.3	M184I	20	M41L, I135M, M184I	20	M41L, I135M, M184I	20	M41L, I135M, M184I
NL-4.3	M184V	ND[a]	ND	15	none	NA	NA[b]
NL-4.3	E138K/M184V	ND	ND	70	H221H/Y	NA	NA
NL-4.3	G190A	30	E138K, G190A	50	ND	NA	NA
NL-4.3	K103N	60	K130N, E138E/K	60	L100I, K103N, E138K, E399E/G	60	K103N, L100I, E138K, E399E/G
NL-4.3	K103N/Y181C	20	K103N, Y181C	30	K103N, Y181C	30	K103N, Y181C
NL-4.3	Y181C	40	Y181C	90	ND	300	A62A/V, Y181C

Baseline mutations that persisted at time of genotypic analysis are underlined. Amino acid substitutions at position 184 in clones containing M184V, E138K/M184I and E138K/M184V reverted to wt, while the amino acid substitution in the M184I clone was retained in the presence of RPV.

[a]ND, not determined.

[b]NA, not applicable.

Table 2: Mutations Selected by ETR and ETR/TFV Drug Pressure After 20-30 Weeks.			
Virus	Baseline Genotype (Subtype)	Week 20-30	
		Drug (nM)	Acquired Mutations
NL-4.3[a]	wt (B)	ETR (250)	L100I, E138G, H221H/Y, A400A/T
NL-4.3[b]	wt (B)	ETR (200)	L100I, E138K
NL-4.3[a]	K103N (B)	ETR (250)	L100I, K103N, I135I/T, H221H/Y, N348I
NL-4.3[b]	K103N (B)	ETR (400)	V90I/V, L100I, K103N, E138E/K
NL-4.3[a]	Y181C (B)	ETR (20)	V179I, Y181C
NL-4.3[b]	Y181C (B)	ETR (500)	I135L, V179F, Y181C, E399G
NL-4.3	K103N/Y181C (B)	ETR (20)	V90I, K103N, V179I, Y181C
NL-4.3	K103N/G190A (B)	ETR (7.5)	K103N, G190A
NL-4.3	G190A (B)	ETR (10)	V90I, V106I, G190A
NL-4.3	E138K (B)	ETR (200)	E138K, V189I, A400A/T
NL-4.3	M184I (B)	ETR (90)	E138E/K, M230L/M
NL-4.3	E138K/M184I (B)	ETR (200)	E138K, V189I
NL-4.3	E138K/M184V (B)	ETR (75)	V118I, E138K, M184V
NL-4.3	M184V (B)	ETR (75)	E138K
4743	G190A (C)	ETR (250)	E138K, V189I/V, G190A, E399E/G
10680	wt (C)	ETR (500)	E138K, G190E/G, K238K/N
10680	Y181C (C)	ETR (5000)	E138V, V179I, Y181C, Y188H
10680	wt (C)	ETR (50) TFV (500)	E138K
10680	Y181C (C)	ETR (500) TFV (500)	E138E/V, Y181C, K238K/N

Genotypic analysis was done at weeks 20, 25 or 30. Baseline mutations that persisted at time of genotypic analysis are underlined. Amino acid substitutions at position 184 in the clones M184V, M184I and E138K/M184I reverted to wt, while the amino acid substitutions in the clone E138K/M184V were retained in the presence of ETR.

Table 3: *In vitro* antiviral activity of NNRTIs against Viruses Containing Various RAMs				
Virus	Mean EC50 ± SD (nM) [FC]			
	ETR	RPV	TMC120	EFV
WT	1.65 ± 0.05	0.85 ± 0.07	1.25 ± 0.07	2.15 ± 0.21
E138V	1.8 ± 0.57 [1.1]	0.75 ± 0.05 [0.9]	1.2 ± 0.14 [1.0]	1.2 ± 0.28 [0.6]
E138K	5.4 ± 0.14 [3.3]	1.8 ± 0.1 [2.1]	3.5 ± 0.2 [2.8]	4.1 ± 0.14 [1.9]
E138A	5.35 ± 0.35 [3.2]	2.1 ± 0.57 [2.5]	4.5 ± 0.1 [3.8]	3.8 ± 0.71 [1.7]
Y181C	9.95 ± 0.35 [6.03]	1.95 ± 0.07 [2.3]	6.3 ± 0.85 [5.3]	5.4 ± 1.0 [2.5]
E138V/Y181C	12 ± 2.8 [7.3]	3.1 ± 0.28 [3.6]	8.15 ± 0.21 [6.5]	4.1 ± 1.3 [1.9]
E138K/Y181C	8.45 ± 1.5 [5.1]	1.8 ± 0.42 [2.1]	11 ± 0.42 [9.2]	2.1 ± 0.42 [1.0]
E138A/Y181C	14.6 ± 0.42 [8.8]	2.85 ± 0.35 [3.4]	14.6 ± 0.56 [12.2]	2.25 ± 0.35 [1.0]

Supplemental Digital Content 1: Primers Used in Site-Directed Mutagenesis Studies for Amplification of Specific Mutations

Mutation	Primer
K103N	Fw 5'-CCTGCAGGGTTAAAACAG**AAC**AAATCAGTAACAGTACTGGA-3' Rev 5'-TCCAGTACTGTTACTGATTT**GTT**CTGTTTTAACCCTGCAGG-3'
Y181C	Fw 5'-CAAAATCCAGACATAGTCATC**TGT**CAATACATGGATGATTTG-3' Rev 5'-CAAATCATCCATGTATTG**ACA**GATGACTATGTCTGGATTTTG-3'
G190A	Fw 5'- CATGGATGATTTGTATGTA**GCA**TCTGACTTAGAAATAGGGC-3' Rev 5'-GCCCTATTTCTAAGTCAGA**TGC**TACATACAAATCATCCATG-3'
E138K	Fw 5'-GCATTCACTATACCTAGTGTAAACAAT**AAG**ACACCAGGGATT-3' Rev 5'-AATCCCTGGTGT**CTT**ATTGTTTACACTAGGTATAGTGAATGC-3'
E138A	Fw 5'-CCATACCTAGTATAAACAAT**GCG**ACACCAGGGATTAGATATC-3' Rev 5'-GATATCTAATCCCTGGTGT**CGC**ATTGTTTATACTAGGTATGG-3'
E138V	Fw 5'-TAGTATAAACAAT**GTG**ACACCAGGGATTAGATAT-3' Rev 5'-ATATCTAATCCCTGGTGT**CAC**ATTGTTTATACTA-3'
M184I	Fw 5'-CATCTATCAATAC**ATT**GATGATTTGTA-3' Rev 5'-TACAAATCATC**AAT**GTATTGATAGATG-3
M184V	Fw 5'-CATCTATCAATAC**GTG**GATGATTTGTA-3' Rev 5'-TACAAATCATC**CAC**GTATTGATAGATG-3'

Supplemental Digital Content 2: Mutations Selected by EFV and EFV/TFV Drug Pressure After 20-30 Weeks.			
Virus	Baseline Genotype (Subtype)	Week 20-30	
		Drug (nM)	Acquired Mutation s
NL-4.3[a]	wt (B)	EFV (1000)	K101E, V108I, V189I/V
NL-4.3[b]	Wt (B)	EFV (200)	K103N, V189I/V, E399G
NL-4.3	K103N (B)	EFV (500)	K103N, Y318F, N348I/N
NL-4.3[a]	Y181C (B)	EFV (20)	V75I, K101Q, V108I, Y181C
NL-4.3[b]	Y181C (B)	EFV (100)	V75L, V106I, Y181C, Y188H, A400A/T
NL-4.3	K103N/Y181C (B)	EFV (50)	K103N, Y181C
NL-4.3	K103N/G190A (B)	EFV (10)	K103N, G190A
NL-4.3	G190A (B)	EFV (7.5)	A62V, V106A, V179D/V, G190A
NL-4.3	E138K (B)	EFV (90)	L100I, K103K/N, E138K
NL-4.3	M184I (B)	EFV (60)	L100I/L, M184I, H188H/Y
NL-4.3	E138K/M184I (B)	EFV (90)	E138K, G190A
NL-4.3	E138K/M184V (B)	EFV (40)	E138K, E399G
NL-4.3	M184V (B)	EFV (50)	Y188C/Y
4743	G190A (C)	EFV (1000)	V106M, G190A, F227F/L, E399K
10680	wt (C)	EFV (10000)	V106M, Y188C, K275R
10680	Y181C (C)	EFV (10000)	A62A/V, K101E/K, V106M, Y181C, F227L, L283L/V
10680	wt (C)	EFV (250) TFV (500)	V106M, F227L
10680	Y181C (C)	EFV (1000) TFV (1000)	V106M, Y181C

Genotypic analysis was done at weeks 20, 25 or 30. Baseline mutations that persisted at time of genotypic analysis are underlined. Amino acid substitutions at position 184 in all clones containing M184V, E138K/M184V and E138K/M184I reverted to wt, while

amino acid substitutions in the M184I clone were retained in the presence of EFV pressure.

Supplemental Digital Content 3: Mutations Selected by Serial Passage Experiments in CBMCs in the Presence of TMC120 Drug Pressure

Virus	Baseline Genotype	Week 20	
		DPV Conc (nM)	Acquired Mutations
NL-4.3	wt	100	E138K, L100I
NL-4.3	E138K	70	L100I, E138K
NL-4.3	E138K/M184I	200	L100I, E138K, M184I
NL-4.3	M184I	80	E138K
NL-4.3	E138K/M184V	90	V108I, E138K
NL-4.3	M184V	70	E138K

Baseline mutations that persisted at time of genotypic analysis are underlined. Amino acid substitutions at position 184 in the clones M184V, M184I and E138K/M184V reverted to wt, while amino acid substitutions in the E138KM184I clone were retained in the presence of TMC120.

Figure 1: Stepwise accumulation of RPV resistance in cell culture. The graph shows the time course of selection of resistant variants in CBMCs using wt virus and virus containing Y181C at baseline. CBMCs were infected with viruses at a low MOI of 0.1 and cultured in the presence of progressively increasing concentrations of drugs starting at a concentration below the EC_{50}.

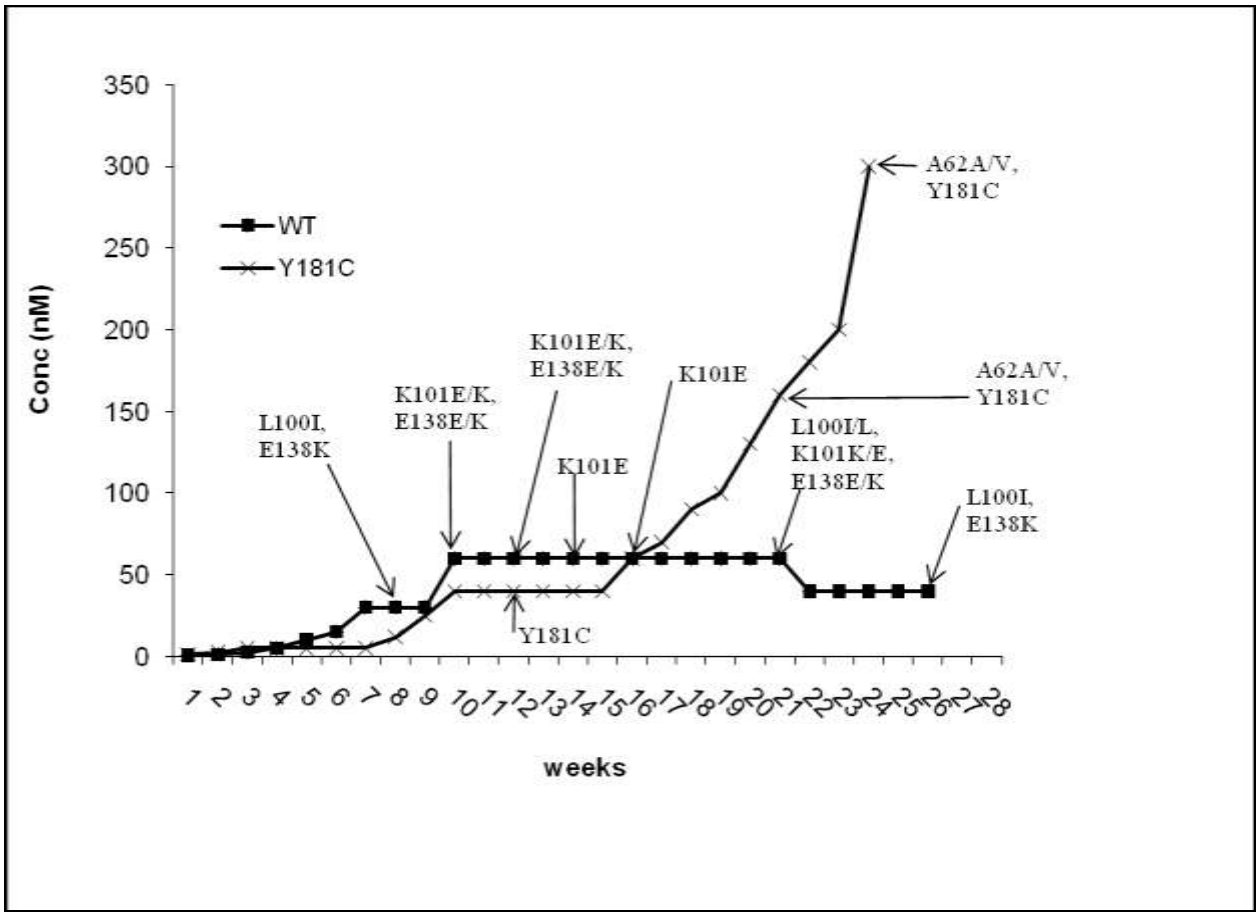
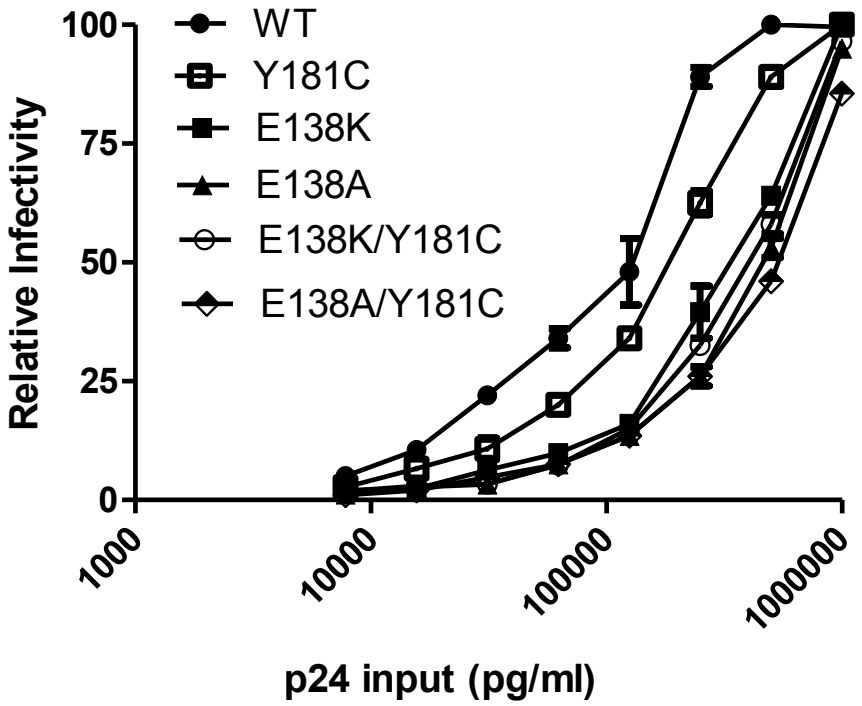

350
300
250
200
150
100
50
0
Conc (nM)
WT
Y181C
L100I,
E138K
K101E/K,
E138E/K
K101E/K,
E138E/K
K101E
K101E
Y181C
A62A/V,
Y181C
L100I/L,
K101K/E,
E138E/K
A62A/V,
Y181C
L100I,
E138K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
weeks

Figure 2: Effect of mutations at position 138 together with Y181C on viral replication capacity. Stocks of both wt and mutated viruses were normalized for p24 and used to infect TZM-bl cells. Luciferase activity was measured at 48 hours post-infection to monitor viral replication. The relative infectivity of wt virus compared to mutated viruses is shown on the Y-axis while the X-axis denotes input of p24.


WT
Y181C
E138K
E138A
E138K/Y181C
E138A/Y181C
Relative Infectivity
100
75
50
25
0
1000
10000
100000
1000000
p24 input (pg/ml)

Chapter 4

Tissue Culture Drug Resistance Analysis of a Novel HIV-1 Protease Inhibitor Termed PL-100 in Non-B HIV-1 Subtypes.

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Abstract

PL-100 is a novel HIV-1 protease inhibitor (PI) that maintains activity against viruses that are resistant to other PIs. To further characterize this compound, we used it to select for drug resistance in tissue culture, using two non-B HIV-1 subtypes, viz. Subtype C and a CRF01_AE recombinant virus. PL-100 selected for both minor and major PI resistance mutations along either of two distinct pathways. One of these involved the V82A and L90M resistance mutations while the other involved a mutation at position T80I, with other mutations being observed at positions M46I/L, I54M, K55R, L76F, P81S and I85V. The resistance patterns in both subtype C and CRF01_AE were similar and an accumulation of at least three mutations in the flap and active sites were required in each case for high-level resistance to occur, demonstrating that PL-100 has a high genetic barrier against the development of drug resistance.

Keywords: HIV-1 subtypes; Drug resistance; Protease inhibition; PL-100.

Introduction

The protease (PR) enzyme of human immunodeficiency virus type 1 (HIV-1) is an excellent therapeutic target since its inhibition prevents the proteolytic processing of the Gag and Gag-Pol polyproteins (Ventoso et al., 2005). Protease inhibitors (PIs) are routinely prescribed for both treatment-naïve and experienced patients and have had a profound impact on HIV-associated disease progression, transmission, and morbidity and mortality (Palella et al., 1998).

Drug resistance is a frequent complication in patients who fail therapy (Luis Jimenez et al., 2005; Turner et al., 2005; Descamps et al., 2009) and many studies have shown that drug-resistant viruses can be sexually transmitted (Brenner et al., 2008; Aghokeng et al., 2009). The problem of drug resistance is compounded by the worldwide dissemination of multiple different subtypes of HIV-1 and the fact that natural polymorphisms in both HIV-1 and HIV-2 can affect the emergence of drug resistance to currently approved drugs (Kantor and Katzenstein, 2003; Stranix et al., 2004; Ntemgwa et al., 2007). Furthermore, polymorphisms within the PR enzyme may not themselves be responsible for resistance but can contribute to the development of high-level resistance if other mutations are present (Vergne et al., 2000; Kantor and Katzenstein, 2003; Velazquez-Campoy et al., 2003; Liu et al., 2007; Bessong, 2008). It is therefore important to understand antiviral activity and drug resistance profiles in viruses of different subtypes.

Recently, a novel PI termed PL-100 was shown to be active against both wild-type and drug-resistant forms of HIV-1 of subtype B origin (Dandache et al., 2007). PL-100 is a lysine-based sulphonamide that was designed on the basis of subtype B PR structural data (Stranix et al., 2004). Although PL-100 demonstrates a high genetic barrier for the development of drug resistance to subtype B viruses (Dandache et al., 2008), little is known about the role that individual polymorphisms in some non-B subtypes might play in regard to the development of drug resistance (Kantor and Katzenstein, 2003; Liu et al., 2007; Ntemgwa et al., 2007). Here we describe *in vitro* development of resistance and the antiviral properties of PL-100 against a panel of wild-type and drug resistant non-B viruses. As examples of non-B subtypes we used subtype C and the circulating recombinant form (CRF) CRF01_AE that represent the most prevalent subtype and CRF in circulation at this time (Hemelaar et al., 2006).

Materials and Methods

Virus isolates, cells and plasmids:

Three HIV-1 CRF01_AE clinical isolates (NI1052, M02138 and NP1525) were obtained from the AIDS Research and Reference Reagent Program of the National Institutes of Health (NIH), Bethesda, MD. Five HIV-1 subtype C (7208, 8032, 8947, 7906 and HB-1) clinical isolates were obtained with informed consent from drug-naive individuals at our clinics in Montreal, Canada. The CRF01_AE isolates have been previously characterized as X4-tropic viruses (Brown et al., 2005) while the subtype C isolates were non-syncytium inducing viruses and considered R5 viruses. MT-2 cells were obtained from

the NIH AIDS Research and Reference Reagent Program. Cord blood mononuclear cells (CBMCs) were obtained through the Department of Obstetrics, Jewish General Hospital, Montreal, Canada. The AG plasmid (p97GH-AG2) was kindly provided by Dr. Masashi Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan.

Drugs:

Nelfinavir (NFV), amprenavir (APV), lopinavir (LPV), atazanavir (ATV), PL-100 and saquinavir (SQV) as gifts from Pfizer, Inc. (San Diego, CA), GlaxoSmithKline (Research Triangle Park, NC), Abbott Laboratories (North Chicago, IL), Bristol-Myers-Squibb, Inc., Ambrilia Biopharma Inc, (Verdun, Quebec, CA) and Roche, Inc., respectively. Efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor, was used as a control drug and was obtained courtesy of BMS Inc.

***In vitro* selection of resistance mutations in CBMCs and MT-2 cells:**

PHA-stimulated CBMCs or MT-2 cells were infected with viruses (multiplicity of infection of 0.1 for CBMCs and 0.01 for MT-2 cells) for 2 hours, incubated at 37ºC, and subsequently washed with RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum and seeded into a 24-well plate at a density of 2.5 x 105 cells per well (Gao et al., 1993). Selection for resistance in CBMCs and MT-2 was performed using increasing concentrations of drugs (PL-100 and APV) at a starting concentration that was below the 50% effective concentration (EC50) of the drugs (Oliveira et al., 2009). As controls, all viruses were simultaneously passaged without drugs. With CBMCs, drug

concentrations were increased at subsequent passages based on RT levels in culture fluids (Loemba et al., 2002; Petrella et al., 2004). With MT-2 cells sub-culturing was performed every 3-4 days and the cells studied for cytopathic effect (Vingerhoets et al., 2005). Virus-containing culture fluids were harvested and kept at -80ºC for subsequent genotypic analysis at the same time that drug concentrations were increased.

Nucleic acid extraction, amplification and sequencing analysis:

Viral RNA was extracted from culture supernatants using the Qiagen QIAamp viral extraction kit (Mississauga, Ontario, Canada). PCR amplification was performed using a previously published protocol (Virco BVBA, Mechelen, Belgium). The resulting PCR-amplified DNA fragments were purified using the QIAquick PCR purification kit and products were used as templates for nucleotide sequencing analysis. Genotyping was performed by sequencing a 325-bp fragment of HIV pol (position 2253-2578) spanning the entire protease (PR) using Virco primers (Virco BVBA Mechelen, Belgium) with a BigDye Terminator sequencing kit (Version 1.1; Applied Biosystems, Forter City, CA) and automated sequencer (ABI Prism 3130 genetic analyzer; Applied Biosystems). The sequence of each sample was compared to that of wild–type (wt) subtype B consensus virus and to data in the Stanford database.

Site-directed mutagenesis and virus production:

The K55R, I85V and K55R/I85V mutations were introduced into the p97GH-AG2 plasmid by site-directed mutagenesis (SDM) using a QuikChange II XL site-directed mutagenesis kit (Stratagene, LaJolla, CA). For SDM of K55R, the forward primer 5'-GGG GGA ATT GGA GGT TTT ATC AGG GTA AGA CAG TAT GAC CAG-3' and reverse primer 5'-CTG GTC ATA CTG TCT TAC CCT GAT AAA ACC TCC AAT TCC CCC-3' were used while for I85V the forward primer 5'-GGA CCT ACA CCT GTC AAC ATA GTT GGA CGA AAT ATG-3' and reverse primer 5'-CAT ATT TCG TCC AAC TAT GTT GAC AGG TGT AGG TCC-3' were employed. Introduction of the various mutations into the plasmid was confirmed by sequencing and DNA ultimately transformed into DH5α cells (Invitrogen) for high-yield of plasmid.

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected with 16μg of plasmid using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. Two days after transfection, supernatants of the transfected cells were clarified by centrifugation at 1500 rpm for 5 min and stored in aliquots at -80°C. Virus production was confirmed by RT assay.

Phenotypic susceptibility to PIs in CBMCs and MT-2 cells:

Drug susceptibility was measured in cell-culture based phenotypic assays as previously described (Salomon et al., 1994; Loemba et al., 2002), to determine the extent to which

PIs (NFV, SQV, APV, LPV, ATV and PL-100) blocked HIV replication *in vitro*. Efavirenz (EFV), a non-nucleoside inhibitor of reverse transcriptase, was used as a control. In brief, cells were infected for 2 hours with either wt or drug selected variants, washed to remove unbound virus and plated in duplicates into 96 well plates containing drugs or not and incubated at 37ºC. For MT-2 cells, cytopathic effect was confirmed on day 4 or 5 and supernatants harvested for RT activity. For CBMCs, cells were fed with fresh media containing appropriate drug dilutions and RT assays were performed at day 7. EC50 concentrations of PIs were determined from RT values.

A preliminary cut-off value had previously been calculated for PL-100 (Dandache et al., 2007; Dandache et al., 2008). The manner in which preliminary cut-off values are calculated is through access to a limited number of clinical samples that have not been exposed to PL-100 but that contain mutations associated with resistance against other members of the PI family of drugs. The EC50 values of these clinical isolates were measured in phenotypic assays in regard to PL-100 and mean fold changes ± 2 standard deviations in EC50 in comparison with laboratory wild-type viruses were calculated. The mean fold-change ±2 standard deviation corresponds to the preliminary biological cut-off value.

Results

Selection of resistance mutations to PL-100 and APV in MT-2 cells:

Polymorphisms are defined as naturally occurring amino acid variations from the subtype B consensus sequence (HXB2) that is present in the Los Alamos HIV database (http://www.hiv.lanl.gov). Three CRF01_AE viruses (NI1052, M02138 and NP1525) were used for selection in MT-2 cells (Table 1). Polymorphisms at positions I13V, E35D, M36I, R41K, H69K and L89M were present in all three CRF01_AE isolates. In addition, K20R and R57K were also present in the M02138 and NI1052 viruses, respectively (Table 1). Some of these polymorphisms are known to contribute to drug resistance for certain PIs (Kantor and Katzenstein, 2003).

In MT-2 cells, mutations emerged through the V82A pathway in the CRF01_AE isolates. The novel mutation K45I was observed with one A/E isolate and this mutation persisted until the end of the selection experiments. The L76F mutation emerged in one A/E isolate between weeks 35 and 66. This mutation was accompanied by other mutations known to be associated with resistance to PIs (Table 1). None of the mutations selected by PL-100 by passage 16 or 17 with isolate M02138 persisted with the exception of L10I. Two additional major PI mutations, L33F and I50V, were selected by APV but not by PL-100.

Genotypic analysis and selection of resistance mutations to PL-100 in CBMCs:

Five subtype C clinical isolates were used for selection in CBMCs with each containing 6-9 polymorphisms at baseline (Table 2). After selection, all viruses passaged without drugs (controls) were sequenced to confirm the absence of new mutations. The results show that two distinct resistance pathways seem to be associated with PL-100 in subtype C in tissue culture drug selection. One pathway consists of active site mutations at either positions 82 and 90 or at both positions, and an alternate pathway, unique to PL-100, utilized a novel active site mutation at position 80 (Tables 2). The T80I mutation occurred with additional changes at active sites 85 and 89 (Table 2). Flap mutations, most often at position 46, accompanied both of these pathway options.

Incidence of mutations selected with PL-100 in CBMC s and MT-2 cells:

For both subtypes, mutations at positions 46 and 82 were the most prevalent, both being observed in 6 of 8 cases (Fig. 1). This was followed by the combinations of M46L/I together with V82A in 3 of 8 cases. The T80I mutation or the combination M46L/I + T80I were present in 2 of 8 cases. The L90M or V82A + L90M or M46L/I + V82A + L90M were each found in one of 8 cases. In addition, the simultaneous presence of mutations at positions 46 and 82 was common in all subtypes. The novel T80I mutation was always found together with a change at position 46. With the exception of T80I, all of the other mutations described have frequently been observed in patients who have failed a variety of regimes containing PI drugs.

***In vitro* phenotypic susceptibility in MT-2 cells:**

We next evaluated the *in vitro* susceptibilities of selected viruses, site-directed mutants, and wt viruses by measuring EC50s of several PIs in a cell-based phenotypic assay. In each case, a wt isolate was used as a reference for the drug susceptibility assays and PL-100-resistant variants were compared with their respective wt isolates to calculate fold-change (FC) for resistance. A lower biological cut-off of 2.5 for PL-100 (arbitrarily chosen cut-off value for this analysis pending clinical availability of the drug) was used. Lower and upper clinical cut-offs were established as 1.2-9.4 for NFV, 1.5-19.5 for APV, 6.1-51.2 for LPV, 2.5-32.5 for ATV 3.1-22.6 for SQV and 3.4 for EFV (Van Houtte et al., 2009; Winters et al., 2009). As shown in Table 3, all of the selected variants displayed cross-resistance to NFV, APV and ATV with the exception of the CRF01_AE isolate (NP1525) containing mutations M46L/K70R/Q92L that confers only moderate resistance to NFV and APV but retained susceptibility to ATV. With one CRF01_AE isolate (M02138), the mutations L10I, K55R, I85V and Q92H were observed by passage 17. This isolate displayed fold changes in drug susceptibility of 8.5, 6.82 and 3.34 for ATV, APV and PL-100, respectively and was susceptible to LPV and NFV (Table 3). To further confirm the biological relevance of the K55R and I85V mutations, site-directed mutants were created in a CRF02_AG plasmid. A very modest reduction in NFV susceptibility (1.9 fold) was seen with I85V (data not shown). Neither of these mutations affected susceptibility to other PIs since the FC in each case was less than the relevant clinical or biological cut-offs. No significant differences were observed when the panel of protease inhibitors was used against the combination K55R/I85V mutation (data not

shown). In the case of clinical isolate NP1525, selection with PL-100 did not result in obvious drug resistance.

Phenotypic susceptibility in CBMCs:

Despite the presence of resistance mutations, all viral isolates retained susceptibility to SQV. All selected viruses showed decreased susceptibility to PL-100. Low and moderate level cross-resistance was observed in NFV, APV, LPV and ATV with higher level resistance being displayed against both NFV and APV (Table 4). The combination M46I/T74S/T80I/V82/I85IV/L89M (isolate 8032) possessed a FC of 20.8 for PL-100 and a FC of 28.7 and 10.2 for APV and ATV respectively. This isolate was susceptible to LPV. The other two isolates with the combinations M46I/V82A/L90M and K43N/M46I/T80I/I85V/L89I were resistant to all PIs tested except SQV.

Discussion

In this study, we show that resistance to PL-100 can emerge via mutations at positions 82 and 90 or alternatively via a T80I substitution that appears to be unique to this drug. We demonstrate the possible role of mutations L76F, K55R and I85V. Although viruses containing K55R, I85V, or K55R/I85V are susceptible to PL-100, Table 3 indicates that viruses containing the mutations selected at passage 17 (i.e. L10I, K55R, I85V, Q92H) showed a 3.3 decrease in susceptibility to PL-100. This might have resulted from combinations of mutations together with secondary PI mutations such as L10I. The K55R

and I85V substitutions might be considered to be secondary PI mutations. The NP1525 variant selected by PL-100 was susceptible to PL-100 with a fold-change of 1.45. Thus our data confirm that PL-100 possess a very high genetic barrier for development of drug resistance.

The most common mutations selected by PL-100 were at positions 46, 82, 90 and 80 in decreasing order of frequency. Although T80I appears to be novel, M46I/L, V82A and L90M have been described in patients failing SQV, NFV, APV, LPV and ATV (Marcelin et al., 2004a; Marcelin et al., 2004b; Mo et al., 2005; Svedhem et al., 2005; Doualla-Bell et al., 2006; Santoro et al., 2009). While the T80I mutation seems to be important, resistance can also occur along a second pathway that is common to other PIs that includes the V82A and L90M mutations. These two pathways appear to be mutually exclusive in all cases. This study demonstrates the importance of using a wide range of viral isolates in drug selection to ensure that the potential for manifestation of a broad array of mutations is fully explored.

We observed similar patterns of resistance to PL-100 with both subtype C and CRF01_AE viruses. A recent *in vitro* selection study showed that resistance may develop slower under PL-100 compared with APV drug pressure in the case of the laboratory-adapted HIV/IIIb (subtype B) virus and that a unique T80I resistance pathway is involved (Dandache et al., 2008). The importance of this pathway is confirmed in the present study.

Our data follow a previous investigation that also identified a L76F mutation in PR (Bold et al., 1998). The role of L76F in PI resistance is yet to be explored. Mutations K55R and I85V were transiently present during PL-100 selection and have been previously observed in cases of PI failure (Svicher et al., 2005; Johnson et al., 2008; Margerison et al., 2008; Descamps et al., 2009; Palma et al., 2009). Site-directed mutagenesis demonstrated, however, that these mutations were susceptible to PL-100 but only impacted moderately on PL-100 susceptibility in the presence of other mutations. K55R and I85V are thought to be able to restore viral replicative capacity (Margerison et al., 2008). Although previous studies with site-directed mutagenesis in the presence of M46I and T80I in an NL4-3 plasmid showed no effect on susceptibility to PL-100 (Dandache et al., 2008) additional experiments are ongoing in our laboratory to study the role of these mutations including L76F and V82A either alone or in combinations on susceptibility to PL-100 and replication capacity using the NL4-3 and AG plasmids.

The genotype of virus 7208 is M46I, V82A and L90M. These are major mutations that decrease susceptibility to saquinavir. The mean fold-change observed in two independent experiments in our phenotypic assay indicated susceptibility to saquinavir. Phenotypic resistance might be of greater value than genotypic data to predict outcome of resistance in some cases. In support of our findings, Piketty et al, (Piketty et al., 1999), showed that patients with baseline mutations at position 46, 82 and 90 had a decreased viral load of fewer than 50 copies/ml after 24 weeks of r/saquinavir therapy. In both MT-2 cells and CBMCs, levels of cross-resistance after selection with PL-100 followed a consistent

pattern. First, both NFV and APV were significantly affected by the mutations selected by PL-100. Second, LPV and ATV were only partially affected by these mutations because the fold-change is just slightly above the lower clinical cut-off and SQV seemed to retain significant activity against viruses that were resistant to PL-100 since the fold-change of the selected viruses did not reach the lower clinical cut-off value. The fact that PL-100 has a distinct resistance profile from other PIs suggests that it might potentially be sequenced with ATV and LPV. Given its overall profile, PL-100 could very well be placed as a first line or second-line therapy for protease-naïve or experienced patients and be a valuable new addition to the HIV drug armamentarium.

Conclusion:

Ultimately, similar patterns of resistance to PL-100 seem to exist in both subtype C and CRF01_AE, despite important differences in baseline polymorphisms among these viruses. High level resistance to PL-100 was observed only in the presence of three or more PI resistance mutations demonstrating the high genetic barrier of this compound. Our observations have also confirmed the importance of a signature T80I mutation that is involved in PL-100 resistance.

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Chapter 4 References

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Table 1

Development of resistance mutations to PL-100 and APV in MT-2 cells.

		CRF01_AE		
Drug	**Passage**	**NI1052**	**M02138**	**NP1525**
	0	I13V, E35D, M36I, R41K, R57K, H69K, L89M	I13V, K20R, E35D, M36I, R41K, H69K, L89M	I13V, E35D, M36I, R41K, H69K, L89M
PL-100	15-17	K43L, K45T, L63V	L10I, K55R, I85V, Q92H	K14R, P79H
	24-30	M46I, V82A	L10I	M46L, K70R
	35-66	L10I, M46I, L76F, V82A	L10I, K45I, I62V, V82A	M46L, K70R, Q92L
APV	45-66	L10F, L33F, M46L, I93V	ND[a]	L10F, I15V, Q18E, L19M, K20R, M46L, 150V, I62V, L63S, C67Y, L89I

[a] ND, not determined

Table 2

Baseline polymorphisms and resistance mutations associated with subtype C viruses in CBMCs

	Week	7208	8032	8947	7906	HB-1
Baseline Polymorphisms	0	T12S, I15V, L19I, E35D, M36I, R41K, L63P, H69K, I93L	I15V, L19T, M36I, N37N/K, R41K/N, L63P, H69K, K70K/R, I93L	L14R, I15V, L19I, M36I, R41K, H69K, I93L	I15V, L19I, M36L, R41K, H69K, L89M	T12S, L19I, M36I/L, R41K, I62I/V, L63P, H69KL89MI93L
PL-100 selected mutations	31	ND[a]	M46M/I, T74S, T80I, L89M	I64V, V82A	ND[a]	ND[a]
PL-100 selected mutations	40	M46I, V82A, L90M	M46I, T74S, T80I, V82I, I85I/V, L89M	I13I/V, I64V, V82A	K43N, M46L, T80I, I85V, L89I	L10R, M46L, V82A

[a] ND, not determined

Table 3:

***In vitro* efficacy in MT-2 cells of various ARVs against CRF01_A/E viruses containing relevant PI resistance mutations.**

Viral isolate	Subtype	PI mutation	Mean EC_{50} (nM) ± SD[a] and Fold Change (FC)					
			NFV	APV	LPV	ATV	PL-100	EFV
Lower and upper clinical cut-offs[b]			1.2-9.4	1.5-19.5	6.1-51.2	2.5-32.5	2.5[c]	3.4
NI1052	A/E	wt	16.5 ± 2	15.0 ± 3	10.1 ± 2	4.0 ± 0.2	9.0 ± 1.8	3.1 ± 0.3
NI1052	A/E	L10I, M46I, L76F, V82A	35.5 ± 7 (2.1)	55.0 ± 3.2 (3.7)	82.0 ± 6 (8.1)	15.0 ± 2 (3.7)	262.0 ± 2 (29.1)	6.2 ± 0.1 (2.0)
M02138	A/E	wt	18.5 ± 0.7	4.7 ± 0.67	2.95 ± 0.3	0.46 ± 0.03	9.03 ± 2	2.9 ± 0.7
M02138 (P 17)[d]	A/E	L10I, K55R, I85V, Q92H	36.0 ± 6 (1.94)	32.7 ± 3 (6.9)	5.5 ± 2 (1.86)	3.9 ± 0.3 (8.5)	30.1 ± 1 (3.3)	ND
M02138	A/E	L10I, K45I, I62V, V82A	81 ± 12 (4.5)	120.0 ± 40 (25.5)	95.5 ± 0.0.7 (32)	19.0 ± 1.4 (41)	289.0 ± 4 (32)	2.5 ± 0.6 (0.86)
NP1525	A/E	wt	15.0 ± 4	10.2 ± 1.7	6.0 ± 2	3.0 ± 0.7	11.0 ± 0.7	2.4 ± 0.1
NP1525	A/E	M46L, K70R, Q92L	19.0 ± 5 (1.3)	49.4 ± 1 (4.8)	11.0 ± 1.1 (1.8)	4.0 ± 2 (1.3)	16.3 ± 6 (1.45)	2.8 ± 0.2 (1.2)

The values represent the means of two independent experiments, each performed in duplicates. Drug susceptibility was expressed as fold-change in EC50, determined by calculating the ratio of EC50 value for mutated and wt virus. (Values in parentheses).

b (Van Houtte et al., 2009) and (Winters et al., 2009).

c Arbitrarily chosen lower cut-off value of PL-100 for this analysis pending availability of clinical data.

d M02138 isolate containing resistance mutations selected at passage 17.

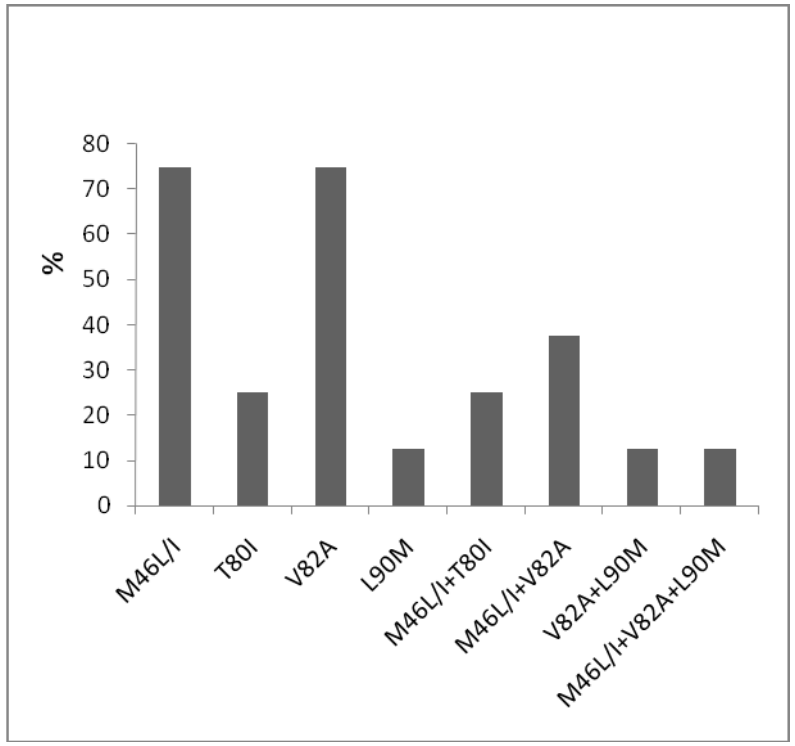
Table 4

***In vitro* efficacy in CBMCs of various ARVs against subtype C viruses containing relevant PI resistance mutations.**

Viral Isolate	Subtype	PI mutation(s)	Mean EC_{50} (nM) ± SD[a] and Fold-Change (FC)						
			NFV	APV	LPV	ATV	PL-100	SQV	EFV
7208	C	Wt	13.0 ± 7.0	12.6 ± 0.1	11.2 ± 3.7	2.4 ± 0.5	9.2 ± 0.5	40.8 ± 0.6	0.31 ± 0.16
7208	C	M46I, V82A, L90M	222.8 ± 20 (17.1)	27.3 ± 23 (2.2)	98.6 ± 15 (8.8)	16.4 ± 12 (6.8)	168.8 ± 21 18.4)	31.0 ± 3.0 (0.8)	0.03 ± 0.01 (0.1)
7906	C	Wt	2.8 ± 0.1	5.1 ± 1.1	1.5 ± 1.0	1.5 ± 1.0	2.2 ± 0.5	15.0 ± 2.0	1.28 ± 0.4
7906	C	K43N, M46L, T80I, I85V, L89I	11.9 ± 2.5 (4.3)	173 ± 15.8 (34.6)	20.8 ± 7.0 (13.9)	14.1 ± 10 (9.4)	129.3 ± 54 (58.8)	22.5 ± 1.0 (0.9)	1.1 ± 0.1 (0.9)
8032	C	Wt	14.7 ± 1.0	39.0 ± 5.0	19.1 ± 19	6.7 ± 0.7	22.2 ± 2.0	46.7 ± 28	0.99 ± 0.08
8032	C	M46I, T74S, T80I, V82L, I85IV, L89M	33.0 ± 19 (2.3)	1120 ± 327 (28.7)	34.5 ± 3.0 (1.8)	68.0 ± 8.0 (10.2)	460.8 ± 121 (20.8)	22.9 ± 9.8 (0.5)	0.51 ± 0.21 (0.5)

[a] The values represents the means of two independent experiments, each performed in duplicates. Drug susceptibility was expressed as fold-change in EC_{50}, determined by calculating the ratio of mutant EC_{50}: wild-type $EC_{50.}$ (values in parentheses).

FIG1: Frequencies of mutations most commonly selected with PL-100. M46L/I and T82A were the most prevalent, being observed in 6 of 8 cases each, followed by the combinations M46L/I + V82A in 3 of 8 cases, T80I and the combination M46L/I + T80I in 2 of 8 cases and L90M, V82A + L90M and M46L/I + V82A + L90M in 1 of 8 cases each.


80
70
60
50
40
30
20
10
0
%
M46L/I
T80I
V82A
L90M
M46L/I+T80I
M46L/I+V82A
V82A+L90M
M46L/I+V82A+L90M

Chapter 5

In Vitro and Structural Evaluation of PL-100 as a Potential Second Generation HIV-1 Protease Inhibitor.

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Abstract

Objectives: HIV-1 protease inhibitors (PIs) are key components of HIV therapy. PL-100 is a novel lysine sulphonamide that demonstrates potent antiviral activity against multi-resistant HIV-1 strains as well as a higher genetic barrier for development of resistance mutations compared to first generation PIs. In the present study, we compared the antiviral activity of PL-100 against HIV-1 subtype B to that of darunavir.

Method: We used tissue culture experiments to evaluate the *in vitro* development of resistance to PL-100 and tested the antiviral activity of several clinically approved PIs against PL-100 selected resistant variants. Structural modeling was also used to compare the binding of PL-100 and darunavir to the HIV-1 protease (PR) enzyme.

Results: PL-100 resistant variants that emerged within 8-48 weeks showed low- to high-level resistance (3.5 to 21.6 fold) to PL-100 but commonly retained susceptibility to darunavir, which, in contrast, did not select for resistance mutations over a period of 40 weeks. Structural modeling demonstrated that binding of PL-100 was predominantly based on polar interactions and delocalised hydrophobic interactions through its diphenyl groups while darunavir has numerous interactions with PR that include hydrogen bonding to PR backbone oxygens at amino acid positions A28, D29 and D30 via di-tetrahydrofuran (di-THF) groups.

Conclusion: Hydrogen bonding contacts and the di-THF group in darunavir, as well as the hydrophobic nature of PL-100, contributes to PI binding and a high genetic barrier for resistance. Redesigning the structure of PL-100 to include a di-THF group might improve it.

Introduction

HIV protease inhibitors (PIs) are an important component of highly active antiretroviral therapy (HAART) (reviewed in).[1] However, the success of HIV treatment is hampered by the emergence of drug resistance [2, 3] and newer PIs with improved pharmacological profiles are needed. Various strategies including structure-based drug design have been used to develop new protease inhibitors (PIs) [4, 5] and one such strategy maximizes the number of hydrogen bonds between a PI and the HIV-1 protease (PR) backbone, leading to activity against drug-resistant HIV. [4, 6] PIs with improved resistance profiles have also been developed using a solvent-anchoring approach [7] and a novel lysine sulphonamide-based molecular core. [8, 9] A survey of five PIs that quantitatively evaluated amounts of bound inhibitor outside the substrate envelope concluded that the exterior volume of the inhibitors correlated with a loss of affinity to mutant PR enzyme. [10] Darunavir is a highly efficient PI that is effective against many HIV variants that are resistant to other PIs [11, 12]. This is attributed to its high binding affinity for PR and the fact that darunavir binds tightly within the substrate envelope. [13]

However, other PIs are needed and PL-100 is a novel lysine-based sulphonamide PI that is potent, specific, and non-toxic, which was designed on the basis of structural data obtained for wild-type (wt) protease variants of HIV-1 group M, subtype B. [14] PL-100 has a favorable resistance profile and is active against many HIV variants that are resistant to other PIs. It is active against multiple HIV subtypes and requires an accumulation of at least three mutations in the flap region and active site of PR in order

for resistance to PL-100 to occur. [15-17] In the present study, we have determined the mutational profile, antiviral activity, and structural binding of PL-100 compared to that of darunavir in HIV-1 subtype B.

Materials and Methods

Virus isolates and cells:

Seven HIV-1 subtype B (5269, 5323, 5331, 5346, 5512, BK132, 5326) clinical isolates were obtained with informed consent from drug-naive individuals at our clinics in Montreal, Canada. MT-2 cells were obtained from the NIH AIDS Research and Reference Reagent Program. Cord blood mononuclear cells (CBMCs) were obtained through the Department of Obstetrics, Jewish General Hospital, Montreal, Canada.

Drugs:

Nelfinavir , amprenavir, lopinavir, atazanavir, PL-100, saquinavir, and darunavir were gifts from Pfizer, Inc. (San Diego, CA), GlaxoSmithKline (Research Triangle Park, NC), Abbott Laboratories (North Chicago, IL), Bristol-Myers Squibb, Inc., Ambrilia Biopharma Inc, (Verdun, Quebec, Canada), Roche, Inc., and Tibotec respectively.

In vitro selection of resistance mutations in CBMCs and MT-2 cells:

In vitro passage experiments were performed in the presence or absence of drug (PL-100, amprenavir and darunavir) as described previously. [17] Virus-containing culture fluids were harvested and kept at -80ºC for subsequent genotypic analysis at the same time that drug concentrations were increased.

Nucleic acid extraction, amplification and sequencing analysis:

Viral RNA was extracted from culture supernatants using the Qiagen QIAamp viral extraction kit (Mississauga, Ontario, Canada). PCR amplification was performed according to a previously published protocol (Virco BVBA, Mechelen, Belgium). The resulting PCR-amplified DNA fragments were purified using the QIAquick PCR purification kit and products were used as templates for nucleotide sequencing analysis. Genotyping was performed by sequencing a 325 bp fragment of HIV *pol* (positions 2253-2578) spanning the entire protease (PR) using Virco primers (Virco BVBA, Mechelen, Belgium) with a BigDye Terminator sequencing kit (version 1.1; Applied Biosystems, Forter City, CA) and automated sequencer (ABI Prism 3130 genetic analyzer; Applied Biosystems). The sequence of each sample was compared with that of the wt subtype B consensus virus and to data in the Stanford database

(http://sierra2.stanford.edu/sierra/servlet/JSierra?action=sequenceInput).

Phenotypic susceptibility to PIs in CBMCs and MT-2 cells:

Antiviral activity was measured in cell culture-based phenotypic assays to determine the susceptibility of HIV-1 to different PIs (nelfinavir, sequinavir, amprenavir, lopinavir, atazanavir, darunavir and PL-100), as described elsewhere. [18] Briefly, cells were infected for 2 hours with either wt or drug- selected variants, washed to remove unbound virus and plated in duplicate into 96-well plates in the presence or absence of drug and incubated at 37ºC. For MT-2 cells, a cytopathic effect was confirmed on day 4 or 5 and supernatants harvested for reverse transcriptase (RT) activity. For CBMCs, cells were fed with fresh medium containing appropriate drug dilutions and RT assays were performed on day 7. The 50% effective concentration (EC_{50}) of PIs was determined from RT values. The preliminary clinical cut off of PL-100 has been described elsewhere. [15]

Simulation of drug docking

The isomeric SMILES description for PL-100 was obtained from the two-dimensional (2D) structure of PL-100 available on PubChem Compound (CID: 513956) and used to generate a three-dimensional (3D) structure using the program AVOGADRO version 1.0.3. [19] The same program was used to perform geometric optimization of unbound PL-100 with 500 steps of steepest descent using the MMFF94 force field. [20] PubChem Compound was also the source of 3D structures for darunavir (CID: 213039) and a different PI, amprenavir (CID: 65016). All ligands were prepared as flexible docking ligands in AutoDock tools. [21] The protein databank (PDB) structures of wt protease 4DQB [22] and multi-drug resistant (MDR) protease (D25N, M36V, I84V) 1RPI [23] were

prepared for docking by removal of cocrystallized ligands. Water molecules were retained for the subsequent docking simulations. The protein files were prepared as receptors using AutoDock tools. Each ligand was docked into the central cavity of the protease dimer using a grid cube measuring 20Å in each of the x, y and z directions in AutoDock Vina [24]. Docking was repeated using DockingServer, [25] which uses Autodock 4 docking parameters and using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method. [21] The Initial position, orientation and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking study was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å was used and quaternion and torsion steps of 5 were applied. A third set of dockings was carried out using SwissDock, [26] an online server that docks ligands to proteins using EADock DSS docking software and analyzes poses by CHARMM c35b1 FACTS. [27] In all cases, the top-ranked results are considered. The molecular graphics programs UCSF Chimera 1.5.3 [28] and PyMol [23] were used for image analysis and processing.

Results:

Development of resistance mutations to PL-100, APV and DRV in CBMCs and MT-2 cells.

To further investigate the unique mutational pathway of PL-100[16], the wt subtype B clone HIV-1/IIIB was selected for drug resistance in CBMCs with both PL-100 and amprenavir

for 48 weeks. Genotypic analysis confirmed the unique resistance pathway of PL-100, such that a novel T80I mutation appeared first followed by M46I and P81S. A novel K45R change was found in one of the isolates; this mutation has also been observed in patients receiving atazanavir. [29] Accumulation of multiple mutations under PL-100 drug pressure in culture was delayed compared to the selections with amprenavir, against which multiple mutations appeared quickly (Table 1).

Five subtype B clinical isolates that contained six or fewer polymorphisms were also used in selections in CBMCs (Table 2). All viruses passaged in the absence of drug (controls) were sequenced to confirm the absence of any additional mutations. The results show that two distinct resistance pathways are associated with PL-100, one of which involves the T80I mutation while the other involves mutations at either positions 82 and 90 or both (Table 2).

Two subtype B clinical isolates (BK132 and 5326) were used for selection in MT-2 cells (Table 2). At baseline, BK132 displayed polymorphisms at positions I64V, V77I and I93L while isolate 5326 harbored polymorphisms at positions E35D, M36I, R41K, L63P, and H69Q. Tissue culture selection with PL-100 yielded the PR mutations R41K/R, M46L, L63P, V82A, L90M in isolate BK132 and L10I, N37S, K45I/K, I54M in isolate 5326 (Table 2).

We also selected for darunavir resistance using two clinical isolates, 5331 and 5346, in CBMCs. After 40 weeks of passage using darunavir concentrations of 0.025 μM and 0.03μM for 5331 and 5346, respectively, no mutations in PR were revealed by genotypic analysis.

Phenotypic susceptibility in CBMCs

All viruses selected in tissue culture were tested for susceptibility to PIs. Lower and upper clinical cut-offs for resistance of approved PIs were previously established as 1.2-9.4 for nelfinavir, 1.5-19.5 for amprenavir, 6.1-51.2 for lopinavir, 2.5-32.5 for atazanavir, 3.1-22.6 for saquinavir and 10-106.9 for darunavir. [30] The *in vitro* antiviral activity of relevant PIs against various PL-100 selected variants was tested in CBMCs at weeks 8, 20, 25 and 48 (Table 3). All viruses containing mutations conferred low- to high-level resistance to PL-100, but retained susceptibility to nelfinavir, lopinavir, saquinavir and darunavir (Table 3). The viruses selected after 8 and 25 weeks were susceptible to atazanavir while those selected after weeks 20 and 48 showed a 2.9- and 2.4-fold decreased susceptibility, respectively, to atazanavir. Susceptibility to amprenavir was decreased by 6.5 and 3.3 fold in the case of variants selected at weeks 20 and 25, respectively, but full activity was retained with viruses selected at weeks 8 and 48.

One subtype B clinical isolate (5331) containing PL-100 mutations selected in CBMCs as well as two additional subtype B clinical isolates (BK132 and 5326) containing PL-

100 mutations selected in MT-2 cells were phenotypically evaluated for PL-100 drug susceptibility (Table 4). In CBMCs, the 5331 isolate was highly resistant to PL-100 compared to other PIs (Table 4). In MT-2 cells, isolates BK132 and 5326 were resistant to all PIs tested except for darunavir, with FCs being above the lower clinical cut-off (Table 4). Viruses containing the T80I mutation displayed only low-level resistance to the other PIs, while viruses containing V82A and L90M conferred high-level cross-resistance against these drugs (Tables 3 and 4).

Structural Modeling of APV, PL-100 and DRV

We used available crystal structures in the protein databases, PDB ID: 4DQB [22] and PDB ID: 1RPI, to try to understand drug-PR interactions and the effect of mutations in the binding pocket on drug binding. [31] The multi drug resistant (MDR) protease displays an enlarged drug-binding domain relative to wt PR (Figure 1).

Using three independent approaches, three drugs (darunavir, amprenavir and PL-100) were docked into the wt structure (Figure 2a-c and Figure 3a-d) and into the MDR protease (Figure 4a-d). Our docking results show that darunavir has numerous interactions with PR (Figure 3b and d), including hydrogen bonding to the backbone oxygens of A28, D29 and D30 via its di-tetrahydrofuran (di-THF) groups (Figure 2b). Amprenavir binds mainly by hydrogen binding interactions with the backbone oxygens of D30 (Figure 2c). The binding of PL-100 was predominantly based on polar

interactions and delocalised hydrophobic interactions (Figure 2a and Figure 3a and d). All three inhibitors bind in the same region (Figure 2a-c) utilizing similar residues through different interactions (Figure 3a-d). Identified PL-100 mutations all affect the hydrophobicity of the pocket but do not specifically alter the binding of the inhibitor core to protease. Docking of all drugs to MDR protease resulted in different docked conformations with severely reduced binding, indicating low reliability of binding when the D25 and V84 mutations were present (Figure 4a-d). The diphenyl group of PL-100 is flexible around its chiral center and can adjust for changes in hydrophobicity fairly easily without losing its core contacts with I51 and D30.

Discussion

Although newer PIs, such as darunavir, have significantly improved response rates in patients with multiple drug resistance, there is still a need for robust new PIs with better pharmacokinetic and tolerability profiles. We previously showed that resistance to PL-100 in non-B HIV-1 subtypes can emerge via mutations at positions 82 and 90 or, alternatively, via a T80I substitution that appears to be unique to this drug. [17]

The present data demonstrate that resistance-conferring mutations were rapidly selected in viruses passaged in the presence of PL-100, which apparently has a lower barrier for drug resistance than darunavir, for which no resistance mutations were selected over 40 weeks. The high genetic barrier and delayed accumulation of darunavir resistance mutations has also been observed by others. [32, 33] Viruses selected under PL-100 drug pressure showed low to high resistance to PL-100 but retained susceptibility to darunavir

and saquinavir. PL-100 selected for mutations at positions R41K/R, M46L, L63P, V82A and L90M in isolate BK132, and L10I, N37S, K45I/K and I54M in isolate 5326. These mutations caused only a slight increase in the fold change (FC) for darunavir.

Dimerization of HIV protease subunits is an essential process in the proteolytic activity of HIV protease. [34, 35] Darunavir can inhibit PR dimerization *in vitro* in addition to blocking PR activity, and this may contribute to its activity against multiple PI-resistant isolates. [36]

Resistance mutations typically reduce the binding affinity of the inhibitor. [37] In the case of protease, more than one resistance mutation is usually needed for resistance to occur (Figure 1). In the case of amprenavir, the hydrogen bonding of amprenavir to the backbone of D30 is important, [4] while the THF ring of darunavir may increase its number of backbone interactions. [4] Here we have shown that darunavir has a superior resistance profile to PL-100 and attribute this to its binding to the A28, D29 and D30 backbone via its di-THF groups (Figure 2a-c). In contrast, PL-100 has diphenyl groups that bind through delocalised hydrophobic interactions. Thus, hydrogen bonding contacts and the presence of the di-THF group in darunavir, as well as the hydrophobic nature of PL-100, are all important factors in drug binding. A high genetic barrier for drug resistance should be important in the design of new PIs. Redesigning the structure of PL-100 to include a di-THF group might improve its binding and antiviral activity.

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Transparency declarations

None to declare

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Table 1: Order of appearance of resistance mutations in HIV-1/IIIB following selection with PL-100 and Amprenavir in CBMCs

Week	PL-100 (µM)	Selected mutations	Week	Amprenavir (µM)	Selected mutations
8	0.1	T80I	15	2.5	L10F, I84V, Q92K
15	1.0	T80I	20	2.5	Not done
18	1.0	T80I	25[a]	2.5	Not done
20	1.0	M46I/M, T80I	25	5.0	L10F, M46I, I84V, Q92K
25[b]	1.0	K45K/R, M46I, T80I, P81S	34	5.0	L10F, M46I, I84V, Q92K
25	2.5	K45R, M46I, T80I, P81S	48	5.0	L10F, M46I, I84V, Q92K
34	5.0	K45R, M46I, T80I, P81S	NA		
48	5.0	K45R, M46I, T80I, P81S	NA		

NA, Not applicable.

[a]Two wells of IIIB virus were passaged at week 25 in the presence of different concentrations of amprenavir (2.5 and 5.0 uM).

[b]Two wells of IIIB virus were passaged at week 25 in the presence of different concentrations of PL-100 (1.0 and 2.5 uM).

Table 2: Baseline polymorphisms and resistance mutations observed upon passage with PL-100 in subtype B viruses

Virus	Baseline polymorphism (WT)	mutations selected in CBMCs[a]	Week/Passage	mutations selected in MT-2 cells[b]
IIIB	None	K45R, M46I, T80I, P81S	48	NA
5269	I15V	L10I, M46L, V82A	48	NA
5323	A41K	M46L, L63P, V82A, L90M	48	NA
5331	K20R, E35D, R41K, A71V, I93L	L10I, L63P, V82A, L90M	48	NA
5346	T12K, I13V, L23Q, L33I, L63P, V77I	L90M	48	NA
5512	Q7L, K14R, L19L/V, K43K/R, I64V, H69H/Y	T12K, I13V, L33I, M46I, L63P, V77I, T80I	48	NA
BK132	I64V, V77I, I93L	NA	15	M46L, V82A
			30	M46L, L63P, V82A, L90M
			66	R41K/R, M46L, L63P, V82A, L90M
5326	E35D, M36I, R41K, L63P, and H69Q	NA	28	L10I, N37S
			42	L10I, N37S, K45I/K, I54M
			66	L10I, N37S, K45I/K, I54M

NA, Not applicable.

[a] The isolates 5269, 5323, 5331, 5346, 5512 and HIV-1/IIIB were passaged in CBMCs over 48 weeks.

[b] The isolates BK132 and 5326 were passaged in MT-2 cells for 66 passages.

Table 3: *In vitro* activity in CBMCs of various PIs against PL-100-selected mutants in HIV/IIIB.

Week	Genotype	Mean EC_{50} (μM) fold change (FC)						
		nelfinavir	amprenavir	lopinavir	atazanavir	PL-100	darunavir	saquinavir
Lower and upper clinical cut offs		1.2-9.4	1.5-19.5	6.1-51.2	2.5-32.5	2.5[a]	10-106.9	3.1-22.6
Wk 0		0.051 ± 0.020	0.164 ± 0.030	0.060 ± 0.010	0.009 ± 0.001	0.043 ± 0.005	0.009 ± 0.001	0.054 ± 0.010
Wk 8	T80I	0.006 ± 0.001 (0.11)	0.324 ± 0.020 (2.00)	0.016 ± 0.010 (0.26)	0.004 ± 0.001 (0.44)	0.150 ± 0.080 (3.49)	0.002 ± 0.001 (0.22)	0.011 ± 0.002 (0.20)
Wk20	M46I/M, T80I	0.038 ± 0.004 (0.74)	1.065 ± 0.050 (6.50)	0.034 ± 0.010 (0.57)	0.026 ± 0.003 (2.90)	0.563 ± 0.160 (13.1)	0.011 ± 0.001 (1.20)	0.028 ± 0.002 (0.52)
Wk25	K45K/R, M46I, T80I, P81S	0.022 ± 0.008 (0.43)	0.541 ± 0.023 (3.30)	0.018 ± 0.010 (0.30)	0.016 ± 0.008 (1.80)	0.641 ± 0.260 (15.0)	0.002 ± 0.001 (0.22)	0.036 ± 0.001 (0.66)
Wk48	K45R, M46I, T80I, P81S	0.029 ± 0.010 (0.56)	0.328 ± 0.010 (2.00)	0.024 ± 0.009 (0.40)	0.022 ± 0.001 (2.40)	0.930 ± 0.170 (21.6)	0.005 ± 0.003 (0.60)	0.065 ± 0.006 (1.20)

[a]Arbitrarily chosen lower cut-off value of PL-100 for this analysis pending availability of clinical data.

Table 4: *In vitro* activity in CBMCs or MT-2 cells of various PIs against viruses containing relevant resistance mutations.

Virus	Mutations	Subtype	Mean EC_{50} (μM) fold change (FC)						
			nelfinavir	amprenavir	lopinavir	atazanavir	PL-100	darunavir	saquinavir
Lower and upper clinical cut offs			1.2-9.4	1.5-19.5	6.1-51.2	2.5-32.5	2.5[a]	10-106.9	3.1-22.6
5331	Wt	B	0.028 ± 0.006	0.035 ± 0.009	0.019 ± 0.009	0.005 ± 0.002	0.017 ± 0.010	0.007 ± 0.002	0.058 ± 0.001
5331	L10I, V82A, L90M	B	0.045 ± 0.002 (1.60)	0.034 ± 0.003 (0.97)	0.039 ± 0.001 (2.05)	0.004 ± 0.001 (0.80)	0.146 ± 0.08 (8.60)	0.002 ± 0.001 (0.29)	0.036 ± 0.009 (0.62)
BK132	wt	B	0.019 ± 0.002	0.020 ± 0.001	0.012 ± 0.0002	0.008 ± 0.001	0.011 ± 0.003	0.005 ± 0.001	ND
BK132	R41K/R, M46L, L63P, V82A, L90M	B	0.082 ± 0.010 (4.30)	0.550 ± 0.020 (27.5)	0.286 ± 0.001 (23.8)	0.062 ± 0.002 (7.79)	0.132 ± 0.030 (12.0)	0.022 ± 0.001 (4.46)	ND
5326	wt	B	0.015 ± 0.003	0.018 ± 0.0001	0.014 ± 0.002	0.007 ± 0.001	0.009 ± 0.002	0.004 ± 0.001	ND
5326	L10I, N37S, K45I/K, I54M	B	0.176 ± 0.002 (11.7)	0.130 ± 0.001 (7.20)	0.080 ± 0.002 (5.70)	0.067 ± 0.003 (9.60)	0.062 ± 0.001 (6.90)	0.028 ± 0.003 (6.90)	ND

ND, Not determined

Polymorphisms at baseline for isolates 5331: K20R, E35D, R41K, A71V and I93L; BK132: I64V, V77I and I93L; 5326: E35D, M36I, R41K, L63P and H69Q.

[a]Arbitrarily chosen lower cut-off value of PL-100 for this analysis pending availability of clinical data.

Figure 1:

Comparison of the size of the inhibitor binding cavity in wt (monomers colored green and light blue) (above) and MDR protease (monomers coloured grey and light brown) (below). The surface of the binding cavity is shown as an opaque surface within the partially transparent dimer in both cases. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*. The figure was generated in PyMOL[23] using protein databank (PDB) files 4DQB[22] and 1RPI[31].

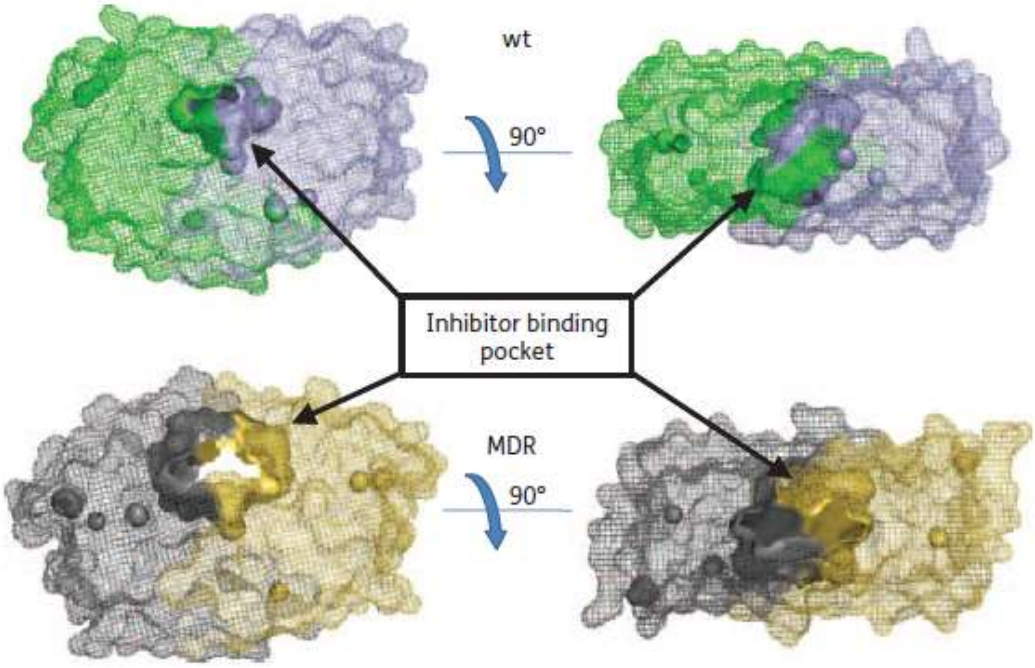

wt
90°
Inhibitor binding pocket
MDR
90°

Figure 2:

PR-inhibitor interactions calculated after docking simulations of (a) PL-100, (b) darunavir and (c) amprenavir to wt PR (grey ribbons) using AutoDock vina.[24] Ligands are shown as transparent space-filling and solid stick models colored based on standard atomic coloration; red shows acidic oxygens and blue represents basic nitrogen atoms; polar hydrogens are shown in white. Hydrogen binding interactions are shown by a solid black line and other residue-ligand contacts are shown by yellow-coloured carbon atoms on the ligand as well as by a solid green line for strong ligand-residue contacts. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*. Determination of residue-ligand contacts and image processing was performed using UCSF Chimera.[28]

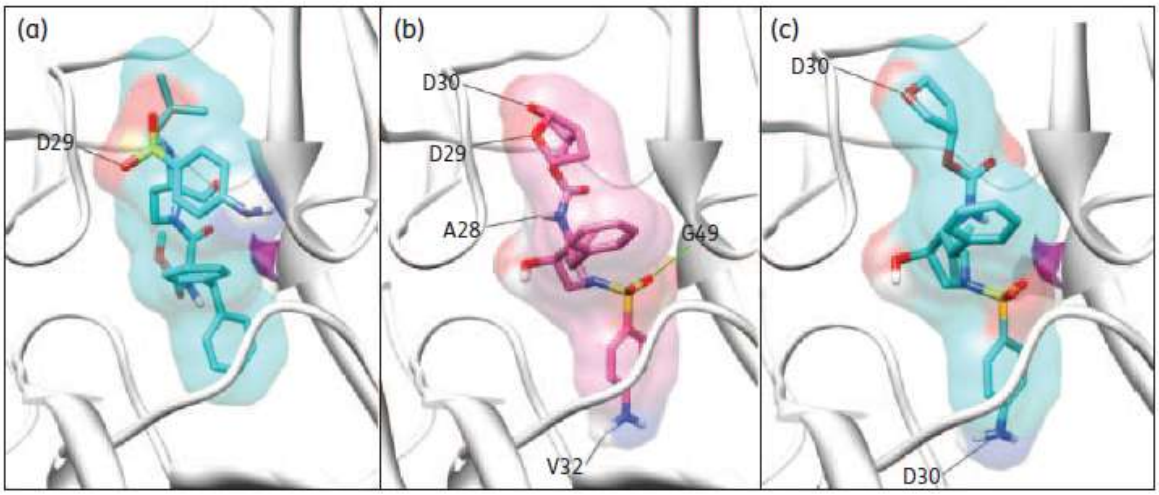
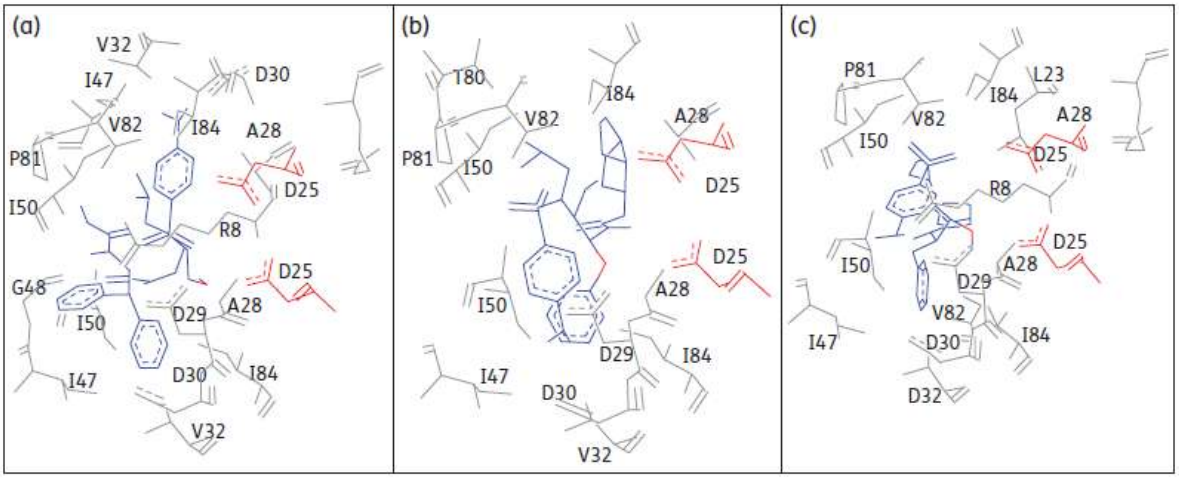

(a)
D29
(b)
D30
D29
A28
G49
V32
(c)
D30
D30

Figure 3:

Extensive wt PR-inhibitor interactions determined using DockingServer [25] for (a) PL-100, (b) darunavir, (c) amprenavir. Wire-trace outlines of inhibitor (dark blue) and protease residues (grey) with catalytic residue D25 are in red. (d) Classification of ligand-residue interactions. Residue colour code: Pink- mutated during selections with a specific drug; green- not mutated during selections but next to position of PR mutated during selections; orange- not mutated or not near mutated position, but previously reported to be involved in drug resistance. This figure appears in colour in the online version of *JAC* and in black and white in the printed version of *JAC*.



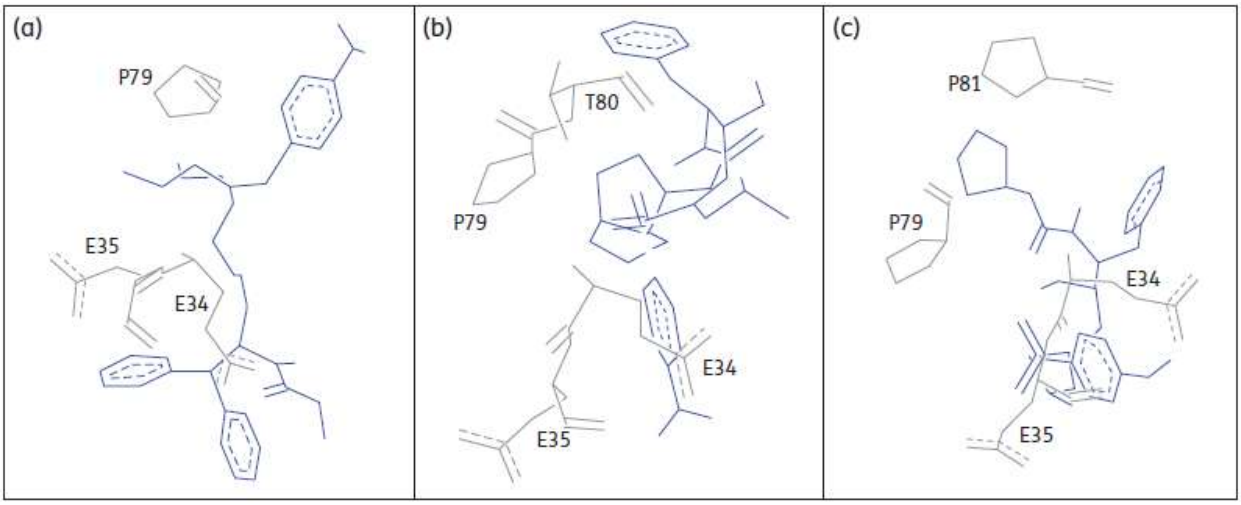
(d)

Ligand	PR–inhibitor interactions			
	H-bonding	Polar	Hydrophobic	Other*
PL-100	G48, D30, I50	D25	A28, V32, I47, I50, P81, V82, I84	R8, D25, D29, D30,
DRV	D29	D25, D30	A28, V32, I47, I50, P81, V82, I84	D25, D29, D30, T80, P81, V82, I 84
APV	D29	R8, D25, D29, D30	V32, I47, I50, I84	D25, A28, D29, D30, L23, I50, P81, V82, I84

*Any of the following interactions; Pi:Pi, cation:Pi or indirect interactions.

Figure 4:

Extensive MDR PR-inhibitor interactions determined using DockingServer [25]for (a) PL-100, (a) darunavir, (a) amprenavir. Wire-trace outlines of inhibitor (dark blue) and PR residues (grey) are shown. (d) Classification of ligand-residue interactions. Residue colour code: Pink- mutated during selections with specific drug; green- not mutated during selections but next to a position mutated during drug selections. This figure appears in color in the online version of *JAC* and in black and white in the printed version of *JAC*.



(d)

Ligand	Calculated PR-inhibitor interactions			
	H-bonds	Polar	Hydrophobic	Other*
PL-100	-	-	P79	E34, E35, P79
DRV	-	-	-	E34, E35, P79, T80
APV	E34, E35	-	P79, P81	E34, E35, P81

* Any of the following interactions; Pi:Pi, cation:Pi or indirect interactions.

Chapter 6

General Discussion

(Sections of this chapter were published in a review "Antiviral Drug Resistance and the Need for Development of New HIV-1 Reverse Transcriptase Inhibitors", authored by Asahchop EL, Wainberg MA, Sloan RD, Tremblay CL. Antimicrobial Agents and Chemotherapy. June 2012).

Until recently, only three antiretroviral drug classes were available for treatment of HIV-1 infected patients; NRTIs, NNRTIs and PIs. Resistance within these classes of drugs and extended cross-resistance among drug classes has also been observed. Thus, the management of multiclass-resistant HIV-1 infection has been a major challenge to clinicians treating HIV-1-infected patients, due to limited options for combination therapy. The advent of new antiretroviral drugs against new targets (fusion inhibitors, integrease inhibitors, R5 coreceptor antagonists) and new-generation drugs within available classes (second generation NNRTIs and PIs with higher genetic barriers for resistance and different resistance profiles) has expanded therapeutic options for patients with multiclass drug-resistant HIV-1. While some of the second generation NNRTIs (ETR and RPV) and PIs (DRV and TPV) have been approved for clinical use, some are under pre-clinical (PL-100) investigation. Drugs against new targets and second generation antiretrovirals maybe useful in salvage therapy, if patients have failed to respond to therapy, due to the presence of resistance mutations selected during first line therapy. We wished to explore the role of naturally occurring polymorphisms

(represented in different subtypes) and baseline resistance mutations in determining the mutational pathways and resistance patterns of some second generation NNRTIs and PIs.

Resistance to ETR and RPV

In Chapter two, as reported by Asahchop et al 2011, we provided a comprehensive *in vitro* analysis of the resistance pattern to ETR in wt clinical isolates of subtype B, subtype C, and CRF02_AG grown over 25 to 30 weeks in CBMCs in increasing concentrations of ETR compared to EFV. Our data show that ETR RAMs emerged after 18 weeks of drug pressure, with the exception of one isolate (BK132) that selected for E138E/K at weeks 11 to 13. The E138K mutation was selected in all isolates and was almost always the first mutation to emerge. The selection of E138K under ETR drug pressure has also been observed *in vitro* and in clinical trials by other groups [250,270]. We also showed that site-directed mutagenesis of E138K revealed low-level resistance to ETR in both pNL4-3 and AG plasmids, with a fold change slightly above the clinical cut-off. Amino acid substitutions at position 138 of the RT have also been described in ETR therapy [270], all of which equally showed low-level resistance to ETR [270,278]. Although the presence of one of these mutations at position 138 on its own is not enough to exclude the use of ETR, its presence may increase the level of resistance to ETR and/or allow further selection of other resistance mutations. Furthermore, genotypic resistance in the DUET studies have confirmed the role of E138 mutations in patients failing ETR, demonstrating an increased FC for ETR over the upper clinical cut-off (CCO) for resistance to ETR (>13). Other ETR RAMs observed in our study included V90I, K101Q, E138K, V179D/E/F, Y181C, V189I, G190E, H221Y, and M230L. There was no difference in the pattern of mutations selected by ETR in B and non-B subtypes.

Although ETR is available in developed countries where subtype B predominates, it will be important to validate the results of the present study in resource limited setting where non-B subtypes circulate. E138K was recently observed as the most frequent NNRTI mutation together with the NRTI mutation M184I/V among HIV-1 naïve patients who failed RPV (an NNRTI of the same family and binding mode as ETR) in combination with either FTC or 3TC in the ECHO and THRIVE trials [279,280]. These phase 3 clinical studies are in agreement with our tissue culture observations and demonstrate the patterns of resistance mutations that would be seen in HIV-1 naïve patients experiencing failure upon ETR treatment in first line therapy. However, ETR is not usually used for treatment of HIV-1 naïve patients; this probably explains differences in patterns of resistance to ETR in our study and the DUET study.

We also observed diminished replication capacity in the case of pNL4-3 and AG viruses containing E138K compared to wt virus and this has been confirmed by other studies [408,409]. The replication capacity of the most frequently observed double mutants in the ECHO and THRIVE studies (E138K/M184I and E138K/M184V) was also found to be comparable to that of wt virus [408,409]. The presence of these double mutants enhanced resistance to ETR and RPV, compared to E138K alone, and suggests a mutual compensation between E138K and the M184I/V mutations [408,409]. The combination of these two mutations results in a highly fit virus and might enhance HIV transmission rates. E138K was also shown to decrease dNTP usage and to compensate for the defect

in dNTP usage and the diminished replication capacity of M184I/V [408]. Another group has analyzed the fitness of these double mutants using growth competition assays in MT-2 cells and showed that the E138K/M184I mutant was less fit than wt or the single mutants [410]. This result contradicts the observation by our group and others. However, additional studies will be necessary to clearly demonstrate how this double mutant impacts on fitness. Although still a subject of further investigation, the public health and epidemiological consequence of E138K/M184I may be detrimental in regards to transmission rate if confirmed to be as fit as wt. Biochemical analysis by our group and others has shown that E138K confers resistance through the p51 subunit of RT, while the presence of E138K and M184I enhanced dNTP usage via the p51 and p66 subunit of RT [411,412]. Our group has also shown that E138K affects the enzymatic activity through the p51 subunit of RT by decreasing RNase H activity and the polymerization rate of RT (Figure 9C) [411]. The mechanism of resistance to ETR or RPV by E138K has been shown to occur through the p51 subunit of RT whereby this mutation leads to a larger increase in dissociation rate of inhibitor, which overcomes the enhanced rate of association (Figure 9B) [412]. Molecular modeling have also revealed that a salt bridge naturally exist between p51(E138) and p66(K101) [412]. This salt bridge has been shown to be disrupted in the presence of E138K, specifically in the p51 subunit of RT, resulting in a large gap at the bottom of the NNRTI binding pocket and repositioning of RPV in the binding pocket [412]. Since E138K of p51 subunit is located in the entrance of the NNRTI binding pocket, this demonstrates that E138K confers resistance to second generation NNRTIs by interrupting with binding at the entrance of the pocket. Recent *in vitro* experiments and analysis of patient PBMCs demonstrated the pre-existence of

E138K and M184I mutations in proviral reservoirs at a relatively high frequency prior to drug exposure as a result of APOBEC3 editing [413]. These mutations may be co-selected in individuals with polymorphisms in APOBEC3 and in a defective HIV Vif variant [413,414]. The implication of these pre-existing mutations selected by APOBEC3 may be the rapid failure of therapy containing RPV or ETR. This further shows the importance of these two mutations and their role in resistance.

We showed that EFV selected for mutations faster than ETR after 12 weeks of EFV drug pressure. The mutations selected included; V106M in subtype C and V106I in subtype B. EFV and ETR specifically selected for G190A and G190E, respectively. This further demonstrates the non-association of G190A with ETR resistance as shown in another study [415]. However, G190E has been shown to be selected by both ETR and RPV [264,278]. Site-directed mutants containing the E138K mutation showed an FC below the CCO of EFV. This is consistent with the non-association of E138K with EFV resistance as observed both in treated patients and in *in vitro* selection experiments. However, one study has observed the selection of E138K after *in vitro* passage experiment of viruses containing mutations at position 135 at baseline [416]. These authors demonstrated FCs for EFV of 2 and 7 for E138K and I135T/E138K, respectively. So far, this is the only study demonstrating the selection of E138K under EFV drug pressure in a background of variants containing amino acid substitutions at position 135 of the RT. It is still unclear why first generation NNRTIs (NVP and EFV) did not select for E138K in a wt virus. One of the possible reasons could be differences in the structure of first (NVP and EFV) and

second (ETR and RPV) generation NNRTIs. Structural modeling studies have shown that second generation NNRTIs bind through the amino acid position 138 in the p51 subunit that is located at the entrance of the binding pocket. Mutations at this position will interfere with binding and reposition the inhibitor. This has only been demonstrated with second generation and not first generation NNRTIs. First generation NNRTIs such as EFV binds to the NNRTI binding pocket using amino acid positions in the p66 subunit of RT [417].

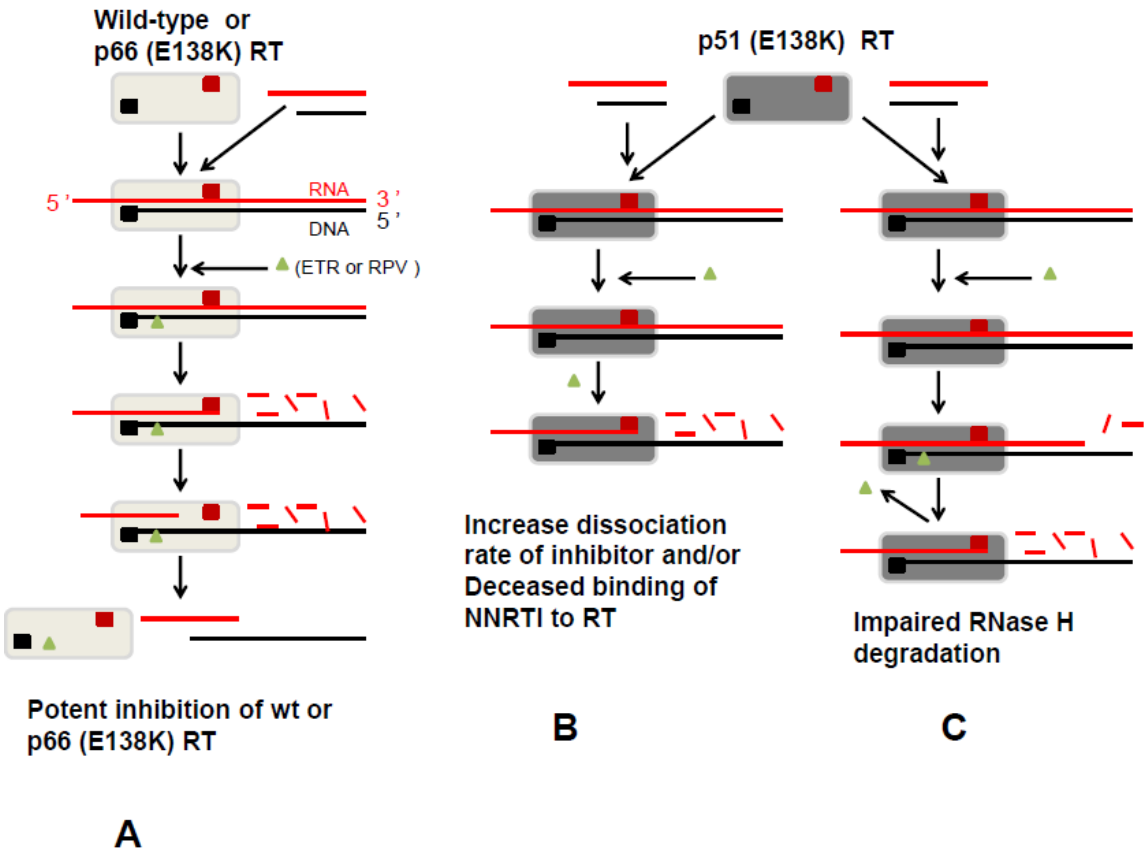


Figure 9: Proposed mechanism of resistance of the E138K mutation to ETR or RPV and impact on enzyme function. (Adapted from Menéndez-Arias L *et al* 2011 [303]). In the presence of wt or p66 (E138K) RT, a potent inhibition is observed (A), while in the presence of p51 (E138K) there is impaired inhibition by large increase in dissociation of the inhibitor and/or decreased binding of NNRTI to RT (B). E138K in the p51 unit affects enzyme function by reducing RNase H activity (C).

In Chapter 3, HIV-1 resistance patterns following selection with ETR and RPV in both wt viruses as well as in viruses containing a variety of NNRTI mutations at baseline was explored. Specifically, the basis for the preferential selection of E138K by ETR in wt viruses compared to the selection of other NNRTI mutations in the DUET clinical studies was examined. In addition, we wished to investigate whether HIV-1 that already contained E138K, M184I/V or E138K plus M184I/V could develop additional resistance mutations in the presence of ETR or RPV.

Our *in vitro* passage experiments showed that E138K was selected by each of wt, K103N- or G190A-containing virus at baseline in the presence of ETR or RPV. In contrast, the exposure of ETR or RPV to subtype B viruses containing Y181C at baseline selected for V179I/F or A62A/V, respectively, but not E138K. In the DUET studies that led to the approval of ETR for use in NNRTI-experienced patients, only three subtype B patients who previously harbored K103N and who developed E138K failed ETR therapy, while one patient who harbored Y181C developed E138V [275]. A different study showed that 12 of 42 ETR failures who harbored mutations at position 181 at baseline

contained at least one new NNRTI mutation but not E138K [418]. In an *in vitro* selection study using a drug of the same family (TMC120), E138K was selected in wt viruses but not in viruses containing Y181C at baseline [287]. Phenotyping of viral clones containing E138K, Y181C or E138K/Y181C showed a fold-change in resistance for Y181C and E138K/Y181C that was not significantly different. Considering that both E138K and Y181C individually confer resistance to either ETR or RPV, one would expect to observe an enhanced fold-change in the presence of both mutations compared to each of them alone. Additionally, the replication capacity of Y181C and E138K/Y181C compared to wt virus was impaired by 1.5 and 3 fold, respectively. Taken together, this implies that in a background of Y181C-containing virus, the emergence of E138K does not give the virus any additional advantage in terms of resistance and/or fitness. Genotyping analyses of samples from the DUET studies have shown that none of the three patients who failed ETR therapy and selected for E138K had a background of Y181C-containing virus [270]. The three patients who selected for E138K had different mutations at baseline. The fact that a larger proportion of patients in the DUET studies were NNRTI-experienced, and possessed Y181C mutation at baseline, could be the basis for the rare selection of E138K. In another clinical study in NNRTI-experienced patients, it was also shown that patients who failed ETR therapy and harbored a background virus containing Y181C selected for mutations at position 179 and not E138K [418]. The present study, together with evidence from other clinical studies, suggests that Y181C may be antagonistic to E138K. As shown here, the combination of Y181C and E138K may lead to a less fit virus compared to virus containing Y181C alone. This could be part of the reason why viruses containing a Y181C background could not select for E138K in the presence of ETR or

RPV since this combination will impair fitness and improve the susceptibility to these drugs.

Biochemical analyses have confirmed this proposed antagonism. In this analysis, it was shown that Y181C conferred antagonism to E138K by decreasing dNTP usage, and impaired enzyme activity and processivity [419]. In the results presented in chapter 2, we showed that E138K was the first mutation to emerge after ETR pressure in most isolates followed by accumulation of Y181C and amino acid substitutions at position 179. In an *in vitro* study, it was also shown that RPV selected for E138K in a viral clone containing V179F/Y181C at baseline [278]. This suggests that the observed antagonism was possibly reversed in the presence of amino acid substitutions at position 179. Biochemical analysis, cell culture and structural modeling studies of the triple mutant E138K/Y181C/V179I/F are needed to help provide a clear picture of how these mutations interact.

The complex formed between RPV and wt RT is stable and comparable to the complex between RPV and K103N mutant RT. In the presence of RPV and mutant RT containing Y181C, there is loss of aromatic interactions resulting in a shift of RPV further into the NNRTI pocket [420]. We thus hypothesize that differences in binding interactions that exist between RPV or ETR and the Y181C RT mutant but not in other mutants is the basis of this antagonism. It is not known if the loss of interaction between the RT mutant containing Y181C and inhibitor enhances the bond that naturally exists between E138 and K101. Additional modeling studies with E138K/Y181C will further explain if RT mutants containing Y181C will prevent the emergence of E138K by stabilizing the salt

bridge between E138 and K101. This will further explain the mechanistic basis of antagonism of Y181C on E138K.

The wt virus under RPV drug pressure showed an intermittent mixture of K101 and E101 that was dominated by E138K at the end of the selection experiment. This could be due to the presence of quasi-species that existed in the viral population. This suggests that K101E and E138K cannot exist in the same viral clone, possibly due to the presence of a salt bridge between K101 and E138 [412]. Both E138K and K101E are resistance mutations associated with decreased susceptibility to ETR and RPV [270,421]. The identification of these two mutations in the same viral clone has not been possible due to that fact that both mutations in a viral clone will establish the same salt bridge as is the case with wt virus. Thus, in a viral population the mutation that results to the most fit virus, either in the presence or absence of drugs, will dominate. So far, fitness studies to compare viruses containing E138K and K101E are still not available. It seems likely that viruses containing E138K have a fitness advantage over K101E because of its predominance both in clinical studies and in cell culture experiments. Fitness studies will be necessary to confirm this. The viral clones containing M184I/V at baseline did not select for NNRTI mutations after exposure to RPV, as observed with ETR in a recent *in vitro* study [411]. This could be due to decreased viral fitness of both clones and difficulties in increasing RPV drug concentration. The results presented here are consistent with those from a previous study, in which viruses containing the M184V mutation were shown to be less likely to select for mutations under drug pressure [422].

Based on the results presented in this thesis and the results of others, we have proposed that different patterns of mutations selected by ETR or RPV may exist in wt virus and in viruses containing some of the most common NNRTI mutations (Figure 10). Panel A represents the E138K pathway while panel B represents the K101E pathway. These two pathways are mutually exclusive. Panel C is a pathway that is specific to Y181C alone. However, the proposed patterns of mutations are not exhaustive. Additional mutations are been identified both in *in vitro* studies and in treated patients.

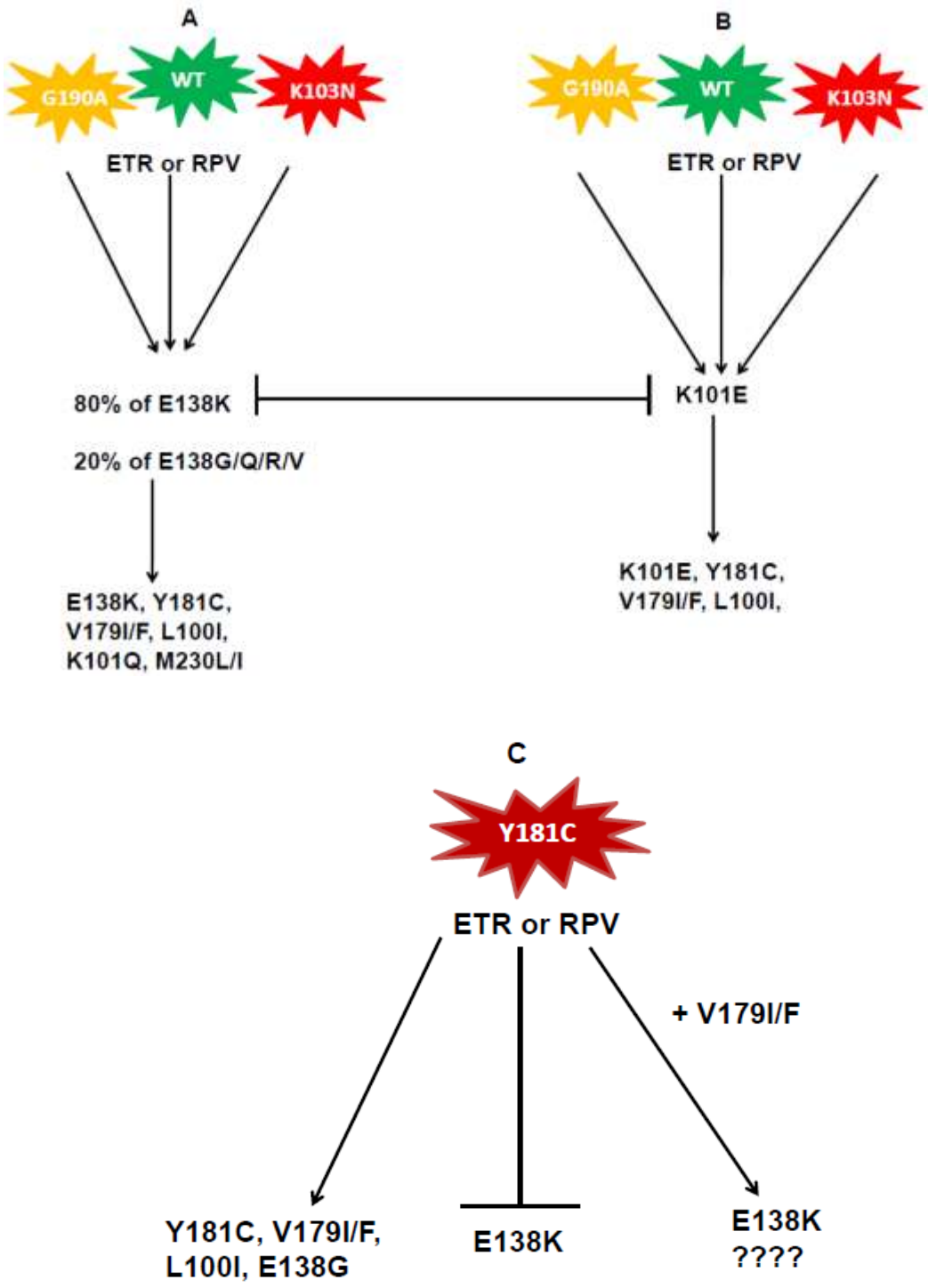


Figure 10: Proposed patterns of emergence of resistance -associated mutations for either ETR or RPV.

A number of studies have observed high level cross-resistance to ETR and RPV [421,423]. In addition, due to similarity in binding, the same mutations are selected in the presence of RPV or ETR, resulting in high-level cross-resistance [278]. In the context of the current study, it seems obvious that patients, who fail RPV because of mutations that are associated with resistance to both ETR and RPV, will be unlikely to derive future benefit from ETR. In a review by Asahchop *et al* 2012, it was reported that "Several studies have researched the theoretical potential of ETR based on the resistance patterns of patients who previously failed NNRTI therapy and accumulated ETR RAMs. These studies have shown a prevalence of more than three ETR RAMs among viral isolates from patients experiencing NNRTI treatment failure, ranging from 4.6% to 10%, while the prevalence of isolates with single ETR RAMs was 17.4% to 35.9% [424,425,426,427]. These studies concluded that there is a low prevalence of ETR resistance at baseline and that patients with prior failure to NNRTIs could potentially benefit from ETR rescue therapy. However, these analyses focused on patients in developed countries that had full access to the most potent antiretroviral drugs who were constantly monitored for viral load and the development of resistance".

In the same study by Asahchop *et al* 2012, the authors reported that, "patients in countries with limited resources often develop resistance faster due to a lack of potent antiretroviral drugs, access to viral load, and drug resistance testing. Some studies in resource limited settings have observed a high prevalence of NNRTI resistance mutations associated with ETR resistance among patients failing an NNRTI containing regimen

[428,429,430]. Using NVP in the failing regimen was associated with intermediate and reduced response to ETR while use of EFV and co-administration of 3TC reduced the risk of ETR resistance [430]. The authors concluded that the frequent occurrence of NNRTI mutations in resource limited settings in which drug resistance testing is rare might compromise the continuous use of ETR and also its use in second line therapy. ETR should be avoided in second line treatment in developing country settings where drug resistance testing is not performed and NVP is used for first-line therapy".

Standard population genotyping is used to routinely detect genotypic resistance in patients failing therapy. However, this technique cannot detect viral variants containing less than 20% of the viral population [384]. Sensitive assays have been shown to detect viral variants as low as 0.1% of the viral population [386,395,431]. The existence of low frequencies of HIV-1 variants in infected patients has been demonstrated to impact susceptibility to antiretrovirals including ETR [392,393]. Transmitted resistance mutations occur frequently in primary HIV-1 infection and most of these, when transmitted, exist as minority variants. Clinical studies that explore the impact of minority variants on second generation NNRTIs (ETR and RPV) are not available. The selection of some resistance mutations in the presence of ETR or RPV is rapid and facilitates the development of additional mutations within the genome. It is likely that the presence of mutations at low frequency before administration of therapy containing either ETR or RPV will result in the accumulation of mutations that will dominate the viral population

and lead to therapy failure. This subject should be further confirmed through clinical studies.

In the present study we used population genotyping to identify resistance mutations. It is likely possible that some ETR and RPV mutations were present at low frequencies such that population genotyping was not sensitive enough to detect the presence of these minority species. A limitation to our study is the fact we did not use sensitive assays such as ultra deep pyrosequencing or allele specific real time PCR to detect low frequency mutants.

RPV is approved for treatment of HIV-1 infected NNRTI-naïve patients while ETR is approved for treatment of NNRTI-experienced patients. Both drugs are of the same family, and possess a similar binding mode within the NNRTI binding pocket and genetic barrier to resistance. At the time of approval of ETR, little was known about its resistance profile. We and others have identified mutations that confer resistance to both ETR and RPV. It is thus obvious that cross-resistance can occur among these drugs. In addition, the frequent use of NVP in developing countries makes it difficult to believe that ETR will be effective in NNRTI-experienced patients. The future use of ETR may be jeopardized if it is used only in NNRTI-experience patients, because people who fail a RPV containing regimen in first line therapy will not benefit from ETR therapy. It should also be noted that the use of two NRTIs in combination with ETR in NNRTI-experienced patients has resulted in therapy failure because of the presence of NRTI and NNRTI mutations at baseline [277,432]. Based on the results presented here and those of others,

ETR could be recommended for treatment of NNRTI-naïve patients. If ETR is considered for second line therapy, genotyping of patient samples is necessary to identify the presence of any mutations associated with ETR failure. In addition, combinations of a protease and an integrase inhibitor could be included in ETR containing regimens for treatment of NNRTI-experienced patients.

Resistance to PL-100 and DRV

In Chapters 4 and 5, we described the resistance profile of PL-100 and compared it to both first and second generation approved PIs. Chapter 4 specifically compares the resistance profile of PL-100 to approved first generation PIs. Asahchop *et al* 2010 reported that "resistance to PL-100 emerged via mutations at positions 82 and 90 or, alternatively, via a T80I substitution that appears to be unique to PL-100. The most common mutations selected by PL-100 were at positions 46, 80, 82, and 90". Amongst these mutations, T80I appears to be novel, while M46I/L, V82A, and L90M have previously been described in patients failing SQV, NFV, APV, LPV and ATV [322,328,433,434,435,436]. Resistance to PL-100 can occur through two pathways: the first (T80I) is important and specific only to PL-100 while the second (V82A and L90M) pathway is common to other PIs (Figure 11). This raises the possibility of cross-resistance in the class and precludes the sequential use of PL-100 and any other first generation PI. The two pathways were mutually exclusive.

Our study in Chapter 4 also demonstrates that the pattern of resistance to PL-100 in subtype C and CRF01_AE was similar and that the development of resistance to PL-100 was delayed compared to that with first-generation PIs, as previously described [437]. The K55R and V85I mutations were intermittently co-selected in tissue culture in the presence of PL-100 drug pressure. Although site-directed mutagenesis of these substitutions either alone or in combination, showed susceptibility to all PIs tested with the exception of NFV, they might be considered to be secondary PI mutations. It is also thought that K55R and V85I are able to restore viral replication capacity [438]. Thus, these mutations might not really have a role in resistance to PIs but rather in restoring viral replication capacity.

The sequential use of PIs in PI-experience patients is limited by the high level of cross-resistance amongst first generation PIs. Although our study in chapter 4 has confirmed that PL-100 possess a slightly higher or equivalent genetic barrier compared to first generation PIs, the emergence of mutations that confer resistance was not significantly delayed in the presence of PL-100. Furthermore, some of the mutations selected by PL-100 are mutations already observed in patients who fail therapy containing a first generation PIs. It therefore seems possible that PL-100 might not be effective in patients who fail therapy containing a first generation PI.

The introduction of the second generation PIs DRV and Tipranavir (TPV) that possesses a high genetic barrier to development of resistance and potent activity against multiple

PI-resistance mutations has dramatically enhanced the response of PI-experienced patients. There is thus the need for robust second generation PIs with activity equivalent to DRV and TPV or even better. As a follow up of Chapter 4, we compared the resistance profile of PL-100 to a second generation PI (DRV) (Chapter 5). Using the lab strain HIV-1/IIIB and clinical isolates we determined the time to development of resistance to PL-100, APV and DRV. Genotypic analysis of the protease gene of the lab strain HIV-1/IIIB showed that the first mutation selected was T80I at week 8. At week 18 and beyond, the mutations K45R, M46I, and P81S were selected. We experienced difficulty in selecting for DRV resistance after 40 weeks using wt clinical isolates. Other groups have also experienced difficulties in selecting for DRV-resistance mutations or for the accumulation of mutations at passage 30 and beyond [439,440]. Phenotypic susceptibility of HIV-1 variants selected under PL-100 drug pressure showed low- to high-level resistance to PL-100, while all variants selected retained susceptibility to DRV and SQV. This is probably due to the high genetic barrier of DRV and the fact that mutations selected by PL-100 are different from DRV-resistance mutations. An important aspect of proteolytic activity of HIV protease is the dimerization of HIV protease subunits [32,112]. DRV can inhibit PR dimerization *in vitro* in addition to blocking PR activity, and this may contribute to its activity against multiple PI-resistant isolates [441].

The Gag cleavage site mutations have been shown to contribute to resistance and loss of fitness in patients failing PI containing regimen [348,350]. Despite this evidence, Gag is not routinely sequenced to monitor the development of resistance in patients failing a PI containing regimen. A limitation to our study is that we did not sequence the *gag* gene to identify mutations selected by any of the PIs used.

Resistance mutations typically reduce binding affinity of an inhibitor by removing an interactive side-chain or by making the active site/inhibitor binding domain more open and solvent exposed; this causes easier dissociation of the inhibitor from the enzyme and thus reduces overall inhibition [117]. In the case of protease, more than one resistance mutation is usually needed for resistance to occur. We have shown that APV possess hydrogen-bonding with the backbone of D30 of protease, and DRV has increased the number of backbone interactions due the presence of its tetrahydrofuran (THF) ring, resulting in higher potency and a higher genetic barrier for resistance. PL-100 has diphenyl groups that bind through delocalized hydrophobic interactions and account for its slightly higher genetic barrier to development of resistance, relative to APV, because the HIV-1 protease has a hydrophobic core. The improved genetic barrier to resistance of PL-100, compared with first generation PIs, is because the diphenyl group of PL100 is flexible around its chiral center and can adjust for changes in hydrophobicity fairly easily without losing its core contacts with I51 and D30. The difficulty of selecting DRV resistant mutants in our cell culture experiments could be due to the presence of a di-THF group that is involved in the binding of the inhibitor to the enzyme. Our results presented here demonstrate that PL-100 might not be of clinical advantage in the treatment of HIV infected patients compared to the use of approved first generation PIs.

Thus, hydrogen bonding contacts and the presence of the di-THF group in DRV, as well as the hydrophobic nature of PL-100, are all important factors in drug binding. A high genetic barrier for drug resistance should be important in the design of new PIs. Redesigning the structure of PL-100 to include a di-THF group might improve its binding and antiviral activity.

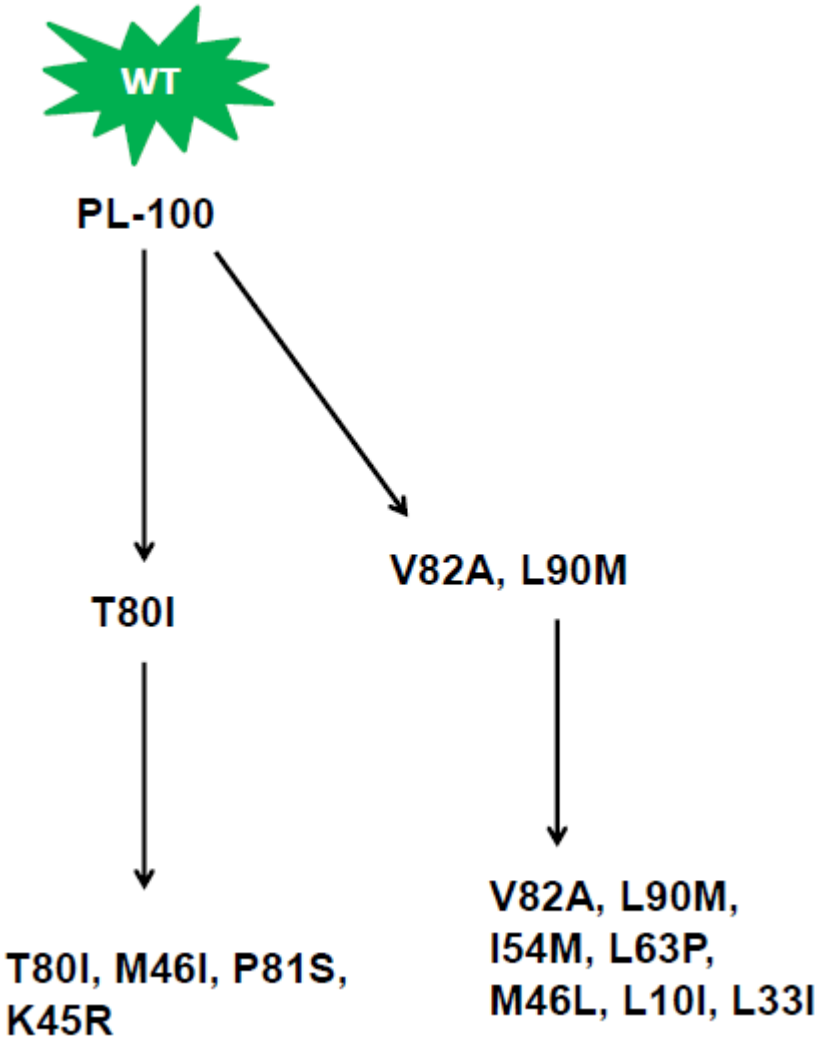


Figure 11: The resistance pathways of PL-100.

Conclusion

We have examined the resistance pattern to second generation NNRTIs (ETR and RPV) and the potential of PL-100 as a second generation PI. Our *in vitro* analysis demonstrated the different patterns of resistance to ETR or RPV in a wt virus compared to a virus containing Y181C at baseline. E138K was the first to emerge and most frequently observed in wt virus of all subtypes tested on exposure to ERT or RPV drug pressure. There was no difference in the pattern of mutations observed in the different subtypes. The fact that viruses containing Y181C mutation at baseline are less likely to develop E138K suggests that Y181C is antagonistic to E138K. Figure 9B shows the proposed mechanism of resistance of E138K to ETR and RPV. PL-100 on its part showed a resistance profile that is comparable or better than the resistance profile of old PIs. *In vitro* and structural modeling studies demonstrated that PL-100 possesses a lower genetic barrier to resistance compared to DRV and suggest that redesigning the structure of PL-100 to include a di-THF group of DRV might improve it genetic barrier.

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