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Resistance and HIV infection: Resistance development against two new drug candidates and natural variability of the cofactor LEDGF/p75

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Chapter 2. Rationale and aims of the study

Chapter 3. Materials and methods

Chapter 4. Results

AIDS	Acquired Immune Deficiency Syndrome
ATR	Ataxia-Telangiectasia and Rad3-related
AZT	3'-Azido-3'-deoxythymidine
BAF	Barrier-to-Autointegration-Factor
bp	base pair
CA	Capsid (protein)
CBP	cAMP response element-Binding Protein
CC_{50}	50% Cytotoxic Concentration
$CCID_{50}$	50% Cell Culture Infective Dose
Cdc2	Cell division control protein 2
CDK9	Cyclin Dependent Kinase 9
cDNA	copy DNA
Chk1	Serine/threonine protein Kinase
CPE	CytoPathic Effect
CVT	Chimeric Virus Technology
DC	Dendritic Cell
DKA	DiKeto Acid
DMSO	DiMethylSulfoxide
DNA	DeoxyriboNucleic Acid
DS	Dextran Sulfate
DTT	DiThioThreitol
EC_{50}	50% Effective Concentration
EDTA	EthyleneDiamine Tetra Acetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
env	Envelope gene
ER	Endoplasmatic Reticulum
ESN	Exposed SeroNegative
FBD	Female Blood Donors
FCS	Fluorescence Correlation Spectroscopy
FDA	Food and Drug Administration
FSW	Female Sex Workers

gag	Group antigen gene
GFP	Green Fluorescence Protein
gp	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HAT	Histone Acetyl-Transferases
HDAC	Histone Deacetylase
HDGF	Hepatoma-Derived Growth Factor
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulfonate)
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigens
HMGA1	High Mobility Group chromosomal protein A1
HMT	Histone MethylTransferases
HTLV	Human T cell Leukemia Virus
IBD	Integrase Binding Domain
IKK	Iκ-B Kinase
IN	Integrase
INBI	Integrase DNA Binding Inhibitor
IniI	Integrase interactor 1
INSTI	Integrase Strand Transfer Inhibitor
IPTG	IsoPropylThioGalactopyranoside
kb	Kilobase
kDa	Kilodalton
LAP2	Lamina-Associated Polypeptide 2
LEDGF	Lens Epithelium-Derived Growth Factor
LEM	LAP2, Emerin and MAN1
LSD	Lysine-Specific Demethylase 1
LSF	Late SV40 Factor
LTR	Long Terminal Repeat
LV	Lentiviral Vector
MA	Matrix (protein)
MOI	Multiplicity Of Infection

MoMLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVB	MultiVesicular Bodies
MW	Molecular Weight
NC	Nucleocapsid (protein)
Nef	Negative regulatory factor
NF-κB	Nuclear Factor-κB
NLS	Nuclear Localization Signal
NRE	Negative Regulatory Element
OBR	Optimized Background Regimen
PAK	P21 Activated Kinases
PBS	Phosphate Buffered Saline
PCAF	P300/CBP-Associated Factor
PCR	Polymerase Chain Reaction
PDP	PyranoDiPyrimidines
PIC	Pre-Integration Comples
pol	Pol gene
PR	Protease
PTAP	Proline - Threonine - Alanine - Proline
P-TEFb	Positive Transcription Elongation Factor b
RNA	RiboNucleic Acid
RNAi	RNA interference
RNaseH	Ribonuclease H
RRE	Rev Response Element
RSV	Rous Sarcoma Virus
RT	Reverse Transcriptase
RV	Retroviral Vector
SCID	Severe Combined ImmunoDeficiency
SDS	Sodium Dodecyl Sulfate
SET	Su(var)3-9, Enhancer of Zeste, Trithorax

SIGN	Specific ICAM-Grabbing Nonintegrin
siRNA	Small interfering RNA
SIV	Simian Immunodeficiency Virus
SN	SeroNegative
SNF5	Sucrose NonFermenting 5
SNP	Single Nucleotide Polymorfisms
SP	SeroPositive
SU	Surface glycoprotein
SWI/SNF	Switch/Sucrose NonFermenting
TAK	Tat Associated Kinase
TAR	Transactivation Responsive Element
Tat	Transactivator of transcription
TM	Transmembrane glycoprotein
TNF-α	Tumor Necrosis Factor α
TPA	Phorbol 12-myristate 13-acetate
TPX	Trapoxin
tRNA	Transfer RNA
TSA	Trichostatin A
Tsg101	Tumor Susceptibility Gene 101
U3	Unique region of the 3' LTR
U5	Unique region of the 5' LTR
Vif	Viral infectivity factor
VPA	Valproic Acid
Vpr	Viral protein R
Vps	Vacuole protein sorting
Vpu	Viral protein U
WT	Wild-Type
YY1	Yin Yang-1

Since the first cases of AIDS were reported in 1981 and the discovery of its etiologic agent, the human immunodeficiency virus (HIV), in 1983, the virus has killed more than 2.9 million people worldwide. About 4.3 million were newly infected in 2006. At the end of 2006 approximately 39.5 million people are living with the disease. Conventional antiretroviral medications consist of 4 different classes of drugs: 1) nucleoside reverse transcriptase (RT) inhibitors, 2) non-nucleoside reverse transcriptase inhibitors, 3) protease inhibitors and 4) one fusion inhibitor. Combinations of these agents provide potent suppression of HIV-1 RNA loads and have dramatically improved clinical outcomes for many patients. Despite the potency of this antiretroviral therapy, the emergence of HIV strains resistant to the presently available anti-HIV drugs is an important factor in therapy failure. Therefore it is extremely important that new compounds are developed that target other steps of the replication cycle of HIV. Whereas the virus can become resistant towards the administered antiretroviral drugs, another kind of resistance, called innate immunity, also occurs. Differences in genome sequence, due to a selective pressure, can render an individual resistant against the development of certain diseases and/or virus infections. Natural resistance to HIV-1 infection, while rare, does exist. Individuals homozygous for a 32 bp deletion in the gene coding for the CCR5 co-receptor of HIV-1 are resistant to infection with an HIV-1 CCR5 strain.

At the onset of my PhD, SPL2923, a polyanionic dendrimer, had been identified in our laboratory as an entry inhibitor of various HIV-1 and HIV-2 strains, including clinical HIV isolates and SIV(mac_{251}). At that time, reverse transcription and integration, however, could not be excluded as possible target steps in the HIV replication cycle of SPL2923. Next to this entry inhibitor, the pyranodipyrimidine V-165 had also been

identified in our laboratory as an integrase inhibitor. Mechanism of action studies revealed that V-165 interferes with DNA-IN complex formation. As such V-165 is the prototype of the integrase binding inhibitors (INBI's). At the same time, LEDGF/p75 was identified in our laboratory as an important co-factor of HIV-1 integration and replication. However, little was known about the natural variability of the gene coding for LEDGF/p75.

My doctoral work focused on the following objectives: 1) Specify the antiviral target and the molecular mode of action of the HIV-1 entry inhibitor SPL2923, 2) Study the effect of mutations found in the *integrase* (*IN*) gene of a HIV-1 strain resistant towards the integrase binding inhibitor V-165, on the enzymatic activity and the susceptibility against the inhibitory effect of several anti-HIV drugs, including V-165, 3) Evaluate if there is an innate immunity associated with mutations in LEDGF/p75 that protects individuals against infection with HIV-1.

The study of HIV-1 resistance development against inhibitors of viral entry, SPL2923 or integration, V-165, included the selection of HIV-1 in the presence of increasing concentrations of inhibitor and the genotypic and phenotypic analysis of this selected strain. Chimeric Virus Technology (CVT) was used for the recombination of the HIV-1 *env* genes *gp120*, *gp41* and *gp160*, and the *integrase* gene.

Resistance selection against the polysulfonic dendrimer SPL2923 resulted in mutations in both *gp120* and *gp41* of the selected strain. No mutations were found in RT or IN. Phenotypic analysis revealed that the mutations in *env* alone were not sufficient to explain the resistance profile of the selected resistant strain. Sequencing of the gene coding for *p17*, the matrix protein, revealed the association of mutations in the matrix

protein with resistance to SPL2923. CVT was established that enabled the recombination of both *p17*, alone or in combination with *gp160*, into a wild type background. By *gp160*-, *p17*- and *p17/gp160*-recombination experiments we proved that the mutations in both the *env* and *gag* genes contribute to the resistance of the selected strain and need to be present simultaneously to reproduce the resistance phenotype towards SPL2923.

Genotypic analysis of PDP-resistant HIV-1 strains revealed mutations in the *IN* gene in an early stage, while mutations in *RT* and *env* appeared at a later stage. Recombinant mutant integrase was purified. In an oligonucleotide-based assay, a slight reduction in over-all enzymatic activity was observed for all the mutant integrase enzymes, except for S230N, which showed WT activity. An IN/DNA interaction assay demonstrated that all the mutant integrases showed a decreased affinity for DNA. Additionally, the mutant enzymes showed a slight resistance to V-165 in this IN/DNA interaction assay. This study revealed that the observed IN mutations interfere with the enzymatic activity and the DNA binding capacity of HIV-1 IN enzymes and that the mutations are responsible for a low-level resistance against PDP. Additional experiments were performed to further analyze the resistance profile of the selected strains. By means of *IN*-, *RT*- or *gp160*-CVT, a quantitative viral adsorption assay and Quantitative PCR analysis of the three HIV DNA species after HIV-1 infection and HIV-1 vector transduction, we conclude that V-165 exploits a multi-modal mechanism of action. However, we still propose integrase binding inhibition as a genuine antiviral target and V-165 as the INBI prototype.

To investigate whether there is an innate immunity due to mutations in LEDGF/p75, DNA sequencing analysis of three different groups was performed: 1) a HIV seronegative

African control group, 2) a HIV exposed seronegative African group of female sex workers and 3) a HIV seropositive African group of female sex workers. Next to mutations in introns, that did not result in an alternatively spliced protein, one mutation in an exon was found. As a consequence, the Q472L mutation was present in LEDGF/p75. This mutation was located outside the integrase binding domain in LEDGF/p75. The Q472L mutation was found in 1 out of the 19 samples of the control group, in 3 out of the 20 samples of the seronegative African group and in 1 out of the 20 samples of the seropositive African group. Statistical analysis revealed that the prevalence of the Q472L mutation in the HIV exposed seronegative group and the seropositive group was not statistically different ($p = 0.61$). Recombinant mutant LEDGF/p75 did not show a difference in binding capacity to IN compared to WT. In a next step, HIV-1 replication was analyzed in LEDGF/p75 knockdown MT-4 cell lines. A 50% reduction in viral replication was observed. Back complementation of WT or mutant(Q472L) LEDGF/p75 restored the viral replication, however no difference in replication was observed in the cell lines back complemented with mutant(Q472L) LEDGF/p75 compared to those back complemented with WT LEDGF/p75. Taken together, these results suggest that the presence of the Q472L mutation in LEDGF/p75 has no effect on the role of LEDGF/p75 in viral replication and does not render a person resistant to HIV infection.

Sinds de ontdekking van HIV begin jaren ‘80 zijn er reeds 39,5 miljoen mensen geïnfecteerd met dit virus. In 2006 zijn er meer dan 2.9 miljoen mensen overleden aan AIDS en ongeveer 4.3 miljoen mensen raakten dat jaar geïnfecteerd met het virus. De huidige therapie is gebaseerd op een combinatie van antivirale middelen, die inwerken op de virale enzymen reverse transcriptase (RT) en protease en op de HIV opname in de cel. Ten gevolge van de toxische nevenwerkingen en het ontstaan van virus dat resistent is aan de toegediende middelen is het noodzakelijk dat er nieuwe remmers ontwikkeld worden die inwerken op andere stappen van de replicatiecyclus. Naast deze resistentie-ontwikkeling van HIV tegenover de gebruikte antiretrovirale middelen is er nog een andere vorm van resistentie, namelijk de natuurlijke immuniteit. Polymorfismen in de genoomsequentie, ontstaan door een selectieve druk, kunnen aanleiding geven tot een cellulaire resistentie tegen het ontwikkelen van bepaalde ziekten of tegen infectie met een viral agens. Natuurlijke resistentie tegen HIV is een bekend fenomeen. Een voorbeeld hiervan is de mutatie in het gen coderend voor de co-receptor voor HIV replicatie CCR5. Een deletie van 32 bp in het deel coderend voor het centrale domein van CCR5, resulteert in een deficiënt proteïne. Personen die homozygoot zijn voor deze mutatie zijn immuun voor infectie met HIV-1 R5 stammen.

Bij de aanvang van mijn doctoraat was SPL2923, een polyanionisch dendrimeer, in ons laboratorium geïdentificeerd als een opname inhibitor die werkzaam is tegen verscheidene HIV-1 en HIV-2 stammen, eveneens tegen verscheidene klinische isolaten en tegen SIV(mac_{251}). Deze molecule inhibeert de replicatie van HIV volgens hetzelfde werkingsmechanisme als dextraansulfaat. Er kon echter niet uitgesloten worden dat SPL2923 ook inwerkt op de RT en/of de integrase (IN) stap. Naast deze opname inhibitor

werden de pyranodipyrimiden (PDP's) in ons laboratorium geïdentificeerd als een nieuwe klasse integrase inhibitoren. Het werkingsmechanisme van deze PDP's is gebaseerd op de inhibitie van de vorming van DNA-IN complexen. V-165, de meest belovende vertegenwoordiger van de PDP's, is dus het prototype van de integrase bindingsinhibitoren (INBI's). In 2003 werd eveneens in ons laboratorium aangetoond dat de transcriptionele co-activator LEDGF/p75 een cellulaire co-factor voor HIV integratie en replicatie is. Er is echter weinig gekend over de natuurlijke variabiliteit van LEDGF/p75.

Mijn doctoraatsthesis behandelt de volgende onderwerpen: 1) Onderzoek naar het antivirale doelwit en het werkingsmechanisme van de HIV opname inhibitor, SPL2923, 2) Studie over de invloed van de integrasemutaties, gevonden in een HIV-1 stam resistent aan V-165, op de enzymatische activiteit en enzymatische gevoeligheid tegenover andere anti-HIV middelen en 3) Nagaan of mutaties in LEDGF/p75 verantwoordelijk zijn voor een natuurlijke bescherming tegen infectie met HIV.

De studie van HIV-1 resistentie-ontwikkeling tegenover inhibitoren van HIV-1 opname of integratie (SPL2923 en V-165 respectievelijk) houdt de selectie in van HIV-1 in de aanwezigheid van een stijgende concentratie aan inhibitor en de genotypische en fenotypische analyse van deze geselecteerde stammen. Met behulp van de Chimere Virus Technologie (CVT) werden er *gp120*-, *gp41*-, *gp160*- en *integrase*-recombinante virusstammen geconstrueerd.

Resistentieselectie tegenover het polyanionische dendrimeer SPL2923 resulteerde in mutaties in zowel *gp120* als *gp41* van de geselecteerde stam. Er werden echter geen mutaties aangetroffen in *RT* of *IN*. Op basis van deze resultaten werd HIV opname als het

antivirale doelwit van SPL2923 in celcultuur geïdentificeerd. Fenotypische analyse toonde aan dat de mutaties in *env* alleen niet voldoende waren om het resistentiepatroon van de geselecteerde stam te verklaren. Genotypische analyse van het gen coderend voor p17, het matrixproteïne, toonde aan dat er eveneens mutaties waren in *p17*. Een nieuwe CVT werd ontwikkeld waarin *p17* alleen, of in combinatie met *gp160*, in een wild type achtergrond kan gerecombineerd worden. *p17*- en *p17*-/*gp160*-recombinatie experimenten toonden aan dat zowel de mutaties in *env* als in *gag* samen aanwezig moeten zijn om de fenotypische resistentie van de geselecteerde stam volledig te kunnen reproduceren.

Genotypische analyse van de V-165 resistente stammen toonde aan dat er mutaties voorkomen in zowel *env*, *RT* als *IN*. De mutaties in *IN* waren reeds vanaf een vroeg tijdspunt aanwezig in de geselecteerde virussen terwijl de mutaties in *env* en *RT* op een later tijdstip verschenen. Recombinant, mutant IN werd opgezuiverd. Alle mutanten, met uitzondering van S230N-IN dat WT activiteit vertoonde, vertoonden een lichte daling in enzymatische activiteit in een over-all oligonucleotide-gebaseerde test. Een IN/DNA interactie test toonde aan dat alle mutanten een verminderde bindingsaffiniteit voor DNA en een beperkte resistentie ten opzichte van V-165 vertoonden. Deze studie toonde aan dat de IN mutaties een invloed hebben op de enzymatische activiteit en de bindingscapaciteit van IN aan DNA, en dat de mutaties verantwoordelijk zijn voor een beperkte resistentie ten opzichte van V-165. Bijkomende studies werden uitgevoerd om de invloed van de mutaties in *env* en *RT* op het resistentie profiel van de SPL2923 resistentie stam verder na te gaan. Aan de hand van de resultaten bekomen met de *IN*-, *RT*- en *gp160*-CVT, een kwantitatieve virus adsorptie test en een kwantitatieve PCR,

specifiek voor de detectie van de 3 verschillende HIV species, na HIV-1 infectie en HIV-1 vector transductie, konden we besluiten dat het werkingsmechanisme van V-165 multi-modaal is. De inhibitie van DNA-IN complex vorming is wel het primaire antivirale doelwit van de INBI, V-165.

Om na te gaan of er, ten gevolge van mutaties in LEDGF/p75, een natuurlijke resistentie tegen infectie met HIV bestaat, werden er 3 groepen stalen, allen van Afikaanse oorsprong, gesequeneerd: 1) een niet-geïnfecteerde controle groep (19), 2) een groep niet-geïnfecteerde, vrouwelijke prostituees (20) en 3) een groep HIV geïnfecteerde, vrouwelijke prostituees (20). Verscheidene mutaties in de intronen van het gen coderend voor LEDGF/p75 werden aangetroffen. Deze mutaties gaven echter geen ontstaan aan een alternatieve splice variant van LEDGF/p75. Naast deze mutaties in de intronen werd er echter ook één mutatie in een exon gevonden. In het eiwit geeft dit aanleiding tot het ontstaan van de mutatie Q472L. Deze mutatie is gelegen buiten de integrase bindingsregio van LEDGF/p75. In de controle groep kwam deze mutatie in 1 op de 19 stalen voor, terwijl in de groep niet-geïnfecteerden de mutatie in 3 op de 20 stalen voorkwam. De mutatie werd ook teruggevonden in de groep HIV geïnfecteerden, namelijk in 1 op de 20 stalen. Statistische analyse toonde echter aan dat de prevalentie van de Q472L mutatie in de niet-geïnfecteerde groep en de geïnfecteerde groep statistisch niet verschillend is ($p = 0.61$). Recombinant, mutant(Q472L) LEDGF/p75 toonde geen verschil in bindingscapaciteit aan voor IN vergeleken met WT LEDGF/p75. Knockdown van LEDGF/p75 in MT-4 cellen resulteerde in een 50% inhibitie in virale replicatie. De virale replicatie kon hersteld worden door back complementatie met WT of mutant(Q472L) LEDGF/p75. Er werd echter geen verschil in virale replicatie

waargenomen in de cellen die mutant(Q471L) LEDGF/p75 tot expressie brachten in vergelijking tot deze die WT LEDGF/p75 tot expressie brachten. Deze resultaten suggereren dat de aanwezigheid van de Q472L mutatie in LEDGF/p75 geen invloed heeft op de rol van LEDGF/p75 tijdens de virale replicatie en een persoon niet beschermt tegen infectie met HIV.

Chapter 1

General introduction

1.1. GENERAL INTRODUCTION

1.1.1. HIV AND AIDS

The human immunodeficiency virus, HIV was discovered in 1983 as the causative agent of Acquired Immune Deficiency Syndrome, AIDS [1-3] and is a member of the lentiviruses, a subfamily of the retroviruses. Retroviruses are enveloped RNA viruses and are characterized by a reverse transcription step in their replication cycle. Unlike other retroviruses, which require cell division for productive infection, lentiviruses can stably integrate their proviral DNA into the genome of dividing as well as non-dividing cells. Based on genome organization and phylogenetic relationship with other lentiviruses, HIV can be divided into 2 types: HIV-1 and HIV-2. Worldwide infection with HIV-1 is more common, however in West-Africa HIV-2 infections are more predominant. The target cells for HIV infection and replication are $CD4^+$ cells, T-helper cells, dendritic cells and macrophages. As a result of the virus-induced destruction and/or functional impairment of these immune cells, immune deficiency occurs, leading to the development of opportunistic infections or malignancy: AIDS. HIV is present in the blood and genital secretions of virtually all infected individuals regardless of whether or not they have symptoms of AIDS. The spread of HIV can occur when these secretions come in contact with tissues such as those lining the vagina, anal area, mouth or eyes or when the skin is ruptured by a cut or puncture from a needle. The most common ways HIV uses to spread throughout the world include sexual contact, needle sharing and mother-to-child transmission during pregnancy, labor or breastfeeding. An estimated 39.5 million people worldwide are living with HIV. Approximately 4.3 million became newly infected and an estimated 2.9 million people lost their lifes to AIDS in 2006. Most people living with

HIV live in the developing world. Bringing the global HIV epidemic under control will not only require more effective approaches to prevent the spread of HIV, but also a broader availability of existing and future antiretroviral drugs (www.unaids.org).

1.1.2. VIROLOGY OF HIV-1

1.1.2.1. Virion structure

The HIV-1 virion, which consists of an envelope surrounding a cone-shaped core, has a diameter of about 110 nm. For a review see [4, 5]. The viral envelope, consisting of a phospholipid bilayer, is derived from the plasma membrane of the producer cell. In the plasma membrane spikes are found that are composed of the surface glycoprotein (SU, gp120) non-covalently bound to the transmembrane glycoprotein (TM, gp41) to form trimeric functional units. Directly underneath the envelope, matrix proteins (MA, p17) form a protein shell. These p17 proteins are in contact with the phospholipid bilayer through myristic acid that is covalently bound to p17. Inside the virion, two viral single stranded RNA copies are stabilized by a ribonucleoprotein complex with the nuclear capsid protein (NC, p7), the three viral enzymes reverse transcriptase (RT), protease (PR) and integrase (IN). This inner nucleoprotein complex is surrounded by a characteristic cone-shaped core consisting of molecules of the capsid protein (CA, p24). Other viral proteins present in the virion are the accessory proteins Vpr (Viral protein R), Nef (Negative regulatory factor) and Vif (Viral infectivity factor).

1.1.2.2. Genome structure

The HIV genome as present in the virion, consists of two single-stranded RNA molecule.

For a review see [4, 6]. By reverse transcription and integration into the host genome, the diploid RNA genome is transformed into one double stranded DNA molecule of 9.8 kb. This provirus is flanked by two long terminal repeat (LTR) sequences (5'- and 3'-LTR), each divided into three regions; the U3, the R and the U5 region (Figure 1). The 5'-LTR harbors *cis*-acting elements involved in regulating viral RNA transcription, that initiates in the U3/R junction. Polyadenylation of transcripts occurs at the R/U5 junction of the 3'-LTR, by the RNA polymerase II. Nine genes are encoded by the provirus, three are common in retroviruses and encode the structural proteins (*gag*), the viral enzymes; integrase, reverse transcriptase and protease (*pol*) and the envelope glycoproteins; gp41 and gp120 (*env*) (Figure 1). The additional six genes encode the regulatory proteins Tat and Rev, and the accessory proteins Vif, Vpr, Vpu (Viral protein U) and Nef (Figure 1). The cellular RNA polymerase II synthesizes three types of transcripts; unspliced (serving as the genomic RNA and as transcripts for the Gag and Gag-Pol polyproteins), multiple spliced (transcripts for regulatory and accessory proteins) and single spliced (transcripts for Env polyprotein) viral mRNA. The *env* gene is transcribed as a single mRNA molecule that is translated in the endoplasmatic reticulum (ER). This gp160 intermediate is modified in the ER where oligomerization to a trimeric structure and glycosylation occurs. This complex is then transported to the Golgi apparatus where it is cleaved into gp120 and gp41 by a cellular endoprotease. The *gag* and *pol* genes are transcribed as a single mRNA molecule and translated into two precursor polypeptides that are processed after budding, in a step called maturation, by the viral protease (PR) to yield separate functional proteins. The Gag-Pol precursor (Pr160gag-pol) is only formed when a ribosomal slip from the *gag* reading frame into a -1 reading frame occurs. The Pr160gag-

pol is cleaved into the proteins reverse transcriptase (RT), protease (PR) and integrase (IN) while the Gag precursor (Pr55gag) is cleaved into the proteins p17 (matrix, MA), p24 (capsid, CA), p7 (nucleocapsid, NC), p6, p2 and p1. p7 or the nucleocapsid (NC) coats the genomic RNA inside the virion core. The primary function of NC is to bind specifically to the packaging signal and deliver full-length viral RNAs into the assembling virion. p6 comprises the C-terminal 51 amino acids of Gag and is important for incorporation of Vpr during viral assembly. Residues 32 - 39 and three hydrophobic residues within a highly conserved sequence motif (Leu41-X42-Ser43-Leu44-Phe45-Gly46) are important for Vpr binding. In Vpr, a predicted α-helical structure located near its N-terminus contains amino acids, is responsible for p6 binding. p6 also helps to mediate efficient particle release. A region of four amino acids (Pro7-Thr8-Ala9-Pro10) has been implicated in this function. p2 and p1 are two spacer peptides, generated upon Gag precursor processing. The spacer peptide p1 is a 16 amino acid protein. Proline residues within this peptide are important for HIV-1 infectivity, protein processing and genomic RNA dimer stability [7]. Different *in vivo* and *in vitro* studies indicate that p2, a spacer peptide of 14 amino acids, regulates the rate of cleavage at the CA/p2 processing site [8]. The highly conserved N terminus of p2, which is predicted to form part of an α-helix that begins in CA, was shown to be essential for proper viral assembly and infectivity [9, 10].

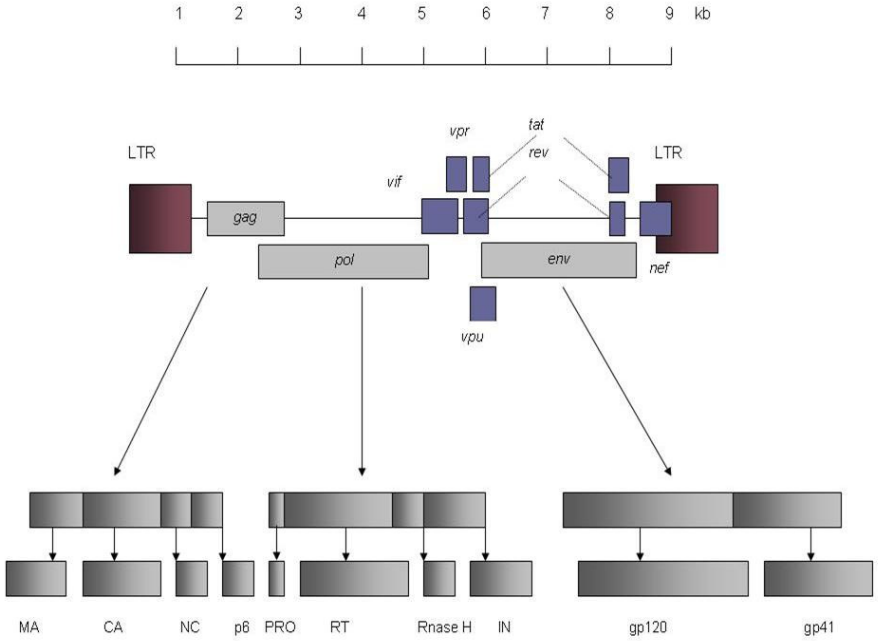

Figure 1: Organization of the HIV-1 genome

The provirus is flanked by two long terminal repeat (LTR) sequences and encodes nine genes. The gag gene encodes the structural proteins matrix (MA), capsid (CA), nucleocapsid (NC) and p6. The viral enzymes integrase (IN), reverse transcriptase (RT) and protease (PR) are encoded by the pol gene while the env gene encodes the envelope glycoproteins gp41 and gp120. The additional six genes encode the regulatory proteins Tat and Rev, and the accessory proteins Vif, Vpr, Vpu and Nef.

1.1.2.3. Structure of the HIV-1 5'-LTR

Verdin *et al.* used different, latently infected cell lines to generate a model of the structure of the 5' HIV-1 long terminal repeat (LTR) under basal condition (Figure 2) [11]. Under this condition, two nucleosomes are present in the 5'-LTR: nuc0 (located partly in the negative regulatory element (NRE); nt 40-200) and nuc1 (located immediately after the transcription start site; nt 465-610). Nucleosomes are composed of about 146 bp of DNA that is wrapped around a central histone octamer which consists of 2 molecules of H2A, H2B, H3 and H4. Two turns of DNA are wrapped around a tripartite structure containing

the $(H3\text{-}H4)_2$ tetramer flanked by 2 H2A-H2B dimers [12]. Two nucleosomes are separated by a linker DNA that is associated with one molecule of H1. Histones contain a C-terminal α-helical domain that is involved in histone-histone interactions and in DNA binding and a N-terminal domain that forms a tail located outside the core particle. The N-terminal tail can undergo different posttranscriptional modifications: phosphorylation, ubiquitination, acetylation, methylation and poly-ADP-ribosylation. The packaging of the DNA in chromatin is an important factor determining initiation and elongation of mRNA transcription. Transcriptionally active regions are characterized by a diffuse structure (euchromatin), while inactive genes are packaged in highly condensed configurations (heterochromatin) [13]. Therefore, for the transcription factors to gain access to the DNA, it is very important that the condensed structure of the chromatin is altered. Different mechanisms able to disrupt these condensed regions have been described. A first mechanism is the acetylation of the histones via the transfer of an acetyl group from acetylcoenzyme A to the lysine residues of the N-terminal tail. Histone acetylation is the result of a dynamic equilibrium between histone acetyltransferases (HAT) and histone deacetylases (HDAC) and is a reversible process. Acetylation of the histones results in the destabilization of the histone-DNA interaction leading to a better accessibility of the DNA for transcription factors. Besides acetylation, methylation of the arginines and the lysines of the N-terminal tail of the histones can occur as well. This process is catalyzed by a family of SET -domain histone methyltransferases (HMT) and has been suggested to be a dead-end modification because no histone demethylases had been identified. However, in 2004 Shi *et al.* discovered LSD1 (Lysine-Specific Demethylase 1), the first histone demethylase that removes the methylgroup from lysine4 from H3 [14]. The

configuration of the histones can also be changed by ATP-dependent chromatin remodeling factors that contain a conserved helicase/ATPase domain. This domain disrupts the DNA-histone interaction which eventually leads to active transcription. In the HIV-1 5'-LTR, two open regions (one in the enhancer/promoter region in U3 and one in the primer binding site in U5, downstream of the site of transcription initiation) are defined by these nucleosomes, nuc0 and nuc1. In these regions transcription factors have been found to bind [15]. The region downstream of the 5'-LTR contains three precisely positioned nucleosomes, referred to as nuc2, nuc3 and nuc4. Transcriptional activation by treatment of these cells with a phorbol ester phorbol 12-myristate 13-acetate (TPA) or Tumor Necrosis Factor (TNF-α) caused a disruption of nuc1 [11]. Treatment of latently infected cell lines with two specific inhibitors of histone deacetylase, trapoxin (TPX) [16] or trichostatin A (TSA) [17] resulted in an increase in virus production in the supernatant. A strong positive correlation between the amount of virus production and the acetylation status of the histones was seen. The only detectable modification was a disruption of nuc1 in the 5'-LTR [18]. Van Lint *et al.* showed that only the expression of a small portion of the cellular genes (2%) was upregulated by TPX or TSA and that consequently the response to TPX and TSA was not the result of a general upregulation of cellular genes [19]. Two possible mechanisms responsible for the repressive activity on HIV-1 transcription by nuc1 are proposed. First, nuc1 may block the binding of transcription factors essential for transcription of the proviral DNA. Second, nuc1 may cause the transcription complex to pause or stop transcribing [19].

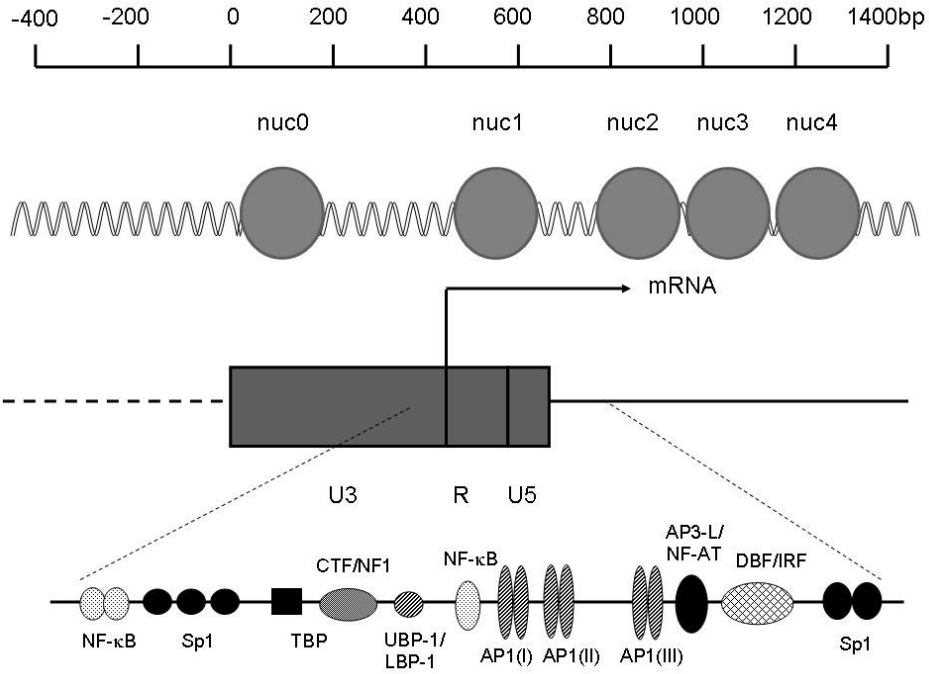

Figure 2: Structure of the HIV-1 5'-LTR

Two nucleosomes are present in the 5'-LTR of HIV-1: nuc0 (located partly in the negative regulatory element (NRE); nt 40-200) and nuc1 (located immediately after the transcription start site; nt 465-610). The region downstream of the 5'-LTR contains three precisely positioned nucleosomes, referred to as nuc2, nuc3 and nuc4. (Adapted from Verdin et al, EMBO, 1993, 12, 3249 - 3259)

1.1.3. REPLICATION CYCLE OF HIV

The HIV-1 replication process can be divided into two distinct phases: the early and the late phase, which are each subdivided into different steps (Figure 3). For a review see [4-6, 20]. The early phase of the replication process starts with the entry of the virus into the host cell. This step is initiated by the aspecific attachment of the virus to cell surface heparan sulfate and is followed by the attachment of the viral gp120 to the cellular CD4-receptor. The interaction with the receptor results in slight conformational changes

in gp120 and CD4 allowing gp120 to expose its co-receptor binding site. The V3 loop of gp120 then binds to its chemokine co-receptor, CCR5 or CXCR4 for respectively the macrophage-tropic (or R5) viruses or the T cell-tropic (or X4) viruses. R5 viruses are associated with the transmission and asymptomatic phases of HIV infection while X4 viruses are more frequently found during the later stages of disease. Due to this binding to the co-receptor, gp41 undergoes conformational changes. The N-terminal section of gp41 forms a fusion peptide that inserts into the cell membrane. In the following step the two α-helices located in the ectodomain of gp41 (one N- and one C-terminal) become positioned in an anti-parallel configuration towards each other and. This allows the HIV core to enter the cell. Once in the cell, the virion undergoes uncoating, a poorly understood process. In a next step, reverse transcription, catalyzed by the viral reverse transcriptase (RT) and primed by $tRNA^{Lys}$, results in the formation of a RNA-DNA molecule. RNaseH cleaves the RNA strand in this heteroduplex and a double-stranded linear viral DNA molecule is produced. The viral DNA, IN, MA, Vpr and RT are present in the pre-integration complex (PIC). In this PIC, the first step of the complex integration process, called '3'-processing', takes place. IN multimers bind to the LTR and remove the terminal GT dinucleotides from each LTR 3'end. The import of the PIC into the nucleus is an energy dependent process which is facilitated by IN, MA, Vpr and the DNA flap, a triple helical DNA domain that is produced during reverse transcription of the plus-strand. Inside the nucleus, the processed 3'-ends of the viral DNA are covalently joined to the 5' ends of cleaved cellular DNA, a step called 'strand transfer' which is again catalyzed by the viral integrase. Strand transfer includes a staggered cleavage of host DNA (5 bases apart) and the insertion of the processed 3'-OH ends of the viral DNA

into the host DNA. The 3'-ends of the target DNA remain unjoined after strand transfer. In the third step of the integration reaction, the '5'-ends joining reaction', the two unpaired nucleotides at the viral 5'-end are removed and the single stranded gaps (5 bases) between the viral and target DNA are repaired by the cellular DNA repair machinery. However, reverse transcriptase and integrase may also play a role in this repair. Integration is the last step in the early phase of the replication cycle of HIV. The late phase begins with transcription of the proviral DNA. This step can be divided into two distinct stages, a Tat-independent and a Tat-dependent one. During the first, Tat-independent phase, only multiply spliced mRNAs are formed. These encode the regulatory proteins Tat, Rev and Nef. In the next Tat-dependent phase Tat binds to an RNA stem loop called the transactivation response (TAR) element and enhances the transcription and the elongation. Tat stimulates the RNA polymerase II activity by recruiting a cellular kinase, TAK (Tat-Associated Kinase or CDK9 (Cyclin Dependent Kinase9)/P-TEFb (Positive Transcription Elongation Factor b)), composed of the catalytic subunit CDK9 and the regulatory subunit Cyclin T1, that activates transcriptional elongation by hyperphophorylation of serine-5 of the RNA polymerase II [21]. Rev binds to the Rev Response Element (RRE), prevents the transcripts from being spliced and recruits cellular proteins involved in nuclear export of unspliced and single spliced mRNAs to the cytoplasm. This complex is then transported through the nuclear pore to the cytosol where it dissociates. The nuclear localization signal of Rev directs the import of Rev back into the nucleus. Translation of these single spliced and unspliced mRNA molecules results in Gag- and Gag-Pol precursor proteins and mature, functional Env proteins. The matrix domain of Pr55gag and Pr160gag-pol polyproteins directs

association with the cellular membrane and interacts with the cytoplasmatic tail of gp41. This interaction is necessary for the incorporation of Env glycoproteins into the virion. The assembly of HIV-1 is thought to occur in plasma membrane microdomains known as lipid rafts that are enriched in cholesterol and glycosphingolipids. The next step in the replication cycle of the virus is particle release or the budding of the virus out of the cell. This process can be viewed as an event in which the cell membrane is broken and resealed to create the viral membrane. Like other enveloped viruses, HIV-1 does not encode its own machinery to do this and therefore must recruit and reprogram cellular proteins to assist in the budding process. Tsg101 (Tumor susceptibility gene 101), a cellular proteins, is required for viral budding. Tsg101 functions in the cellular vacuole protein sorting (Vps) pathway, where it plays a central role in selecting cargo for incorporation into vesicles that bud into late endosomal compartments called multivesicular bodies (MVB). In viral budding, the protein could have an analogous role, however, at a different site, the plasma membrane. Tsg101 binds to the Late domain (PTAP = proline - threonine - alanine - proline) in p6 and thereby links the p6 late domain to the Vps machinery and facilitates the final stages of virus release. The factor(s) that catalyze the 'pinching-off' step are as yet unidentified, however, this release preferentially occurs at sites of cell-to-cell contact. Only the unspliced genomic RNA, which contains the Ψ encapsidation signal, will be packaged into the virion. After budding of the virions into the extracellular space, the Gag and Gag-Pol polyproteins are cleaved by protease (PR) in a process called maturation to produce the independent enzymes as well as the structural proteins. Finally, the structural proteins rearrange to form the infectious virus particle.

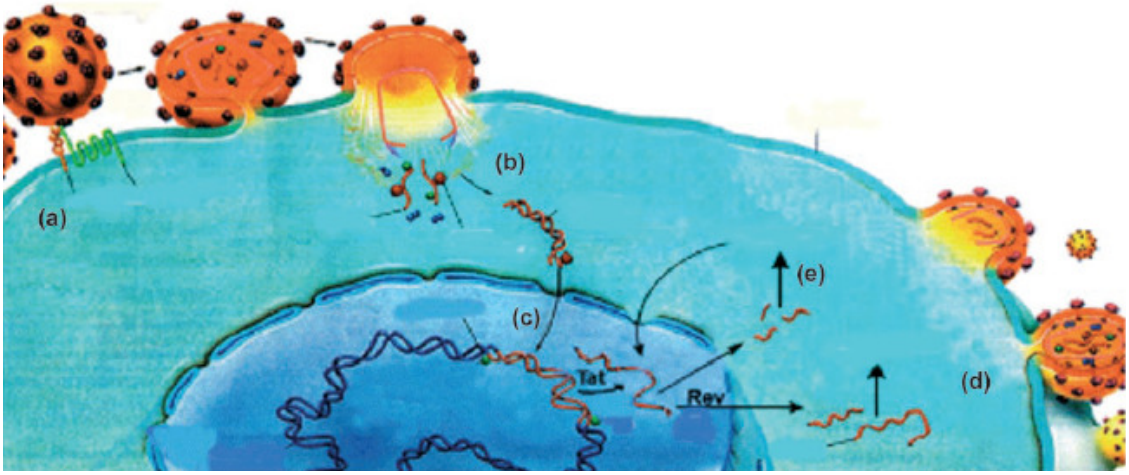

Figure 3: Replication cycle of HIV-1

HIV-1 replication can be divided into 2 distinct phases. The early phase begins with the aspecific attachment of the virus to cell surface heparan sulfate and comprises the entry step (step a), the reverse transcription (step b) and the integration of the proviral DNA (step c). The late phase includes all events from transcription of the integrated DNA (facilitated by the regulatory proteins Tat and Rev, which are themselves viral gene products that return to the nucleus; step e) to budding (step d) and maturation and results in the formation of infectious viral particles. (Adapted from Meadows and Gervay-Hague, ChemMedChem, 2006, 1, 16 - 29)

1.1.4. CELLULAR CO-FACTORS OF HIV REPLICATION AND INTEGRATION

The replication of HIV-1 in the human cell is a complex mechanism. Now it becomes more and more clear that the virus also makes use of cellular proteins and pathways to complete its replication. The identification and characterization of these cellular co-factors will lead to a better understanding of the viral replication and may eventually lead to the development of new antiretroviral compounds targeting these interactions with cellular co-factors. To day, different co-factors of HIV-1 replication have already been identified, however, for some of them, their exact role in the replication cycle of HIV still

has to be elucidated.

1.1.4.1. Cellular co-receptors

One of the first cellular co-factors used in the replication cycle of HIV are the cellular receptor CD4 and co-receptors CXCR4 or CCR5. The first efforts at targeting cellular co-factors for antiretroviral therapy have focused on these receptors. A 32 bp deletion in the gene coding for the CCR5 co-receptor of HIV-1 results in a receptor that is severely truncated and cannot be detected at the cell surface. Individuals, homozygous for this deletion, are resistant to infection with an HIV-1 CCR5 strain [22].

1.1.4.2. Cellular co-factors of Nef

Co-immunoprecipitation of the viral accessory protein Nef revealed that Nef associates with a cellular serine-kinase of 65 kDa. This ATP binding protein is serologically and functionally related to the group I family of p21 activated kinases (PAK's), known mediators of cellular signal transduction [23]. This association of Nef with the serine-kinase correlates with enhanced virion infectivity and efficient proviral DNA synthesis [24]. Another study showed that depletion of Pak1 by siRNA (small interfering RNA) strongly inhibited HIV infection and decreased the level of integrated provirus. In addition, overexpression of Pak1 enhanced HIV infectivity [25]. These results implicate Pak1 as being the dominant PAK involved in HIV infection.

1.1.4.3. Cellular co-factors of Vpr

The HIV-1 accessory protein Vpr induces an arrest in the G2 phase of the cell cycle in

infected cells. This results in an increased transcriptional activity of the LTR. Vpr-induced G_2 arrest depends on signaling events analogous to the DNA damage response. Specifically, it requires activation of the ATR (ataxia-telangiectasia and Rad3-related) mediated checkpoint signal pathway, although, the exact mechanism by which Vpr activates ATR is not known. The activation of ATR, a cellular kinase, by Vpr in turn activates the cellular kinase Chk1 (Serine/threonine protein kinase). Further activation of the ATR/Chk1 cascade leads to inhibition of another cellular kinase, Cdc2 (Cell division control protein 2), the key regulator of the G2/M transition, which in turn results in an arrest of the cells in the G2 phase of the cell cycle [26]. The kinase Wee1 has been proposed as the immediate inhibitor of Cdc2 [27].

1.1.4.4. Cellular co-factors affecting viral budding

Another host factor is encoded by the tumor susceptibility gene 101. This gene encodes the host cellular protein, Tsg101, that is appropriated by HIV-1 in the budding process of viral particles from the infected cell. Overexpression of the N-terminal domain of Tsg101 inhibits HIV-1 budding by blocking the p6 late domain function [28], while depletion of endogenous Tsg101 using a siRNA approach inhibits virus release [29].

1.1.4.5. Cellular co-factors of integrase

Besides these, cellular co-factors for HIV-1 integration have also been identified. Among them are the barrier-to-autointegration-factor (BAF), the High Mobility Group chromosomal protein A1 (HMGA1), Integrase interactor 1 (IniI) and Lens epithelium-derived growth factor (LEDGF/p75).

Barrier-to-autointegration-factor (BAF) was found in the PIC of HIV-1 by co-immunoprecipitation of integrase using anti-BAF antibodies [30]. BAF is able to restore the PIC activity of HIV-1 *in vitro* after salt-inactivation [31]. However, BAF does not stimulate the activity of integrase *in vitro* [32]. BAF is also a cellular component of the PIC of Moloney Murine Leukemia Virus (MoMLV) [33]. It blocks autointegration, by compacting the viral DNA in the PIC and stimulates intermolecular integration *in vitro* [34]. BAF, when complexed with DNA, interacts with Lamina-Associated Polypeptide 2 (LAP2) a member of the LEM (as for LAP2, Emerin and MAN1) family of the inner nuclear membrane and nucleoplasmic proteins, in uninfected cells [35]. Recently, lamina-associated polypeptide 2α (LAP2α) has been defined to be present in the PIC of MoMLV. LAP2α stabilizes the association of BAF with the PIC to stimulate intermolecular integration and inhibit autointegration. Knock-down of LAP2α resulted in a dramatic inhibition in viral replication [36]. This demonstrates a critical contribution of BAF and LAP2α to the nucleoprotein organization of the PIC and to viral replication. However, the exact function of LAP2α-BAF-viral DNA in MoMLV (and HIV-1) *in vivo* integration still remains to be determined. A recent report showed by RNAi that BAF and emerin, an integral inner-nuclear-envelope protein and another binding partner of BAF, are necessary for HIV-1 infection [37]. BAF was required for the association of viral cDNA with emerin and for the ability for emerin to support virus infection. However, emerin and BAF did not facilitate cDNA integration but, rather, were required for the appropriate localization of viral cDNA before chromatin engagement. Therefore, emerin, which bridges the interface between the inner nuclear envelope and chromatin, may be necessary for chromatin engagement by viral cDNA before integration.

High mobility group chromosomal protein A1 (HMGA1), a non-histone DNA-binding protein, has been shown to restore the HIV-1 PIC activity *in vitro* [38], although, its stimulating activity was 500 fold lower than that of BAF. However, a study by Beitzel *et al.* showed that chicken cells lacking HMGA1 are neither deficient for cell growth nor for retroviral integration [39]. Now evidence suggests that HMGA1 might have a role in HIV-1 transcription [40, 41].

Integrase interactor 1 (IniI) was found to interact with HIV-1 integrase in a yeast-two-hybrid screen [42]. The protein is the human homolog of the yeast SNF5 (Sucrose nonfermenting 5), a transcriptional activator and a component of the SWI/SNF (Switch/Sucrose nonfermenting) chromatin remodeling complex [43] and has been shown to be part of the mamalian SWI/SNF complex [44]. IniI activates HIV-1 integrase DNA-joining activity *in vitro* [42]. Studies demonstrated that IniI was exported from the nucleus in infected cells and co-localized with incoming viral particles, suggesting that it might also be involved in the early steps of HIV replication [45]. The minimal IN interaction domain of IniI, S6, acts as a specific *trans*-dominant inhibitor of the late steps of the viral replication cycle upon cellular overexpression, indicating a possible role of IniI during the post-integration steps of HIV-1 replication, enhancing the release of virus particles [46]. At present it is unclear whether IniI is really essential for HIV-1 integration. Although it has been found to enhance the integrase DNA-joining activity *in vitro*, no strong evidence for a possible role of IniI in the HIV-1 replication *in vivo* has been provided. Based on the present study on the S6-fragment, evidence for a possible role of IniI during the post-integration steps of HIV-1 replication is stronger. However, given the observed cellular relocalization of IniI during HIV-1 infection, a role for

virus-associated IniI during the early steps of HIV-1 infection, such as PIC nuclear import and/or integration, also remains possible. Maroun *et al*. recently reported that by interacting with IN, SNF5/Ini1 indeed interferes with early steps of HIV-1 infection [47].

Lens epithelium-derived growth factor (LEDGF/p75) was identified as a HIV-1 integrase-interacting protein by co-immunoprecipitation of nuclear extracts of cells stably overexpressing HIV-1 integrase from a synthetic gene [48].

What is known about its importance for HIV replication? Besides encoding p75 the *LEDGF* gene also encodes a smaller splice variant, p52, which lacks the 205 C-terminal amino acids of p75 but contains 8 additional amino acids. p52 however does not show affinity for the HIV-1 integrase. Recombinant LEDGF/p75 enhances the strand transfer activity and the binding of HIV-1 IN to DNA [48, 49]. LEDGF/p75 proved to be essential for nuclear localization and chromosomal association of HIV-1 IN since transient and stable RNA-interference (RNAi)-mediated knockdown of endogenous LEDGF/p75 expression abolished nuclear/chromosomal localization of integrase [50, 51]. Busschots *et al.* reported that the binding of LEDGF/p75 to IN is lentiviral specific; *in vitro* LEDGF/p75 only binds to HIV-1, HIV-2, SIV_{mac} and FIV integrases but not to the MoMLV, RSV (Rous Sarcoma Virus) or HTLV-2 (Human T cell Leukemia Virus) integrases [49]. LEDGF/p75 is probably present in the preintegration complex [51]. To investigate the role of LEDGF/p75 in viral HIV-1 replication, LEDGF/p75 levels were suppressed by RNAi and stable HelaP4 LEDGF/p75 knockdown cell lines were generated. After transient and stable knock-down a respectively three- to fivefold and two- to fourfold inhibition of HIV-1(NL4.3) replication was seen. Real time quantitative PCR analysis pinpointed the block to the integration step whereas no reduction in nuclear

import was observed. Back-complementation of LEDGF/p75 in these knockdown cell lines restored the viral replication to nearly wild-type [52] demonstrating the role of LEDGF/p75 in HIV replication. In addition, a study by Llano *et al.* showed that silencing of LEDGF/p75 by using a lentiviral based RNAi approach in T cells resulted in a reduced viral replication [53]. Re-expression of LEDGF/p75 in these cell lines rescued viral replication.

LEDGF/p75, 530 AA long, is a member of the hepatoma-derived growth factor (HDGF) family and contains, like all members of this family, a conserved N-terminal PWWP-domain (AA 1-91) thought to be involved in protein-protein interactions [54] and chromatin binding [55]. Besides the PWWP domain, LEDGF/p75 also contains a nuclear localization signal (NLS) (AA 148-156). A single amino acid change in the NLS, Lys155Ala, was sufficient to exclude the mutant LEDGF/p75 from the nucleus [56]. A conserved domain of LEDGF/p75 that is necessary and sufficient for the binding of LEDGF/p75 to the HIV-1 integrase was mapped to just 83 amino acids, spanning the residues 347 - 429: the integrase binding domain or IBD. This IBD is essential but not sufficient to enhance the strand transfer activity of HIV-1 IN [57]. Moreover, HIV-1 replication was completely abrogated by overexpression of an eGFP-IBD fusion protein [58].

What is known about the cellular role of LEDGF/p75? During the course of purifying the general transcriptional co-activator PC4, a ~75 kDa polypeptide was co-purified. This polypeptide was identified as p75 or the positive transcription co-factor 4 (PC4)-interacting protein. p75 showed modest co-activator properties and interacts with general transcription factors and the transcription activation domain of VP16. However,

p52, the splice variant of p75, showed more pronounced co-activator properties and interaction with general transcription factors and the transcription activation domain of VP16 [59]. In other reports LEDGF/p75 has been described as a growth factor [60] and a factor that plays a protective role against cellular stress [61].

For the co-factors of HIV-1 integration described here, BAF has not been validated as a direct co-factor of HIV-1 integrase *in vivo* and both HMGA1 and IniI probably have roles in post-integration events. Therefore, LEDGF/p75 is the first *in vivo* validated cellular co-factor that is essential for HIV-1 integration.

1.1.5. CURRENT ANTI-HIV THERAPY: HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)

Currently, there are four classes of drugs approved by the FDA (Food and Drug Administration) for the treatment of HIV infected persons: 1) nucleoside reverse transcriptase inhibitors, 2) nucleotide reverse transcriptase inhibitors, 3) non-nucleoside reverse transcriptase inhibitors, 4) protease inhibitors and 5) one fusion inhibitor. Initially, treatment of infected individuals consisted of a single inhibitor class, named monotherapy. In 1996, combination therapy, which consists of three or more drugs from two or more different classes of inhibitor, was introduced. This regimen, called Highly Active Retroviral Therapy (HAART), resulted in substantial reductions in HIV-related morbidity and mortality and a subsequent restoration of the $CD4^+$ cells in the peripheral blood [62, 63]. However, replication competent viruses could be isolated from patients receiving HAART with a RNA plasma level below detectable level [64, 65]. This means that viral replication is incompletely suppressed in patients receiving HAART. This

incomplete suppression of viral replication is unfortunately not the only drawback of HAART. Patient adherence, toxicity and viral resistance are other challenges facing HAART. Toxicity is a major concern in the administration of HAART. The tendency of the drugs to elicit such adverse side effects as severe lipodystrophy, glucose intolerance, diabetes mellitus, high cholesterol, cardiovascular complications, … has posed a significant barrier to treatment [66] [67]. Drug resistance is a consequence of incomplete suppression of viral replication. The high replication rate of HIV, the high error-prone nature of RT and the absence of a 3' to 5' exonuclease proofreading activity cause HIV to exhibit enormous genetic variability. Mutation variants accumulate that display replication advantages in the presence of antiretroviral compounds. This leads to diminished drug efficacy and gradually renders each component of the regimen inactive [68]. These drug resistant mutations can then be transferred to others [69, 70]. Such patients are more likely to fail their first treatment regimen. Whereas drug regimens are more simple now than in previous years, pills still must be taken twice a day with a strict consideration to meals. Violation of these regimens can lead to incomplete suppression. Numerous studies have documented a correlation between successful suppression and adherence to medication schedules [71, 72]. Therefore, patient adherence is an important issue affecting the emerging of resistant viruses.

These challenges have prompted the search for new drugs targeting alternative steps of the replication cycle and new therapeutic strategies to control viral replication [73]. The novel antiretroviral compounds are preferentially directed against new viral or cellular targets to reduce the risk for cross-resistance. These compounds are eagerly awaited for and may prove beneficial for the growing number of HIV-infected individuals who have

developed resistance to the currently available RT and protease inhibitors. An attractive new target for anti-HIV therapy is entry, the first step in the replication cycle of HIV, since blocking entry should lead to suppression of infectivity and replication. Next to entry, integration of the viral genome into the cellular chromosome is also an attractive target for anti-HIV therapy. During HIV replication, integration, an essential step in the replication cycle of HIV, is catalyzed by the viral integrase. After integration the proviral DNA is replicated and genetically transmitted as part of the cellular genome. Thus, integration defines a point of no return in the life cycle of HIV. Moreover, no known functional homologue in human cells is known [74].

1.1.5.1. Entry inhibitors

Numerous entry inhibitors acting at different stages of the entry process have been reported, of which a growing number has recently been introduced into clinical trials or is in preclinical development. The search for agents that prevent HIV-1 entry has focused on a) shielding off the positively charged sites in the V3 loop of gp120, thereby blocking the attachment to cell surface heparan sulfate b) blocking the interaction of gp120 with the cellular receptor CD4 c) blocking the secondary interaction of gp120 with the cellular co-receptors CCR5 or CXCR4 and d) the formation of the fusion-active gp41.

Typical polyanionic compounds, like dextran sulfate (DS), inhibit HIV binding to its target cells [75]. They exert their anti-HIV activity by shielding off the positively charged sites in the V3 loop of gp120, thereby blocking the attachment to cell surface heparan sulfate [76]. Dextran sulfate is now being evaluated as a microbicide to prevent the sexual transmission of HIV-1. Recent Phase I/II clinical studies have confirmed the safety and

acceptability of dextran sulfate as a vaginal microbicide [77]. Commencing in 1992, the Biomolecular Research Institute (BRI) first investigated dendrimers as protein mimics for pharmaceutical applications, before licensing the technology to Starpharma Holdings Limited (SPL). The dendrimer SPL2923 (Figure 7), also known as BRI2923, shares an analogous mode of action due to its polysulfonated periphery. Dendrimers are highly branched macromolecules that are built up in generations from a reactive core group by the use of branched building blocks to give spherical molecules. SPL2923 consists of a fourth generation polyamidoamine (PAMAM) dendrimer scaffold built from an ammonia core, which is fully capped on the surface with 24 naphthyldisulfonic acids. SPL2923 was found to inhibit the replication of HIV-1(III_B) at an EC_{50} of 0.3 µg/ml. This compound was also effective against various other HIV-1 strains, including clinical isolates, HIV-2 strains, simian immunodeficiency virus SIV(MAC_{251}), and HIV-1 strains that were resistant to reverse transcriptase inhibitors [78]. The dendrimers are now being extensively studied for their use as microbicide [79].

Many molecules are able to inhibit gp120-CD4 binding and they all have different structures and mechanisms of action. PRO 542, developed by Progenics, is a tetravalent soluble recombinant antibody-like fusion protein that contains four IgG2 molecules in which the variable fragments of both light chains are substituted with the gp120 binding-domain of CD4, i.e. the D1/D2 domain of CD4. PRO 542 effectively neutralized a panel of laboratory-adapted strains and primary isolates of HIV-1, including strains with different tropisms and isolated from different stages of the disease, at concentrations that should be readily achieved *in vivo* [80]. PRO 542 also protects against infection by primary isolates in the human peripheral blood lymphocyte (hu-PBL) SCID mouse model

of HIV-1 infection [81]. It is one of the gp120-CD4 binding inhibitors in more advanced stages of clinical development. Phase I trials concluded that the compound is well tolerated without dose-limiting toxicities [82]. Currently it is being evaluated in phase II clinical trials. Preliminary evidence of antiviral activity was observed as reductions in both plasma HIV RNA and plasma viremia. A statistically significant acute reduction in plasma virus load was observed after administration of a single 10 mg/kg dose of PRO 542, and 1 subject experienced a >2 log reduction in HIV RNA at 1 - 2 weeks after injection. Smaller virus load reductions were observed at lower doses [83]. TNX-355, a compound of Tanox/Biogen, is a non-immunosuppressive monoclonal antibody that binds to a unique epitope in domain 2 of the CD4 molecule [84]. The compound blocks soluble CD4-induced conformational changes in the envelope glycoproteins of HIV-1 and HIV-1 infection of $CD4^+$ cells [85]. The antiviral activities of TNX-355 against a variety of laboratory-derived and clinical strains of HIV-1 ranged from 0.13 to 2.0 µg/ml when the antibody was present for only the initial 18 h of the culture period. When the antibody was replenished throughout the culture period, the mean EC_{50}s were significantly lower [86]. Clinical trial phase II results showed that HIV-infected patients, who have tried and failed other drug regimens, and received TNX-355 in combination with an optimized background regimen (OBR), had a considerably greater reduction in viral load and a statistically significant increase in $CD4^+$ cells than did patients given placebo in combination with OBR (Lewis S. The XVI International AIDS Conference, Toronto, August 17, 2006). Another peptide mimicking the D1 domain of the CD4 receptor is CD4M33. The compounds induced conformational changes in gp120 that are similar to those initiated by soluble CD4. CD4M33 effectively inhibited infection by

different HIV-1 strains, including HIV-1(HXB_2), HIV-1(IIIB), HIV-1(YU2), and HIV-1(BaL) both in continuous cell lines, in co-receptor-transfected HeLa cells and in PBMC. Importantly, CD4M33 also inhibited infection by primary HIV-1 isolates, including pure CCR5-using isolates, dual-tropic isolates and an isolate that uses CCR5, CCR3, and CXCR4 for entry. EC_{50} values ranged from 1.7 - 151 µg/ml [87]. The conserved CD4-binding pocket on gp120 is a target for BMS-806, another HIV binding inhibitor, developed by Bristol-Meyers Squib. Binding of BMS-806 to gp120 is specific, reversible and co-receptor independent. Preclinical development of this inhibitor is ongoing. BMS-806 has excellent potency against a panel of HIV-1 laboratory strains, with a median EC_{50} of 12 nM (range 0.9 - 743 nM), and is effective against R5 and X4 HIV-1 strains and R5/X4 HIV-1 strains [88]. The compound showed good pharmaceutical properties [88, 89]. BMS-906 has been replaced by BMS-488043, a derivative based on the structure-activity-relationship studies. BMS-488043 is a novel, oral, small-molecule attachment inhibitor of HIV-1 that blocks viral entry by preventing the binding of the viral envelope protein gp120 to cellular CD4 receptors on the surface of T cells. *In vitro* susceptibility assays indicate that BMS-488043 is effective against both X4 and R5 HIV-1 laboratory strains, with potent activity against subtype B clinical isolates (median EC_{50} of 37 nM). It is inactive against HIV-2, other RNA viruses, and is non-cytotoxic against multiple cell lines. (Lin PF *et al*., 11th Conference on Retroviruses and Opportunistic Infections, San Francisco, abstract 534, 2004). In healthy adult volunteers the compound demonstrated promising safety, tolerability and pharmacokinetics when administered for up to 14 days (Hanna G *et al*., 11th Conference on Retroviruses and Opportunistic Infections, San Francisco, abstract 141, 2004).

BMS-488043 appeared generally safe and well tolerated, with no serious adverse effects or participant discontinuations observed in phase II clinical trials (Hanna G *et al*. 11th Conference on Retroviruses and Opportunistic Infections, San Francisco, 2004, abstract 535). In this study, patients who received either 800 or 1800 mg of drug responded with viral load reductions of approximately 1 and 1.2 $\log_{10}$ copies/ml, respectively, over 10 days of therapy.

In addition, co-receptor antagonists can inhibit HIV-1 replication. Schering-Plough developed SCH-C, an orally bioavailable CCR5 antagonist which has a broad and potent antiviral activity *in vitro* against primary HIV-1 isolates that use CCR5 as their entry coreceptor, with mean 50% inhibitory concentrations ranging between 0.4 and 9 nM [90]. SCH-C demonstrated good pharmacokinetics, safety and tolerability besides a clear decrease in HIV RNA in phase I/II clinical trials (Reynes J. *et al*., 9th Conference on Retroviruses and Opportunistic Infections, Seattle, oral abstract 1, 2002). SCH-C has been shown to prevent HIV-1 entry in the hu-PBMC-SCID mouse model [90]. The compound has been replaced by SCH-D. SCH-D acts in a similar mode, but shows higher antiviral potency. In a PBMC infection assay with a panel of 30 R5-tropic HIV-1 isolates from different regions of the world, representing diverse genetic clades SCH-D showed potently inhibitory activity against all the viral isolates tested, with geometric mean EC_{50}s ranging between 0.04 and 2.3 nM. As expected, viruses capable of using the CXCR4 coreceptor for infection were not significantly inhibited in primary PBMC cultures [91]. Schurmann and colleagues presented compelling data on this CCR5 inhibitor. The compound has been shown in a study of drug-naive patients to have potent anti-HIV activity when dosed at 10, 25, or 50 mg twice daily. The best results were obtained at the

2 higher doses, with viral load decreases as great as ~1.3 $\log_{10}$ copies/ml (Schurmann D. *et al*., 11th Conference on Retroviruses and Opportunistic Infections, SCH D: antiviral activity of a CCR5 receptor antagonist, San Francisco, California. Abstract 140LB, 2004). PRO 140, another entry inhibitor of Progenics, is an anti-CCR5 monoclonal antibody and binds to several sites of the CCR5 ectodomain. Low nanomolar concentrations of PRO 140 inhibited infection of PBMC by R5 primary HIV-1 isolates representing subtypes A to C, E, and F with a median EC_{90} value of 16 nM. Interestingly, significant inhibition of a dualtropic subtype C virus was also observed. There were no obvious genetic-subtype-dependent differences in viral sensitivities [92]. Phase I/II clinical trials with PRO 140 are currently ongoing. Other anti-CCR5 agents are TAK-779 [93], TAK-220 [94], 2 entry inhibitors of Takeda, GW-873140 (Glaxo SmithKline) [95] and UK 427,857 (Pfizer) [96].

A number of polycationic molecules were found to interact electrostatically with the negatively charged amino acid residues of CXCR4. The majority of these compounds are high molecular weight peptidic agents, *e.g*. T22 [97], T134 [98], T140 [99] and the Tat protein analogues, ALX40-4C [100] and CGP64222 [101]. Examples of low molecular weight CXCR4 antagonists are the highly potent and selective bicyclams [102-104] of Anormed, represented by AMD3100. AMD3100 has an EC_{50} of 1 to 10 ng/ml against different strains of HIV-1, including clinical isolates [105]. The compound has been evaluated in Phase II clinical trials (Schols D. *et al.*, 9th Conference on Retroviruses and Opportunistic Infections, Seattle, oral abstract 2, 2002) but has not been further pursued for the treatment of HIV infection essentially because the lack of oral bioavailability. The clinical use of AMD3100 is now re-oriented towards stem cell mobilization for

transplantation of stem cells in cancer patients (non-Hodgkin lymphoma and multiple myeloma) [106, 107]. AMD070 is a second generation CXCR4 antagonist of lower molecular weight than AMD3100 but with a similar binding site. AMD070 was found to strongly inhibited virus infectivity at an EC_{50} of 1-10 nM. The compound inhibited the replication of X4 HIV-1 and HIV-2 laboratory strains, primary clinical isolates and drug-resistant viruses in different $CD4^+$ T-cell lines, CXCR4-transfected cell lines and PBMCs. AMD070 had no activity against R5 HIV-1 variants. However, R5X4 and R3R5X4 HIV strains, which were able to use CCR3 and/or CCR5, in addition to CXCR4, for entering transfected cells, were prevented from infecting PBMCs in the presence of AMD070 [108]. It is currently the leading AMD derivative under clinical investigation. Preliminary data on AMD070 shows that the drug is active, generally safe and well tolerated in HIV patients. The study involved dosing of AMD070 twice daily for 10 consecutive days. Activity data from the first 8 HIV patients enrolled in this first dose cohort show that 4 of the 8 patients had significant reductions in CXCR4 viral load with an average reduction of 1.3 log (Anormed, Press release, March 17, 2006). From the group of HIV entry inhibitors that interfere with the hairpin formation and membrane fusion, enfuvirtide, also referred to as T-20 and distributed by Trimeris as Fuzeon, has been approved by the FDA to be included in anti-HIV combination regimens [109, 110].

T-20, the only entry inhibitor currently approved by the FDA, is a synthetic 36-amino acid peptide segment corresponding to residues 127-162 of the ectodomain of gp41, i.e. the envelope HR2 domain. T-20 binds to the *N*-terminal fusion peptide of the pre-hairpin intermediate and prevents the subsequent formation of the fusion-active hairpin conformation [111]. A second generation fusion inhibitor, T-1249, which binds to a

slightly different region of HIV gp41 protein than T-20, has already been identified. T-1249 has greater potency *in vitro*, compared with T-20, and elicits activity against most T-20-resistant isolates [112]. The compound demonstrated a strong dose-response in patients with multi-drug resistant strains [113, 114]. However, the clinical development of this compound was discontinued due to formulation problems [115].

1.1.5.2. Integrase inhibitors

The first decade of research on effective inhibitors of the viral integrase yielded different mechanistic classes of compounds (for reviews, see [116-119]): 1) nucleotides and analogs, 2) hydroxylated aromatic compounds, 3) agents interacting with DNA, and 4) peptides and antibodies. However, most of these compounds did not exhibit antiviral activity in cell culture or were too toxic. For most of the IN inhibitors with antiviral activity in cell culture, it was not unambiguously shown that integration was the sole target. Both the G-quartets [120] and L-chicoric acid derivatives [121], IN inhibitors with proven antiviral activity, were shown to target viral entry as well in cell culture [122, 123]. Thus far only 2 classes of integrase inhibitors have been identified, the integrase strand transfer inhibitors (INSTI's) and the integrase binding inhibitors (INBI's).

The identification of a series of diketo acids (DKA) as strand transfer inhibitors that prevent integration and HIV-1 replication in cell culture, provided the first proof of principle for HIV-1 integrase inhibitors as antiviral agents [124]. Their mode of action is based on the ability to bind selectively to the integrase complexed with the viral (or donor) DNA and to compete with the host (or target) DNA [125, 126]. A first diketo acid, L-708,906 (Figure 4A), showed *in vitro* activity against various HIV-1, HIV-2 and SIV

strains [127, 128]. The compound was active at an EC_{50} of 12.4 or 24.5 μM against HIV-1(III_B) or HIV-1(NL4.3), respectively. The EC_{50} for inhibition of HIV-2(ROD) was 62.1 μM, and for inhibition of SIV(MAC_{251}) it was 22.6 μM. L-708,906 was also active against the replication of HIV-1 clinical strains in PBMCs (EC_{50}, 16.0 μM). L-708,906 retained its activity when tested against drug-resistant HIV-1 strains [128]. Shionogi & Co Ltd replaced the carboxylic acid group of DKA with isosteric moieties, such as a tetrazole or a triazole group. Out of more than 300 of these analogues, S-1360 was selected for further study. The compound inhibited strand transfer and viral replication in the micromolar range [129]. The EC_{50} of S-1360 against HIV-1(III_B) in the MT-4/MTT assay was 200 nM. S-1360 also demonstrated potent antiviral activity against a variety of clinical isolates with a mean EC_{50} of 140 nM. S-1360 was active against both X4 tropic and R5 tropic strains, and against known NRTI, NNRTI, and PI drug-resistant variants (9^{th} Conference on Retroviruses and Opportunistic Infections, Seattle, 2002, Yoshinaga *et al.*, Oral Abstract Session 4, S-1360: in Vitro Activity of a New HIV-1 Integrase Inhibitor in Clinical Development). S-1360 (Figure 4B) was the first integrase inhibitor to reach clinical studies. The results of this study indicated that S-1360 was generally well tolerated and had an acceptable pharmacokinetic profile. S-1360 displayed antiviral activity with an efficiency of the same order of magnitude as that of AZT [130]. However, phase II clinical trials with S-1360 were halted secondary to toxicity concerns. Glaxo SmithKline is investigating a sister compound of S-1360, GSK-810871, that is now in preclinical development [119]. The Merck group developed a series of metabolically more stable heterocyclic compounds, represented by L-870,810, that contains a 8-hydroxy-[1,6]-naphthyridine-7-carboxamide pharmacophore as a substitute

for the 1,3-DKA moiety [131]. The compound inhibited the replication of HIV-1(III_B) with an EC_{95} of 15 nM and was also active against SIV (EC_{95} of 7 - 15 nM), HIV-2 (EC_{95} of 8 nM) and multidrug-resistant viruses. L-870,810 also inhibited HIV-1 clinical isolates and exhibited comparable activity against non-syncytia and syncytia viruses from clades A - D and F [132]. In a double-blind, placebo-controlled, randomized trial in 30 HIV-1 infected patients, L-870,810 monotherapy resulted in a 50-fold reduction in viral load, but clinical studies were halted due to liver and kidney toxicity observed in dogs. Its sister molecule, L-870,812, an inhibitor of HIV-1 and SIV integrase, has shown efficacy in rhesus macaques infected with simian-human immunodeficiency virus (SHIV) [133]. The Merck compound L-900,612 (MK-0518) (Figure 4D) is a new strand transfer inhibitor that inhibits HIV replication in cell culture. The compound was broadly active against primary isolates from different subtypes and different tropism with EC_{50} values ranging from 6 - 50 nM (Data presented at the 14th International AIDS Conference, Toronto, 2006, Mike Miller for the HIV-1 Integrase Inhibitor Discovery Team, Biochemical and antiviral activity of MK Biochemical and antiviral activity of MK-0518, a potent HIV integrase inhibitor). At the 13th Conference on Retroviruses and Opportunistic Infections a multisite, placebo-controlled phase II clinical trial of MK-0518 which involved 167 people infected with multidrug-resistant HIV was described. MK-0518 lowered the level of HIV to below 400 copies per ml in 80% of the treated participants. The drug was generally well-tolerated with similar adverse events and side effects to those reported in the placebo arm. The promising clinical data obtained with MK-0518 thus advanced the compound along the developmental pipeline entering phase III trials (Grinsztejn B. *et al.* and the Protocol 005 Study Team. 13th Conference on Retroviruses and Opportunistic

Infections, Denver, abstract LB159, 2006), [134].

GS-9137 (Figure 4E) (formally JTK-303, identified by Japan Tobacco Inc. and licensed to Gilead in 2005) is a dihydroquinoline-3-carboxylic acid strand transfer inhibitor of integrase [135]. GS-9137 showed potent antiviral activity against different laboratory strains, with EC_{50} ranging from 0.1 to 0.7 nM. The compound also showed potent antiviral activity against several clinical isolates with an average EC_{50} of 0.62 nM for HIV-1 isolates and an EC_{50} of 0.53 nM for a HIV-2 clinical isolate (Data presented at the 13th Conference on Retroviruses and Opportunistic Infections, Denver, 2006, Matsuzaki *et al.*, Poster 508, JTK-303/GS-9137, a Novel Small Molecule Inhibitor of HIV-1 Integrase: Anti-HIV Activity Profile and Pharmacokinetics in Animals). At the same conference, Gilead Sciences presented data on the antiviral activity of the experimental integrase inhibitor GS-9137, in treatment-naive and experienced patients. GS-9137 monotherapy demonstrated substantial antiviral activity and was well tolerated in all dosage arms (DeJesus E. *et al*. and Gilead Sci., 13th Conference on Retroviruses and Opportunistic Infections, Denver, abstract LB160, 2006), [136].

Next to the diketo acids, a series of 5*H*-pyrano[2,3-d:-6,5-d']dipyrimidines (PDPs) has been identified as a second class of IN inhibitors [137]. The most potent congener of this class, V-165 (Figure 4C), the 5-(4-nitrophenyl)-2,8-dithiol-4,6-dihydroxy-5*H*-pyrano[2,3-d:-6,5-d']dipyrimidine, inhibited HIV-1 replication in cell culture and IN activity in an oligonucleotide-based enzymatic assay at micromolar concentrations. V-165 was active against HIV-1(III_B) at an EC_{50} of 8.9 μM. The compound was also active against HIV-1(NL4.3 and L1), HIV-2(ROD and EHO), and SIV(MAC251), at EC_{50} values ranging from 3.7 to 30 μM. V-165 retained antiviral activity against virus

strains that were resistant towards the viral entry antagonists dextran sulphate or the bicyclam AMD3100 and also proved active against several strains resistant to inhibitors of reverse transcription. Using a series of quantitative PCRs on cells transduced with HIV-1 vectors, V-165 was shown to inhibit integration without marked effect on viral DNA synthesis. Mechanism of action studies revealed that V-165 interferes with DNA-IN complex formation [138]. As such V-165 is the prototype of the integrase binding inhibitors (INBI's) [118].

The styrylquinolines (SQLs) have been described as another group of integrase inhibitors. The most promising SQLs inhibit HIV-1 integrase *in vitro* at micromolar or submicromolar concentrations and block HIV replication in CEM cells, with no significant cellular toxicity in a 5-day period assay [139]. These compounds compete with the LTR substrate for integrase binding in vitro. FZ41 is the most potent compound from this group [140]. Cell experiments suggest that these compounds inhibit HIV replication at one or several steps prior to integration [141].

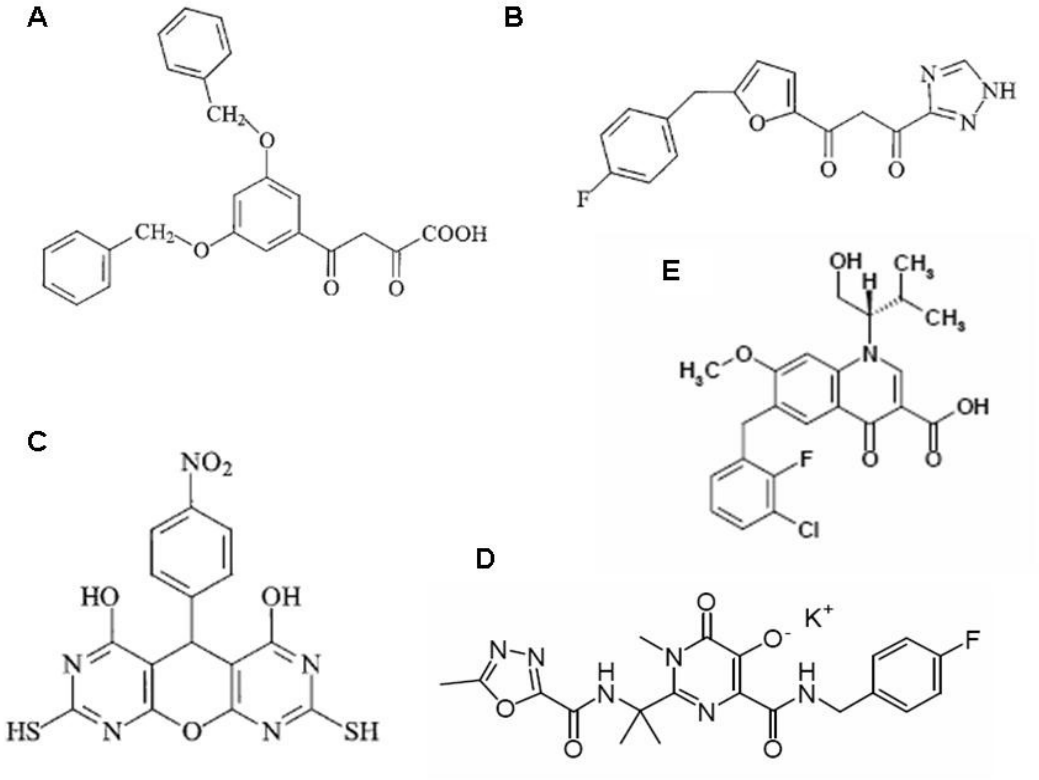

Figure 4: Chemical structures of the HIV-1 IN inhibitors (A) L-708,906, (B) S-1360, (C) V-165, (D) MK-0518 and (E) GS-9137.

1.2. HIV LATENCY

1.2.1. INTRODUCTION

HIV-1 infection is characterized by continuous viral replication throughout the disease. Antiretroviral therapy can suppress plasma HIV RNA concentrations below the detectable limit, but the major obstacle to eradicate HIV-1 is the establishment of a latent infection. These latent reservoirs are mainly composed of latently infected resting memory $CD4^+$ T cells that carry an integrated provirus that is transcriptionally silent. It is now clear that latent reservoirs of HIV-1 can persist for years in the presence of HAART. The extremely long half-life of these cells combined with a tight control of HIV-1

expression, makes this reservoir ideally suitable to maintain hidden copies of the virus, which are in turn able to trigger a novel systemic infection upon discontinuation of therapy.

1.2.2. RESERVOIRS OF HIV-1 LATENCY

1.2.2.1. Pre-integration latency

Resting $CD4^+$ T-lymphocytes are not highly susceptible for HIV infection. Entry occurs quite normally in these cells but once in the cell, the virus encounters blocks at different steps of the replication cycle. Zack *et al.* showed that after HIV-1 infection of isolated resting primary blood lymphocytes (PBL) from a non-infected donor there was an efficient initiation of reverse transcription but 24 hrs post infection incomplete reverse transcribed DNA product was still found [142]. Other reports however, have demonstrated that in $CD4^+$ T cells isolated from infected individuals only fully reverse transcribed DNA products are found [143]. In this context Pierson *et al.* performed experiments by which they could prove that the reverse transcription in $CD4^+$ T cells isolated from infected patients is delayed (up to 3 days) but results in fully transcribed DNA product. These proviral DNA products were very labile and decayed with a half life of 1 day, pointing to a significant decay of viral DNA during RT. Some of these DNA products were in a functional pre-integration complex (PIC) because GC cleavage at the 5'end, which is only possible in a functional PIC, was observed [144]. The slow completion of this reverse transcription process is probably due to a low dNTP pool in the $CD4^+$ T cells. Addition of exogenous deoxyribonucleosides to T cells in the G_0 or G_{1a} phase increases the rate at which reverse transcription proceeds [145]. Besides the block

at the level of reverse transcription the PIC faces problems to enter into the nucleus [146].

1.2.2.2. Post-integration latency

In addition to the labile form of latency, resting $CD4^+$ T cells that have an integrated copy of proviral DNA have been detected *in vivo* [147]. Since integration requires activation of the resting T cell, post-integration latency results from the return of an infected, activated T cell to the resting state. Activated T cells are highly susceptible to HIV infection and typically die quickly as a result of the cytopathic effect of the virus or the cytotoxic activity of the host cell. Some of them however survive long enough to revert to inactive cells with a memory phenotype (characterized by CD45RO) and give rise to a resting $CD4^+$ T-lymphocyte that carries an integrated provirus [143] (Figure 5). In response to antigen stimulation these resting T cell undergo proliferation and differentiation and give rise to effector cells. These cells die quickly but a small part of them survives again and gives rise to a new pool of inactive $CD4^+$ T-lymphocytes that carry an integrated provirus.

During asymptomatic HIV-1 infection, the fraction of resting $CD4^+$ T cells that carries an integrated HIV-1 provirus only counts for a small fraction (<0.05%) of the resting $CD4^+$ T cells population. This fraction is similar in blood and lymph nodes. The largest fraction of the infected, resting $CD4^+$ T cells consists of cells having linear, unintegrated viral DNA (~5%). Upon mitogenic stimulation, infectious virus can be recovered from a small fraction of the stably as well as from the unstably latently infected resting $CD4^+$ T cells [143]. Although the population of latently infected $CD4^+$ T cells having a integrated copy of viral DNA that is replication competent is small, we should not underestimate the

importance of this fraction because these memory $CD4^+$ T cells can survive for months and possibly even years [148, 149].

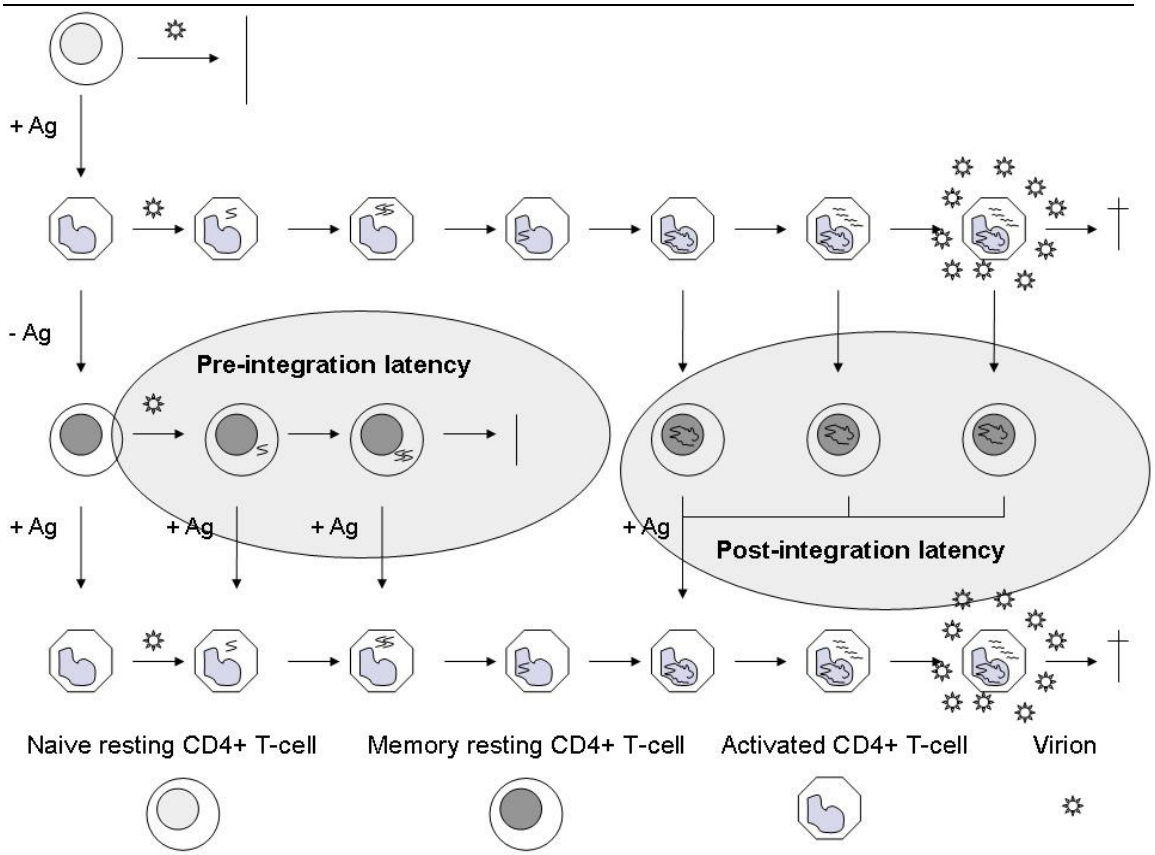

Figure 5: Generation of the different forms of HIV-1 latency

Resting, naïve $CD4^+$ T cells are poorly infectable by the commonly transmitted forms of HIV-1 owing to the absence of the CCR5 co-receptor. Resting, memory $CD4^+$ T cells are susceptible to infection with HIV-1. However, reverse transcription is slow and the virus encounters a block at nuclear import. This will result in a labile, pre-integration form of HIV-1 latency. Upon antigen stimulation, these cells become activated and viral replication can proceed. Activated $CD4^+$ T cells are highly susceptible to HIV-1 infection and allow continuous replication of HIV-1. Although these cells die quickly, some survive and revert to memory $CD4^+$ T cells which contain an integrated copy of proviral DNA. This form of latency is called post-integration latency. These cells can survive in the body for months or even years. (Adapted from Lassen et al., Trends in Molecular Medicine, 2004, 10, 525 - 531)

1.2.2.3. Other viral reservoirs

In addition to the $CD4^+$ T-lymphocytes, dendritic cells (DC) and macrophages are also considered to be reservoirs for latent HIV-1 infection. However, their role in long-term viral persistence has yet to be demonstrated. DCs express low levels of CD4 and coreceptors CXCR4 or CCR5. In addition, DCs can internalize a virus via the DC-specific ICAM-grabbing non-integrin (DC-SIGN) lectin [150]. After capture, virions are efficiently transmitted to T cells in *trans* by locally concentrating virus and receptors during the formation of the so-called infectious synapse [151]. These viruses are not protected within these DC and are degraded within a few hours in lysosomal compartments of the cell [152]. Macrophages can carry a mature viral particle in their late endosomes [153]. These viral particles can persist and retain infectivity for several weeks in these cells. The persistence of intracellular virions does however not require the viral accessory proteins Vpu and Nef [154].

1.2.3. MOLECULAR MECHANISMS BEHIND LATENCY

To study the molecular mechanisms underlying latent HIV-1 infection we need a good model. This however is not so straightforward. Four possible ways to study latency have been proposed, but they all have their individual disadvantages. A first model makes use of a cell line carrying one (ACH2) or two (U1) integrated quiescent proviruses. These latently infected cell lines have been made by infecting lymphoblasts with HIV and by selecting the cells that survive and thus resist viral replication. These cells however are characterized by mutations in the provirus, in cellular genes or at the site of integration [155, 156] and thus not entirely correspond with the natural population of latently

infected cells in infected individuals. A second model exploits HIV derived vectors [157]. These transduced T cell lines though remain activated and continue proliferating and therefore hardly resemble the quiescent, latently infected cells *in vivo*. Alternatively, as third approach, one could use a mouse model. The Severe Combined Immunodeficiency (SCID)-hu (Thy/Liv) mouse carries a source of human hematopoietic progenitor cells and human fetal thymus to provide a microenvironment for HIV infection [158]. The exact extent to which it can be applied to mimic natural infection is not known. A fourth model, the SIV macaque model reportedly shows persistence of the virus in resting $CD4^+$ T cells with many similarities to the human situation but the protocol for infection of these monkeys is so complex and impractical that this model is only used for preclinical evaluation of novel strategies to target viral reservoirs [159].

Nevertheless, different possible explanations have been proposed for the molecular mechanisms behind HIV-1 latency. A first possible explanation is the presence of transcriptional repressors. Two ubiquitous host transcription factors Yin Yang-1 (YY1) and late SV40 factor (LSF) cooperatively recruit histone deacetylase 1 (HDAC1) to the HIV-1 LTR and inhibit transcription [160]. Pyrrole-imidazole polyamides, which block the binding of the LSF-YY1 complex to the LTR promoter and consequently the recruitment of HDAC1 close to nuc-1, have been shown to induce reactivation of HIV-1 expression [161]. A second possible explanation is the absence in resting $CD4^+$ T cells of the active form of the host transcriptional activators that are necessary for HIV-1 gene expression. Nuclear Factor-κB (NF-κB), a heterodimer composed of p50 and p65, is a key host transcription factor for LTR activation. It interacts with two highly conserved binding sites found in the viral LTR and so promotes transcriptional activation [162].

NF-κB is in resting T cells sequestered in the cytoplasma, bound to IκB. Upon cellular activation or stimulation by TNF-α, NF-κB is released from IκB by the protein kinase pathway. The IκB kinase (IKK) is activated and phosphorylates IκB, leading to its degradation through the ubiquitin-proteasome pathway. NF-κB then accumulates in the nucleus and activates its target gene [163]. However, an interaction of the p65 unit of NF-κB with the HDAC1 [164] or HDAC3 [165] has been reported. Another possible explanation is a premature termination of HIV-1 transcripts due to the absence of the viral transactivator protein (Tat) and Tat-associated host factors. Tat activates HIV-1 transcription through interaction with a *cis*-acting RNA element (TAR) present at the 5' end of all nascent HIV-1 transcripts [166]. Tat works at the level of elongation, not initiation. In the absence of Tat, HIV transcription is highly inefficient because the assembled RNA polymerase II complex cannot move efficiently on the viral DNA. Tat stimulates the RNA polymerase II activity by recruiting a cellular kinase, TAK (Tat-associated kinase or CDK9/P-TEFb), composed of the catalytic subunit CDK9 and the regulatory subunit Cyclin T1, that activates transcriptional elongation by hyperphophorylation of serine-5 of the RNA polymerase II [21]. Cyclin T1 enhances the affinity and the specificity of Tat to TAR binding [167]. The levels of CDK9, Cyclin T1 and TAK activity are low in resting T cells but increase in response to activating stimuli [168]. Tat also recruits the transcriptional co-activators p300 and cAMP response element (CREB)-binding protein (CBP), that are histone acetyl-transferases (HAT) [169]. p300 and CBP exhibit strong sequence similarities, conserve similar functions and are functional homologues. Hence they are frequently referred to as p300/CBP. p300/CBP acetylates the lysine residue in the amino-terminal domains of the core histones [170].

Besides acetylating the histones, p300/CBP also acetylates Tat at Lys50 in the TAR binding domain. Acetylation at Lys50 of Tat promotes the dissociation of Tat from TAR [171] and the interaction with the bromodomain of the transcriptional co-activators p300/CBP-associated factor (PCAF) [172]. PCAF acetylates Tat in the activation domain at Lys28 which leads to enhanced Tat binding to TAK [171]. Protein interaction studies demonstrate a direct interaction between RNA polymerase II and the histone acetyltransferases p300 and PCAF. Importantly, p300 interacts specifically with the non-phosphorylated, initiation-competent form of RNA polymerase II. In contrast, PCAF interacts with the elongation-competent, phosphorylated form of RNA polymerase II [173]. The site of integration of the provirus in the host genome can also be responsible for the latency. A lot a contradictions exist concerning this topic. In an *in vitro* study Jordan *et al.* infected a transformed T cell line and then selected for clones with reversibly, non-productive infection. These clones showed a preferential integration into centromeric heterochromatin, known to be repressive for transcription [174]. This result contrasts sharply with the *in vitro* study of Schröder *et al.* who found that active genes were preferential integration targets for HIV-1, particularly genes that were activated in cells after infection [175]. A recent *in vivo* analysis of the integration sites in resting $CD4^+$ T cells was reported. Integration into centromeric regions was not seen. Instead, HIV integration into transcriptionally active regions was favored. HIV-1 integrated in introns of genes that were actively expressed in resting $CD4^+$ T cells. HIV-1 sequences might even be included in the primary transcripts of the host genes [176]. Thus, the absence of virus production in infected resting $CD4^+$ T cells can not be the result of proviral integration into regions that are intrinsically repressive for transcription.

However, only a small fraction of the latent $CD4^+$ T cells can produce replication-competent virus following cellular activation and it remains possible that the integration sites in these cells have unique characteristics. Post-transcriptional mechanisms involving the failure to export unspliced HIV-1 RNA due to the absence of sufficient levels of the viral protein Rev are also proposed as a molecular mechanism of HIV-1 latency [177].

1.2.4. THERAPEUTIC STRATEGIES TO OVERCOME LATENCY

Although highly active antiretroviral therapy (HAART) is able to decrease plasma HIV RNA levels below the limits of detection and to restore immune function, HIV can not be eradicated from patients. Intensive antiretroviral therapy in combination with T cell activation has failed to demonstrate a significant and persistent decline of the latent viral reservoirs, which appear small but stable and contain both wild-type and drug-resistant virus [178]. A therapeutic compound able to induce the expression of quiescent proviruses without activating the resting cells might allow depletion of latently HIV-infected cells. The activation of the resting latently infected cells should be avoided to prevent the generation of new target cells for the new synthesized virus. One possible approach is to combine valproic acid (VPA), an inhibitor of histone deacetylation with HAART. VPA is capable of inducing outgrowth of HIV-1 from resting $CD4^+$ T cells derived from infected patients without full activation of the cells [179]. *In vivo*, treatment with VPA in combination with HAART accelerated the clearance of HIV from resting $CD4^+$ T cells [180]. Other interesting compounds that can be used in combination with HAART to eliminate latency are prostatin or the human cytokine interleukin-7. However,

these compounds have been demonstrated to reactivate latent HIV-1 although, in the absence of cellular proliferation [181, 182].

Chapter 2

Rationale and aims of the study

Our group studies the molecular virology of HIV integration and develops novel anti-HIV drugs against new targets, i.e. virus entry, integration and integrase/LEDGF interaction. During my doctoral work I have addressed multiple questions related to antiviral or cellular resistance.

2.1. HIV RESISTANCE DEVELOPMENT

In order to predict the future *in vivo* resistance development against new inhibitors in patients, study of the development of antiviral resistance *in vitro* is required. For new compounds reported to inhibit HIV replication, the antiviral target in the replication cycle of HIV always needs to be confirmed and the molecular mode of action needs to be identified. *In vitro* selection of resistant virus is an important tool in mechanism of action studies of anti-HIV drugs. Viral resistance testing is also helpful in the clinical management of HIV-1 infected patients and may inform the clinician on therapy failure due to the emergence of resistant strains. Therefore, the development of phenotypic assays to directly measure the ability of clinical HIV isolates to grow in the presence of an HIV inhibitor is required. Next to these phenotypic assays, enzymatic assays for antiviral targets are a useful *in vitro* tool to study the influence of the mutations on the enzymatic activity and susceptibility profile of a mutant enzyme against antiviral drugs. We studied antiviral resistance against a novel entry inhibitor, SPL2923, and a compound that inhibits HIV integration, V-165.

2.1.1. HIV RESISTANCE DEVELOPMENT AGAINST SPL2923

The aim of this study was to specify the antiviral target and the molecular mode of action

of the HIV-1 entry inhibitor SPL2923. Genetic studies with HIV-1 *gag* and/or *env* mutants revealed an interaction between the cytoplasmatic tail of gp41 and the matrix protein (MA) [183-185]. Since the virus obtained after recombination of the *gp160* gene of the HIV-1 strain resistant to SPL2923 did not yield the same resistance profile against SPL2923 as the selected strain, we investigated whether mutations in the *gag* gene were in addition responsible for the observed resistance profile.

2.1.2. HIV RESISTANCE DEVELOPMENT AGAINST V-165

Antiviral resistance development against the pyranodipyrimidine V-165 resulted in mutations in the *IN*, *RT* and *env* gene. We wanted to investigate the effect of the observed mutations in the *integrase* gene on the enzymatic activity and the susceptibility against the inhibitory effect of several anti-HIV drugs, including V-165.

2.2. NATURAL RESISTANCE TO HIV-1 INFECTION

The human genome consists of 3.10^9 base pairs, that differ among the population. This makes every individual, except for identical twins, different. Differences in the genome can render an individual resistant or less susceptible for the development of certain diseases and/or virus infection. This phenomenon is called **innate immunity**. Various examples of innate immunity are known. A heterozygous state of a variant of the heamoglobin gene, causing sickle cell trait (genotype HbSA), confers a high degree of resistance to malaria [186]. A 32 bp deletion in the gene coding for the CCR5 co-receptor of HIV-1 results in a receptor that is severely truncated and cannot be detected at the cell surface [22]. Individuals, homozygous for this deletion, are resistant to infection with an

HIV-1 CCR5 strain. Several studies investigation the distribution of homozygosity and heterozygosity of the deleted allele of different populations have reported the same finding: an enrichment of the homozygous Δ32 CCR5 genotype in highly HIV exposed persons who remain uninfected [187, 188]. However, most (96% according to one estimate [189]) highly exposed HIV-seronegative persons are not homozygous for the Δ32 CCR5 allele. Moreover, among exposed but persistently HIV seronegative persons in Africa and Thailand, no single person positive for the Δ32 CCR5 allele has been found [190]. These data suggest that other mechanisms may contribute to remain seronegative despite high HIV exposure: chemokine-related mechanisms, such as altered CCR5 levels on the cell surface or the level of circulating chemokines, the involvement of immune system genes, such as those of the Human Leukocyte Antigens (HLA) system, the involvement of immune responses, such as those of cytotoxic T cells or mutations in cofactors or restriction factors that may alter the susceptibility to infection (for a review see [191]). The relative importance of these mechanisms in populations with differing modes of exposure or genetic backgrounds needs to be elucidated. We analysed the genetic variability of LEDGF/p75, an essential co-factor of HIV-1 integration, discovered in our laboratory.

2.2.1. NATURAL VARIABILITY OF LEDGF/p75

LEDGF/p75 has been identified as an important co-factor of HIV-1 integration and replication. However, little is known about the natural variability of the gene coding for LEDGF. We have started to address the question whether there is an innate immunity associated with mutations in LEDGF that protects individuals against HIV-1infection.

Chapter 3

Materials and methods

3.1. CELLS

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, supplied by Invitrogen, Merelbeke, Belgium), supplemented with 10% heat inactivated fetal calf serum (Harlan Sera-Lab Ltd., International Medical, Brussels, Belgium) and cultured at 37°C in a humidified atmosphere containing 5% CO_2.

MT-4 cells [192] were maintained in RPMI1640 medium (Gibco BRL, supplied by Invitrogen, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal calf serum (Harlan Sera-Lab Ltd., International Medical, Brussels, Belgium), 2 mM L-glutamine, 0.1% sodium bicarbonate and 20 µg/ml of gentamycin.

Generation and selection of stable MT-4 LEDGF/p75 knockdown cells (MT-4/LEDGF-KD) is described elsewhere (Hombrouck *et al.*, submitted for publication). Briefly, MT-4/LEDGF-KD were generated using lentiviral vector transfer plasmid encoding a shRNA targeting LEDGF/p75 driven by a mU6 promotor and a CMV driven eGFP-ZEOCIN fusion gene [193]. 1 x 10^5 MT-4 cells were transduced at an MOI of 1 and selected with 200 µg/ml zeocine (Invitrogen, Merelbeke, Belgium). Finally, the 10% of the highest MT-4shp75 eGFP expressor cells were sorted, till a total amount of 1 x 10^5 cells was reached, using the FACSVantage (BD Biosciences, Erembodegem, Belgium) [58]. A similar approach was used for the generation of the MT-4/LEDGF-mmKD cell lines. Four mutations were introduced in the short hairpin that was expressed from the transferplasmid of the lentiviral vector used. MT-4/LEDGF-KD and MT-4/LEDGF-mmKD cells were cultured as described for the MT-4 cells, however, the medium was additionally supplemented with 200 µg/ml zeocine (Invitrogen, Merelbeke, Belgium).

MT-4 LEDGF/p75 knockdown cells backcomplemented with WT (MT-4/LEDGF-KD-BC) or mutant(Q472L) LEDGF/p75 (MT-4/LEDGF-KD-BCQ472L) were cultured as described for the MT-4/LEDGF-KD cells but in addition 0.25 µg/ml puromycin was added to the medium (Invitrogen, Merelbeke, Belgium). All the cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

3.2. STUDY SUBJECTS

Blood from 10 Caucasian HIV seronegative Belgian individuals was collected. 19 seronegative female blood donors (FBD) were enrolled at the blood transfusion centre in Abidjan, Côte d'Ivoire. 20 HIV exposed seronegative (ESN) and 20 seropositive (SP) female sex workers (FSW) were enrolled at a confidential FSW clinic in Abidjan, Côte d'Ivoire, between June 1998 and May 2000. The women were followed as part of a clinical trial testing the efficacy of a nonoxynol-9 microbicide gel [194]. Whole blood was drawn in EDTA tubes (BD Biosciences, Erembodegem, Belgium). Plasma was separated from whole blood by centrifugation and tested for HIV by ELISA and Western blot, and confirmed by HIV RT-PCR. PBMC were separated from whole blood by gradient centrifugation and stored in liquid nitrogen. Samples were obtained via Institute of Tropical Medicine, Antwerp, Belgium.

3.3. COMPOUNDS

SPL2923 was synthesized as described in Witvrouw *et al*. [78]. Dextran Sulfate (DS) (average molecular weight, 5,000) was purchased from Sigma (Bornem, Belgium). AMD3100 was provided by G. Bridger (AnorMED, Langley, BC, Canada) and was

synthesized as described earlier [195]. Fuseon (T-20) was purchased from Roche Diagnostics (Vilvoorde, Belgium). 3'-Azido-3'-deoxythymidine (AZT) was synthesized according to the method described by Horwitz *et al*. [196]. Delavirdine was obtained from B. Bruce (Pharmacia and Upjohn, Kalamazoo, MI). Saquinavir was a gift from N. Roberts (Roche Products Limited, Welwyn Garden City, UK). V-165 was obtained from Ampharm Inc. (Ramsey, NJ). Nevirapine was obtained from Boehringer Ingelheim (Ridgefield, CN). Ritonavir was obtained from J.M. Leonard (Abbott laboratories, Abbott Park, IL). All compounds were dissolved in dimethylsulfoxide (DMSO), except DS and T-20, at 10 mg/ml. DS was dissolved in milli-Q water and T-20 in PBS.

3.4. VIRUS STRAINS

The plasmid pNL4.3 [197], a molecular clone consisting of the plasmid pUC18, wherein the complete HIV-1 genome flanked with chromosomal DNA is inserted, was obtained from the National Institutes of Health (Bethesda, MD).

The origin of the HIV-1(III_B) strain has been described elsewhere [198]. NL4.3/SPL2923, NL4.3/DS and NL4.3/T-20 have previously been selected by serial passage of HIV-1(NL4.3) in the presence of increasing concentrations of SPL2923, DS or T-20 respectively [78, 199, 200].

NL4.3 and III_B strains resistant towards the integrase inhibitor V-165 were selected. The resistance selection of HIV-1(NL4.3) and HIV-1(III_B) against V-165 was initiated at a low multiplicity of infection (MOI = 0.01) in MT-4 cells and a drug concentration equal to the 50% effective concentration (EC_{50}), as determined in the MT-4/MTT assay (EC_{50} = 3 μM). Every 3 to 4 days the MT-4 cell culture was monitored for the appearance of

HIV-induced cytopathic effect (CPE). When CPE was observed, the cell free culture supernatant was used to infect fresh, uninfected MT-4 cells in the presence of an equal or higher concentration of the compound. When no virus breakthrough was observed, the infected cell culture was subcultivated in the presence of the same concentration of compound. The corresponding selected virus strains are further referred to as NL4.3/V-165 and III_B/V-165.

3.5. PLASMIDS

The bacterial expression plasmids pRP1012 (Dr. R. H. A. Plasterk, Dutch Cancer Institute, Amsterdam, The Netherlands), encoding HIV-1 IN (strain HTLV-III), or pCP-Nat75, encoding LEDGF/p75, [50] were used for bacterial expression of His_6-tagged IN or non-tagged LEDGF/p75, respectively.

Site-directed mutagenesis to generate the mutant expression plasmids was performed using the QuickChangeTM Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Briefly, the WT bacterial expression plasmid and two synthetic primers, complementary to each other and containing the mutation (referred to as MUT_F and MUT_R), were used (Table 1). The primer sets were extended during temperature cycling by means of *Pwo* DNA polymerase (Roche Diagnostics, Mannheim, Germany). The PCR conditions were as follows: denaturation for 2 min at 95°C was followed by 16 cycles of amplification consisting of 30 sec at 95°C, 60 sec at 55°C and 10 min at 68°C. Subsequently, the PCR product was digested with *Dpn*I, which selects for the synthesized DNA containing the mutations. The pRP1012 expression plasmid containing the single mutation T206S was further mutagenized to obtain the double mutant. The presence of

the expected mutations was confirmed by DNA sequencing.

To construct the lentiviral transfer plasmids pCombi_LEDGF_IRES_Puro and pCombi_LEDGF(Q472L)_IRES_Puro, eGFP was removed from the plasmid pCombi_eGFP_IRES_Puro by *Bam*HI-*Spe*I digestion and replaced by *Bam*HI-LEDGF-*Xba*I or *Bam*HI-LEDGF(Q472L)-*Xba*I, respectively. These fragments were obtained by PCR amplification using the primers LEDGF-forw and LEDGF-rev using respectively pEF1-LEDGF-Back [51] or pEF1-LEDGF(Q472L)-Back as a template. Subsequent to amplification the resulting products were digested with *Bam*HI and *Spe*I.

Table 1: Primers used for the construction of the different plasmids

N155L_F	5'-CCA GGG CGT AGT AGA ATC TAT GCT TAA AGA ATT AAA GAA AAT TAT AGG AC-3'
N155L_R	5'-GTC CTA TAA TTT TCT TTA ATT CTT TAA GCA TAG ATT CTA CTA CGC CCT GG-3'
I161T _F	5'-GAA TAA AGA ATT AAA GAA AAC TAT AGG ACA GGT AAG AGA TCA GGC-3'
I161T_R	5'-GCC TGA TCT CTT ACC TGT CCT ATA GTT TTC TTT AAT TCT TTA TTC-3'
V165I_F	5'-GAA AAT TAT AGG ACA GAT AAG AGA TCA GGC TGA ACA TCT TAA GAC-3'
V165I_R	5'-GTC TTA AGA TGT TCA GCC TGA TCT CTT ATC TGT CCT ATA ATT TTC-3'
T206S_F	5'-GGG GAA AGA ATA GTA GAC ATA ATA GCA TCA GAC ATA CAA ACT AAA G-3'
T206S_R	5'-CTT TAG TTT GTA TGT CTG ATG CTA TTA TGT CTA CTA TTC TTT CCC C-3'
S230N_F	5'-CGG GTT TAT TAC AGG GAC AAC AGA AAT CCA CTT TGG AAA G-3'
S230N_R	5'-CTT TCC AAA GTG GAT TTC TGT TGT CCC TGT AAT AAA CCC G-3'
Q472L_F	5'-AAA AGC TAG AGA AGG AGC TCA CAG GGT CAA AGA C-3'
Q472L_R	5'-GTC TTT GAC CCT GTG AGC TCC TTC TCT AGC TTT T-'3
LEDGF-forw	5'-GGC GGG ATC CAG ACA CCA TGA CTC GCG ATT TCA AAC C-'3
LEDGF-rev	5'-CAG GTC TAG ACT AGT TAT CTA GTG TAG AAT CCT TC-'3

3.6. LENTIVIRAL VECTOR PRODUCTION

Lentiviral vector production was performed as described earlier [157] with minor modifications [201]. Briefly, WT and mutant(Q472L) Combi_LEDGF_IRES_Puro

vector particles were produced by triple, transient transfection of 293T cells with a second generation packaging plasmid lacking the *vif*, *vpr*, *vpu* and *nef* genes (pCMVΔR8.91) [202], a plasmid encoding the envelope of vesicular stomatitis virus (pMDG) [202] and the WT or mutant(Q472L) pCombi_LEDGF_IRES_Puro transfer plasmid. 24 h prior to transfection, 6×10^6 cells 293T cells were seeded in a Ø 8,5 cm cell culture dish in OPTIMEM with Glutamax (Gibco BRL, supplied by Invitrogen, Merelbeke, Belgium) supplemented with 2% fetal calf serum. The next day, 20 µg of transfer plasmid, 10 µg of pCMVΔR8.91, 5 µg of pMDG and 150 mM NaCl were mixed to a total volume of 700 µl. An equal volume containing 100 µl of a 10 mM polyethylenimine (PEI) stock solution and 600 µl of 150 mM NaCl was added slowly to the DNA mixture. Following 15 min of incubation at room temperature, the medium was taken of the 293T cells, the DNA-PEI complexes were added to 5 ml of OPTIMEM with Glutamax without serum and put on the cells. After overnight incubation at 37°C in a 5% CO_2 humidified atmosphere, the medium was replaced. Supernatant was harvested at day 2 and 3 post-transfection and filtered through a 0.45 µm pore-size filter (Sartorius, Minisart, Göttingen, Germany). The filtered vector particles were concentrated using Vivaspin 15 50 000 MW (Vivascience, Hannover, Germany) at 2500 g. Vector containing medium was stored at -80°C.

3.7. GENERATION OF THE MT-4/LEDGF-KD CELL LINE STABLY EXPRESSING WT OR Q472L-LEDGF/p75

To analyze the effect of Q472L-LEDGF/p75 expression on viral replication, MT-4/LEDGF-KD cells were transduced with Combi_LEDGF_IRES_Puro vector. 24 hrs

prior to transduction cells were seeded in a 96 well plate at a density of 60 000 cells/well. The next days, the cells were transduced with WT or mutant(Q472L) Combi LEDGF IRES Puro vector. After overnight incubation at 37°C in a 5% CO_2 humidified atmosphere, the medium was replaced. Five days after transduction, the cells were selected with puromycin (0.25 µg/ml). At different time-points during selection LEDGF/p75 expression was analysed with Western blotting.

3.8. WESTERN BLOTTING

Whole-cell extracts of the different cell lines were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad, München, Germany). Membranes were blocked with milk powder in PBS supplemented with 0.1% Tween 20. Membranes were exposed overnight to specific antibodies against LEDGF/p75 (BD Biosciences, Erembodegem, Belgium), washed with PBS supplemented with 0.1% Tween 20 for about 1 hr and followed by 3 hrs exposure to goat anti-mouse antibodies conjugated with horseradish peroxidase (Dako, Glostrup, Denmark). Detection was performed using chemiluminescence (ECL^+) (Amersham Biosciences, Piscataway, NJ). Equal loading was controlled using α-tubulin antibodies (Sigma, Bornem, Belgium) followed by goat anti-mouse-HRP antibodies (Dako, Glostrup, Denmark).

3.9. HIV-1 INFECTION

Infection of MT-4 cells with HIV-1(NL4.3) was performed with 1 x 10^6 cells in 1 ml medium at MOIs of 0.1 and 0.5 for 4 hrs. After 4 hrs cells were washed three times with

PBS and resuspended in 10 ml RPMI1640 medium supplemented with 2 mM L-glutamine, 0.1% sodium bicarbonate and 20 µg/ml of gentamycin. Supernatants was collected and HIV-1 replication was monitored by quantifying p24 antigen in the supernatant via ELISA (Alliance HIV-1 p24 ELISA kit, PerkinElmer, Zaventem, Belgium).

3.10. PCR AMPLIFICATION OF DIFFERENT GENES

For sequencing of the resistant HIV-1 strains a DNA extraction of proviral DNA was performed using the QIAamp blood Kit (Qiagen, Westburg, Leusden, The Netherlands). PCR reactions were performed using the Expand™ High Fidelity PCR system (Boehringer Mannheim, Roche, Germany). Primers are listed in Table 2.

The gene coding for gp120 was amplified in a nested PCR. An outer PCR reaction, using the primers AV310 and AV311 was followed by an inner PCR reaction using the primers AV312 and AV313. The outer cycling conditions were as follows: a first denaturation step of 2 min at 95°C followed by 40 cycles of 15 sec at 95°C, 30 sec at 50°C and 2 min at 72°C. A final extension was performed at 72°C for 10 min. For the inner cycling, the following conditions were used: after 2 min denaturation at 95°C, 30 cycles of 15 sec at 95°C, 30 sec at 58°C and 2 min at 72°C were performed followed by 10 min extension at 72°C.

The gene coding for gp41 was amplified using the primers AV320 and AV321. The cycling conditions were as follows: denaturation for 2 min at 95°C followed by 40 cycles consisting of 15 sec at 95°C, 30 sec at 55°C and 2 min at 68°C. A final extension was performed at 72°C for 10 min. The gene coding for gp160 was amplified using the

primers AV310 and AV319. The cycling conditions were as follows: denaturation for 2 min at 95°C followed by 40 cycles consisting of 15 sec at 95°C, 30 sec at 55°C and 3 min 40 sec at 68°C. A final extension was performed at 72°C for 10 min.

A fragment of *gag* encoding p17 and a part of p24 was PCR amplified using the primers AV14 and BVR2. The cycling conditions were as follows: denaturation for 2 min at 95°C followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C and 90 sec at 72°C. A final extension was performed at 72°C for 10 min.

The gene coding for integrase was PCR amplified using the primers HP4149 and IN-PCRC. The PCR conditions were as follows: denaturation for 2 min at 95°C, followed by 40 cycles of amplification consisting of 30 sec at 95°C, 30 sec at 52°C and 90 sec at 72°C. A final extension was performed at 72°C for 1 0min.

The gene encoding RT was PCR amplified using the primers RT1 and RT2. The PCR conditions were as follows: denaturation for 2 min at 95°C was followed by 35 cycles of amplification consisting of 15 sec at 95°C, 60 sec at 65°C and 90 sec at 72°C and a final extension at 72°C for 10 min.

For sequencing of *LEDGF/p75*, genomic DNA and total RNA was extracted from 0.5 x 10^6 PBMC cells using Trizol (Invitrogen, Merelbeke, Belgium). The DNA and RNA pellets were resuspended in 35 µl mQ. cDNA synthesis from the extracted RNA was performed using ThermoScript RT-PCR-system (Invitrogen, Merelbeke, Belgium) and oligo(dT) primers. The PCR conditions were as follow: 5 min at 65°C and 60 min at 50°C followed by 15 min at 70°C. cDNA samples were stored at -20°C. A fragment of LEDGF/p75, consisting of the integrase binding domain, was amplified from this cDNA using the primers LEDGF-F1 and LEDGF4. The cycling conditions were as follows:

denaturation for 10 min at 95°C followed by 35 cycles of 30 sec at 95°C, 1 min at 50°C and 90 sec at 68°C. A final extension step was performed at 72°C for 10 min. A fragment of *LEDGF/p75*, consisting of the integrase binding domain, was amplified from the extracted DNA by PCR using the primers LEDGF1 and LEDGF3. The cycling conditions were as follows: denaturation for 10 min at 95°C followed by 35 cycles of 30 sec at 95°C, 1 min at 50°C and 10 min at 68°C. A final extension step was performed at 72°C for 10 min.

Table 2: Primers used for PCR amplification of different genes

gene		
gp120	**AV310**	5'-AGC AGG ACA TAA T/CAA GGT AGG-3'
	AV311	5'-CTA CTT TAT ATT TAT ATA ATT CAC TTC TCC-3'
	AV312	5'-AGA A/GGA C/TAG ATG GAA CAA GCC CCA G-3'
	AV313	5'-TCC TTC ATA TTT CCT CCT CCA GGT C-3'
gp41	**AV320**	5'-ATT GTA/G GAG GA/GG AAT TTT TCT ACT G-3'
	AV321	5' TTG CTA/G CTT GTG ATT GCT/C CCA TG-3'
gp160	**AV310**	5'-AGC AGG ACA TAA T/CAA GGT AGG-3'
	AV319	5'-GCT G/C CC TTA/G TAA GTC ATT GGT CT-3'
p17/p24	**AV14**	5'-CTC TCT CGA CGC AGG ACT CGG CTT GCT GAA-3'
	BVR2	5'-GCC AA/GA TC/TT TCC CTA AAA AAT TAG CC-3'
IN	**HP4149**	5'-CAT GGG TAC CAG CAC ACA AAG G-3'
	IN-PCRC	5'-CCC AAA TGC CAG TCT CTT TCT CCT G-3'
RT	**RT1**	5'-GTA GAA TTC TGT TGA CTC AGA TTG G-3'
	RT2	5'-GAT AAG CTT GGG CCT TAT CTA TTC CAT-3'
LEDGF	**LEDGF-F1**	5'-GCC AGA AGT TAA GAA AGT GGA GAA G-3'
	LEDGF4	5'-GGA TTT ACC AGA CTG TCT TTT CAC TG-3'
	LEDGF1	5'-TTG GGC TCA AAG CAT TAA TCC-3'
	LEDGF3	5'-CTC TGA AGG ATT CTA CAC TAG ATA AC-3'

3.11. SEQUENCING OF DIFFERENT GENES

For sequencing of the different genes PCR products were first purified using the PCR purification kit (Qiagen, Westburg, Leusden, The Netherlands). To carry out the sequencing reaction, the ABI PRISM™ Dye terminator cycle sequencing core kit (Perkin Elmer, Brussels, Belgium) was used. The primers are listed in Table 3.

The primers used to sequence the *gp41* gene were: AV322, AV32, AV324, AV326, AV327, AV328, AV329, AV330 and AV331. The primers used to sequence the *gp120* gene were: AV304, AV305, AV306, AV307, AV308, AV309 and AV313. The primers used to sequence the *gag* gene were: AV58, AV13, AV103, AV26, AV159 and BVR3. The primers used to sequence *integrase* were: IN-PCRA, IN-SEQ3, IN-SEQ1 and IN-PCRB. The primers used to sequence the *RT* gene were: AV36, AV44, AV59, AV181, AV191 and MW3. The primers used to sequence the cDNA fragment of *LEDGF/p75* were: LEDGF-F1, LEDGF-S3 and LEDGF-R1. The primers used to sequence *LEDGF/p75* were: Seq1, Seq2, Seq3, Seq4, S9 and R1. The samples were loaded on the ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Brussels, Belgium). The sequences were analyzed using the software program Vector NTI Suite 7 (InforMax Inc., Oxford, UK).

Table 3: Primers used for sequencing of different genes

gene		
gp41	**AV322**	5'-AAG CAA TGT ATG CCC CTC C-3'
	AV323	5'-CTG CTC CC/TA AGA ACC CAA-3'
	AV324	5'-GGC AAA GAG AAG AGT GGT-3'
	AV326	5'-TTG GGG T/CTG CTC TGG AAA AC-3'
	AV327	5'-TTT TAT ATA CCA CAG CCA-3'
	AV328	5'-ATA ATG ATA GTA GGA GG-3'
	AV329	5'-GTC CCA GAA GTT CCA CA-3'
	AV330	5'-GGA G/ACC TGT GCC TCT TCA-3'
	AV331	5'-TCT CAT TCT TTC CCT TA-3'

gp120	**AV304**	5'-ACA TGT GGA AAA ATG ACA TGG T-3'
	AV305	5'-GAG TGG GGT TAA TTT TAC ACA TGG-3'
	AV306	5'-TGT CAG CAC AGT ACA ATG TAC ACA 3'
	AV307	5'-TCT TCT TCT GCT AGA CTG CCA T-3'
	AV308	5'-TCC TCA GGA GGG GAC CCA GAA ATT-3'
	AV309	5'-CAG TAG AAA AAT TCC CCT CCA CA-3'
	AV313	5'-TCC TTC ATA TTT CCT CCT CCA GGT C-3'
gag	**AV58**	5'-GGG TGC GAG AGC GTC-3'
	AV13	5'-CTG CGA ATC GTT CTA GCT CCC TGC TTG CCC-3'
	AV103	5'-GCC ATA TCA CCT AGA ACT TT-3'
	AV26	5'-GCT ATG TCA CTT CCC CTT GGT TCT C-3'
	AV159	5'-GGG ATT AAA TAA AAT AGT AAG-3'
	BVR3	5'-TTT CCA ACA GCC CTT TTT CCT AG-3'
IN	**IN-PCRA**	5'-GGA GGA AAT GAA CAA GTA GAT-3'
	IN-SEQ3	5'-GGA TAT ATA GAA GCA GAA GTT A-3'
	IN-SEQ1	5'-TTA AGA TGT TCA GCC TGA TCT-3'
	IN-PCRB	5'-CCT GAA ACA TAC ATA TGG T-3'
RT	**AV36**	5'-CAG TAC TGG ATG TGG GTG ATG-3'
	AV44	5'-TAC TAG GTA TGG TAA ATG CAG T-3'
	AV59	5'-GGG GCA AGG CCA ATG GAC-3'
	AV181	5'-TTC ATT TCC TCC AAT TCC TTT GTG-3'
	AV191	5'-CTT GAT AAA TTT GAT ATG TCC ATT G-3'
	MW3	5'-TAT GTA GGA TCT GAC TTA GAA ATA GGG C-3'
LEDGF/p75		
cDNA	**LEDGF-F1**	5'-GCC AGA AGT TAA GAA AGT GGA GAA G-3'
	LEDGF-S3	5'-CTT CAA AGG ATA CAT GC-3'
	LEDGF-R1	5'-GTC AGG TAA TCA TGG AAA AGT CTA C-3'
DNA	**Seq1**	5'-GCC AGA TAT GAT TTA ATC TAG C-3'
	Seq2	5'-GCC TGT ATA TAG AAA TAC TGG-3'
	Seq3	5'-GTG ATT TCA AGT CAT GTG GAT T-3'
	Seq4	5'-GGT GAA AAT CTG ATG GGC C-3'
	S9	5'-CTT CAA AGG ATA CAT GC-3'
	R1	5'-GTC AGG TAA TCA TGG AAA AGT CTA C-3'

3.12. RECOMBINATION EXPERIMENTS

MT-4 cells were cultured at a density of 5.0 x 10^5 cells/ml one day prior to transfection. The next day, cells were pelleted and resuspended in phosphate-buffered saline at a concentration of 3.125 x 10^6 cells/ml. For each transfection 2.5 x 10^6 cells (0.8 ml) were

used. Transfections were performed by electroporation using an EASYJECT (Eurogentec, Seraing, Belgium) and electroporation cuvettes (Eurogentec, Seraing, Belgium).

For *gp120*-recombination experiments, MT-4 cells were cotransfected with 10 µg of the linearized *gp120*-deleted pNL4.3 clone [200] and 2 µg purified AV312-AV313 PCR product (PCR Purification Kit, Qiagen, Westburg, Leusden, The Netherlands).

For *gp41*-recombination experiments, cells were cotransfected with 10 µg of the linearized *gp41*-deleted pNL4.3 clone [200] and 2 µg purified AV320-AV321 PCR product, whereas 2 µg of the AV310-AV319 PCR product was co-transfected with 10 µg of the *gp160*-deleted pNL4.3 clone [200] in *gp160*-recombination experiments.

3.13. DRUG SUSCEPTIBILITY ASSAY

To determine the inhibitory effect of antiviral drugs on the HIV-induced CPE in human lymphocyte MT-4 cell culture the MT-4/MTT-assay was used [203]. This assay is based on the reduction of the yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan derivative, which can be measured spectrophotometrically. The 50% cell culture infective dose ($CCID_{50}$) of the different HIV strains was determined by titration of the virus stock using MT-4 cells. For the drug-susceptibility assays MT-4 cells were infected with 100-300 $CCID_{50}$ of the virus stock in the presence of five-fold serial dilutions of the antiviral drugs. The concentration of the compound achieving 50% protection against the CPE of the HIV strain, which is defined as the EC_{50}, was determined.

3.14. PRODUCTION AND PURIFICATION OF RECOMBINANT PROTEINS

Recombinant IN or LEDGF/p75 was produced by transforming PC1, with the wild-type or mutant, IN or LEDGF/p75 bacterial expression plasmids. PC1 is a phage T1 resistant *Escherichia coli* lacking Endo I, a non-specific endonuclease for duplex DNA. It was obtained by P1 phagemediated transduction of the [Delta]*endA*::TcR mutation from BT333 into BL21(DE3)(pLysS) (Novagen, Madison, WI) [204]. Cells were grown at 30°C in 1 l of LB medium. At an absorbance of 0.7, measured at 600 nm, isopropylthiogalactopyranoside (IPTG) to a final concentration of 1 mM was added and cells were allowed to grow for an additional 3 hrs.

For the extraction of recombinant His_6-tagged IN, bacteria were pelleted and resuspended in cold buffer containing 50 mM Tris (pH 7.2), 1 M NaCl, 7.5 mM CHAPS and 0.1 mM PMSF and lysed by french press. The resulting suspension was centrifuged at 30000*g* for 30 min. The supernatant containing IN was loaded onto 3 ml of Ni-NTA (nitrilotriacetic acid)-chelating resin (Qiagen, Westburg, Leusden, The Netherlands) in the presence of 20 mM imidazole. The column was washed 2 times with 50 mM Tris (pH 7.2), 1 M NaCl, 7.5 mM CHAPS and 20 mM imidazole and 1 time with 50 mM Tris (pH 7.2), 1 M NaCl, 7.5 mM CHAPS and 40 mM imidazole. The IN protein was eluted in 50 mM Tris (pH 7.2), 1 M NaCl, 7.5 mM CHAPS and a gradient of imidazole (70 mM - 200 mM). Fractions were analyzed with sodium dodecyl sulfate-(SDS) polyacrylamide gel electrophoresis, and IN was detected by Coomassiestaining. Fractions containing IN were pooled and NaCl concentration was adjusted to 200 mM. The sample was loaded on a 3 ml P-Cell column. The column was washed with 50 mM Tris (pH 7.2), 7.5 mM CHAPS

and 150 mM NaCl. The IN protein was eluted in 50 mM Tris (pH 7.2), 7.5 mM CHAPS and a gradient of NaCl of 200 mM - 1000 mM. The peak fractions were pooled, NaCl concentration was adjusted to 1 M.

For the extraction of non-tagged LEDGF/p75, bacteria were pelleted and resuspended in cold buffer containing 25 mM Tris (pH 7.0), 500 mM NaCl, 10 mM DTT and 0.1 mM PMSF and lysed by sonication. The resulting suspension was centrifuged at 30000*g* for 30 min. The supernatants obtained by centrifugation of the lysate was passed through a 5 ml HiTrap Heparine column (Amersham Biosciences, Piscataway, NJ). The LEDGF/p75 protein was eluted by a gradient of NaCl (400 mM - 1100 mM) in 30 mM Tris (pH 7.0). Fractions were analyzed with SDS-PAGE and LEDGF/p75 was detected by Coomassiestaining. Peak fractions containing LEDGF/p75 were pooled and further purified on a 5 ml HiTrap Sepharose column (Amersham Biosciences, Piscataway, NJ). The protein was eluted by a gradient of NaCl (200 mM - 600 mM) in 50 mM NaPi (pH 7.2). After these purification steps, the fractions containing recombinant IN or LEDGF/p75 were analyzed with SDS-PAGE and protein were detected by Coomassiestaining. The purified proteins were further concentrated by ultrafiltration using Vivaspin 15R (Vivascience, Hannover, Germany) to a final concentration of 2 mg/ml. Finally, the purified proteins were supplemented with 5 mM dithiothreitol (DTT) and 10% glycerol or 10% glycerol alone for IN or LEDGF/p75, respectively, and frozen at -80°C. All protein concentrations were measured using the Bradford assay (Bio-Rad, München, Germany).

3.15. ENZYMATIC INTEGRASE ASSAYS

3.15.1. THE OVER-ALL INTEGRASE ASSAY

To determine the specific activity of the mutant integrase enzymes we used an over-all integrase assay making use of a radio-active labeled DNA oligo. The substrate and target DNA used in this enzymatic over-all integration assay consists of the HPLC-purified deoxyoligonucleotides, INT1 (5'-TGT GGA AAA TCT CTA GCA GT) and INT2 (5'-ACT GCT AGA GAT TTT CCA CA), corresponding to the U5 end of the HIV-1 LTR (Amersham Biosciences, Piscataway, NJ). The oligonucleotide INT1 were purified through a 20% denaturing polyacrylamide/urea gel and radioactively labeled at the 5'-end using polynucleotide T4 kinase and [γ-^{32}P]ATP (Amersham Biosciences, Piscataway, NJ). The DNA substrate and target for IN reactions was made by annealing INT1 and INT2. An equimolar mixture of the two oligonucleotides in the presence of 100 mM NaCl was heated shortly at 95°C and allowed to cool slowly to room temperature. The enzymatic integration reactions were carried out as described previously ([205] and [206]). Briefly, the final reaction mixture contained 20 mM HEPES (pH 7.5), 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 0.5% (v/v) polyethylene glycol 8000, 15% DMSO, 0.2 pmol of the oligonucleotide substrate and 1 µl of diluted IN in a final volume of 10 µl. The IN was diluted in 750 mM NaCl, 10 mM Tris (pH 7.6), 10% glycerol and 1 mM β-mercaptoethanol. Reactions were started by the addition of the enzyme and allowed to proceed for 1 hr at 37°C. Reactions were stopped by the addition of formamide loading buffer (95% formamide, 0.1% xylene cyanol, 0.1% xylene cyanol, 0.1% bromophenol blue and 0.1% sodium dodecyl sulfate). Samples were loaded on a 15% denaturating polyacrylamide/ureum gel (Figure 6). The extent of 3'-processing or DNA strand transfer

was based on measuring the respective amounts of -2 bands or strand transfer products relative to the intensity of the total radioactivity present in the lane. These data were determined using the OptiQuant Acquisition and Analysis software (Perkin Elmer Corporate, Fremont, CA).

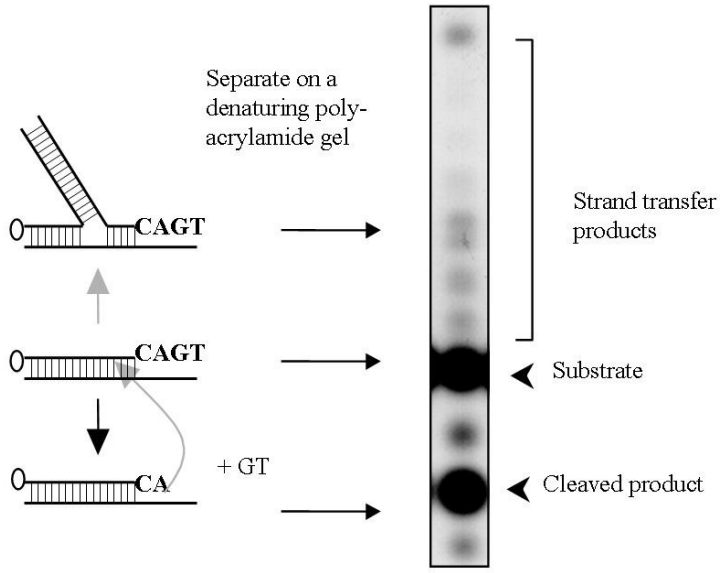

Figure 6: Analysis of the integration reaction using radiolabeled oligonucleotides

A schematic representation (left panel) and an autoradiogram (right panel) of the integrase-mediated reactions is presented. A 20-base pair oligonucleotide substrate, consisting of the terminal U5 LTR sequence is used to evaluate the integrase-mediated reactions. The 3'-processing reaction cleaves off the terminal GT dinucleotide (formation of the -2 band) and this cleaved substrate will be covalently inserted into another DNA oligonucleotide in the strand transfer reaction. O: [γ-^{32}P], radioactive label.

3.15.2. THE OVER-ALL INTEGRASE ASSAY USING AN ENZYME-LINKED IMMUNOSORBENT ASSAY

To determine the susceptibility of the different integrase enzymes towards different compounds we optimized an over-all integrase assay using an enzyme-linked

immunosorbent assay. This assay makes use of an oligonucleotide substrate of which one oligo (5'-ACT GCT AGA GAT TTT CCA CAC TGA CTA AAA GGG TC-3') is labeled at the 3' end with biotin and the other oligo (5-GAC CCT TTT AGT CAG TGT GGA AAA TCT CTA GCA TG-3') is labeled at the 5' end with digoxigenin. Oligos were annealed by heating shortly an equimolar mixture of the two oligonucleotides in the presence of 400 mM NaCl at 95°C and allowed to cool down slowly to room temperature. The different integrase enzymes were diluted to the same specific activity in 750 mM NaCl, 10 mM Tris pH 7.6, 10% glycerol and 1 mM β-mercaptoethanol. To perform the over-all integration reaction 4 µl diluted integrase (corresponding to a concentration of WT integrase of 1.6 µM) and 4 µl annealed oligos (7 nM) were added to a final reaction volume of 40 µl containing 10 mM $MgCl_2$, 5 mM DTT, 20 mM HEPES (pH 7.5), 5% PEG and 15% DMSO. The reaction was carried out for 1 hr at 37°C. This over-all integrase reaction was follow by an immunosorbent assay on an avidin coated plate [207].

3.15.3. IN-DNA BINDING ASSAY USING FCS

To determine to capacity of the mutant integrase enzymes for DNA, we used FCS. For the DNA binding assay, based on FCS, the synthetic oligonucleotides INT1 and INT2 were used. INT1 was fluorescently labeled with a tetramethylrhodamine derivative (Tamra) (Molecular Probes, Leisden, The Netherlands) at the 5' end. These oligonucleotides were purified further through a 20% denaturing polyacrylamide/urea gel. For DNA substrate preparation, equimolar amounts of complementary oligonucleotides were annealed in 20 mM HEPES (pH 7.5), containing 100 mM NaCl.

The samples were incubated at 80°C for 1 min and allowed to cool down to 20°C over the course of approximately 90 min. The final DNA concentration of the fluorescent dsDNA was determined using the ConfoCorI. In the binding assay, the DNA substrate concentration was kept constant at 30 nM, while the IN concentration varied between 0 and 1.6 µM. After an incubation of the samples for 10 min at room temperature in the reactionbuffer containing 20 mM Tris (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 10% dimethyl sulfoxide and 5% polyethylene glycol 8000, measurements were performed during 60 sec. A commercial FCS setup (ConfoCor I of Zeiss-EVOTEC) was used as described previously [208, 209]. The laser beam was focused at about 180 µm above the bottom of the Nunc cuvettes (Nalge Nunc International, Naperville, IL) in a typical volume of 10 µl. Each sample was measured 10 times. The data electronics and software (Borland Delphi) were used as described earlier [210]. The data were analyzed as described before, using the quantile plot analysis method [211].

3.15.4. IN-HIS_6-TAG PULL-DOWN ASSAY

To determine the binding capacity of LEDGF/p75 to IN we used an IN His_6-tag pull-down assay. Therefore, Ni-NTA (nitrilotriacetic acid)-chelating resins (Qiagen, Westburg, Leusden, The Netherlands) were washed by gentle centrifugation with 1 ml bindingsbuffer containing 400 mM NaCl, 25 mM Tris (pH 7.4), 0.1% Nonidet P-40, 25 mM Imidazole and 1 mM $MgCl_2$. Samples containing 20 µl Ni-NTA-chelating resin (settled volume), 20 µl bindingsbuffer and 6 µg C-terminal His_6-tagged HIV-1-IN were incubated on ice for 10 min. After this incubationstep 6 µg LEDGF/p75 and 4 µg BSA was added to the sample together with 200 µl bindingsbuffer and rocked at 4°C for 3 to 4

hrs. The samples were washed thrice with 300 µl bindingsbuffer by gentle centrifugation. Bound proteins were eluted with 20 µl elutionbuffer (bindingsbuffer supplemented with 200 mM imidazole and 1% SDS). Samples were supplemented with SDS-page sample buffer and 40 mM DTT and analyzed on SDS-PAGE followed by Coomassiestaining.

Chapter 4

Results

4.1. HIV RESISTANCE DEVELOPMENT

The current therapy for the treatment of HIV-1 infection in infected patients is based on a combination of several antiviral agents targeting multiple steps of the HIV-1 life cycle. Drugs that have been formally approved for the treatment of HIV-infected patients belong to four classes, known as nucleoside reverse transcriptase (NRTI), nonnucleoside reverse transcriptase (NNRTI), protease (PI) and fusion inhibitors. Highly active antiretroviral therapy (HAART) has remarkably reduced the mortality caused by HIV in the developed world. Nevertheless, due to low-level residual replication and the genetic flexibility of the virus, drug-resistant HIV strains emerge in treated patients. Moreover, transmission of HIV drug-resistant strains has been recognized as a serious threat to the efficacy of current antiretroviral therapy. In this context, both the understanding and control of antiviral resistance and the continuous development of new antiretroviral agents are warranted.

4.1.1. HIV RESISTANCE DEVELOPMENT AGAINST SPL2923

Results presented in this chapter have been published in:

Mutations in both *env* and *gag* genes are required for HIV-1 resistance to the polysulfonic dendrimer SPL2923, as corroborated by chimeric virus technology. Antivir Chem Chemother. 2005: 16: 253-66. Hantson, A., Fikkert, V., Van Remoortel, B., Pannecouque, C., Cherepanov, P., Matthews, B., Holan, G., De Clercq, E., Vandamme, A.M., Debyser, Z. and Witvrouw, M.

An attractive target for anti-HIV therapy is entry, since blocking entry should lead to suppression of infectivity and replication. HIV entry into its target cells is initiated by the aspecific attachment of the virus to cell surface heparan sulfate followed by the interaction of the viral protein gp120 with the CD4 receptor on the cell surface and the interaction of gp120 with the co-receptor. SPL2923, a polyanionic dendrimer, has been previously identified as an inhibitor of various HIV-1 and HIV-2 laboratory strains, clinical HIV isolates and SIV(mac_{251}). Time-of-addition experiments revealed that the compound inhibited the HIV-1 replication according to a mechanism similar as dextran sulfate (DS). However, at higher concentrations the compound was found to interact at the time coinciding with the moment of reverse transcription. SPL2923 could penetrate the cell and showed activity in enzymatic RT and IN assays. Reverse transcription and integration could however not be excluded as possible targets of SPL2923 [78]. DS exerts its anti-HIV activity by shielding off the positively charged sites in the V3 loop of gp120, thereby blocking the attachment to cell surface heparan sulfate [76]. The dendrimer SPL2923 shares an analogous mode of action due to its polysulfonated periphery. Dendrimers are highly branched macromolecules that are built up in generations from a

reactive core group by the use of branched building blocks to give spherical molecules. SPL2923 consists of a fourth generation polyamidoamine (PAMAM) dendrimer scaffold built from an ammonia core, which is fully capped on the surface with 24 naphthyldisulfonic acids (Figure 7). A SPL2923 drug-resistant strain (NL4.3/SPL2923) was generated by *in vitro* selection of HIV-1(NL4.3) in the presence of the compound and mutations were reported in the gene coding for *gp120* whereas no mutations were found in *RT* or *integrase* [78].

Here, we further analyzed the (cross-)resistance profile of the NL4.3/SPL2923 strain. We used *env*-Chimeric Virus Technology (CVT) [200] to determine to what extent the *env* mutations are accountable for the phenotypic resistance of NL4.3/SPL2923. Unexpectedly, the reduced sensitivity to SPL2923 was not reproduced by the recombination of *gp120*, *gp41* or *gp160* of NL4.3/SPL2923 in a wild-type background. Therefore, we had to develop a novel CVT assay in which the *gag* gene alone or in combination with *gp160* could be cloned into a wild-type background, the *gag*-chimeric virus technology. The virus obtained after recombination of the *gag* and the *gp160* genes displayed the same phenotypic resistance as the selected strain. Apparently, regions outside *env* play a role in the phenotypic resistance of NL4.3/SPL2923 towards SPL2923.

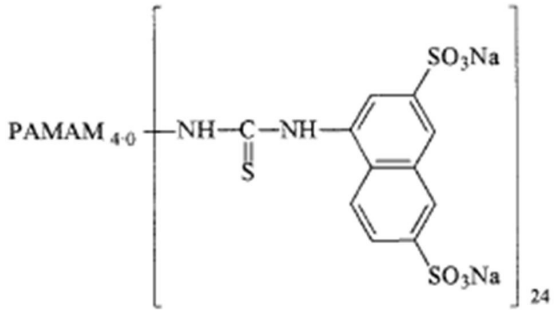

Figure 7: Chemical structure of SPL2923

SPL2923 consists of a fourth generation polyamidoamine (PAMAM) dendrimer scaffold built from an ammonia core, which is fully capped on the surface with 24 naphthyldisulfonic acids.

4.1.1.1. Genotypic analysis of the *gp160*, the *IN* and the *RT* gene of the SPL2923 drug-resistant strain

We (re)analysed the genes coding for *gp160*, *IN* and *RT* of the earlier selected NL4.3 strain resistant to SPL2923, DS or T-20 for mutations. Genotypic analysis revealed multiple mutations in the *gp120* and the *gp41* gene of NL4.3/SPL2923 in comparison to the wild-type HIV-1(NL4.3) strain (Table 4). *Gp120* displayed the following mutations; F147L, V154V/E, K292Q, F323Y, 366-370ΔFNSTW, R389E and F393V, while *gp41* displayed the mutations A22V, L33S, P216L and A308T. No mutations were found in the gene coding for IN and RT.

Table 4: Mutations in gp120 and gp41 of different resistant HIV-1(NL4.3) strains

	Amino acid position in gp120															
	115	136	147	154	247	271	280	292	295	299	323	325	366-370	389	393	460
NL4.3 WT[a]	S	S	F	V	F	N	Q	K	N	A	F	N	FNSTW	R	F	K
NL4.3/SPL2923[b]	-	-	L	V/E	-	-	-	Q	-	-	Y	-	deletion	E	V	-
NL4.3/DS[c]	N	N	-	-	I	E	H	-	D	-	-	K	deletion	T	-	N
NL4.3/T-20[d]	-	-	-	-	-	-	-	-	-	T	-	-	deletion	-	-	-

	Amino acid position in gp41								
	2	22	33	43	126	210	213	216	308
NL4.3 WT[a]	V	A	L	N	N	L	P	P	A
NL4.3/SPL2923[b]	-	V	S	-	-	-	-	L	T
NL4.3/DS[c]	V/M	-	-	-	K	L/I	P/L	-	-
NL4.3/T-20[d]	-	-	S	K	-	-	-	-	-

[a] NL4.3 wild type strain

[b] HIV-1(NL4.3) strain selected in MT-4 cells in the presence of SPL2923

[c] HIV-1(NL4.3) strain selected in MT-4 cells in the presence of DS [199]

[d] HIV-1(NL4.3) strain selected in MT-4 cells in the presence of T-20 [200]

4.1.1.2. Evaluation of phenotypic (cross)-resistance of NL4.3/SPL2923

In a next step we determined the antiviral activity of SPL2923 against the virus strains HIV-1(NL4.3) and NL4.3/SPL2923 in MT-4 cells. In parallel, the sensitivity of both strains for the HIV entry inhibitor dextran sulfate (DS), the CXCR4 antagonist AMD3100, the fusion inhibitor T-20, the nucleoside RT inhibitor AZT, the non-nucleoside RT inhibitor delavirdine and the PR inhibitor saquinavir was determined in the MT-4/MTT assay. NL4.3/SPL2923 displayed a reduced susceptibility to SPL2923 compared to the WT HIV-1(NL4.3) strain (7.1-fold increase in EC_{50}), while cross-resistance was observed towards the DS and T-20 (163.6- and 39.5-fold increase in

EC_{50}, respectively) (Table 6). The sensitivity of NL4.3/SPL2923 to AMD3100, AZT, delavirdine and saquinavir was comparable to the sensitivity of the wild-type strain (1.6-, 2.0-, 1.8- and 1.0-fold increase in EC_{50}, respectively) (Table 6 and data not shown).

4.1.1.3. *Env*-Chimeric Virus Technology (*Env*-CVT)

To see to what extent the mutations found in *gp160* were responsible for the observed (cross)-resistance profile we performed *env*-recombination experiments. *Gp120*, *gp41* or *gp160* of the wild-type and the selected, resistant NL4.3 strain were cloned into a wild type background. These recombinant viruses were referred to as Rgp120/NL4.3 and Rgp120/SPL2923, Rgp41/NL4.3 and Rgp41/SPL2923 or Rgp160/NL4.3 and Rgp160/SPL2923, respectively. The recombined *env*-sequences were verified to be identical to those of the respective HIV-1(NL4.3) or NL4.3/SPL2923 strains after recombination. The antiviral susceptibility of the recombined strains for SPL2923, DS, AMD3100, T-20 and AZT was determined. *Gp120*-recombination of NL4.3/SPL2923 resulted in a strain with sensitivity comparable to Rgp120/NL4.3 for all compounds evaluated, with the exception of DS (13.2-fold reduced sensitivity). A respectively 11.4- and 29-fold reduced susceptibility for DS and T-20 and WT sensitivity towards all other compounds was observed for the strain Rgp41/SPL2923. The *gp160*-recombined NL4.3/SPL2923 strain displayed a comparable loss in susceptibility for the compounds DS (91.0-fold) and T-20 (22.1-fold) as compared to the originally selected strain; however, only a 2.5-fold reduced sensitivity was observed for SPL2923 (Table 6).

4.1.1.4. Genotypic analysis of the *gag* gene of drug-resistant strains

Because recombination of the resistant *env*-gene in a wild-type background did not render the resulting recombinant virus (Rgp160/SPL2923) resistant to SPL2923, other mutations than those found in *gp160* had to be responsible for the observed resistance-profile. We speculated that mutations in *p17* might contribute to this resistance-profile. Sequencing analysis of the *p17* gene of the strain selected in the presence of SPL2923 revealed the following substitutions; E11K, K25R, V34I and G61R. In addition, the mixture A/E was found at position 92 of the p24-encoding region of gag. The mutations E11K and V34I were also present in the *p17* gene of the complete virus population or part of the virus population of the virus strain resistant to the entry inhibitors dextran sulfate (DS) or T-20; NL4.3/DS and NL4.3/T-20, respectively (Table 5).

Table 5: Mutations found in *gag* of different resistant HIV-1(NL4.3) strains

	Amino acid position in gag				
	p17				**p24**
	11	25	34	61	92
NL4.3 WT[a]	E	K	V	G	A
NL4.3/SPL2923[b]	K	R	I	R	A/E
NL4.3/DS[c]	K	-	I	-	-
NL4.3/T-20[d]	E/K	-	V/I	-	-

[a] NL4.3 wild type strain

[b] HIV-1(NL4.3) strain selected in MT-4 cells in the presence of SPL2923

[c] HIV-1(NL4.3) strain selected in MT-4 cells in the presence of DS [199]

[d] HIV-1(NL4.3) strain selected in MT-4 cells in the presence of T-20 [200]

4.1.1.5. *Gag*-Chimeric Virus Technology (*gag*-CVT)

To evaluate the influence of the mutations found in *gag* on the susceptibility of the NL4.3/SPL2923 strain for SPL2923 we developed an assay in which the *gag* gene alone or in combination with *gp160* could be cloned into a wild-type background, the

gag-chimeric virus technology.

4.1.1.5.1. Construction of the p17- and the p17/gp160-deleted clone

For the construction of the *p17*-deleted pNL4.3 clone, pNL4.3 was digested with the restriction enzymes *Bss*HII and *Spe*I. Since part of the packaging signal was deleted by this digest, a linker sequence, designed to contain the packaging signal and the novel *Mlu*I restriction site besides both the *Bss*HII and *Spe*I restriction sites, was created by means of PCR amplification. To construct the linker sequence, a 126-nucleotide base pair fragment of pNL4.3 was amplified using the primers AV14 and PC-VAB (5'-ACT AGT AGT TCC TGC TAT GTC ACG CGT CTC TCT CCT TCT AGC C-3'). PC-VAB was specifically designed to insert the *Mlu*I and *Spe*I restriction sites in the amplicon. Therefore, this primer contains besides the packaging signal, a tail sequence comprising both restriction sites. The cycling conditions were as follows: denaturation for 10 min at 95°C followed by 40 cycles consisting of 15 sec at 95°C, 30 sec at 55°C and 30 sec at 68°C. A final extension was performed at 72°C for 10 min. The resulting PCR fragment was digested with the restriction endonucleases *Bss*HII and *Spe*I and subsequently purified by phenol/chloroform extraction. This linker sequence was ligated into the vector to recircelize the plasmid.

The *p17/gp160*-deleted clone was generated in analogy with the previously described clones. The *gp160*-deleted clone described in Fikkert *et al.* [200] was digested with the restriction enzymes *Bss*HII and *Spe*I (Figure 8) and the linker sequence as described above, was ligated into the vector in order to recircularize the *p17/gp160*-deleted plasmid.

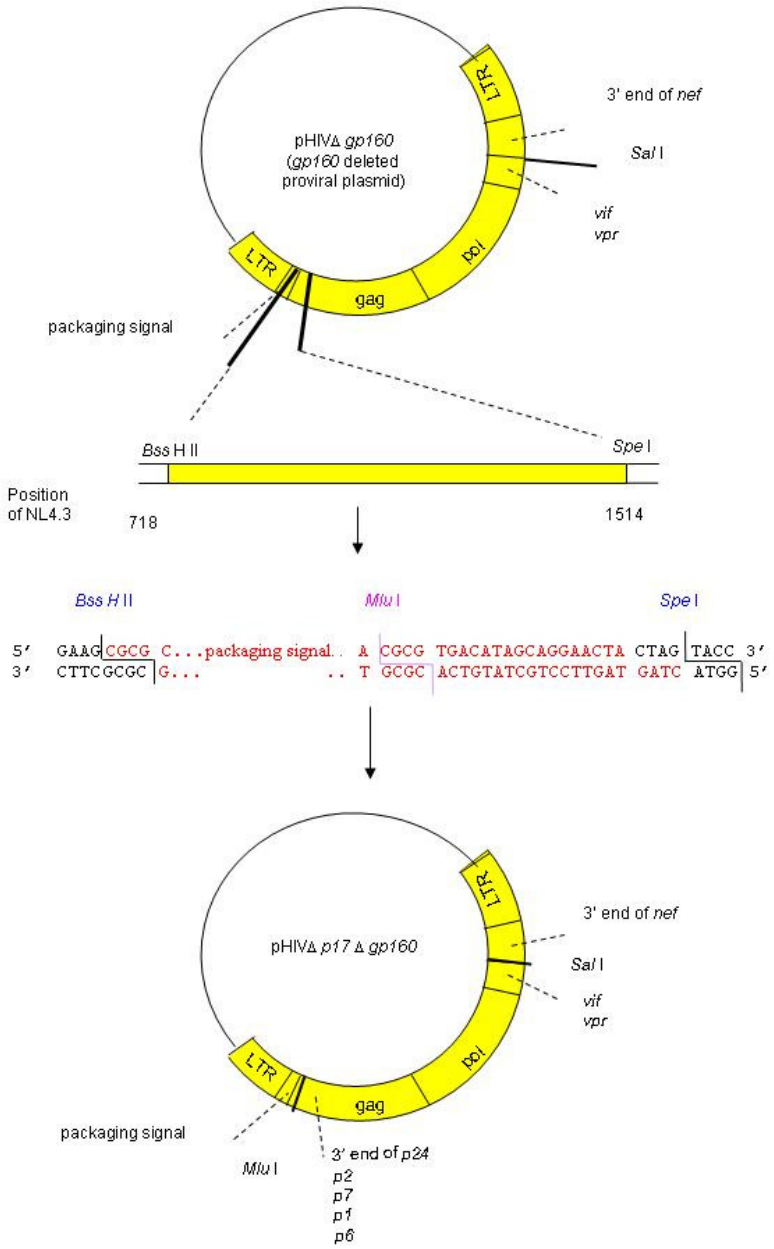

Figure 8: Construction of the *p17/gp160*-deleted clone

To generate the p17/gp160-deleted clone, the gp160-deleted pNL4.3 clone was digested with BssHI and SpeI. A linker sequence containing the MluI restriction site and the packaging signal was designed and subsequently ligated into the vector. The p17-deleted clone was constructed in analogy using the full-length pNL4.3 clone

4.1.1.5.2. p17- and p17/gp160-recombination experiments

For *p17*-recombination experiments, MT-4 cells were co-transfected with 10 µg *Mlu*I linearized *p17*-deleted pNL4.3 clone and 2 µg purified AV14-BVR2 PCR product (PCR Purification Kit, Qiagen). Recombination of the PCR amplified *gag*-region (*p17* and the 5' part of *p24*) derived from the HIV-1(NL4.3) and NL4.3/SPL2923 strains in the clone deleted for this region resulted in the *p17*-recombined strains Rp17/NL4.3 and Rp17/SPL2923. Two possible approaches for *p17/gp160*-recombination experiments were evaluated. A first approach consisted of the co-transfection of MT-4 cells with the *p17/gp160*-deleted pNL4.3 clone linearized at the position of the *p17* deletion, the *p17/gp160*-deleted pNL4.3 clone linearized at the position of the *gp160* deletion and both *p17* and *gp160* PCR products. In a second approach, the *p17/gp160*-deleted pNL4.3 clone was digested at both the position of the *p17* and the *gp160* deletion preceding co-transfection with the PCR-derived *p17* and *gp160* sequences. For the first approach of *p17/gp160*-recombination experiments, the cells were co-transfected with 7 µg of *Mlu*I linearized *p17/gp160*-deleted pNL4.3 clone, 7 µg of *Sal*I linearized *p17/gp160*-deleted pNL4.3 clone, 2 µg purified AV14-BVR2 PCR product and 2 µg purified AV310-AV319 PCR product, while for the second approach, the cells were co-transfected with 15 µg of *Mlu*I and *Sal*I digested *p17/gp160*-deleted pNL4.3 clone, 2 µg purified AV14-BVR2 PCR product and 2 µg purified AV310-AV319 PCR product. The electroporation conditions used for all transfections were 250 V and 1500 µF. The next day, the cells were centrifuged and resuspended in 10 ml culture medium and cultured at 37°C in a humidified atmosphere with 5% CO_2. The recombinant virus was harvested by centrifugation, once full CPE was microscopically observed in the culture (about 6 days

post-transfection). Aliquots of 1 ml were stored at -80°C. Recombination of the PCR amplified *gag*-region (*p17* and the 5' part of *p24*) and *env*-region (*gp160*) derived from the HIV-1(NL4.3) and NL4.3/SPL2923 strains in the clones deleted for these regions resulted in the *p17/gp160*-recombined strains Rp17gp160/NL4.3 and Rp17gp160/SPL2923, respectively. Two possible approaches for *p17/gp160*-recombination experiments were evaluated. A first approach consisted of the co-transfection of MT-4 cells with the *p17/gp160*-deleted pNL4.3 clone linearized at the position of the *p17* deletion, the *p17/gp160*-deleted pNL4.3 clone linearized at the position of the *gp160* deletion and both *p17* and *gp160* PCR products. In a second approach, the *p17/gp160*-deleted pNL4.3 clone was digested at both the position of the *p17* and the *gp160* deletion preceding co-transfection with the PCR-derived *p17* and *gp160* sequences. We obtained proof-of-principle that both approaches resulted in productive virus infection. Though, the second approach resulted faster in a productive virus infection than the first approach.

4.1.1.5.3. Phenotypic analysis of the p17- and p17/gp160-recombined strains

Phenotypical analysis of the different *gag*-recombined strains revealed that the introduction of the *p17* mutations of NL4.3/SPL2923 in wild-type background did not render the resulting virus less sensitive than the wild-type strain. The loss in sensitivity for SPL2923 of the Rp17gp160/SPL2923 strain with respect to the Rp17gp160/NL4.3 strain mirrored the decreased sensitivity of the corresponding parental selected strain (NL4.3/SPL2923) as compared to HIV-1(NL4.3) for all antiviral compounds evaluated,

that is 5.1-, 73.6-, 1.4-, 27.5-, and 0.5-fold increase in EC_{50} for SPL2923, DS, AMD3100, T-20 and AZT, respectively (Table 6).

Table 6: Susceptibility of NL4.3/SPL2923 and its different recombined strains

	EC_{50}[a] (µg/ml) (fold increase in EC_{50})[b]				
	Entry inhibitors				RT inhibitor
	SPL2923	**DS**	**AMD3100**	**T-20**	**AZT**
NL4.3[c]	0.31 ± 0.20	0.28 ± 0.24	0.015 ± 0.014	0.055 ± 0.047	0.0011 ± 0.0005
NL4.3/SPL2923[d]	2.20 ± 1.19	45.8 ± 22.5	0.025 ± 0.025	2.17 ± 0.81	0.0022 ± 0.0019
	(7.1)[b]	**(163.6)**	(1.6)	**(39.5)**	(2.0)
Rgp120/NL4.3[e]	0.23 ± 0.17	0.29 ± 0.30	0.013 ± 0.005	0.32 ± 0.21	0.0025 ± 0.0008
Rgp120/SPL2923[f]	0.43 ± 0.27	3.82 ± 2.83	0.007 ± 0.003	0.19 ± 0.12	0.0025 ± 0.0008
	(1.9)	**(13.2)**	(0.5)	(0.6)	(1.0)
Rgp41/NL4.3[g]	0.20 ± 0.09	0.14 ± 0.08	0.004 ± 0.002	0.020 ± 0.004	0.0030 ± 0.0021
Rgp41/SPL2923[h]	0.27 ± 0.17	1.6 ± 0.09	0.008 ± 0.005	0.58 ± 0.012	0.0016 ± 0.0011
	(1.4)	**(11.4)**	(2.0)	**(29.0)**	(1.9)
Rgp160/NL4.3[i]	0.29 ± 0.20	0.29 ± 0.26	0.012 ± 0.006	0.028 ± 0.005	0.0022 ± 0.0011
Rgp160/SPL2923[j]	0.73 ± 0.14	26.4 ± 20.9	0.023 ± 0.008	0.62 ± 0.34	0.0033 ± 0.0028
	(2.5)	**(91.0)**	(1.9)	**(22.1)**	(1.5)
Rp17/NL4.3[k]	0.69 ± 0.23	1.54	0.028	0.22	0.0013
Rp17/SPL2923[l]	0.72 ± 0.31	2.11	0.029	0.17	0.0014
	(1.0)	(1.4)	(1.0)	(0.8)	(1.0)
Rp17gp160/NL4.3[m]	0.42 ± 0.006	0.40 ± 0.34	0.014 ± 0.007	0.026 ± 0.006	0.0010 ± 0.0005
Rp17gp160/SPL2923[n]	2.14 ± 0.69	29.45 ± 15.20	0.020 ± 0.011	0.72 ± 0.24	0.0005 ± 0.0002
	(5.1)	**(73.6)**	(1.4)	**(27.5)**	(0.5)

[a] 50% inhibitory concentration or concentration required to inhibit the cytopathic effect (CPE) of HIV by 50% in MT-4 cells

[b] Fold increase in EC_{50} of the compound against the *in vitro* selected or recombined NL4.3/SPL2923 strain compared to the EC_{50} of the compound against the parental HIV-1(NL4.3) or recombined HIV-1(NL4.3) strain, respectively

[c] HIV-1(NL4.3) wild type strain

[d] HIV-1(NL4.3) strain *in vitro* selected in the presence of SPL2923

[e-g-i-k-m] HIV-1(NL4.3) wild-type strain recombined with the *gp120*, *gp41*, *gp160*, *p17* and *p17* and *gp160* genes of HIV-1(NL4.3)

[f-h-j-l-n] HIV-1(NL4.3) wild-type strain recombined with the *gp120*, *gp41*, *gp160*, *p17* and *p17* and *gp160* genes of NL4.3/SPL2923

Data represent mean values ± standard deviation, for at least three separate experiments

Statistical significant ($p < .025$) fold increase in EC_{50} in bold

4.1.1.6. Conclusion

A HIV-1(NL4.3) strain resistant to the inhibitor SPL2923 (NL4.3/SPL2923) was selected in cell culture by passaging HIV-1(NL4.3) in the presence of increasing concentrations of the compound. Phenotypic analysis of NL4.3/SPL2923 showed a 7.1-fold reduced susceptibility to SPL2923 and a pronounced cross-resistance to the entry inhibitors DS (163.6-fold) and T-20 (39.5-fold). However, NL4.3/SPL2923 did not show reduced susceptibility to RT inhibitors. Several mutations in the *gp120* and *gp41* genes of NL4.3/SPL2923 were identified in comparison with the wild-type HIV-1(NL4.3) strain, whereas no mutations were found in the *RT* or *IN* genes. To define to what extent the mutations in the *env*-gene were responsible for the phenotypic (cross-) resistance profile of NL4.3/SPL2923, *env*-CVT was performed. This technique places the *gp120*-, *gp41*- and *gp160*-sequences derived from this resistant strain into a wild-type background. *Gp160*-recombination reproduced the cross-resistance of NL4.3/SPL2923 towards DS, while recombination of *gp120* or *gp41* only partly reproduced this cross-resistance profile. In contrast, the mutations in *gp41* of NL4.3/SPL2923 were sufficient to explain the cross-resistance to T-20. Unexpectedly, the reduced susceptibility to SPL2923 was not fully reproduced after recombination of the *gp160*-gene derived from NL4.3/SPL2923 into wild-type background. The search for mutations in NL4.3/SPL2923 in viral genes other than *env* revealed several mutations in the gene encoding the matrix protein or p17 and one mutation in the gene coding for the capsid protein or p24. In order to analyze the impact of the *gag* mutations on their own and in combination with the mutations in *env* on the phenotypic resistance towards SPL2923, we developed a *p17*- and a *p17/gp160*-CVT. Phenotypic analysis of the *p17*- and *p17/gp160*-recombined

strains derived from NL4.3/SPL2923 revealed that the mutations in both *env*- and *gag*-genes have to be present to completely reproduce the resistant phenotype of NL4.3/SPL2923 towards SPL2923.

4.1.2. ANTIVIRAL RESISTANCE DEVELOPMENT AGAINST V-165

Results presented in this chapter will be published in:

Resistance of human immunodeficiency virus type 1 to the pyranodipyrimidine V-165. Hombrouck, A., Hantson, A., Van Remoortel, B., Michiels, M., Vercammen, J., Rhodes, J., Tetz, V., Engelborghs, Y., Christ, F., Debyser Z. and Witvrouw, M., submitted to Journal of Virology.

After integration of HIV-1 into the genome of the host cell the proviral DNA is replicated and genetically transmitted as part of the cellular genome. Therefore, integration defines a point of no return in the establishment of infection by HIV. Since no human counterpart of the enzyme is known, there is substantial interest in developing effective and selective inhibitors of HIV integrase [74]. The first decade of research on effective inhibitors of the viral integrase yielded different mechanistic classes of compounds (for reviews, see [116-118]: 1) nucleotides and analogs, 2) hydroxylated aromatic compounds, 3) agents interacting with DNA, and 4) peptides and antibodies. However, most of these compounds did not exhibit antiviral activity or were too toxic in cell culture. For most of the IN inhibitors with antiviral activity in cell culture, it was not unambiguously shown that integration was the sole target. The identification of a series of diketo acids (DKA) as strand transfer inhibitors (INSTI's) that prevent integration and HIV-1 replication in cell culture, provided the first proof of principle for HIV-1 integrase inhibitors as antiviral agents [124]. Their mode of action is based on the ability to bind selectively to the integrase complexed with the viral (or donor) DNA and to compete with the host (or target) DNA [125, 126]. Next to the diketo acids, a series of 5*H*-pyrano[2,3-d:-6,5-d']dipyrimidines (PDPs) has been identified as a second class of

IN inhibitors [137]. The molecules differ in the substituents at position 2 and 8 (X); 4 and 6 (Y) and in the para-substituent on the phenyl group (Z). The antiviral activity is dependent on the X, Y and Z substituents. PDPs interfere with the replication of various HIV-1, HIV-2 and SIV strains in cell culture. The most potent congener of this class is V-165 (Figure 9). Mechanism of action studies revealed that V-165 interferes with DNA-IN complex formation [137]. As such V-165 is the prototype of the integrase binding inhibitors (INBI's).

We investigated the resistance development of HIV-1 towards the PDP V-165. Therefore HIV-1 was grown in the presence of increasing concentrations of V-165. Mutations in *integrase*, *reverse transcriptase* and *gp160* were found. Different mutant integrase enzymes were constructed and purified. We studied the impact of the observed IN mutations at the enzymatic activity and the (reduced) susceptibility towards V-165.

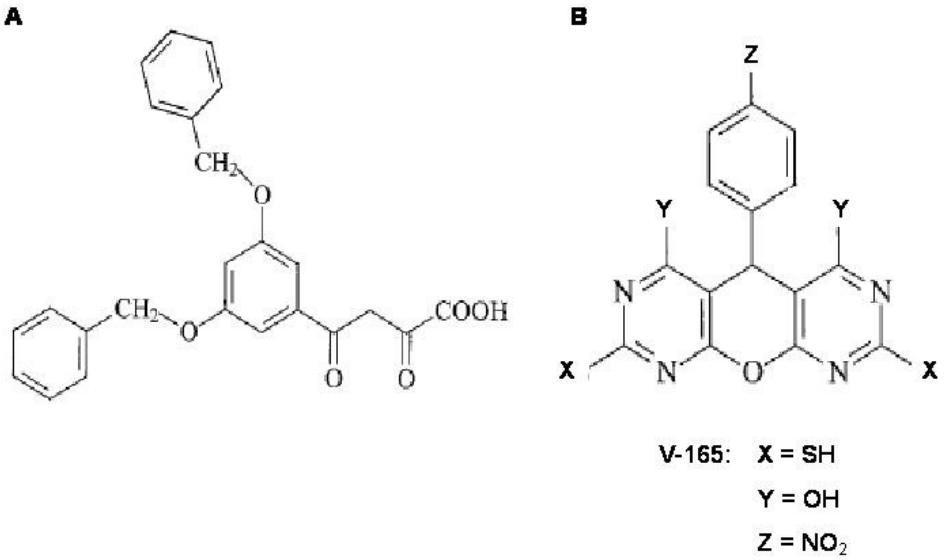

Figure 9: Chemical structure of (A) L-708,906 and (B) PDP

4.1.2.1. Selection of HIV-1(NL4.3) and HIV-1(III_B) strains resistant to the pyranodipyrimidine V-165

To further investigate the mechanism of action of V-165, HIV-1(NL4.3) and HIV-1(III_B) strains resistant towards the pyranodipyrimidine V-165 were selected by serial passaging (83 and 93 passages, respectively) the strains in the presence of increasing concentrations of V-165. These drug-resistant strains are referred to as NL3.4/V-165 and III_B/V-165, respectively. The V-165 resistant strains were selected at a final concentration of 36 µg/ml. A higher concentration of V-165 proved to be cytotoxic. These strains are referred to as NL4.3/V-165 and III_B/V-165, respectively

4.1.2.2. Progressive accumulation of mutations in the *IN,* the *gp160* and the *RT* gene of the V-165 resistant strains

The resistant strains were analysed for mutations in the *IN*, *RT* and *gp160* gene (Table 7). Several mutations were detected in the different coding regions in comparison with the sequence of the equivalent wild-type HIV-1 strain. For the NL4.3/V-165 strains, after 15 passages in the presence of V-165, the T206S and the S230N mutations were detected in the *IN* gene, while mutations in the other genes appeared at a later stage. A29S, T168N, R272T, N293N/D and Δ364-368FNSTW occurred in the *gp120* gene in the virus population after 83 passages. In the *gp41* gene the L33S mutation appeared after 63 passages of the virus. Also, the *RT* gene of the selected HIV-1(NL4.3) virus contained several mutations. After 63 passages the mutation K70R appeared, while after 83 passages the mutations T69N, K70R and T215Y/S/F were detected in the *RT* gene.

For the III_B/V-165 strains, the mutation V165I appeared in the *IN* gene, however, even

after 93 passages, this mutation only occurred in 50% of the virus population. In gp120 the following mutations were found: T108I, Δ109-110NT, F145L, K148A, Q278H, I288V, A297T, N308A/V, A314T, Δ364-368FNSTW, P390L and S438P. Gp41 held the mutations N113D, A156T, S157N, N163D, and I270T. Also, mutations in RT were found, these include D67N, K70R, Q197K, E203E/K and K451R. No mutations were identified in the *gag* gene of the viruses selected in the presence of V-165.

Table 7: Mutations in gp120, gp41, IN and RT of NL4.3/V-165 and IIIB/V-165

	Amino acid position in gp120											
	29	108	109-110	145	148	168	272	278	288	293	297	308
NL4.3 WT[a] IIIB WT[b]	A	T	NT	F	K	T	R	Q	I	N	A	N
NL4.3/V-165[c]	S	-	-	-	-	N	T	-	-	D	-	-
IIIB/V165[d]	-	I	deletion	L	A	-	-	H	V	-	T	A/V

	Amino acid position in gp120				**Amino acid position in gp41**					
	314	364-368	390	428	33	113	156	157	163	270
NL4.3 WT[a] IIIB WT[b]	A	FNSTW	P	S	L	N	A	S	N	I
NL4.3/V-165[c]	-	deletion	-	-	S	-	-	-	-	-
IIIB/V165[d]	T	deletion	L	P	-	D	T	N	D	T

	Amino acid position in IN			**Amino acid position in RT**						
	165	206	230	67	69	70	197	203	215	451
NL4.3 WT[a] IIIB WT[b]	V	T	S	D	T	K	Q	E	T	K
NL4.3/V-165	-	S	N	-	N	R	-	-	Y/S/F	-
IIIB/V165	V/I	-	-	N	-	R	K	E/K	-	R

[a] HIV-1(NL4.3) wild type strain

[b] HIV-1(IIIB) wild type strain

[c] HIV-1(NL4.3) strain *in vitro* selected in the presence of V-165

[c] HIV-1(IIIB) strain *in vitro* selected in the presence of V-165

4.1.2.3. Evaluation of phenotypic (cross)-resistance of the different selected HIV-1 strains

To verify whether the HIV-1 strains selected in the presence of increasing PDP concentrations were indeed less susceptible to the inhibitory effect of the drug, we determined the antiviral activity of V-165 against the strains HIV-1(NL4.3), NL4.3/V-165, HIV-1(III_B) and III_B/V-165. The susceptibility of the selected strains towards the entry inhibitor dextran sulfate, the CXCR4 antagonist AMD3100, the fusion inhibitor T-20, the nucleoside RT inhibitor AZT, the non-nucleoside RT inhibitor nevirapine, the protease inhibitor ritonavir and the integrase strand transfer inhibitor (INSTI) L-870,810 was measured as well. The strains NL4.3/V-165 and III_B/V-165 showed a respectively 7.0- and >7.7-fold reduced susceptibility towards the compound used for selection, V-165. Unexpectedly, both strains also showed a reduced susceptibility to AMD3100 (22.7- and 13.2-fold increase in EC_{50}, respectively), T-20 (24.5-fold increase in EC_{50} and not determined) and the NRTI AZT (9.6- and 8.3-fold increase in EC_{50}, respectively). The other evaluated inhibitors, including the HIV integrase inhibitor L-870,810, retained activity against the resistant strains (Table 8).

Table 8: Phenotypic analysis of the NL4.3/V-165 and III_B/V-165 strains

	EC_{50}[a] (µg/ml) (fold increase in EC_{50})[b]			
	Entry inhibitor		**RT inhibitor**	**IN inhibitor**
	AMD3100	**T-20**	**AZT**	**V-165**
HIV-1(NL4.3)[c]	0.004 ± 0.001	0.288 ± 0.095	0.008 ± 0.002	7.084 ± 3.082
HIV-1(IIIB)[d]	0.017 ± 0.001	nd[g]	0.008 ± 0.001	5.190 ± 1.570
NL4.3/V-165[e]	0.091 ± 0.021 **(22.7)[b]**	7.040 ± 0.181 **(24.5)**	0.080 ± 0.055 **(9.6)**	49.394 ± 1.048 **(7.0)**
IIIB/V-165[f]	0.224 ± 0.120 **(13.2)**	nd	0.066 ± 0.023 **(8.3)**	>39.880 ± 12.340 **(>7.7)**

[a] 50% inhibitory concentration or concentration required to inhibit the cytopathic effect (CPE) of HIV by 50% in MT-4 cells

[b] Fold increase in EC_{50} of the compound against the *in vitro* selected strains compared with the EC_{50} of the compound against the parental strain

[c-d] HIV-1(NL4.3) and HIV-1(III_B) wild type strains respectively

[e-f] HIV-1(NL4.3) and HIV-1(III_B) strains selected in the presence of V-165 respectively

[g] nd = not determined

Data represent mean values ± standard deviation, for at least three separate experiments

Statistical significant (p< .025) fold increase inEC_{50} in bold

4.1.2.4. Enzymatic activity of mutant integrases

To study the impact of these IN mutations on enzymatic activity and drug susceptibility, mutant integrase was produced by site-directed mutagenesis. The single mutant integrase enzymes IN-N155L, IN-I161T, IN-V165I, IN-S230N, IN-T206S and the double mutant IN-T206S/S230N were purified in parallel with wild type HIV-1 IN. IN-N155L was predicted to confer resistance towards V-165 by computer modeling (Courtesy of Drs. A. Voet, KULeuven) whereas the mutation IN-I161T appeared in a PDP-analogue resistant strain (data not shown). The enzymatic activities were determined in the oligonucleotide-based over-all integration assay. The IN-S230N mutant displayed WT

enzymatic activity, the IN-N155L only displayed 3'-processing activity whereas all the other mutants showed a 2-fold reduction in enzymatic activity.

To determine the ability of the different enzymes to bind DNA, fluorescence correlation spectroscopy (FCS) was used. FCS is based on the statistical analysis of fluorescence fluctuations, which are caused by fluorescent molecules diffusing in and out of a well defined volume. Using FCS Vercammen *et al.* [211] have studied the association of integrase to fluorescently labeled oligonucleotides. The binding of integrase to these fluorescent oligonucleotides results in the formation of high molecular mass and can be seen as the appearance of bright spikes during fluorescence correlation spectroscopy measurements (Figure 10). Using statistical methods the binding constant of integrase to DNA can be calculated. In the ongoing search for integrase inhibitors, the inhibition of the binding of integrase to viral DNA is considered a potential target. To investigate the binding characteristics of HIV-1 integrase, we can also use this FCS-based method to determine the binding constant in the presence of the compound.

As shown in Table 8 the mutant enzymes displayed a decreased affinity for DNA. Compared to WT integrase IN-N155L, IN-I161T, IN-V165I, IN-T206S and IN-T206S/S230N showed at 3.1-, 10.0-, 4.3-, 2.0- and 2.1-fold decrease in affinity, respectively.

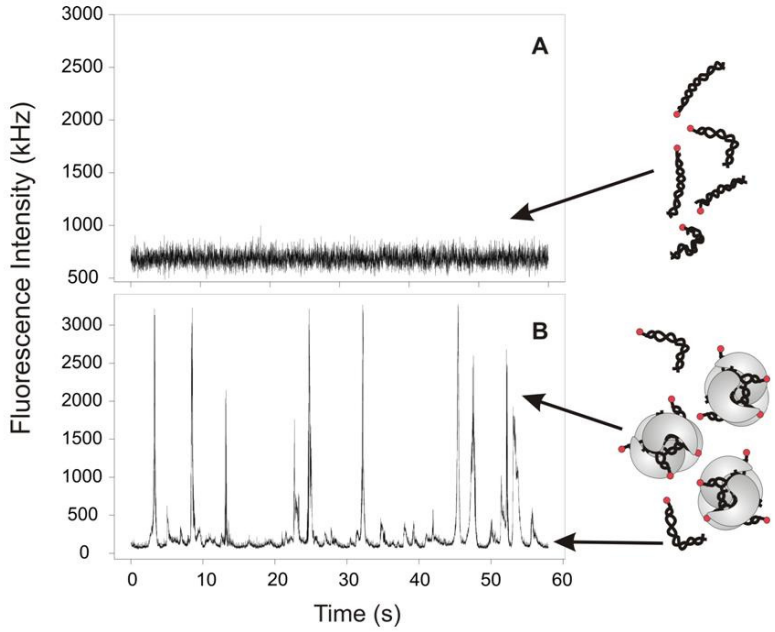

Figure 10: Fluorescence correlation spectroscopy (FCS): Addition of HIV-1 integrase to fluorescently labeled DNA induces spikes in the fluorescence signal

The movement of fluorescently labeled DNA molecules in an open volume causes a fluctuation signal, which can be recorded over short time periods (A). When these labeled LTR DNA fragments are added to HIV-1 integrase, fluorescent spikes are seen in the fluorescence signal, due to the formation of big DNA-integrase complexes. The remaining signal, which is not included in the spikes, can be assigned to the free DNA, still present in the solution (B). By measuring this fluorescence signal the amount of free and bound DNA can be calculated.

4.1.2.5. Susceptibility of the different mutant integrase enzymes towards the integrase inhibitors V-165 and L-870,810

We next determined the sensitivity of mutant IN enzymes to the inhibitory effect of V-165 in an over-all integrase ELISA assay. In this assay, the IN-N155L mutant could not be tested, due to a lack of strand transfer activity. We normalized the different enzyme preparations for equal enzymatic activity. V-165 inhibited the mutants IN-I161T,

IN-V165I, IN-T206S, IN-S230N and IN-T206S/S230N to 0.7, 0.8, 0.9, 1.3 and 1.6-fold lower extent than WT IN, respectively. No cross-resistance towards the naphthyridine analogue, L-870,810, was observed.

In an FCS-based IN-DNA binding assay, we determined the association constant of the inhibitor (K_I) of WT and mutant IN for binding of IN to DNA. The mutant enzymes showed low level resistance to V-165. IN-N155L, IN-I161T, IN-V165I, IN-T206S and IN-T206S/S230N were 1.8-, 2.1-, 1.6-, 2.1- and 2.3-fold less susceptible to V-165, respectively (Table 9). The cross-resistance towards the integrase inhibitor L-870,810 could not be tested in this assay, because an integrase strand transfer inhibitor has no effect on the binding of integrase to DNA.

Table 9: Affinity of IN enzymes for DNA and inhibition of DNA/IN interaction by V-165

	K_a[a] (μM^{-1}) **(Fold decreased affinity for DNA)**[c]	K_I[b] (μM) **(Fold resistance)**[d]
IN-WT	5.00 ± 0.12	0.26 ± 0.05
IN-N155L	1.62 ± 0.16 **(3.1)**[c]	0.48 ± 0.07 **(1.8)**[d]
IN-I161T	0.50 ± 0.03 **(10.0)**	0.56 ± 0.06 **(2.1)**
IN-V165I	1.17 ± 0.06 **(4.3)**	0.41 ± 0.11 **(1.6)**
IN-T206S	2.53 ± 0.23 **(2.0)**	0.55 ± 0.09 **(2.1)**
IN-T206S/S230N	2.44 ± 0.15 **(2.1)**	0.60 ± 0.15 **(2.3)**

[a] K_a represents the association constant of IN for DNA as measured by FCS

[b] K_I is the association constant of the inhibitor

[c] Fold decrease in K_a of the mutant IN compared with WT IN

[d] Fold increase in K_I of the mutant IN compared with WT IN

Data represent mean values ± standard deviation, for at least three separate experiments

4.1.2.6. Conclusion

First, PDP-resistant HIV-1(NL4.3) and HIV-1(III_B) strains were selected by growing the virus in the presence of increasing concentrations of V-165 for several weeks. By genotyping, mutations in the *IN* gene were detected in an early stage, while mutations in the other genes (*RT* and *env*) appeared at a later stage. No mutations in *gag* were found. 100% of the population of the NL4.3/V-165 strain carried the mutations T206S and S230N in the *IN* gene, while the mutation V165I in *IN* only appeared in 50% of the population of the III_B/V-165 strain. Phenotypic analysis of these resistant strains (NL4.3/V-165 and III_B/V-165) revealed a 7.0- and >7.7-fold reduced susceptibility towards V-165 but also a reduced susceptibility to the entry inhibitors AMD3100 and T-20 and the RT inhibitor AZT. No cross-resistance towards other evaluated inhibitors was observed. To determine the effect of the mutations on enzymatic level the single mutants IN-WT, IN-N155L, IN-I161T, IN-V165I, IN-S230N, IN-T206S and the double mutant IN-T206S/S230N were purified. IN-N155L was predicted to confer resistance towards V-165 by computer modeling whereas resistance selection with a PDP-analogue revealed the mutation IN-I161T. We evaluated the effect of the different IN mutations on enzymatic activity and drug susceptibility. In an oligonucleotide-based assay, the IN-S230N mutation did not impair over-all integration activity, the IN-N155L displayed only 3'-processing activity whereas all the other mutants displayed a 2-fold reduction in over-all enzymatic activity. In the over-all integration reaction, V-165 inhibited the mutants IN-I161T, IN-V165I, IN-T206S, IN-S230N and IN-T206S/S230N to a 0.7-, 0.8-, 0.9-, 1.3- and 1.6-fold lower extent than WT IN, respectively. No cross-resistance towards the naphthyridine analogue L-870,810 was observed. FCS analysis demonstrated

that all the mutant integrases showed a decreased affinity for DNA. Additionally, the mutant enzymes IN-N155L, IN-I161T, IN-V165I, IN-T206S and IN-T206S/S230N showed a 1.8-, 2.1-, 1.6-, 2.1- and 2.3-fold resistance to V-165 in this IN/DNA interaction assay. This study revealed that the observed IN mutations interfere with the enzymatic activity and the DNA binding capacity of HIV-1 IN enzymes and that the mutations are responsible for a low-level resistance against PDP.

4.2. NATURAL RESISTANCE TO HIV-1 INFECTION

Two phenomena have indicated that natural resistance to HIV-1 infection, while rare, does exist. First, there are individuals who have been exposed to HIV, in some cases repeatedly and over long periods of time, who have remained uninfected. Such 'highly exposed non-infected' persons have been reported among sex workers [212, 213], individuals having unprotected sex with seropositive partners [214], infants born from HIV infected mothers [215, 216], health workers with accidental occupational exposure [217, 218], intravenous drug users using contaminated needles [219] and hemophiliacs exposed to HIV-1 infected blood [220]. Second, there are individuals who have become infected with HIV but whose disease has not progressed or has progressed very slowly. 'Long-term non-progressors' have been identified among various groups, including homosexual men, women, IV drug users and children [221]. Some of the same genetic mutations have been found in both 'highly exposed non-infected' persons and 'long-term non-progressors', suggesting a unifying theory for both conditions, namely that host traits that prevent or hinder HIV-1 infection into cells will reduce the likelihood of infection and, should infection occur, slow or entirely eliminate the development of serious diseases. In this chapter we address the question if there is a natural resistance to HIV-1 infection associated with mutations in LEDGF/p75.

4.2.1. NATURAL VARIABILITY OF LEDGF/p75

Lens epithelium-derived growth factor (LEDGF/p75) was first identified as a HIV-1 integrase-interacting protein by co-immunoprecipitation of HIV-1 integrase. Recombinant LEDGF/p75 has been shown to enhance the strand transfer activity and the binding of HIV-1 IN to DNA [48, 49]. p52 is a splice variant of p75 which lacks the 205 C-terminal amino acids of p75 but does not show affinity for the HIV-1 integrase. LEDGF/p75 is essential for nuclear localization and chromosomal association of HIV-1 IN [50, 51]. To investigate the role of LEDGF/p75 in HIV-1 replication, HelaP4 LEDGF/p75 knockdown cell lines were constructed. In these knockdown cell lines a inhibition of HIV-1 replication was seen. Back-complementation of LEDGF/p75 in these knockdown cell lines restored the viral replication to nearly wild-type [52]. LEDGF/p75, 530 AA long protein, contains a conserved N-terminal PWWP-domain (AA 1-91) that is thought to be involved in protein-protein interactions [54] and chromatin binding [55], a nuclear localization signal (NLS) (AA 148-156) [56, 222] and a conserved domain that is necessary and sufficient for the binding of LEDGF/p75 to the HIV-1 integrase; the integrase binding domain or IBD [57]. p75 showed modest co-activator properties and interacted with general transcription factors and the transcription activation domain of VP16 [59]. In other reports LEDGF/p75 is described as a growth factor [60] and it plays a protective role against cellular stress [61].

Little is known about the natural variability of the gene coding for LEDGF. Is there an innate immunity encoded by mutations/polymorphisms in LEDGF/p75 that protects individuals against infection with HIV-1 or is there a correlation between disease progression and natural variants of LEDGF/p75? The first question has been addressed

in this part of the work.

4.2.1.1. Sequencing analysis of PBMC samples of a Caucasian population

Genomic DNA extracted from 0.5 x 10^6 PBMC of 10 HIV seronegative (SN) Caucasian individuals was sequenced. Sequencing analysis of a 5666 base pair fragment of *LEDGF/p75* (39797-45462; corresponding to position in PSIP1; PC4 and SFRS1 interacting protein 1 (Genbank accession number NC_000009)) containing the integrase binding domain (IDB) revealed several polymorphisms in *LEDGF/p75* (Table 10). At position 41250 a cytosine was predominant in this population, however three out of ten carried a mixture of cytosine and adenine. At position 42503, in all individuals except in three, where a mixture of guanine and adenine was found, a guanine was present. All individuals carried a homozygote or heterozygote AAG-deletion polymorphism at position 42566-42568. These polymorphisms are all located in introns of the *gene*. No polymorphisms were found in exons of the sequenced fragment.

Table 10: Genotypic analysis of *LEDGF/p75* of a seronegative Caucasian population

	position in *LEDGF/p75*		
	41250	42503	42566-AAG-42568
sample 1	**C**	**G**	**homozygote Δ**
sample 2	**C**	**G**	**homozygote Δ**
sample 3	**C**	**G**	**homozygote Δ**
sample 4	**C**	**G**	**homozygote Δ**
sample 5	**C**	**G**	**homozygote Δ**
sample 6	**A/C**[a]	**A/G**	**heterozygote Δ**
sample 7	**A/C**	**A/G**	**heterozygote Δ**
sample 8	**C**	**G**	**homozygote Δ**
sample 9	**C**	**G**	**homozygote Δ**
sample 10	**A/C**	**A/G**	**heterozygote Δ**

[a] Heterozygote for the polymorphism

4.2.1.2. Sequencing analysis of PBMC samples of two HIV seronegative African populations

Genomic DNA extracted from 0.5 x 10^6 PBMC of 19 seronegative African female blood donors (FBD) (control) and 20 HIV exposed seronegative (ESN) female sex workers (FSW), was sequenced. The same polymorphisms as in the Caucasian group were found, however two extra polymorphisms in an intron were present. At position 42433, an adenine or a mixture of an adenine and a thymine was found, whereas at position 42809 a thymidine or a cytosine or a mixture of both was present. In addition to these polymorphisms in introns, one heterozygote polymorphism in exon 13 of *LEDGF/p75* appeared. This polymorphism was located outside the IBD. This polymorphism was present in 1 out of 19 individuals of the SN FBD and in 3 out of 20 individuals of the ESN FSW (Table 11). As a result, the LEDGF/p75 protein contained a leucine instead of a glutamine at position 472 (Figure 11). The occurrence of a leucine at position 472 was confirmed by mRNA (messenger RNA) sequencing of these samples.

Polymorphisms in the introns can give rise to an alternative splice donor or splice acceptor site, which then results in an alternatively spliced protein. To predict if the observed polymorphisms in the intron could give rise to alternative splice donor or splice acceptor sites we used a software tool (www.fruitfly.org/seq_tools/splice.html). With this tool, no additional splice donor or splice acceptor sites were predicted to occur, nor did any of the splice donor or splice acceptor sites seemed to disappear.

Table 11: Genotypic analysis of *LEDGF* of two seronegative African populations

	position in *LEDGF/p75*					
	INTRON					EXON
	41250	42433	42503	42566-42568	42809	42350
Seronegative FBD						
sample 1	A	A	A	AAG	nd[b]	A
sample 2	**A/C**[a]	A	**A/G**	**heterozygote Δ**	nd	A
sample 3	A	A	A	AAG	nd	A
sample 4	A	A	A	AAG	nd	A
sample 5	**A/C**	A	**A/G**	**heterozygote Δ**	nd	A
sample 6	A	A	A	AAG	**T**	A
sample 7	A	A	A	AAG	**T**	A
sample 8	A	A	A	AAG	C	A
sample 9	A	**A/T**	A	AAG	nd	A
sample 10	A	**A/T**	A	AAG	C	A
sample 11	**A/C**	A	**A/G**	**heterozygote Δ**	nd	A
sample 12	**A/C**	**A/T**	**A/G**	**heterozygote Δ**	nd	A
sample 13	**A/C**	A	**A/G**	**heterozygote Δ**	nd	A
sample 14	A	A	A	AAG	**T/C**	A
sample 15	A	A	A	AAG	nd	A
sample 16	**A/C**	A	**A/G**	**heterozygote Δ**	nd	A
sample 17	A	A	**A/G**	**heterozygote Δ**	nd	A
sample 18	**C**	A	**G**	**homozygote Δ**	nd	**A/T**
sample 19	**A/C**	A	**A/G**	**heterozygote Δ**	nd	A
Seronegative FSW						
sample 1	A	A	A	AAG	**T**	A
sample 2	A	A	A	AAG	nd	A
sample 3	A	A	A	AAG	C	A
sample 4	A	A	A	AAG	nd	A
sample 5	**A/C**	A	**A/G**	**heterozygote Δ**	nd	A
sample 6	**C**	A	**G**	**homozygote Δ**	C	**A/T**
sample 7	A	**A/T**	A	AAG	nd	A
sample 8	**A/C**	A	nd	nd	nd	A
sample 9	A	A	**A/G**	**heterozygote Δ**	nd	A
sample 10	A	A	A	AAG	nd	A
sample 11	**C**	A	nd	nd	nd	**A/T**
sample 12	A	A	**A/G**	**heterozygote Δ**	nd	A
sample 13	**A/C**	A	**A/G**	**heterozygote Δ**	nd	**A/T**
sample 14	**A/C**	**A/T**	**A/G**	**heterozygote Δ**	nd	A
sample 15	**C**	**A/T**	**A/G**	**heterozygote Δ**	nd	A
sample 16	A	A	nd	nd	nd	A
sample 17	**A/C**	A	**A/G**	**heterozygote Δ**	nd	A
sample 18	A	A	**A/G**	**heterozygote Δ**	nd	A
sample 19	A	A	**A/G**	**heterozygote Δ**	nd	A
sample 20	A	**A/T**	**A/G**	AAG	nd	A

[a] Heterozygote for the mutation

[b] nd = not determined

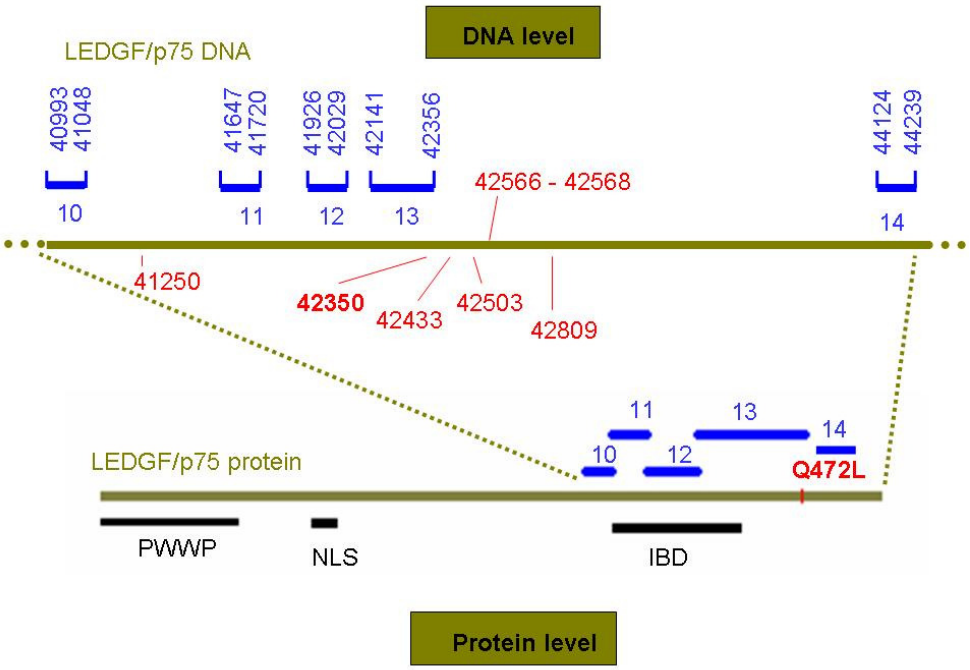

Figure 11: Position of the polymorphisms in the LEDGF gene and at mRNA level

Five intron polymorphisms were found in the different, analyzed PBMC populations, while one polymorphism in exon 13, outside the IBD region was found. At the protein level this resulted in a leucine at position 472.

4.2.1.3. Sequencing analysis of PBMC samples of a HIV seropositive African population

The Q472L mutation found in exon 13 in LEDGF/p75 in the seronegative African population could have a protective function against HIV infection. Therefore, as a positive control group, genomic DNA extracted from 0.5 x 10^6 PBMC cells of 20 African HIV seropositive (SP) female sex workers was sequenced. The same polymorphisms as found in the ESN FSW population were found in this SP FSW population. No additional mutations in the introns or exons were found. The heterozygote mutation Q472L was detected in only 1 out of the 20 samples (Table 12).

4.2.1.4. Statistical analysis

Statistical analysis using the Fisher's exact test was performed, to analyze if there was a statistical difference between the prevalence of the Q472L mutation in the HIV exposed seronegative group female sex workers and the seropositive group female sex workers. However, the prevalence in both groups was not statistically different (p = 0.61).

Table 12: Genotypic analysis of *LEDGF* in seropositive African female sex workers

	position in *LEDGF/p75*					
	INTRON					EXON
	41250	42433	42503	42566-42568	42809	42350
sample 1	A[a]	**T**	A	AAG	nd[b]	A
sample 2	A	A	A	AAG	nd	A
sample 3	A	**T**	A	AAG	nd	A
sample 4	A	**A/T**	A	AAG	nd	A
sample 5	**A/C**	A	**A/G**	**heterozygote Δ**	nd	A
sample 6	A	A	**A/G**	**heterozygote Δ**	nd	A
sample 7	A	**T**	A	AAG	nd	A
sample 8	A	**A/T**	A	AAG	nd	A
sample 9	A	**T**	A	AAG	**T**	A
sample 10	A	**A/T**	A	AAG	nd	A
sample 11	**C**	A	**G**	**homozygote Δ**	nd	A
sample 12	**A/C**	A	**A/G**	**heterozygote Δ**	nd	A
sample 13	**A/C**	A	**A/G**	**heterozygote Δ**	nd	**A/T**
sample 14	A	**A/T**	A	AAG	nd	A
sample 15	A	A	**A/G**	**heterozygote Δ**	nd	A
sample 16	A	A	**A/G**	**heterozygote Δ**	nd	A
sample 17	A	**A/T**	A	AAG	A	A
sample 18	**A/C**	**A/T**	**A/G**	**heterozygote Δ**	nd	A
sample 19	A	A	A	AAG	**T**	A
sample 20	A	A	nd	nd	nd	A

[a] Heterozygote for the mutation

[b] nd = not determined

4.2.1.5. His_6-tag pull-down assay

To evaluate the effect of the Q472L polymorphism in LEDGF/p75 on the binding capacity of LEDGF/p75 to integrase, a bacterial expression plasmid expressing the Q472L-LEDGF/p75 was constructed (pCP-Nat75-Q472L). The WT and mutant LEDGF/p75 were expressed in bacteria, purified, and tested for binding to integrase in a IN-His_6-tag pull-down assay. No difference in binding capacity to IN was seen for the Q472L-LEDGF/p75 compared with the WT LEDGF/p75 (Figure 12).

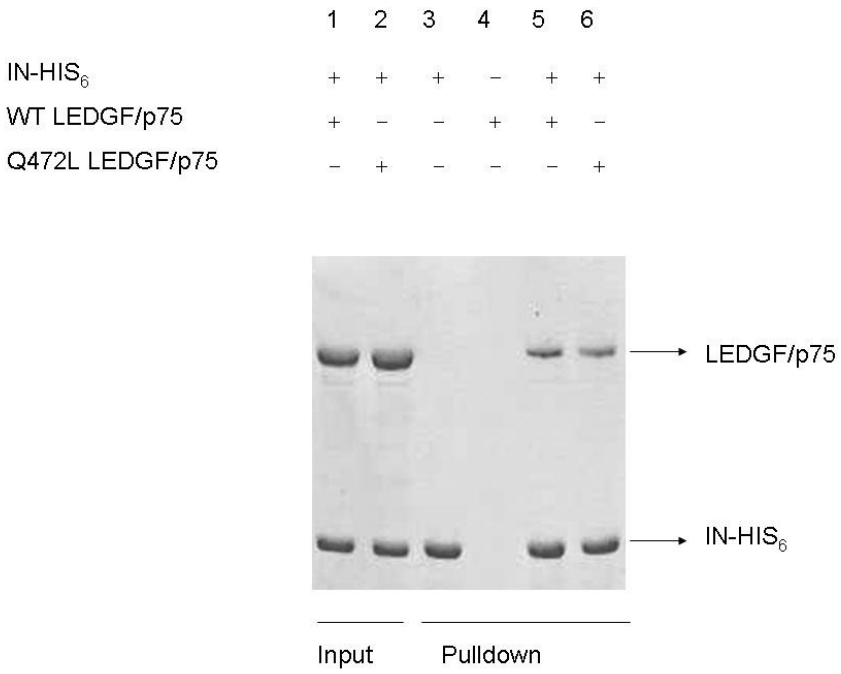

Figure 12: IN-His_6-tag pull-down assay

Lanes 1 and 2 contain input quantities of IN-His_6 and wild-type (WT) or Q472L-LEDGF/p75, respectively. Lanes 3 contains proteins bound to Ni-NTA agarose beads after incubation with IN-His_6. Lane 4 contains proteins bound to Ni-NTA agarose beads after incubation with WT LEDGF/p75. Lanes 5 and 6 contain proteins bound to Ni-NTA agarose beads after incubation with IN-His_6 and WT LEDGF/p75 or Q472L LEDGF/p75. Proteins bound to the Ni-NTA agarose beads were separated on SDS-PAGE and stained with Coomassie.

4.2.1.6. Generation of a MT-4/LEDGF-KD-BC cell line expressing Q472L-LEDGF/p75

To evaluate the effect of the polymorphism on HIV-1 replication, polyclonal MT-4/LEDGF-KD-back-complementation cell lines expressing WT or mutant(Q472L) LEDGF/p75 were selected after transduction of the stable LEDGF/p75 knockdown cell line, MT-4/LEDGF-KD with WT or mutant(Q472L) Combi LEDGF IRES Puro vector, respectively (Figure 13). These cell lines are referred to as MT-4/LEDGF-KD-BC and MT-4/LEDGF-KD-BCQ472L, respectively.

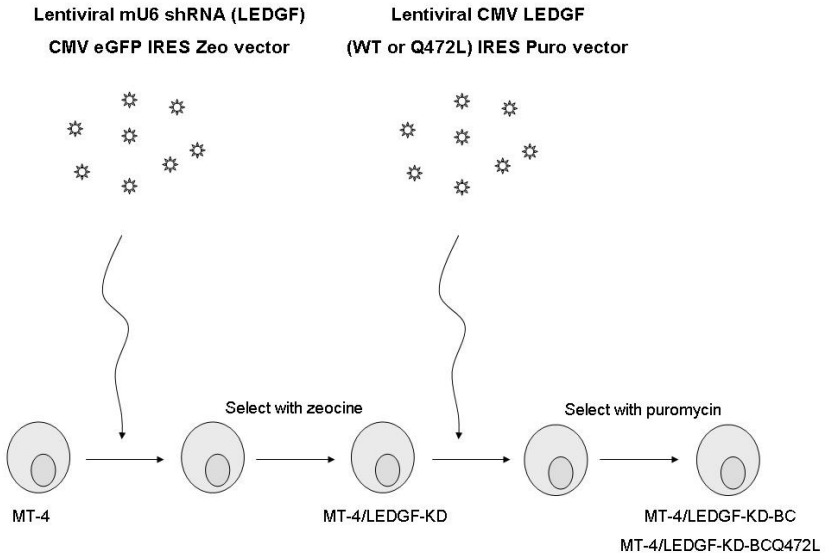

Figure 13: Generation of a MT-4/LEDGF-KD cell line back complemented with WT or mutant(Q472L) LEDGF/p75

MT-4/LEDGF-KD were generated by transducing MT-4 cells with a lentiviral vector encoding a shRNA targeting LEDGF/p75 driven by a mU6 promotor and a CMV driven eGFP-ZEOCIN fusion gene. Five days after transduction the cells were selected with zeocine. These MT-4/LEDGF-KD cells were transduced with Lentiviral Combi_LEDGF_IRES_Puro vector to generate the MT-4/LEDGF-KD-BC and MT-4/LEDGF-KD-BCQ472L cell line. Five days after transduction the cells were selected with puromycin.

20 and 40 days after selection, the expression of LEDGF/p75 was monitored by Western blotting. Stable expression of WT and mutant(Q472L) LEDGF/p75 was clearly evidenced. The levels of LEDGF/p75 in the back complemented cell lines were slightly higher than the level of LEDGF/p75 in the control cell line (MT-4/LEDGF-mmKD). Figure 14 shows the level of LEDGF/p75 expression in the different cell lines at day 40.

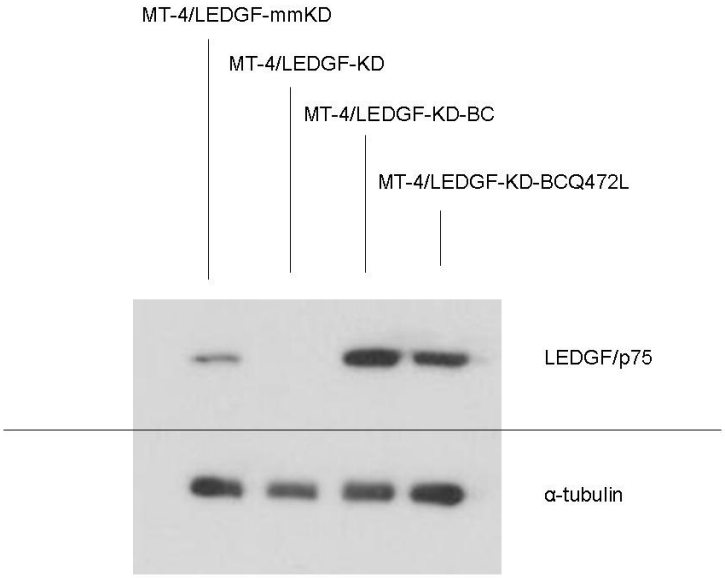

Figure 14: Western blot analysis of LEDGF/p75 expression 40 days after selection
Whole-cell extracts of the different cell lines were loaded on SDS-page and the LEDGF/p75 expression level was verified by Western blotting.

4.2.1.7. Effect of Q472L-LEDGF/p75 expression on viral replication

To analyze the effect of mutant(Q472L) LEDGF/p75 expression on HIV-1 replication, we infected the MT-4/LEDGF-mmKD, MT-4/LEDGF-KD, MT-4/LEDGF-KD-BC and MT-4/LEDGF-KD-BCQ472L cell lines in parallel with HIV-1(NL4.3) at a MOI of 0.1. Supernatants was harvested at 3, 4, 5, 7 and 8 days post infection and the p24 antigen was measured by ELISA. We observed a 2.5-fold reduction in p24 antigen production in the polyclonal LEDGF/p75 knockdown cell line (MT-4/LEDGF-KD) compared with the

control cell line (MT-4/LEDGF-mmKD) (Figure 15). Viral replication was restored to above control level in the polyclonal MT-4/LEDGF-KD-BC. No difference in viral replication, however, was observed in the cell line back complemented with mutant(Q472L) LEDGF/p75 (MT-4/LEDGF-KD-BCQ472L) compared to the cell line back complemented with WT LEDGF/p75 (MT-4/LEDGF-KD-BC) (Figure 15).

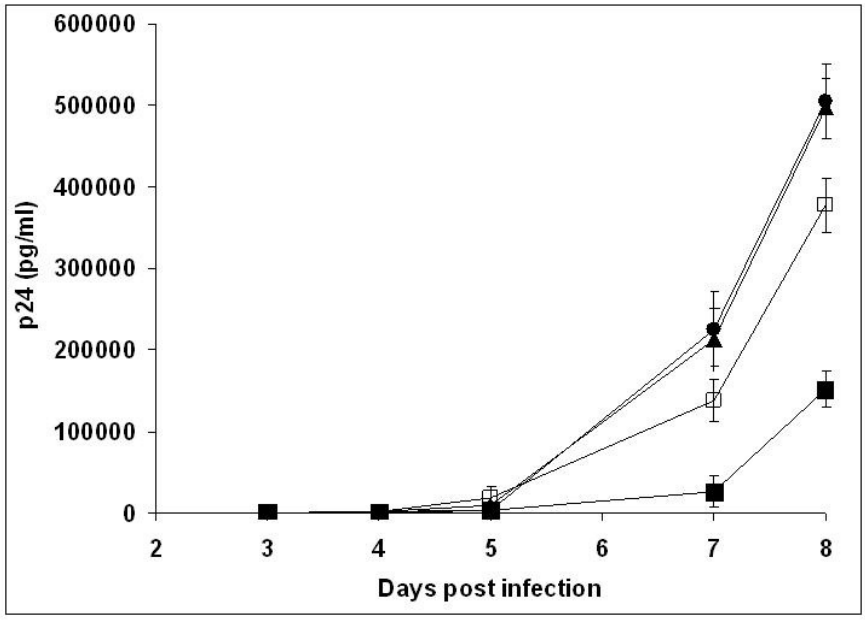

Figure 15: Effect of Q472L-LEDGF/p75 expression on viral replication

MT-4/LEDGF-mmKD (□), MT-4/LEDGF-KD (■), MT-4/LEDGF-KD-BC (▲) and MT-4/LEDGF-KD-BCQ472L (●) cells were infected with HIV-1(NL4.3) at an MOI of 0.1. p24 antigen production in the supernatants 3, 4, 5, 7 and 8 days after infection was measured by ELISA. HIV-1 replication is impaired in LEDGF/p75 knockdown cell lines while replication is restored in the LEDGF/p75 knockdown cells that are back complemented with WT or (Q472L)LEDGF. Data represent mean values ± standard deviation, for 3 separate experiments.

4.2.1.8. Conclusion

Sequencing of part of the gene coding for LEDGF/p75 of a seronegative Caucasian population revealed several polymorphisms in intron sequences, compared with the

reference sequence: A41250A/C, A42503A/G and 42566-42568ΔAAG. Two additional intron polymorphisms were found by sequencing analysis of African populations: A42433A/T and C42809T. Computer analysis of these polymorphisms predicted that no additional splice donor or splice acceptor sites were created and none of the existing sites disappeared. In addition to these polymorphisms in the introns one polymorphism in exon 13 was found in some of the African samples. This polymorphism was present in 1 of the 19 individual of the seronegative group female blood donors (control) and in 3 of the 20 individuals of the HIV exposed seronegative group female sex workers. This exon polymorphism resulted in a LEDGF/p75 protein containing a leucine at position 472 in stead of a glutamine. The presence of a leucine at position 472 was confirmed by mRNA sequencing. Sequencing of a third African group revealed that this polymorphism was also present in African female sex workers infected with HIV. However, in this group the mutation only occurred in 1 out of 20 samples. Statistical analysis revealed that there was no statistically significant difference between the prevalence of this mutation in the seronegative and the seropositive group female sex workers ($p = 0.61$). Although this polymorphism was located outside the IBD, we wanted to investigate the influence of this polymorphism on the ability of LEDGF/p75 to bind to integrase. Therefore mutant recombinant Q472L-LEDGF/p75 was purified. In an IN-His_6-tag pull down assay no difference in the binding capacity of Q472L-LEDGF/p75 to integrase was detected when compared to the binding capacity of WT LEDGF/p75. Next, MT-4/LEDGF-KD cell lines were constructed that express WT or mutant(Q472L) LEDGF/p75 to see if the mutant LEDGF/p75 has an effect on the viral replication of HIV-1. Western blotting showed that the expression of LEDGF/p75 was stable over time. MT-4/LEDGF-mmKD,

MT-4/LEDGF-KD, MT-4/LEDGF-KD-BC and MT-4/LEDGF-KD-BCQ472L cell lines were infected with HIV-1(NL4.3). A 2.5-fold reduction in viral replication could be observed in the polyclonal LEDGF/p75 knockdown cell line (MT-4/LEDGF-KD) compared with the control cell line (MT-4/LEDGF-mmKD). Viral replication could be restored by back complementation of WT or mutant(Q472L) LEDGF/p75 in these cells.

Chapter 5

Discussion

5.1. HIV RESISTANCE DEVELOPMENT

5.1.1. ANTIVIRAL RESISTANCE DEVELOPMENT AGAINST SPL2923

Earlier studies regarding the mode of action of polyanionic dendrimers pointed to the interaction with gp120 as the basis of the anti-HIV activity of the negatively charged dendrimers. However, it appeared that the RT or integrase steps could not be excluded as antiviral targets of SPL2923 [78]. To identify the target in cell culture, an HIV-1 strain was selected in the presence of SPL2923. Several mutations were identified in *gp120* and *gp41* of NL4.3/SPL2923, whereas no mutations were identified in the genes encoding the reverse transcriptase or integrase. Therefore RT and integrase can be excluded as important antiviral targets at concentrations of dendrimer used for selection. Results from time-of-addition experiments at a SPL2923 concentration of 100 fold the EC_{50} indicated that the compound has to be present at the time of infection in order to inhibit HIV replication. In contrary, when a concentration of 500 fold the EC_{50} was used in a time-of-addition experiment, SPL2923 was found to interact at a time coinciding with the moment of reverse transcription. The final selected strain was able to grow in the presence of 20 µg/ml SPL2923, a concentration 60 fold higher than required to inhibit the replication of wild-type HIV-1 by 50%. In addition, this high concentration, 500 fold EC_{50}, is not relevant when considering the clinical use of the compound. In conclusion, at normal clinical concentrations the compound will never interfere with the reverse transcription or integration step of the HIV-1 replication cycle.

Since recombination of *gp160* of the SPL2923 resistant strain into a WT background resulted in a virus with a cross-resistance profile comparable with the cross-resistance profile of the original selected strain, we can conclude that the mutations in *gp160* were

sufficient to explain the cross-resistance profile of the SPL2923 resistant selected strain. However, the *gp160*-recombined NL4.3/SPL2923 strains displayed no significant reduction in susceptibility (up to 2.5-fold) to the compound SPL2923. Therefore we can conclude that the resistance against SPL2923 itself is only partially attributed to mutations in *gp160* and that mutation in other genes might contribute.

Genetic studies of HIV-1 *gag* and *env* mutants imply interactions between the cytoplasmatic domain of gp41 and the matrix protein (MA). These interactions occur in order to mediate Env glycoprotein incorporation into the virions. In a study by Freed *et al.* the mutations L12E and L30E in the MA were found to impair virus replication as a result of a defect in Env incorporation. This block could be reversed by pseudotyping with heterologous retroviral envelope glycoproteins with a short cytoplasmatic tail. Deletion mutations in the cytoplasmatic tail of HIV-1 gp41 could as well restore this phenotype [183]. In addition, a V to I mutation at AA 34 in the MA of the virus also compensated for these L12E and L30E mutations. In contrast, the MA mutation V34E by itself was associated with a reduction in gp120 content of the virions [184]. Another study by Murakami and colleagues showed that small deletions in α-helix 2 (between amino acid residues 277-296) of the cytoplasmatic domain of gp41 impaired virus infectivity. This impaired virus infectivity was the result of a disruption of env glycoprotein incorporation into the virion. These replication defects could however be reversed by introducing the V34I in the MA of the virus [185]. These observations indicate an important role of the amino acids at positions 12 and 34 in the MA and of the α-helix 2 of the cytoplasmatic domain of gp41.

The binding of the capsid proteins (CA) to cyclophilin A (CyPA), a cellular enzyme

which catalyze a rate-limiting step in the folding of host proteins, has been studied by Franke *et al*. As a result of this binding, CyPA is incorporated into the virion. The interaction between CA and CyPA occurs via contact with the proline-rich domain of CA. Blocking CyPA incorporation by mutating CA results in a decrease in virus infectivity [223]. It has also been suggested that CyPA promotes disassembly of the viral core during uncoating by weakening the association of the CA proteins [224]. In addition, after incorporation CyPA may relocate to the viral surface during maturation, where the exposed CyPA can mediate viral entry by attachment to heparans [225] or CD147 [226]. Cyclosporine A has a high affinity for CyPA and thereby prevents the incorporation of CyPA into the virion. A HIV-1 strain was selected in the presence of cyclosporine A. The cyclosporine resistant selected strain displayed mutations in the CA: A92E or G94D. As a result, these drug-resistant mutant viruses do not require virion-associated CyPA to initiate infection [227].

Based on these findings, the gag protein represented the most likely candidate to affect resistance against SPL2923. Indeed, sequencing analysis of *gag* of NL4.3/SPL2923 revealed several mutations in the MA and one mutation in the CA. The mutations in the MA result in an overall change towards more basic amino acids. The amino acids at position 25 is located in the basic domain of p17 which is known to play an important role in membrane targeting [228]. Genetic analysis of a NL4.3 strain resistant to DS or T-20 (NL4.3/DS and NL4.3/T-20, respectively) also revealed some mutations in the MA of these viruses. However, since recombination of *gp120* of the NL4.3/DS strain and *gp41* of the NL4.3/T-20 strain into wild-type background could fully reproduces the resistant phenotypes of both strains, mutations outside these regions as such do not seem

to affect the phenotypic resistance of both strains to DS or T-20 [200].

To investigate the impact of the mutations revealed in *gag* on the phenotypic resistance of the NL4.3/SPL2923 strain towards SPL2923, new recombination assays were developed, wherein *p17* and the 5' part of *p24* by itself or in combination with *gp160* could be placed in the wild-type backbone of HIV-1(NL4.3). However, the low percentage of homologous recombination after transfection reduces the likelihood of recombination of 2 discontinuous regions (*p17* and *gp160*). For this reason, 2 different approaches for the *p17/gp160*-recombination assay were evaluated. In a first approach, MT-4 cells were co-transfected with the *p17/gp160*-deleted clone linearized at the *p17*-deletion, the *p17/gp160*-deleted clone linearized at *gp160*-deletion and the PCR-derived *p17*- and *gp160*-sequences. In this way, the *p17*- and *gp160*-sequences each recombine in their homologous regions on different plasmids (unless linearization is not essential for homologous recombination) and viable virus particles will only be generated by an additional recombination step during viral replication. This approach requires the packaging of one RNA strand containing the *p17*-recombined HIV sequence, still lacking *gp160,* and the other RNA strand containing the *gp160*-recombined HIV sequence, still lacking *p17*, into the virion. In order to achieve the packaging of the HIV genome that lacks *p17*, the *p17/gp160*-deleted vector has been completed for the packaging signal via the reintroduction of a linker sequence. In a second approach, MT-4 cells were co-transfected with the *p17/gp160*-deleted plasmid linearized at the *p17*-deletion as well as at the *gp160*-deletion and the PCR-derived *p17*- and *gp160*-sequences. This approach required two recombination events to occur in the same *p17/gp160*-deleted plasmid. In this case however, the disruption of the packaging signal does not affect recombination.

We obtained proof-of-principle that both approaches resulted in productive virus infection. The second approach though had a higher probability to result in infective virus production compared to the first approach. Phenotypic analysis of the NL4.3/SPL2923 *p17*-recombined strain revealed that the observed mutations in MA and CA on their own do not have an effect on the (cross-)resistance against SPL2923, DS and T-20. On the contrary, *p17/gp160*-recombination resulted in a viral strain with a similar resistance profile as the original NL4.3/SPL2923 strain, implying that the mutations in *gag* (*p17* and *p24*) have to be present in combination with the *env*-mutations in order to fully reproduce the resistant phenotype. However, these *gag* mutations did not contribute to the cross-resistance of NL4.3/SPL2923 to DS and T-20.

Mutations in *gag* have never before been reported to be associated with resistance towards entry inhibitors. However, additional mutations in the p7/p1 and the p1/p6 cleavage site have been observed in strains resistant to protease inhibitors. Deficient gag-polyprotein processing by a mutant protease could be alleviated by mutations in *gag* proteolytic cleavage sites [229]. Additional *gag* mutations have also been observed in clinical HIV-1 isolates from patients receiving protease inhibitors [230]. Duplication of a proline-rich p6 PTAP motif has been identified to be associated with resistance to a nucleoside RT inhibitor *in vivo* [231].

This is the first reported phenotypic recombination assay that is able to assess the impact of mutations in discontinuous genes on a viral phenotype. This opens perspectives for the further development of different resistance assays, wherein the phenotypic effect of mutations in separated genes for the viral resistance against antiretroviral compounds can be evaluated.

The more we learn about HIV resistance development against different classes of anti-HIV compounds, the more it becomes clear that the mechanisms responsible for HIV resistance development are multifaceted and additional genes may be involved besides the genes encoding the protein directly targeted by the antiretroviral agent.

5.1.2. ANTIVIRAL RESISTANCE DEVELOPMENT AGAINST V-165

As part of the preclinical studies with PDP, an early integrase binding inhibitor (INBI) prototype, we have investigated the development of HIV resistance against V-165 in cell culture. PDP-resistant HIV-1 strains were selected by growing the virus in the presence of increasing concentrations of V-165 for several weeks. A NL4.3 and a III_B strain resistant towards V-165 were selected, NL4.3/V-165 and III_B/V-165 respectively. For the NL4.3/V-165 strain, the mutations T206S and S230N were detected in the gene coding for integrase. These mutations occurred as early as 8 weeks in the presence of V-165. For the III_B/V-165 strain, the mutation V165I appeared in *IN*. However, even after 93 passages, this mutation only occurred in half of the virus population. Next to mutations in the *IN* gene, numerous mutations were identified in the gene coding *gp160* and *RT* of the strains selected in the presence of V-165. Some of the mutations that arose in *gp120* are already known to be associated with resistance towards AMD3100 and entry inhibitors in general (F145L, R272T, Q278H, I288V, N293D, A297T, the 364-368 FNSTW deletion, P390L and S438P) [200, 232]. The mutation L33S in *gp41* appeared after 63 passages in the presence of V-165. This mutation has previously been described to be associated with resistance to T-20 [200]. Both the D67N, K70R and the T215Y/F mutation in the RT gene are known NRTI resistance mutations [233]. These mutations are so-called TAM's or thymidine associated mutations. The presence of these mutations in the RTs causes the RT to remove more efficiently the chain terminating residue from the growing DNA chain [234]. While the mutations in *IN* occurred in the early phase of the selection proces, most of the *gp160* and the *RT* mutations were only identified in a later phase of the selection process. Accordingly, at low selective pressure, mutations were primarily

detected in the *IN* gene of the selected strain. When V-165 inhibitory pressure was increased, mutations were also detected in the *gp160* and the *RT* gene of the passaged virus. No mutations were identified in the *gag* encoding region of the selected viruses. NL4.3/V-165 and III_B/V-165 were respectively 7- and >7.7-fold less susceptible to V-165 compared to the wild-type strain.

We evaluated the effect of the different mutations, N155L, I161T, V165I, T206S, S230N and T206S/S230N, on the enzymatic activity and the drug susceptibility of recombinant integrase. The mutation N155L was predicted to confer resistance to V-165 by computer modeling of the interaction of V-165 with IN (Drs. A. Voet, KULeuven) while the mutation I161T was selected in a HIV-1 strain resistant towards a PDP-analogue. The mutations N155L, I161T, V165I and T206S are located in the catalytic core of the IN enzyme whereas the S230N mutation is positioned in the DNA binding site of the enzyme (Figure 16).

In an oligonucleotide-based assay, the S230N mutation did not impair over-all integration activity, whereas the IN-I161T, IN-V165I, IN-T206S and the T206S/S230N mutants displayed a 2-fold reduction in over-all enzymatic activity. IN-N155L only displayed 3'-processing activity. In the same oligonucleotide-based assay V-165 inhibited the overall activity of the integrase mutants IN-I161T, IN-V165I, IN-T206S, IN-S230N and IN-T206S/S230N to a 0.7, 0.8, 0.9, 1.3 and 1.6-fold lower extent than the overall activity of the WT IN, respectively. No cross-resistance towards the naphthyridine analogue, L-870,810, was observed. The PDPs act specifically on the binding of IN to the DNA. To determine the ability of the different mutant integrase enzymes to bind to DNA, we used fluorescence correlation spectroscopy. All mutant integrases showed a decreased affinity

for DNA. Additionally, the mutant enzymes showed only low level resistance to the inhibitory effect of V-165. 1t appears that mutations in the PDP binding site of integrase also interfere with the enzymatic activity of the integrase. This may explain the difficulty in selecting PDP-resistant strains in cell culture. Previous reports on HIV resistance development against diketo acids (DKA) also pointed out a high barrier to select resistance. Mutations in IN were only observed following 3 months of selection in the presence of the respective DKA [129, 132, 235]. Also, the DKA selected IN mutants appeared to have a decreased enzymatic activity [124, 235] and a lowered replication kinetics [124, 129, 235].

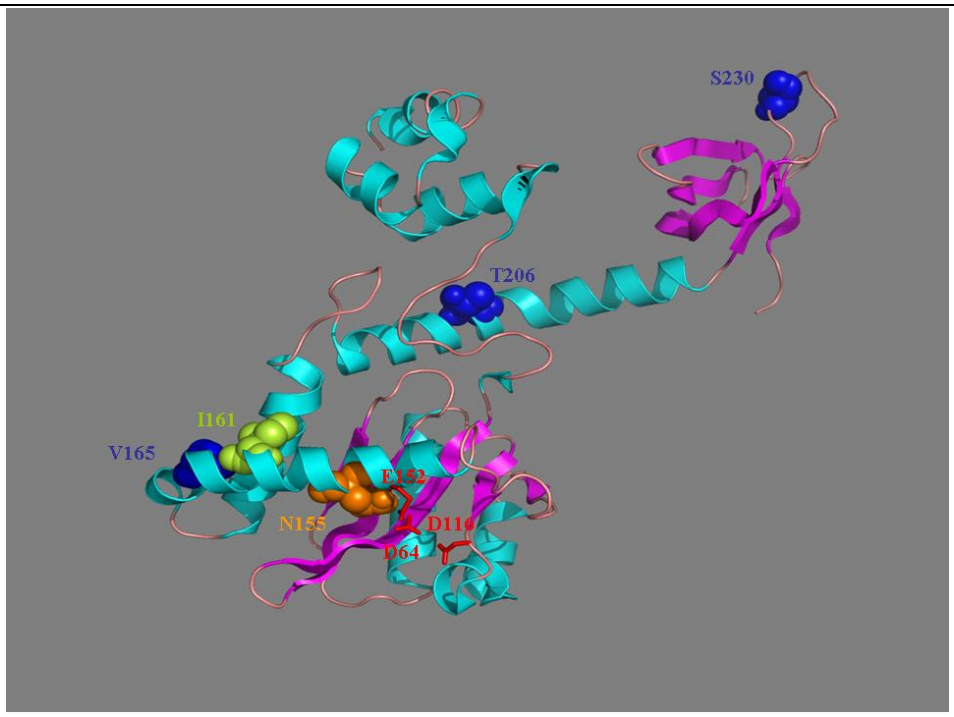

Figure 16: 3D-model of HIV-1 integrase and the mutations found in integrase

Integrase is composed of one polypeptide chain that folds into three distinct functional domains: the N-terminal domain (residues 1–50), the catalytic core domain (residues 50–212) and the C-terminal DNA binding domain (residues 212–288). The highly conserved residues in the catalytic core, Asp64, Asp116 and Glu152, commonly referred to as the 'DDE motif', are marked in red.

In previous studies, the integrase mutations T206S, S230N and V165I were found, in combination with other integrase mutations, as polymorphisms in a population of North American clinical isolates from HIV seropositive patients [236]. These polymorphisms, in combination with the other present integrase polymorphisms in that strain, however, did not affect the 3'-processing activity of the enzyme nor the replication kinetics of the virus and did not alter the viruses resistant towards the inhibitor L-chicoric acid [236]. This does not exclude their potential relevance in resistance to IN inhibitors. The mutation V165I has been described by Fikkert *et al*. to emerge in a HIV-1 strain resistant towards the integrase inhibitor S-1360 [129], whereas the mutation S230R occurred in a HIV-1 strain resistant towards L-708,906. The single mutation S230R did not appear to impair the specific enzymatic activity of recombinant integrase [235]. In contrast, the mutant IN-N155L was reported to show a 5-fold reduction in 3'-processing activity and a 5-fold decrease in over-all integration reaction [237].

In order to further assess the significance of each set of mutations in the IN, RT or gp160 gene for the observed resistance profile, recombinant strains carrying the wild-type HIV-1(NL4.3) or the resistant IN, RT or gp160 gene in a wild-type HIV-1(NL4.3) background were constructed. The antiviral susceptibility of these different recombinant strains was determined using the MT-4/MTT assay (data not shown: Doctoral thesis in preparation A. Hombrouck). By recombining IN into the WT background we could not fully reproduce the observed phenotypic resistance profile of the corresponding parental selected strain. The RT recombined viral strains reflected the observed loss in susceptibility of the V-165 selected strains to the NRTI AZT, implying a role for these mutations in the observed cross-resistance to AZT. They did not seem to be responsible

for the observed loss in susceptibility against V-165. We do not know the reason why the specific TAM mutations are selected in the presence of V-165. Perhaps V-165 by virtue of its dipyrimidine structure may affect nucleotide incorporation in the growing cDNA chain during reverse transcription, thereby functioning as a chain terminator. In a previous study, it was shown that V-165 does inhibit HIV RT activity *in vitro* at micromolar concentration [137], however by performing Q-PCR assays, we could not detect a significant inhibitory effect of V-165 on HIV cDNA synthesis in cell culture. The *env* recombined viral strains displayed a nearly similar loss in sensitivity to T-20 and AMD3100 as their original *in vitro* selected strains, indicating that the mutations in the recombined region are sufficient to reproduce the (cross-)resistant phenotype of the strains selected in the presence of V-165. In addition, these strains also exhibited a partial loss in sensitivity to V-165. These results suggest that the mutations selected in the envelope of the resistant virus strains might play a role in antiviral resistance development against V-165 at high concentrations of the compound. To elucidate this matter, several experiments were carried out to further investigate the antiviral effect of V-165 on viral entry (data not shown: Doctoral thesis in preparation A. Hombrouck). Previously, viral entry was excluded as antiviral target of V-165 in cell culture. In a lentiviral transduction experiment, it was shown that integration was the major antiviral target [137]. However, since the vectors used in these experiments were VSV-G pseudotyped, inhibition of HIV-1 entry was not assessed for. We revisited this Q-PCR analysis in a comparative study after HIV-1 infection and HIV-1 vector transduction in the presence or absence of V-165. After transduction with HIV-1 vector, no clear inhibition of DNA synthesis was detected in the presence of V-165. Furthermore, there

was a pronounced defect in the generation of proviral DNA, while no substantial increase in 2-LTR circles was measured. These results are highly consistent with our previous report and corroborate integration as a genuine target for PDP. However, during HIV-1 infection of MT-4 cells, inhibition of viral DNA synthesis by V-165 was clearly evidenced at 6 hours post-infection. Next to this quantitative PCR analysis, a quantitative viral adsorption assay was carried out. Apparently, V-165 affects the adsorption of the virus to the cell, although the compound is much less potent than a typical adsorption inhibitor such as dextran sulphate. Taken all the available data into account, we can conclude that at high concentrations V-165 affect HIV-1 entry. However, when HIV-1 entry is not assayed for, the inhibitory activity of V-165 on integration becomes apparent. This comparative study warns us for the use of a pseudotyped lentiviral vector transduction assay to identify the target of an experimental antiviral compound in cell culture.

In conclusion, this set of experiments illustrates the usefulness of studying antiviral resistance development to pinpoint the viral target(s) of a compound. This has already been illustrated for the integrase inhibitors L-chicoric acid [123] and Zintevir [122], for which antiviral resistance studies indicated that these compounds also affect viral entry. For V-165, our results revealed a multi-modal mechanism of action. V-165 inhibits viral entry at high concentrations but also inhibits integration as indicated by the decrease in integrated proviruses after transduction with VSV-G pseudotyped lentiviral vectors. Although the physicochemical properties of V-165 are not optimal, which results in problems with batch variation and stability, and although V-165 is poorly recovered from human hepatic microsomal matrix and 1% BSA, we still propose integrase binding

inhibition as a genuine antiviral target and V-165 as the INBI prototype. The quest for more stable and potent derivatives of V-165 that specifically target binding of integrase to viral DNA should therefore be pursued.

5.2. NATURAL RESISTANCE TO HIV-1 INFECTION

5.2.1. NATURAL VARIABILITY OF LEDGF/p75

LEDGF/p75 has been identified and validated in our laboratory as an essential co-factor for HIV integration and replication. However, not much is known about the natural variability of the gene coding for LEDGF/p75. We also wondered if there are mutations in LEDGF/p75 that can render a person resistant or less susceptible for infection with HIV. To analyze this, we sequenced a part of the gene coding for LEDGF/p75, namely the region spanning the integrase binding domain (IBD; amino acid residues 347 - 429) of LEDGF/p75. Four groups were analyzed, namely, 1) Caucasian seronegative individuals, 2) seronegative African female blood donors, 3) African HIV exposed seronegative female sex workers and 4) African seropositive female sex workers. Multiple mutations were found in the introns of the gene. Mutation at base pair position 41250 and 42503 and a deletion mutation at position 42566 - 42568 were found in the Caucasian group, while 2 additional mutations in the introns were found in the Africans; at position 42433 and 42809. The nucleotide differences at position 41250, 42503 and 42809 have already been described as single nucleotide polymorphisms (SNP) in LEDGF/p75, while the mutation at position 42433 and the deletion mutant at position 42566 - 42568 constitute new, unknown SNP's (www.ncbi.nlm.nih.gov/projects/SNP and www.hapmap.org). The mutations in the introns may account for an alternative splice variant of LEDGF/p75 if the introduction of the mutation induce alternative splice donor or splice acceptor sites. We used a software tool (www.fruitfly.org/seq_tools/splice.html) to predict splice donor and splice acceptor sites in the mutant genes. No additional splice donor or splice acceptor sites were predicted, nor did any of the splice donor or splice

acceptor sites disappear. This result was confirmed by sequencing the mRNA of some of the PBMC samples. We can conclude that the mutations in the introns of the gene coding for LEDGF/p75 are not responsible for a natural resistance to infection with HIV, because they do not give rise to a mutant LEDGF/p75 protein. Besides these mutations in the introns, a single mutation was found in exon 13. This mutation (Q472L) substitutes a glutamine at position 472 of LEDGF/p75 by a leucine. The mutation was present in none of the 10 Caucasian samples, in 1 out of 19 African seronegative female blood donors, in 3 out of 20 African HIV exposed seronegative female sex workers and in 1 out of 20 African seropositive female sex workers. This accounts for a frequency of respectively 0, 5.3, 15.0 and 5.0%. In order to determine the statistical difference between the prevalence of this mutation in the African seronegative and seropositive group female sex workers (15.0 versus 5.0%) a Fisher's exact test was performed. The observed difference did not reach statistical significance. This study should be repeated on much larger groups is order to obtain more statistical relevance. Three exon mutations in LEDGF/p75 have been described; S116R, S275F and L478V (www.ncbi.nlm.nih.gov/projects/SNP and www.hapmap.org). These mutations are located outside the IBD.

Although the Q472L mutation was located outside the IBD and the prevalence of the mutation in both groups female sex workers was not statistical different, we wanted to analyze the effect of the Q472L mutation on the binding of recombinant LEDGF/p75 to recombinant integrase. Therefore, an IN-His_6-Tag pull-down assay was performed. No difference in the binding capacity to IN was seen for the mutant Q472L-LEDGF/p75 compared with the WT LEDGF/p75.

To analyze the effect of the Q472L mutation on HIV-1 replication, polyclonal

MT-4/LEDGF-KD-back-complementation (BC) cell lines expressing WT or mutant(Q472L) LEDGF/p75 were generated. We infected these MT-4/LEDGF-mmKD, MT-4/LEDGF-KD, MT-4/LEDGF-KD-BC and MT-4/LEDGF-KD-BCQ472L cell lines in parallel with HIV-1(NL4.3). Viral replication was determined by measuring the amount of p24 antigen in the supernatants. A 2.5-fold reduction in viral replication was observed in the polyclonal LEDGF/p75 knockdown cell line (MT-4/LEDGF-KD) compared with the control cell line (MT-4/LEDGF-mmKD). Viral replication was restored to above control level in the polyclonal MT-4/LEDGF-KD-BC cell line. However, no difference in viral replication was observed in the cell line back complemented with mutant(Q472L) LEDGF/p75 (MT-4/LEDGF-KD-BCQ472L) compared to the cell line back complemented with WT LEDGF/p75 (MT-4/LEDGF-KD-BC).

Together with the lack of statistical difference in the prevalence of the Q472L mutation between the group seronegative female sex workers and the group seropositive female sex workers, these results, obtained from the IN-His_6-Tag pull-down assay and the back complementation experiment, suggest that the presence of the Q472L mutation in LEDGF/p75 may have no effect on the role of LEDGF/p75 in viral replication. However, an effect of the presence of the Q472L mutation on the progression to AIDS has not been investigated and can therefore not be excluded.

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Curriculum Vitae

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Date of birth:	12/05/1978
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Marital status:	Married to Joris Van der Auwera
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Work experience

2007 - present **Quintiles Belgium, Vilvoorde**
Clinical Research Associate

2002 - 2006 **Katholieke Universiteit Leuven, Leuven, Belgium**
PhD student
Department of Molecular and Cellular Medicine
Division of Molecular Medicine
Promoter: Prof. Dr. M. Witvrouw
Co-promoter: Prof. Dr. Z. Debyser
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IWT-scholarship 2002 - 2007

Title: Resistance and HIV infection: Resistance development against two new drug candidates and natural variability of the cofactor LEDGF/p75

Education

1998 - 2001 **Katholieke Universiteit Leuven, Leuven, Belgium**
Ingenieursjaren Bio-Ingenieur in de Cel- en Genbiotechnologie
Magna cum Laude

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Master thesis

Katholieke Universiteit Leuven, Leuven, Belgium
Department of Microbiology and Immunology
Promoter: Prof. Dr. E. De Clercq
Co-promoter: Prof. Dr. M. Witvrouw

Title: Genotypic and phenotypic resistance of HIV against binding and fusion inhibitors

Supervision of Master students

2005 - 2006	Supervisor of Master thesis: Biomedical Sciences, KULeuven Christel Desadeleer
	Title: HIV resistance development against integrase inhibitors
2003 - 2004	Supervisor of Master thesis: Biomedical Sciences, KULeuven Nathalie Lambrechts
	Title: Optimalisation and validation of a quantitative HIV RT-PCR

Laboratory skills

Cell culture	Transfection, Vector production, Transduction, Production stable cell lines, FACS-analysis, Immunofluorescence
Virology (HIV)	Infection, Resistance, Chimeric Virus Technology, p24-ELISA
Molecular Biology	RNA: (Q-)RT-PCR, Ribonuclease protection assay (α-P^{32})
	DNA: (Q-)PCR, Sequencing, Cloning, Site Directed Mutagenesis
	Protein (HIV-IN): Protein purification, Activity assays (ELISA, radioactive (γ-P^{32}) IN-assay), Protein-protein interactions

Publications in international reviewed journals

Fikkert V, Cherepanov P, Van Laethem K, **Hantson A**, Van Remoortel B, Pannecouque C, De Clercq E, Debyser Z, Vandamme AM, Witvrouw M. (2002) env chimeric virus technology for evaluating human immunodeficiency virus

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Hombrouck A, **Hantson A**, Van Remoortel B, Vercammen J, Rhodes D, Tetz V, Engelborghs Y, Christ F, Debyser Z en Witvrouw M. Resistance of human immunodeficiency virus type 1 to the pyranodipyrimidine V-165. Submitted for publication to Journal of Virology

Presentations at international conferences

12th Conference on Retroviruses and Opportunistic Infections (CROI)
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Hantson A, Hombrouck A, Voet A, Vercammen J, Engelborghs Y, De Mayer M, Witvrouw M en Debyser Z, Importance of aminoacids V165, T206, S230 and N155 for the interaction of pyranodipyrimidines with HIV-1 integrase as revealed by molecular docking and resistance selection.

Language Skills

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