THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

HIV-2 protease as a chemotherapeutic target

by

Dr. Mohamed Mahdi

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List of Abbreviations

AIDS	acquired immune deficiency syndrome
APV	amprenavir
ATV	atazanavir
CA	capsid protein
CCR5	C-C chemokine receptor type 5 cDNA
CMV	cytomegalovirus
CYP2C19	cytochrome P450 2C19
CYP3A	cytochrome P450 3A
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DRV	darunavir
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESCRT	endosomal sorting complexes required for transport
EVPSPS	cellular class E vacuolar protein sorting proteins
FBS	fetal bovine serum
FI	fusion inhibitor
FPV	fosamprenavir
GFP	green fluorescent protein
HAART	highly active anti-retroviral therapy
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
IC_{50}	half maximal inhibitory concentration

INSTI	integrase strand transfer inhibitor
INV	indinavir
K_{cat}	catalytic constant
K_i	inhibition constant
K_m	Michaelis constant
LPV	lopinavir
LTR	long terminal repeat
MA	matrix protein
Myr	myristoylation
NC	nucleocapsid protein
NEF	negative regulatory factor
NFV	nelfinavir
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitors
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEI	polyethyleneimine
PI	protease inhibitor
PI(4,5)P2	phosphatidylinositol-4,5-bisphosphate
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
PR	protease
RNA	ribonucleic acid
RSV	Rous sarcoma virus
RP-HPLC	reversed phase high performance liquid chromatography
RRE	rev response element
RT	reverse transcriptase
RTV	ritonavir

SCID	severe combined immunodeficiency disorder
SDS	sodium dodecyl sulfate
SIN	self-inactivating
SIV/MAC	simian immunodeficiency virus/macaque
SQV	saquinavir
SU	surface protein
TAR	Tat-responsive region
TAT	transcriptional trans-activator
TFA	trifluoroacetic acid
TM	transmembrane
TPV	tipranavir
TRIMα	tripartite motif protein 5 α
TRIS	tris aminomethane
VIF	viral infectivity factor
VPR	viral protein R
VPU	viral protein U
VPX	viral protein X
WHO	World health organization

INTRODUCTION

The human immunodeficiency virus type 2 (HIV-2) is a member of the lentivirus genus, of the *retroviridae* family. Current evidence indicates that it has descended from the simian immunodeficiency virus sooty mangabey (SIV/sm). Since the discovery of the virus in 1986, along with HIV-1, it had claimed the lives of millions of patients suffering from acquired immune deficiency syndrome (AIDS) worldwide. HIV-2 bears a striking genomic resemblance to HIV-1, and apart from variability in the genetic sequence accounting for the presence of unique accessory genes; such as viral protein X (*vpx*) in HIV-2 and viral protein U (*vpu*) in HIV-1, in addition to sequence and phenotypic differences between their envelope proteins, the two viruses share a similar structural genomic architecture. Clinically, HIV-2 can be distinguished from its counterpart by its decreased infectivity and attenuated pathogenicity, patients infected with HIV-2 tend to be long-term non-progressors, due to the fact that the rate of progression from infection to the development of AIDS tends to be slow and prolonged [1]. HIV-2 has a much lower prevalence than its counterpart, and for decades, the virus had remained relatively contained in the West African region, with statistical data on its prevalence widely lacking. However, recently, many reports have indicated that the virus is spreading beyond its geographical confines, particularity into Europe [2,3].

HIV-2 protease (PR) is a homodimeric aspartyl protease, composed of two identical subunits, each having 99 amino acid residues. Two aspartic acid residues within the active site; as part of the Asp-Thr-Gly motif, carry out the crucial step of viral protein and polyprotein processing, resulting in mature infectious virions. Protease inhibitors (PIs) are considered major pillars as part of Highly Active Anti-Retroviral Therapy (HAART) in the treatment of HIV infection and AIDS. Most of them are substrate analogues, binding to and inhibiting the viral enzyme. Given the then limited geographical distribution and lower pathogenicity of HIV-2, all the currently approved PIs were essentially designed against HIV-1, and their

effects on HIV-2 protease had never been thoroughly characterized. There is about 39–48% similarity in amino acid sequences between HIV-1 and HIV-2 proteases [4], depending on the viral subtype being studied. Therefore, this polymorphism has the potential to substantially affect the specificity of the protease for peptide substrates and inhibitors, and indeed, may render an otherwise very potent inhibitor on HIV-1 protease obsolete. So far, few studies have examined the efficacy of some of the clinically used protease inhibitors on HIV-2, either in enzymatic or phenotypic susceptibility assays [5-8], with some conflicting results. The problem in accurately determining the efficacy of a given inhibitor stems from the fact that many factors can influence the analysis; such as the stability and level of purity of the enzyme in enzymatic experiments, the type of cells and methods used in cell culture assays, as well as the viral strain under study. Moreover, some of the phenotypic susceptibility assays done so far relied on viruses isolated from patients under HAART, which probably alters the sequence of the protease by means of primary and secondary mutations, again influencing the results obtained.

Treatment-associated mutations are commonly encountered in patients receiving HAART [9,10]. In regards to the viral protease, as a consequence of amino acid substitution(s) in the substrate binding pocket or in a nearby site, the binding affinity of the inhibitors may become substantially reduced, leading to a decrease in the affinity to the inhibitor or even resistance. Many HIV-1 PI-associated mutations have been described in the literature, yet their effect on the susceptibility of HIV-2 protease to the inhibitors remains understudied. Of the currently known treatment-associated mutations observed in HIV-2 protease, I54M and L90M have been shown to be implicated in the reduced susceptibility to certain inhibitors [5,7]. Their effects; however, have never been characterized in association with all of the inhibitors, neither enzymatically, nor in cell culture.

Since PIs are major constituents of anti-retroviral combination therapies, we believe that it is of vital importance to thoroughly characterize the susceptibility of HIV-2 protease to the inhibitors designed originally for HIV-1. Our aim was to study the efficacy of the currently used PIs using the same HIV-2 protease coding sequence, in both enzymatic and cell culture assays. Using such a standardized approach would eliminate many confounding factors that come to mind when interpreting results of previous studies, such as divergence of viral strains, variability in methodologies and types of cell culture. We believe the strength of this study is to provide data about all PIs with the same model, which will in turn provide accurate results about the inhibitor`s efficacy, and help tailor the treatment for HIV-2 infection. Moreover, as data on HIV-2 protease treatment-associated mutations were mostly lacking, we studied the combined effect of two commonly encountered mutations on the susceptibility of the protease, assaying for all of the inhibitors using our modular system.

THEORETICAL BACKGROUND

Origin of the Human Immunodeficiency Virus type -2 (HIV-2)

HIV-2 is a lentivirus, belonging to the *orthoretroviridae* group of the retrovirus family. SIV, HIV-1 and HIV-2; the so called primate lentiviruses, share a similar genetic architecture, testament to their common ancestral origin [11]. Phylogenetic analysis revealed that both HIV-1 and HIV-2 emerged as a result of recombination events between ancestral simian viruses, resulting in two or more separate cross-species transmissions from chimpanzees and mangabeys. According to molecular clock analysis studies, these events occurred possibly many centuries ago [12,13]. HIV-2 is found to be closely related to SIV isolated from macaques and sooty mangabey monkeys. It is worth mentioning that current evidence suggests that SIV macaque (SIV/mac) came about as a result of an unintentional transmission of SIV from sooty mangabeys to macaques in captivity, hence the similarity between the two viruses. Using molecular cloning and sequencing analysis, an ancestral origin of HIV-2 from SIV/sm was first proposed in 1989 [14], shortly thereafter, viral isolates from patients in West Africa were found to resemble locally circulating simian viruses infecting sooty mangabeys and rhesus macaques [15]. More evidence of the SIV/sm-HIV-2 lineage is supported by the fact that SIV/sm from infected monkeys was capable of infecting laboratory workers after accidental exposure, resulting in seroconversion and persistent seropositivity after the exposure [16]. The infected personnel, however, were asymptomatic, with no clinical or laboratory evidence of immunodeficiency, and the virus exhibited low replication rate, and was difficult to culture. The similarity in the clinical picture between SIV/sm and HIV-2 infection therefore serves as more proof of their lineage.

It has been reported that HIV-2 was first isolated in 1986 from West African patients who presented with symptoms of AIDS [17]. It is yet not clear how the virus crossed over from primates to humans, but given the fact that sooty mangabeys are frequently hunted in many areas of West Africa, it is only plausible that the cross-over must have occurred through cutaneous exposure to infected primate blood or body fluid, resulting in the unsuspecting hunters being exposed to such a highly divergent simian virus [18]. One study conducted in Sierra Leone found that 7 out of 12 sooty mangabey bush meat samples being sold in a market were infected with SIV/sm, although the viruses were highly divergent, despite the samples originating from the same geographical region [19]. It is now believed that the first cross-species transmission of HIV-2 probably occurred in the former Portuguese colony Guinea-Bissau during the first half of the 20th century [20], this was then followed by a rapid spread of the virus within the region in the period between 1960-1970 [21].

HIV-2 genetic diversity

To date, eight distinct lineages (groups) of HIV-2 have been characterized and designated alphabetical letters A-H [22-24] (Table 1). These lineages are thought to individually represent independent cross-species transmission of the primate virus to humans. Groups A and B have been classically described as the epidemic groups, responsible for the majority of infections, with A being spread throughout West Africa and B predominating in Cote d'Ivoire [25,26]. Groups C and H; on the other hand, are the non-epidemic subtypes, representing sporadic transmission of the virus with limited spread. Groups C, G, H are currently localized to Cote d'Ivoire, group D resembles a SIV/sm strain from Liberia, and groups E, F were isolated from patients native to Sierra Leone [13,15,27,28]. Divergence between HIV-2 groups is attributed to the variability in *gag*, *pol* and *env* sequences. It has been reported that

up to 25% sequence variability exists between the groups, with groups E and F being the most divergent strains according to *gag* and *env* sequences, respectively [22,29].

Table 1. **Geographical distribution of HIV-2 groups**. * CRF: circulating recombinant form, ** isolated from immigrant patients originating from Sierra Leone [30].

Group	Distribution	Remarks
A	Spread throughout West Africa, isolated cases in France, Portugal, India, USA, Brazil, and Korea. AB CRF* isolated in Japan	Epidemic, worldwide distribution
B	Cote d'Ivoire , isolated cases in France	Epidemic, regionally pandemic
C	Cote d'Ivoire , Liberia	Sporadic
D	Liberia	Sporadic
E	Sierra Leone, USA**	Sporadic
F	Sierra Leone, USA**	Sporadic
G	Cote d'Ivoire	Sporadic
H	Cote d'Ivoire	Sporadic

Pathogenicity of HIV-2 varies significantly depending on the virulence of the group in question. For example, groups C, D, E and G are generally known for their sporadic nature, difficulty in isolation and replication in culture, and attenuated course of infection, as well as the lack of immunosuppression in infected individuals. Groups A, B, F and H on the other hand were indeed associated with reduced CD4+ T-cell counts, high viral loads and the development of immunodeficiency, they are also more easily isolated from infected individuals and readily replicable in tissue culture [23,29,30].

Epidemiology of HIV-2

Since its discovery, the virus had been confined to the West African region, predominantly in Guinea-Bissau, Cote d'Ivoire, Sierra Leone, Mali, Nigeria, The Gambia, Senegal and Cape Verde. Epidemiological data; however, on the prevalence of HIV-2 remain widely lacking, with yet no WHO estimates of the epidemic. Rough estimates put the global prevalence of HIV-2 at 1-2 million, though these estimates are grossly confounded by the fact that epidemiological statistics are not available from the countries mostly affected, in addition to inclusion of patients who are dually infected with both HIV-1 and HIV-2 [2,31].

The ending of the colonial era, population migration, as well as advances in international trade have all been implicated in the worldwide spread of HIV-2 beyond its geographical confines, especially into Europe, with countries such as France and Portugal reporting the incidence of HIV-2 being continuously on the rise [32,33]. Available statistical data show that HIV-2 infection comprised 2.3% and 1.8% of AIDS cases in Portugal and France, respectively [33,34].

In the past decade, an interesting pattern of decline in HIV-2 prevalence has been observed in the endemic West African nations, particularly among the younger population. In Guinea – Bissau for instance, prevalence of HIV-2 infection decreased from 7.4% to 4.4% between 1996-2006, with a concomitantly increased prevalence of HIV-1 (2.3% to 4.6%) [35]. This change in prevalence may perhaps be explained by the attenuated infectivity, decreased vertical transmission, and lower shedding in genital secretions of HIV-2, as compared to HIV-1 [36,37]. Additionally, HIV-2 infected individuals are usually long-term non-progressors to AIDS, with low levels of viremia and usually normal CD4+ T-cell counts. This probably acted as a confounding factor in the analysis, as these individuals were only serotyped after the development of AIDS as they advanced in age, which explains why the change in

prevalence pattern was only observed in the younger population. Moreover, as a result of long-term non-progression, these individuals were perhaps more susceptible to HIV-1 infection *ipso facto*, due to the already present socio-economic risk factors.

HIV-2 genome

Structural genes

Sharing a common ancestry, HIV-1 and HIV-2 share a common genomic organization (Figure 1). The structural retroviral genes *gag, pol and env* comprise the body of the genome, bracketed by the long terminal repeats (LTRs) on each side.

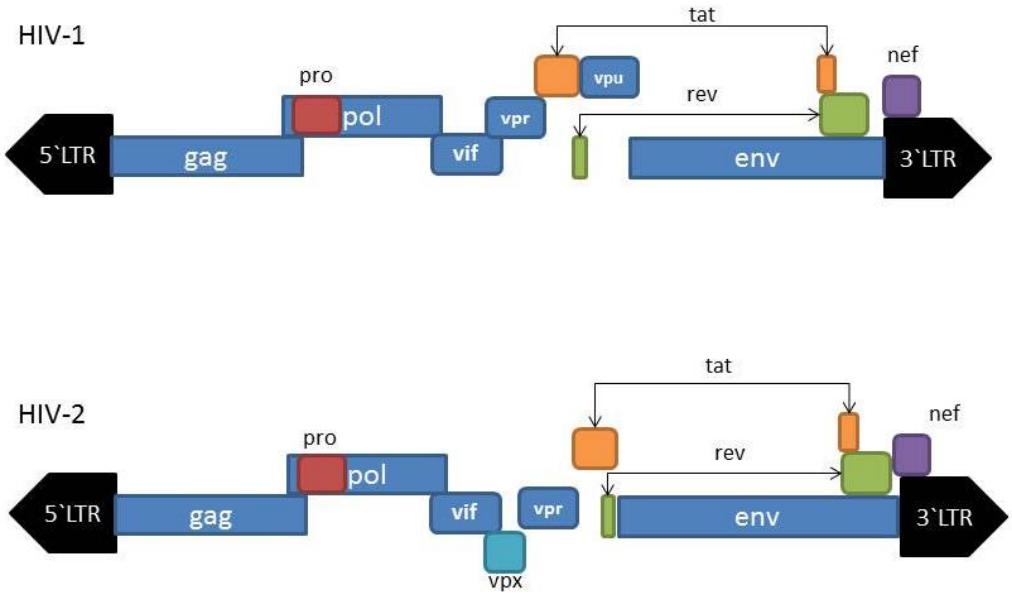


Figure 1: **Comparison of the genomic organization of HIV-1 and HIV-2.**

In the following paragraphs, a brief description of structural genes as well as the regulatory and accessory genes will be provided, shedding some light on currently known differences

between HIV-1 and HIV-2 genome wise. A schematic representation of the structure of HIV-2 is provided in figure 2.

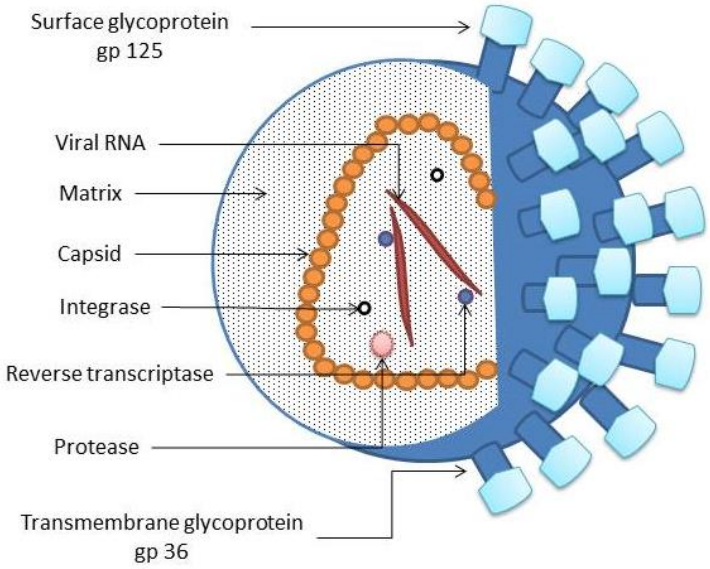


Figure 2: **Structure of HIV-2.**

The *gag* encodes internal structural proteins that are expressed as a precursor polypeptide. Post-translationally, proteolytic processing of the precursor protein results in the formation of the amino-terminal matrix protein (MA) p16, the phosphorylated capsid protein (CA) p26 and the nucleocapsid protein (NC) p12, a process that occurs during, or shortly after budding from the host cell, mediated by the viral protease [38]. These core proteins are essential for the assembly of viral particles, moreover, it was shown that the *gag*-encoded polyprotein is capable of self-assembly, independent of other viral components. In fact, researchers were able to produce virus-like particles using only *gag* encoded polyprotein and recombinant vaccinia virus in mammalian cells [39].

The MA domain of Gag becomes N-terminally myristoylated (myr), a process essential for binding of Gag to the cellular plasma membrane [40]. Targeting of HIV Gag to the plasma membrane also relies on a complex interaction between the MA domain and the cellular factor phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) that is associated with the inner leaflet of the membrane [41,42]. It was shown that the myr group is sequestered in the MA protein, while being properly exposed in the Gag precursor. This has led to the widely accepted proposition that a myristoyl switch mechanism is responsible for regulating the interaction between Gag and the membrane [43]. HIV- 1 and HIV-2 MA structures were found to be very similar, and parallel to HIV-1, targeting of HIV-2 Gag is also mediated by PI(4,5)P2, however, the myr switch mechanism of HIV-2 was found to be much weaker than that of HIV-1.This finding may help explain the somewhat less stable association of HIV-2 Gag proteins with the plasma membrane [42]. Additionally, it also sheds light on the fact that many strains of HIV-2 were shown to be unable to assemble and bud from yeast cells, as a result of Gag being easily dissociated from the membrane as well as failing to form high-order multimers at the budding site, in contrast to HIV-1 which efficiently produced viral particles within the same cell line [44].

Capsid domain (CA) of the Gag protein is located centrally, it is thought that this domain plays an essential role in particle assembly, possibly through mediating Gag-Gag interactions [45]. Capsid protein is responsible for the structural stability of the virion, forming a conical structure encapsulating the viral RNA and core-associated protein complex. Defects in the core structure have been implicated in the impairment of viral reverse-transcription and viral infectivity [46,47]. Structurally, CA protein is divided into 2 domains, a proline rich N-terminal domain that has been linked to viral maturation and cellular cyclophilin incorporation, and a C-terminal domain mediating Gag-Gag interactions. Cyclophilin A; a peptidyl-prolyl isomerase, binds the proline-rich loop located on the surface of HIV capsid.

Binding of cyclophilin to the capsid protein is thought to protect HIV from cellular restriction factors, such as the tripartite motif protein 5 α (TRIM5α) [48,49]. In contrast to HIV-1, HIV-2 is generally more susceptible to the human TRIM5α restriction factor, which has little or no effect on the former.

The nucleocapsid domain of Gag as well as the mature form of the protein are vital to the viral replication cycle. They are involved in chaperoning the viral RNA and reverse transcriptase throughout transcription, mediating primer tRNA annealing, RNA packaging, assisting in virion assembly, and proviral integration [50-52]. Both nucleocapsid proteins of HIV-1 and HIV-2 (NCp7 and NCp8, respectively) contain highly conserved zinc fingers, with N-terminal domain of NCp8 being significantly shorter with less positively charged residues. This structural difference is perhaps responsible for the observed reduced chaperoning activity of NCp8 of HIV-2 as compared to HIV-1 [53]. Theoretically, one might conclude that the reduced activity of the nucleocapsid may negatively influence the reverse-transcription and replication dynamics of HIV-2; however, taking the complexity and pathogenicity of HIV-2 into consideration, it is wise to assume that other viral proteins may compensate for the sub-optimal chaperoning property of NCp8.

The *env* gene codes for the surface-expressed 160 kDa Env glycoprotein, which mediates the viral attachment, fusion and entry through the cell membrane. Post-translationally, it is cleaved into gp 125 and gp 36 in case of HIV-2, and gp 120, gp 41 in HIV-1. These glycoproteins trimerize to form a knobbed spike structure on the viral surface, with gp 125 mediating interaction with host receptors. Thereafter; following a conformational change, gp 36 brings about fusion with the cell membrane [54,55]. Despite structural similarity, HIV-1 and HIV-2 only share a 35-40% similarity in amino acid sequence of the Env proteins, this sequence and phenotypic variability perhaps is accountable for the lower fusion temperature threshold (25 vs 31 ºC), and insensitivity to target membrane glycophospholipid composition

observed with HIV-2 envelope glycoproteins [56,57]. Moreover, HIV-2 Env has been shown to exhibit a significantly faster fusion kinetics as compared to HIV-1, as a result of a rapid CD4-induced conformational change of the surface (SU) protein, exposing the CXCR4 binding site for fusion [58].

The viral *pro* and *pol* genes encode for the viral enzymes; protease, reverse transcriptase and the integrase. The enzymes are translated as part of the Gag-Pro-Pol precursor fusion protein, and are thereafter processed by the viral protease into mature proteins. The reverse transcriptase (RT) mediates the reverse-transcription step required for creating double stranded DNA from a single stranded viral RNA. It harbors two enzymatic activities: a DNA polymerase utilizing either DNA or an RNA template, and an RNAse H, that degrades RNA only when it is part of the DNA/RNA heteroduplex during reverse-transcription. HIV-2 RT is shorter by 1 amino acid, and there is a 61 % homology between HIV-1 and HIV-2 RT amino acid residues [59]. RTs from both viruses were found to mediate similar activities; however, it was found that HIV-2 RT is slightly less active and less processive than HIV-1 RT (about 75 % of HIV-1 RT`s activity) [60].

Integrase is required for the integration of retroviral cDNA into the host cell genome. The literature describes 65 % similarity in amino acid sequence between HIV-1 and 2 [61]. It is interesting to note that while the length of HIV-1 integrase is constant (288 amino acids), that of HIV-2 is variable, depending on the position of the C-terminal stop codon [62].

Regulatory genes

Parallel to HIV-1, HIV-2 contains two regulatory genes; *tat* and *rev*, coding for the two viral trans-activator proteins Tat and Rev. The transcriptional trans-activator (Tat) functions as a potent activator of HIV gene expression, the protein is translated from two exons within the central region and *env* gene. It binds to Tat-responsive region (TAR); a highly conserved RNA stem-loop structure present at the 5` end of HIV RNA, this interaction serves to augment both LTR-directed transcriptional initiation as well as elongation of viral RNA [63,64]. HIV-2 Tat is considerably larger than that of HIV-1 (130 vs 86 amino acids long, respectively), and apart from some conserved domains, little homology exists between the two proteins [65]. Functionally speaking, it is interesting to note that HIV-1 Tat is able to trans-activate both HIV-1 and HIV-2 LTRs with equal efficiencies, while HIV-2 Tat activates its heterologous counterpart less efficiently [65].

Another regulatory gene *rev* encodes for the trans-activating protein Rev. This protein is synthesized at the early stages of infection, and functions as an efficient transporter of un-spliced, singly-spliced, as well as the viral genomic RNAs from the nucleus to the cytoplasm of host cells. It is only when a sufficient amount of multiply-spliced mRNA coding for Rev begins to accumulate in the cytoplasm, that the infection reaches the late productive phase, otherwise, the infection remains in the latent phase [66]. To be able to carry out its function, Rev must bind to a complex segment of viral RNA named Rev response element (RRE), located in all viral RNAs requiring Rev transport for expression in the host cell cytoplasm [55]. Other functions of Rev have been found to be counteracting the effects of *cis*-acting inhibitory elements in *gag*, *pol* and *env* of HIV; through shunting those RNAs towards export from the nucleus, and increasing the translation efficiency of RRE-containing RNAs in the cytoplasm [67,68]. An important difference between Rev proteins of HIV-1 and 2; just as in the case of Tat, is that although HIV-1 Rev is capable of facilitating nuclear export of viral

RNAs of both HIV-1 and 2, HIV-2 Rev is functionally impaired in HIV-1 context. Contrary to previous hypothesis, both Rev 1 and Rev 2 are capable of recognizing their respective RRE counterpart [69], and the explanation for this non-reciprocal regulation lies in the fact that HIV-2 Rev`s interaction with HIV-1 RREs leads to aberrant multimerization of Rev on the heterologous RNA sequence; a feature required for the proper functioning of Rev. Defect in such multimerization severely impairs the interactivity and exporting ability of Rev [70].

Accessory genes

Negative regulatory factor (Nef) is a highly conserved accessory protein that is encoded by the *nef* gene, a single exon extending into the 3`LTR region. Nef is predominantly expressed during the early stages of infection, and its presence confers virulence to the virus. On one hand, Nef acts as a down-regulator of cell surface receptors; such as CD4 and CD28 on T-helper cells, by increasing the rate of their endocytosis and lysosomal degradation [71], as well as major histocompatibility complex-I and II (MHC-1,II) present on antigen presenting cells [72]. On the other hand, Nef was found to maintain a state of T-cell activation, mediated through increased T-cell signaling [73], in addition to increasing viral infectivity, as evident by the earlier expression of viral proteins and enhanced replication kinetics in Nef^{+} virions [74]. The precise mechanisms by which this boost in infectivity is governed remains unclear.

HIV-2 Nef has not yet been thoroughly characterized; however, a study was able to detect a difference between HIV-1 and HIV-2 Nef proteins in utilizing intracellular signal transduction pathways during pathogenesis. HIV-1 for example interacted with tyrosine-protein kinase HCK, while HIV-2 Nef was bound to an adaptor protein Nefin-1, and showed weak or no interaction with HCK [75].

Viral proteins R and X (Vpr and Vpx) are two accessory viral proteins encoded by their respective related genes *vpr* and *vpx*. These proteins are present in the viral particle, and have been found to function in the early stage of viral replication [55]. In case of HIV-1, Vpr has been associated with nuclear localization of the pre-integration complex, inhibition of cellular proliferation, and induction of differentiation, in addition to serving as a transcriptional activator of viral genome [76,77]. There is a 45 % homology in amino acid sequence between HIV-1 and HIV-2 Vpr proteins. Structural and functional data remain scarce regarding Vpr of HIV-2, and although HIV-2 Vpr has been associated with cell cycle arrest in G2 state; just as in the case of HIV-1, this effect is attenuated in comparison to HIV-1, due to the short half-life of HIV-2 Vpr. Moreover, HIV-2 Vpr was found to be necessary for efficient viral budding. Other functions of Vpr remain to be established in case of HIV-2 [78].

Vpx is an accessory protein found only in HIV-2 and its ancestor SIV/sm. Sharing 22 % similarity in amino acid sequence with HIV-2 Vpr, it has been proposed that *vpx* came to exist as a gene duplication of *vpr*. Vpx has been found to be necessary for the efficient nuclear import of the pre-integration complex, similar to Vpr [78]. All in all, Vpr and Vpx of HIV-2 are thought to augment the viral replication cycle, and may as well play key roles in neutralizing intrinsic host cellular restriction factors that limit or inhibit replication of the virus [79].

Viral infectivity factor Vif is a protein encoded by *vif* gene, downstream of *pol*. This protein is conserved among all lentiviruses, except for the equine infectious anemia virus (EIAV), which lacks the *vif* open reading frame [80]. Vif has been found to be an important determinant of viral infectivity, as its knock-down has been shown to hamper early stages of infection; such as impairment of reverse-transcription, and defect in virion particle assembly as a result of impaired Env incorporation [81]. The literature describes 25 % homology in Vif amino acid sequence between HIV-1 and 2, so far; besides the sequence polymorphism,

another difference exists between the two proteins in terms of cellular localization. In contrast to HIV-1 Vif, which has been found to localize predominantly in the cytoplasm, Vif of HIV-2 was consistently present in the nucleus 18 hours after transfection of HeLa cells. Whether or not the protein re-localized to the cytoplasm thereafter, or even the significance of such difference in localization remains to be elucidated [82]. It is important to note that Vif was found to counteract the actions of the cellular restriction factor APOBEC3G; a cytidine deaminase that restricts viral infectivity by mutating retroviral DNA, by means of triggering massive deamination of deoxycytidine to deoxyuridine leading to viral destruction [83]. Vif mediates its action through ubiquitination of APOBEC3G, tagging it for subsequent proteasomal degradation [84]. APOBEC3G showed less restriction of HIV-2 compared to HIV-1 when Vif was knocked out from both viruses. This could indicate that maybe other factors, or perhaps even HIV-2 Gag proteins are somewhat involved in counteracting APOBEC3G [82,85].

Life cycle of HIV

A schematic representation of the life cycle is provided in figure 3. Initially; in the attachment phase, the virus must first gain proximity to the cell surface. It has been shown that Env plays and important role bringing HIV into close contact with the cell surface receptor for attachment to occur, through various interactions with cell-surface heparan sulfate proteoglycans, α4β7 integrin or pattern recognition receptors [86]. Once in contact, the surface glycoprotein binds the cell surface receptor CD4, where Phe43 residue of CD4 partially fills a hydrophobic cavity within the SU. This interaction brings about a bridging sheet between the inner and outer domains of the surface glycoprotein monomers, which in turn changes its conformation to expose a binding site for a secondary cell surface receptor.

Interaction of this secondary receptor with the fusion peptide; located at the amino terminus of the transmembrane protein (TM), triggers a complex rearrangement of the transmembrane protein, leading to tethering of the viral and host membranes, and the formation of the fusion pore [87]. It is still unclear; however, whether fusion occurs at the cell surface or within endocytotic vesicles. Previous studies demonstrated that HIV entry occurs in a pH-independent manner; moreover, some researchers proved that endocytosis is required for compete fusion to occur, albeit pH-independent, and in specific cell lines [86,88].

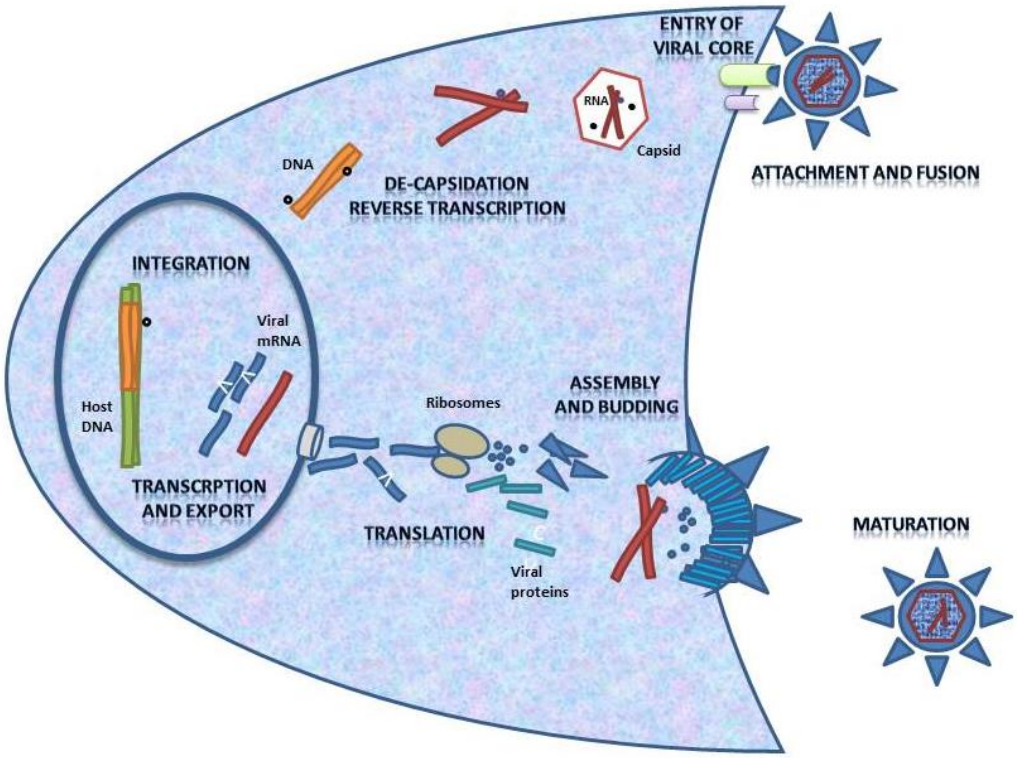


Figure 3: **Life cycle of HIV**.

Fusion is followed by entry of the viral core; composed of the viral RNA and replication enzymes encased by the capsid protein, while the envelope remains within the cell membrane. Thereafter, the viral capsid dissociates in a step referred to as de-capsidation or un-coating. Although still a matter of debate, the precise cellular location where un-coating occurs

remains unclear, with previous consensus being that it happens immediately following viral entry into the cytoplasm, recent suggestions however hint to the idea that un-coating occurs several hours after entry, possibly after reverse-transcription, and close to the nuclear pore [89].

Reverse-transcription is mediated by the viral RT enzyme, a heterodimer that harbors two functionally active sites: an N-terminal DNA dependent DNA polymerase that can use either RNA or DNA template, and a C-terminal RNAse; a nuclease that digests the RNA strand within the RNA-DNA duplex that forms during the process of reverse-transcription. End result of reverse-transcription is the conversion of the viral RNA into double stranded DNA that is complementary to the viral RNA, with the exception of the presence of terminal duplications in the DNA that are not present in the template RNA. These duplications are termed long terminal repeats, that are essential for viral integration into host cell DNA and viral genomic transcription [55]. Integration of viral DNA into host genome is then mediated by the viral integrase enzyme, that carries out the 3` processing activity of viral and chromosomal DNA, and the strand transfer step required to integrate the viral DNA. Integration is also assisted by cellular host enzymes that repair the strand gaps created by the 3` end processing, establishing an integrated proviral genome. This marks the end of the early phase of infection. Using host transcription factors and RNA polymerase, transcription of viral genome is initiated from the U3 promoter region embedded within the LTR region, and the regulatory protein Tat maintains efficient elongation as discussed earlier. Viral mRNAs are then exported from the nucleus into the cytoplasm, where they are translated. Regulatory protein Rev is required for the export of un-spliced and singly-spliced viral mRNAs.

Following expression of viral proteins, assembly begins of virus-like particles at the cellular membrane, a process in which Gag polyprotein domains play a vital role. Once assembly is complete, virus-like particles then bud out from the cell membrane, mediated by the late

domain of Gag and cellular class E vacuolar protein sorting proteins (EVPSPS); which are subunits of Endosomal Sorting Complex Required for Transport complexes (ESCRT), that also carry out the similar function of abscission of membrane during cell division [90].

Finally, although the precise timing is still unknown, the viral protease cleaves the immature precursor viral polyproteins into individual proteins, resulting in the reorganization of the viral structure and the formation of functional structural proteins and active viral enzymes. This viral maturation mediated by the viral protease is thought to take place during or right after budding of the virus, either way, this step of the viral cycle is a crucial determinant of viral infectivity, as immature virus-like particles were consistently found to be non-infectious [91,92].

Aspects in HIV-2 life cycle

Research on HIV-2; and particularly on the viral life cycle, is scarce. Although the life cycle is thought to be similar; if not identical, between HIV-1 and 2, some interesting points regarding HIV-2 deserve mentioning. To begin with, beside utilizing CD4 cell surface receptor and co-receptors CCR5 or CXCR4; additionally, HIV-2 has been shown to use a broader spectrum of co-receptors; such as CCR1, CCR2b, CCR3, CCR8, CXCR1, GPRI, APJ, and US28 among others. HIV-2 was also able to infect cells deficient in CD4 receptor *in vitro*. This variability in co-receptor utilization would theoretically be advantageous to HIV-2 boosting its infectivity; interestingly, broad co-receptor utilization of HIV-2 has not been linked to increased pathogenicity [93,94].

Replication dynamics of HIV-2 have also been found to slightly differ from that of HIV-1. Plasma viral load and DNA load in patients infected with HIV-2 is considerably lower,

indicating a significantly decreased replication rate. Furthermore, HIV-2 is associated with an acute “surge” in viral production right after infection, followed by a prolonged latency stage, while HIV-1 shows a lower level of replication, albeit steady-state through time, at least in some cell lines [94]. HIV-2 infection is classically associated with a prolonged symptomless clinical course, and although the exact mechanism for the virus`s preference for this long latency stage remains unknown, several factors may possibly be implicated; such as the presence of different regulatory elements within the promoters of the LTR region in HIV-2, as well as the structure of the LTRs themselves. LTRs in HIV-2 are indeed significantly larger, and contain complex structures as compared to those of HIV-1 [95]. Whether or not other differences exist in replication kinetics between the two viruses, and if some of the observed difference can be attributed to either sequence variability in the viral proteins or the presence of unique accessory genes remains to be elucidated.

The viral protease

Structural characteristics

The viral protease is a homodimeric enzyme, belonging to the aspartic group of proteases. It is comprised of two identical monomers, each containing 99 amino acid residues (Figure 4). The structure of the PR is of a predominantly β sheet-organization, and as in the case with all aspartic proteinases, two aspartic acid residues placed in the highly conserved motif Asp-Thr-Gly carry out the proteolytic processing through a coordination of a water molecule, leading to the hydrolysis of the target peptide bond.

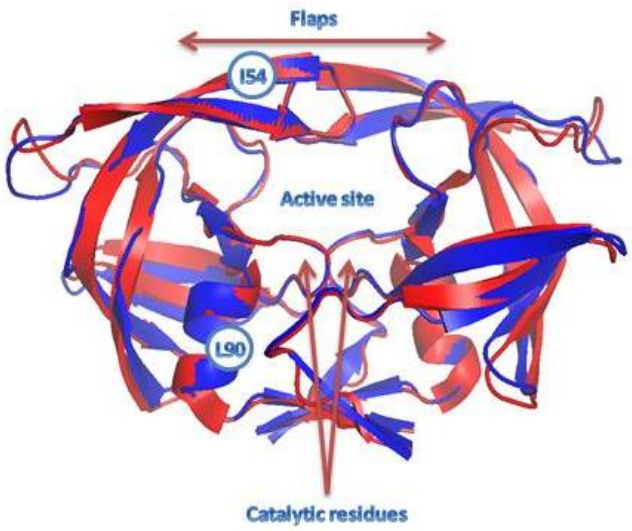


Figure 4: **Superimposed crystal structures of HIV-1 and HIV-2 proteases**. HIV-1 protease (5HVP.pdb) is indicated by blue, while HIV-2 protease (3EBZ.pdb) by red color. Residues commonly associated with treatment-associated resistance are also labelled.

By convention, the amino acid residues on the substrate are designated P4-P3-P2-P1-P1`-P2` -P3`-P4`, where the peptide bond between P1 and P1` is the scissile bond. Correspondingly, their respective binding sites in the enzyme are denoted with the letter S. Structural analysis of the protease revealed that the aspartic acid residues in the active site are positioned in a loop approaching the center of the dimer, positioned at the hydrophobic core of the enzyme. Once bound to the substrate, the viral PR undergoes structural changes as indicated by movement of the flap region; a segment composed of antiparallel β sheet with a β–turn extending over the substrate binding cleft present at the top of the dimer, as well as internal movements within the core of PR [55]. To carry out its proteolytic processing, the PR must interact with the substrate over at least seven amino acid residues, from P4 to P3`, as evidence suggests that shorter peptides interacting with PR are either processed less efficiently or not processed at all. Studies of HIV PR in association with inhibitors found that the PR interacts with the peptide backbone of the inhibitor, as well as the side chains extending into the subsites; moreover, the inhibitor binds to the enzyme in an extended β conformation,

facilitated by a series of hydrogen bond interactions between C=O and NH groups of the inhibitor and the PR [96]. While the viral PR is a symmetric dimer, it is interesting to note that it binds the substrate in an asymmetric manner, as minor differences where observed when studying interactions between the amino half and the carboxyl half of the bound inhibitor in association with the PR; additionally, subsites were characterized in the PR for substrate side chains P3 through P3′, with interactions of the P4 and P4′ side chains being much weaker [97]. It is now evident that cleavage site sequences of retroviral polyprotein substrates recognized by the viral protease are quite variable; however, certain features are common to most precursor processing sites; such as the presence of a small amino acid at P2 and P4, glutamic or basic amino acid at the P3 position, glutamine or glutamic acid in P2` (in case of primate lentiviruses), and hydrophobic amino acids at P1-P1` (Figure 5). Aromatic amino acids are generally excluded from P3` [55].

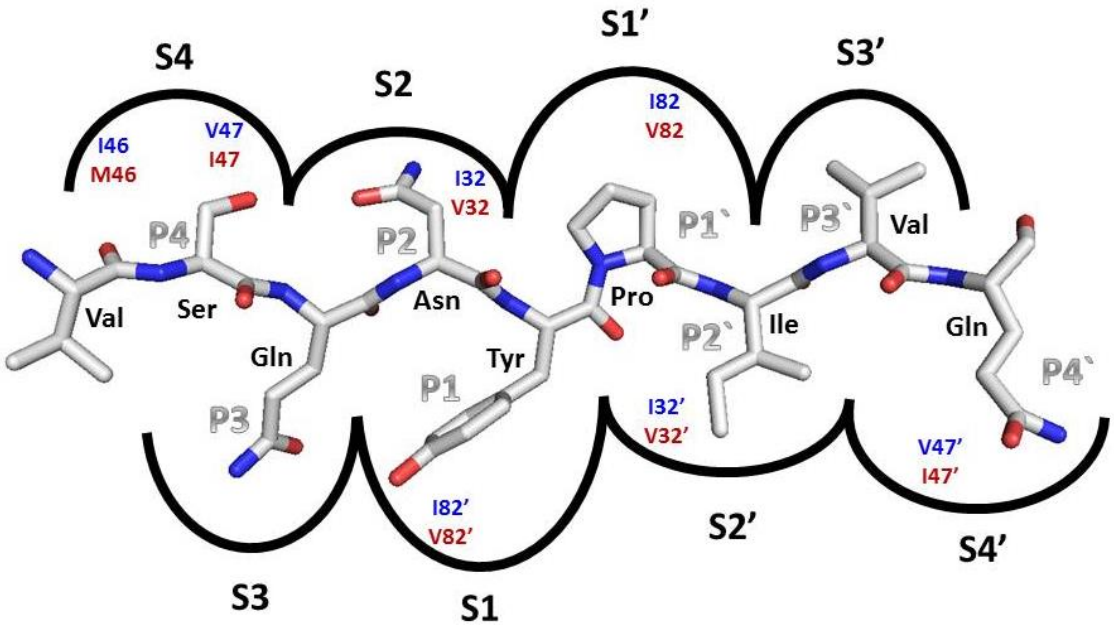


Figure 5: **Schematic representation of HIV PR with a substrate representing matrix↓capsid cleavage site.** Substrate binding pockets in the PR are indicated by black curves and labeled with S, the sizes of which relatively correspond to the size of the pocket. P sites on the substrate are labeled with grey color. Amino acid polymorphisms in the substrate binding site of PR are also shown; blue color denotes HIV-2 while red indicates HIV-1. The figure was constructed with the aid of Dr. János András Mótyán using PyMOL software (based on Tozser J., 2010) [98].

Expression and autoprocessing

HIV-2 protease is encoded by a 297 long nucleotide sequence embedded in the *pol* reading frame, and is expressed as part of Gag-Pro-Pol precursor polyprotein. This fusion polyprotein is brought about as a result of translational bypassing of the termination codon present at the 3` terminus of *gag* reading frame, consequently, translation continues into adjacent *pro* and *pol* reading frames, resulting in the fused proteins. As in the case with HIV-1 and the majority of retroviruses, this translational bypassing mechanism is the result of a ribosomal frame-shifting; where occasional ribosomes shift backward one nucleotide towards the 5` (–1 frame-shift), leaving the downstream termination codon of *gag* reading frame and shifting into an overlapping portion of the *pro-pol* reading frame. Evidence for this frame-shifting mechanism comes from studies that demonstrated the presence of a heptanucleotide slippery sequence that was able to mediate a basal level of frame-shifting [55,99].

To carry out its proteolytic processing of viral proteins, PR must first be liberated from the Gag-Pro-Pol polyprotein in a process termed autoprocessing; a controlled process by which the precursor PR self-catalyzes its cleavage from the polyprotein, forming free mature PR that goes on to process other viral proteins. Within the Gag-Pro-Pol polyprotein, PR is flanked at the N-terminus by a trans-frame region (referred to as p6* site) and the reverse transcriptase at the C-terminus. It has been shown that blocking the N-terminal cleavage site of PR resulted in a severe reduction of its proteolytic ability, while alteration of its C-terminal cleavage site had little or no effect on its activity [100,101]. Thus, it quickly became apparent that N-terminal cleavage plays a crucial role in autoprocessing of PR. It is worth noting that despite having similar amino acid sequence, the catalytic property of the precursor and mature PR differ profoundly, this is shown by the limited catalytic ability of the precursor protein in contrast to the mature form [102], and although both proteins share the same catalytic site, explanation of the aforementioned difference lies in the finding that the N-terminal extension peptide (p6*)

may perhaps hinder the activity of the precursor PR by destabilizing the dimer formation, leading to only transient interactions between the precursor PR and its substrate [103]. The exact mechanism of autoprocessing of HIV PR demands further exploration; however, certain amino acid residues were shown to have a role in the regulation of autoproteolysis, such as H69, C67 and C95. Previous studies have demonstrated that H69E exerts limited effect on autoprocessing in the presence of C67/C95; however, when these cysteine residues were substituted (C67A/C95A), H69E severely impaired autoprocessing [104]. It is interesting to note that autoprocessing does not occur immediately after the synthesis of Gag-Pro-Pol polyprotein; instead, it certainly occurs right before virion release. Factors implicated in regulating this feature are still largely unknown, and whether or not the initiation of autoprocessing is activated by an increase in the concentration of Gag-Pro-Pol polyproteins at the assembly site; promoting dimerization of PR, remains debatable. While the general consensus is that precursor PR probably initiates its autoprocessing through cleavages at the amino and/or carboxy termini freeing PR dimers, it is still hard to explain how this dimerization is achievable given the presence of p6* peptide upstream of the PR functioning as a dimer destabilizer [103].

Substrate specificity

The viral protease is crucial to the viral life cycle, processing polyprotein precursors post-translationally into mature active proteins. To carry out its function, PR interacts with ten unique sites within the viral polyproteins, and the substrate must bind to the enzyme in an extended anti-parallel β strand conformation, while the amino acid side chains of the substrate extend in opposite directions within the substrate-enzyme complex [105]. Substrate specificity was found to be largely determined by residues clustering around the P2-P3` subsites [106],

and as previously mentioned, despite its symmetry, the enzyme recognizes and binds asymmetric substrate sites present in Gag-Pro-Pol polyproteins. The amino acid sequence distribution within the active site was found to vary in both size and charge, it is therefore believed that the viral protease interacts with the substrate on the basis of a characteristic shape adopted by the substrate, rather than recognizing a specific amino acid sequence [107].

Hydrophobic residues comprise the majority of the substrate binding site, with the exception of Asp25 and Asp29. Arg8, Asp30 and Lys45 are able to interact with polar side chains of long peptides [108]. During catalysis, the peptide allocates to the active site residues 25-29 and two flap regions, this is facilitated by the formation of hydrogen bonds. Asp25 and Asp29 are also found to form hydrogen bonds with the main chain peptide. Crystallographic studies of HIV-1 protease in complex with substrates revealed that in order for the protease to achieve specificity when in contact with the substrate, P1 and P3 must interact on the un-primed site of the cleavage cleft forming a toroid, and on the P1` and P3`sites enough space must be present to accommodate water cluster molecules. Disruption of this toroid formation or interference with water presence in these sites is therefore detrimental to the activity of the protease [107]. Studies on substrate catalysis by PR revealed the existence of two types of cleavage sites: Type I; characterized by the presence of an aromatic residue at P1 and proline at the P1` site, and type II sites; typically characterized by the presence of hydrophobic residues at P1 and P1`. There is a preference for Asn residue at P2 and β-branched hydrophobic residue at P2` in the case of type I cleavage sites. In type II cleavage sites however, a β-branched residue is preferable at P2, while P2` residue is usually Glu or Gln. Additionally, S4 subsite was found to preferably bind small hydrophilic residues. S3-S3` subsites are relatively large, and generally hydrophobic. The size of the corresponding P residue interacting with the subsites; in addition to its hydrophobicity, were found to play a major role in substrate specificity of the enzyme [98].

As studies on HIV-2 PR substrate specificity remain widely lacking, few differences can be found in the literature. For example, modeling experiments have revealed that S1/S1` subsites in HIV-2 PR tend to favor smaller P1 side chains in the peptide, which may have an effect on hydrolysis of certain substrates [109]. The presence of Cys or Asn residue in P1 results in a boosted hydrolysis of the substrate by HIV-2 protease. Thr, and hydrophobic amino acids such as Phe, Gly, and Ile on the other hand remarkably decreased the relative activity of the protease [110]. Generally, it is safe to assume that substrate specificity of HIV-2 PR is thought to be similar to that of HIV-1, and their kinetic parameters are rather comparable [111].

Mechanism of resistance

It is now believed that resistance mutations occurring in patients receiving protease inhibitor-based regimes are a result of a gradual stepwise process. Given the lack of a proofreading capability of the viral RT, and the accelerated rate of viral replication, the ground is set for virion variants with PR harboring the resistance mutations to be selected. Initially, primary mutations are selected first. These mutations result in the alteration of the substrate binding site, thus interfering with the binding of the inhibitor either directly or indirectly. Thereafter, additional mutations accumulate in the viral protease; termed secondary mutations, with the end result of inferring high level or resistance against the inhibitor [112].

Resistance mutations generally involve hydrophobic residues of the PR. Currently marketed PIs are competitive active site binders, they are classically a lot smaller and of a different shape than the PR`s natural substrates. Their functional groups are on average similar, coming into contact with the same residues of the PR active site. This similarity led to the finding that overlaying the inhibitors on the substrate envelope would result in common locations;

particularity between P3 and P2` subsites, where the inhibitors tend to interact with specific residues in the viral PR. It is the mutation of these residues that result in multi-drug resistant PRs, with the exception of L90M mutation which does not come into contact with the inhibitors, yet it is associated with multi-drug resistance [113]. Moreover, visualizing how an inhibitor may fit within the substrate envelope using *in silico* studies has become a useful predictive tool to analyze the extent of the inhibitor`s susceptibility to resistance mutations [114]. Interestingly, primary resistance mutations against the inhibitors also decrease the affinity of PR to its natural substrates, negatively affecting the replicative efficiency of the virus. However, HIV; impressively, compensates for this aftereffect by generating compensatory mutations that enable the PR to cleave its natural substrates while remaining resistant to the drug, a form of adaptive fitness. Those compensatory mutations are quite indistinguishable from the secondary mutations, and the mechanism by which they exert their effect is still unclear [112,115].

PI-associated resistance mutations

Polymorphism in the amino acid sequence between HIV-1 and 2 PR varies depending on the virus strain in question, and stems from 50-60 % difference in the nucleotide sequences of the two viral enzymes. It is now clear that these natural polymorphisms in HIV-2 PR can be held accountable for the accelerated development of resistance to certain inhibitors [5], in fact, polymorphic amino acids found in wild-type HIV-2 protease have been associated with resistance to PIs in HIV-1 (Figure 6). Generally speaking, treatment-associated changes in HIV-2 PR gene tend to develop in sites parallel to those that can be found in HIV-1 PR. Other mutational motifs associated with resistance to certain inhibitors include 62A and 99F under indinavir and nelfinavir pressure, with or without an additional 90M mutation. 50V and 82L mutations tend to confer resistance against amprenavir and tipranavir respectively. Moreover,

occurrence of 54M solely or in association with 84V, 90M or 99F have been found to confer multi-drug resistance to the HIV-2 PR [5].

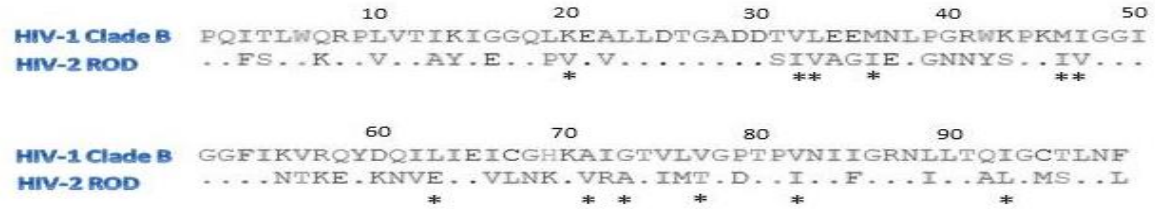


Figure 6: **Protease sequence alignment**. HIV-1 B clade protease sequence in alignment with HIV-2 ROD protease. Asterix indicate polymorphic amino acids in HIV-2 associated with resistance to HIV-1 PIs.

I54M and L90M mutations

The effects of I54M and L90M single mutations have already been described previously, based on the amprenavir complexes of HIV-1 PR and its drug resistant mutants. There is; however, a lack of structural and analytical data for these mutations regarding HIV-2 PR. I54 is positioned in the flap region of the protease, and while it is not exposed to the active cavity, it forms hydrophobic interactions with the neighboring flap and loop residues. Substitution of isoleucine in the 54th position to methionine is a non-polymorphic mutation that was found to be selected primarily by fosamprenavir, lopinavir and darunavir, as well as causing a cross-resistance to nelfinavir, indinavir and saquinavir in case of HIV-1 PR. L90M on the other hand is present in the hydrophobic core of the protease, near the catalytic aspartate residues; but outside of the active site. This mutation is primarily selected by saquinavir, nelfinavir, indinavir and lopinavir. Additionally, it is implicated in reducing the susceptibility of HIV-1 PR to most of the other inhibitors, with the exception of tipranavir and darunavir [116].

Regarding the implication of these primary mutations in the resistance of HIV-2 PR to the inhibitors, only a handful of studies aimed at characterizing the effect of either mutation in association with a select few PIs. I54M was associated with resistance to saquinavir and possibly darunavir, also resistance to lopinavir was implied if I54M was an adjuvant to I84V and L90M. Furthermore, L90M results in resistance to saquinavir; and possibly lopinavir, if found in association with I84V and either I54M or I82F [117].

Even though these mutations do not occur in the active site, it is thought that substitution of the native amino acid to methionine introduces a longer side chain, resulting in a shift of the main chain atoms relative to their original positions, in addition to the formation of new van der Waals forces and hydrophobic interactions with neighboring residues. This in turn results in the alteration of the substrate binding pocket and the stability of the dimer–inhibitor association, leading to decreased efficacy or even resistance to the inhibitor(s).

Highly active anti-retroviral therapy (HAART)

The standard of care for HIV infection and AIDS is the use of a combination of usually 3 anti-viral drugs that target different steps in the viral replication cycle. This combination of drugs is referred to as Highly Active Anti-retroviral Therapy, which has the potential to reduce mortality and morbidity among HIV infected patients. Currently, six classes of anti-retroviral drugs exist; Nucleoside reverse transcriptase inhibitors (NRTIs), Non-nucleoside reverse transcriptase inhibitors (NNRTIs), Protease inhibitors (PIs), Integrase strand transfer inhibitors (INSTIs), Fusion inhibitors (FIs) and Chemokine receptor antagonists (CCR5 antagonists). NRTIs are deoxyribonucleosides that are structural analogues of the RTs natural substrates, although lacking the 3′-hydroxyl group. During reverse-transcription, by competing with the analogous deoxynucleotides triphosphates, they are incorporated into the

newly synthesized DNA strand, resulting in the termination of DNA synthesis. NNRTIs on the other hand target the reverse transcriptase enzyme, and act by binding into a hydrophobic pocket of the enzyme that is in proximity to the catalytic site, resulting in a conformational change of the enzyme and, therefore, interfere with substrate hydrolysis. INSTIs interfere with the viral DNA double strand integration within the host DNA, they are in fact inhibitors that bind tightly to the active site of the integrase enzyme, altering the catalytic triad through chelating divalent metal ions. The only currently approved FI targets the fusion process of the virus with the cell membrane by competitively binding to the transmembrane part of the viral glycoprotein, blocking the formation of the post-fusion structure. CCR5 antagonists bind to the co-receptor C-C chemokine receptor type 5, preventing viral entry into the cell. Unfortunately, many viral variants; particularly HIV-2, have adapted to bypass the necessity to utilize this co-receptor, making their use in HIV-2 infection questionable [118]. Only one CCR5 antagonist has been approved for use in HIV treatment thus far.

The guidelines suggest combination therapies with three NRTIs or two NRTIs plus one PI as an initial therapeutic approach against HIV-2. INSTIs have recently been shown to exhibit potent efficacy against HIV-2 [119] . It has become evident that the use of NNRTIs as part of HAART in combating HIV-2 infection is highly discouraged, as a result of natural polymorphisms in the HIV-2 RT gene sequence, which alters the binding pocket significantly reducing the binding of the inhibitor, thereby leading to resistance, especially against first generation NNRTIs [120]. Therefore, as PI-based regimens constitute a major and effective part in the treatment of HIV infected patients, it is essential to characterize their efficacy against HIV-2.

Protease inhibitors

Ten protease inhibitors are approved so far by the Food and Drug Administration (FDA), out of which nine remain in production today (Table 2). Protease inhibitors are typically classified into first and second generation inhibitors, with second generation inhibitors specifically designed to tackle HIV-1 resistance that quickly emerged with first generation drugs, as well as to improve the bioavailability, dosing frequency, and minimize the commonly encountered side effects.

Table 2. Clinically approved protease inhibitors.

Inhibitor	Abbreviation	Trade name	Remarks
Saquinavir	SQV	Invirase	
Ritonavir	RTV	Norvir	Used as booster drug Combination therapy
Indinavir	INV	Crixivan	
Nelfinavir	NFV	Viracept	
Amprenavir	APV	Agenerase	Discontinued
Lopinavir/+Ritonavir	LPV	Kaletra, Aluvia	Second-generation Fixed-dose combination therapy
Atazanavir	ATV	Reyataz	Second-generation
Fosamprenavir	FPV	Telzir, Lexiva	Second-generation
Tipranavir	TPV	Aptivus	Second-generation Non-peptidic inhibitor
Darunavir	DRV	Prezista	Second-generation Non-peptidic inhibitor

Most of the inhibitors; with the exception TPV and DRV, are actually peptidomimetic, mimicking the enzyme`s natural substrates. Peptidomimetic PIs contain a hydroxyethylene group at their core, in order to protect them from being catalyzed by the viral protease. TPV on the other hand; being a non-peptidomimetic inhibitor, contains a dihydropyrone ring as a central scaffold.

SQV was the first to be approved in the US for use in combination therapy in treatment naïve patients. Due to low efficacy and susceptibility to treatment-associated mutations when used as monotherapy, it is now used as a part of triple combination therapies, which was shown to maximize the clinical benefits of the drug. However, low bioavailability of SQV as a result of rapid metabolism by hepatic enzymes necessitates its frequent dosing with high fat content meals.

The first PI to be approved in Europe was RTV, its prolonged absorption and bioavailability gave it an edge over SQV even when used as monotherapy in treatment naïve patients. Prolonged use however rendered the drug ineffective due to the occurrence of resistance mutations. Moreover, RTV`s many side effects were poorly tolerated by patients, resulting in poor compliance and increased toxicity. Apart from being initially developed as a PI, it also serves as a potent inhibitor of the intestinal and liver CYP3A complex, thus, nowadays RTV is primarily used as a booster drug for other PI's that are eliminated by CYP3A metabolism, and has long been successfully used in combination with IDV and SQV, enhancing their bioavailability and potentiating their activity [121]. When used as booster drug, RTV is used in a sub-optimal dosage to reduce its toxicity, relying on the boosted drug to act as the main protease inhibitor.

LPV is marketed in a formulation that combines LPV and RTV as a booster. A single daily use, enhanced bioavailability and the prolonged time for the development of resistance mutations made it preferable among other PIs. ATV was approved for a single daily dose, and is generally used in combination therapies either as a standalone or in association with the booster drug RTV. Noticeably, ATV inhibits UDP glucuronyl transferase, resulting in clinically detectable, yet usually asymptomatic hyperbillirubinaemia. When used in booster combination with RTV, however, this side effect is significantly attenuated.

IDV, APV and NFV also showed potent activity against the viral protease, and are both licensed for use in monotherapy as well as in combination therapy. Although their efficacy is hampered by the somewhat limited bioavailability, moderate to severe side effects, such as nephrotoxicity and diarrhea in case of IDV and NFV respectively, and their susceptibility to resistance mutations. FPV is the pro-drug of APV, thanks to its longer bioavailability and lower pill burden (APV required patients to take 2x8 capsules) it has now successfully replaced APV in clinical applications. When used with RTV as a booster drug, FPV was even more efficacious, although diarrhea remains a troublesome side effect to patients.

TPV and DRV are non-peptidic, sulphonamide-containing protease inhibitors. These molecules are characterized by their flexibility in binding into the active site of the enzyme, giving them an advantage against peptidomimetic inhibitors should a mutation occur. TPV induces cytochrome P450 expression, resulting in the enhancement of its own metabolism; therefore, an elevated boosting with RTV is required when used. DRV is the latest approved PI, which was proven to be efficacious on multi-drug resistant strains, usually administered with RTV as a booster. Its side effects are significantly limited compared to other PIs [122].

Some protease inhibitors are available as sulfate salts; such as indinavir and atazanavir, this formulation substantially improves the drug`s absorption and bioavailability.

It is important to note that the currently approved PIs are essentially designed for HIV-1, and their association with the HIV-2 viral protease had not been thoroughly characterized. Apart from a handful of phenotypic susceptibility assays and studies documenting response to PI-based regimens in HIV-2 infected individuals, the efficacy of PIs against HIV-2 PR remain unestablished.

Lentiviral vectors and their application in clinical research

Given their ability of integration into the host genome, and hijacking the cellular transcription machinery for their own propagation, retroviral vectors have long been used as efficient tools for delivering genes into target cells. They provided numerous prospects in clinical research and gene therapy, since the publishing of the infamous study documenting a therapeutic approach to combat severe combined immunodeficiency disorder (SCID) by gene transfer using gamma-based retroviral vectors [123]. The utilization of retroviral vectors seemed prosperous in the field of gene therapy clinical research, in combating immunological and hereditary disorders; however, the safety of such methodology soon became a concern. The possible generation of replication-competent viruses, germline alterations, as well as insertional mutagenesis were valid issues [124,125]. HIV-based lentiviral vectors have proven to be very powerful instruments in clinical research and genetic engineering, transferring genes into mammalian cells in a stable and reliable manner, leading to long-term gene expression [126]. They are capable of shuttling large genes, as well as transducing non-dividing or slowly-dividing cells. Such desirable properties were the reason HIV-based lentiviral vectors quickly superseded earlier retroviral vectors, which required prior activation of the cells before transduction [127]. Over the past decades, the biosafety and efficiency of the vectors have significantly increased, by means of modification of the vectors through elimination of accessory non-essential virulence genes, and incorporation of dedicated promoters. Second generation vectors are based on a single packaging plasmid encoding the structural genes *gag* and *pol*, and the regulatory genes *rev* and *tat*. A transfer vector with a defective lentiviral genome is used to carry the gene of interest, usually in conjugation with a strong promoter, while another plasmid encodes for the envelope proteins. In third generation vectors, the biosafety is further improved as a result of splitting the packaging vector into two separate plasmids, one encoding *rev*, and the other one encoding *gag* and *pol*. They are Tat

independent, due to replacement of the LTR U3 region by a stable efficient promoter such as that of cytomegalovirus (CMV) or Rous sarcoma virus (RSV) [128,129]. It is worth mentioning that despite being safer, third generation vectors yield lower viral titers and can be troublesome to work with, as a result of using an additional plasmid.

Self-inactivation (SIN) is an additional biosafety measure of recent lentiviral vectors, a feature in which the transcriptional enhancers present in the 3`LTR region are deleted, resulting in the LTR of the integrated provirus being defective, hence, no longer acting as a transcriptional promoter. Therefore, only the internal cassette is transcribed, and the newly formed virions are rendered replication-incompetent after a single cycle of infection.

The glycoproteins from the vesicular stomatitis virus G (VSV.G) are among the most commonly used envelope glycoproteins in lentiviral vectors. The virus has been shown to enter the cells though pH-dependent endocytosis, following the binding to phosphatidyl serine cell surface receptor [130]. Pseudotyping of lentiviral vectors with VSV.G confers a wide spectrum of cell tropism to the virions, allowing for various research and clinical applications of the lentiviral vectors carrying the gene of interest. As previously mentioned, the peculiar replication dynamics of HIV-2 in terms of attenuated pathogenicity and the preference for latent integration, merits its consideration as a useful tool for gene transfer. Perhaps, then, it is worth exploring the applicability of HIV-2-based lentiviral vectors in the field of gene therapy.

Scope of the study

Compared to HIV-1, relatively limited information is available regarding HIV-2. Differences do exist between the two viruses in terms of unique accessory genes, sequence polymorphism as well as replication dynamics. Treatment of HIV infection relies on a combination of anti-retroviral drugs, using two or more classes, one of which is PIs. Clinically used PIs have been developed to inhibit HIV-1 protease, and their effect against HIV-2 protease remains understudied. It is essential to understand that current treatment protocols are generally based on the susceptibility of HIV-1 to the inhibitors, and given the sequence polymorphism between the two viral proteases, inhibitors may exert a variable effect on HIV-2 protease [5,131]. A major obstacle in treatment of HIV infection and AIDS is the development of treatment-associated mutations. Mutations associated with PIs have been well characterized in regards to HIV-1; however, studies about these mutations and their effect on the susceptibility of HIV-2 protease to inhibitors are widely lacking [132]. Therefore, we wanted to carry out an in-depth study of HIV-2 protease and its susceptibility to clinically used inhibitors, in addition to characterizing some of the treatment-associated mutations that tend to develop in patients receiving PI-based regimens.

Aim 1: Development of an HIV-2 protease modular system

1. To optimize transfection and transduction efficiencies of a 2nd generation self-inactivating lentiviral vector system.
2. Introduction of unique restriction sites into the coding sequence of HIV-2 protease to allow for the interchange of the protease sequence between the cell culture lentiviral vector and an *in vitro* expression plasmid.
3. Optimization of HIV-2 protease expression *in vitro*, and characterization of its autodegradation/autoinactivation properties.

Aim 2: Inhibition profiling of currently used PIs using our HIV-2 protease modular system

1. To study the efficacy of PIs *in vitro*. Efficacy of the inhibitors was determined by calculating IC_{50} and K_i values obtained from activity measurements using the HPLC method.
2. To study the efficacy of PIs in cell culture. IC_{50} values determined by measuring the activity of RT enzyme obtained from culture supernatants, after treatment of cells with different concentrations of the inhibitor.
3. Analysis of correlation between data obtained from enzymatic and cell culture experiments.

Aim 3: Effects of I54M and L90M double mutation on the susceptibility of the protease to the inhibitors

1. Introduction of the double mutation into the *in vitro* expression plasmid. Following kinetic characterization, cloning into HIV-2 CGP vector for cell culture experiments.
2. Inhibition profiling of PIs using the double mutant protease in cell culture as well as enzymatic assays.

MATERIALS AND METHODS

HIV-2 vector system

To carry out our analysis, we utilized a self-inactivating, second generation lentiviral vector system, based on the ROD strain of HIV-2 (Figure 7). HIV-2 CGP served as a structural protein expression construct, CRU5SINCGW served as an HIV-2 vector with GFP expression, and pMD.G coded for Vesicular stomatitis virus envelope protein. HIV-2 CGP and CRU5SINCGW were a kind gift from Joseph P. Dougherty at the Robert Wood Johnson Medical School [133]. The long terminal repeat region was altered, to render the virus replication-incompetent after only a single cycle of infection. SIN type of retroviral vectors proved to be a safe alternative in the studies of transfection and transduction, the elements responsible for pathogenesis have been eliminated, while the *cis*-acting elements essential for virus propagation have been retained.

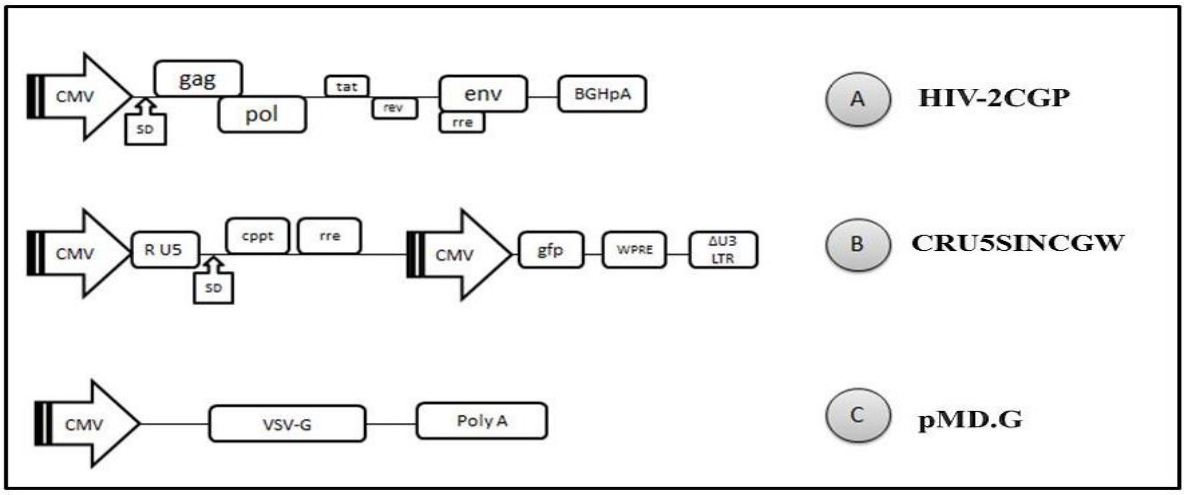


Figure 7: **HIV-2 2nd generation lentiviral vector system used in cell culture experiments**. A: HIV-2 CGP, a CMV driven gag-pro-pol expression construct. B: CRU5SINCGW, HIV-2 based minimal vector with a GFP expression cassette. C: pMD.G a plasmid coding for VSV envelope protein. CMV: human cytomegalovirus immediate early promoter; SD: splice donor site; rre: rev response element. BGHpA: bovine growth hormone polyadenylation signal. U3-R-U5; retroviral long terminal repeats. cppt; central polypurine tract. GFP; green fluorescence protein. WPRE; woodchuck hepatitis virus post-transcriptional regulatory element. VSV-G; vesicular stomatitis virus G protein. PolyA; polyadenylation site.

Cassette construction

We have used the protease coding sequence within the HIV-2 CGP as template (Table 3). Site-directed mutagenesis was done according to QuikChange mutagenesis protocol (Stratagene, La Jolla, CA, USA), utilizing designed oligonucleotide primers to introduce unique restriction sites AgeI and AfeI at 5' and 3' of the HIV-2 CGP protease coding region, respectively. The silent mutations were 8 amino acids apart from the ends of the protease coding sequence. NdeI and BamHI restriction sites were then attached to its 5' and 3' end respectively using PCR with the aid of designed oligonucleotides. Thereafter, the entire region was ligated into a pET11a expression plasmid (Invitrogen) for bacterial expression. Success of the mutagenesis and ligation were verified by restriction endonuclease enzyme digestion and DNA sequencing.

Table 3. **Sequence of HIV-2 CGP protease and oligonucleotide primers used in the construction of the cassette.**

Structure	Sequence
Protease coding sequence	5'- CCT CAA TTC TCT CTT TGG AAA AGA CCA GTA GTC ACA GCA TAC ATT GAG GGT CAG CCA GTA GAA GTT TTG TTA GAC ACG GGA GCT GAC GAC TCA ATA GTA GCA GGA ATA GAG TTA GGA AAC AAT TAT AGC CCA AAA ATA GTA GGG GGA ATA GGG GGA TTC ATA AAT ACC AAG GAA TAT AAA AAT GTA GAA ATA GAA GTT CTA AAT AAA AAG GTA CGG GCC ACC ATA ATG ACA GGC GAC ACC CCA ATC AAC ATT TTT GGC AGA AAT ATT CTG ACA GCC TTA GGC ATG TCA TTA AAT CTA -3'
Protease amino acid sequence	**P Q F S L W K R P V V T A Y I E G Q P V E V L L D T G A D D S I V A G I E L G N N Y S P K I V G G I G G F I N T K E Y K N V E I E V L N K K V R A T I M T G D T P I N I F G R N I L T A L G M S L N L**
Primers used in silent mutagenesis	**AgeI**: 5'-CTC TCT TTG GAA AAG ACC GGT AGT CAC AGC ATA C-3' **AfeI**: 5'-GGC AGA AAT ATT CTG ACA GCG CTA GGC ATG TCA TTA AAT CTA C-3'
Oligonucleotides for NdeI-BamHI restriction sites	**NdeI**: 5'-CTT CAT ATG CCT CAA TTC TCT CTT TGG AAA AGA CCG G-3' **BamH1**: 5'-CGA TCC GTA CAG TAA TTT AGA TAC TCC TAG GGC G-3'

Double mutant protease

We have obtained synthetic HIV-2 protease coding sequence harboring the two unique restriction sites AgeI and AfeI, in addition to the I54M and L90M mutations (A162G, C268A) from GenScript (GenScript USA Inc., Piscataway, NJ, USA). The sequence was also ligated into pET11a expression plasmid. Upon reception, PCR sequencing and restriction analysis were used to verify the plasmid. AgeI and AfeI endonucleases were then used to restrict the sequence for subsequent ligation into the HIV-2 CGP vector for cell culture experiments.

Transfection and transduction assays

Using an HIV-1 transfection protocol [134], 293T human embryonic kidney cells (Invitrogen) were seeded in T-75 flask in 15 ml DMEM (Sigma-Aldrich) supplemented with 10 % FBS, 1 % glutamine and 1 % penicillin-streptomycin. The day before transfection, cells were passaged in order to achieve 70 % confluency the next day. After achieving 70 % confluency (5-6 x 10^6 cells/ml), a total of 45 µg plasmid DNA was used for the transfection of cells using polyethylenimine (PEI). Cells were then incubated at 37 °C, 5 % CO_2 in 5 ml 1 % FBS containing DMEM without antibiotics. After incubation for 6 hours, the medium was replaced by 15 ml DMEM containing 10 % FBS, 1 % glutamine, 1 % penicillin-streptomycin. We then collected the medium after 24, 48 and 72 hours, filtered it through a 0.45 µm polyvinylidene fluoride filter (Millipore), followed by concentration of the virus by ultracentrifugation (100000 g, 2 hours, 4 °C). The pellet containing viral particles was then dissolved in 200 µl phosphate-buffered saline (PBS) and stored at -70 °C until use. ELISA-based colorimetric reverse transcriptase assay (catalog No. 11468120910; Roche Applied Science, Mannheim, Germany) was then used to detect the amount of RT in the viral samples. Regarding the infectivity (transduction) assays, we have opted to use a 96-well plate to achieve maximum

transduction efficiency. 293T cells were plated in 96-wells plate in 200 µl DMEM supplemented with 10 % FBS, 1 % glutamine and 1 % penicillin-streptomycin. At 50 % confluency (2.5-3 x 10^4 cells/ml), cells in 50 µl DMEM were infected with viral particles containing 10-57 ng reverse transcriptase as determined by the reverse transcriptase colorimetric assay per well. On the next day the medium was supplemented with 120 µl of DMEM containing 20 % FBS, 2 % glutamine, 2 % penicillin-streptomycin, followed by incubation of the cells at 37 °C, 5 % CO_2 for 5-6 days. Cells were then scraped off by vigorous pipetting and fixed in PBS containing 1 % formaldehyde. Infected cells were then checked under fluorescence microscope (Axiom 200) for the presence of green fluorescent protein (GFP). For quantitative analysis, the cells were counted by flow cytometry (FACS Calibur, BD Bioscience) to determine the percentage of GFP positivity in 5000 cells.

In vitro protease expression and purification

pET11a plasmid containing the protease coding sequence was expressed in a culture of 100 ml of E. *coli* BL21(DE3) (Invitrogen) cells in Luria-Bertani medium, that was supplemented with 100 µg/ml ampicillin. When the absorbance reached 0.8-1.0 OD_{600nm}, the culture was induced with a final concentration of 1 mM isopropyl-β-D-1-thiogalactopyranoside for 3 hours. Cells were harvested by centrifugation at 5500 g (Beckman centrifuge, JA-14 rotor), 20 minutes at 4 °C and the pellet was then stored at 4 °C overnight. The following day the pellet was dissolved in 8 ml buffer A (50 mM Tris, 10 mM DTT, 10 mM EDTA, pH 8.0), after the addition of 4 mg of lysozyme the cells were disrupted using the sonication method (Branson Sonicator, 3 x 3 minutes at 40 % energy, 4 °C) and then centrifuged at 48000 g (JA-20 rotor) for 20 minutes at 4 °C (step 1). The pellet was re-suspended in 8 ml buffer B (50 mM Tris, 10 mM DTT, 10 mM EDTA, 1 M urea, 0.5% Triton X-100, pH 8.0) followed by another

centrifugation (step 2). On the following step the pellet was re-suspended in 8 ml buffer A and a repeat centrifugation was carried out (step 3). The insoluble fraction was then solubilized in 5 ml buffer C (50 mM Tris, 10 mM DTT 5 mM EDTA, 7.5 M guanidine-HCl, pH 8.0) (step 4) [135]. The protease was then purified from this fraction using reversed-phase high performance liquid chromatography (RP-HPLC) with the aid of an ÄKTA purifier (Amersham Pharmacia Biotech) using a POROS 20 R2 (PE Biosystems, PerSeptive Biosystems) C_{18} column. A linear gradient from 99.95 % water and 0.05 % trifluroacetic acid (TFA) to 40 % acetonitrile and 0.05 % TFA over a period of 25 minutes was applied at a flow rate of 1 ml/min at room temperature. Following the elution, the peak fractions were immediately lyophilized with Eppendorf concentrator plus and then solubilized in buffer D (20 mM PIPES, 1 mM EDTA, 100 mM NaCl, 10 % glycerol, 0.5 % Nonident P40, pH 7.0) then pooled. The pooled fraction was dialyzed against the same buffer overnight and then concentrated using Amicon Ultra-4 centrifugal filter units (Millipore). Protein concentration of fractions was determined by the Bradford assay (Bio-Rad).

Activity assays of the protease

We have determined the activity of the protease using an HPLC-based method, in complex with an oligopeptide substrate MSLNL↓PVAKV that represents the protease/reverse transcriptase cleavage site in HIV-2 [136]. In the catalytic reaction, 10 µl buffer E (0.5 M phosphate, 10 mM DTT, 4 M NaCl, 10 % glycerol, pH 5.6), 5 µl substrate (2 mg/ml); dissolved in water, and 5 µl purified protease were added. After incubation for 1 hour at 37 °C, the reaction was stopped by the addition of 180 µl 1 % TFA. Using HPLC, the product and substrate peaks were separated by using a water-acetonitrile gradient in the presence of

0.05 % TFA. Kinetic parameters were then calculated for the enzyme-substrate complex using Prism Graphpad software.

Study of autodegradation/autoinactivation

In able to examine the stability of the purified protease and its susceptibility to autodegradation/autoinactivation, the active protease dialyzed at 4 °C against buffer D (pH 7.0) was incubated at 37 °C for various time intervals, thereafter, activity of the enzyme was measured as described for the activity assays. Similar experiments were carried out with purified protease dialyzed in 50 mM Na-acetate and 50 mM NaCl, (pH 5.0) [135]. For SDS-polyacrylamide gel analysis, 15 µl of protease was incubated at 37 °C for multiple time intervals, then run on 16 % SDS gel, densitometry was then used to determine the density of the different protease bands using AlphaImager HP system software.

Enzymatic inhibition assays

After expression and purification of the protease, and the determination of its kinetic parameters using the aforementioned oligopeptide, we carried out inhibition profiling of clinically used PIs *in vitro*. To perform our experiments, serial dilutions were prepared from the inhibitors using dimethyl sulfoxide (DMSO) in concentrations ranging from 10 nM to 50 µM. The catalytic reactions contained 10 µl buffer E (0.5 M phosphate, 10 mM DTT, 4 M NaCl, 10% glycerol, pH 5.6), 4.8 µl substrate, 5 µl purified protease and 0.2 µl inhibitor in DMSO or DMSO alone; which served as a control, followed by incubation at 37 ºC for 1 h. The concentration of the protease was adjusted to achieve less than 20% substrate hydrolysis. Following incubation of the mixture at 37 ºC for 1 h, 180 µl of 1% TFA was added to

terminate the reactions. Thereafter, HPLC measurements were used to determine the inhibitor's IC_{50} by measuring the decrease in substrate hydrolysis. The inhibitory constant K_i was then calculated from IC_{50} using the formula $K_i = (IC_{50}- E/2)/(S/K_m + 1)$, in which E is the active enzyme concentration, S is the substrate concentration and K_m is the Michaelis constant.

Inhibition profiling in cell culture

Using the transfection protocol described previously, after transfecting 293T human embryonic kidney cells with a total of 45 µg plasmid DNA using PEI, the cells were incubated at 37 °C, 5% CO_2 in 5 ml 1% FBS containing DMEM without antibiotics for 5–6 h. After incubation, cells were split and transferred into a 96-well plate containing serial dilutions of the inhibitor ranging from 3.2 nM to 100 µM in a total volume of 200 µl DMEM/well, supplemented with 10% FBS, 1% glutamine and 1% penicillin-streptomycin. After 3 days incubation at 37 °C, the virus-containing medium was collected from the wells, briefly centrifuged to remove cellular debris, and 10 µl samples were taken from each corresponding well. We then used the reverse transcriptase colorimetric assay to determine the IC_{50} values from triplicate measurements. This ELISA-based method quantitatively determines RT activity in cell culture. Incubation of samples with the reaction mixture was carried out for 17–18 hours; this was a slight modification to the manufacturer's protocol in order to get accurate results using the colorimetric assay, as it allows for sufficient detection and quantification of reverse transcriptase.

Protease inhibitors

The protease inhibitors darunavir, saquinavir, lopinavir, tipranavir, indinavir sulfate and atazanavir sulfate were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. Ritonavir was obtained from Abbott laboratories, nelfinavir obtained from Agouron, indinavir from Merck, atazanavir from *Bristol*-Myers Squibb, and amprenavir from *Vertex* Pharmaceuticals Inc.

Statistical analysis of correlation

To assess the correlation of IC_{50} values obtained from enzymatic and cell culture inhibition assays, Prism Graphpad software was used to calculate Pearson coefficient, based on the assumption that both X and Y values are sampled from populations that follow a Gaussian distribution. In addition, two-tailed P value was calculated to verify the significance of correlation. Moreover, normal distribution of the data was tested with the Shapiro–Wilk test. Differences of the IC_{50} values obtained from *in vitro* enzymatic and cell culture assays were calculated for both enzymes. The datasets did not follow the normal distribution; thus, the non-parametric Wilcoxon-test was applied. The null hypothesis (H_0) was that IC_{50} values obtained had the same mean rank, and the alternative hypothesis (H_1) was that the mean ranks of the IC_{50} values differed according to the determination method used, using a type I error of 0.05. Due to the small sample size, the Monte-Carlo permutation (based on 99,999 random assignments) were applied to control the asymptotic probability value of the tests [137,138]. Effect size quantifies the size of the difference between two groups of data (*i.e.*, data obtained by enzymatic and cell culture assays) in a standardized and comparable form. It ranges from −1 to +1, where 0 means that the methods have no effect on the IC_{50} values; values approaching −1 or +1 indicate larger magnitude [139,140]. Statistical tests were performed with PAST 3.09 software [141].

RESULTS

Construction of HIV-2 Protease cassette

Using site directed mutagenesis, silent mutations were introduced to include the unique restriction sites AgeI and AfeI at 5' and 3' of the HIV-2 CGP protease coding region, respectively. PCR was then used to amplify the complete protease sequence from HIV-2 CGP vector with NdeI and BamHI restriction sites attached to its 5` and 3` end respectively. The whole region was then cloned into pET11a plasmid for *in vitro* expression. More detail about the construction of the cassette is provided in the *Materials and methods* section. The unique restriction sites AgeI and AfeI were purposefully engineered to be 8 amino acids apart from the termini, to allow for the interchange of different protease coding segments between the cell culture HIV-2 CGP vector and the *in vitro* pET11a plasmid. The reason for carefully placing the unique restriction sites was due to the fact that analysis of HIV-2 protease sequences has shown that the majority of strains harboring treatment-associated resistance mutations comprise a single or multiple amino acid changes that fall within that region [117,142]. A schematic representation of the modular cassette system is provided in figure 8.

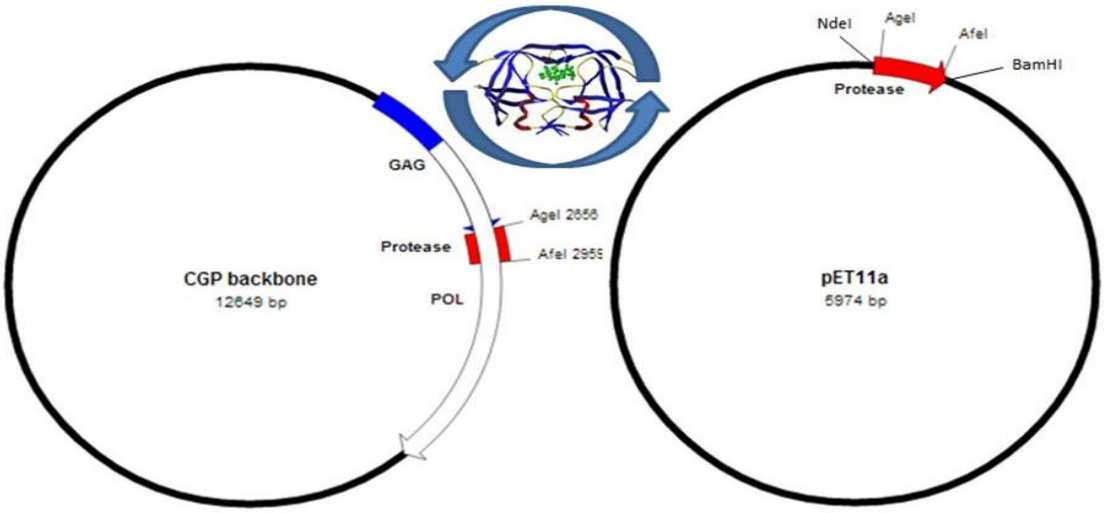


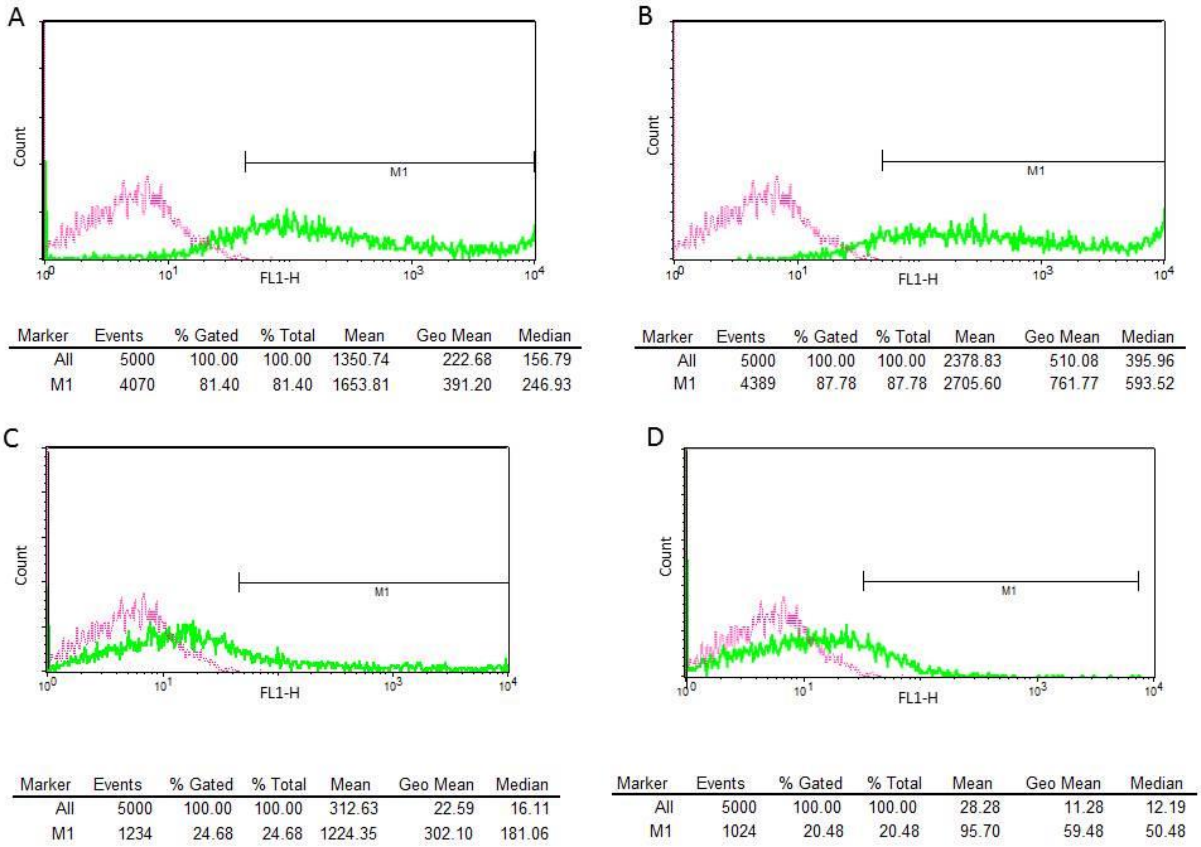
Figure 8: **Schematic representation of the modular system**. Protease coding sequence can be interchanged between the lentiviral HIV-2 CGP vector and the bacterial expression plasmid pET11a.

Upon reception of the protease coding sequence harboring the I54M-L90M double mutation, pET11a plasmid was verified by PCR sequencing and restriction endonucleases. Thereafter, the mutant protease was restricted and ligated into HIV-2 CGP cell culture vector for analysis.

Testing the modified HIV-2 CGP vector

Following the silent mutagenesis, we wanted to examine whether or not the change in the nucleotide sequence introduced by the silent mutations affected the transfection and/or transduction efficiency of the vectors. In parallel, transfection and transduction experiments were carried out using both the wild-type and modified HIV-2 CGP vectors, efficiency of the modified vector was comparable to that of the wild-type (Figure 9). Using RT colorimetric assay, the virus concentration obtained from transfection experiments was determined, and varied between 0.5-0.8 ng/µl RT, and FACS measurements detected more than 80% positivity of GFP cells for both vectors. In transduction experiments, infectivity of wild-type and modified virions was comparable, using only 10 ng of virus as detected by FACS measurements. It is worth mentioning that percentage of positive GFP cells was related

directly to the concentration of recombinant virus used. Calculation of viral titers by multiplying the cell number, the percentage of GFP and the dilution factor from transduction experiments yielded infectious unit/ml (IU/ml) of $2.2x10^6$ for the wild-type, and 1.8×10^6 for the modified vector, those results fall within the expected transduction efficiency of HIV-2 derived SIN vectors in adherent cell lines [133].



Marker	Events	% Gated	% Total	Mean	Geo Mean	Median
All	5000	100.00	100.00	1350.74	222.68	156.79
M1	4070	81.40	81.40	1653.81	391.20	246.93

Marker	Events	% Gated	% Total	Mean	Geo Mean	Median
All	5000	100.00	100.00	2378.83	510.08	395.96
M1	4389	87.78	87.78	2705.60	761.77	593.52

Marker	Events	% Gated	% Total	Mean	Geo Mean	Median
All	5000	100.00	100.00	312.63	22.59	16.11
M1	1234	24.68	24.68	1224.35	302.10	181.06

Marker	Events	% Gated	% Total	Mean	Geo Mean	Median
All	5000	100.00	100.00	28.28	11.28	12.19
M1	1024	20.48	20.48	95.70	59.48	50.48

Figure 9: **Comparison of the wild-type and modified HIV-2 CGP vectors**. Results of flow cytometric detection of green fluorescent protein. A: number of gated cells following transfection using wild-type HIV-2 vector. B: number of gated cells following transfection with the vector modified for the cassette system. C: transduction using wild-type vector. D: transduction using the modified vector. A minimum of three experiments were carried and the data shown are representative samples of results acquired. Pink color indicates control cells.

Expression and purification

The *in vitro* expression plasmid pET11a containing HIV-2 protease coding region was expressed in E.*coli* BL21(DE3) competent cells. Cells were lysed using the sonication method and the insoluble fraction was dissolved in a guanidine-HCl-containing buffer. The protease was then purified with the aid of RP-HPLC using a C18 column. Following the elution, peak fractions were immediately lyophilized with Eppendorf concentrator plus and then solubilized in PIPES buffer (pH 7.0) (Figure 10). We have experienced that PIPES buffer (pH 7.0) considerably preserves the activity of the protease, and enables for prolonged use of the fractions, even after multiple freeze/thaw cycles, more details are to be mentioned in the examination of the autodegradation/autoinactivation section of this thesis. Pure (>90 %) fractions were then pooled, and dialyzed in the same PIPES based buffer (pH 7.0), concentration of the pooled fractions ranged from 1-3.5 mg/ml as measured by Bradford assay. Western blotting was also used to verify the protease using monoclonal antibody. The expression and purification protocols were identical for both the wild-type as well as the double mutant protease.

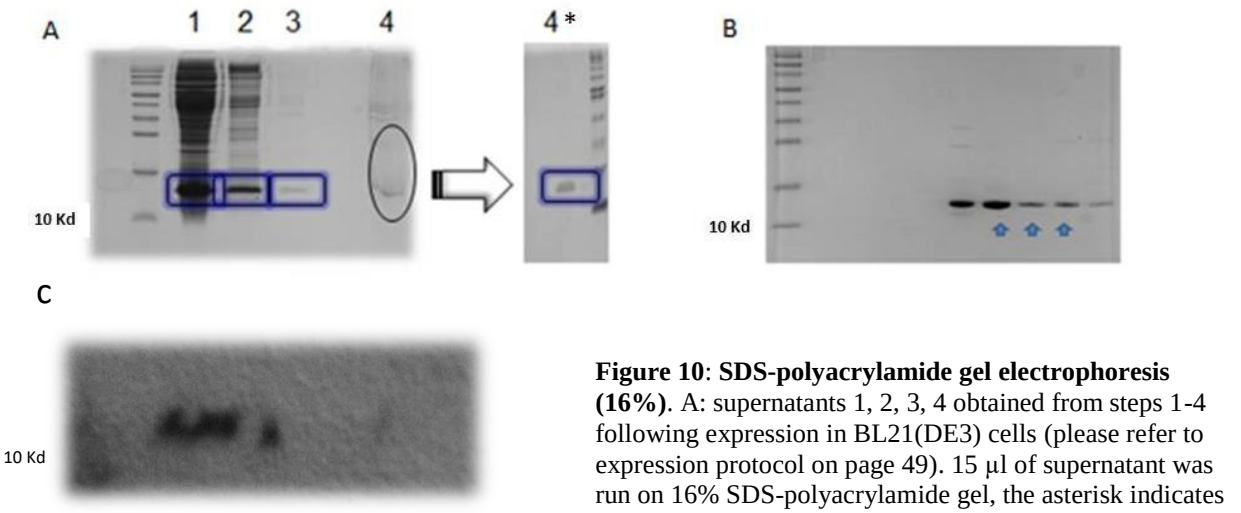


Figure 10: **SDS-polyacrylamide gel electrophoresis (16%)**. A: supernatants 1, 2, 3, 4 obtained from steps 1-4 following expression in BL21(DE3) cells (please refer to expression protocol on page 49). 15 µl of supernatant was run on 16% SDS-polyacrylamide gel, the asterisk indicates the solubilized pellet after 1:15 dilution with water to assist visualization (due to the presence of high GnHCl content). B: protease fractions after reversed-phase high performance liquid chromatography, arrows indicate the pooled fractions. C: Western blot analysis of HIV-2 pooled PR fraction using monoclonal antibody.

Kinetic assays

Activity of the purified protease was determined by incubating it with the oligopeptide substrate MSLNL↓PVAKV representing the protease/reverse transcriptase cleavage site in HIV-2. The HPLC method was used to analyze the activity of the enzyme, by analyzing the change in product and substrate peaks (Figure 11). Once activity of the purified fractions was confirmed, experiments to determine kinetic parameters for the enzyme and substrate were carried out. In case of the wild-type protease, K_m=0.012 ± 0.002 mM, k_{cat} = 0.91 ± 0.02 s^{-1}, k_{cat}/K_m=75.8 ± 12.7 $mM^{-1}s^{-1}$, these values were very similar to those previously determined for a chemically synthesized, wild-type enzyme, refolded using the same protocol [136]. Based on the protein content and activity of the protease samples, the folding efficiency was approximated to be 10–15%. For the double mutant protease harboring I54M and L90M mutations, the values were: K_m = 0.07 ± 0.01 mM, k_{cat} = 0.88 ± 0.09 s^{-1}, k_{cat}/K_m = 12.2 ± 2 $mM^{-1}s^{-1}$. It is apparent from the kinetic results that the introduced mutations caused a substantial increase of the K_m value without affecting the turnover number.

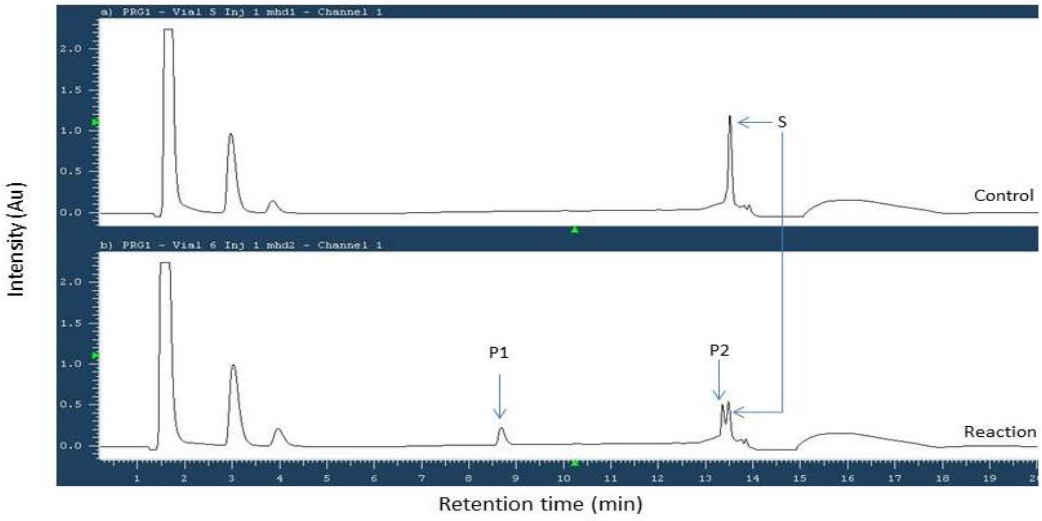


Figure 11: **Activity assay. A**ctivity determination of the purified pooled protease; P1, P2: cleavage products, S:substrate.

Characterizing the autoinactivation/autodegradation properties of HIV-2 protease

As described in the introductory section, it is thought that the viral protease domain within the Gag-Pro-Pol polyprotein, facilitates a cascade of proteolytic reactions that ultimately lead to the formation of the free mature protease, which in turn mediates its role in processing the viral proteins. Following its formation, this intrinsic autolytic ability of the mature viral protease is speculated to play a major role in its autodegradation, perhaps initially at an exposed amino-terminal strand/loop, as previously described [143]. This autodegradation appears to be responsible for the accelerated loss of the enzyme`s activity, and has proved to be a major hindrance to *in vitro* studies. In our experiments, incubation of the protease in an acidic (pH 5.0) buffer; typically used previously to dialyze HIV proteases, resulted in a rapid loss of the enzyme activity, with only minimal activity detected after only 12 hours of incubation at 37 ºC. Using a neutral (pH 7.0) PIPES buffer, however, resulted in a significant prolongation of the enzyme`s activity, where the enzyme maintained almost half of its activity after a 24 hour incubation at 37 ºC (Figure 12). Moreover, using this buffer facilitated the prolonged use of protease fractions, without substantial loss of the enzyme`s activity after multiple freeze/thaw cycles, some fractions maintained their activity after more than a year of incubation in -20 ºC. It is interesting to note that as our SDS-gel analysis of autodegradation/autoinactivation did not show substantial protein degradation following either refolding protocol; in pH 5.0 and pH 7.0 buffers. Therefore, in our assays the loss of enzymatic activity appears to be mainly the consequence of autoinactivation rather than autodegradation.

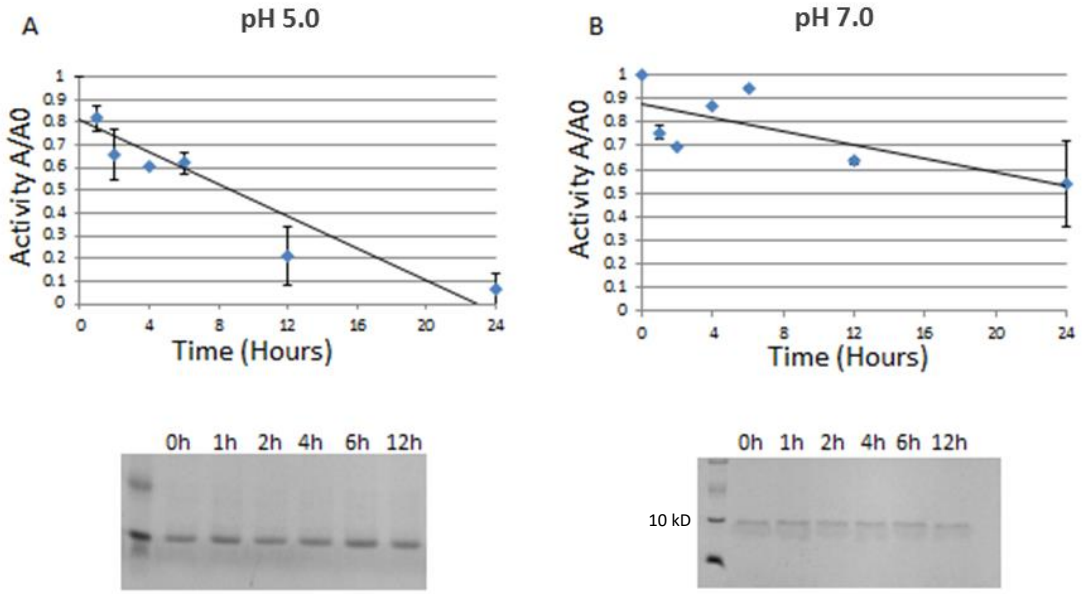


Figure 12: **Studies on autodegradation/autoinactivation of HIV-2 protease**. A: autodegradation analysis in a buffer composed of 50 mM sodium acetate and 50 mM sodium chloride (pH 5.0). B: autodegradation in buffer D (pH 7.0). X axis represents the incubation time in hours; Y axis represents A/A0 the ratio of active protein A at a given time to the total active protein A0 at time 50. Autodegradation characterization was also performed on 16% SDS gel electrophoresis, the corresponding gel pictures are shown, the seemingly double banding seen in SDS-gel picture of B is due to artefact caused by the PR buffer composition.

In vitro susceptibility assays

Following the expression, purification and determination of activity of the wild-type and double mutant proteases, inhibition profiling assays were performed using the HPLC method in triplicate measurements. In case of the wild-type HIV-2, the majority of the PIs (with the exception of nelfinavir, tipranavir and amprenavir) showed good inhibition efficacy against HIV-2 protease (Table 4). Amprenavir, tipranavir and nelfinavir had the highest K_i values (2.4, 1.3 and 1 nM, respectively); and hence were comparatively weak inhibitors of the protease. Indinavir sulfate and lopinavir, on the other hand, had the lowest K_i (0.03 nM), followed by darunavir, atazanavir sulfate and saquinavir (K_i = 0.05, 0.09, and 0.09 nM, respectively). It is noteworthy that we could not detect any difference between indinavir/atazanavir and their sulfate derivatives, as the values were identical; therefore, our assays only included the sulfated form of the drugs. In regards to HIV-2 protease harboring

the I54M and L90M treatment-associated mutations, it became apparent that introducing the mutations significantly decreased the efficacy of the inhibitors, with the exception of tipranavir, which remained indifferent to the mutations. The highest fold increase in K_i was observed in the case of ritonavir (>100 fold), followed by nelfinavir, darunavir and saquinavir (>20 fold). Indinavir sulfate, atazanavir sulfate and lopinavir showed an increase in K_i of more than 10 fold, while amprenavir increased by nine fold.

Table 4. **Results obtained from enzymatic assay to evaluate the efficacy of protease inhibitors.** Using the wild-type and a protease harboring I54M-L90M mutations. Data represent mean values ± SD.

Inhibitor	K_i (nM)	K_i (nM)	Fold Increase (K_i)
	Wild-Type	Double Mutant (I54M-L90M)	
Lopinavir	0.03 ± 0.001	0.3 ± 0.02	10
Indinavir	0.03 ± 0.020	0.3 ± 0.07	10
Darunavir	0.05 ± 0.005	1.1 ± 0.1	22
Saquinavir	0.09 ± 0.001	1.9 ± 0.2	21
Atazanavir	0.09 ± 0.030	1.5 ± 0.04	17
Ritonavir	0.12 ± 0.075	13.0 ± 2.4	108
Nelfinavir	1.0 ± 0.3	26.3 ± 2	26
Tipranavir	1.3 ± 0.6	0.6 ± 0.2	0.5
Amprenavir	2.4 ± 1.9	20.8 ± 1.2	9

Inhibition profiling in cell culture

Having optimized our transfection protocols, we chose to determine the efficacy of the inhibitors by means of measuring the change in RT activity, using the ELISA based colorimetric RT assay. Commonly, either luciferase activity or GFP fluorescence can be measured after infection of target cells; alternatively though, RT activity can be quantified from the supernatant as a measure of mature, infectious particles [5,133,144]. Since our HIV-2 vector system is a self-inactivating one, only single cycle phenotypic assays can be

measured; therefore, we have decided to base our measurements on RT activity, being a significantly more sensitive measure as compared to GFP fluorescence.

In case of the wild-type protease, darunavir and lopinavir were very potent inhibitors (IC_{50} = 0.4 ± 0.05 and 0.1 ± 0.01 μM, respectively), followed by saquinavir (IC_{50} = 1.3 ± 0.2 μM), indinavir sulfate (IC_{50}= 1.4 ± 0.3 μM) and nelfinavir (IC_{50}= 2.7 ± 0.9). Tipranavir had an IC_{50} of 3.7 ± 0.6 μM, and the IC_{50} of atazanavir sulfate was 5.9 ± 0.5 μM, while amprenavir was notably less efficient in blocking the viral enzyme (IC_{50} = 69 ± 9 μM).

Assays carried out for the double mutant protease resulted in a decrease of the inhibition efficacy for most of the inhibitors. More than 40 fold and >30 fold increase in IC_{50} was observed in case of darunavir and lopinavir, respectively, and IC_{50} of indinavir sulfate increased by >10 fold. The double mutation resulted in a significant IC_{50} increase of atazanavir sulfate, nelfinavir and saquinavir. As predicted, the double mutation did not have an effect in case of tipranavir. Amprenavir failed to inhibit the double mutant protease, its IC_{50} value was unmeasurable (Table 5).

Table 5. **Results obtained from cell culture inhibition profiling assays.** Evaluation o**f** the efficacy of protease inhibitors, using the wild-type and a protease harboring I54M-L90M mutations. Data represent mean values ± SD.

Inhibitor	IC_{50} (μM)	IC_{50} (μM)	Fold Increase
	Wild-Type	Double Mutant (I54M-L90M)	
Lopinavir	0.1 ± 0.01	3.1 ± 1.1	31
Darunavir	0.4 ± 0.05	19 ± 2	47
Saquinavir	1.3 ± 0.2	3.5 ± 1.2	3
Indinavir sulfate	1.4 ± 0.3	17 ± 2	12
Nelfinavir	2.7 ± 0.9	5.1 ± 1.7	2
Tipranavir	3.7 ± 0.6	5.8 ± 1.7	1.5
Atazanavir sulfate	5.9 ± 0.5	29 ± 1	5
Ritonavir	7.1 ± 0.7	15 ± 1	2
Amprenavir	69 ± 9	>100	–

Correlation analysis

In order to prove the usefulness of the modular system, correlational analysis was carried out between results obtained from *in vitro* inhibition profiling and cell culture assays. To avoid statistical bias, nelfinavir and ritonavir had to be excluded from our correlation analysis due to their unique pharmacodynamic properties, given the fact that HEK 293T cell line was found to express cytochrome P450 enzyme complexes; albeit in low concentration [145,146]. Correlating the *in vitro* enzymatic assays to those performed in cell culture yielded a Pearson's correlation coefficient of 0.89 ($p = 0.006$) and 0.96 ($p = 0.001$) for the wild-type and the double mutant, respectively (Figure 13). Moreover, since these data are non-normally distributed, further statistical analysis showed that there were no significant difference between the values obtained from both assays; ($p > 0.05$) (wild-type: $z = 1.35$ and $p = 0.22$; I54M-L90M mutant: $z = 0.51$ and $p = 0.69$). However, based on the effect size values, the magnitude of the difference was slightly higher in case of the wild-type (effect size value was 0.36 for the wild-type and 0.13 for the double mutant protease).

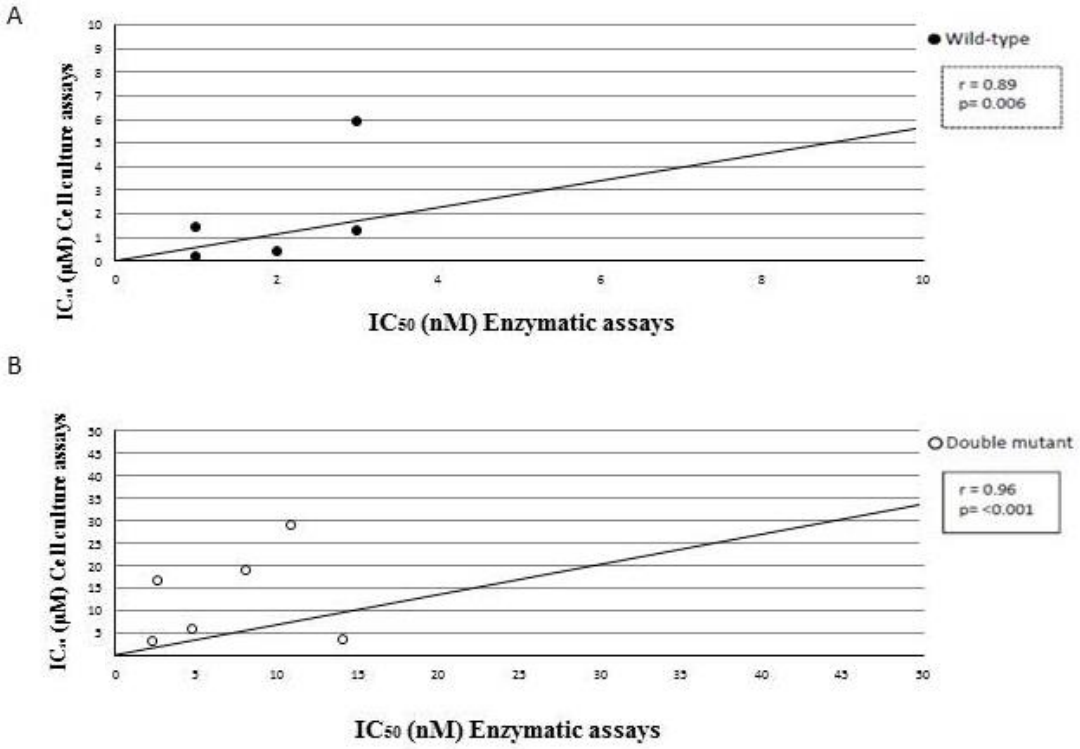


Figure 13: **Linear correlation analysis.** Correlation of IC_{50} obtained from *in vitro* enzymatic and cell culture assays using both the A: wild-type and B: double mutant protease. As mentioned previously nelfinavir and ritonavir were excluded from the analysis due to their unique biotransformation properties in cell culture. Correlation in case of the wild-type is indicated by a dotted line, while that of the double mutant is shown by a continuous line. P values were calculated at 95% confidence intervals.

DISCUSSION

HIV-2 has not received its fair share of clinical research, despite being a causative agent of AIDS. Its decreased pathogenicity and attenuated clinical course, in addition to its geographical confinement were all factors implicated in the scarcity of research on the virus, focusing more on HIV-1 as the major pathogen. Within the previous decade, there were many reports about the increasing incidence of HIV-2 infection in Europe, India, the Americas and even Japan [2,117,147]. Conceivably, the ending of the colonial era, and the return of the colonizers many decades ago, as well as mass population migration are thought to have resulted in the spread of the virus, as many of the countries reporting the increased incidence had socio-economic ties to West African countries. Despite its prolonged clinical course, once the replication momentum of HIV-2 is gained, the virus is nevertheless capable of causing AIDS in infected individuals, that is clinically indistinguishable from that caused by HIV-1 [1,148]. It is therefore of vital importance to distinguish between the two viruses, and establish evidence-based treatment protocols that are tailored against HIV-2, especially given the fact that clinical data on mono-HIV-2 infection are scant.

As described earlier, treatment of HIV infection and AIDS rely on a combination of two or more classes of anti-retroviral drugs, this approach aims at maximizing the efficacy of treatment, decreasing the viral load, and bypassing the viral resistance that quickly emerges in case of drug monotherapy; which still remains a major obstacle in therapy. PIs are considered major constituents in these combination therapies; moreover, recently, evidence has suggested that they may also be used as mono-therapeutic agents for maintenance therapy and also prophylactically [149,150]. Compared to HIV-1, very few crystal structures of the viral protease have been studied in complex with the inhibitors, this indicates that the PIs currently used were essentially designed against HIV-1, and their inhibition efficacy against HIV-2 protease remains to be characterized. Indeed, very few studies measured the susceptibility of

HIV-2 to PIs, the majority of them were phenotypic susceptibility assays that relied on isolating the virus from patients under treatment [5-8] . A major limitation of studies on protease inhibitors is the variability in results obtained, depending on the type of the assay used, the cell culture, as well as the virus strain isolated, which makes an accurate determination of the inhibitor's true efficacy troublesome.

Our goal was to construct a modular HIV-2 protease system that would enable for reliable determination of the efficacy of PIs in blocking the viral enzyme. Following the construction of the cassette system, we have confirmed that the silent mutations did not interfere with the transfection/transduction efficiency of the modified vector, PCR sequencing also confirmed that there was no alteration in the C-terminal region of *gag* as a result of mutagenesis. *In vitro*, optimization of the expression protocol and using PIPES (pH 7.0) buffer greatly prolonged the activity of the protease, possibly as a result of limiting its autodegradation /auto-inactivation, as evident from our comparative analysis in both buffer systems. As described earlier, autodegradation is a major factor in the attributed loss of activity of the viral enzyme, hence interfering with *in vitro* analysis. Throughout the literature, many methods aimed at decreasing the autodegradation; such as the use of catalytic-site inhibitors, storing the protease at a sub-optimal pH, or even modifying the implicated amino acid cleavage sites (such as the Leu5-Trp6 residues in case of HIV-1) [143]. Once the kinetic parameters were determined, our enzymatic assays indicated that amprenavir, tipranavir and nelfinavir were less effective in blocking the protease as compared to the rest of the inhibitors. We are only aware of one study that analyzed the susceptibility of HIV-2 protease kinetically [6]. Some of the values were comparable, however, tipranavir had a significantly higher K_i value; therefore, we are inclined to conclude that strain variability, the level of purity of the enzyme, and perhaps the use of a different substrate for the analysis are all reasons to blame for this discrepancy. Lopinavir, indinavir sulfate, darunavir and saquinavir on the other hand were very effective

inhibitors. Atazanavir sulfate had been shown to exert a variable and somewhat decreased efficacy on different HIV-2 isolates [151,152], in our *in vitro* assays however, it showed adequate inhibition of the protease, for its efficacy was comparable to that of saquinavir.

Our cell culture experiments were based on the utilization of SIN-2nd generation lentiviral vectors that had a high safety profile, and yielded adequate amounts of VSV-pseudotyped virions. Given the fact that the viral protease is essential to the processing of the viral polyproteins in the late stage of infection, and since SIN vectors are only capable of single cycle infections, inhibition profiling of protease inhibitors was performed in the transfection stage. Our methodology relied on detection of RT activity from the culture supernatant, which we trust to be an accurate method for the detection of mature virions, considering the fact that the viral protease is known to be required for the maturation of the RT enzyme [153]. When we carried out inhibition profiling, it was apparent that the values obtained were significantly higher than those obtained from the few other phenotypic susceptibility assays on HIV-2. As mentioned earlier, we have attributed this to be a result of the variability in methodology and type of cell culture used. Cell culture inhibition profiling was consistent with results obtained from enzymatic assays. Lopinavir and darunavir were very potent in inhibiting the protease at very low concentrations, followed by saquinavir, indinavir sulfate, and nelfinavir, while amprenavir had the lowest efficacy. Interestingly, despite exhibiting strong potency in enzymatic assays, atazanavir sulfate required higher concentration in cell culture, and ritonavir was second to amprenavir in terms of lowest efficacy. Beside its anti-protease effect, rintonavir is also a potent inhibitor of CYP3A, primarily used alongside other protease inhibitors that suffer rapid metabolism by the cytochrome system; hence, acting as a booster drug to enhance their bioavailability and potentiate their activity. We therefore speculate the seemingly decreased efficacy in cell culture to be a result of interaction between the cells and

the inhibitor. The noticeably better inhibition efficacy of nelfinavir in cell culture can be explained by the fact that nelfinavir is metabolized mainly to nelfinavir hydroxy-tert-butylamide (M8) by the P450 enzyme complexes in the liver; particularly CYP2C19. This bioactive metabolite had been shown to exhibit a potent antiviral activity that is comparable to that of the parent drug [154], hence it exhibited potency comparable to that of indinavir in cell culture.

To the best of our knowledge, there are no structural data available for I54M and/or L90M mutants of HIV-2 PR in complex with the inhibitors. We chose to study these mutations because of their implication in resistance to some PIs, as evident from data collected from patients failing HAART [7,116]. Neither residue is directly associated with the active site, therefore, it is believed that their substitution to methionine decreases the efficacy of the inhibitor as a result of alteration of the substrate binding pocket. *In vitro*, this double mutation resulted in a significant decrease in inhibition efficacy to all of the inhibitors based on the calculation of K_i values, with the exception of tipranavir. Ritonavir was the most affected (> 100 fold increase in K_i), followed by nelfinavir, darunavir and saquinavir (> 20 fold). Susceptibility to lopinavir, indinavir, and amprenavir was also decreased.
Inhibition profiling in cell culture revealed that I54M and L90M double mutation had the greatest effect in case of darunavir and lopinavir (>40 fold and >30 fold increase in IC_{50}, respectively), the IC_{50} of indinavir sulfate increased by >10 fold, additionally, the double mutation resulted in a significant increase in the IC_{50} of atazanavir sulfate, saquinavir and ritonavir (5, 3 and 2 fold, respectively). It appears that the mutation did not have a major effect in case of susceptibility to nelfinavir and tipranavir. Perhaps this is to be attributed to the nature of the drugs structure and their association with the viral protease, such enzyme-drug complex data are really lacking in regards to HIV-2 protease. Tipranavir; along with

darunavir are non-peptidic, sulfonamide containing protease inhibitors, that were designed specifically to tackle treatment-associated mutations, exhibiting more flexibility in binding to the enzyme. It was shown that L90M mutation exerts little or no effect on the susceptibility to tipranavir in HIV-1 isolates, while I54M was associated with reduced susceptibility to the inhibitor [116][116][116][116][116]. It is therefore conceivable that in case of tipranavir, the combined effect of the mutations did not disrupt the main chain hydrogen bonds. This however cannot be said about darunavir, as our results indicate that the double mutation greatly interfered with the enzyme-inhibitor complex.

Our correlation analysis proved that results obtained from both assays are comparable. Both linear correlation and the non-parametric Wilcoxon-test for data showing non-normal distribution revealed that data from *in vitro* enzymatic profiling and cell culture assays may indeed be used comparably, a testament to the reliability of the modular system.

SUMMARY

In summary, we presented a comprehensive analysis of HIV-2 protease and its susceptibility to the currently marketed PIs. Such data are really scarce, and given the ever increasing incidence of HIV-2 infection, it is wise to characterize the viral responsiveness to combination therapies containing PIs. Having optimized the expression and stability of the viral protease, we can conclude from our comparative enzymatic and cell culture inhibition profiling that lopinavir, darunavir, indinavir sulfate and saquinavir are indeed highly potent HIV-2 protease inhibitors. Tipranavir and nelfinavir on the other hand, showed a significantly lower efficacy when compared to the others. While amprenavir consistently was proven to be a weak inhibitor, the variable results obtained for atazanavir sulfate; enzymatically and in cell culture, incline us to doubt its reliability when used by HIV-2 infected patients. I54M-L90M mutations reduced the susceptibility of the protease to most of the inhibitors to variable degrees, with the exception of tipranavir. Perhaps the use of tipranavir in patients with such identified mutations is advantageous after all. We are confident that the use of such a standardized system capable of carrying out comparative analysis will prove beneficial in tailoring the use of protease inhibitors in HIV-2 infected patients.

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List of publications related to the dissertation

1. **Mahdi, M.**, Szojka, Z., Mótyán, J.A., Tőzsér, J.: Inhibition Profiling of Retroviral Protease Inhibitors Using an HIV-2 Modular System.
Viruses. 7 (12), 6152-6162, 2015.
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PLoS One. 9 (11), 13 p., 2014.
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List of other publications

3. Constantin T., Rákóczi É., Ponyi A., Ambrus C., Kádár K., Vastagh I., Dajnoki A., Tóth B., Bokrétás G., Müller V., Katona M., Csíkós M., Fiedler O., Széchey R., Varga E., Rudas G., Kertész A., Molnár S., Kárpáti S., Nagy V., Magyar P., **Mahdi M.**, Németh K., Bereczki D., Garami M., Erdős M., Maródi L., Fekete G.: Fabry-betegség: Diagnosztikai útmutató.
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OTHER POSTERS

2015: M. Mahdi, Zs. Szojka, and J. Tőzsér. Evaluating the efficacy of protease inhibitors against HIV-2, and the effect of the I54M-L90M double mutation. 27th International Workshop on Retroviral Pathogenesis. Mülheim an der Ruhr, Germany.

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