

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

HIV-2 protease as a chemotherapeutic target

by

Mohamed Mahdi MD

Supervisor: Prof. József Tózsér PhD, DSc



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The Examination took place at Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen at 11:00 a.m., 16th of February, 2015.

Head of the Defense Committee: Prof. Gábor Szabó MD, PhD, DSc

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13:00, 5th of September, 2016.

Introduction

HIV-2: origin and groups

The human immunodeficiency virus type 2 (HIV-2) is a member of the lentivirus genus, of the *retroviridae* family. 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, possibly many centuries ago. While HIV-1 has descended from the simian immunodeficiency virus (SIV) infecting chimpanzee (SIV/cpz), HIV-2 is found to be closely related to SIV isolated from macaques and sooty mangabey monkeys (SIV/mac, and SIV/sm). To date, eight distinct lineages (groups) of HIV-2 have been characterized and designated alphabetical letters A-H, these lineages are thought to individually represent independent cross-species transmission of the primate virus to humans. 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. Pathogenicity of HIV-2 varies significantly depending on the virulence of the group in question, with groups C, D, E and G generally being known for their sporadic nature, attenuated course of infection, as well as the lack of immunosuppression in infected individuals, while groups A, B, F and H on the other hand being indeed associated with reduced CD4⁺ T-cell counts, high viral loads, and the development of acquired immunodeficiency syndrome (AIDS).

Epidemiology and clinical characteristics of HIV-2

Since the isolation of the virus in 1986, along with HIV-1, it had claimed the lives of millions of patients suffering from AIDS worldwide. 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; however, 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. For long, HIV-2 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. Recent studies however, have detected the worldwide spread of the virus 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. Available statistical data show that HIV-2 infection comprised 2.3% and 1.8% of AIDS cases in Portugal and France, respectively.

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. Plasma viral load and DNA load in patients infected with HIV-2 is considerably lower than that seen in HIV-1 infection, 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. Nevertheless; and for yet undetermined reasons, once the replication momentum of HIV-2 is gained, a rapid clinical progression to AIDS ensues, with appearance of symptoms indistinguishable from those caused by HIV-1.

The viral protease

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. It is a homodimeric enzyme, belonging to the aspartic group of proteases, comprised of two identical monomers, each containing 99 amino acid residues. 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. The viral protease is essential to the viral life cycle, by cleaving 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.

Protease inhibitors as part of 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 (HAART), which has the potential to reduce mortality and morbidity among HIV infected patients. Protease inhibitors (PIs) are considered major pillars as part of HAART in the treatment of HIV infection and AIDS. Most of them are substrate analogues, binding to and inhibiting the viral enzyme. Ten protease inhibitors are approved so far by the Food and Drug Administration (FDA), out of which nine remain in

production today, 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. Given the then limited geographical distribution and lower pathogenicity of HIV-2, 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. 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. 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.

PI-associated resistance mutations

Treatment-associated mutations are commonly encountered in patients receiving HAART. 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. There is about 39–48 % similarity in amino acid sequences between HIV-1 and HIV-2 proteases, 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. 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. I54 is positioned in the flap region of the protease, L90 on the other hand is present in the hydrophobic core of the protease, near the catalytic aspartate residues; but outside of the active site. Even though these mutations do not occur in the active site, it is thought that substitution of the native amino acid to methionine 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).

AIMS

Compared to HIV-1, relatively limited information is available regarding HIV-2. Treatment of HIV infection relies on a combination of anti-retroviral drugs, using two or more classes, one of which is PIs. 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. 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.

Our aims were achieved by carrying out the following:

- 1: Development of an HIV-2 protease modular cassette system that allows for the analysis of HIV-2 protease in enzymatic, as well as in cell culture experiments.
- 2: Inhibition profiling of currently used PIs using our HIV-2 protease modular system.
- 3: Examination of the effects of I54M and L90M double mutation on the susceptibility of the viral protease to the inhibitors.

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. 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.

Cassette construction

Using the protease coding sequence within the HIV-2 CGP as template, 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. 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.

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. 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

293T human embryonic kidney cells (Invitrogen) were seeded in T-75 flask in 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. Cells were transfected using *polyethylenimine (PEI)*, thereafter incubated at 37 °C, 5 % CO₂ in 1 % FBS containing DMEM without antibiotics. After incubation for 6 hours, the medium was replaced by fresh DMEM containing 10 % FBS, 1 % glutamine, 1 % penicillin-streptomycin. We then collected the medium after 24, 48 and 72 hours, followed by concentration of the virus by ultracentrifugation. The pellet containing viral particles was then dissolved in 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, 293T cells were plated in 96-wells plate in DMEM supplemented with 10 % FBS, 1 % glutamine and 1 % penicillin-streptomycin. At 50 % confluency, cells were infected with viral particles containing 10-57 ng reverse transcriptase/well as determined by the reverse transcriptase colorimetric assay per well. On the next day the medium was supplemented with fresh DMEM containing 20 % FBS, 2 % glutamine, 2 % penicillin-streptomycin, followed by incubation of the cells at 37 °C, 5 % CO₂ 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 *E. coli* BL21(DE3) (Invitrogen) cells in Luria-Bertani medium, that was supplemented with 100 µg/ml ampicillin. The culture was induced with a final concentration of 1 mM isopropyl-β-D-1-thiogalactopyranoside for 3 hours. Cells were harvested by centrifugation (Beckman centrifuge, JA-14 rotor), and the pellet was then stored at 4 °C overnight. Cells were then disrupted by the sonication method, and multiple centrifugation steps were carried out in different buffers to extract the protease from the insoluble fractions. The protease was then purified 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) C₁₈ column. Following the elution, the peak fractions were immediately lyophilized with Eppendorf concentrator plus and then solubilized in pH 7.0 buffer, 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, using an oligopeptide substrate MSLNL↓PVAKV that represents the protease/reverse transcriptase cleavage site in HIV-2. In the catalytic reaction, 10 µl phosphate buffer, 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

To be able to examine the stability of the purified protease and its susceptibility to autodegradation/autoinactivation, the active protease dialyzed at 4 °C against pH 7.0 buffer 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). 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.

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.*

Enzymatic inhibition assays

Serial dilutions were prepared from the inhibitors using dimethyl sulfoxide (DMSO) in concentrations ranging from 50 μ M to 10 nM. The catalytic reactions contained 10 μ l phosphate buffer, 4.8 μ l substrate, 5 μ l purified protease and 0.2 μ l inhibitor in DMSO or DMSO alone; which served as a control, followed by incubation at 37 °C for 1 h. The concentration of the protease was adjusted to achieve less than 20 % substrate hydrolysis. Following incubation of the mixture at 37 °C for 1 h, 180 μ l 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 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 50 μ M to 3.2 nM in a total volume of 200 μ l 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 μ l samples were taken from each corresponding well. We then used the reverse transcriptase colorimetric assay to determine the IC_{50} values from triplicate measurements.

Statistical analysis of correlation

To assess the correlation of IC₅₀ 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 IC₅₀ 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 (H₀) was that IC₅₀ values obtained had the same mean rank, and the alternative hypothesis (H₁) was that the mean ranks of the IC₅₀ 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. 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 IC₅₀ values; values approaching -1 or +1 indicate larger magnitude. Statistical tests were performed with PAST 3.09 software.

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. 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.

Testing the modified HIV-2 CGP vector

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. Using RT colorimetric assay, the virus concentration obtained from transfection experiments was determined, and varied between 0.5-0.8 ng/ μ l RT, and FACS measurements detected more than 80 % positivity of GFP cells for both vectors. 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 2.2×10^6 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.

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. In case of the wild-type protease, $K_m = 0.012 \pm 0.002$ mM, $k_{cat} = 0.91 \pm 0.02$ s⁻¹, $k_{cat}/K_m = 75.8 \pm 12.7$ mM⁻¹s⁻¹. 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: $K_m = 0.07 \pm 0.01$ mM, $k_{cat} = 0.88 \pm 0.09$ s⁻¹, $k_{cat}/K_m = 12.2 \pm 2$ mM⁻¹s⁻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.

Characterizing the autoinactivation/autodegradation properties of HIV-2 protease

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, presumably 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 °C. Using a neutral (pH 7.0) 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 °C. 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 °C. 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.

***In vitro* susceptibility assays**

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. Amprenavir, tipranavir and nelfinavir had the highest K_i values (2.4, 1.3 and 1.0 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 ($K_i = 0.05, 0.09, \text{ and } 0.09$ nM, respectively). 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.

Inhibition profiling in cell culture

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

Correlation analysis

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. 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).

DISCUSSION

Since PIs are considered major constituents of anti-retroviral combination therapies, and given the pronounced polymorphism in amino acid sequence between HIV-1 and 2 proteases, it is 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. 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 already under treatment. 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. *In vitro*, optimization of the expression protocol and using piperazine-based (pH 7.0) buffer greatly prolonged the activity of the protease, possibly as a result of limiting its autodegradation /autoinactivation, as evident from our comparative analysis in both buffer systems. Our enzymatic assays indicated that amprenavir, tipranavir and nelfinavir were less effective in blocking the protease as compared to the rest of the inhibitors. Lopinavir, indinavir sulfate, darunavir, saquinavir and atazanavir sulfate on the other hand were very effective inhibitors.

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.

In vitro, I54M-L90M 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.

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., Tózsér, J.: Inhibition Profiling of Retroviral Protease Inhibitors Using an HIV-2 Modular System.
Viruses. 7 (12), 6152-6162, 2015.
DOI: <http://dx.doi.org/10.3390/v7122931>
IF:3.353 (2014)
2. Mahdi, M., Matúz, K., Tóth, F., Tózsér, J.: A Modular System to Evaluate the Efficacy of Protease Inhibitors against HIV-2.
PLoS One. 9 (11), 13 p., 2014.
DOI: <http://dx.doi.org/10.1371/journal.pone.0113221>
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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., Széchehy 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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Total IF of journals (all publications): 6,587

Total IF of journals (publications related to the dissertation): 6,587

The Candidate's publication data submitted to the IDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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LIST OF PRESENTATIONS

2015: M. Mahdi, Zs. Szojka, and J. Tózsér. 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.

2015: M. Mahdi, Zs. Szojka, and J. Tózsér. Inhibition profiling of currently used protease inhibitors using an HIV-2 modular system, and the effect of the I54M-L90M double mutation. 40th annual meeting on Retroviruses. Cold Spring Harbor, NY, USA.

2014: M. Mahdi, Zs. Szojka, K. Matuz, F. Tóth and J. Tózsér. A modular system to study the effect of protease inhibitors on the action of Human Immunodeficiency Virus type 2 (HIV-2). Hungarian Molecular Life Sciences. Debrecen, Hungary.

2013: M. Mahdi, Z.s. Szojka, K. Matuz and J. Tózsér. HIV-2 protease as a chemotherapeutic target. Hungarian Molecular Life Sciences. Siofok, Hungary (Poster)

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