THESIS OF PHD DISSERTATION

Design and characterization of macromolecular inhibitors of human immundeficiency virus 1 protease

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Debrecen, 2008

1. INTRODUCTION

1.1. Retroviruses

The retroviruses, have positive double-stranded RNA genome, belong to the Retroviridae family. The Lentivirus genus, which includes HIV-1, is in the Orthoretrovirinae subfamily. The virions are 80–100 nm in diameter, and their outer lipid envelope incorporates and displays the viral glycoproteins. The functional Env is a homotrimeric protein and each monomer contains heterodimer of surface protein and transmembrane protein. The matrix and capsid proteins build the internal protein core. The shape and location of the core are characteristic for various genera of the family. The viral genom, which is associated with nucleocapsid proteins and is located in the core, encodes three enzymes which are essential for the viral replication: protease, reverse transcriptase and integrase.

Retroviruses are broadly divided into two categories: simple and complex, distinguishable by the organization of their genomes. In its simplest form, as seen for example in murine leukemia virus (MLV), retroviral replication requires only three distinct virus-encoded genes. These are the *gag* gene, which encodes the structural proteins, the *pol* gene, which encodes the enzymes, and *env*, which encodes the envelope glycoproteins. In the integrated DNA provirus, these three genes are invariably arranged in the same order (5'-*gag-pol-env*-3') and are flanked by the characteristic long terminal repeats (LTRs) generated during the process of reverse transcription. The LTRs contain enhancer and promoter elements required for efficient transcription of the retroviral genome and also contain sequences important for efficient mRNA polyadenylation within the 3' LTR.

HIV-1 and all members of the Lentivirus genus encode several gene products in addition to the characteristic retroviral *gag*, *pol*, and *env* genes. At least two of these proteins, Tat and Rev, were shown to act in trans to regulate the gene expression. The Tat protein act as a potent activator of HIV-1 LTR-specific transcription, therefore establishes a strong positive feedback. This action of Tat results in the accumulation of Rev protein which then inhibits the further synthesis of the multiply spliced regulatory mRNAs and activates the expression of unspliced and single spliced mRNAs that encode the viral structural proteins.

1.2. The retroviral life cycle

The retroviral life cycle can be divided into two phases, namely the early and the late phase. In the begining of the early phase, the virus enters to the cell with direct fusion or receptor mediated endocytosis. The reverse transcription occurs in the entering capsid structure. A

preintegration complex is formed, composed of the genomic DNA and some of the protein components of the capsid, which enters the nucleus. The integration of the viral DNA into the host genome is mediated by the integrase. The late phase starts with the transcription of viral DNA into RNA. A single spliced mRNA serves as a template for Env synthesis. The Gag proteins assemble at the membrane where the Env proteins are concentrated and the assembled particle buds from the membrane. The viral protease cleaves Gag and Gag-Pro-Pol polyproteins at limited number of sites, producing a condensed, cone-shaped core of „mature" infectious particle. This function of PR is essential for virus replication.

1.3. The HIV-1 protease

Retroviral proteinases consists of 99-138 residues and their molecular weight is 11-15 kDa. These enzymes exhibit characteristic features typical of cellular aspartic proteases and there is a close homology in the first and secondary structure between retroviral proteinases and one domain of cellular aspartic proteinases. They contain many β-sheets and one or two short α-helices depending on the enzyme. The N- and C-terminal regions of the two monomers form a four stranded β-sheet.

There are three specific regions in the enzyme: region of the catalytic triad (-Asp-Thr/Ser-Gly-) which is characteristic of aspartic proteinases and found close to the N-terminal end; flap region, which is rather flexible and bends to the substrate during the formation of the enzyme-substrate complex; dimerisation region, which is mainly composed by the N- and C-terminal sequences.

The HIV-1 PR is maximally active at acidic pH (4.5-6.5) and also active at high ionic strength (1-2 M NaCl) in vitro like cellular aspartyl proteases. The exact sites of PR cleavage have been identified for a number of viral polyproteins. Comparison of these cleavage sites shows no unique sequence of amino acids; however, several amino acids occur repeatedly, and the sequences are quite hydrophobic.

Several groups have designed modifications of the original peptide assays, which used HPLC and sometimes radioactivity to detect cleavage products. One of these employed peptide substrates with N-terminal proline, and fluorescamine to detect the formation of new N-terminal primary amine. A fluorogenic method for measuring protease activity utilizing a synthetic peptide with an acceptor and a quenching molecule attached to either end of the substrate was first described for HIV-1 PR. The sequence of the substrate was based on the HIV-1 MA↓CA cleavage site, and it contained two covalently modified residues, a Glu modified with the fluorophore 5-((2-aminoethyl)amino)naphtalene-1-sulfonic acid (EDANS)

and a Lys modified with the acceptor chromophore 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL). The acceptor molecule was chosen for overlap of its absorbance with the emission spectrum of the fluorophore, resulting in quenching of the fluorophore, through resonance energy transfer. As a result of proteolytic cleavage, the full fluorescence quantum yield of EDANS is restored.

1.4. Inhibitors of HIV-1 protease

The initial targeting of the PR focused on small molecule inhibitors, especially on substrate-based peptidomimetic inhibitors: all HIV-1 PR inhibitors currently used in therapy belong to this class. However, resistance against these drugs develops rapidly, and emerging virus strains are often cross-resistant to other PR inhibitors. Alternative strategies have also been suggested, like the development of non-peptidomimetic compounds targeting the active site or functionally important sites other than the active site, i.e. the dimerization interface or the flap region. Furthermore, the PR may also be targeted by macromolecular inhibitors that could provide higher interaction surface and hence a lower chance for the development of resistance. Designing defective PR monomers and expressing them in the infected cells has been demonstrated to be an effective method: similar strategy worked for Gag, Tat, Rev, Env and Vpr proteins of HIV-1. Small molecule PR inhibitors contribute substantially to the side effects of highly active anti-retroviral therapy (HAART): insulin resistance, dyslipidemia, lipodystrophy, atherosclerosis. Because of the different chemical nature of these macromolecular inhibitors, they are not expected to exert the abovementioned side effects.

2. OBJECTIVES

The HIV-1 PR is responsible for the processing of viral Gag and Gag-(Pro)-Pol polyproteins during maturation, hence catalyzing essential steps of virion replication. Therefore the PR has proved to be an effective target for antiretroviral therapy of AIDS. Various PR inhibitors are now in clinical use, however, resistance usually develops rapidly against these compounds, so development of new, broad specificity inhibitors is important. Our aims were:

- To develop a microtiter plate fluorescent assay for various retroviral proteases to compare their activities and inhibition profiles. This assay could be an alternative method beside the conventional HPLC assay.
- To design and produce novel macromolecular inhibitors which are expected to be less sensitive to the emergence of resistance and can be used in gene therapy approach. In contrast to previous studies we would prefer the hydrophilic residues in our strategy to increase the solubility and also to achieve higher effective concentration.

3. MATERIALS AND METHODS

3.1. Oligopeptides

Oligopeptides were synthesized by solid-phase peptide synthesis on a Model 430A automated peptide synthesizer or on the Vega Coupler 250C using Boc chemistry, and were purified by reversed-phase high-performance liquid chromatography. The sequences of the peptides were checked with Beckman 6300 amino acid analyser. The EDANS/DABCYL containing peptides and the fluorescent control peptide RE(EDANS) were synthesized by Dr. Ivo Blaha.

3.2. HPLC assay

Purified HIV-1 and HTLV-1 proteases were prepared as described previously. The protein concentrations for the PR preparations were determined by amino acid analysis. The activity of proteases were measured in 20 µl volume. The active amount of the enzymes were determined by active site titration. Compound 3 and IB-268 were used as inhibitors for HIV-1 and HTLV-1 proteinases, respectively. The reaction mixture was incubated at 37 °C for 1 hour and the reaction was stopped by the addition of 180 µl 1% TFA. Substrates and cleavage products were separated using an increasing water-acetonitrile gradient (0-100%) in the presence of 0.05% TFA. Kinetic parameters were determined by fitting data obtained at less 20% substrate hydrolysis to the Michaelis-Menten equation by using Fig.P program. The standard errors of the kinetic parameters were below 20%. The catalytic constants were calculated using the active enzyme amounts detremined by active site titration.

3.3. Fluorescent microtiter plate assay

The PR assays were performed in microtiter plate wells in 200 µl reaction volume. The 110 µl reaction mixture was preincubated at 37 °C for 5 min and the reaction was initiated by the addition of 90 µl fluorescent peptide substrate. The increase of fluorescence was detected at 460 nm, using 355 nm excitation wavelength in a Wallac 1420 Victor2 fluorimeter luminometer at 37 °C. Inhibition assays were performed in the same way, but the reaction mixture contained 2 µl DMSO or inhibitor solution diluted in DMSO. Inner filter effect was determined by measuring the fluorescence as a function of the concentration for RE(EDANS), at the substrate concentration range used for the kinetic measurements. Data analysis was performed with the program KiDet (an in house Fortran program), which fitted lines to the fluorescent intensity versus time data, corrected them with the inner filter effect

then calculated the k_{cat} and K_M values with nonlinear regression analysis of Michaelis–Menten equation. Inhibition constants as well as the active enzyme concentrations were calculated with the same program using the equation of Williams and Morrison. To validate the fluorescent method, 100 µl aliquot was removed at the end of measurement, the reaction was stopped by the addition of 100 µl stop solution and the samples were subjected to reversed-phase HPLC separation.

3.4. Molecular modeling

Mutated residues were introduced into a high-resolution crystal structure of HIV-1 PR or into the previously minimized structures with Sybyl program. All 20 natural amino acids were changed in the selected position of monomer B. Intermonomer interaction energy was calculated for each mutated structure as the difference of the total energy of the dimer and the sum of the total energies of each monomer. Based on favorable inter- and intramolecular energies, mutants were selected and subjected to the next round of in silico mutagenesis at a different position. The structures were examined on a Silicon Graphics Fuel computer graphics system.

3.5. Site-directed mutagenesis of recombinant HIV-1 PR

The clone coding for the stabilized HIV-1 PR in a pET expression vector was used as template for the mutagenesis. Mutants were generated by the QuikChange mutagenesis protocol with the appropriate oligonucleotide pairs.

3.6. Protein expression and purification

Freshly prepared Escherichia coli BL21 (DE3) culture bearing one of the plasmid constructs was grown at 37°C and induced for expression with 1 mM IPTG for 3.5 hours. Cells were harvested by centrifugation and lysed by sonication on ice. The lysate was centrifuged and suspended in lysis buffer containing 3 M urea and sonicated again. This washing step was repeated three times to obtain purified inclusion bodies. The final pellet was dissolved in denaturation buffer. Proteins were purified by high-performance liquid chromatography (HPLC) using acetonitrile gradient in water, in the presence of 0.05% trifluoroacetic acid. Purity of selected fractions was assessed by sodium dodecyl sulphate–polyacryl amide gel electrophoresis using 16% polyacrylamide gels. Solvents of the collected fractions containing the PR or a mutant PR were removed by SpeedVac Concentrator SVC 100H. Dried proteins were dissolved in 6 M guanidine-HCl.

3.7. In vitro examination of macromolecular inhibitors

PR assays were performed as described in 3.3. Briefly, 10 µl of denaturated protein solution containing the active and inactive proteins in various ratios in 6 M guanidine-HCl solution was added into microtiter plate wells. The mixture was preincubated at 37°C for 15 minutes and the reaction was initiated by the addition fluorescent peptide substrate. A truncated PR incapable of dimerization (PR_{5-95}), bearing deletions of terminal residues 1–4 and 96–99 was used as a control protein. Activity percentage relative to the control protein was calculated and plotted against the ratio of inactive:active proteins using the SigmaPlot program.

3.8. Detection of heterodimer-formation

Mutants and N-terminal hexahistidine-tagged wild type PR in 6 M guanidine-HCl solution were mixed in 1:1 molar ratio or were applied alone in the case of control samples. ProBond Ni-NTA agarose equilibrated in the suitable buffer. The suspension was incubated on ice for 30 minutes. The resin was separated by centrifugation and washed three times with the equilibrating buffer. The proteins in the supernatant were analyzed by SDS–PAGE using Coomassie Brilliant Blue staining.

3.9. NMR-spectroscopy

Uniformly ^{15}N-labeled PR_{RE} and PR_{RER} proteins for NMR experiments were prepared by growing E. coli BL21 (DE3) bearing the appropriate plasmid and were purified from inclusion bodies using reversephase HPLC. Proteins were dialyzed extensively against 30–50 mM formic acid or 7 mM HCl and concentrated to ~2 mg/ml using Millipore YM-10 centriprep and centricon concentrators. Four samples namely ^{15}N - PR_{RE}, ^{15}N - PR_{RER} and a 1:1 mixture of ^{15}N - PR_{RE}:unlabeled PR and ^{15}N - PR_{RER}:unlabeled PR, were prepared for acquiring ^{1}H–^{15}N Heteronuclear Single Quantum Coherence (HSQC) spectra. Proteins were prepared according to the Ishima's quench protocol of protein folding.

3.10. Modification of plasmids of HIV-1 vector system

The original HIV-1 vector system (a kind gift from Dr Didier Trono) was modified to introduce a more efficient promoter and to contain sequences of mutant PRs. CMV promoter sequence was amplified by polymerase chain reaction (PCR) and was inserted between the *BamHI* and *MluI* restriction sites in the plasmid pWOX-GFP, upstream to the green fluorescent protein initiation codon. The PR coding fragment from the pMDLg/pRRE was

cloned between *PstI* and *StyI* restriction sites of pT7Blue-3 plasmid. Site-directed mutagenesis was performed using the QuikChange protocol. Mutated fragments were cloned back into the pMDLg/pRRE plasmid. N- and C-terminal truncations to mimic PR5–95 coding region were created by overlapping PCR.

3.11. Production of viral particles

Viral particles were produced by transient transfection into 293T cells. A total of 35.5 μg plasmid was used for the transfection: 10 μg pWOX-CMV-GFP (transfer vector plasmid), 6.5 μg pMDLg/pRRE (packaging plasmid containing wildtype PR sequence), 2.5 μg pRSV.rev (Rev coding plasmid), 3.5 μg pMD.G (VSV-G envelope protein-coding plasmid) and 13 μg pMDLg/pRRE-M (plasmid containing a mutant PR sequence) or salmon sperm DNA. The conditioned media containing virus particles were collected after another 24, 48 and 72 h and concentrated by ultracentrifugation. The viral pellet was reconstituted in phosphate-buffered saline solution, and stored at -70°C. To determine the total amount of capsid protein (p24) by enzyme-linked immunosorbent assay the uncleaved Gag was digested with PR after the virus lysis.

3.12. Infectivity assay

For infection, 293T cells were seeded in 24-well plates. After overnight incubation, cells were infected with viral particles containing equal amount (24 ng) of capsid protein. After two days, the medium was completed with DMEM containing double the amount of FBS, glutamine and antibiotics. On the seventh day, cells were scraped, washed with PBS and 10 000 cells were counted by flow cytometer to determine the number of GFP-positive cells.

4. RESULTS AND DISCUSSION

4.1. Development of a microtiter plate fluorescent assay for inhibition studies on retroviral proteases

4.1.1. Assay of modified cleavage site peptides by HTLV-1 and HIV-1 proteases using an HPLC assay

It was shown previously that the peptide representing the HTLV-1 CA↓NC cleavage site (KTKVL↓VVQPK) was an efficient substrate of both proteinases. We have selected this substrate for further modifications. The fluorescent donor group EDANS, and a quenching acceptor group DABCYL were built into the N- and C-terminal part of the peptide, respectively, to obtain the peptide designated FSP-354. This peptide was assayed as a substrate of the HTLV-1 PR by the conventional HPLC method, to compare its kinetic parameters to that of the unmodified substrate. The K_M value of the substrate was low, similar to the original peptide, but the catalytic constant was much lower, together yielding an approximately 200-fold lower specificity constant (k_{cat}/ K_M). Substitution of P1 Leu to Phe in this sequence (yielding FSP-404) did not alter substantially the kinetic parameters for HTLV-1 PR, although it more than doubled the specificity constant when introduced into the original substrate sequence. Our previous study indicated that the HTLV-1 PR may require more residues at the N-terminus than HIV-1 PR, therefore another version of the P1 Phe substituted peptide was synthesized, in which the N-terminal sequence was extended by one more residue, at the expense of shortening the C-terminal part. However, this peptide (FSP-405) was a much worse substrate for HTLV-1 PR, than FSP-404, indicating that the proper length at the C-terminus is also important for efficient catalysis. Interestingly, FSP-354 and FSP-404 were much better substrates for HIV-1 PR than for HTLV-1 PR, even though their nonfluorescent analogs were substantially better substrates for the HTLV-1 PR.

We have also tested a donor/acceptor substrate version of the HIV-1 MA↓CA (VSQNY↓PIVQ) cleavage site. Similar to the unmodified substrate, the fluorescent peptide was not hydrolyzed by the HTLV-1 PR, while it was an even better substrate of the HIV-1 proteinase than the unmodified one.

4.1.2. Calibration of the inner filter effect for the fluorescent plate reader assays

In the case of high substrate concentartion it would happen that the DABCYL of an ucleaved substrate molecule can absorb the fluorescence of the EDANS of a cleaved substrate molecule. This phenomenon is the inner filter effect, which need to be corrected in enzymatic

measurements using intramolecular fluorescence energy transfer (FRET)-based proteinase substrates. Here we present an alternative method for inner filter calibration in which a small modified peptide, RE(EDANS) was used to mimic the cleavage products. At each substrate concentration of fluorescent substrates, the fluorescence of RE(EDANS) peptide was measured as a function of its concentration and the decrease in slope was converted to a correction factor, by which the individually measured fluorescent intensity values were divided.

4.1.3. Fluorescent microtiter plate assay

The fluorescent substrates were also assayed in a microtiter plate assay, in which the appearance of the fluorescence of the EDANS group was detected after cleavage. The conditions of the fluorescent assay were somewhat different from the conditions of the HPLC method, since the high salt concentration (2 M), which was previously found to be optimal for the retroviral proteinases interfered with the fluorescent detection. To validate the fluorescence microtiter plate method, aliquots were injected at the end time point of the measurements, and the specificity constants were calculated based on peak quantitation, as done in the conventional HPLC assays. The values obtained with the HPLC quantitation agreed with the values obtained with the fluorescent readings corrected with the inner filter effect.

4.1.4. Comparison of the inhibition profile of HTLV-1 and HIV-1 proteases

We have selected FSP-354 for assay of various inhibitors designed against HIV-1 PR or designed on the basis of HTLV-1 cleavage sites. Only Indinavir was able to inhibit HTLV-1 PR in the studied concentration range among the tested HIV-1 PR inhibitors used in clinical practice. We have tested two statine-containing peptides based on HTLV-1 cleavage sites as inhibitors of the HTLV-1 PR in the fluorescent assay. Two new compounds, containing reduced peptide bonds in place of the cleavable bond were also assayed. These were so far the best inhibitors of the HTLV-1 PR we have tested.

4.2. Examination of macromolecular inhibitors of HIV-1 protease

4.2.1. In silico mutagenesis of HIV-1 protease

Maximal interaction energy between wild type PR and the inhibitory mutant is expected when the surface of the mutant monomer is similar to that of the substrate-bound wild type monomer. However, it is very difficult to mimic properly the surface of a two-chain

complex (PR subunit plus peptide substrate) with a single-chain mutant monomer, therefore we attempted only to prevent binding of substrate by building a physical barrier into the substrate-binding pocket. The polarity of this barrier was not as important as in the former strategy (mimicry of substrate-bound enzyme surface), therefore we had the freedom to choose charged or hydrophilic residues to improve the stability and the solubility of the mutant monomer.

Molecular mechanics calculations were run to calculate the intermonomer interaction energy of heterodimeric PR and the total energy of mutated monomer with substitutions of all 20 natural amino acids at position 25. Substitution of the catalytic residue (Asp25) must be the first one that should be considered for the creation of an inactive monomer. Not surprisingly, positively charged residues (Lys, Arg) gave the best interaction energy values; however, only Trp showed better monomer energy than the original Asp. The Asp25Lys and the Asp25Arg mutations fitted well into our working hypothesis, therefore both were selected for further in silico mutagenesis study. The next considered position was 49, which is located near the tip of the flap at the top of the substrate-binding site. Gly49 could be found in the wild type monomer and it could be changed even to the largest residue (Trp) without any detrimental effect to the desired inhibition. Our calculations also showed that large hydrophobic residues (Phe, Tyr) and positively charged residues (Lys, Arg) gave good intermolecular interaction energy values. The former residues were neglected because their hydrophobicities did not fit into our design strategy. The positively charged residues may promote good ion-pairs with the catalytic aspartate of the wild type monomer; however, they might repel the positively charged residue introduced at position 25 in the same monomer. The Arg25–Glu49 double mutant (called PR_{RE}) was selected for further rounds of computational mutagenesis and for in vitro experiments. The next candidate residue for mutagenesis was Ile50, following the pioneering work of Craik and co-workers. Ile50 is located in the tip of the flap, and forms a hydrophobic cover on the top of a substrate or an inhibitor. Our calculations showed that Arg and Lys in this position could result in favorable intermonomer interaction energy. The long and flexible chain of Arg/Lys may interact with the catalytic aspartate or with Asp30 resulting in good ionic interactions. Monomer having Arg50 had more favorable total energy than that of monomer bearing Lys50, therefore the Arg25-Glu49-Arg50 triple mutant (called PR_{RER}) was also selected for in vitro experiments.

To generate a monomer having an enhanced hydrophobic active-site/flap interface, a modified version of the PR_{KWW} mutant was constructed by changing Asp25 to hydrophobic Trp. Trp25 had the best monomer energy in our calculation and may interact well with Trp49

and Trp50 through aromatic–aromatic stacking. In this mutant (PR_{WWW}), all mutated residues were changed to hydrophobic ones to fulfill the requirements of a hydrophobic strategy.

4.2.2. In vitro inhibition experiments

The selected mutations were introduced into the PR sequence. Proteins were expressed in *E. coli* and purified from inclusion bodies. The activity of PR in the presence of variable amounts of mutant proteins was measured using a microtiter plate-based fluorescent method and activity relative to the same amount of dimerization-defective $PR_{5–95}$ mutant was calculated. $PR_{5–95}$, which contains deletions of the N- and C-terminal residues, was shown to adopt a monomer fold, but is inactive because of its inability to form dimer. The PR_{WWW} mutant showed a weak dosedependent inhibition, while the PR_{RE} and the PR_{RER} mutants showed similar inhibition as the previously described PR_{KWW} mutant.

To test whether the inhibition was caused by the specific interaction between PR and mutant monomers, we showed that heterodimeric PRs were formed in the 1:1 mixture of His_6-tagged X_{28}-PR and PR_{RER} or PR_{RE} mutant proteins, as it was shown previously for PR_{KWW} mutant by Craik and co-workers.

4.2.3. NMR experiments

Ishima's protocol was used to probe monomer folding/dimerization of PR_{RE} and PR_{RER} proteins and to assess if they have the potential to form heterodimers with wild type PR. The HSQC spectrum of ^{15}N-labeled PR_{RER} in the absence of wt PR is typical of a folded monomer. In addition, some signals indicate the presence of unfolded fraction of the protein. In contrast, in the presence of equal proportion of unlabeled wt PR, some signals characteristic of the PR dimer become apparent. Although additional NMR experiments are required to assign signals of all of the shifted peaks of PR_{RER} in the presence of wt PR, this observation is clearly indicative of heterodimer formation of PR_{RER} with wt PR as only the labeled PR_{RER} will be visible in the spectrum. Similar NMR experiments were carried out using ^{15}N -labeled PR_{RE}. Differently from the PR_{RER} spectrum, the ^{1}H-^{15}N HSQC spectrum of PR_{RE} in the absence of PR exhibited very weak signals and signals corresponding to a folded protein were not evident. Remarkably, in the presence of wt PR, PR_{RE} is fully folded, exhibiting peaks similar to those of PR_{RER}, indicating that the PR_{RE} most likely forms a dimer when folded in the presence of wt PR.

4.2.4. To test of infectivity inhibition

To test the infectivity of viral particles containing wt and mutant PRs, we used a third-generation HIV-1 vector system. It consists of four separate plasmids. pWOX-GFP is the vector used to introduce the foreign gene (here GFP). We modified the internal promoter of the original construct by the insertion of CMV promoter upstream to the GFP gene. The resultant pWOX-CMV-GFP vector produced substantially higher fluorescent signal than that of the original one in our assay system. pMDLg/pRRE is the packaging construct coding for Gag and GagPol polyproteins in the transfected cells under the control of CMV promoter, in the presence of Rev protein, which is encoded in a separate construct of pRSV.rev. The last construct, pMD.G, encodes G protein of vesicular stomatitis virus, a heterologous envelope to pseudotype viral particles. pMDLg/pRRE plasmid was modified by site-directed mutagenesis to harbor the desired mutations. 293T cells were transfected by equal amounts of plasmids of the various constructs and the composition of viral particles were analyzed by western blot using anti-CA antibody. The Gag (and presumably GagPol) expression was very low in constructs harboring PR_{RE} and $PR_{5–95}$ mutants. Wild type PR completely processed Gag and GagPol polyproteins; however, there was no sign for polyprotein processing in viral particles containing PR_{RER} and PR_{KWW} protein. 293T cells were also transfected by plasmid mixtures encoding the mutant and wt PRs in a DNA ratio of 2:1. Concentrations of virus stocks were set to be equal based on p24 assay. 293T cells were infected by viral particles and the quantity of the infected cells was measured by cell sorting based on the fluorescent signal of GFP-containing cells. We used salmon sperm DNA for control experiments, in which 97% of the cells were infected, which showed the effectiveness of our infection protocol. Infectivity of viral particles containing PR_{KWW} or PR_{RER} proteins were drastically reduced.

5. SUMMARY

We have developed a high throughput microtiter plate fluorescent assay which can be a good alternative to the traditional HPLC-based protease assay, since it is faster and is able to measure several parallel samples. Our assay was validated by using the traditional method and applied to HIV-1 and HTLV-1 proteases. Several new fluorescent substrates containing EDANS/DABCYL groups were designed and tested. We have also developed an alternative method for the inner filter correction. Inhibition profiles of HIV-1 and HTLV-1 PRs were compared using various clinically used HIV-1 PR inhibitors and new HTLV-1 PR inhibitors were also designed and tested. Later, the new method has also been applied successfully for additional retroviral proteases.

Furthermore, we have designed and investigated defective HIV-1 proteases which contained mutations in the active site and in the substrate binding site. After the *in silico* mutagenesis, we prepared and purified selected proteins for *in vitro* as well as in cell culture experiments to determine their inhibitory effect on wild-type HIV-1 PR. Mutants containing charged residues showed dose-dependent, specific, trans-dominant inhibitory effect in our experiments, and they represent a new generation of macromolecular inhibitors against HIV-1 PR. Heterodimerization could be detected, for the first time, between a wild-type retroviral PR and trans-dominant negative mutants by NMR spectroscopy using our novel hydrophilic constructs and our facile protein folding protocol. The Asp25Arg and the Gly49Glu mutations introduced into analogous positions of other retroviral PRs may exert a similar inhibitory effect on the corresponding wild-type PR. These residues target the catalytic aspartate, thus it may be considered a general strategy for all retroviral PRs.

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