Final Report

Results:

(Publications supported by this OTKA grant are cited in bold).

1. Retroviral protease expression and characterization

To facilitate the comparative capsid and nucleocapsid cleavage specificity studies, we have performed specificity studies on various retroviral proteases (**Eizert et al., 2008**) and reviewed various aspects of the field (**Menendez and Tözsér, 2008, Weber et al., 2009, Tözsér, 2010**). We have also cloned HIV-2 protease (PR), but due to its self-degrading characteristics, stabilizing modifications of the enzyme are required (our unpublished results). Due to these difficulties, a triple mutant protease in which the substrate binding site residues of a stabilized HIV-1 PR are replaced by those differing in HIV-2 PR appear to provide a solution (**Tie et al., 2012**). As in the period of the grant timeframe a new potential human retrovirus, xenotropic murine leukemia virus-related virus has been discovered; we have been involved in the characterization of the proteolytic enzyme of this virus (**Li et al., 2011**). Although the virus later was characterized as a product of *in vitro* recombination, as XMRV PR and Moloney murine leukemia virus (MMLV) PR appeared to have very similar specificity, XMRV PR characterization is expected to contribute to the better understanding of MMLV PR function and characteristics. An important aspect of this project was to extend our studies to the human T-cell lymphotropic viruses. Therefore we reviewed the field (**Bagossi et al., 2009**, **Boross et al., 2009**) as well as studied in detail the HTLV-1 PR, including its dimerization characteristics (**Kádas et al., 2008**)

2. Study of the proteolytic processing of wild-type and mutant HIV-1 capsid proteins.

It has been established previously that the CA protein of HIV-1 is a substrate of the viral protease, although the determined cleavage sites were different in experiments using different techniques for identification (Tőzsér et. al., 2003; Rumlova et. al., 2003). Using an MS/MS-based identification, we have verified the previously found cleavage sites in CA as well as identified a new cleavage site between Leu43 and Ser44 (data not shown). To make proteolytically resistant as well as more susceptible CA mutants, we have introduced several mutations into a recombinant his-tagged CA coding plasmid. The first sets of generated mutants are summarized in Table 1.

In vitro cleavage of HIV-1 CA mutants		Expected effect:	Observed effect:
Ala22↓Trp23			
Ala22	Ala23	Inhibition	Inhibition
Ala77↓Ala78			
Pro77	Ala78	Inhibition	Inhibition
Ala77	Val78	Enhancement	Enhancement
Leu189↓Leu190			
Phe189	Leu190	Enhancement	Enhancement
Ile189	Leu190	Inhibition	No alteration
Pro189	Leu190	Inhibition	Inhibition
Leu189	Lys190	Inhibition	Inhibition
Ile189	Lys190	Inhibition	Inhibition
Multiple mutants			
2x: Val78/Phe189		Enhancement	Enhancement
3x: Ala23/Pro77/Pro189		Inhibition	Inhibition
4x: Ala23/Pro77/Ile189/Lys190		Inhibition	Inhibition

Table 1. Cleavage site CA mutants and the effect of mutations on the proteolytic susceptibility.

Mutant proteins were expressed in *E. coli*, purified by using nickel chelate affinity chromatography, then subjected to proteolytic processing by HIV-1 protease. Cleavage products were separated by SDS-PAGE and identified using mass spectrometry analysis of the cut-off bands. As the cleavage was still not completely abolished by the applied mutations, further multiple mutants were generated, W23A/L43A/S44G/A77P/W117A/L189P/ L205A, as well as W23A/L43A/S44G/A77P/W117A/L189I/L190K/L205A. Surprisingly, while these mutants were substantially more resistant towards proteolysis compared to the previous ones, the cleavage was still not completely prevented (Figure 1), suggesting that still unidentified mutations exist in the CA protein.
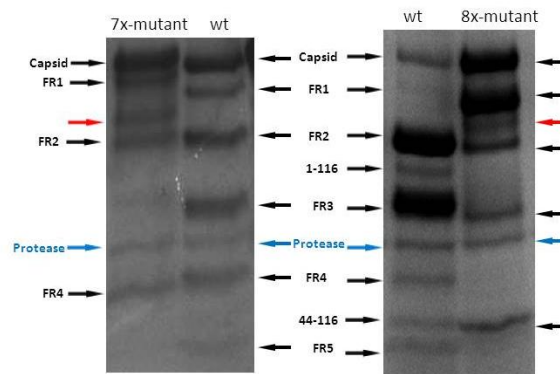

Figure 1. Cleavage of CA mutants by HIV-1 PR. 7x-mutant: W23A/L43A/S44G/A77P/W117A/L189P/L205A, 8x-mutant: W23A/L43A/S44G/A77P/W117A/L189I/L190K/L205A. Mutant proteins were incubated with HIV-1 PR then the reaction mixtures were separated using SDS-PAGE. Protein bands were identified using MS and MS/MS techniques.

3. Study of the effect of HIV-1 capsid cleavage site mutations on viral infectivity.

To be able to use a recombinant HIV-1-based lentiviral system for infectivity study, we modified a previously published third generation self-inactivating lentiviral system and utilized it to characterize *trans*-dominant negative HIV-1 PR inhibitor forms (**Miklossy et al., 2008**). We have adopted this system to study the *in vitro* characterized capsid (CA) mutations on the infectivity of HIV-1. 293FT cells were transfected by the vectors to produce virions, and the supernatant of the cells was collected and concentrated with ultracentrifugation or ultrafiltration. The amount of viruses produced was determined by p24 ELISA assay and then 293T cells were transduced with identical amount of p24. GFP expression of cells was analyzed by cytometry to compare the infectivity of the mutant virions to that of the wild type.
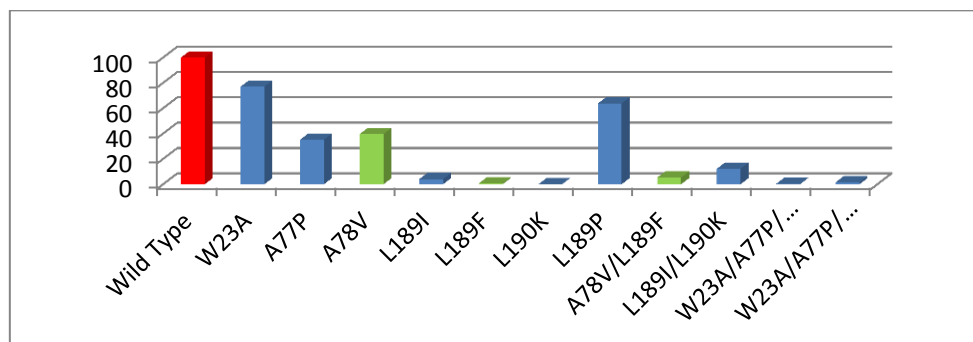

Figure 2. Effect of capsid mutations on the infectivity of recombinant HIV-1 virus. (red: wild-type, green: proteolysis-facilitating mutations, blue: proteolysis-inhibiting mutations.)

Infectivity assays (Figure 2) suggested, that both enhancing and proteolysis inhibitory mutations caused decrease of the infectivity. To exclude the aberrant capsid structure formation as the cause of the reduced infectivity, CD spectra were collected for the purified proteins. While alteration of the secondary structures was observed in some cases, it did not correlate with the infectivity: for example the completely noninfectious 4x mutant had a secondary structure very similar to that of the wild-type protein (data not shown). We have also cloned and expressed HTLV-1 and MMLV CA proteins, but they did not appear to be a substrate of the respective protease. To study the equine infectious anemia virus (EIAV) capsid mutants we have developed a cell line stably expressing GFP under the viral LTR, and the infection of the cells can be followed by GFP expression (data not shown).

4. Studies on the nucleocapsid protein processing of retroviruses.

While oligopeptides (EIAV NC-1 and NC-2) having the sequence of the cleavage site in the first and second cysteine arrays of the nucleocapsid (NC) protein of EIAV were previously found to be hydrolyzed by EIAV PR at the expected -Cys↓Tyr- and -Cys↓Phe- bonds (Tözsér et al., 1993, Figure 3). An oligopeptide representing the analogous regions (NC-1) was also found to be hydrolyzed by HIV-1 PR, however, cleavage occurred at the -Phe↓Asn- bond instead of the predicted -Cys↓Phe- bond, while a peptide representing the region homologous to EIAV NC-2 was not hydrolyzed (Tözsér et al., 1993). Later the NC-2 cleavage site was identified to be at the last Cys of the second zinc finger instead at the first one (Tözsér et al., 2004). To determine whether peptides representing the corresponding sequences in other retroviruses could be substrates to the respective PR, peptides having the corresponding sequences in AMV were assayed as substrates of the AMV PR. Although the generally low activity of the AMV PR (Tözsér et al., 1996) prohibited the determination of the exact kinetic parameters, both peptides were processed by the enzyme, at positions similar to those found in case of EIAV. These data imply that cleavability of NC sequences around the cysteines might not be restricted to lentiviruses.

| | site | peptide[a] | site of cleavage by the PR of | | |
			EIAV	HIV-1	AMV
EIAV	NC-1	AAQTCYNCGK[b]	TC↓YN	–	N.D.
EIAV	NC-2	APKVCFKCKQ[b]	VC↓FK	–	N.D.
HIV-1	NC-1	KMVKCFNCGK[b]	CF↓NC	CF↓NC	N.D.
HIV-2	NC-1	KPIKCWNCGK	CW↓NC	CW↓NC	CW↓NC
RSV	NC-1	SRGLCYTCGS	LC↓YT	N.D.	CY↓TC
RSV	NC-2	SRERCQLCNG	–	N.D.	CW↓NC
RSV	NC-1	SRGLCYTCGS	LC↓YT	N.D.	CY↓TC
RSV	NC-2	SRERCQLCNG	–	N.D.	CQ↓LC
MMLV	NC-1	DRDQCAYCKE	CA↓YC	–	DQ↓CA

Figure 3. Cleavage of oligopeptides having zinc finger sequences by retroviral proteinases. [a]The Cys residues of the Zn finger are underlined, while the site of cleavage is indicated by an arrow.[b]Cleavage of these peptides by EIAV PR and their own PR have been reported previously (Tözsér et al., 1993).[c]This cleavage site is at the last Cys residue of the second zinc finger (Tözsér et al., 2004). N.D., not determined.

Furthermore, we have also studied the NC cleavage of MMLV PR. The peptide representing the predicted cleavage site was a substrate of EIAV and AMV PRs (Figure 3), it was not processed by MMLV PR (data not shown). We have cloned the MMLV NC protein into a bacterial expression clone and studied its processing by MMLV PR. Interestingly, while it appeared to be a good substrate of the protease; the identified cleavage site was not in the zinc finger region (data not shown).

5. Design of mutant NC-1 cleavage sites with various susceptibility toward proteolysis.

In our previous study (Tözsér et al., 2004) we have designed a limited number of NC-1 mutants, having altered susceptibility towards HIV-1 PR. We have selected Asn17 (P1' residue in the wild-type sequence) for mutagenesis, based on the following reasons: (i) unfavorable changes only in the P2-P2' region of a peptide substrate could completely abolish hydrolysis (ii) the two cysteines (Cys15 and Cys18) are crucial for zinc coordination required for most of the established NC functions while Phe16 was found to be essential for the packaging of viral RNA. The limited set of P1' modified substrates was extended to include all types of amino acid residues (Table 2).

	Site	Peptide	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
1.	wild type:	KIVKCF↓NCGK[a]	0.43	0.017	0.040
2.	Asn17Phe	KIVKCF↓FCGK[a]	0.02	0.90	59.6
3.	Asn17Leu	KIVKCF↓LCGK[a]	0.17	0.196	1.153
4.	Asn17Val	KIVKCF↓VCGK	0.45	0.284	0.632
5.	Asn17Ala	KIVKCF↓ACGK[a]	0.32	0.064	0.200
6.	Asn17Cys	KIVKCF↓CCGK	1.52	0.247	0.162
7.	Asn17Thr	KIVKCF↓TCGK	0.31	0.017	0.054
8.	Asn17Gln	KIVKCF↓QCGK	0.67	0.035	0.052
9.	Asn17Ser	KIVKCF↓SCGK	0.63	0.004	0.006
10.	Asn17Arg	KIVKCF↓RCGK	1.05	0.020	0.019
11.	Asn17Lys	KIVKCF↓KCGK[a]	0.86	0.005	0.006
12.	Asn17Glu	KIVKCF↓ECGK	1.21	0.013	0.011
13.	Asn1Asp	KIVKCF↓DCGK		not hydrolyzed	
14.	Asn17Gly	KIVKCF↓GCGK[a]	1.34	0.002	0.001

Table 2. Proteolytic processing of substituted oligopeptides representing the first cleavage site in the nucleocapsid protein. [a]These values were published previously (Tözsér et al., 2004)

Using the recombinant viral system described for the CA mutant experiments, we have introduced three representative mutations into the HIV-1 NC protein of the virus and studied the viral infectivity. These mutations substantially reduced the infectivity of the viruses (Figure 4)
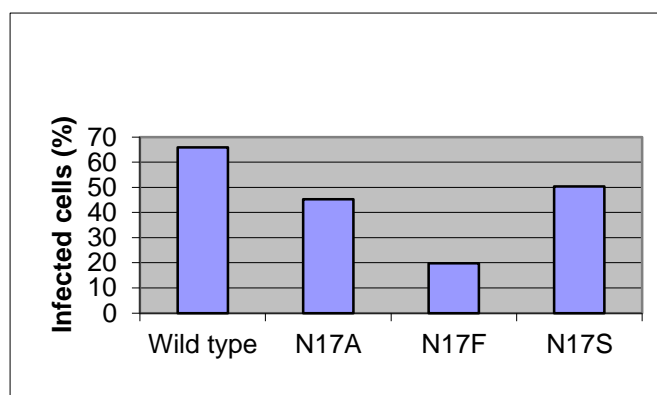

Figure 4. Infectivity of recombinant HIV-1 virions harboring NC protein mutations. Average of two experiments are shown. Cells were infected with virions having equal p24 amount.

6. Design of NC-1 cleavage revertants:

To verify the *in vivo* importance of the NC cleavage, the design and availability of "revertant" mutants might be very useful, in which the internal positions of the proximal zinc-finger (CFGC motif) is not changed, nevertheless, cleavability within this region is regained by introducing mutations into close vicinity. The primary target positions for creation of such revertants should be the residues adjacent to the CFGC motif, since proper side chains in these positions might substitute for the loss of interaction energy caused by the mutation of Asn17 to Gly. Based on previous detailed specificity studies on the HIV-1 PR, we have selected beta branched residues or the flexible and also branched Leu as substituting residues. Peptides having such mutations are listed in Table 3.

	Peptide	Cleavage site	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m [a] ($mM^{-1}s^{-1}$)	k_{cat}/K_m [b] ($mM^{-1}s^{-1}$)
1.	KIVTC**F**G**C**GK	-		< 0.01		
2.	RKIVIC**F**G**C**GK<u>R</u>	IC↓FG	0.035	0.034	0.97	0.51
3.	KIV**K**IC**F**G**C**GK	IC↓FG	0.070	0.025	0.35	0.48
4.	RKIV**K**C**F**G**C**I**K**<u>R</u>	-		< 0.01		
5.	RKIV**L**C**F**G**C**GK<u>R</u>	VL↓CF, LC↓FG		not determined		
6.	<u>R</u>KIV**I**C**F**G**C**I**K**<u>R</u>	IC↓FG	< 0.010	0.015	-	0.48

Table 3. Proteolytic processing of multiply substituted NC-1 cleavage site peptides. Residues substituted in the NC-1 cleavage site sequence are in bold, while the C-terminal arginin added to increase peptide solubility is underlined. [a]Specificity constant calculated from the K_m and k_{cat} values. [b]Specificity constant determined using a competition assay with RPGNF↓LQSRP Gag cleavage site peptide.

We plan to introduce representatives of these "revertant" mutations into the NC of our recombinant HIV-1 virus and study the effect on viral infectivity.

7. Analysis of HIV-1 particles/processing in the host cells in the early phase of the infection

To analyze HIV-1 particles by mass spectrometric methods, N^{15}-Cys-labeled recombinant HIV-1 virions were produced in 293FT cells. After six passages the cells incorporated the heavy cysteines into their proteins. These "heavy" cells were used for recombinant HIV-1 virus production using our published protocol (**Miklossy et al., 2008**). The amount of the produced viruses was determined using an HIV-1 p24 antigen ELISA kit. The labeled viruses were used to infect 293T cells. In these experiments we planned to follow the fate of the viral proteins based on their heavy cysteine content. After 0, 4 and 12 hours the infected cells were collected and trypsinized to remove attached virions, and then cells were lysed in RIPA buffer in the presence of a protease inhibitor cocktail. In order to separate the nuclear and cytosolic fractions the Nuclei EZ prep nuclei isolation kit (Sigma) was utilized. The cytosolic and nuclear fractions were acetone precipitated, and then the precipitates were redissolved in ammonium bicarbonate and analyzed with ESI-FTICR mass spectrometer (Bruker). MALDI-TOF and FT-ICR mass spectrometry measurements were done but unfortunately, due to high PEG (polyethylene glycol) contamination the signals for the HIV-1 particles were not unambigously visible; the PEG ionized much more efficiently and suppressed the protein signals. As no PEG was knowingly used in the protocol, we wanted to figure out its source. The provider of the nuclear fragment separation kit did not disclose the composition of the buffers utilized, however, MALDI-MS analysis of their lysis buffer verified high amount of PEG (Figure 5). At this point we plan to repeat the experiment avoiding the use of the isolation kit.
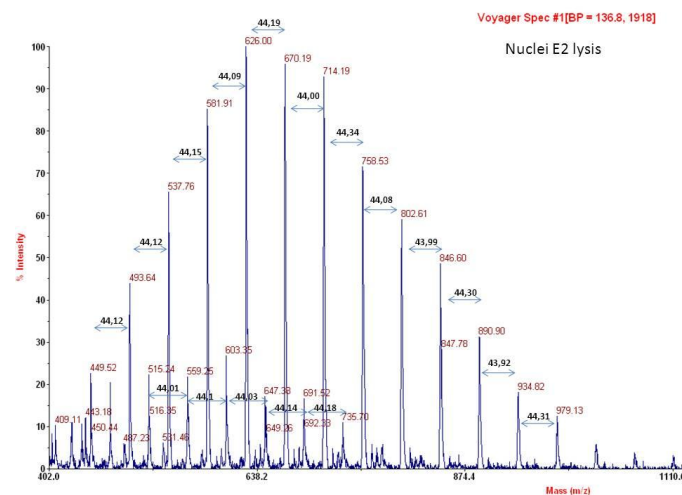

Figure 5. Verification of PEG contamination in the buffer utilized for nuclear fraction preparation. The 44 Da differences are characteristic to the presence of PEG

We have also established the experimental system for MMLV infection and EIAV infection.

8. Analysis of the host cell proteins in the early phase of the infection

In these experiments, non-labeled recombinant HIV-1 viruses were produced and quantified as described (**Miklossy et. al. 2008**), and utilized to infect 293T cells. After 0, 4

and 12 hours the infected cells were harvested and treated as described above. Cell lysates were centrifuged and the supernatants were used for protein sample preparation. The cleared cell lysates were acetone precipitated, precipitates were redissolved in ammonium bicarbonate and digested with trypsin. The tryptic fragments were analyzed on an ESI-LTQ-Orbitrap (Thermo Scientific). The acquired MS/MS spectra were processed with Proteome Discoverer 1.2.0. software (Thermo Scientific) and used for protein identification and also for relative protein quantification. For protein identification the human IPI database (ipi.HUMAN.v3.76_T33VStd) was used, the precursor ion mass tolerance was 10 ppm, the fragment ion mass tolerance was 0.5 Da. For protein quantification the areas under the curve of the precursor ions were used. As a result, substantial changes were observed in the cellular proteome during the time-course of the infections, as exemplified in Figure 6. Analysis of the results is still under way. Among many others, the levels of stress proteins appear to substantially increase in the early phase of infection. These type proteins were previously found to be overexpressed in the virion-producing cells (Wheeler et al., 2007).
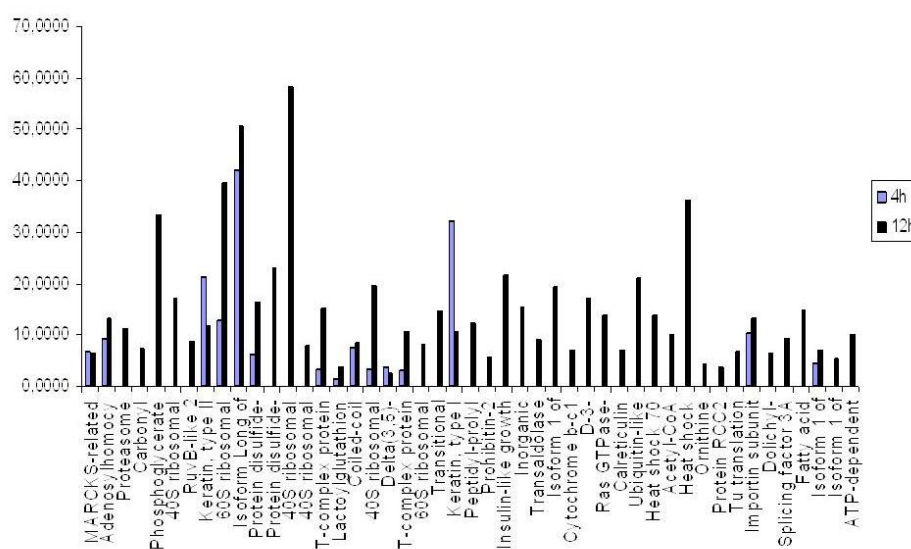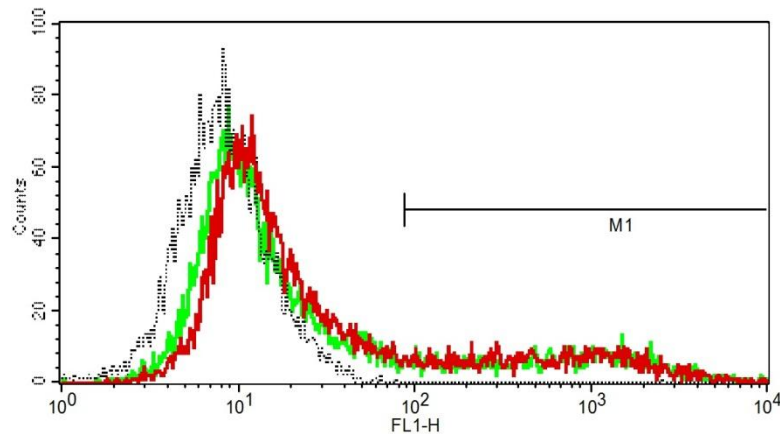

Figure 6. Quantitative proteome changes as a consequence of HIV-1 infection. As an example, differences observed at 4 and 12 hours after infection are shown (only part of the results).

9. Study the effect of protease inhibitors in the early-phase of viral replication

To study the effect of protease inhibitors on the early phase of viral replication, HIV-1 PR inhibitor saquinavir (10 µM final concentration) and UK88947 (24µM final concentration) were added 1 hour prior to transfection or together with the virus (Nagy et. al., 1994). Based on the cytometric analyses, these (very high) concentrations of inhibitors failed to inhibit infectivity of the virus, as exemplified with the UK88947 experiment (Figure 7). However, a specific EIAV PR inhibitor HBY-786 appeared to be able to significantly inhibit the GFP expression of our reporter cell line (data not shown).

B

Fig. 7. Effect of UK-88947 on viral infectivity – Emission profiles (grey: control, red: wild type virus-infected cells, green: inhibitor treated, virus-infected cells)

In another experiment, we have treated the cells with hydroxychloroquine. Hydroxychloroquine -being a weak base- accumulates in the organelles having low pH and increases the pH of these compartments. When 15 µM hydroxychloroquine was added together with the virus, the infectivity of the virus was reduced by 20% (Figure 8), suggesting that acidification of the lysosomes may contribute to the infection of VSV-G pseudotyped virions. We have obtained similar results using EIAV infection of our reporter cell line (data not shown). To study if this acidification is related to the effect of lysosomal aspartic proteases, we have also treated the cells with pepstatin A, a general aspartic protease inhibitor.
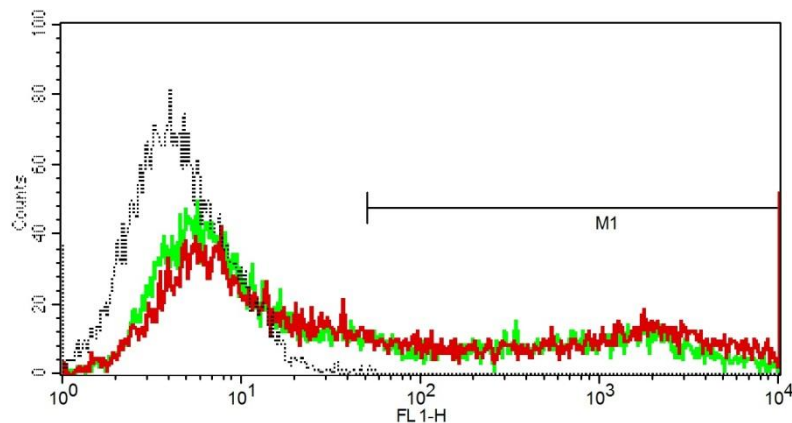

Fig. 9. Effect of chloroquin on the viral infectivity (grey: control, red: wild type virus-infected cells, green: chroroquin-treated, virus-infected cells)

A candidate protease to be involved in virion disassembly within the lysosomes is the aspartic protease Cathepsin D, which is only weakly inhibited by pepstatin A (Gacko et. al., 2007). In our experiment pepstatin A (100 µM) added together with the virus reduced viral infectivity by 40%, (Figure 10), however the pretreatment of the cells one day before infection (data not shown).
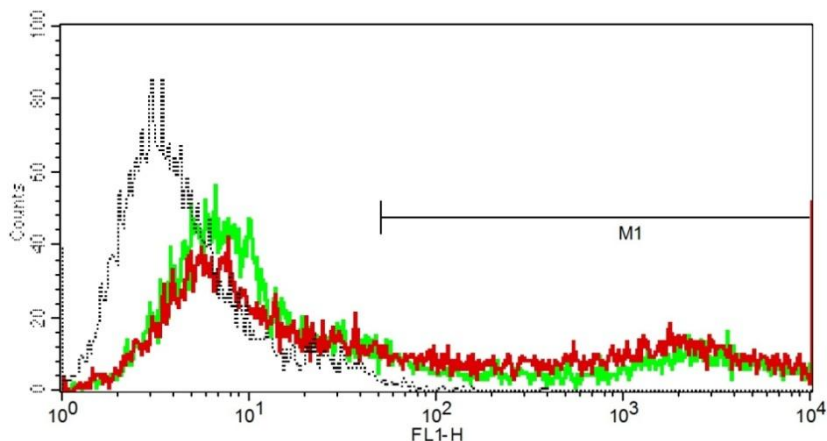
Fig. 10.: effect of pepstatin A on the viral infectivity. (grey: control, red: wild type virus-infected cells, green: pepstatin-treated, virus-infected cells)

Based on these results further experiments are required to clarify the effect and molecular targets of aspartyl protease inhibitors in the early-phase of retroviral life-cycle.

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