Detailed scientific report of the research (NKFIH 125238)

Analysis of networks of host proteins in the early time points following HIV transduction.

We aimed to study the early responses of HEK293T cells to transduction with VSV.G pseudotyped human immunodeficiency virus type 1 (HIV-1) virion, within the 0–12 h time interval. The cell lysates were subjected to label-free mass-spectrometry-based proteomic analysis, and the generated data were analyzed using a network model. Both qualitative and quantitative data were used to examine weighted networks and functional sub-networks (**Csősz et al., 2019**).

The proteomic landscape of the early stage of HIV-1-based transduction (0, 4 and 12 h post-infection) was determined by label-free proteomics, and the mass spectrometry proteomics data have been deposited into the ProteomeXchange Consortium. A qualitative analysis was carried out to detect newly expressed or down-regulated proteins, completed by statistical analysis in order to examine changes in the amount of proteins.

To explore the possible functional associations, we have searched for the available protein-protein interactions of the quantified proteins in our datasets, as well. Generation of weighted networks, and analysis of functional sub-networks revealed that the dynamics of protein level changes in sub-networks is different in HIV-1 transduced samples 12 h post-infection. In the very early stages of infection, proteins involved in translation, transcription and DNA condensation were upregulated. Examination of the binary networks resulted in two protein clusters, one comprising proteins having a role in translation and ribosome biogenesis, and the other containing proteins with a role in RNA splicing.

As a continuation of this work one of our further aims was to characterize the remodeling of the cellular proteome and transcriptome in early time-points following virus entry upon transduction of cells (HEK293, THP-1 and Jurkat) using VSV-G pseudotyped HIV virions. Initially, HEK293 cells in T-25 flasks were transduced with HIV-1 and HIV-2 pseudovirions. At 0, 2 h, 8 h, 12 h, and 26 h time-points, cells were washed, harvested and prepared for quantification by mass spectrometry using Thermo Orbitrap Fusion (Thermo Scientific) mass spectrometer. In parallel, transcriptomic changes occurring at the same time-points as a result of HIV infection was also analyzed using Illumina NextSeq 500 sequencer NGS platform. Our results show that At 2 h time-point, thrombospondin 1, collagens (COL1A2, COL3A1) and eukaryotic translation factors, in addition to various genes coding for long-non-coding RNA were significantly upregulated after HIV-2 transduction, compared to HIV-1 (**Linkner et al., 2021**). The manuscript summarizing our findings is ready for submission.

Moreover, interpretation of the results at 8, 12 and 26 h, as well as examination of proteo-transcriptomic alterations in immune (THP-1 and Jurkat) cell lines is currently underway. Our findings are expected to shed more light on the complex life cycle of HIV, and even more so for the lesser studied HIV-2.

Inhibitory Effects of HIV-2 Vpx on Replication of HIV-1.

Dual infection with HIV-1 and HIV-2 viruses has long been described in the literature, but the molecular mechanism behind the interviral interaction was not explored. We hypothesized that interference at the viral accessory/regulatory protein level may be responsible for the attenuated pathogenicity of HIV-1 observed in dually infected patients, therefore, we aimed to identify the protein(s) that may be responsible for interference (**Mahdi et al., 2018**).

Initially, we tested the transfection and transduction efficiencies of HIV-1 and HIV-2 vectors and pseudovirions, respectively, followed by simultaneous transduction of HEK-293T cells with HIV-1 and HIV-2 pseudovirions. The remarkable decrease of the HIV-1-related fluorescent signal indicated that pre-transduction with HIV-2 pseudovirions significantly protects HEK cells against HIV-1 transduction.

To investigate the effects of inactivation of the accessory/regulatory proteins, HIV-2 accessory/regulatory proteins were systematically inactivated by site-directed mutagenesis, the modified genes included protease, Tat, Vif, Ref, Vpx, and Vpr. HEK293T cells were transfected

with HIV-2 vectors coding for the inactivated or functionally restricted protein of interest, followed by transduction with HIV-1 in sequential experiments.

A significant decrease of HIV-1 infectivity was observed in cells that were primarily transfected with HIV-2 vectors carrying a defective protease, Tat, Rev, Vif, or Vpr, while the infectivity increased when defective *vpx* gene was applied. This suggested that HIV-2 Vpx may mediates the dampening of the infectivity of HIV-1 (HIV-1 transduction). The results of experiments performed with a functionally restricted Vpx mutant (K68A-R70A) proved our findings and indicated that the inactivated Vpx protein is unable to impair the transduction efficiency of HIV-1.

We found that Vpx interferes with reverse transcription of HIV-1 as well as virion production, because in the presence of Vpx the copy number of the LTR junctions was significantly reduced. Vpx was found to be able to incorporate into HIV-1 virions, resulting in a detrimental effect on virus infectivity. Considering the interaction of between Vpx and host restriction factor SAMHD1, the experiments were performed using activated THP-1 cells, as well.

Our *in vitro* studies highlighted the functional importance of Vpx (**Mahdi et al., 2018**), which was characterized in details by investigation of protein-protein interactions of HIV-2 Vpx with cellular and possibly HIV-1 proteins in dual infection experiments, using in-vitro proteomics techniques and proximity ligation assays, in addition to studies of the changes in the cellular transcriptome induced by HIV-2 Vpx (**Mahdi et al., 2020a**). The manuscript summarizing our findings is being prepared for submission.

Y44A Mutation in the Acidic Domain of HIV-2 Tat Impairs Viral Reverse Transcription and LTR-Transactivation.

While investigating the interviral interference between HIV-1 and HIV-2, we studied the effects of pre-transduction of cells with HIV-2 prior to transduction with HIV-1, using modified HIV-2 vectors. The vectors carried modified regulatory and accessory proteins which were functionally inactivated by site-directed mutagenesis (**Mahdi et al., 2018**). The studied genes included protease, Tat, Rev, Vif, and Vpr. To inactivate the proteins, mutations known from the literature were chosen, except for Tat, for which a new mutation was designed (**Szojka et al., 2020**).

To design the mutation of HIV-2 Tat, sequence- and structure-based *in silico* analyses were performed. First, the predictions were performed based on protein sequences and homology model structures in order to identify residues that may be potentially targeted by site-directed mutagenesis. After primary mapping, some selected residues were further analyzed and Y44A mutation was chosen for inactivation, as it was predicted to potentially alter the secondary structure of the protein, and distort the ordered region. The effects of Y44A mutation were compared to those of Y55A mutation, which was known to inactivate HIV-2 Tat. We found that Y44A mutation results in loss of HIV-2 Tat's function (**Mahdi et al., 2018**), but we decided to further characterize Y44A mutation *in vitro*, and explore how the Y44A mutant HIV-2 Tat mutation affects the activity and expression of the reverse transcriptase, and Tat-dependent LTR (long terminal repeat) transactivation.

As compared to the wild-type, transduction with Tat-mutant pseudovirions significantly decreased GFP positivity, the decreased transactivation was observed for both Y44A and Y55A mutants, which were detected both in the pseudovirions and in the lysates of GHOST(3) cells, using immunoblot. An ELISA-based colorimetric assay showed a significantly diminished reverse transcriptase activity for both mutants, revealing the detrimental effect of the mutations on the activity. This effect was due to the lack of reverse transcriptase in the presence of Y44A mutant Tat in the pseudovirions, in contrast to the reverse transcriptase, Tat mutations did not alter the amount of capsid and Tat in the pseudovirions. Proximity ligation assay (PLA) was also used to detect HIV-2 Tat in the pseudovirions, which showed that Vpx and Tat proteins are also incorporated into the virions, indicating their interaction.

Our results imply that the applied *in silico* methods may be useful for mutation design, however, the limitations and possible uncertainties of the simple prediction methods and structural models need to be considered. The Y44A mutation - designed by sequence- and structure based methods - was found to inactivate HIV-2 Tat, the *in vitro* analyses revealed that the acidic domain has a role in its transactivation function and contributes to the stability and activity of the reverse transcriptase and Tat-dependent LTR transactivation.

Functional Study of the Retrotransposon-Derived Human PEG10 Protease.

Our aim was the biochemical characterization of the paternally expressed gene 10 (PEG10) protease using *in vitro* and *in silico* methods (**Golda et al., 2020a**) (**Golda et al., 2020b**). The cDNA sequence was modified by insertional mutagenesis to produce a frameshift-mutant RF1/RF2-encoding sequence, and autoproteolytic cleavage site mutants and catalytic site mutants were also prepared. First mutations were expected to abolish self-processing, while the latter ones were expected to fully inactivate the protease. Proteolytic processing of the PEG10 RF1/RF2 fusion protein expressed in bacterial expression system was observed, but the S26A fireman's-grip mutant also showed similar processing, which indicated that this cleavage was not catalyzed by the PEG10 protease. Therefore, we switched to eukaryotic protein expression.

Western-blot analyses showed a higher molecular weight for the PEG10 RF1/RF2 protein overexpressed in 293T human cells which was assumed to be due to a possible posttranslational modification. The *in silico* analyses implied ubiquitination of the protein, which was proved *in vitro* by Western-blot analysis, as well. *In silico* analyses implied that the structural characteristics of the protease domain are highly similar to those of retroviral-like proteases (Mótyán et al., 2020), the mutations designed based on the proposed model also proved this, the mutations of the active site (fireman's-grip) abolished the autoproteolytic activity. The PEG10 RF1/RF2 protein was found to retain its autoproteolytic activity during evolution (*cis*-activity), despite using a recombinant protein substrate – designed to contain the putative cleavage site – the *trans*-activity was not detected, which implied that the autoproteolysis may block the *trans*-activity. We proved that the PEG10 protease, but not cellular proteases, are responsible for the processing of the RF1/RF2 protein, for which the optimal pH was close to neutral. Inhibition studies revealed that pepstatin A, lopinavir, nelfinavir, saquinavir, darunavir, and tipranavir do notprevent autoproteolysis. Based on our results, PEG10 exhibits natural resistance against multiple HIV-1 protease inhibitors, similarly to yeast Ty1 retrotransposon protease (Gazda et al., 2020) and the human retroviral-Like aspartic protease 1 (ASPRV1) (Golda et al., 2020c).

To elucidate the functional importance of *PEG10* in mammalian cells, we studied proliferation and viability of HEK293T and HaCaT cells that were transfected with plasmids coding for different PEG10 constructs. Our results implied that RF1/RF2 overexpression results in increased proliferation, but resulted in a detrimental effect on cell viability. Our results indicate the important role of PEG10 in the regulation of proliferation of cells, and possibly indicating its contribution to the inhibition of apoptosis.

Molecular modeling suggested that the structure of PEG10 protease is highly similar to those of other eukaryotic retroviral-like proteases that were studied recently by our research group, including the yeast Ty1 retrotransposon protease (Gazda et al., 2020), the human retroviral-Like aspartic protease 1 (ASPRV1) (Golda et al., 2020c), and the DNA-damage inducible protein 1 and 2 (Ddi1 and Ddi2) (Mótyán et al., 2020), buth the main structural characteristics of PEG10 protease (**Golda et al., 2020a**) may be different from those of most retroviral proteases (Mótyán et al., 2020). These results initiated the *in vitro* investigation of dimer interfaces, we have designed dimer interface mutants of ASPRV1 protease and studied the catalytic properties *in vitro*. The effects of mutations on the activities were determined *in vitro*, we predicted the changes upon mutations on protein structure *in silico*, and the investigation of the effects of the protein structure *in vitro* remains to be performed. Our results are expected to reveal the structure-function relationships we proposed for retroviral-like proteases (Mótyán et al., 2020), and may support comparative analysis of PEG10, ASPRV1, Ty1 and Ddi proteases. Additionally, the already existing PEG10 expression

constructs enable for studies on the Gag-like domain, which may contribute to the formation of virus-like particles *via* assembly of PEG10 (Abed et al., 2019). We have already started to investigate the proposed role of PEG10 protease domain in the vesicle budding, using cell culture-based experiments.

Biochemical Characterization, Specificity and Inhibition Studies of HTLV-1, HTLV-2, and HTLV-3 Proteases

The aim of this study was the *in vitro* comparative analysis of human T-lymphotropic virus type 1, 2 and 3 (HTLV-1, -2 and -3) proteases (**Kassay et al., 2021**). The proteases were expressed in BL21(DE3) *E.coli* cells with or without fusion tag. The untagged PRs were used to study main enzymatic characteristics and specificities of each enzyme, while MBP-fused forms were used to study the effects of mutations on autoproteolysis.

Biochemical characteristics of the untagged enzymes were investigated using synthetic oligopeptide substrates representing the wild-type and modified variants of naturally occurring cleavage sites of HTLV-1, HTLV-2, and HTLV-3 proteases, and those of other retroviruses were also tested. We determined differences between the catalytic efficiencies and dependence of activities on pH, temperature, and ionic strength (NaCl concentration). The effects of different inhibitors on enzyme activity were also tested, inclduing HIV-1 PR inhibitors (atazanavir, darunavir, DMP-323, indinavir, ritonavir, and saquinavir) and IB-268 and IB-269 inhibitors that were previously designed against HTLV-1 PR. For HIV-1 protease inhibitors, only moderate inhibition was observed, revealing that these inhibitors are not effective against HTLV proteases, while IB-269 was found to be the most effective against both HTLV-2 and HTLV-3 proteases than against HTLV-1 protease.

For the comparative analysis of amino acid preferences, activity measurements were performed to determine relative activities of HTLV PRs using a series of oligopeptide substrates. Each substrate was modified from the wild-type HTLV-1 CA/NC (KTKVL*VVQPK) substrate by shortening its length (P5-P5′, P4-P5′, and P3-P5′) or by modifying single positions (P4, P3, P2, P1, or P1′). These sets of substrates were used previously to characterize the specificity of HTLV-1 protease (Sperka et al., 2007), thus, our results obtained for HTLV-2 and -3 proteases are comparable with those ones obtained for HTLV-1 protease. The octa- and nonapeptides were found to be less effective substrates for both HTLV-2 and HTLV-3 proteases as compared to the decapeptide, which suggested that these proteases - similarly to HTLV-1 protease (Sperka et al., 2007) - also have extended substrate-binding sites. The HTLV-1 protease was found previously to have external substrate binding sites which enable interactions with P12-P5 (and P5′-P12′) substrate residues (Laco et al., 2007), our results provide first experimental evidence for the existence of these sites in HTLV-2 and HTLV-3 PRs. Specificity studies using peptides representing HIV-1, EIAV, RSV, MMTV, MPMV, BLV, and MuLV PR cleavage sites and P4, P3, P2, P1, or P1′-modified HTLV-1 CA/NC peptides implied a narrower specificity for HTLV-2 and -3 proteases than for HTLV-1 protease.

Our results also implied that HTLV proteases are more sensitive towards binding site mutations as compared to HIV-1 protease, which is in agreement with their different replication strategies. While HIV-1 underwent more rapid evolution, and its protease became highly tolerant for mutations under the selective pressure, the HTLV viruses - replicating predominantly in the DNA form - show a much more rigid substrate specificity of the protease.

The higher sensitivities of HTLV proteases towards binding site mutations, compared to HIV-1 protease, implied that deltaretroviuses (including HTLV and bovine leukemia virus, BLV) may have lower mutation intolerance. Based on our observations (**Kassay et al., 2021**) we have started to perform comparative autoprocessing studies of HTLV and BLV proteases, by studying the autoprocessing capabilities of MBP-fused enzymes that carry such ligand binding site mutations (the modified residues correspond those of the wild-type or drug-resistant HIV-1 protease) which have not been studies previously.

Analysis of the efficacy of HIV protease inhibitors against SARS-CoV-2's main protease.

We have tested the inhibitory potentials of multiple HIV-1 PR inhibitors - that have been approved for therapeutic use in the antiretroviral therapies - against Ty1 (Gazda et al., 2020), ASPRV1 (Golda et al., 2020c), PEG10 (**Golda et al., 2020a**), and HTLV-1, -2, and -3 proteases (**Kassay et al., 2021**). The pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that has resulted in millions of infections worldwide made the identification of effective antivirals necessary, and repurposing of available antivirals gained HIV protease inhibitors as considerable drugs. Therefore, the available set of HIV protease inhibitors was used to test their efficiecies against SARS-CoV-2 Mpro (**Mahdi et al., 2020b**), since inhibition profiling of HIV-1 protease inhibitors against SARS-CoV-2 Mpro has not been attempted *in vitro, up to the date of* our studies.

We carried out an in-depth analysis of the efficacy of these molecules against the main protease of SARS-CoV-2 (Mpro). For this, both cell culture-based and *in vitro* enzymatic assays were designed. The *in vitro* assay was based on the use of recombinant Mpro, which was expressed in BL21(DE3) bacterial cells and synthetic oligopeptide substrate – representing the nautral autoproteolytic cleavage site of SARS-CoV-2 polyprotein - was used for activity measurements. For cell culture-based assays, a dark-to-bright recombinant GFP fluorescent reporter protein substrate was designed. Of the approved HIV-1 protease inhibitors lopinavir, ritonavir, darunavir, saquinavir, and atazanavir were able to inhibit the viral protease in cell culture, albeit in concentrations much higher than their achievable plasma levels, given their current drug formulations. While inhibition by lopinavir was attributed to its cytotoxicity, ritonavir was the most effective of the panel, with IC50 of 13.7 μM. None of the inhibitors showed significant inhibition of SARS-CoV-2 Mpro in our *in vitro* enzymatic assays up to 100 μM concentration.

Our results implied that targeting of SARS-CoV-2 Mpro by some of the HIV protease inhibitors might be of limited clinical potential, given the high concentration of the drugs required to achieve significant inhibition. Therefore, given their weak inhibition, any potential beneficial effect of the protease inhibitors in COVID-19 context might perhaps be attributed to acting on other molecular target(s), rather than SARS-CoV-2 Mpro. However, the tested inhibitors were found to have no or only limited therapeutic potential, our work resulted in the setup of such experimental systems which may be applied to test other potential inhibitors against SARS-CoV-2 Mpro, and the reporter substrate-based cell culture assay may be adapted to study other proteolytic enzymes, as well.

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