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Abstract

Title of Thesis: Quest for New Drugs Against HIV-1 Multi-Drug Resistant Proteases

Kasey Vernon, Master of Science, 2020

Thesis Mentor: Dr. Richard Y. Zhao, Professor and Division Head of Molecular Pathology, Department of Pathology, University of Maryland School of Medicine

HIV-1 protease inhibitors (PIs) are the most potent class of drugs in combinational antiretroviral therapies (cART). However, the current therapies are still not adequate due to the emergence of drug resistance. There is still a need for development of more potent PIs. The objective of this study was to search for multi-drug resistant inhibitors to combat HIV-1 and viral resistant proteases. Our lab developed a fission yeast (*Schizosaccharomyces pombe*) cell-based system that allows large-scale or high-throughput drug screening. We first used a cell growth-based assay to test a drug candidate. We then used a HIV-1 enzymatic assay to confirm it. We explored four batches of potential anti-HIV compounds as well as nine FDA approved PIs. All FDA-approved PIs potentially inhibited HIV-1 PR with 3 small molecule compounds showed minimal inhibitory effects. Results of this study demonstrated the use of fission yeast cell-based system as a means to discover new PIs.

Quest for New Drugs Against HIV-1 Multi-Drug Resistant Proteases

by
Kasey Vernon

Thesis submitted to the Faculty of the Graduate School of the
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of the requirements for the degree of
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“Girlfriends” who also keep me on my toes and being my sounding board through the good and not so great parts of this process.

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List of Abbreviations

APV	Amprenavir
ATV	Atazanavir
cART	combinational antiretroviral therapy
dr	drug resistant
DRV	Darunavir
GFP	Green Fluorescent Protein
HIV-1	Human Immunodeficiency virus type 1
IDV	Indinavir
LPV	Lopinavir
mdr	multi-drug resistant
NFV	Nelfinavir
PI	Protease Inhibitor
PMG	Pombe Glutamate Media
PR	Protease
RTV	Ritanavir
SQV	Saquinavir
TPV	Tipranavir
Vpr	Viral Protein R

Introduction

The Human Immunodeficiency virus type 1 (HIV-1) has continued to be a global public health problem. HIV-1 primarily infects CD4+ T cells which are an essential component of the human immune system (H. Li et al., 2017). At the culmination of 2019, there were approximately 38 million people living with HIV and 1.7 million newly infected. The number of individuals infected, however has decreased by 39% and the morbidity rate 51% due to combination anti-retroviral therapies (cART) (World Health Organization, July 2019). This approach of drug therapy has shown to be effective in suppressing the virus and offer improving quality of life (World Health Organization, July 2019). However, the current therapies are still not adequate. The development of new anti-retroviral therapies is essential to address obstacles such as drug resistance, drug toxicity, and HIV neurocognitive disorders (González, 2017). There are currently six classifications for anti-retroviral drugs which are entry inhibitors, fusion inhibitors, integrase inhibitors, non-nucleotide reverse transcriptase inhibitors and protease inhibitors (PI) (Greene et al., 2008). The most potent class of anti-HIV drugs are PIs (Benko et al., 2016; Yang et al., 2012). According to the World Health Organization, cART will always include at least one PI.

HIV-1 Protease and Protease Inhibitors

HIV-1 Protease (PR) is a target for anti-HIV therapies due to the important role played during viral reproduction. HIV-1 PR is an aspartic protease that typically presents as a homodimer with the subunits containing 99 amino acids (Ghosh et al., 2016; Wlodawer et al., 1989). The primary functions of HIV-1 PR is to proteolyze the viral Gag-Pol polyprotein for the production of viral enzymes such as, reverse transcriptase,

PR, integrase and structural proteins, as well as for the maturation of infectious viral particles (Benko et al., 2016; Rumlová et al., 2014). Proteolysis of Gag by protease occurs during the early stage maturation in which the intact full length Gag precursor polyprotein is cleaved by the viral protease into functional subunits (Su et al., 2019).The Gag polyprotein consists of component matrix (MA), capsid (CA), nucleocapsid (NC), p6, and two spaced peptides p1 and p2 (Su et al., 2019). HIV-1 PR has been shown to induce necrotic and apoptotic cell death in CD4+ T cells. CD4+ depletion is a key indicator of HIV-infection ((Benko et al., 2016; Rumlová et al., 2014). PR is essential for viral replication and survival. Current FDA approved PIs were designed to impersonate natural HIV-1 PR in order to compete at the active enzyme binding site. PIs block activity in a competitive manner with Gag for protease binding (Su et al., 2019). Over time viral gene mutations can cause morphology changes to the active site making the PIs unsuccessful (Benko et al., 2017). Gag cleavage site mutations contribute directly to PI resistance and non-cleavage site mutations contribute by compensating for the loss of viral fitness reducing its proteolytic functions (Su et al., 2019). The recognition of the cleavage site by protease is likely to be based on the asymmetric three-dimensional structures that would fit into the substrate-binding pocket of protease (Su et al., 2019). For example, our lab isolated M10 and M11 multi-drug resistant HIV strains. They are resistant to all current FDA-approved PI drugs. M10 and M11 are a clear reason why drug resistance is still a problem (**Table 1**) (Benko et al., 2017). When a PI drug is used in combination, HIV-1 viral loads could be reduced to the lowest possible level (Benko et al., 2016). In this study we will search for new small molecule inhibitors to combat HIV-1 and against HIV-1 viral multi-drug resistant proteases.

Table 1: Mutational and multidrug resistant profiles of proteases isolated from HIV-infected patients

Mutation Status	Nonsynonymous gene mutations found in the *PR* gene	Known resistance to PI drugs	Reference
WT	None	None	Benko, Z. et al., 2016
M7	V32I, L33I, M36I, I54V, A71V, G73S, L90M	**IDV, SQV, RTV, NFV, ATV** (APV, FOS, LPV, TPV)	Benko, Z. et al., 2017
M10	L10I, I13V, K20R, L33I, M36I, I54M, A71T, G73S, I84V, L90M	**APV, FOS, IDV, SQV, RTV, NFV, ATV, TPV** (LPV)	Benko, Z. et al., 2017
M11	L10F, L33F, M46I, I54L, H69K, A71V, G73S, V77I, V82T, I84V, L90M	**APV, FOS, IDV, SQV, RTV, NFV, ATV, TPV** (LPV)	Benko, Z. et al., 2017

Note: The three mutant HIV-1 PRs were isolated from the plasma samples of HIV-infected patients who were cared at the University of Maryland Medical Center. They carried seven (M7), ten (M10) and eleven (M11) *PR* gene mutations, respectively. The wild type (WT) PR was derived from pNL4-3. The drug resistant profiles were generated in a CAP/CLIA accredited hospital laboratory as part of the clinical reports by using the ViroSeq HIV-1 Genotyping System (Abbott Molecular, Chicago, IL). Amprenavir; FOS, Fosamprenavir; IDV, Indinavir; SQV, Saquinavir; LPV, Lopinavir+Ritonavir; RTV, Ritonavir; NFV, Nelfinavir; ATV, Atazanavir; TPV, Tipranavir; Drugs in parenthesis indicate possible drug resistance.

Fission Yeast

Fission yeast (*Schizosaccharomyces pombe*) is a single-cell eukaryote and is divided by binary fission that produces a daughter cell with equal size to the mother cell (G. Li & Zhao, 2018; Zhao, 2017). Similar to multicellular eukaryotes, they have nuclei and other membrane bound organelles such as mitochondria, Golgi apparatus and a network of membranous tubules within the endoplasmic reticulum (Zhao, 2017). Cellular functions such as cell proliferation, cell cycle regulation, cell transport, and cell death/apoptosis are highly conserved from yeast to higher eukaryotes (Zhao, 2017). They grow rapidly with a doubling time of 3-5 hours and lends itself to easy molecular manipulation (Forsburg & Rhind, 2006; Zhao, 2017). The length of a cell is an indicator of its position within the

cell life cycle (Forsburg & Rhind, 2006). Fission yeast has been a model system for our lab to study the effect of HIV-1 viral protein R (Vpr) on cell proliferation, cell cycle G2/M regulation, and cell/death apoptosis over two decades (Benko et al., 2017). In a previous study in our lab, it was shown that the wild type HIV-1 PR proteolyze the HIV-1 viral substrate in fission yeast in the same way it does in mammalian cells (Benko et al., 2017). This aide the establishment of the fission yeast cell-based system as a high throughput screening system for the search of potential PI's.

Hypothesis

Fission yeast cell-based HIV-1 PR expression system will produce at least a minimum inhibition to discover novel PIs against wild type and drug resistant M10 and M11 proteases.

Objectives

Search for multi-drug resistant inhibitors to combat HIV-1 and viral resistant proteases.

Materials and Methods

Cells and Viruses

RE294 wild-type (WT) yeast cell line, is a derivative of SP223 which was generated by our lab and contains a single integrated copy of the HIV-1 PR gene at the *nmt1* gene locus (Benko et al., 2016) HIV-1 PR gene expression is under the control of an inducible no message in thiamine (*nmt1)* promoter (Benko et al., 2016). Pombe Glutamate Medium (PMG) supplemented with adenine, uracil, leucine, thiamine (20μM), and G418, was used for yeast cell growth and plasmid selections. ZB011 multi-drug resistant (mdr) yeast cell was generated by cloning M11 HIV-1 drPR gene isolated by our

lab in PYZ2N plasmid (Benko et al., 2017). ZB011 was grown under the same condition except Zeocin was used as the selection marker instead of G418 (Benko et al., 2016).

HIV Protease Inhibitors

In the last two decades, structure based design has led to the discovery of nine FDA approved PI's (**Figure 1**) (Ali et al., 2010). They were rationally designed to mimic the transition state of the polyprotein substrates of HIV-1 protease (Ali et al., 2010). The PIs represent the most potent anti-HIV drugs and have a high genetic barrier against resistance (Ali et al., 2010; Aoki et al., 2018).The current nine FDA approved PIs are Saquinavir (SQV), Indinavir (IDV), Ritonavir (RTV), Nelfinavir (NFV), Amprenavir (APV), Lopinavir (LPV), Atazanavir (ATV), Tipranavir (TPV), and Darunavir (DRV) (Ali et al., 2010). SQV was the first HIV-1 PI approved by the FDA in 1995 discovered by Roche (Ali et al., 2010). Included in the first generation of PI's is SQV, RTV, IDV, NFV and APV (Ghosh et al., 2016). Second- generation included LPV, ATV, TPV, and the third-generation includes DRV (Ghosh et al., 2016). SQV and RTV prevent the cleavage of the viral polyproteins resulting in the formation of immature noninfectious virus particles. IDV, ATV and TPV, block virus maturation and causes the formation of immature, noninfectious virions. NFV, APV, LPV, DRV prevent the cleavage of the gag and gag-pol polyprotein resulting in immature noninfectious virus. Darunavir is the current first line therapy and highly effective against both wild-type and some of the moderate PI-resistant HIV (Aoki et al., 2018; Benko et al., 2016). Due to the its development having a P2 ligand "backbone binding" design concept contributes to its high genetic barriers against drug resistance (Ghosh et al., 2007). Therefore, it will be

used as a control against the potential anti-HIV compounds. FDA approved protease inhibitors were obtained from the NIH AIDS Reagent Program **(Table 2)**.

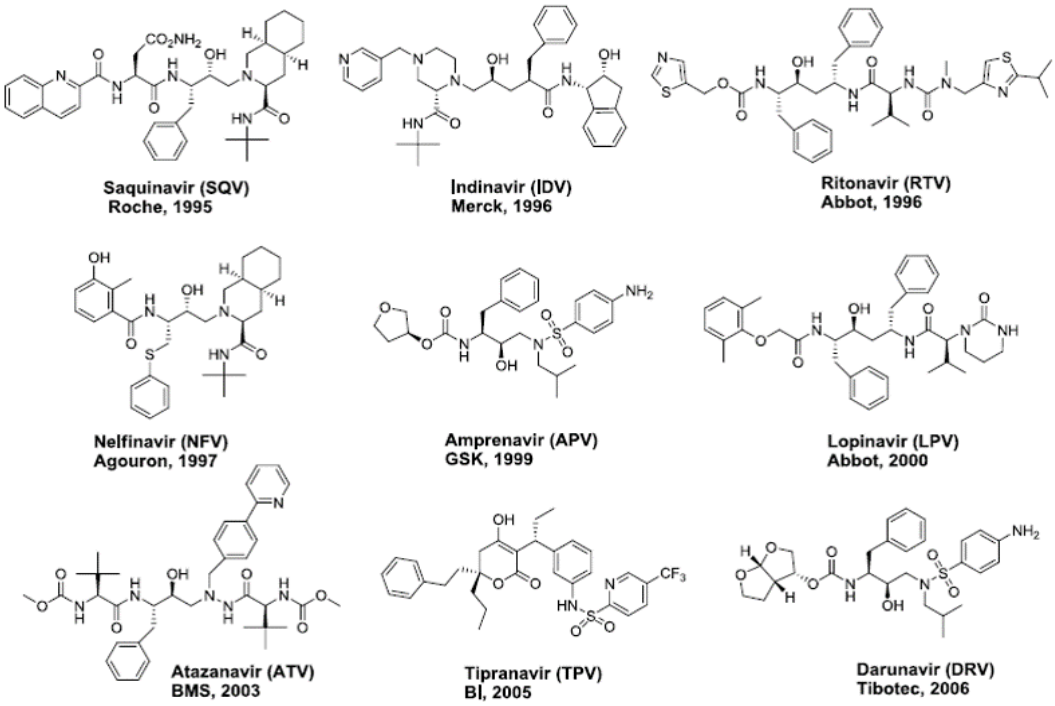


Figure 1. FDA approved HIV-1 Protease Inhibitors (Ali et al., 2010)

Table 2: FDA approved Protease Inhibitors Used in WT fission yeast cell-based system		
Protease Inhibitors	Abbreviation	References
Saquinavir	SQV	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Saquinavir.
Indinavir	IDV	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Indinavir Sulfate from NIAID, DAIDS (cat# 8145).
Ritonavir	RTV	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Ritonavir from NIAID, DAIDS (cat# 4622).
Nelfinavir	NFV	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Nelfinavir.
Amprenavir	APV	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Amprenavir from NIAID, DAIDS (cat# 8148).
Lopinavir	LPV	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Lopinavir from NIAID, DAIDS (cat# 9481).
Atazanavir	ATV	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Atazanavir Sulfate from NIAID, DAIDS (cat# 10003).
Tipranavir	TPV	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Tipranavir from NIAID, DAIDS (cat# 11285).
Darunavir	DRV	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Darunavir (Cat# 11447) from Tibotec, Inc.

Possible Anti-HIV Compounds

Potential protease inhibitor drug compounds were obtained from National Institute of Allergy and Infectious Disease (NIAID) (Dr. Mohamed Nasr). All compounds are small molecule compounds and blind testing was done on compounds. There are 4 total batches of compounds that were received. Batch one has 18 compounds **(Table 3)**. Batch two has 21 compounds **(Table 4)**. Batches three has 40 compounds **(Table 5)**, and batch four has 40 compounds, **(Table 6)**.

Table 3: Compounds Received from NIH (Batch 1)	
Sample ID	NSC Number
NCI-1-1	NSC125066
NCI-1-2	NSC152429
NCI-1-3	NSC226667
NCI-1-4	NSC278358
NCI-1-5	NSC318516
NCI-1-6	NSC349452
NCI-1-7	NSC376128
NCI-1-8	NSC638606
NCI-1-9	NSC667520
NCI-1-10	NSC672462
NCI-1-11	NSC686303
NCI-1-12	NSC719475
NCI-1-13	NSC721354
NCI-1-14	NSC729477
NCI-1-15	NSC735062
NCI-1-16	NSC745104
NCI-1-17	NSC746847
NCI-1-18	NSC758252

Note: NSC number is numeric identifier for substances submitted to the National Cancer Institute (NCI) for testing and evaluation. All these compounds were provided by Mohamed Nasr, Ph.D., Drug Development and Clinical Sciences Branch, Division of AIDS, NIH.

Table 4: Compounds Received from NIH (Batch 2)	
Sample ID	NSC Number
NCI-2-1	13502-J/1
NCI-2-2	14385-S/1
NCI-2-3	14417-C/1
NCI-2-4	16765-G/1
NCI-2-5	29724-Q/1
NCI-2-6	43121-C/1
NCI-2-7	44652-R/2
NCI-2-8	46605-P/1
NCI-2-9	55825-M/1
NCI-2-10	60849-W/2
NCI-2-11	63027-P/1
NCI-2-12	80815-Y/1
NCI-2-13	89268-N/5
NCI-2-14	95222-K/1
NCI-2-15	101808-T/1
NCI-2-16	106326-G/1
NCI-2-17	251193-T/1
NCI-2-18	382843-R/1
NCI-2-19	683339-S/1
NCI-2-20	683340-T/1
NCI-2-21	758247-P/4

Note: NSC number is numeric identifier for substances submitted to the National Cancer Institute (NCI) for testing and evaluation. All these compounds were provided by Mohamed Nasr, Ph.D., Drug Development and Clinical Sciences Branch, Division of AIDS, NIH.

Table 5: Compounds Received from NIH (Batch 3)	
Sample ID	NSC Number
NCI-3-1	3053-Y/52
NCI-3-2	9519-C/1
NCI-3-3	18268-O/6
NCI-3-4	29723-P/1
NCI-3-5	29725-R/1
NCI-3-6	41869-R/1
NCI-3-7	45735-V/3
NCI-3-8	48077-P/2
NCI-3-9	61586-X/2
NCI-3-10	72301-U/1
NCI-3-11	78844-I/2
NCI-3-12	78845-J/2
NCI-3-13	78846-K/2
NCI-3-14	78866-H/1
NCI-3-15	78868-J/1
NCI-3-16	79022-Z/1
NCI-3-17	81722-L/2
NCI-3-18	85252-W/1

NCI-3-19	85254-Y/1
NCI-3-20	87221-N/2
NCI-3-21	87222-O/2
NCI-3-22	89178-P/1
NCI-3-23	89624-Y/3
NCI-3-24	93639-O/1
NCI-3-25	93743-A/1
NCI-3-26	95090-Q/2
NCI-3-27	96353-O/4
NCI-3-28	96700-Q/1
NCI-3-29	96703-T/1
NCI-3-30	96710-A/1
NCI-3-31	96720-N/1
NCI-3-32	98946-I/1
NCI-3-33	106254-A/1
NCI-3-34	106257-G/1
NCI-3-35	106623-C/1
NCI-3-36	107660-G/8
NCI-3-37	108044-W/1
NCI-3-38	111885-W/1
NCI-3-39	112903-F/2
NCI-3-40	112954-K/1

Note: NSC number is numeric identifier for substances submitted to the National Cancer Institute (NCI) for testing and evaluation. All these compounds were provided by Mohamed Nasr, Ph.D., Drug Development and Clinical Sciences Branch, Division of AIDS, NIH.

Table 6: Compounds Received from NIH (Batch 4)	
Sample ID	NSC Number
NCI-4-1	668590-M/1
NCI-4-2	668873-T/1
NCI-4-3	668881-C/1
NCI-4-4	668889-M/1
NCI-4-5	668892-P/1
NCI-4-6	668898-V/1
NCI-4-7	669656-U/1
NCI-4-8	669670-L/1
NCI-4-9	669701-T/1
NCI-4-10	669703-V/1
NCI-4-11	669704-W/1
NCI-4-12	669714-J/1
NCI-4-13	669778-C/1
NCI-4-14	669812-P/1
NCI-4-15	670359-K/1
NCI-4-16	670869-O/1
NCI-4-17	672142-W/1

NCI-4-18	672454-M/1
NCI-4-19	672456-O/1
NCI-4-20	672457-P/1
NCI-4-21	672462-U/1
NCI-4-22	672660-L/1
NCI-4-23	672663-O/1
NCI-4-24	672671-W/1
NCI-4-25	672916-O/1
NCI-4-26	679540-O/1
NCI-4-27	679678-O/1
NCI-4-28	679679-P/1
NCI-4-29	679680-Q/1
NCI-4-30	679682-S/1
NCI-4-31	679683-T/1
NCI-4-32	679685-V/1
NCI-4-33	680834-U/1
NCI-4-34	681948-H/1
NCI-4-35	681957-Q/1
NCI-4-36	688366-I/1
NCI-4-37	688534-P/1
NCI-4-38	694857-N/1
NCI-4-39	694866-W/1
NCI-4-40	694868-Y/1

Note: NSC number is numeric identifier for substances submitted to the National Cancer Institute (NCI) for testing and evaluation. All these compounds were provided by Mohamed Nasr, Ph.D., Drug Development and Clinical Sciences Branch, Division of AIDS, NIH.

Induction of HIV PR Gene Expression in Fission Yeast Cells

Fission yeast cells were grown in PMG media supplemented with adenine, uracil, and leucine. To achieve gene expression cells were grown in the presences of 20µM Thiamine in PMG supplemented media at 30°C with constant shaking of 250–300 rpm for 24hrs. G418 antibody was also added. Cells were washed three times with distilled water and diluted to a final concentration with PR gene "off". RE294 cells and compounds added to 96 well plate at concentration of approximately $2x10^5$ cells/mL. Cell growth was measured by optical density 600nm using a spectrophotometer at 0hr, 48hr and 72hr (Benko et al., 2016).

Colony Formation Assay

A fission yeast colony-formation assay was used to investigate the effect of the PR gene expression on fission yeast cell proliferation and viability with the nine FDA PI's. The nine FDA PIs at 150 µM concentration, Thiamine at 20µM concentration and a Mock sample were plated in six well plates. Pombe Glutamate Medium (PMG) liquid media supplemented with adenine, uracil, leucine, and G418, was used then added to create agar plates with PR "gene on". RE294 yeast culture was prepared the same way as described about for the yeast cell growth. The inhibitory effect of the FDA PIs on colony formation was evaluated four days after plating by comparing growth of colonies of the drug treated well to that of the controls.

Measurement of IC50

IC_{50} is the concentration of an inhibitor where the response is reduced by half (Aoki et al., 2018). Dose-dependent curves in fission yeast for all nine FDA approved PIs were established and calculated for the IC_{50}. It was used as a standard reference for comparison with other PI compounds and further establish the fission yeast model in screening potential PIs. RE294 cells were prepared and treated with the protease inhibitors as described previously in the induction of the PR gene expression. I used a 3x dilution for the concentration dosage for all protease inhibitors and measure cell growth by optical density at 600 nm using a spectrophotometer at 72 hr. Data were analyzed using GraphPad Prism.

Measurement of HIV PR Enzymatic Activity in Fission Yeast

"Green fluorescent protein (GFP) re-localization assay" was developed to measure proteolytic activities of HIV-1 PR (Benko et al., 2017). To examine the PR enzymatic activity, the RE294 fission yeast cells were prepared as described above and collected 20hr post gene induction and drug treatment with the nine FDA approved PI's (Benko et al., 2016). The $_{wt}$PR and $_{mdr}$PR are generated in a fission yeast expression vector pYZ3N. GFP and VPR are connected via a polypeptide linkage and will contain a PR cleavage sequence, MA↓CA (DSQNY↓PIVQ) (Benko et al., 2017). Separation of HIV-1 PR cleavage appears predominantly as a "ring-like" structure localized on the nuclear membrane in fission yeast (Benko et al., 2017). GFP is used for fluorescent detection and it typically disperses throughout fission yeast cells (Benko et al., 2017). The appearance of the cells will confirm PR cleavage.

Results

FDA Protease Inhibitors cleave PR gene expression in fission yeast leading to yeast cell colony formation and cell growth

In this study we wanted to investigate the effect of the PR gene expression on fission yeast cell proliferation and viability in the presences the nine FDA PI's. We wanted to demonstrate the inhibitory of effects of the established PI's using a fission yeast colony-formation assay. FDA approved PI's will be added to PMG media to make selectively inhibit yeast cell growth. RE294 (WT) yeast strain will be cultured and plated at $4x10^4$ cell/mL. In the presence of thiamine, the *nmt1* promoter repressed "gene off" and when thiamine is removed from the growth media, the promoter is fully activated "gene on" **(Figure 2A)** (Benko et al., 2016). The inhibitory effects were visualized after four days of incubation at 37°C. No cell growth was observed due to PR gene expression suppressing growth. In **Figure 2B**, we have shown that when in the PR "gene on" for all of the samples for the know protease inhibitors, gene expression is blocked. In blocking the PR gene expression, yeast colony formation is induced resulting in yeast cell proliferation. Inhibitors effectively blocked the PR gene expression in the wild type fission yeast. The addition on Dimethyl Sulfoxide (DMSO) in **Figure 2B** was to insure it is not reacting with the drug compounds as this is the diluent used to store compounds.

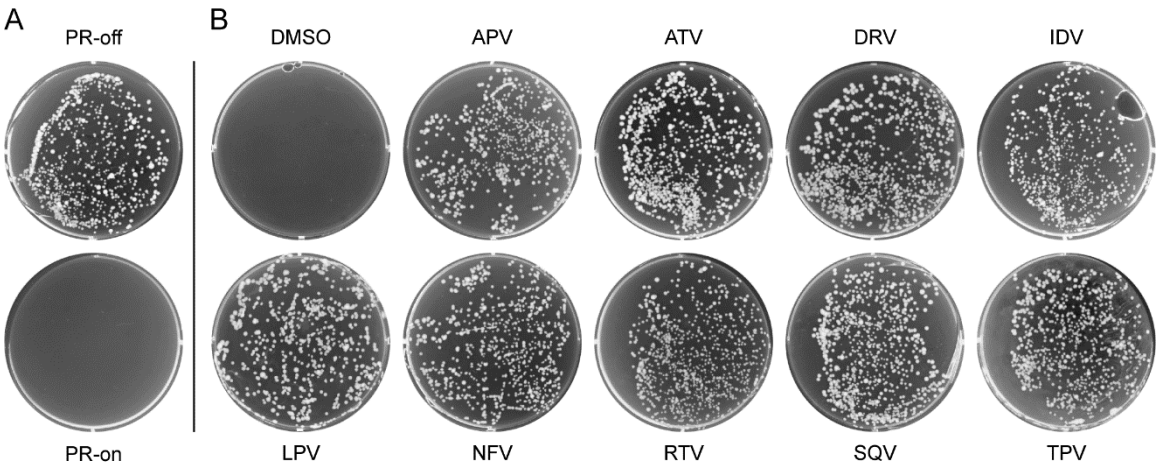


Figure 2. Colony Formation and cell growth **(A)** RE294 growth with HIV-1 PR-on and PR-off expression of the HIV-1 PR gene (Benko et al., 2016); **(B)** Inhibition of HIV-1 PR induced by FDA Protease Inhibitors. Figure generated by Dr. Jiantao Zhang.

Anti-HIV compounds show minimal inhibitory effect in fission yeast cell-based system

In order to observe the potential inhibitory effects of the small molecule compounds, a yeast growth curve will also be used. RE294 yeast cells will be treated with drug compounds at a concentration of 150 µM and will be measured for cell growth at 0hr, 48hr and 72hr. Four batches of potential anti-HIV compounds were tested to evaluate their inhibitory effect. Thiamine (20 µM) which proliferates yeast cell growth and DRV will be used as the control to compare against the potential PI's. In previous study the inhibitory effect of FDA approved PI on mdr protease was established using the fission yeast cell based system (Benko et al., 2017). Batch one has 18 compounds. Batch two has 21 compounds, batches three and four have 40 compounds each of potential protease inhibitors. In **Figures 3-6**, the inhibitory effects of the potential protease inhibitors are shown. In some cases, the compounds were insoluble in multiple diluents such as Dimethyl Sulfoxide (DMSO) and acid. Heating the compounds was also used to address

solubility. Due to solubility issues, a 0hr reading was taken and samples were not reported if the absorbance reading was a 2x fold change to that of the average of the mock sample. In Batch one, compounds 1-2, 1-6 and 1-10 are showing minimal inhibitory effect in the RE294 yeast cells. Although compound 1-12 appears to show minimal inhibitory effect, due to the decrease in growth from the 48hr to the 72hr it is not a protease inhibitor. The decrease in growth can be an indicator of drug toxicity. No significance testing was performed on the data; however, the increase in yeast cell growth over time is indicative of its minimal inhibitory capabilities. This also demonstrates the usage of fission yeast as a means to identify potential protease inhibitors even at a minimal level. Of the other potential anti-HIV compounds, none of them showed any significant inhibitory effect on the WT fission yeast. The three compounds that showed minimal inhibitory effect will need to be further evaluated in the mdr fission yeast, ZB011.

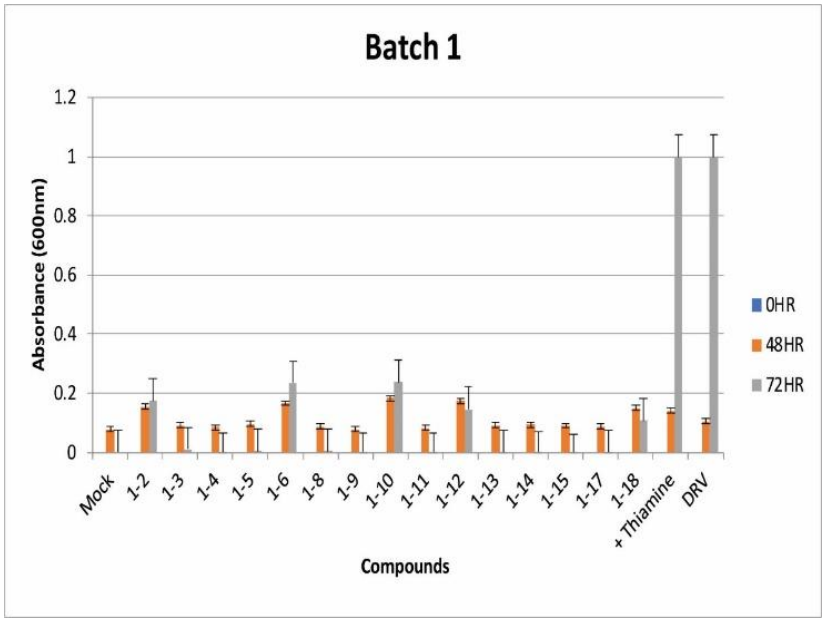
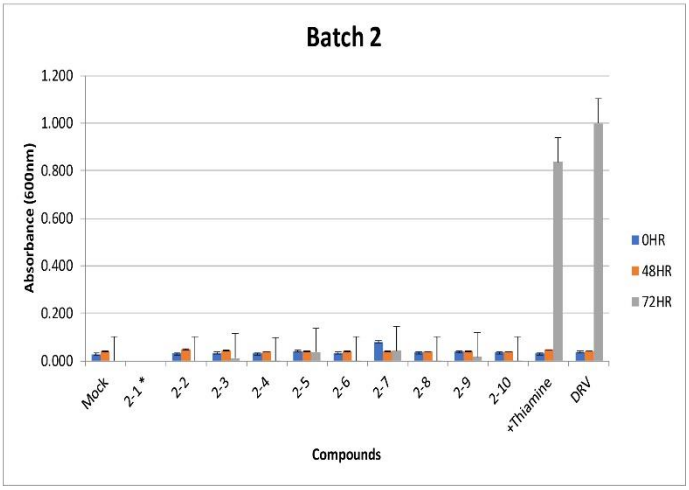


Figure 3**.** Inhibitory effects of NCI Batch 1 small molecule compounds on WT fission yeast measured at 0, 48 and 72 hr. Compounds 1-18.

A



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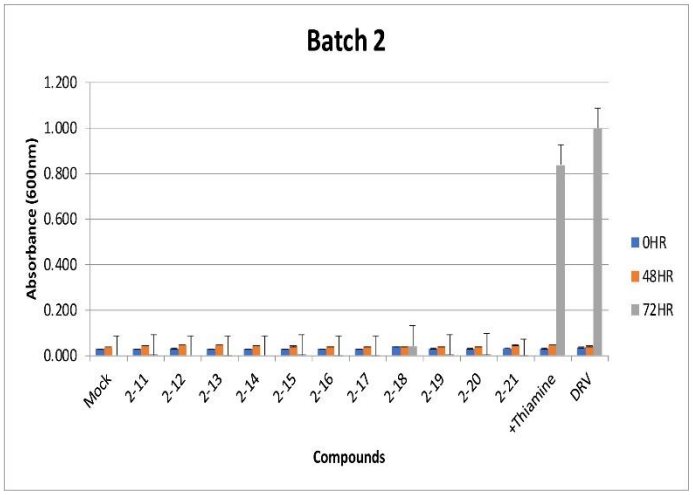
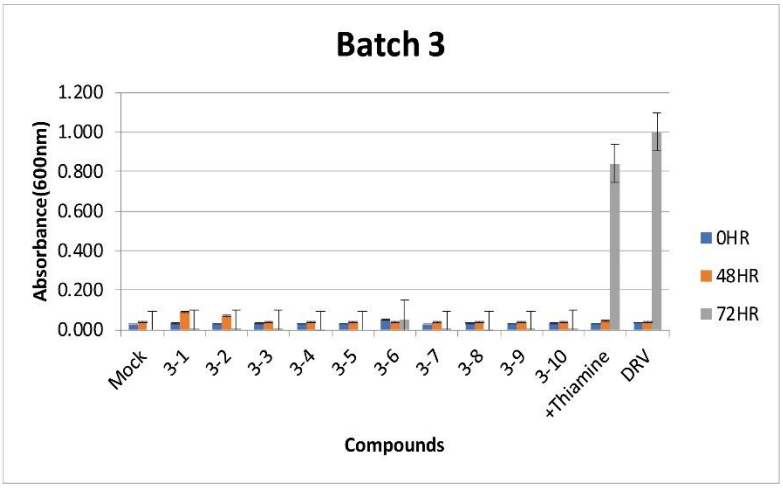


Note: *Compounds are insoluble, so data was inconclusive and removed from reporting

Figure 4**.** Inhibitory effects of NCI Batch 2 small molecule compounds on WT fission yeast measured at 0, 48 and 72 hr. **(A)** Compounds 1-10 **(B)** Compounds 11-21.

Figure 5**.** Inhibitory effects of NCI Batch 3 small molecule compounds on WT fission yeast measured at 0, 48 and 72 hr. **(A)** Compounds 1-10 **(B)** Compounds 11-20 **(C)** Compounds 21-30 **(D)** Compounds 31-40.

A



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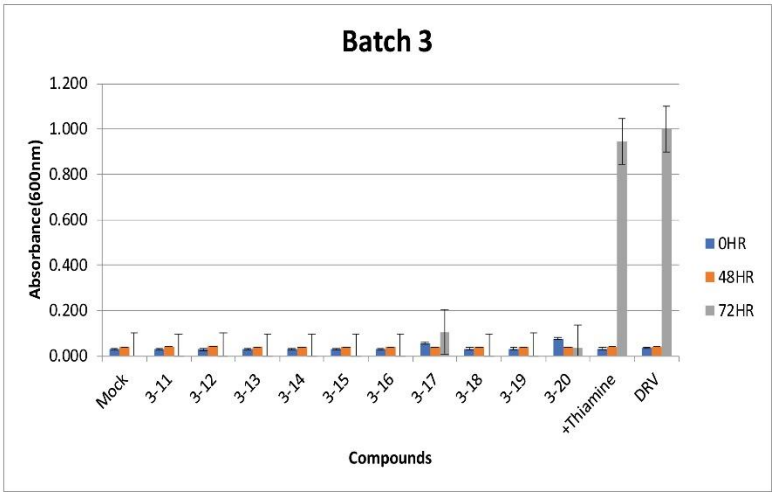
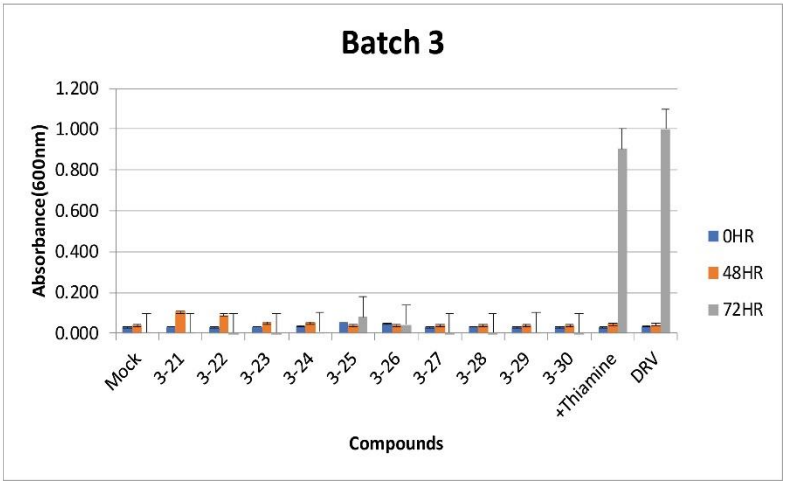
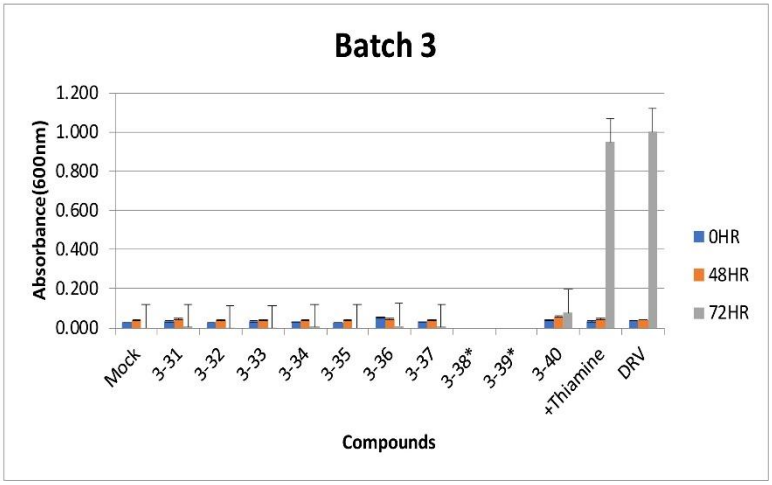


Figure 5 continued

C



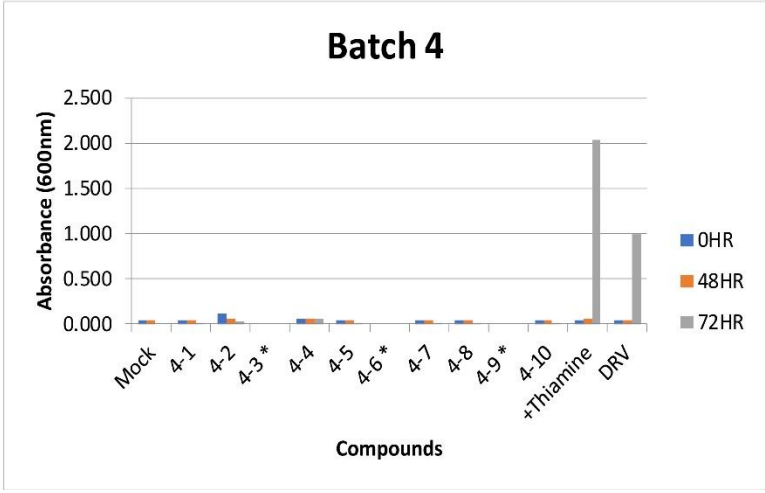
D



Note: *Compounds are insoluble, so data was inconclusive and removed from reporting

Figure 6**.** Inhibitory effects of NCI Batch 4 small molecule compounds on WT fission yeast measured at 0, 48 and 72 hr. **(A)** Compounds 1-10 **(B)** Compounds 11-20 **(C)** Compounds 21-30 **(D)** Compounds 31-40.

A



B

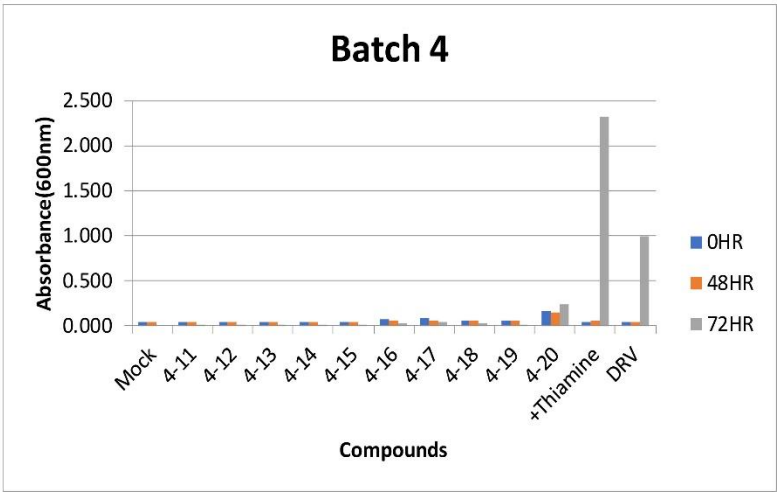
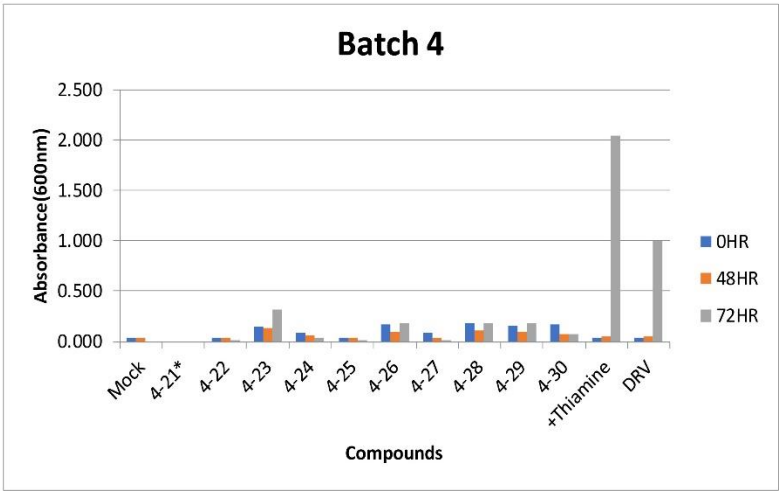


Figure 6 continued

C



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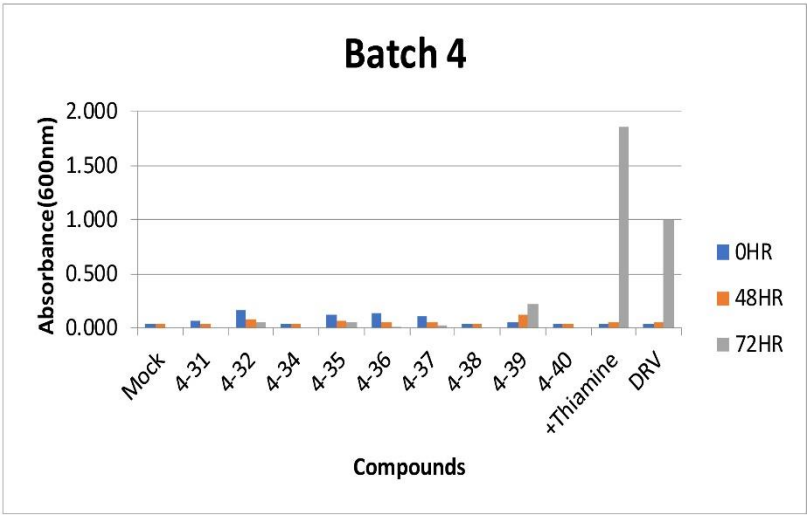


Note: *Compounds are insoluble, so data was inconclusive and removed from reporting

FDA Protease Inhibitors IC_{50} Determination by Dose-Dependent Curve

The determination of the IC_{50} for protease inhibitors will establish a comparison threshold for potential protease inhibitors. It demonstrates the efficacy of the drug

compound or drug potency. It will also demonstrate at which concentrations the protease inhibitors start to exhibit functioning. DRV is currently the first line therapy for HIV-1 protease class of drugs (Aoki et al., 2018; Benko et al., 2016). In this study the RE294 yeast cell culture was treated with concentrations 150, 100, 30, 10, 3, 1, 0.3 and 0.1µM and measured at 72 hours for cell growth. In this study originally, we started with the pill form of the drugs and used the used DMSO as the solvent. Upon performing the growth curve, we discovered that Amprenavir is the pure drug and Fosamprenavir, pill form, is a pro-drug which is metabolized in the body. Upon that discovery, we moved to use the pure drug form of all the compounds. The dose-dependent curves and IC_{50} determination by the linear regression of the curve is shown in **Figure 7**. All of the FDA approved inhibitors showed effects on the RE294 yeast cells. The drug compounds do differ in efficacy which can also be observed by the dose dependent curves which is to expected. The concentrations at which the drug at 50% will cause inhibition will be correlated to mammalian cell lines which has long been established.

Figure 7**:** FDA Protease Inhibitors dose-dependent curve determining the IC_{50} linear regression measured at 72 hr. post treatment **(A)** APV **(B)** ATV **(C)** DRV **(D)** IDV **(E)** LPV **(F)** NFV **(G)** RTV **(H)** SQV **(I)** TPV.

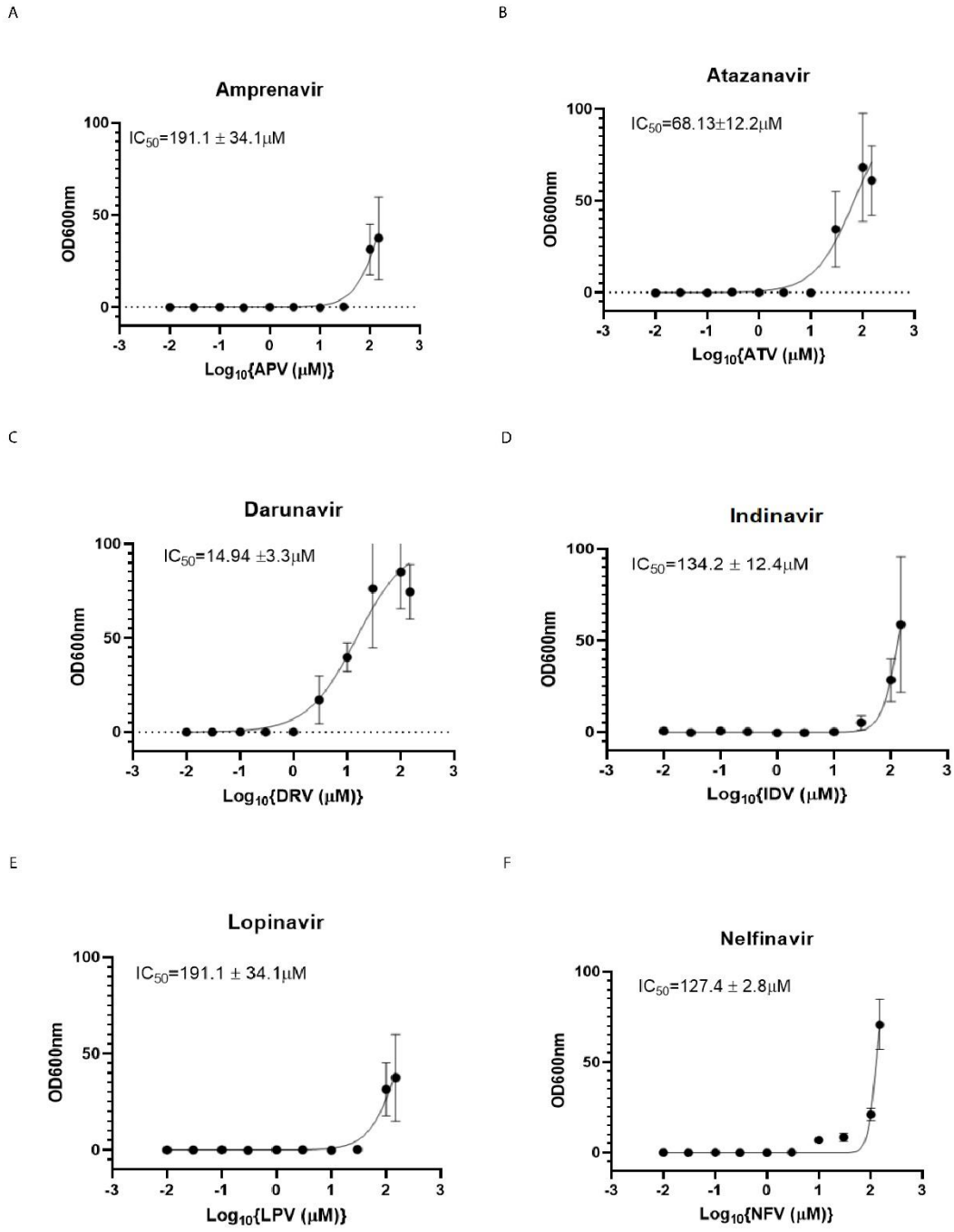
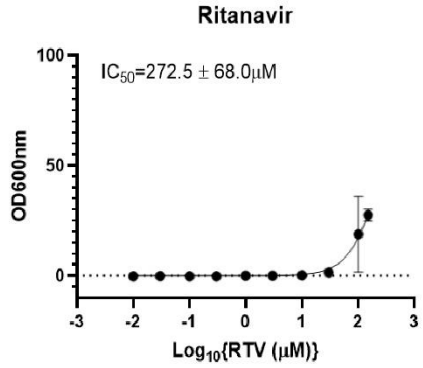
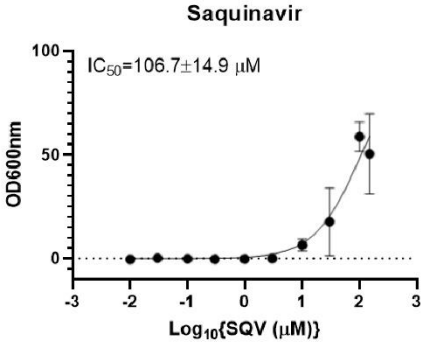


Figure 7 continued

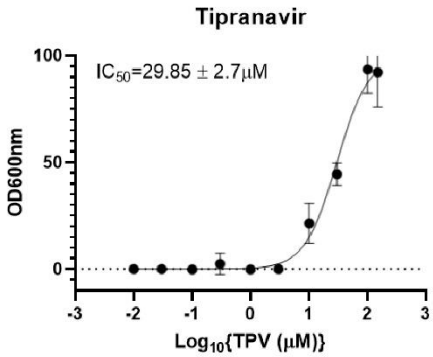
G



H



I



Protease Inhibitors target specific enzymatic protein cleavage by HIV-1 PR in fission yeast

In previous studies our lab has shown that $_{wt}$PR cleaved the indigenous MA↓CA (DSQNY↓PIVQ) viral targets in the fission yeast as it does in HIV-1 infection in mammalian cell (Benko et al., 2016). The aim with this experiment was to demonstrate the proteolytic effect of HIV-1 protease cleavage in the presence of protease inhibitors. Our lab developed a "GFP re-localization assay" that allowed us to specifically measure proteolytic activities of HIV-1 PR in fission yeast (Benko et al., 2016). Separation of HIV-1 PR cleavage appears predominantly as a "ring-like" structure localized on the nuclear membrane in fission yeast shown in **Figure 8** (Benko et al., 2017). GFP is used for fluorescent detection and it typically disperses throughout fission yeast cells (Benko et al., 2017). GFP enzymatic assay will be used as a confirmatory assay to the colony formation assay. In **Figure 9A**, the polypeptide substrate linker without the addition of the PR gene shows the "ring like" structure indicating no cleavage due to the absence of PR. With the addition of the PR gene at the bottom of **Figure 9A**, it demonstrates gene function being expressed with GFP being uniformly disturbed throughout the cell. In **Figure 9B**, all were tested in the condition of the polypeptide substrate linker with the addition of PR gene. We demonstrated PR cleavage at the enzymatic level in all nine of the protease inhibitors shown in **Figure 9B**. The "ring like" structure is present in all nine of the protease inhibitors. This confirms that the protease inhibitor effectively inhibited RE294 cell proliferation and PR cleavage by GFP localization in the nuclear membrane.

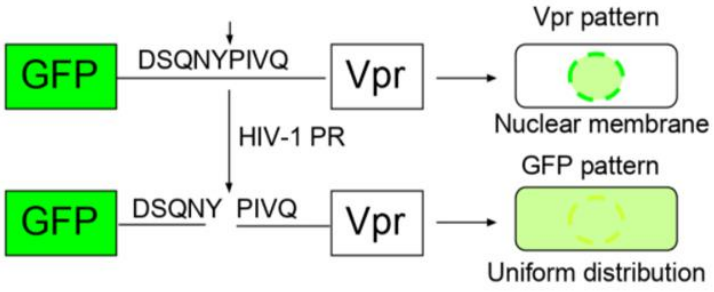


Figure 8**:** schematic drawing to show how the proteolytic test was designed to measure the HIV-1 PR-mediated cleavages of the GFP-MA-Vpr fusion protein constructs in fission yeast (Benko et al., 2017).

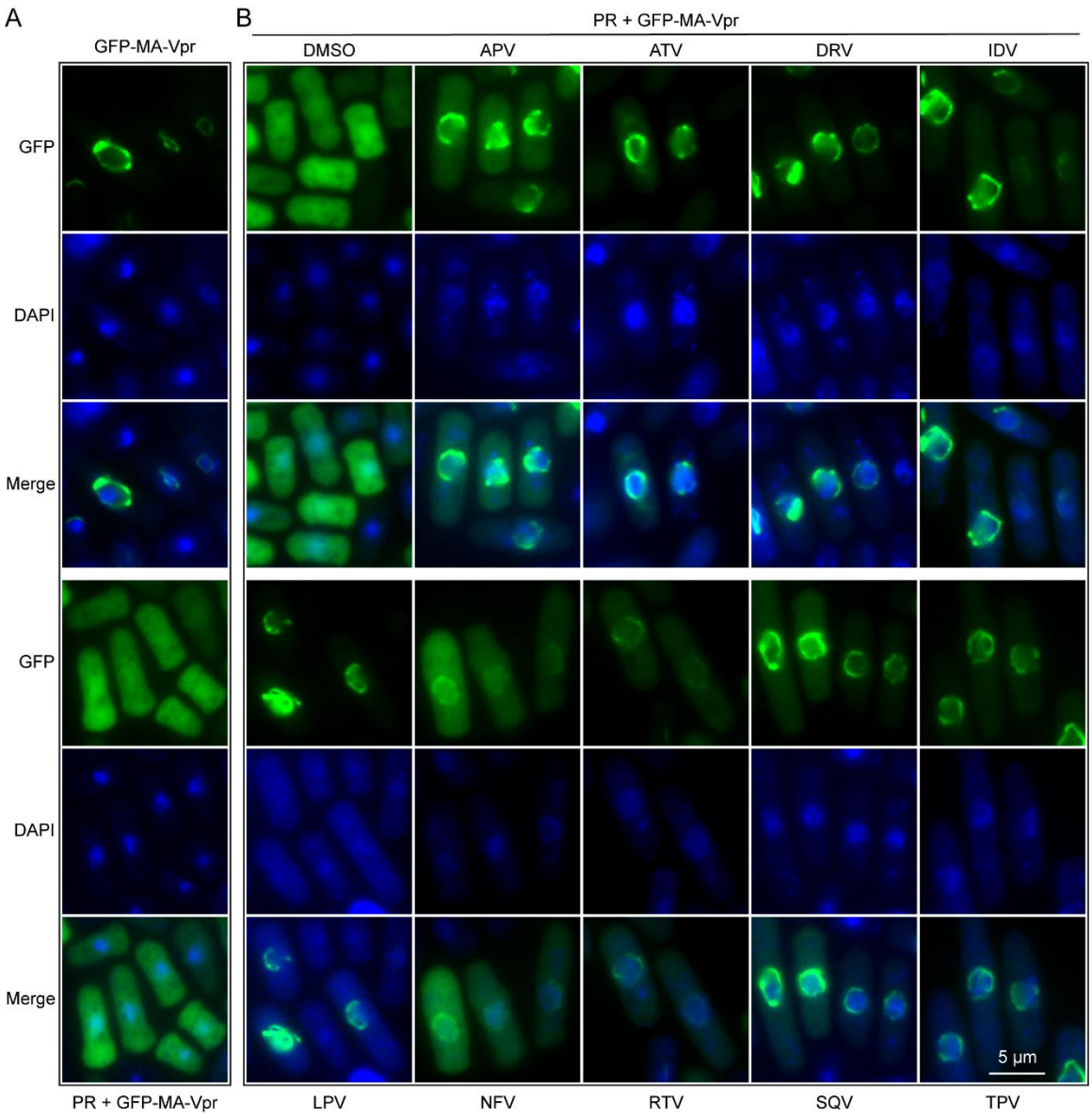


Figure 9: GFP enzymatic cleavage of PR induced by FDA protease (A) GFP and GFP cut1, $_{wt}$PR cleaved the indigenous MA↓CA (DSQNY↓PIVQ) (B) Protease Inhibitors PR enzymatic activity with GFP, DAPI, and a merge of the staining. Figure generated by Dr. Jiantao Zhang.

Discussion

In this study we were able to demonstrate the efficacy of the current approved protease inhibitors in a fission yeast cell-based system. PR gene integration in the fission yeast, RE294 cells, facilitated PR gene expression which was established in a previous study done by our lab (Benko et al., 2016). The fission yeast model is an established model system and makes it a great candidate for identifying potential anti-HIV therapeutics. We showed that in Batch one there were three compounds, 1-2, 1-6 and 1-10, that show minimal inhibition in the WT fission yeast. A dose-dependent IC_{50} curve was created to be as a comparison for more potential anti-HIV therapeutics and demonstrated the concentration efficacy for the current approved HIV therapies. Furthermore, we were able to show the PR-cleavage through the GFP enzymatic assay in the WT fission yeast. This demonstrated the mechanism by which the cell proliferation is facilitated. It will be key in identifying protease inhibitors from the other HIV-1 drug classes.

In the fission yeast cell growth assay minimal inhibition was observed in 3 of the compounds in Batch one of the small molecule compounds from our collaborator Dr. Nasr at NIAID. The minimal inhibition in batch one compounds, 1-2, 1-6 and 1-10 should now be evaluated in the mdr fission yeast strain. Although the inhibition is minimal, this information could be beneficial to our collaborator in the structural design of future small molecule anti-HIV drug candidates. A comparison between current FDA approved protease inhibitors and these compounds could have similarities further confirming the functional groups that aide in being an effective competitive inhibitor.

This data could further push us towards finding potential compounds that will be able to work in the M10 and M11 mdr fission yeast isolates.

The fission yeast cell-based system has been further established as a viable candidate for PI drug discovery. It was previously done using the wild-type and multi-drug resistant protease yeast strains using IDV. The presence of the PR gene in the wild-type and multi-drug in yeast strains was done by western blot in previous study conducted in our lab (Benko et al., 2017) When IDV was added to the agar plates producing $_{mdr}$PR and $_{wt}$PR with increasing concentrations colony formation was restored only on the $_{wt}$PR producing plate (Benko et al., 2017). This was possible through PR cleavage in WT fission yeast cell-based system visually shown through the GFP enzymatic assay. Expression without PR gene will appear in a "ring-like" structure and in the presence of PR gene GFP will separate from VPR due to protease cleavage and will appear uniform throughout (Benko et al., 2016). The success in demonstrating efficacy with IDV lead us to complete the PI drug class with this model system. The development of new anti-retroviral therapies is essential to address obstacles that contribute the multi-drug resistance. on RE294 fission yeast cells. Structure based design has led to the discovery of nine FDA approved PI's (Ali et al., 2010). This is the first time all nine FDA approved PI have been evaluated in the fission yeast cell model as opposed to the established data in mammalian cell lines.

In the future, the potential PIs discovered from this study should be confirmed in mammalian cells to confirm the same mechanisms are working as seeing fission yeast. A downfall of this study was that we were not able to show any efficacy on several compounds due to insolubility. In the future this should also be addressed as we are possibly missing out on some potential anti-HIV compounds. This data is also something

our collaborator's at NIAID can take in to account during the designing of future candidates. On our labs part, a different methodology might be ideal to deal with this occurrence and should be further evaluated. Also, multi-drug resistant isolates should be integrated into a mammalian cell line as well to be consistent across the board. This model system could potentially be effective to discover antiviral drugs against other viral disease as new viral diseases such as COVID-19 caused by SARS-CoV-2 are still emerging and drug resistance is still occurring.

Conclusion

In this study we were able to prove our hypothesis that the fission yeast cell based system could be used to minimally detect protease inhibition. All FDA-approved PIs inhibited wild type HIV-1 PR in fission yeast RE294 strain. Also, three small molecule compounds showed minimal inhibitory effects against wild type HIV-1 PR. However, none of the compounds were tested against ZB011, the M11 multidrug resistant PR. This study demonstrated the use of fission yeast cell-based system as a means to discover new PIs. However, more searches are needed to find PIs against multidrug resistant HIV-1 PRs such as M11. As stated previously, emergence of viral drug resistance is still a major issue and there is a continued need for more drug therapies.

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