Sabbatical Final Progress Report

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Prepared for TUMS research review committee

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Background:

Human immunodeficiency virus (HIV) infection leads to acquired immunodeficiency syndrome (AIDS), which has caused a pandemic that has led to major health challenges in developed, as well as developing countries. Despite the success of current antiviral therapy (ART), which can keep HIV genome copy numbers below detection level among infected patients, eradication of HIV has remained elusive.

The chief obstacle in achieving patient's cure through viral eradication is the ability of HIV to establish latency in target cells, predominantly in resting CD_4^+ T cells. HIV latency, which involves viral integration into host chromosome, allows virus to escape the immune response, as well as antiviral therapy. HIV latent cells may harbor replication-competent virus, but since they express no HIV antigens, they remain undetected by the immune response. Consequently, soon after discontinuation of HIV therapy, virus often rebounds, and HIV disease ensues.

Our laboratory investigates mechanisms that regulate proviral HIV latency, and has devised various genetic and epigenetic strategies to modulate HIV latency in infected T cells. These strategies include using HIV latency reversing agents (LRA), as well as latency promoting agents (LPA), which aim at ultimately regulating HIV latency and block activation of latent virus in tissue reservoirs of HIV-infected individuals. We envision that, in due course, control of clinical HIV latency will enable patient's immune system to overcome HIV disease, and deliver a

functional cure for AIDS. These strategies represent important novel approaches towards fighting the global AIDS epidemic.

List of Abbreviations

ADEH	L-aspartic acid dimethyl ester hydrochloride
ART	Anti-retroviral therapy
AIDS	Acquired immunodeficiency syndrome
EDTA	Ethylenediaminetetra acetic acid
eEF1α	Eukaryotic elongation factor 1 alpha
eGFP	Enhanced green fluorescent protein
FFA	Flufenamic acid
GLI	Glimepiride
HIV	Human immunodeficiency virus
LPA	Latency promoting agent
LTR	Long terminal repeat
mdHIV	Mini-dual human immunodeficiency virus reporter
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear Factor Kappa B
OME	Omeprazole
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative polymerase chain reaction
RES	Resveratrol
RPMI-1640	Roswell Park Memorial Institute 1640 media
shRNA	Small hairpin RNA
TAR	Trans-activation response
TAT	Trans-activator of transcription
TNFα	Tumour necrosis factor alpha

Research Final Progress Report:

My research project involved understand the genetic and epigenetic regulation of HIV-LTR transcription and examining the activity of several Latency Promoting Agents (LPAs) towards

studying various aspects of "Lock & Block" strategy to control HIV latency. These research efforts will ultimately help for treatment of patients with latent infection with HIV. My project focused on several LPAs that can selectively control HIV-LTR activity of latently infected T cells. Our initial studies had delineated the epigenetic factors that play pivotal roles in giving rise to differences in latency patterns among various populations of HIV–infected CD4 (+) Jurkat T cell clones. In addition, we have developed several cellular clones of Jurkat-tat (J-Tat) cells, which express HIV trans-activating protein (tat) protein constitutively. Tat protein is a HIV trans-activating protein important in transcription of HIV-LTR and generation of full-length mRNA for productive HIV replication. So far, an HIV reporter construct has been developed in our lab for use in flow cytometric analysis. Figure 1 shows a double-labeled HIV reporter mini-virus (mdHIV), which has the green fluorescence protein (GFP), and dsRed to indicate HIV integration as well as HIV-LTR transcription activity, respectively. The GFP label in mdHIV is under control of elongation factor (eEF-1a) promoter, and dsRed expression is under control of HIV-LTR promoter. The mdHIV virus allows for expansion of productively HIV-infected cells, with no apparent toxic effect. In addition, our lab has grown and cultured 50 distinct clones of J-tat cells and has found that each clone responds differently to various viral and cellular activators.

The main goal of my project was to advance our knowledge of HIV latency in transformed T cells *in vitro,* and initially my strategy focused on using well-characterized clones of transformed CD4 + T Jurkat-tat cells as a model to study specific epigenetic aspects of HIV latency. To determine the clinical relevance of findings, ultimately, these observations made using transformed cell cultures will be compared and verified in primary CD4+ T cells, obtained from peripheral blood mononuclear cells (PBMC). Below, the following has been achieved in my research project which has made good progress, and we anticipate the results will be published in the next several months.

shRNA screen for latency associated genes: Our team carried out shRNA mediated gene knockdown, which involves introduction of small interfering RNAs (siRNAs) into cultures HIV-infected T lymphocyte clones to efficiently knock down expression of 4,600 target genes for which modulating FDA-approved drugs are available. These experiments were aimed at assessing the differences in expression of the target genes among latent cells versus actively infected cells. shRNA constructs were used for long term, stable gene silencing and lentiviral MISSION® TRC (The RNAi Consortium) was used for delivery of shRNA, since lentiviruses readily integrate the

shRNA into the genome to achieve stable gene silencing with less biased solution than clonal selection.

VSV-G envelope pseudotyped lentiviral particles contained shRNA and efficiently transduce lentiviral particles into mammalian cell lines. The amount of lentiviral particles needed for transduction was optimized by using the MISSION® TurboGFP control transduction particles, which express a green fluorescent protein marker used to monitor experimental design and serves as a positive control. Quantitative Real-Time PCR (qPCR) assay was used to evaluate target gene expression and knockdown. qPCR assay for mRNA transcript assay was used to quantitate amplified products using fluorescent reporter molecules. As in conventional PCR, target DNA templates were amplified and at each cycle, fluorescent signals were monitored real time for relative quantification. Our team analyzed and compared cellular gene expression among actively HIV infected versus latent cells in three mdHIV infected Jurkat-Tat (J-Tat) clones 11, 90, and 131. The gene expression analysis compared the 3 subpopulations of cells that display active (+/+), silenced (-/-), or latent (+/-) flow cytometric profiles. The fluorescence of the reporter molecules is measured and quantified by **qPCR analysis** ; typically using SYBR® Green that intercalates between the bases in double-stranded DNA, or probes designed to bind a specific sequence on the DNA. There are two major quantification methods for qPCR data; relative quantification, the more common method, uses ΔΔCt information, by which the expression or abundance ratio of the target gene in the sample is determined, compared to a control gene, and normalized with the expression ratio of a reference gene. Since, the efficiency E values for target and reference genes differ, this method also accounts for the differences in E values, and is commonly employed in gene expression analysis.

My project was a part of ongoing comprehensive project in our laboratory, which investigates the various aspects of the molecular mechanisms of HIV latency, and it involved examining HIV latency promoting activity (LPA) in J-Tat lymphocytes infected with a VSV-pseudotyped minivirus double-labeled HIV (mdHIV) construct. This mdHIV construct has the green fluorescent protein (eGFP) gene, as well as dsRed gene to indicate HIV integration and LTR activity, respectively. The mdHIV eGFP gene expression is under control of eEF-1a promoter, whereas its dsRed gene is under control of HIV-LTR promoter. The mdHIV model allows for the long-term culture and expansion of latently HIV-infected J-Tat cells, which are analyzed by flow cytometry, without apparent cytopathic effects.

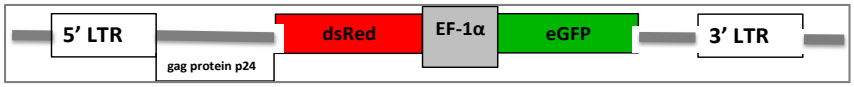


Figure 1. The minivirus double-labeled (mdHIV) reporter construct with gag and LTRs intact.

As shown in Figure 1, mdHIV has been developed for identification and characterization of latently infected cells using mdHIV capable of a single cycle of replication. FACS analysis of J-Tat cells revealed that cells infected with mdHIV respond at various degrees to stimulation by substances like phorbol myristate acetate (PMA) and Ionomycin (IO). Below is an illustration of the analysis methodology (Figure 2A) and four representative J-Tat clones, which show the various patterns of HIV activation and label expression in infected cells after treatment with PMA, as compared with mock-treated cells (Figure 2B).

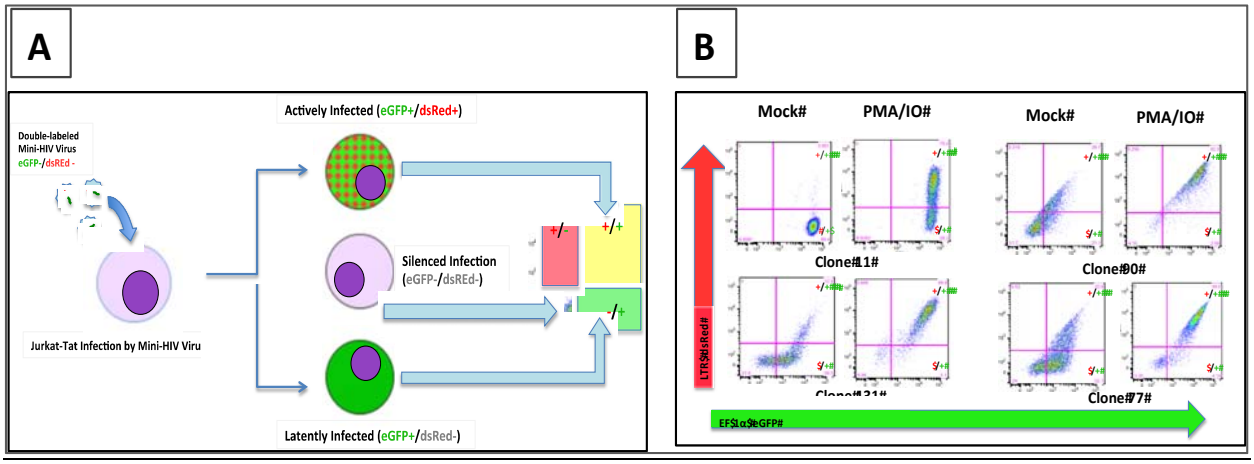


Figure 2. **A**. Schematic illustration of flow cytometric analysis of Jurkat-Tat cells infected with mdHIV. **B.** Representative J-tat clones showing a diverse HIV expression profile due to variation in LTR promoter activity induced by PMA/IO (phorbol myristate acetate/ionomycin).

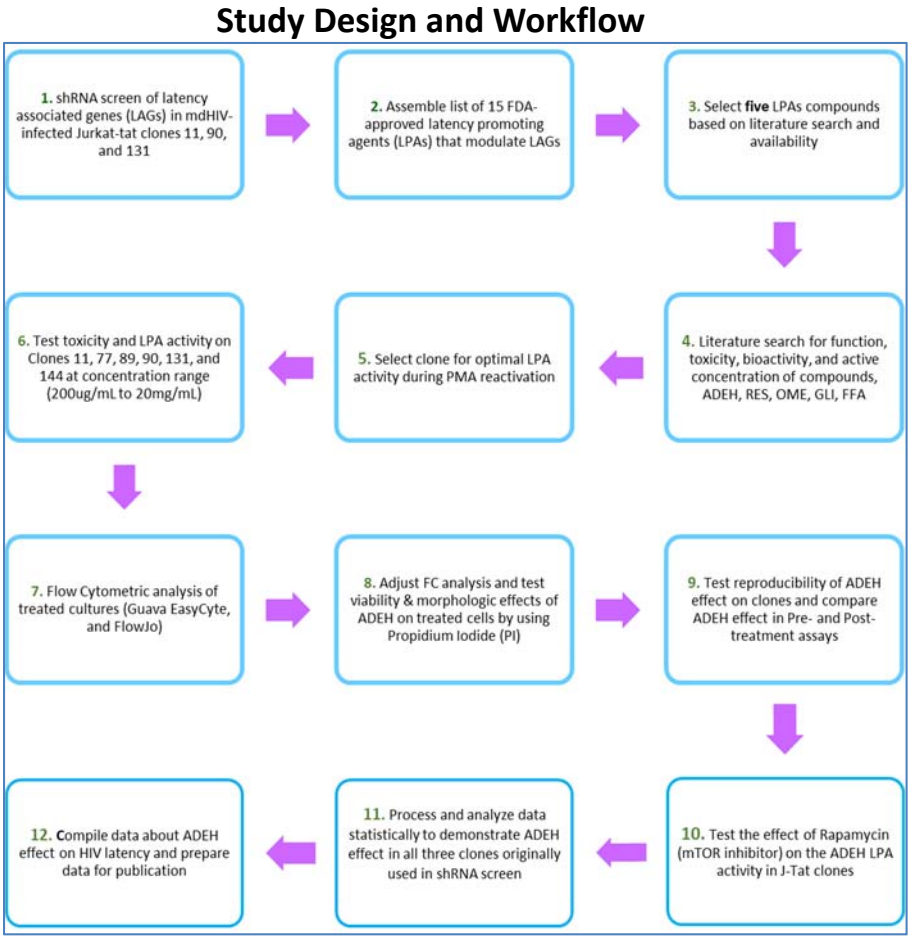


Figure 3. Study design and workflow for investigation of latency promoting activity (LPA) of five agents.

Figure 3 demonstrates our current strategy and workflow of our project, which includes the essential steps involved towards achieving the main goals of my project.

A summary of goals that were achieved is shown below:

1) Literature search regarding active concentration, solvent, so forth**. Table 1** summarizes the results of this search.
2) Cultured J-tat clones 11, 89, 90, 131, and 144 to obtain complete flow cytometry (FC) profile and establish reproducible basal levels of mdHIV latency in each clone
    a. Prepared stocks of these clones in liquid Nitrogen for future use.
3) Established the FC profile of the above J-tat clones at PMA-induced as well as basal (mock-treated) conditions in order to confirm that latent mdHIV is inducible at various PMA concentrations in these clones.
    a. *In vitro* reactivation of mdHIV were carried out by PMA, Ionomycin (I/O), and TNF-a, in enriched RPMI-10 medium.
        i. Tested 4h vs 24h treatment for PMA and TNF-a
            1. In most of these experiments, TNF-a did not show a strong effect, probably due to degradation of TNF-a preparation. Later, research verified that it had expired and was no longer active.
        ii. Tested 24h treatment for various combinations of I/O plus PMA at different

concentrations ranging between and 50nM and 5nM

iii. Tested LPA activity of Omeprazole (OME), Resveratrol (RES), Aspartic acid analog (L-Aspartic acid dimethyl ester hydrochloride; ADEH), Flufenamic acid (FAF), and Glimepiride (GLI).
iv. Tested various concentrations of the LPAs on several clones
v. Tested two various LPAs incubation times on two J-Tat clones
vi. Determined the optimal conditions for LPA activity by testing simultaneous, Pre- and Post ADEH treatment of cultures prior to PMA induction.

4) We held three in person meetings, and two virtual meetings with all research team members to decide on the following; (daily meetings with individual members were also held)
    i. Presented, reviewed, and discussed the preliminary data
    ii. Discussed strategy and experimental design
        1. Decided on the direction of project for the next 3 months.
        2. Selected ADEH for detailed LPA activity study for the next few months
            a. The LPA compound ADEH was selected for the focus of the study, because Aspartic acid in critically involved in mTOR cellular internal signaling pathway, which is a main area of interest in our laboratory.
        3. Checked the LPA activity OF ADEH on RGH HIV in Jurkat cells (acute infection model).

5) I also tested the LPA activity of OME using both latent mdHIV Jurkat cell models.

Table-1. List of potential latency promoting agents (LPAs) investigated in this study.

No	Gene	Full Name	Reagent	Activity	Solubility, stability and storage	Reference	Cell type	Concentration	Solvent
1	PTGS1	Prostaglandin-Endoperoxide Synthase 1	Resveratrol (RES)	Inhibit	Solubility: 16mg/mL (sigma), 50mg/mL (Cayman chemicals), 65mg/mL (ENZO) in DMSO, insoluble in water. Stability in solution: stable for 3 months in DMSO at -20°C, store in the dark	2016	Jurkat	<25µM not toxic,	DMSO
						2002	Jurkat	IC50=13.4µM (caused a 50% drop in proliferation)	Not Known
2	PANX 1	Pannexin-1	Flufenamic acid (FAF)	Inhibit	Solubility: 40mg/mL (Cayman), 28mg/mL (TOCRIS) in DMSO; Stability in solution: 2 years in DMSO at -80°C (SelleckChem)	2017	T84 cells (colon cancer)	5-50µM	DMSO
						1999	LnCaP (cell line)	50-100µM	DMSO
3	CLCN 2	Chloride voltage-gated channel 2	Omeprazole (OME)	Inhibit ?	Solubility: 19mg/mL (sigma), 30mg/mL (Cayman) in DMSO Stability in solution: 2 years in DMSO at -80°C, photosensitive, hydroscopic	2007	Jurkat	0-200µM	Normal saline (0.9% NaCl)
						2004	Jurkat	100-1000µM	PEG, NaHCO3
4	KCNJ 1	Potassium rectifying channel subfamily J1	Glimepiride (GLI)	Inhibit	Solubility: >10mg/mL (sigma), 3mg/mL Stability in solution: 2 years in DMSO at -80°C (SelleckChem)	2014	RAW 264 (mouse macrophage cell line)	0.3-5µM	proposed DMSO
5	ASPA	Aspartoacylase	Aspartic acid (A), or Analog, L-Aspartic acid dimethyl ester hydrochloride; (ADEH)	Inhibit ?	Solubility: 5mg/mL in water, 50mg/mL in 0.5M HCl (with heat) or 1M NaOH (sigma), 8mg/mL in water, INSOLUBLE IN DMSO	2016	HepG2, HLE (hepatoma) Huh-7	1mM, 2mM, 3mM	proposed DMSO

To assess whether a 2h pre-treatment of Aspartic acid analog, ADEH (10mM) has any effect on the viability of J-Tat cells, culture samples were analysed by flow cytometry (FC) after a 24h incubation with 5nM PMA. Prior to analysis, samples were treated with FC buffer containing phosphate buffered saline (PBS), 1% fetal calf serum (FBS), 1% EDTA, and 1:10,000 propidium Iodide (PI). Figure 4 shows the comparison of FC profiles of the latently infected J-Tat clone 131 cells that have been induced with 5nM PMA for 24h at 37C after PI staining. Using FlowJo^{R} software, cells stained with PI (i.e PI^{+} cells) were excluded from analysis, because dead cells commonly stain more rapidly and stronger with PI than the live cells. As shown in Figure 4, data

suggests that ADEH treatment does not change the viability of HIV infected J-Tat clone 131 cells, but changes the morphology and gating of these cells. Therefore, from that point on, PI staining was established as a routine procedure, prior to all FC analysis in this project.

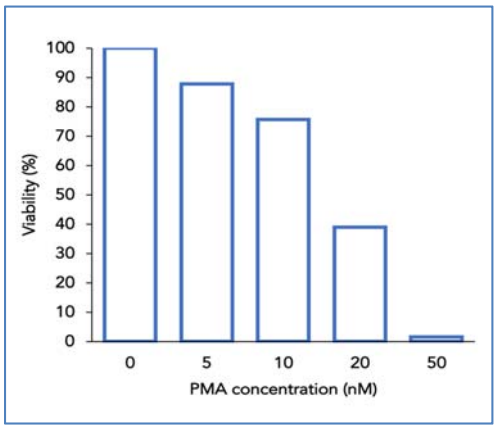


FIGURE 4. Assessment of PMA toxicity. Clone 77 was treated with 0-50nM PMA for 24h and viability measured with trypan blue on a BioRad cell counter. 50nM PMA resulted in almost no viable cells while cultures treated with 5-10nM PMA maintained viabilities > 75%. Values are normalized to the untreated sample which was set to 100%.

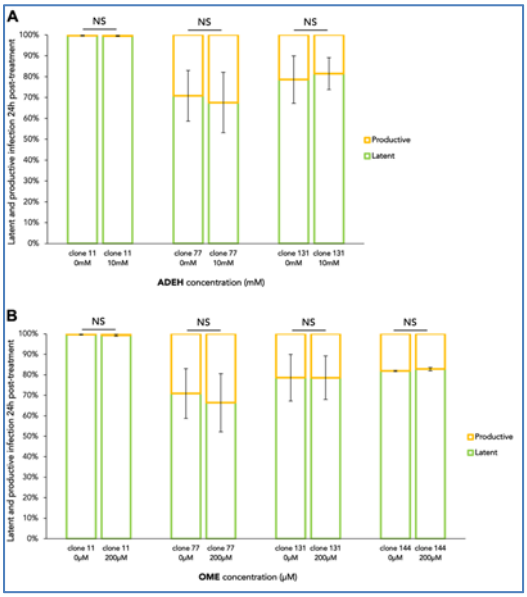


FIGURE 5. Verification of effects of ADEH and OME on basal activity in Jurkat-Tat clones. (A) ADEH or (B) OME treatments had no effect on fluorescence and were not significant (NS), P>0.05. Error bars represent standard error of the mean (SEM). All samples were compared with untreated controls, and analyzed using a two-sample t-test assuming equal variances.

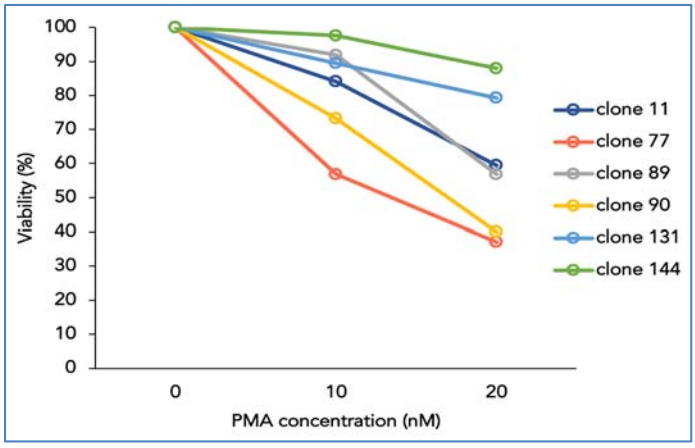


FIGURE 6. Assessment of clone-specific sensitivity to PMA. Clones 11, 77, 89, 90, 131, and 144 were assessed for their reaction to PMA at 0, 10, and 20nM. Clones 131 and 144 maintained the highest viabilities (> 80%), while clones 77 and 90 demonstrated the greatest sensitivity to PMA. Values are normalized to the untreated control which was set to 100%.

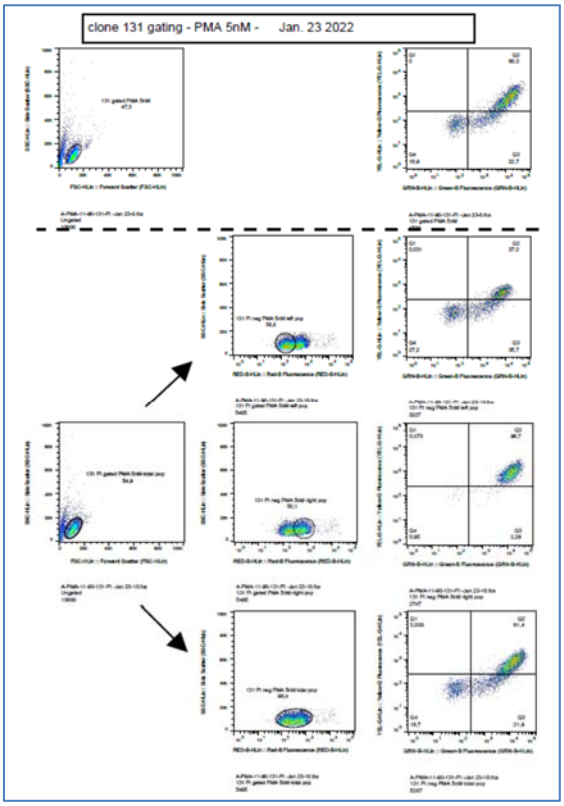


Figure 7. Comparison of flow cytometric analysis of cells of J-Tat clone 131 after treatment with 10mM of aspartic acid analog (ADEH) after a 2h pre-treatment. Profile of various sub-populations are shown using Propidium Iodide exclusion gating parameters.

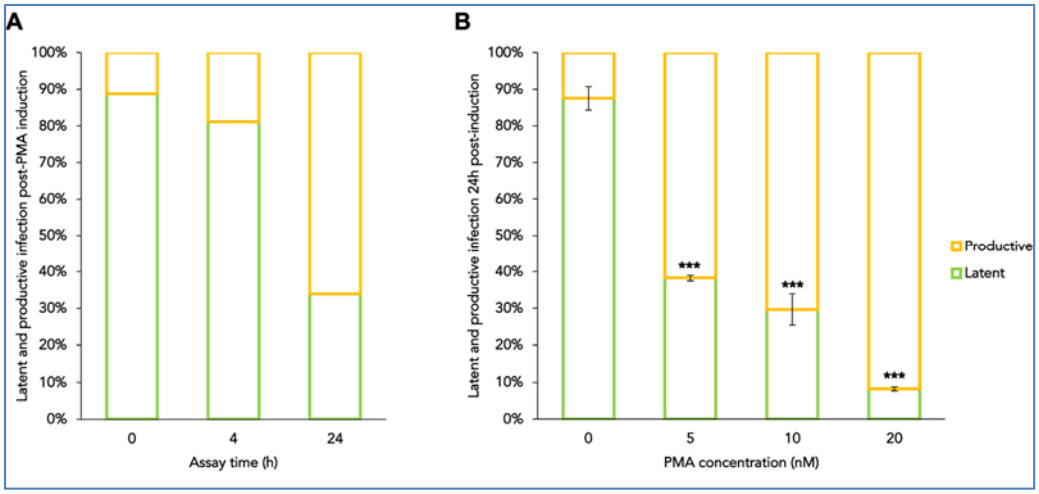


FIGURE 8. Time and dose-dependent induction by PMA. (A) Time-course assay of PMA induction at 10nM in clone 77. 4h did not yield sufficient induction of productive infection. (B) Dose response of clone 77 to PMA induction. Cultures were treated with 5, 10, or 20nM PMA and fluorescence read after 24h. All concentrations yielded significant induction, however, 20nM also caused noticeable toxicity and 5 or 10nM PMA induced HIV with minimal toxic effects. Graphs indicate the proportion of productive infection (dsRed+/eGFP+) relative to latent infection (dsRed-/eGFP+) as shown in yellow and green, respectively. Error bars represent SEM, and a two-sample t-test was used to compare PMA and untreated samples assuming equal variances. ***P<0.0005.

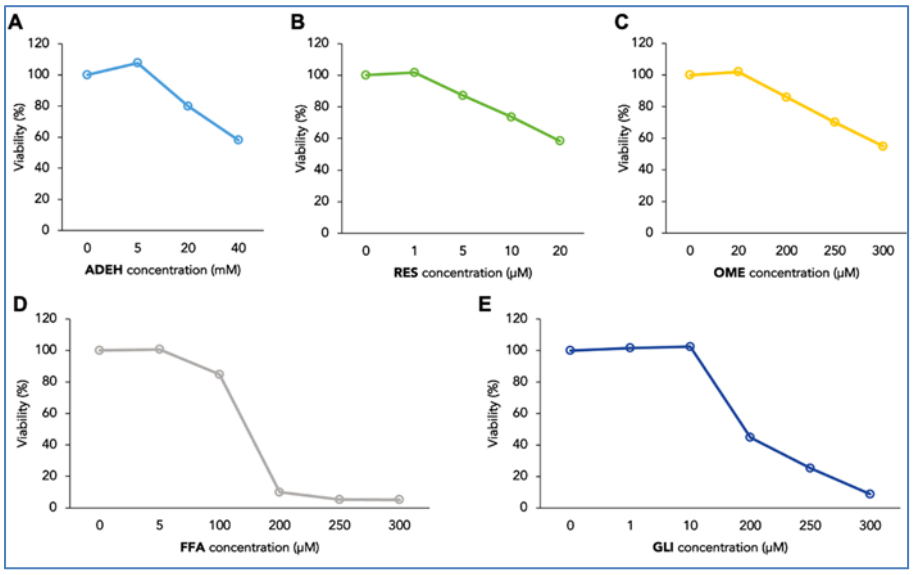


FIGURE 9. Toxicity of LPA agents on clone 144. (A) ADEH 0-40mM. (B) RES 0-20μM. (C) OME 0-300μM. (D) FFA 0-300μM. (E) GLI 0-300μM. Treatments that resulted in viabilities greater than 75% were considered non-toxic levels, which included ADEH at 5-20mM, RES at 1-5μM, OME at 20-200μM, FFA at 5-100μM, and GLI at 1-10μM.

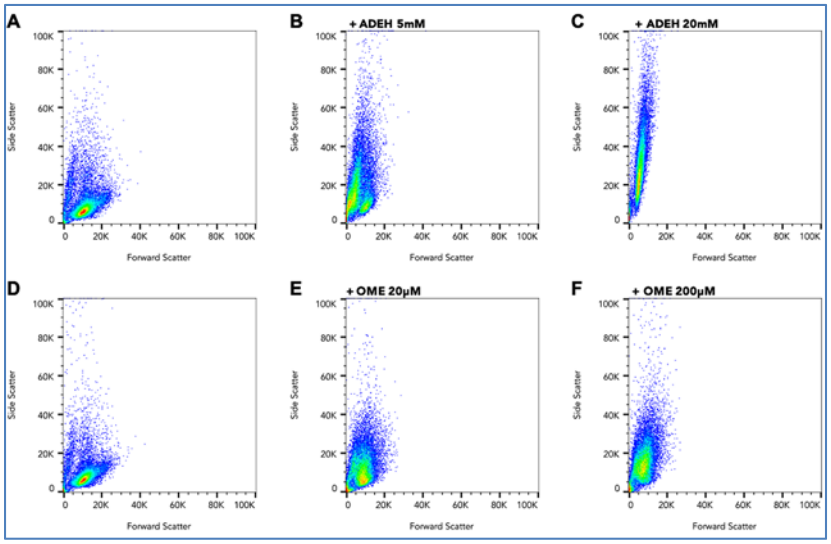


FIGURE 101. The effect of ADEH or OME treatment on gating of cells during FC analysis. ADEH and OME alter the morphology of cells. (A) Untreated clone 144 cells show typical pattern of side scatter and forward scatter dispersion. (B) Treatment with 5mM ADEH causes reduced forward scatter, i.e., reduced size. (C) Treatment with 20mM ADEH decreases forward scatter and increases side scatter, i.e., increased granularity and reduced size. (D) Untreated clone 144 with usual pattern of side and forward scatter. (E) Treatment with 20µM OME also alters profile. (F) Treatment with 200µM OME causes profile changes and obscures gating selection.(challenge)

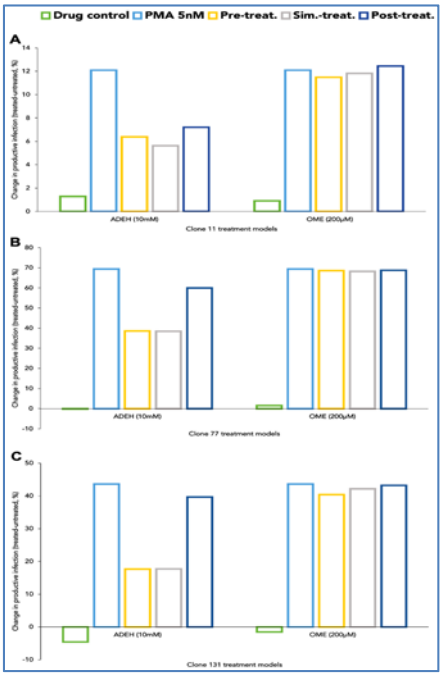


FIGURE 11. Assessment of alternate treatment models. Controls were either treated with 10mM ADEH, 200µM OME, or 5nM PMA alone and activity measured 24h later. Experimental samples either underwent a 2h pre-treatment with ADEH or OME followed by stimulation with PMA (Pre-treat.), simultaneous treatment with ADEH or OME and PMA (Sim.-treat.), or PMA stimulation for 2h followed by addition of ADEH or OME (Post-treat). All values are depicted as proportions of latent infection and productive infection events and are normalized to an untreated control (set at 0 on graph). (A) clone 11, (B) clone 77, (C) clone 131.

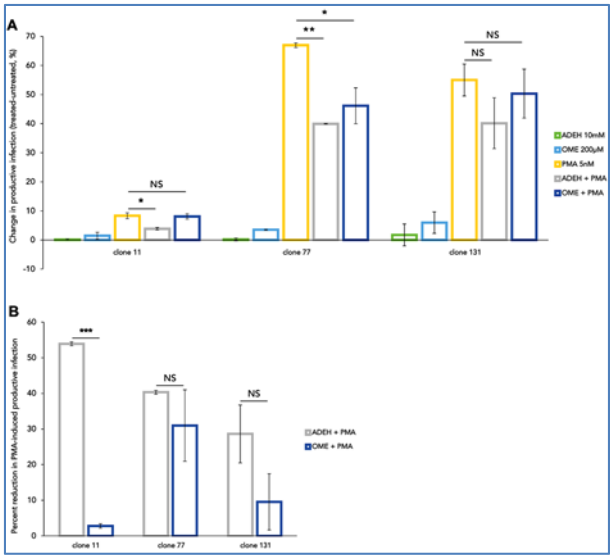


FIGURE 12. Prevention of PMA-mediated activation of mdHIV clones by preincubation with ADEH or OME. (A) Change in productive infection frequencies measured by eGFP and dsRed fluorescence, proportionate to the total of productive and latent infection events. Stimulation by 5nM PMA shows the greatest increase in productive infection while treatment with 200µM OME or 10nM ADEH alone causes no significant difference. Preincubation with ADEH or OME shows some prevention of activation by PMA. (B) Data shown as percent reductions in PMA-induced infection from panel A. Pre-treatment with ADEH shows higher activity than OME at preventing a shift towards mdHIV productive infection. Error bars represent SEM, and comparisons performed with a t-test assuming equal variances. NS, not significant. *P<0.05, **P<0.005, ***P<0.0005.

In order to measure the LPA activity of ADEH and Omeprazole (OME) on PMA-activated J-Tat cells, clone 77 and 131 cultures were pre-treated with either ADEH (10mM), or OME (200uM) for 2 hours prior to PMA (5nM) induction. After 20-24 hours, culture samples were harvested, centrifuged (3min at 2500rpm). After decanting the sample supernatant fluid, the cellular pellets were then re-suspended in 500uL of FC buffer with PI (1:10,000), and analyzed by EasyCyte (Guava) Flow Cytometer. The effect of re-incubation of ADEH and OME in cultures of clones 11, 77, and 131 cells as percentage shift of activated (+/+) cells to latent (+/-) as a proportion of the PMA-activated cell population, the LPA activity. Regarding project goals, the data for two compounds (ADEH and OME) on three J-tat clones have been processed and compiled during the past nine months. Additional experiments are underway to investigate the LPA link to mTOR cell signaling pathway in Jurkat as well as J-Tat cells. Our team plans to investigate the following aspects of the project in near future:

1. Rapamycin activity effect on the LPA activity.
2. Effect on infected Jurkat-Tat cells acutely infected with RGH virus.

In order to assess whether a 2h pre-treatment of Aspartic acid analog, ADEH (10mM) has any

effect on the viability of J-Tat cells, culture samples were analysed by flow cytometry (FC) after a 24h incubation with 5nM PMA. Prior to FC analysis, samples were treated with FC buffer containing phosphate buffered saline (PBS), 1% fetal calf serum (FBS), 1% EDTA, and 1:10,000 propidium Iodide (PI). Figure 13 shows the comparison of FC profiles of the latently infected J-Tat clone 131 cells that have been induced with 5nM PMA for 24h at 37C after PI staining. Using FlowJo^{R} software, cells stained with PI (i.e PI^{+} cells) were excluded from analysis, because dead cells commonly stain more rapidly and stronger with PI than the live cells. As shown in Figure 4, data suggests that ADEH treatment does not change the viability of HIV infected J-Tat clone 131 cells, but changes the morphology and gating of these cells. Therefore, for the remaining part of this project, PI staining was established as routine procedure, prior to all FC analysis.

To measure the LPA activity of ADEH and Omeprazole (OME) on PMA-activated J-Tat cells, clone 77 and 131 cultures were pre-treated with either ADEH (10mM), or OME (200uM) for 2 hours prior to PMA (5nM) induction. After 20-24 hours, culture samples were harvested, centrifuged (3min at 2500rpm). After decanting the supernatant fluid, the cellular pellets were then re-suspended in 500uL of FC buffer with PI (1:10,000), and analyzed by EasyCyte (Guava) Flow Cytometer. Figure 13 demonstrates representative FC profile of samples from cultures that were treated with either ADEH (10mM), or OME (200uM) versus mock-treated culture samples. As shown in Figure 14, data indicates that both ADEH and OME have some latency promoting activity (LPA) in clone 77, as well as clone 131 cultures that were pre-treated for 2 hours. LPA is defined by marked reduction in percentage of activated (+/+), concomitant with an increase in percentage of latent (+/-) cells in a culture sample's FC profile, as compared to mock-treated (untreated) cultures. The LPA effect was detected under both 5nM and 20nM PMA induction conditions. Importantly, LPA activity of ADEH and OME were prominent only in PMA stimulated cells, and there was minimal LPA activity in latently infected J-Tat cells that were treated with ADEH or OME alone.

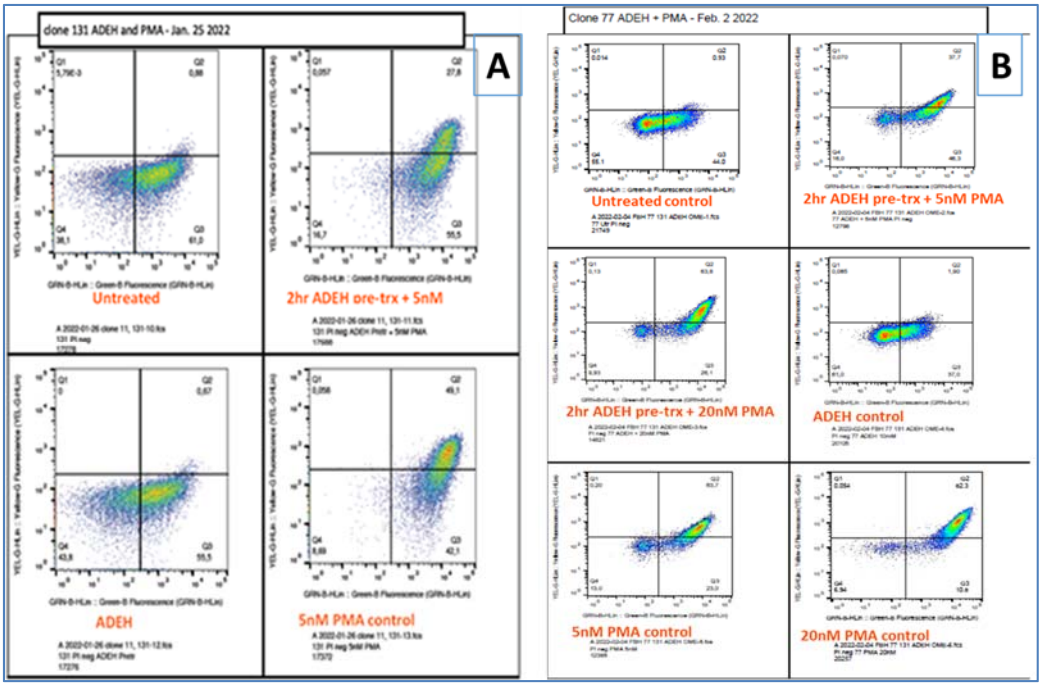
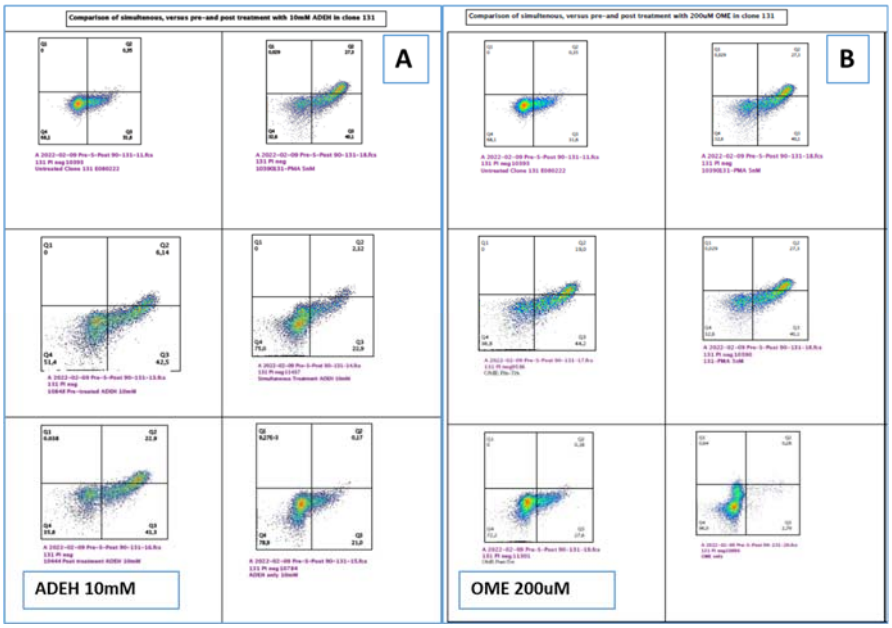


Figure 13. Comparison of LPA activity of ADEH in **(A)** Clone 77 and **(B)** Clone 131 cells. Flow Cytometric (FC) profile of mdHIV-infected J-Tat clones 77 **(B)** and 131 **(A)** cells pre-treated with either ADEH (10mM) for 2h prior to induction by a 24h PMA (5nM or 20nM) treatment.

Moreover, to assess the optimal conditions and timing for ADEH or OME LPA activity in J-tat cells, clone 131 cultures were pre-treated with either ADEH (10mM) and OME (200uM) for 2h prior to a 24h reactivation with PMA (5nM). FC profile of samples were compared with samples from cultures that were simultaneously, or post-treated (2h) with the same concentrations of ADEH (Fig. 6A) or OME (Fig.6B). Data shown in Figure 14 indicates that although 10mM ADEH has some level of LPA activity under all three conditions, ADEH pre-treatment shows the maximum or optimal LPA activity (Fig. 6A). Likewise, pre-treatment with OME was the optimal condition for detection of LPA activity in clone 131 cells (Fig. 6B). To ensure reproducibility, these preliminary observations are currently being repeated and verified by my research teammates. In addition, they plan to use various *in vitro* models, including RGH-HIV acute infection, and primary CD_4^+ T lymphocyte models, to verify the validity of the LPA activity under various conditions.

a.



b.

Figure 14. Comparative flow cytometric analysis of the LPA activity of ADEH **(A)** and OME **(B)** mdHIV infected J-Tat clone 131 cultures. FC profile of mock-treated cultures were compared with cultures treated simultaneously with either ADEH (10mM) and OME (200uM), as well as the pre- and post-treated cultures after a 24h reactivation with PMA (5nM).

Below, Figure 15 summarizes data from several experiments testing the LPA activity of ADEH and OME in cultures of three different J-Tat clones (11, 77, and 131). Data is presented in terms of percentage of activated +/+ cells (Q2 in FC profile) in pre-treated cultures, after stimulation of the respective cultures with either 5nM or 20nM of PMA for 24h. The percentage of cells with activated HIV-LTR (+/+) is a markedly reduced after pre-treatment with either ADEH (10mM) or OME (200uM), in all three clones. The highest and lowest percentage reduction of (+/+) cells was detected in clones 77 and 11, respectively. ADEH and OME also displayed a prominent LPA effect in clone 131 cultures by reducing the percentage of the activated HIV-LTR (+/+) cells.

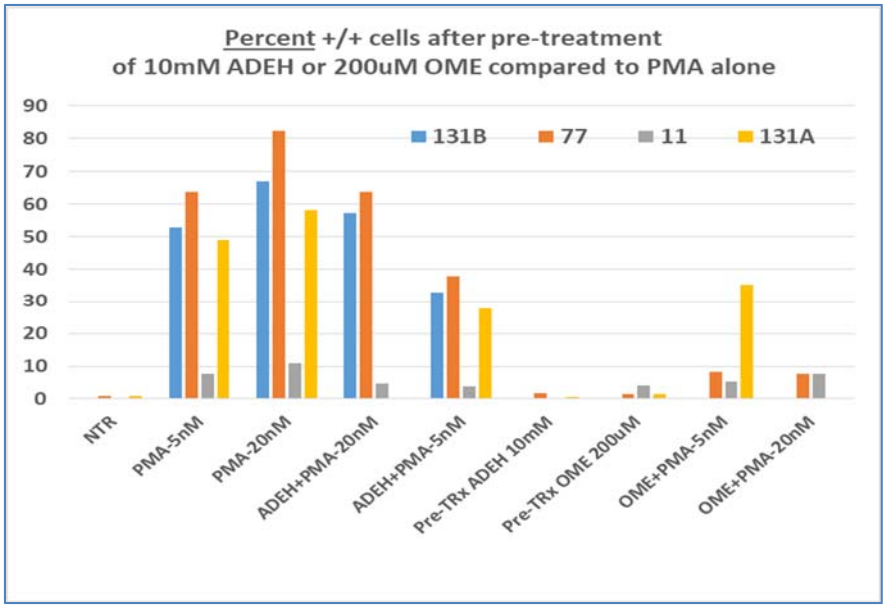


Figure 15. Comparison of LPA activity of Aspartic acid analog (ADEH) and Omeprazole (OME) in dmHIV-infected J-Tat clone 131 cultures pre-treated with ADEH or OME for 2 hours prior to a 24h PMA (5nM) induction.

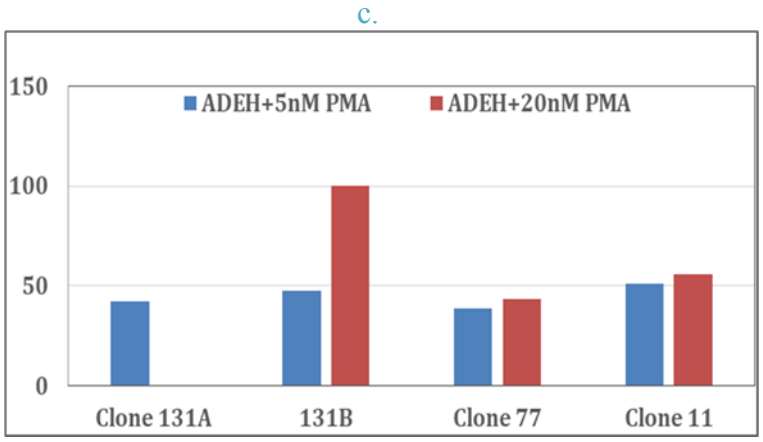


Figure 16. Comparison of percent reduction of activated +/+ cells (Q2 in FC profile) in various mdHIV-infected J-Tat cultures as an indicator of LPA activity of ADEH after a 24h induction by either 5nM or 20nM PMA. Three different J-Tat cultures (clones 11, 7, and 131) underwent a 2h pre-treatment ADEH (10mM) prior to PMA induction. Samples 131A and 131B represent data from two separate experiments.

Figure 16 summarizes the LPA activity of ADEH and OME in cultures of clones 11, 77, and 131 cells as percentage shift of activated (+/+) cells to latent (+/-) as a proportion of the PMA-activated cell population, i.e. a Q2 to Q3 % shift in FC profile. Data is presented for ADEH pre-treated cultures, after 24h stimulation of each culture with either 5nM or 20nM of PMA. As shown in Figure 8, the LPA activity of ADEH is similar in clone 11 and 77 cells at about 45 and 50%, respectively, while similar LPA activity is detected both 5nM as well as 20nM PMA stimulation for

both clones. However, for clone 131 cultures, ADEH shows almost 100% LPA activity under 20nM PMA induction, (Figure 8). Since clone 11 and 131 cells were among the cultures that were used in the original shRNA screen, they remain the focus of further studies in the coming months.

Data regarding the LPA activity of two agents (ADEH and OME) on three J-tat clones have been processed and compiled. Additional experiments are underway to investigate the LPA link to mTOR cell signaling pathway in Jurkat as well as J-Tat cells. Furthermore, *in silico* analysis regarding LPA activity of ADEH on additional clones, in underway. My laboratory team has planned additional experiments, including further *in silico* analysis regarding LPA activity of ADEH on other clones, such as clone 90, and effects on primary T cells and PBMC's. Ultimately, these research efforts will help treatment of patients with latent infection with HIV and control of AIDS pandemic. My senior research advisor (Dr. Ivan Sadowski) plans to publish the results of my research project in the near future.

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I really appreciate your continuous support and kind consideration.

Applicant Name and signature

Farhad Bonakdar Hashemi, PhD

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