RRI- LFKRI

Lipid-conjugated HIV-1 Fusion Inhibitor Exhibits Enhanced Potency and Increased Serum Half-life

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Research Plan

A. Question or Problem being addressed

Human immunodeficiency virus (HIV) is the pathogen that causes acquired immune deficiency syndrome (AIDS). Introduction of highly active anti-retroviral therapy, which uses a combination of drugs, has had a remarkable impact on the AIDS epidemic by converting it to a manageable illness from what was once considered a fatal disease (Becerra, 2016). Despite this success, more than 36 million people are currently living with AIDS and nearly 2 million people get newly infected each year (www.unaids.org). The major obstacle in making an appreciable dent in this high infection rate is drug resistance, side effects of the drugs and patient compliance (Vandamme, 1999). Therefore, there is an urgent need to develop new drugs, especially against targets critically important for HIV-1 life cycle, particularly entry into the target cell (Figure 1). HIV entry into the target cell is initiated by the binding of the viral envelope glycoprotein (Env) surface subunit gp120 to the receptor CD4 and a coreceptor (CCR5 or CXCR4) on the target cell, triggering a cascade of conformational changes of the transmembrane subunit gp41 (Eckert, 2001, Colman, 2003). The C-terminal heptad repeat (CHR) and N-terminal heptad repeat (NHR) of gp41 interact with each other (Figure 2) to form a stable six-helix bundle (6-HB), in which three CHR helices pack into the

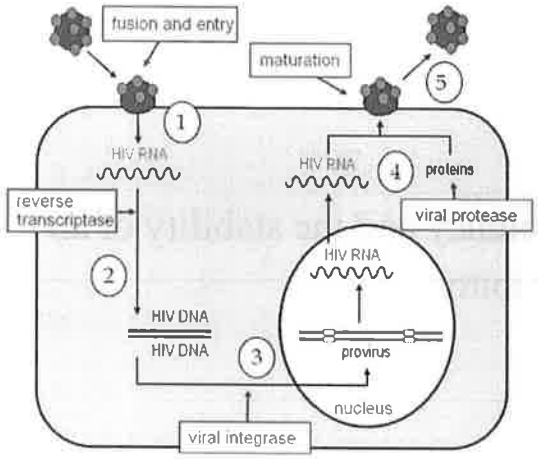

Figure 1. Drug Targets of HIV Life-cycle

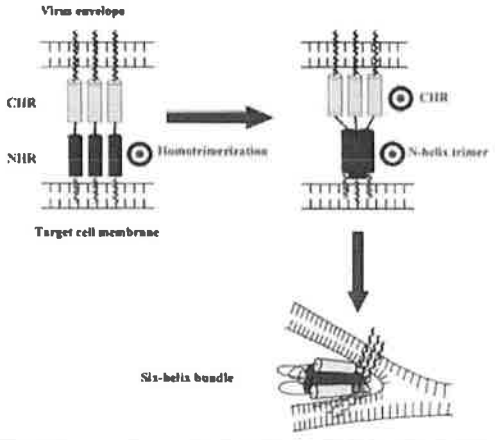

Figure 2. Formation of 6-HB during viral entry

hydrophobic grooves on the NHR trimer core in an antiparallel way to pull viral and target cell membranes into proximity for fusion (Chan, 1997, Weissenhorn, 1997). The crystal structure of the 6-HB identified a deep pocket on the C-terminal portion of NHR helices, which is penetrated by three hydrophobic residues from the pocket-binding domain (PBD) of the CHR helix (Tan, 1997). This pocket region critically determines the stability of the 6-HB core and the fusogenic activity of gp41, which offers an ideal target site for anti-HIV fusion agents (Chan, 1998). Peptides corresponding to both the NHR and CHR peptide regions of gp41 possess potent anti-HIV activities *in vitro* and *in vivo* (Zhang, 2015). Both peptides act as decoys to block 6-HB formation during fusion-inducing conformational changes of gp41. One of the CHR peptides, T20 (enfuvirtide [Fuzeon]; Genentech), was approved by the U.S. FDA as the first HIV fusion/entry inhibitor (Lalezarri, 2003). T20 can bind to the gp41 NHR trimer to block the formation of 6-HB. However, the clinical application of this entry inhibitor is limited because it has an extremely short half-life, which necessitates multiple doses and can induce drug-resistant mutants in the treated patients (Baldwin, 2004, Greenberg, 2004). Therefore, new strategies and concepts to extend the half-life of such inhibitors and enhance potency are urgently required to develop next-generation drugs that target the HIV-1 fusion process.

Rationale: The lipid rafts of the host T cell has been shown to be enriched in the receptor (CD4) and co-receptors (CCR5 and CXCR4) for HIV (Campbell, 2001; Popik, 2002) making them the site where HIV fusion occurs. The rationale behind this project is to target these sites of active fusion by directing the fusion inhibitor to these membrane domains by the incorporation of a lipid moiety into the fusion inhibitor. Homing of the inhibitor to these sites on the T cell will increase the local concentrations of the drug, thereby increasing its potency. Without special

modifications to increase their half-lives, most peptides are cleared from the serum in the range of minutes to two hours by host metabolism and renal elimination. Thus, addition of a lipid motif may prolong the half-life of the peptide inhibitor through binding to serum albumin, as shown by drug design targeting other diseases.

B. Goals/Expected Outcomes/Hypotheses

Goals: are to develop the next generation anti-fusion inhibitor. The only licensed fusion inhibitor T20 suffers from low potency and short half-life thus requiring use of high dosages taken many times a day which results in low patient compliance. Additionally, T20 resistant mutants have been increasingly reported. Therefore, there is an urgent need to develop new anti-fusion drugs, which will effectively prevent HIV entry into the target cell, at low dosage. The current front-runner is YIK, a T20 peptide with three modifications- addition of a two amino acid (MT) hook at the N-terminus, an IDL tail and mutagenesis of amino acid residue 639 from Thr to Ile (last year's project). The specific goal of this project will be to enhance the stability of the YIK inhibitor and increase its local concentration at the site of viral membrane fusion by introduction of a lipid motif into YIK. Two different lipid motifs, palmitic acid and cholesterol will be tested to compare efficacy.

Hypothesis: Modification of YIK by incorporating a lipid motif to its C-terminus will increase the half-life of the inhibitor and enhance its potency by targeting the lipid rafts of the host T cell to yield a more potent anti-HIV fusion inhibitor.

C. PROCEDURES

Lipidated Peptides

- The peptides will be designed to contain a lysine residue at their C termini with a Dde side chain-protecting group to enable the conjugation of a lipid motif
- Peptides will be synthesized with purity >95%
- Concentrations of the peptides in PBS will be measured By NanodropTM 2000 Spectrophotometers (Thermo Fisher Scientific Inc., Waltham, MA, USA) and calculated based on a theoretical molar-extinction coefficient according to the peptide sequences

Circular dichroism (CD) and thermal midpoint (Tm) analysis (Ji, 1999)

- CD spectroscopy will be performed to determine the conformation of N- and YTK/YIK complexes as previously described.
- Briefly, each of the C-peptides (10 μM) will be mixed with N46 at ratio of 1:1.
- After incubation at 37 °C for 30 min, the samples will be then cooled down to 4 °C.
- The CD spectra of each sample will be acquired on a Jasco spectropolarimeter (Model J-715, Jasco Inc., Japan) at 4 °C using a 5 nm bandwidth, 0.1 nm resolution, 0.1 cm path length, and a 5.0 sec averaging time.
- Spectra will be corrected by the subtraction of a blank corresponding to the solvent composition of each sample.
- Thermal midpoint analysis will be performed at 222 nm by applying a thermal gradient of 5 °C/min. The melting curve will be smoothed and the midpoint of the thermal unfolding transition (Tm) values will be calculated using Jasco software utilities as previously described.

Inhibition of 6-HB Formation by Inhibitory Peptides In Vitro (Chong, 2015).

- The inhibitory activity of peptides on 6-HB formation will be measured by a modified ELISA.
- Briefly, a 96-well polystyrene plate (Costar, Corning Inc., Corning, NY, USA) will be coated with 50 mL 2 mg/mL NY364 (a polyclonal antibody for HIV-1 gp41 subunit) in 0.1 M Tris buffer (pH 8.8).
- A mixture of 1 mM N46 and C-peptide with graded concentrations will be added to the coated plate after incubation at 37°C for 30 min.
- After another 1-hour incubation at 37°C, the plate will be washed with washing buffer (PBS containing 0.1% Tween 20) three times and refilled by 50 mL 1 mg/mL NC-1 (a monoclonal antibody specific for HIV-1 gp41 6-HB
- 50 mL of horseradish peroxidase (HRP)-labeled rabbit anti-mouse antibody (Sigma, St. Louis, MO, USA) (1:4000 diluted) will be added to the wells of plate, followed by incubation for 1 h and washing.
- Finally, the substrate 3,3,5,5-tetramethylbenzidine (TMB, Sigma, St. Louis, MO, USA) will be added.
- Absorbance at 450 nm (A450) will be tested By an ELISA reader (Ultra 384, Tecan, NC, USA).

Inhibition of Env-Mediated Cell–Cell Fusion Assay by Inhibitory Peptides (Chong, 2015)

- A dye transfer assay will be used to detect HIV-1 Env-mediated cell-cell fusion

- One mL of 2 X 10^4/mL H9 cells (expressing Env protein) will be labeled with 2.5 mL of 1 nM fluorescent reagent, Calcein AM (Molecular Probes, Inc., Eugene, Oregon), and incubated at 37 ^{0}C for 30 min.
- For each well of the 96-well plate, 50 mL 2 X 10^4/mL labeled H9-Env cells will be then incubated with 100 mL 1 X 10^5/mL MT-2 cells at 37 ^{0}C for 2 h in the presence or absence of the tested peptide at graded concentrations.
- The fused and unfused Calcein-labeled HIV-1 IIIB cells will be counted under an inverted fluorescence microscope (Zeiss, Oberkochen, Germany). The IC50 values will be calculated by using the Calcusyn computer program (Biosoft, Ferguson, MO, USA).

Inhibition of HIV-1 Pseudovirus Infection by Inhibitory Peptides (Lu, 2012)

- Inhibitory activities of peptides on infection by pseudovirus HIV-1 strain will be measured
- For each well of the 96-well plate, 10^4 MT-2 cells with graded concentrations of peptide will be infected by 100mL TCID50 of the HIV-1 pseudovirus
- After overnight culture, the medium will be replaced with fresh RPMI 1640 medium containing 10% FBS. 50 ml of culture supernatant will be collected from each well on the fourth day for MT-2 cells
- The supernatant will be mixed with equal volumes of 5% Triton X-100 to release p24.
- The p24 antigen will be detected by sandwich ELISA using an anti-p24 antibody as capture antibody and a high titer biotin-tagged anti-P24 antibody as secondary antibody, followed by addition of streptavidin-peroxidase followed by incubation for 1 h and washing.

- Finally, the substrate 3,3,5,5-tetramethylbenzidine (TMB, Sigma, St. Louis, MO, USA) will be added.
- Absorbance at 450 nm (A450) will be tested by an ELISA reader (Ultra 384, Tecan, NC, USA).
- IC50 values will be calculated using the Calcusyn software program (Biosoft, Ferguson, MO, USA).

Analysis of the Half-life of Peptide Inhibitors

- Serum samples will be obtained from mice administered the various peptides at time points ranging from 0-16 h
- Anti-HIV-1 activities of the samples will be determined in the same way as described above using the p24 /pseudovirus assay.
- The highest dilution-fold of the serum causing 50% inhibition of infection (IC_{50}) will be calculated.

Cytotoxicity of YIK-C16 (Yang et al, 2018)

- The cytotoxic effects of HP23-E6-IDL, YIK, and YIK-C16 to MT-2 and M7 cells will be determined using MT-2 and M-7 cell lines (ATCC).
- A peptide at graded concentration will be incubated with 2×10^5/mL cells at 37°C for 3 days before adding 10 μL of CCK8 reagent.

- After another round of incubation at 37°C for 2 h, A450 will be measured with the Multi-Detection Microplate Reader.
- Cell viability will be calculated by dividing A450 of untreated cells by A450 of cells treated with a peptide.

Data Analysis

- Excel worksheets will be used to record all data and generate inhibition curves
- For each reading, averages and standard deviation will be calculated and used on the inhibition graphs
- The lines of best fit will be drawn using the SigmaPlot 10.1 program (Systat Software, Inc., Chicago, IL).
- Readout of all assays will be IC50 values wherein concentration of peptide required to achieve an inhibition of 50% will be used to compare efficacy of peptides
- A software program Calcusyn (Biosoft, Ferguson, MO, USA) will be used to calculate IC50 values
- Statistical tests to analyze if differences between IC50 values obtained among the 3 inhibitory peptides will be done using two-tailed unpaired t-test or ANOVA test, where appropriate. $p \leq 0.05$ will be considered statistically significant.

D. Bibliography

Baldwin, C. E., Sanders, R. W., Deng, Y., Jurriaans, S., Lange, J. M., Lu, M., & Berkhout, B. (2004). Emergence of a Drug-Dependent Human Immunodeficiency Virus Type 1 Variant during Therapy with the T20 Fusion Inhibitor. *Journal of Virology, 78*(22), 12428-12437. doi:10.1128/jvi.78.22.12428-12437.2004

Becerra, J. C., Bildstein, L. S., & Gach, J. S. (2016). Recent Insights into the HIV/AIDS Pandemic. *Microbial Cell,3*(9), 450-474. doi:10.15698/mic2016.09.529

Campbell, S.m, S.m Crowe, and J. Mak. "Lipid Rafts and HIV-1: From Viral Entry to Assembly of Progeny Virions." *Journal of Clinical Virology* 22.3 (2001): 217-27.

Chan, D. C., Chutkowski, C. T., & Kim, P. S. (1998). Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. *Proceedings of the National Academy of Sciences, 95*(26), 15613-15617. doi:10.1073/pnas.95.26.15613

Chan, D., Fass, D., Berger, J., & Kim, P. (1997). Hiv Gp41 Core Structure. *Cell.* doi:10.2210/pdb1aik/pdb

Chong, H., Qiu, Z., Su, Y., Yang, L., & He, Y. (2015). Design of a highly potent HIV-1 fusion inhibitor targeting the gp41 pocket. *Aids,29*(1), 13-21. doi:10.1097/qad.0000000000000498

Colman, P. M., & Lawrence, M. C. (2003). The structural biology of type I viral membrane fusion. *Nature Reviews Molecular Cell Biology,4*(4), 309-319. doi:10.1038/nrm1076

Eckert, D. M., & Kim, P. S. (2001). Mechanisms of Viral Membrane Fusion and Its Inhibition. *Annual Review of Biochemistry,70*(1), 777-810. doi: 10.1146/annurev.biochem.70.1.777

Greenberg, M. L., & Cammack, N. (2004). Resistance to enfuvirtide, the first HIV fusion inhibitor. *Journal of Antimicrobial Chemotherapy,54*(2), 333-340. doi:10.1093/jac/dkh330

Ji, H., Shu, W., Burling, F., Jiang, S., & Lu, M. (1999). Inhibition Of Hiv-1 Infectivity By The Gp41 Core: Role Of A Conserved Hydrophobic Cavity In Membrane Fusion. *Journal of Virology*. doi:10.2210/pdb1qr8/pdb

Lalezari, J. P., Henry, K., O'Hearn, M., & Montaner, J. S. (2003). Enfuvirtide, an HIV-1 Fusion Inhibitor, for Drug-Resistant HIV Infection in North and South America. *New England Journal of Medicine,349*(11), 1100-1100. doi:10.1056/nejm200309113491126

Lu, L., Tong, P., Yu, X., Pan, C., Zou, P., Chen, Y., & Jiang, S. (2012). HIV-1 variants with a single-point mutation in the gp41 pocket region exhibiting different susceptibility to HIV fusion inhibitors with pocket- or membrane-binding domain. *Biochimica Et*

Biophysica Acta (BBA) - Biomembranes,1818(12), 2950-2957. doi: 10.1016/j.bbamem.2012.07.020

Popik, W., T. M. Alce, and W.-C. Au. "Human Immunodeficiency Virus Type 1 Uses Lipid Raft-Colocalized CD4 and Chemokine Receptors for Productive Entry into CD4+ T Cells." *Journal of Virology* 76.10 (2002): 4709-722.

Tan, K., Lu, M., & Wang, J. (1997). Atomic Structure of A Thermostable Subdomain Of Hiv-1 Gp41. *Proceedings of the National Academy of Sciences of the United States of America*. doi:10.2210/pdb1szt/pdb

Vandamme, M., Laethem, K. V., & Clercq, E. D. (1999). Managing Resistance to Anti-HIV Drugs. *Drugs,57*(3), 337-361. doi:10.2165/00003495-199957030-00006

Weissenhorn, W., Dessen, A., Harrison, S., Skehel, J., & Wiley, D. (1997). Atomic Structure Of The Ectodomain From Hiv-1 Gp41. *Nature*. doi:10.2210/pdb1env/pdb

Yang, Wenqian, Zhiwu Sun, Chen Hua, Qian Wang, Wei Xu, Qiwen Deng, Yanbin Pan, Lu, and Shibo Jiang. "Chidamide, a Histone Deacetylase Inhibitor-based Anticancer Drug, Effectively Reactivates Latent HIV-1 Provirus." *Microbes and Infection* 20.9-10 (2018): 626-34.

Zhang, D., Li, W., & Jiang, S. (2015). Peptide fusion inhibitors targeting the HIV-1 gp41: A patent review (2009-2014). *Expert Opinion on Therapeutic Patents*. doi: 10.1517/ 13543776 .2014

Zheng, B., Wang, K., Lu, L., Yu, F., Cheng, M., Jiang, S., . . . Cai, L. (2014). Hydrophobic mutations in buried polar residues enhance HIV-1 gp41 N-terminal heptad repeat–C-terminal heptad repeat interactions and C-peptides' anti-HIV activity. *Aids,28*(9), 1251-1260. doi:10.1097/qad.0000000000000255

SAFETY INFORMATION

1. HUMAN PARTICIPANTS RESEARCH

Does not apply

2. VERTEBRATE ANIMAL RESEARCH

Serum samples provided by the laboratory at LFKRI, no direct handling of mice, no animals were euthanized for the project.

3. POTENTIALLY HAZARDOUS BIOLOGICAL AGENTS

- Pseudo-virus of HIV-1 will be used, to make culture supernatants, made in the mentor's lab, it is a 1-cycle virus, handled by mentor: BSL-2
- T-Cell lines- H9 and MT-2 will be used- BSL-2 (from ATCC)

Safety Precautions:

Personal Protective Equipment will always be worn, including gloves, lab coats, eye goggles

All work with cell lines will be conducted in Biosafety Laminar Air Flow Cabinets.

All material will be bleached and autoclaved

4. HAZARDOUS CHEMICALS, ACTIVITIES AND DEVICES

Does not apply