Continuation/Research Progression Projects Form (7)

Required for projects that are a continuation/progression in the same field of study as a previous project.
This form must be accompanied by the previous year's abstract and Research Plan/Project Summary.

Student's Name(s) GISELLE RASQUINHA

To be completed by Student Researcher: List all components of the current project that make it new and different from previous research. The information must be on the form; use an additional form for previous year and earlier projects.

Components	Current Research Project	Previous Research Project: Year: 2018-2019
1. Title	Lipid Conjugation Yields Novel HIV-1 Fusion Inhibitor that Demonstrates Improved Efficacy and Prolonged Serum Half-life	The enhancement of HIV-1 fusion inhibitor potency by key residue mutagenesis and C-terminal anchor addition
2. Change in goal/ purpose/objective	To construct a novel HIV-1 fusion inhibitor by lipidation via adding palmitic acid and analyze the effect on anti-fusion activity	To mutate a key amino acid residue that was involved in the binding to gp41 and to analyze if it affected fusion of HIV-1
3. Changes in methodology	Besides the 3 assays, several new assays were used in this study, including cytotoxicity assays, ex vivo stability assays to measure half-life of the inhibitor and melting curve analysis of the complex formed between inhibitor and native gp41 to look for change in affinity between lipidated and non-lipidated inhibitor	3 main assays were used to assess potency: cell-cell fusion assay, 6 helical bundle formation assay and P24 sandwich ELISA assay.
4. Variable studied	Effect of conjugation of a C-16 lipid moiety on potency and half-life of inhibitor	Mutagenesis of a key interacting amino acid residue and adding a three amino acid tail
5. Additional changes	This project used many new assays as readout of increased potency/half-life. The main goal was to increase the half-life of the inhibitor so novel ex-vivo assays were designed to quantify this parameter.	

Attached are:

☑ Abstract and Research Plan/Project Summary, Year 2018-2019

I hereby certify that the above information is correct and that the current year Abstract & Certification and project display board properly reflect work done only in the current year.

GISELLE RASQUINHA — Student's Printed Name(s)

Signature

October 28, 2019 — Date of Signature (mm/dd/yy)

The enhancement of HIV-1 fusion inhibitor potency by key residue mutagenesis and C-terminal anchor addition

Giselle Rasquinha 2018 -2019

ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) Env transmembrane subunit GP41, which contains the fusion peptide and the N- and C-terminal heptad repeats (NHR and CHR) mediates fusion between HIV and the T-cell. The six-helix bundle (6-HB) that is formed between the NHR and CHR domains, brings the viral and cell membranes into proximity for fusion. T20 (Enfuvirtide), a 36-residue peptide derived from the CHR, is the only clinically available HIV-1 fusion inhibitor. However, it easily induces drug resistance and has low potency, which urgently calls for next-generation drugs. The reference inhibitor used in this study was HP23, an altered T20 peptide, that is the current fusion inhibitor front runner. HP23 was modified to strengthen interaction with the NHR by adding IDL (Ile-Asp-Leu) to its C-terminus to form YTK and mutating aa 693 from Thr to Ile on YTK to form YIK. Inhibition of 6-HB formation, cell-cell fusion and infection assays were used to assess anti-HIV potency of YTK and YIK. YIK was 10-fold better than YTK and 30-fold more potent in inhibiting 6HB formation. Both peptides were twice as efficient as preventing cell-cell fusion and 3-fold better at inhibiting viral infection than HP23, with YIK performing marginally better than YTK in these two assays. These findings suggest that addition of an anchor–tail to the C-terminus of a CHR peptide along with mutations of key residues may increase the anti-HIV efficacy.

The enhancement of HIV-1 fusion inhibitor potency by key residue mutagenesis and C-terminal anchor addition

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2018-2019

Research Plan

A. RATIONALE

Enfuvirtide (T20), is the only FDA licensed drug to prevent HIV-T cell fusion. It is a 36 amino acid peptide derived from the C-terminal heptad repeat (CHR) region of HIV-1 transmembrane protein gp41 that binds to the N-terminal heptad repeat (NHR) region of gp41 thus preventing the formation of the viral fusion core, the gp41 six helix bundle (6-HB). T20 suffers low potency, requiring use of high dosages taken twice 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 prevent HIV entry into the target cell. The current front-runner is HP23, a modified T20 peptide with addition of a two amino acid (MT) hook at the N-terminus. Similar modifications of the C-terminus of the inhibitory peptide may increase stability of interaction between inhibitor and NHR of gp41, thus increasing its anti-fusion ability.

B. HYPOTHESIS

Modifications of HP23 including addition of an IDL (Ile-Asp-Leu) anchor to its C-terminus, to allow additional binding of the inhibitor to the shallow pocket in the N-terminal region of the gp41 NHR-trimer, forming HP23-YTK and mutation of the amino acid residue 639 of HP23-YTK from Thr to Ile to form HP23-YIK to facilitate stronger interaction with the NHR region, will yield more potent anti-HIV fusion inhibitors.

C. RESEARCH METHODS AND CONCLUSIONS

PROCEDURES

Peptides

- Peptides were synthesized with purity >95%.
- Concentrations of the peptides in PBS were 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.

Inhibition of 6-HB Formation by Inhibitory Peptides In Vitro

- The inhibitory activity of peptides on 6-HB formation was measured by a modified ELISA.
- Briefly, a 96-well polystyrene plate (Costar, Corning Inc., Corning, NY, USA) was coated with 50 mL 2 mg/mL NY364 (a polyclonal antibody for HIV-1 gp41 subunit) in 0.1MTris buffer (pH 8.8).
- A mixture of 1 mM N46 and C-peptide with graded concentrations was added to the coated plate after incubation at 37°C for 30 min.
- After another 1-hour incubation at 37°C, the plate was 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) were 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) was added.
- Absorbance at 450 nm (A450) was tested By an ELISA reader (Ultra 384, Tecan, NC, USA).

Inhibition of Env-Mediated Cell–Cell Fusion Assay by Inhibitory Peptides

- A dye transfer assay was used to detect HIV-1 Env-mediated cell-cell fusion
- One mL of 2 X 10^4/mL H9 cells (expressing Env protein) were 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 were 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 were counted under an inverted fluorescence microscope (Zeiss, Oberkochen, Germany). The IC50 values were calculated by using the Calcusyn computer program (Biosoft, Ferguson, MO, USA).

Inhibition of HIV-1 Pseudovirus Infection by Inhibitory Peptides

- Inhibitory activities of peptides on infection by pseudovirus HIV-1 strain
- For each well of the 96-well plate, 10^4 MT-2 cells with graded concentrations of peptide were infected by 100mL TCID50 of the HIV-1 pseudovirus
- After overnight culture, the medium was replaced with fresh RPMI 1640 medium containing 10% FBS. 50 ml of culture supernatant was collected from each well on the fourth day for MT-2 cells
- The supernatant was mixed with equal volumes of 5% Triton X-100 to release p24.
- The p24 antigen was 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) was added.
- Absorbance at 450 nm (A450) was tested By an ELISA reader (Ultra 384, Tecan, NC, USA).
- IC50 values were calculated using the Calcusyn software program (Biosoft, Ferguson, MO, USA).

Data Analysis

- Excel worksheets were used to record all data and generate inhibition curves
- For each reading, averages and standard deviation was calculated and used on the inhibition graphs
- The lines of best fit were drawn using the SigmaPlot 10.1 program (Systat Software, Inc., Chicago, IL).

- Readout of all assays was IC50 values wherein concentration of peptide required to achieve an inhibition of 50% was used to compare efficacy of peptides
- A software program Calcusyn (Biosoft, Ferguson, MO, USA) was used to calculate IC50 values
- Statistical tests to analyze if differences between IC50 values obtained among the 3 inhibitory peptides were done using