**Descriptive Title**

**Date:**

**Objective:** (1-2 sentence description of experiment objective/hypothesis)

# A. Protocol:

(General description of protocol)

*Note: remove blue example and replace with appropriate description.*

# *Example*

1. *CD34+ NOD/scid-IL-2Rγnull (NSG) mice (Hu-NSG) will be infected with HIV-1ADA and treated 2 weeks later (based on viral load) with a combination of nanoformulations of NMCAB (integrase inhibitor), myristoylated-lamivudine (M3TC), myristoylated-abacavir (MABC) (nucleoside reverse transcriptase inhibitors) and rilpivirine (RPV; non-nucleoside reverse transcriptase inhibitor) until the viral load in plasma becomes undetectable.*
2. *Then the mice will be injected with AAV6-CRISPR/CAS9 targeting CCR5 to look for its ablation and allowed for 4 more weeks to look for any viral rebound (at this stage, 5 mice will be sacrificed to look for CCR5 expression in major tissue reservoirs).*
3. *The mice will then be injected with AAV9-CRISPR/CAS9 targeting LTR-Gag region and will be measured for viral elimination after 8 more weeks.*
4. *Plasma viral load, CD4/CD8 T-cell counts and drug levels will be determined 2, 6, 10, 14, and 18 weeks after infection.*
5. *Animals will be sacrificed 20 weeks after infection and blood and tissues will be collected for immune cell quantitation, viral RNA/DNA in fractionated cells, immunohistochemistry, and drug levels.*

**B. Animals and Treatment Groups**

**Animals:** Species, strain, sex, age

**Administration route and method:** Describe route of injection, site of injection, syringe and needle size

*Example: Intramuscular (IM), caudal thigh muscle. IM injection will be administered using a BD Micro-Fine™ IV needle (0.35 mm (28G) x 12.7 mm (1/2”) and 0.5 mL insulin syringes.*

**Dosages:** provide mg active drug equivalents/kg body weight; provide HIV infection dose; provide other treatment doses

*Example: 45 mg CAB-equivalents/kg*

**Dose frequency:** provide how often dose (drug / HIV innoculant) will be given and over what time frame

**Dose Quality checks:**

**Pre-dose:** 10 µl of each dosing solution will be added to 990 µl MS-grade methanol prior to animal injections

**Post-dose:** 10 µl of each dosing solution will be added to 990 µl MS-grade methanol immediately after all animals are injected

Store pre- and post-dose samples at -80˚C for drug quantitation

Drug in pre- and post-dose formulations will be quantitated by HPLC and/or LC-MS/MS

**C. Human cells reconstitution:** (describe source of human cells for reconstitution; provide number of human cells for reconstitution)

**D. Preparation of treatments and formulations:**

Describe descriptions of preparation of HIV dose, drug formulation (s) (including preparation buffer and drug/excipient ratios), other treatment modalities such as AAV-CRISPR/Cas9 constructs

**E. Description of procedures**

Provide a detailed description of the procedures to be done during the study, including infection, treatments, timing of sample collections, what samples will be collected, viral load determinations, experiment conclusion, tissues to be collected and processed

# F. Experimental design:

1. **Preparation of drugs and constructs:**

*Note: remove blue example and replace with appropriate description.*

|  |  |  |
| --- | --- | --- |
| **Formulation** | **Preparation Process** | **Drug dose (mg/kg)** |
| *NMDTG* | *Homogenization* | *45* |
| *NM3TC* | *Homogenization* | *40* |
| *NMABC* | *Homogenization* | *40* |
| *NRPV* | *Homogenization* | *45* |
| *AAV6-Cas9 for CCR5* | *As obtained from Temple University* | *100 ul/mouse* |
| *AAV9-Cas9 for LTR-Gag* |  | *50-100 ul/mouse* |

*Example: The nanoformulations will be prepared by high-pressure homogenization without purification and the suspension will be diluted in sterile water on the day of administration. The nanosuspensions will be characterized for size, charge and PDI following production and just prior to injection. A* ***40-50 µl volume for each nanoformulation combination (NMCAB/NRPV and NM3TC/NMABC)*** *will be administered by IM injection in opposing thigh muscles. AAV-Cas9 will be injected as per instruction from* ***Temple University Research team****.*

# Experimental Scheme and Groups of animals

Provide a schematic timeline for treatment and sample collection

*Example:*



1. **Blood/Plasma Collection**

*Note: remove blue example and replace with appropriate description.*

*Example: Blood will be collected on weeks 0, 2, 6, 10, 14 and 18 for plasma drug levels and for cell phenotyping analysis by flow cytometry. On weeks 2, 6, 10, 14 and 18 one aliquot of plasma will be saved for viral load detection. Once we achieve undetectable viral load in the plasma mice will be injected with AAV-Cas9 construct.*

# Tissue Collection

Liver, spleen, kidneys, lungs, brain, lymph nodes (neck for drug levels), gut and muscle for drug levels and immunohistology will be collected at the time of sacrifice for infected animals.

# Immunohistochemistry

For HLA-DR and HIV-1 p24 detection in spleen, lung, brain and lymph nodes

# Viral load, LC-MS/MS, PCR and serum chemistry analyses

**Viral load:** Blood will be collected at mentioned time points post-treatment by facial vein bleed and plasma will be separated after centrifugation. Plasma will be stored at -80˚C and will be used for viral load determination.

**Serum chemistry (endpoint of experiment):** For serum chemistry analysis 110 µl of serum from the final blood collection will be aliquoted into a clean 0.5 ml micro centrifuge tube and stored at -80˚C until analysis. The remaining serum will be stored (-80˚C) in a 1.7 ml micro centrifuge tube for drug analysis.

**Drug levels**: Blood and tissues will be collected at time of sacrifice for LC-MS/MS analysis of drug levels. Each organ will be weighed and the weight recorded at the time it is removed. One-half of each of these tissues will be fixed for histopathology analysis and the other half will be frozen at -80˚C for drug analysis.

**Viral detection by semi-nested real-time qPCR:** HIV-1 RNA and DNA will be determined in spleen, bone marrow, liver, lung, gut and brain by real time PCR assay, using specific primers. Human CD45+ measurements and GAPDH will be used for normalization purposes.

**Viral detection by droplet digital PCR (ddPCR):** HIV-1 DNA will be determined in spleen, bone marrow, liver, lung, gut and brain by ddPCR assay, using specific primers and probes, the sensitivity of this assay is 1-2 copies. Human CD45+ measurements will be used for normalization purposes.

**RNAscope analysis:** Viral RNA will be measured in 5 μm thick paraffin embedded spleen and lymph node tissue sections using antisense probe V-HIV1- Clade-B (Cat no 416111) targeting 854-8291 bp of HIV-1NL4-3*.* Human peptidylprolyl Isomerase B (PPIB) will be used as positive control for the tissue analyzed. All reagents will be from Advanced Cellular Diagnostics, Newark, CA.

# Flow cytometry analysis

Flow analysis will be done immediately after the blood and organ collection. A fraction of spleen tissue, bone marrow and 50 µl of whole blood sample will be used for cell markers staining. In Flow analysis, human pan-CD45, CD3, CD4, CD8, CD14 and CD19 antibody markers will be used.

# Immunohistochemistry

For histological evaluation spleens, livers, lungs, kidneys, and lymph nodes will be removed immediately after euthanasia and fixed with 4% paraformaldehyde overnight followed by processing in the tissue processor Shandon Citadel 1000 (Thermo Electron Corporation), according to the instrument instructions, and then embedded in paraffin. Sections 5-micron- thick will be cut from the paraffin blocks, mounted on glass slides and subjected to immunohistochemical staining with mouse monoclonal antibodies for HLA-DQ/DP/DR (clone CR3/43, 1:100) and HIV-1 p24 (clone Kal-1, 1:10) antibodies from Dako. The polymer-based HRP- and AP-conjugated anti-mouse and anti-rabbit Dako EnVision systems will be used as secondary detection reagents and developed with 3,3'-diaminobenzidine (DAB). All paraffin- embedded sections will be counterstained with Mayer’s hematoxylin. Images will be obtained by an Optronics digital camera fixed to a Nikon Eclipse E800 (Nikon Instruments, Melville, NY) using MagnaFire 2.0 software.