**Descriptive Title**

**Date:**

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

1. **Protocol:**

(General description of protocol)

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

*Example: Plasma and tissue drug levels will be determined in female NSG mice following intramuscular (IM, caudal thigh muscle) administration of NCAB, NMCAB, and NM2CAB.*

1. *Nanoformulations will be administered by IM injection (caudal thigh muscle) to female NSG mice at* ***45 mg CAB-eq./kg*** *dose at a volume of 40 µL/25 g mouse on Day 0.*
2. *Mice will be sacrificed on Days 14, 28, 42, and 364 following treatment.*
3. *Blood collection: Day 1, 7, 14, and weekly onwards up to day 364*
4. *Vaginal Fluids/Wash collection: Day 14, 28, 42 and 364.*
5. *Tissues (vaginal, rectal, liver, lungs, spleen, kidneys, brain, gut, and muscle) will be collected for drug analysis on Days 14, 28, 42, and 364. Portion of tissues at day 364 will be fixed and processed for histopathological evaluation.*
6. **Treatment Formulation(s)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Formulation** | **Preparation Process** | **Excipient(s)** | **Formulation Drug concentration (mg/ml)** | **Target Particle Size (nm)** |
|  |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |

Describe preparation of drug formulation, including preparation buffer and drug/excipient ratios.

*Example: For all, drug (CAB, MCAB, or M2CAB) and P407 will be combined in endotoxin-free water at a ratio of 100:10 and homogenized by high-pressure Avestin C3 homogenizer. Particle size will be determined by DLS with a target size between 300 and 400 nm and PDI around 0.2. After homogenization the formulation will be used without further purification.*

*For all, nanoformulations as drug suspensions will be diluted using the appropriate buffer to the final drug concentration for injection. Final drug content will be determined using UPLC-UV/Vis. Pre- and post-dose injection samples will be collected for size, charge, and PDI as determined by DLS. Pre- and post-dose injection samples will be collected for drug concentration analysis by UPLC-UV/Vis and UPLC-MS/MS.*

Describe preparation of control vehicle solution.

1. **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.*

 **Dosage:** provide mg active drug equivalents/kg body weight

*Example: 45 mg CAB-equivalents/kg*

**Dose frequency:** provide how often dose 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

**Experimental Design:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Group** | **Treatment** | **Animal Number** | **Blood Collection Day(s)** | **Sacrifice Day(s)** |
| *Examples:**1* | *NCAB* | *20* | *Day 1, 7, 14, 21, 28, 35, 42,**49, and 56* | *Day 14, 28, 42, and 56* |
| *2* | *NMCAB* | *20* | *Day 1, 7, 14, 21, 28, 35, 42,**49, and 56* | *Day 14, 28, 42, and 56* |
| *3* | *NM2CAB* | *20* | *Day 1, 7, 14, 21, 28, 35, 42,**49, and 56* | *Day 14, 28, 42, and 56* |
| *4* | *Control* | *5* | *Day 1, 7, 14, 21, 28, 35, 42,**49, and 56* | *Day 56* |

**Animal groups at each sacrifice timepoint and number of mice at each timepoint:**

*Example:*

*Day 14: NCAB – 5 mice, NMCAB – 5 mice; NSCAB – 5 mice; Total – 15 mice*

*Day 28: NCAB – 5 mice, NMCAB – 5 mice; NSCAB – 5 mice; Total – 15 mice*

*Day 42: NCAB – 5 mice, NMCAB – 5 mice; NSCAB – 5 mice, Total – 15 mice*

*Day 364: NCAB – 5 mice, NMCAB – 5 mice; NSCAB – 5 mice; Control – 5 mice; Total – 20 mice*

**Total animals for study:** (provide total number of animals needed)

**Parameters to be evaluated:** (describe which parameters will be evaluated and at what timepoints)

*Example: The mice will be administered a single 45 mg/kg CAB eq. dose of NCAB or NMCAB or NM2CAB in the caudal thigh muscle on the right/left hind leg. If there is a 10% decrease in body weight (BW) and/or abnormal behavior in a mouse, that mouse will be terminated at that point. Body weights will be measured before the treatment and then weekly to follow up. Toxicity will be assessed by histopathological evaluation of the liver, spleen, gut, kidneys, lung and brain, and a serum chemistry analysis.*

**Basal diet and water:** (check information with Comparative Medicine)

*Example: Pelleted Teklad 7012 (Frederick, MD) and sterile water (company name?) will be used.*

**Quarantine and Group assignment:**

All animals in the experiment will be quarantined and acclimated to the animal room for at least **1 week**. Upon arrival, animals will be housed 5 per cage. Approximately one week after arrival, the animals will be weighed and given a detailed physical examination.

*Example of additional description: Those that appear healthy will be randomized by weight into 12 groups of 5 mice each. Animals will be housed 5/cage. Each animal will be assigned a unique identification number and will be identified by a tail marking. Cage cards with the appropriate data will be placed on all cages.*

1. **Blood collection**

*Example: Blood will be collected at days 1 and 7, and weekly up to day 364 in heparin coated blood collection tubes (source). Samples will be collected from submandibular vein (cheek bleed) by using lancets (MEDIpoint 5, Inc., Mineola, NY)*

1. Whole blood for drug analysis: 25 μL of whole blood will be added to 1 mL LC-MS grade methanol for prodrug (MCAB or SCAB) measurements. Samples will be stored at -80˚C until analysis.
2. Whole blood for complete blood counts will be collected in EDTA-coated tubes (source)
3. Plasma for serum chemistry and drug anlaysis. Plasma will be collected from the remaining collected blood by centrifugation at 2,000g for 8 min. Samples will be stored at -80˚C until drug analysis or serum chemistry analysis.
4. **Tissue collection**

Tissues (liver, spleen, lymph nodes (inquinal) lungs, kidneys, brain, gut, vaginal, testes, rectal, heart and muscle) will be collected at sacrifice days Whole tissue weights will be recorded Tissue sections will be fixed in 10% formalin and the remainder stored at -800 C for drug quantitation.

1. **Histology (at sacrifice):**

*Example: The lungs, liver, spleen, gut, kidneys and brain will be removed and portion of each tissue will be placed in 10% buffered formalin. Following fixation, tissues will be processed for paraffin embedding. Approximately 5-micron sections will be cut and mounted on glass slides. Tissue sections will be stained with hematoxylin and eosin. Tissues sections will be examined by Dr. S. Cohen.*

1. **Serum Chemistry:**

An aliquot of the plasma samples (100 µL) collected at the end of study will be stored at -80˚C until analysis. Samples will be analyzed using a VetScan comprehensive diagnostic profile disc and a VetScan VS-2 Veterinary Blood Chemistry analyzer (Abaxis Veterinary Diagnostics, Union City, CA, USA).

1. **Complete Blood Counts:**

Blood will be collected into K+/EDTA tubes for hematology analysis using a Abaxis HM5 Veterinary Hematology Blood analyzer (Abaxis Veterinary Diagnostics, Union City, CA, USA). At least XX µl of blood will be needed for analysis.