**Transplant Date: October 13, 2021**

**Protocol Number:** AMI-018-006

**Study Number:** AMI-018-006-132

**Title of Study:** Compare performance of TNAO0010 at 0.125X, 0.25X or 0.5X dose with Ambys pHH lot AM010P versus TNAO0010 at 0.25X with DJW in FRG rats.

**Primary Investigators:** Lisa Wilson

**Number of Animals:** 40. **Species:** Rat. **Strain:** SD FRG. **Gender:** Male & Female. **Age or weight range: 5-**6 weeks

**Purpose**:

In IACUC protocol AMI-018-006, the focus of the set of experiments is to utilize the immunodeficient rat model of hereditary tyrosinemia type 1 (HT1) - which are deficient in the enzyme fumarylacetoacetate hydrolase (FAH) – as the model for expanding human hepatocytes. Hepatocytes that are FAH+ have a selective growth advantage over FAH- hepatocyte; therefore, we hypothesize that FAH+ human hepatocytes will grow and repopulate the FAH-KO rat liver. Proof-of-concept has already been demonstrated in the mouse model of HT1 (see Azuma et al. 2007 PMID: 17664939).

The efficiency of the FRG rat bioreactor as it relates to health of the animal, expansion of the human hepatocytes and the viability of the cells at the time of perfusion and purification is dependent on 3 main variables: the Ad:uPA preconditioning agent, the human cadaveric cells and the NTBC cycling to expand the transplanted cells. One purpose of AMI-018-006-132 will be to test that pHH master cell bank, AM010P, generated at Ambys is able to generate humanized FRG rats that meet the criteria for the downstream CMC processing to generate the lots of huFRG derived human hepatocytes necessary for future studies. AM010P cell bank was found to be contaminated with bacteria at some point during the processing to the time of cryopreservation. After consideration of the antibiotics in the cell bank media, Gentamicin/Amphotericin-B (GA)], and the prophylactic SMZ-TMP administered to FRG rats in the drinking water, the risk for deleterious effects of contaminated cells could be minimized.

We have demonstrated that the dose of Ad:uPA is critical for efficient engraftment, early expansion and survival of the animals post-transplant. Due the unknown variability in each Ad:uPA lot received from the manufacture, variability in the lots potentially are influencing early engraftment and overall health of the bioreactor – too high a dose and the human cells expand aggressively, and the liver and animals’ health is impaired, too little a dose and the initial engraftment is limited, and the overall expansion is decreased. It is imperative that we optimize the potency of each the Ad:uPA lot with the pHH lot prior to initiation of these critical IND enabling studies. Another purpose for this study AMI-018-006-132 is to test three doses of TNAO0010, 0.625E7, 1.25E7 and 2.5E7 pfu/gram of body weight in transplantation with the proposed pHH lot, AM010P and use DJW as the pHH control also with 1.25E7 TNAO0010 as the preconditioning agent for use in future studies.

All FRG rats will be on 5LJ5 diet

All FRG rats will begin on 16 mg/L NTBC + 820µg/mL Equisul in the drinking water

**Experiment Outline:**

N=40 FRG rats

Animals will be held for at least one week to acclimate following arrival.

Animals will be socially housed. On the rare case where one animal remains in a cage without a partner, additional cage enrichment (non-food) will be provided.

Animals will be divided into 2 groups, A and B, with equal number of males and females.

Using body weights collected the same day as preconditioning the rederived FRG rats from Envigo will be dosed with Ad-uPA 24h ± 2h prior to transplant and placed on the drinking water as specified in the NTBC cycling protocol located on the Ambys G-drive. On the day of transplant, the cryopreserved validated commercial PHH Lot will be prepared following the standard protocol used for transplantation.

**Day -1:** In the AM, ALL rats will be dosed using Vector Biolabs titer of TNAO0010 at **3.0E+11pfu/mL**. The dose will be calculated to be ~2.5E7 or 1.25 pfu/gram of body weight Ad-UPA by IV tail injection. Ad-uPA will be provided to the IVS team for tail vein dosing. The NTBC drinking water will be changed as indicated in the chart.

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| **Group** | **# of FRG rats** | **Ad:uPA dose (pfu/g of BW) IV by tail vein** | **Ad:uPA lot** | **pHH Donor** | **Cell dose by laparotomy into the splenic pulp** | **NTBC Cycle ID** |
| A | 8 | 1.25E7 | TNAO0010 | DJW | 2.5E6 viable cell/100g of BW | 38 |
| B | 8 | 2.5E7 | TNAO0010 | AM010P | 5E6 viable cell/100g of BW | 38 |
| C | 8 | 1.25E7 | TNAO0010 | AM010P | 5E6 viable cell/100g of BW | 38 |
| D | 8 | 0.625E7 | TNAO0010 | AM010P | 5E6 viable cell/100g of BW | 38 |

**Day 0:** The volume of hepatocytes suspension determined by body weight will be in 1.5ml Eppendorf tubes and transferred on ice. Hepatocytes will remain on ice until time of injection. At time of injection, hepatocytes will be gently pipetted up/down x3 with P1000 pipet and sterile P1000 tip to get the cells in suspension (hepatocytes are large and will quickly pellet to the bottom of the tube). Hepatocytes will be drawn into a sterile 1cc syringe with a 27G needle and injected into the spleen via laparotomy method.

**Day 1 onwards:** Animals will be put on the short NTBC cycle as indicated above in the chart and as indicated in the NTBC water cycle sheet located on the Ambys’ G drive.

All animals post-transplant will be offered the nutritionally balanced Supreme mini treats supplement at the time of body weight collection as positive reinforcement (~1-2 pellets) and for supplementation of additional calories and vitamins at a dose of 6-8 pellets per animal.

The IVS team will record the date and quantity of supplement administration, and clinical observational scores.

**Day 7 ± 2 days**: Serum will be isolated from retro-orbital blood collected from each animal in group A, D and E to access the quantitation of uPA expression in the blood.

Collection method: Induce a surgical plane of anesthesia with 3-5% isoflurane and from the vena cava collect ~500µL into a serum separator to isolate serum for blood uPA quantitation. Serum will be dispensed into 3 equal volume aliquots into sterile 1.5mL microfuge tubes before storing at -20C.

**Day 30 – 90**: All animals, collect 5µL whole blood for human albumin measurements via tail vein, tail or saphenous vein nick at the specified dates on the chart above and the NTBC water cycle sheet located on the Ambys’ G drive. **PI will communicate:**

**Day 50 huAlb readout:** hAlbumin **≤ 350µg/mL** will be removed from study and euthanized.

**Day 70 huAlb readout:** hAlbumin **≤ 1000µg/mL** will be removed from study and euthanized.

**Day 0-125:** Body weights and clinical scoring will be measured and documented up to three times each week.

**Day >125:** IVS to notify PI and Perfusion Team of any remaining animals.

**Animal care and welfare:**

Refer to **the document titled “VETERINARY CLINICAL CARE AND REPORTING FOR FRG RATS”** for the evaluation and documentation of clinical status and palliative treatments**.** In reference to the group A and B assignments above, animals will be supplemented based on their group assignments.

For veterinary health and surveillance screening, the Attending Veterinarian may request aseptic sample collection of tissues in lieu of, prior to or following perfusion without interference to primary study objectives, under general anesthesia. Once all samples have been collected, the animal will be euthanized via exsanguination/vital organ removal.

**Humanized FRG rats for perfusion, hepatocyte isolation and human hepatocyte purifica**

For animals with clinical scores ≥ 3 at ≥ 70 days and h-Alb ≥ 2500 µg/mL (higher the better), may be offered to the Product Development team for optimization of processes involving liver perfusion (refer to the document titled “Perfusion Procedure Guideline: Portal Vein and Inferior Vena Cava Approaches), hepatocyte isolation, human hepatocyte purification and in vitro characterization.

**Additional Veterinary Testing:**

For veterinary health and surveillance screening, the Attending Veterinarian may request aseptic sample collection of tissues in lieu of, prior to or following perfusion without interference to primary study objectives, under general anesthesia. Once all samples have been collected, the animal will be euthanized via exsanguination/vital organ removal.

**Humanized FRG rats for blood collection and liver harvest**

Animals with a CS score of 2.5 and human albumin concentrations of ≥ 2,500µg/mL may be scheduled for blood and tissue harvest of the liver for IHC staining to assess human hepatocyte repopulation.

**Humanized FRG rats for perfusion, hepatocyte isolation and human hepatocyte purification:**

At ≥ 71 days post-transplant with human albumin concentrations of ≥ 2,500µg/mL, animals may be offered to the Product Development team for optimization of processes involving liver perfusion (refer to the document titled “Perfusion Procedure Guideline: Portal Vein and Inferior Vena Cava Approaches), hepatocyte isolation, human hepatocyte purification and in vitro characterization.

Once the cannula has been inserted into the caudal vena cava or portal vein, secured and blood starts to fill the head, ≥ 500µL whole blood will be collected for sera isolation. Alternatively, the blood may be collected from the tail vein based on a 50µL aliquot of sera will be dispensed into a pre-labeled sterile 1.5 mL tube for Tyrosine and Succinylacetone quantitation. Remaining sera will be transferred to a second pre-labeled sterile 1.5mL tube. Both aliquots should be stored at -20C until shipped for analysis.

Principal Investigator: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Head of Pharmacology (or alternate): \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Appendix A: Processing blood, urine and tissue samples**

Blood collection

* Using Isoflurane, anesthetize the animal to a surgical plane of anesthesia (Induction 4-5%, maintenance 2-3%). Confirm with a firm toe pinch on both hind feet.
* Using operating scissors or other appropriately sized scissors, cut through the skin and muscle to open the peritoneal cavity.
* Using gauze, sweep the intestines out of the body cavity to expose the inferior vena cava.
* Using 5 mL (rat) syringe and 25 G needle puncture the inferior vena cava and slowly withdraw 4mL of blood from rat. Be careful to not collapse the vein by creating too much vacuum pressure.

Serum:

* Dispense ≤ 2mL whole blood volume into 4mL serum separator tube and allow to clot for at least 1 hour at room temperature.
* Centrifuge at 10,000 rpm for 5 minutes.
* Dispense 220µL into 4 X 1.5 sterile mL tube, store at -80C

Plasma:

* Dispense ≥ 1mL of whole blood in a 4mLK2EDTA anticoagulation micro tube (do not overfill or blood will clot).
* Gently invert 3 times (do not shake) to facilitate complete mixing with anticoagulant.
* Centrifuge at 10,000 rpm for 5 minutes.
* Dispense 80µL into 4 X 1.5 sterile mL tube, store at -80C

Urine during Necropsy

* Following collection of blood, collect urine (if present) via cystocentesis. Urine to be placed in labeled freezer compatible tube and store at -20oC

Collection of Liver

* Using blunt tipped scissors (curved if possible) separate the liver from the diaphragm, blood vessels and connective tissue and remove the liver from the body cavity.
* Make 4-5, 1cm nicks across the liver to facilitate fixation.
* Place whole liver in bottle of 10% NBF.

Collection of the Kidney

* Remove the adrenal glands, located in the fat cranial to the kidneys.
* Dissect the kidneys away from the fat and cut at the juncture of the kidney with the renal artery and vein.
* Transect the left kidney (transverse section) with a scalpel blade or straight razor.
* Longitudinally bisect the right kidney at the midline.
* Place into a bottle with 10% NBF