Cysteinyl Leukotriene (cysLT) Induced Retinal Vascular Permeability Model

Key Points:

- The **cysteinyl leukotriene (cysLT) induced retinal vascular permeability model** allows for investigation of potential therapeutics for the treatment of retinal vascular leakage associated with diabetic retinopathy [1].
- CysLTs are bioactive lipids synthesised from cell membrane arachidonic acid via a 5-hydroxyeicosatetraenoic acid (5-HETE) intermediate [2-5]. 5- HETE is increased in the vitreous of diabetic patients which correlates with the progression of DR [6].
- CysLTs contribute to retinal capillary degeneration, chronic inflammation and diabetic retinopathy in murine diabetic models [7, 8].
- Brown Norway rats are selected for retinal vascular permeability assays because of higher levels of permeability observed in this strain, reportedly due to increased levels of retinal VEGF [9].
- Blood–retinal barrier breakdown is expressed as micrograms of Evans blue per g of retinal dry weight calculated as previously described [1, 10, 11].

Animals:

House male Brown Norway rats (BN/RijHsd: 250-300g) in pairs in polypropylene cages with free access to a standard maintenance diet (e.g. Harlan Teklad 2018). Maintain all animals at 21±4°C and 55±20% humidity on a normal light/dark cycle (lights on: 07:00 h - 19:00 h). Habituate animals to their environment and diet for approximately two weeks prior to the commencement of study.

Drugs:

CysLTs are from Cayman Chemical: Leukotriene C4 (CAY 20210 50 µg) and leukotriene D4 (CAY 20310 50 µg). The cysLT cocktail is made up of 1:1 combination of Leukotriene C4 and leukotriene D4 dissolved in sterile saline prior to dosing.

Experimental procedures:

Eight eyes are required per treatment for statistical significance.

Administration of vehicle and cysLT should be randomised such that as much as possible the number of cysLT dose applications and vehicle to the left and right eyes will be equal.

Treatment (intravitreal) Eye (left/right)	**Treatment (intravitreal) Eye (left/right)**	**N**
Vehicle (16.6% ethanol; 5 µl)	cysLT (30 µM in 16.6% ethanol, 5 µl)	4
cysLT (30 µM in 16.6% ethanol, 5 µl)	Vehicle (16.6% ethanol; 5 µl)	4

On Day 1 animals receive intravitreal administration 5 µl total volume, as detailed in the table above.

Intravitreal dosing:

Weigh and dose animals subcutaneously with analgesia (Carprofen 5mg/kg (5mg/ml solution)) at least 30 minutes prior to the scheduled time of intravitreal dosing.

Apply dilating agents to the eyes (e.g. Minims® tropicamide 1%, eye drops solution) 5 to 10 minutes prior to scheduled time of intravitreal dosing.

Anaesthetise animals with isoflurane. Monitor animals subsequent to induction of anaesthesia and ensure that animals are anesthetised (e.g. absence of corneal reflex) prior to the following procedures being undertaken. Administer one drop of tetracaine hydrochloride 0.1% to each eye once the loss of corneal reflex has been confirmed; this is to provide local ocular anaesthesia for 10 to 20 minutes.

Perform intravitreal injections in the anaesthetised animals with the aid of a surgical microscope.

Dosing of vehicle/cysteinyl leukotrienes is by intravitreal injection to the vitreous body via the pars plana. Use a 30 gauge needle to make an incision in the sclera just behind the corneo-scleral limbus.

Use a Hamilton (34 Gauge, 13 mm, point style 4; ESSLAB, UK) flexifil bevelled needle, attached to a Hamilton nanofil syringe, going in to the eye at a shallow angle to carry out the intravitreal injection. Deliver treatment of 5µl to the incision site and leave the needle in place for 30 seconds following the injection, to equalise pressure, before withdrawing the syringe. Then inject the other eye using the same method.

Clean needles with sterile distilled water prior to and between injections.

Apply Visco Tears (lubricant) to the eyes immediately post injection and as required until the rat regains consciousness; this is to prevent the cornea drying out.

Record the time the intravitreal dose was administered.

Place animals in a clean cage with food and water, with a warming lamp and heat pad, and monitor constantly whilst recovering from anaesthesia.

Only return animals to their home cages when fully conscious and only mildly ataxic.

Monitor animals for the presence of any obvious signs of blood in the vitreous. If any is noted, terminate the animal by a Schedule 1 method. (Follow the procedure as described in your own project licence).

Evans blue testing:

Evans blue testing occurs on Day 2, approximately 24 hours post intravitreal dosing.

To aid i.v dosing place the animals in a heated chamber (37°C) prior to administration of Evans blue. Administer Evans blue (45 mg/kg i.v. (30 mg/ml in PBS, filtered through a 0.80 µm filter), pre warmed to 37°C) via lateral tail vein injection over 10 seconds.

Immediately after injection with Evans blue, animals will turn visibly blue, confirming uptake and distribution of the dye.

Two hours after Evans blue dosing, administer animals with a terminal dose of anaesthetic (pentobarbital; Euthatal® solution 1 ml i.p.), then open the abdomen and chest cavity.

Section a prominent vein (e.g. vena cava) and take a blood sample immediately (0.3 ml taken in a Sarstedt CB300 LH tube).

Centrifuge immediately at 5,000 rpm for 5 min.

Take a 100 µl plasma aliquot (supernatant) and store frozen at -80°C prior to determination of Evans blue concentration.

Immediately after the blood sample is taken perfuse animals with citrate buffer (pH 3.5; pre warmed to 37°C) via the heart.

Perfuse for 5 minutes by a pump delivering 17.5ml citrate buffer per minute.

After perfusion, bilaterally enucleate all animals and dissect retinas under a microscope.

Retinal Evans blue:

Blot retinas dry and place into pre labelled and pre-weighed (to 4 decimal places) eppendorf vials (one per retina). Weigh contents to 4 decimal places.

Freeze vials containing retinas on dry ice and then to -80 °C prior to overnight freeze-drying.

After freeze drying record the final dry weight of the retinas.

Crush the retinas into 120 µl of formamide.

Extract the Evans blue from the retinas by incubating the vials at 70°C for 18 hours.

Following incubation, centrifuge vials at 15,000 rpm for 5 minutes and measure the absorbance of the supernatant in a microplate reader at 620 nm and 740 nm.

Determine the OD of the retinal Evans blue extracts three times and then calculate the average.

Determine the extract concentration against the same volume of a standard curve of Evans blue in formamide and express results as micrograms of Evans blue per g of retinal dry weight.

Plasma Evans blue:

Thaw plasma samples on ice.

Dilute plasma sample 50 X in formamide.

Measure the absorbance in a microplate reader at 620 nm and 740 nm.

Determine the OD of the plasma Evans blue extracts three times and then calculate the average.

The plasma concentration is determined against the same volume of a standard curve of Evans blue in formamide and results expressed as micrograms of Evans blue per microlitre of plasma.

Calculate retinal vascular permeability in units of µl plasma x g retinal dry weight^{-1}. hr^{-1} using the following equation;

$$\text{RVP} = \frac{\text{Evans blue (µg) / pooled retinal dry weight (g)}}{\text{Evans blue concentration (µg / µl) x circulation time (hr)}}$$

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