Real Science Review:
Pre-clinical Trials of New Pain Reliever Drug

FFA Student, Ray: "Mr. Mac, did you know that one of my quarter horses can't compete in next month's rodeo, because he has really sore feet. The veterinarian says he has something called laminitis. What is that all about? People sometimes get sore feet, but I don't think it is ever like this.

FFA Teacher, McPherson: laminitis is unique to horses, because their feet take a lot of pounding. The inner anatomy of the hoof is unique. Laminitis is apparently very painful to horses, and they may become permanently lame.

Original Report: A. G. P. Guedes, F. Aristizabal, A. thereSole, A. Adedeji, R. Brosnan, H. Knych, J. Yang, S.-H. Hwang, C. Morisseau, B. D. Hammock. Pharmacokinetics and antinociceptive effects of the soluble epoxide hydrolase inhibitor t -TUCB in horses with experimentally induced radiocarpal synovitis. *J. of Veterinary Pharmacology and Therapeutics* **2018,** *41* (2) , 230-238. DOI: 10.1111/jvp.12463.

*Drs. Guedes and Hammock have filed for a patent on the soluble epoxide hydrolase inhibitor to treat laminitis in horses. They have assigned the patent to the University of California, where Dr. Guedes is a faculty member in the college of Veterinary Medicine at U. California, Davis.

Revising author: W. R. Klemm

Vocabulary Used in the Original Report

Analgesia: absence of pain

Antinociceptive: pain relieving ("Nociceptive" means causing a discomfort that in humans we would call "pain." Scientists apply the term to lower animals that receive injury that humans would consider painful if it happened to them. Pain can only be perceived when conscious, and we cannot know if lower animals have the capacity for consciousness (just being awake is not the same as being consciously aware).

Blinding, blinded: situation where the person collecting data does not know the treatment conditions at the time. Purpose is to reduce unintended bias.

Carpus/carpi: the area of the foreleg just above the hoof (similar to the wrist in humans).

Epoxide: a class of chemicals in which the molecule contain a ring of two carbons and one oxygen atom, arranged like an equilateral triangle. Each carbon atom has an attached oxygen or

chain of carbon atoms. “Super glue” contains epoxy groups, but the rest of the molecule is nothing like the epoxy compounds being discussed here in horses.

Hydrolase: “Hydro” refers to water; “lase” refers to breaking down. A hydrolase is any enzyme that uses water to break a chemical bond, which typically results in dividing a larger molecule to smaller molecules. An epoxy hydrolase uses water to break down molecules containing epoxy groups.

Laminitis: an extremely painful inflammation inside the hoof of horses.

Lipopolysaccharide: a large molecule that contains a lipid bound to a number of sugar molecules bonded together.

Mg/kg: milligrams of drug per kilogram of body weight. This is the standard way of stating drug dose.

Plasma half–life: a measure of how long a chemical lasts in the body. It is the post-administration time at which half of the chemical is still present in the blood.

Radiocarpal joint: the joint between the radius and carpal bones.

Rescue Analgesia: injection of a pain reliever immediately after relevant pain data are collected.

A New Treatment for Laminitis in the Horse

Abstract

This study tested the effectiveness of a possible new drug treatment for horse laminitis. Seven adult horses were injected with a lipopolysaccharide irritant into the radiocarpal joint to induce a temporary irritation. Then, the horses were injected intravenously with the drug being tested, an epoxide hydrolase inhibitor, t-TUCB. Tests with various doses of t-TUCB were repeated in the same horses at three-week intervals. Two investigators, blind to the treatment given, assigned pain scores (at rest and at trot), degree of lameness, and touch sensitivity scores before and up to 48-hours after TUCB injection. Pain, lameness, and sensitivity-to-touch scores were significantly lower with the 1 mg/kg dose of drug, but not at lower doses. Plasma half-life of the 1 mg/kg dose was 24 +/- 5 hours.

Introduction: Questions to Answer

1. If there was a hypothesis, either stated or implied, what was it?

2. How well did the authors justify doing this study?

3. What are some other related ideas that they did not test?

Introduction

A common way to relieving pain in horses (*and humans*) is to use drugs that inhibit the enzyme cyclooxygenase (COX). COX is part of a metabolic pathway that generates painful lipids (Guedes, 2017). At the same time, another pathway generates certain anti-inflammatory fatty acids. However, these fatty acids are rapidly destroyed by another enzyme, epoxy hydrolase.

Studies in rodents revealed that inhibition of epoxy hydrolase prevents destruction of the anti-inflammatory fatty acids (see Figure 1). These can then counteract the pain caused by COX (references were listed). Preliminary unpublished studies in horses with laminitis suggested that inhibiting epoxy hydrolase relieved pain. This present study is part of a preclinical trial of various doses of an epoxy hydrolase inhibitor on pain relief.

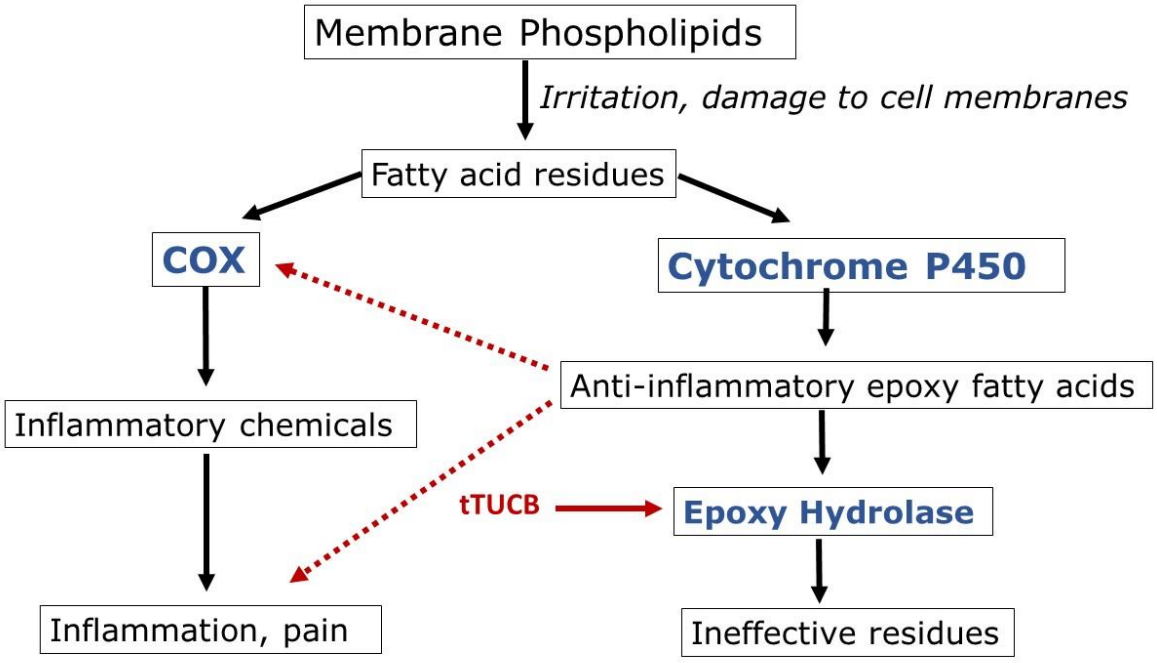

Figure 1. Trauma and irritation enable membrane phospholipids to break into smaller fatty acid residues that activate two further metabolic pathways. The COX pathway generates inflammatory chemicals known as prostaglandins, while the cytochrome p450 pathway produces anti-inflammatory epoxy fatty acids. However, these anti-inflammatory fatty acids cannot produce their effect because they are destroyed by epoxy hydrolase (EH). The theory is that a drug that could inhibit EH would prevent the destruction of the anti-inflammatory epoxy compounds. Epoxy compounds could then accumulate and reduce inflammation and pain (diagram redrawn and simplified from original).

Think About It!

In your notebook, put in reminders for:

- The two metabolic pathways activated when cell membranes are damaged.
- The inflammatory chemicals produce in the COX pathway.
- The anti-inflammatory chemicals produced in the cytochrome pathway.
- The significance of inhibiting the epoxy hydrolase in the cytochrome pathway.

Methods: Questions to Answer

1. What acts as a control group by receiving no treatment? What is the purpose for having this group? How well does it serve that purpose?

2. What factors (variables) that might affect the results are not considered?

3. What are the advantages and disadvantages of the procedures and equipment used?

Material and methods

Animals and Study Design

The carpal joints of seven horses were injected with irritant chemical. Then, horses were injected intravenously with various doses of a drug, t-TUCB, or the drug's solvent alone as a control. A gap of three weeks between each treatment allowed elimination of drugs and the natural reversal of induced pain. Clinical and lameness examinations before testing established that horses were healthy. We conducted blood tests were before and after each treatment. The test design was:

- Randomized (random sequence of drug or placebo)
- Crossover (repeat treatment on same animal),
- Blinded (researchers did not know whether drug or placebo were given).

Synthesis and Preparation of t-TUCB

We synthesized the drug under test, t-TUCB, according to previously published methods (references were listed). The day before each experiment, t-TUCB was dissolved in dimethyl sulfoxide) to final concentrations of 3, 30, or 90 mg/ml, was filter-sterilized with 0.2 μm pore size sterilizing-grade membranes and placed into sterile 10 ml silicone-coated glass tubes. These tubes were kept upright at room temperature in the laboratory until usage the next day. The solubility of t-TUCB in DMSO at room temperature was confirmed via liquid chromatography–mass spectrometry (LC-MS).

Baseline Data Collection

The day before the experiment, the hairs over the dorsal aspect of both carpi were clipped, and baseline data were collected. After overnight fasting, we placed a catheter in both jugular veins following skin desensitization with 1.5 ml 2% lidocaine. One catheter was for administering t-TUCB or placebo and was subsequently removed. The other catheter was for blood sampling and administering sedatives. This catheter was removed 24 hr after beginning the experiment.

Pain Model

Our model induced reversible pain and lameness by injecting an irritant into the radiocarpal joint. This method was selected because other reports found it useful in studies of pain relief in horses (references were cited). Another reason was that a similar approach was used in earlier rodent studies that tested t-TUCB for anti-inflammatory and pain-relieving effects (references were cited). Before injecting the irritant, horses were sedated with 0.2–0.5 mg/kg xylazine, and the skin over both carpi was surgically scrubbed using povidone-iodine antiseptic soap. One radiocarpal joint was injected with 3 μg of the irritant lipopolysaccharide (LPS) from Escherichia coli (source: Sigma-Aldrich Co.) freshly prepared sterile to 1.5 μg/ml in 0.9% NaCl. The first injected joint was randomly assigned; subsequent injections alternated between joints.

Treatments

All syringes were prepared to contain the same volume of solution (0.009 ml/kg). Treatments consisted of 0.009 ml/kg DMSO (vehicle control) or 0.03, 0.1, 0.3, or 1.0 mg/kg t-TUCB.

Immediately after LPS injection, we drew 10 ml of blood was from the jugular vein catheter, and administered the t-TUCB intravenously over 30–45 seconds. The catheter was then flushed with the aspirated blood and then with 5 ml of heparinized saline to ensure administration of the full dose.

Measuring t-TUCB tissue concentrations and distribution

We measured plasma drug levels by liquid chromatography/mass spectral methods as previously described (Tsai et al., 2010). Statistical calculations were performed with commercially available software, using conventional techniques (Graphpad Prism version 5). Data from the 0.3 mg/kg of t-TUCB were omitted, because plasma levels were too low to be measured.

Test Methods for Lameness and Analgesia

The main variables of interest were pain and lameness scores. Outcomes were assessed before and at 2, 4, 8, 12, 24, 36, and 48 hr after LPS and treatment. Sequence of procedures was always the same (blood sampling, physical exams, pain and lameness scoring, and joint fluid collection). Physical examination included heart (HR) and respiratory (RR) rates, mean arterial pressure (MAP), and rectal temperature. Carpal joint circumference was determined as an index of swelling with measuring tape positioned at the level of the accessory carpal bone.

Two investigators blinded to treatment independently assigned pain and lameness scores at the predetermined time points. Pain scores were assigned with a visual scale (VS) for three different conditions (at rest in the stall, walking, and then trotting in a straight line) and then averaged to

form a final score. The VS corresponds to a 100 mm line representing the range of possible pain (0 = "no pain" on the left and 100 = "worst possible pain" on the right). The evaluator places a mark on this line corresponding to the pain severity, and the distance from the left extreme to this mark corresponds to the VS score. Observations of general demeanor (facial expression, position of ears, interest in surroundings) and weight bearing on the LPS-injected leg formed the basis for the VS scoring. The VS was shown to be highly reliable when assessing lameness in horses, especially when used by experienced individuals (references were cited).

At the 12-hr evaluation time point, horses with VS > 50 mm at rest and walk received intravenously 4 mg/kg phenylbutazone (Equi-Phar phenylbutazone injection; Vedco Inc.) to rescue the horse from pain. The cut-off for rescue analgesia was similar or slightly more stringent to that previously reported in this same pain model (references cited). The dose of phenylbutazone was selected primarily on the basis of its clinical use (references cited).

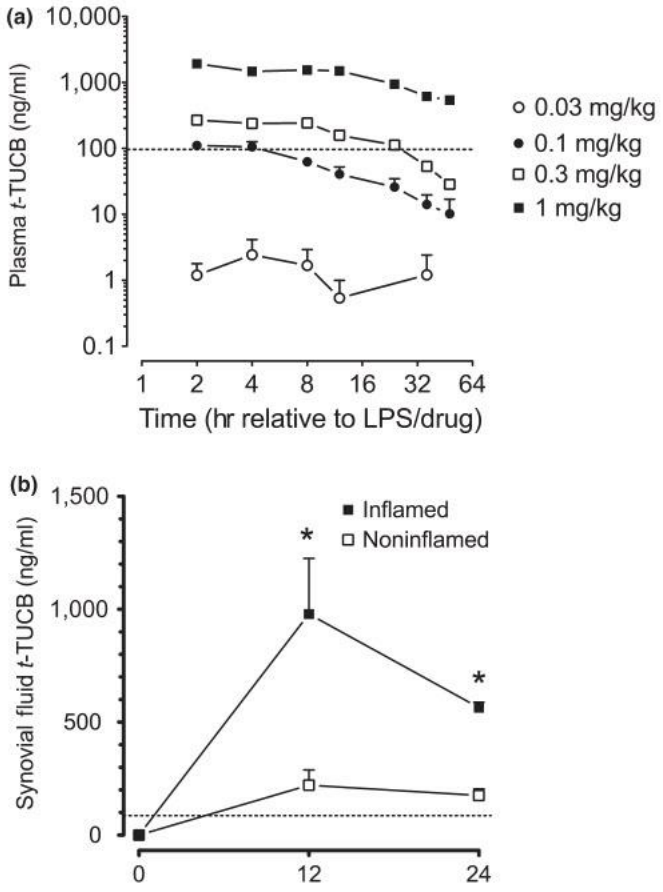

Figure 2. T-TUCB levels in plasma (a) and radio-carpal joint fluid (b) at various times after treatment. In synovial fluid, the higher drug levels were significantly higher (*) in inflamed joints at both times.

Results

Two horses had to be withdrawn early from the study for reasons unrelated to this study (they were needed for teaching purposes). Therefore, the number of horses per treatment varied.

Physical Examination Variables

There were no treatment effects on these variables (data not shown here).

Drug Blood and Carpal Joint Levels

The measured plasma and joint concentration of t-TUCB are shown in Fig. 2. Plasma half-life of the 1 mg/kg dose was 24 +/- 5 hours.

Pain and Lameness Scores

The highest dose of t-TUCB markedly reduced both pain and lameness scores at statistically significant levels at the highest dose level of 1 mg/kg (Figure 3).

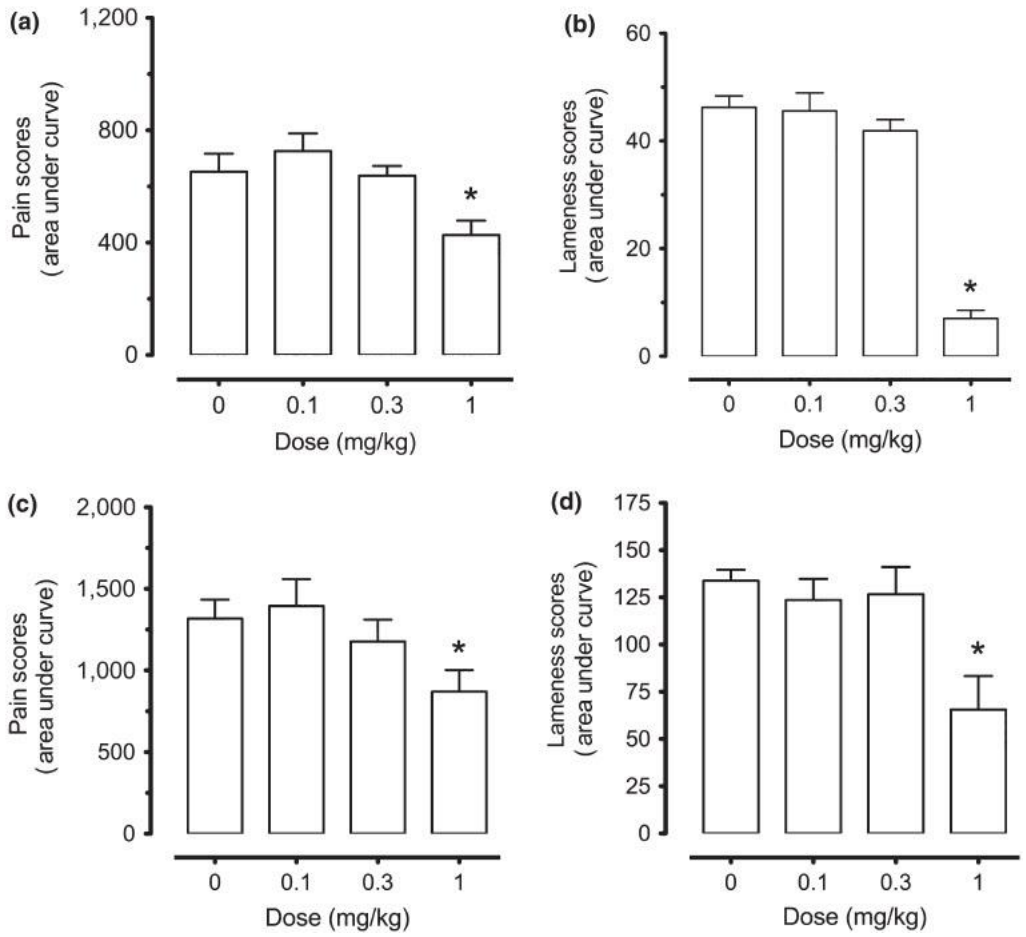

Figure 3. Dose-response data for pain and lameness scores during 0-12 hours after injection (top two panels) and 0-48 hours (bottom two panels).

Results: Questions to Answer

1. Do the results support the hypothesis or not? How convincing is that support?

2. Do you notice anything of possible importance in the data that authors failed to mention?

3. Is the variation in data large enough to suggest that some unknown variables interfere with reliable results? What might these be?

4. How big is the 'treatment' effect? Is it large enough to be of much practical importance?

Joint Fluid Cells and Proteins

The protein concentration and leukocyte numbers increased markedly and statistically significantly after LPS administration in all treatments at the 12- and 24-hr evaluation time points compared to baseline (data not shown here). There were significant increases in % of neutrophils and a significant decrease in % of small and large mononuclear cells after LPS injection. There were no significant *t*-TUCB treatment effects on blood cells as compared to control.

Discussion

This study used an inflammatory joint pain model to assess the antinociceptive and anti-inflammatory effects of a range of doses of the epoxy hydrolase inhibitor t-TUCB. In this model, 1 mg/kg t-TUCB produced significant antinociceptive effects, as indicated by decreased response to touch) as well as in pain and lameness scores. There was little anti-inflammatory activity as evaluated by the effects on joint swelling or inflammatory blood cell numbers and protein concentration in the joint fluid. These results indicate that drug-induced inhibition of EH may releive inflammatory joint pain in horses.

It is worth noting that the levels of t-TUCB were significantly higher in the joint fluid of the inflamed joint compared to the noninflamed contralateral joint. However, we have no explanation.

In a prior study of rats, t-TUCB doses as low as 0.1 mg/kg significantly reduced mechanical hyperalgesia to injection of irritant LPS into the foot pads (reference cited). A prior study of horses being treated with long-term clinical laminitis showed that adding 0.1 mg/kg of t-TUCB significantly reduced pain-indicating behaviors (references cited). Notably, this low dose was not effective in the present study.

Experimental paradigm (species, pain phenotype, diet, health status, concurrent COX inhibitors) may have profound influence on the kind and amount of pain-inducing fatty acids and thus the response to treatment with epoxy hydrolase inhibitors. This makes direct comparisons between studies difficult.

The current study has several potential limitations to be considered. First, the small sample size for the 0.3 mg/kg t-TUCB treatment could have produced a false-negative result in pain and lameness scores. Second, the cut-off point for rescue analgesia was arbitrarily selected, although it was similar to a previous study using this same model (references cited). It is unlikely that the rescue analgesia with phenylbutazone at the 12-hr time point was a significant confounding factor because the pain and lameness findings were the same whether the data were analyzed for the first 12 hr (i.e., before rescue analgesia) or for the entire 48-hr period.

Lastly, xylazine sedation likely did not affect pain and lameness scores at early time points (2 and 4 hr), because it has a short duration of action (reference cited), especially at the low doses used in this study.

In conclusion, our results indicate that inhibition of epoxy hydrolases may have a role in decreasing inflammatory joint pain and lameness in horses. Future studies are needed for understanding t-

Discussion: Questions to Answer

1. Summarize how the authors discussed the results in terms of their original hypothesis.

2. Did the authors point out ideas that go beyond the hypothesis?

3. What ideas for future research did the authors generate?

4. What ideas for future research do you generate?

5. How would you state the "so what" or take-home lesson?

TUCB, and for the role and mechanisms of inflammatory fatty acids and epoxy hydrolases in joint pain.

Conflict of Interest Statement

A. G. P. Guedes, C. Morisseau, S-H. Hwang and B. D. Hammock are authors of composition of matter and/or use patents in this area. B. D. Hammock is the founder of EicOsis. This company is moving she inhibitors through clinical trials for treating pain, hypertension, inflammation, and other disorders. However, this study is independent from the company.

References

Identification of the references can be found in the original report and are not necessary for our purposes here.