Soluble Epoxide Hydrolase Inhibition Is Antinociceptive in a Mouse Model of Diabetic Neuropathy

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Abstract: Neuropathic pain is currently an insufficiently treated clinical condition. There remains a critical need for efficacious therapies without severe side effects to treat the uniquely persistent and tonic pain of neuropathy. Inhibitors of the soluble epoxide hydrolase (sEH) enzyme that stabilize endogenous epoxy fatty acids have demonstrated antihyperalgesia in clinical chronic inflammatory pain and modeled neuropathic pain. Recently, the conditioned place preference assay has been used to evaluate the tonic nature of neuropathy in several animal models. The current experiments use the conditioned place preference assay alongside withdrawal thresholds to investigate the antihyperalgesic efficacy of sEH inhibitors in a murine model of diabetic neuropathy. Here, the sEH inhibitor trans-4-[4-(3-trifluoromethoxyphenyl-1-ureido)-cyclohexyloxy]-benzoic acid (t-TUCB) at 10 mg/kg induced a robust place preference in diabetic neuropathic mice representative of pain relief. Importantly, this effect was absent both in control mice and in sEH-knockout mice at the same dose, indicating that t-TUCB is not positively reinforcing or rewarding. When compared to gabapentin, t-TUCB elicited a similar degree of withdrawal threshold improvement without the same degree of spontaneous locomotion decline in neuropathic mice. Overall, these experiments show that inhibiting the sEH attenuates chronic pain and offers an alternative to current side-effect-limited therapies to meet this clinical need.

Perspective: These experiments demonstrate antihyperalgesia in a murine chronic pain model mediated by inhibiting the sEH enzyme. The results of this study indicate that inhibiting the sEH is a promising alternative for blocking chronic pain.

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Key words: Neuropathic pain, conditioned place preference, antihyperalgesia, soluble epoxide hydrolase, epoxy fatty acids.

Neuropathy is a debilitating condition currently with no adequate therapy. Despite decades of research investigating alternatives to treat chronic pain, few improvements have been made and it remains a largely unmet clinical need. This need is becoming urgent as the diabetic population increases worldwide and neuropathy occurs in 1 of every 2 patients. The pain of diabetic neuropathy is due to nerve damage that progresses to central sensitization of the nervous system characterized by ectopic and idiopathic firing of nerves. This pain is felt both as hyperalgesia, a hypersensitivity to painful stimuli, and as allodynia, a painful response to innocuous stimulation. The arachidonic acid cascade has been exploited for decades to alter pain sensation and inflammation. Several classes of enzymes, including cyclooxygenase, lipoxygenase, and cytochrome P450 oxidase (P450), metabolize the parent polysaturated fatty acids into bioactive lipid metabolites. The most well-known metabolites are the prostaglandins formed by cyclooxygenase enzymes and,
in particular, prostaglandin E₂, a sensitizing and directly acting algogen. Recently, the importance of epoxy fatty acids as signaling mediators and their functional significance in nociception has received attention. The epoxy fatty acids formed by P450 enzymes are chemically stable, though rapidly degraded by the soluble epoxide hydrolase (sEH) enzyme. Small molecule inhibitors of sEH have been used to stabilize and elevate levels of these natural molecules in vivo, allowing observation of their antinociceptive activity. Inhibiting the sEH has been shown to be antihyperalgesic in models of inflammatory pain.\(^{15,16}\) These experiments used a quantitative metabolomic profile to show that this antihyperalgesia was correlated to changes in the epoxy fatty acid substrate to corresponding diol product ratios after sEH inhibition. Additionally, application of exogenous epoxyeicosatrienoic acids formed from arachidonic acid has been shown to block pain in rodents.\(^{17}\) Subsequently, the epoxides of docosahexaenoic acid and eicosapentaenoic acid have also demonstrated antihyperalgesia in modeled pain.\(^{26}\)

Because epoxy fatty acid metabolites of all 3 classes have been shown to be substrates of sEH, inhibiting this enzyme is a uniquely suited strategy for eliciting antihyperalgesia. Recently, sEH inhibition also successfully treated a clinical case of severe chronic neuropathic pain in equine laminitis.\(^{13}\) Here, we test the antihyperalgesic efficacy of sEH inhibitors in a model of chronic pain, specifically diabetic neuropathy.

The von Frey assay is a traditional measure of allodynia using a narrow filament to probe for increased sensitivity to innocuous mechanical stimulation. However, clinical descriptions of diabetic neuropathy often include a tonic, persistent pain that is not stimulus evoked.\(^{1,2}\) Although pin prick assays are still used clinically, these assays measure the response to an acutely applied stimulus and thus do not represent the tonic nature of neuropathy.\(^{4,25}\) Consequently there are limitations to using only withdrawal threshold assays as measures in modeled neuropathic pain.\(^{32}\) Recently the conditioned place preference (CPP) paradigm has been used to address these limitations when investigating neuropathic pain.\(^{10,17,28}\) The CPP uses a nonevoked and drug-free testing paradigm to assess pain. It has therefore been suggested that the CPP assay allows for a better assessment of tonic pain.\(^{10,17,34}\) An added advantage of the CPP assay in testing analgesics is its ability to assess both the negative (relief of a pain status) and positive (rewarding) reinforcing effects of compounds associated with environmental cues.\(^{5,32,36}\)

Here, we employed the CPP assay to determine the effects of sEH inhibition on diabetic neuropathy. We then used the CPP assay to test for reward or positive reinforcement associated with the small molecule sEH inhibitor in both wild-type and sEH-null mice.

**Methods**

**Animals**

All procedures and animal care adhered to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were performed in accordance with the protocols approved by the Animal Use and Care Committee of the University of California, Davis. Great care was taken to minimize suffering of the animals and to reduce the number of animals used. Experiments on wild-type mice used groups of male C57BL/6 mice (20–22 g) purchased from Charles River Laboratories (Wilmington, MA). Experiments on sEH-knockout mice used mice on a 129X1/SvJ_C57BL/6 background, backcrossed over 10 generations with targeted disruption of the Ephx2 gene and maintained at the facilities of the University of California, Davis.\(^{30}\) Both wild-type and sEH-null mice were housed under standard conditions (25°C) in a fixed 12-hour light/dark cycle with ad libitum food and water.

To induce diabetes, wild-type mice were injected with 150 mg/kg streptozocin intraperitoneally.\(^{9}\) After 1 week, the mice were assessed for a decrease in hind paw mechanical withdrawal thresholds indicating allodynia and were tested for their blood glucose levels via tail vein blood.

For quantification of the sEH inhibitor, whole blood was collected per Liu et al.\(^{23}\) Briefly, 10 μL whole blood was taken via the tail vein with a pipette tip rinsed with 7.5% K₂ ethylenediaminetetraacetic acid and added to 50 μL deionized water before and 15, 30, 60 and 90 minutes after dosing with the inhibitor. Samples were taken from 8 mice per group and the mean plasma concentration ± standard error of the mean (SEM) is reported.

**Chemicals**

The sEH inhibitor trans-4-[4-(3-trifluoromethoxy-phenyl-1-ureido)-cyclohexyloxy]-benzoic acid (t-TUCB; also referred to as uc1728) used for the experiments was synthesized and characterized in-house as previously described.\(^{14}\) The sEH inhibitor t-TUCB has a mass of 438.3 g/mol, a 1.6 clogP, and 212.2°C melting point. It is soluble in water at 5 μg/mL but up to 10 mg/mL in polyethylene glycol (PEG400). As a high-melting-point crystal, it dissolves slowly in water and thus is first dissolved in an organic cosolvent. t-TUCB is a potent low-nanomolar inhibitor in vitro on murine recombinant sEH enzyme determined with an α-cyanocarbonate substrate in a fluorescent assay.\(^{22,38}\) Doses of t-TUCB were formulated in PEG400 for the experiments and injected subcutaneously. Morphine sulfate and gabapentin purchased from Fisher Scientific (Waltham, MA) were diluted in saline and injected subcutaneously for morphine and intraperitoneally for gabapentin.

**Nociceptive and Motor Skill Bioassays**

A CPP apparatus was based on the method of Carr et al with modifications.\(^{6}\) The apparatus is a 30 × 16 × 20-cm rectangular acrylic box with distinct visual patterns and a floor with tactilely distinct sides of equal size. The patterned visual cues avoided light and dark solid visual environments that influence conditioning, giving the basal preference of rodents for dark conditions. Mice were first habituated to the box for 30-minute sessions at the same time of day on 2 consecutive days. For the preconditioning measurement, mice were placed into the apparatus with access to both chambers and...
observed for 30 minutes. Preconditioning was followed by 3 conditioning days on which the vehicle was counter-balanced daily with the compounds (sEH inhibitor, gabapentin, or morphine), each for 30-minute intervals. Briefly, the mice received an injection of vehicle and were immediately placed in the box isolated to 1 chamber in the morning. At least 4 hours after the vehicle was injected, the same mice were injected with sEH inhibitor or control drugs (gabapentin, morphine) as appropriate and immediately isolated to the counterbalanced chamber. On the day after this, mice were tested for preference by being placed in the box with free access to both chambers and observed drug free for 30 minutes. The preconditioning day and test day were videoed and analyzed with custom software that tracked the mouse and calculated the time spent in both chambers of the box. Measurements were calculated as test minus preconditioning time in the drug-paired chamber. There was a slight bias in the baseline of all mice tested, and therefore mice were conditioned with vehicle to their preferred chamber and compounds to the nonpreferred chamber. Increased time spent in a chamber (eg, increased time in the drug-paired, nonpreferred chamber) indicated preference for that chamber. The PEG400 vehicle was tested in a separate group of mice following the same procedure for test compounds. The vehicle control group received PEG400 counterbalanced to the nonpreferred chamber at least 4 hours after sham injection and placement in the preferred chamber. Several preliminary tests to refine the experimental conditions showed no difference in PEG400 vehicle or saline injections compared to naive mice.

For the von Frey assay, an electronic von Frey anesthesiometer (IITC, Woodland Hills, CA) fitted with a 0–90 g probe arm was used to quantify allodynia in the diabetic mice. Mice were placed in clear acrylic chambers on a steel mesh floor. The hind paw of the mouse was probed through the mesh with a rigid tip probe connected to an electronic readout pressure meter displaying the grams of force required to elicit a hind paw withdrawal. The withdrawal thresholds were measured 3 times per mouse at 1-minute intervals for each point. The scores are reported as the percentage of diabetic baseline values (normalized to 100%) on the day of treatment averaged per group ± SEM.

**Statistics**

Data were analyzed using SigmaPlot 11.0 for windows (Systat Software Inc, San Jose, CA). The applied statistical methods are reported in the Results section, with P values ≤ 0.05 considered significant.

**Results**

**Dose Response of t-TUCB in the CPP Assay**

Before the CPP assay, allodynia was confirmed in the diabetic mice. The average was a 63% decrease in mechanical withdrawal thresholds compared with prediabetic baselines. We then tested t-TUCB in these mice to assess the negative reinforcing effects of sEH inhibition in the CPP assay. The effects were measured and calculated as the amount of time spent in the drug-paired chamber at the postconditioning test minus the preconditioning time in the drug-paired chamber. Increased time in the drug-paired chamber indicated preference for that chamber. Diabetic C57/B6 male mice showed no place preference for the vehicle-paired chamber in the CPP assay (Fig 1). The dose range of 1, 3, and 10 mg/kg/d t-TUCB exhibited a clear dose-response relationship in this assay. The 1 mg/kg/d dose of t-TUCB showed no statistically significant change for time spent in the drug-paired chamber (Fig 1). An intermediate dose of 3 mg/kg/d resulted in an increased CPP that was significant when compared to vehicle controls (t-test: t [10] = 2.493, P = 0.032, n = 6). However, when the entire dose range was analyzed for the effect of dose on CPP acquisition with a 1-way analysis of variance (ANOVA), 10 mg/kg/d t-TUCB remained the only statistically significant dose compared to vehicle controls. At this dose, t-TUCB induced a robust place preference for the drug-paired chamber in neuropathic mice, indicating antihyperalgesia (all t-TUCB doses, 1-way ANOVA, Holm-Sidak method: F[3, 20] = 5.141, P = 0.008 vs vehicle, n = 5–7). Gabapentin, a positive control for diabetic neuropathy, produced an expected place preference in the diabetic mice (t-test: t[12] = 2.307, P = 0.040 vs vehicle, n = 6–8; Fig 1).

**Effects of t-TUCB in Withdrawal Threshold and Open-Field Assays**

Separate groups of diabetic mice were subsequently treated with a single administration of t-TUCB (10 mg/kg) or gabapentin (100 mg/kg) or vehicle and tested in of a 16-square-grid clean floor with slight modifications from Luria et al.24 The open-field assay was tested for 2 minutes once before injection as the diabetic baseline, and then at 30 and 60 minutes postinjection. Mice were tested for 2 minutes and returned to their home cage for each interval. The open-field assay was scored manually, with the score a combination of vertical rears and lines crossed completely with both hind paws. Scores are reported as the percentage of diabetic baseline values compared to both chambers and observed drug free for 30 minutes.
Effects of t-TUCB in Controls and Blood Concentration Assessment

Figure 1. Repeated administration of t-TUCB relieves diabetic neuropathy in the CPP assay. Increasing the dose of t-TUCB results in a clear dose-response relationship in the CPP assay depicted as the change in time (test-preconditioning) spent in the drug-paired chamber. The 10 mg/kg/d dose of t-TUCB significantly increases the CPP for the drug-paired chamber (*P = .008). The positive control gabapentin (100 mg/kg/d) induces a drug-paired chamber preference compared to the vehicle (8P = .040). However, the 10 mg/kg/d dose of t-TUCB outperformed the positive control gabapentin at a 10-fold lower dose.

Effects of t-TUCB on sEH Knockout Mice in the CPP Assay

We then used sEH-knockout mice to address the possibility that the sEH inhibitor could have a positively reinforcing effect unrelated to the inhibition of the sEH enzyme. As expected, there was no effect of the PEG400 vehicle on place preference in the sEH-knockout mice (Fig 4). When administration of t-TUCB at 10 mg/kg/d for 3 days was tested per the post hoc test, there was no significant increase compared to vehicle in the time spent in the t-TUCB-paired chamber. Morphine was also tested in sEH-knockout mice to ensure their capacity to respond to a positive control, and it induced a robust place preference (1-way ANOVA, Holm-Sidak method; F[2, 18] = 10.988, P ≤ .001, n = 6–8; Fig 4). The lack of CPP in sEH-knockout mice is consistent with results in nondiabetic wild-type controls, indicating that there are no positive reinforcing effects of t-TUCB.

Discussion

This study demonstrates that inhibiting the soluble epoxide hydrolase enzyme can attenuate chronic pain in a model of murine diabetic neuropathy. The sEH inhibitor t-TUCB exhibits a clear dose-response relationship and equals the effects of gabapentin, a first-line therapy for this condition. Additionally, t-TUCB does not induce place preference in control or sEH-knockout mice, indicating that there are no rewarding side effects of inhibition of the sEH enzyme or the small molecule inhibitor itself.

Withdrawal Thresholds and Open-Field Assays

The use of withdrawal threshold assays is a standard protocol for determining pain-like behavior in rodent models. We used the von Frey assay to verify allodynia
in the diabetic mice for this study, which assesses sensitivity to innocuous mechanical stimulation. We also tested the sEH inhibitors with this reflex withdrawal assay to confirm the CPP results. In these studies, administration of the sEH inhibitor to neuropathic mice significantly improved their mechanical withdrawal thresholds. We compared 10 mg/kg t-TUCB to 100 mg/kg gabapentin, both of which effectively increased neuropathic withdrawal thresholds. The side effects of gabapentin including sedation and loss of motor activity have been previously established in mice. It is possible that the motor skill impairment may also affect reflex withdrawals. Here, we used 100 mg/kg gabapentin, which was reported as a minimally effective dose in murine neuropathic pain models.18,20 However, a slightly lower dose of 90 mg/kg has been shown to reduce both rotarod performance and spontaneous motor activity in mice.11 In contrast to these effects of gabapentin, 10 mg/kg t-TUCB–treated mice previously displayed no observable effects on sedation or loss of spontaneous

Figure 2. A single dose of t-TUCB relieves pain similar to gabapentin. (A) A single 10 mg/kg dose of t-TUCB increases mechanical withdrawal threshold scores from painful diabetic BLs normalized to 100% (*P ≤ .001). A single dose of gabapentin 100 mg/kg also increases mechanical withdrawal thresholds (#P ≤ .001). Gabapentin analgesia is not significantly increased compared to t-TUCB (P = .647). (B) The analgesia mediated by a single dose of gabapentin also correlates with a decrease in open-field activity at 60 minutes postadministration compared to t-TUCB (†P = .030). Abbreviation: BL, baseline.

Figure 3. Repeated administration of t-TUCB is not active in absence of pain state. (A) In control mice, the vehicle does not significantly alter place preference. Similarly in nondiabetic controls, t-TUCB at 10 mg/kg/d for 3 days does not induce a preference for the drug-paired chamber (n.s., P = .931). (B) Although no place preference is induced, the blood concentration of t-TUCB is well above the IC50 for the duration of the CPP assay. A single 10 mg/kg administration of the inhibitor results in a blood concentration 40× the IC50 after 15 minutes and continues to increase to more than 100× the IC50 by 90 minutes.
animals. Although the basis for the different result in a change from the basal pain-like behavior in diabetic administration could be associated with pain relief and spontaneous movement associated with some of the antinociceptive activity of exogenous eicosatrienoic acids and other epoxy fatty acids may be mediated through the endogenous opioid system. There is an indication that the unexpected trend of -TUCB does not share the side effect of sedation with morphine or hypoalgesia in the von Frey assay. The CPP is an alternative for assessing the ongoing tonic pain of neuropathy by using nonevoked conditions to measure pain relief. This is compared to reflex withdrawal tests that use acutely applied stimulus to measure response. The CPP results in these experiments have revealed that sEH inhibition relieves diabetic neuropathic pain in mice in a dose-dependent manner. There was a positive dose-response relationship, although 1 mg/kg -TUCB did not induce a CPP. However, the 10 mg/kg/d dose of -TUCB was effective in reducing pain-related behavior evidenced by a robust drug-paired chamber preference in neuropathic mice. It was surprising that only the high dose of -TUCB was statistically significant because sEH inhibitors typically have biological outcomes at much lower doses than those used in these experiments. However, previous use of -TUCB in the rat also demonstrated 10 mg/kg to be the minimum significantly effective dose in the diabetic neuropathy model compared to the lower doses that were effective on inflammatory pain. The plasma concentrations of -TUCB in mice measured in this study do not reach a maximum within 30 minutes, which is equivalent to the duration of the conditioning in the CPP assay. However, at 10 mg/kg, a single administration of -TUCB reached maximal efficacy by 30 minutes in diabetic mice in the von Frey assay, and a single 10-mg/kg dose reached more than 60× the murine IC50 in control mice within this time period. The difference in effective dose compared to other outcomes could also be due to the requirement of conditioning in the CPP assay. As described by Bardo et al, the necessity for temporal contiguity to achieve a place preference was accounted for in this study by immediately placing the mice in the drug-paired chamber after injection. The onset of the drug effect, in this case alleviating pain, was demonstrated by both increased withdrawal thresholds in the von Frey assay and induced place preference in the CPP assay within 30 minutes of administration. The positive control for these experiments, gabapentin, is a first-line therapy to treat human diabetic neuropathy. Gabapentin has been found minimally effective after nerve ligation induced neuropathy at 100 mg/kg. Therefore, a dose of 100 mg/kg gabapentin was chosen for the positive control. Gabapentin induced a statistically significant preference for the drug-paired chamber in diabetic mice. The lack of effect in control mice administered 100 mg/kg gabapentin has been previously observed. The previous studies differed in the model and assay parameters they employed compared to the current experiments. To our knowledge, the experiments here are the first to examine diabetic neuropathy in the CPP assay. Although the 100 mg/kg dose of gabapentin induced a place preference in diabetic neuropathic mice, it was outperformed by a 10-fold lower dose of the sEH inhibitor. The determination of a minimal effective dose of gabapentin in this model was not the object of these experiments. However, previously a matching dose of 10 mg/kg of gabapentin was ineffective against streptozocin-induced neuropathy in mice using a tail-flick assay. Furthermore, these observations in mice are consistent with a recently reported equine study in which an sEH inhibitor reversed the severe inflammatory and neuropathic pain associated with terminal laminitis. In this case, the patient failed to respond to the standard of care steroid, nonsteroidal anti-inflammatory drug, and gabapentin treatment. The

**CPP Negative Reinforcement**

The CPP is an alternative for assessing the ongoing tonic pain of neuropathy by using nonevoked conditions to measure pain relief. This is compared to reflex withdrawal tests that use acutely applied stimulus to measure response. The CPP results in these experiments have
complete recovery of the thoroughbred suggests that the efficacy of sEH inhibition reported in mice in the CPP assay extends beyond rodent models.

**CPP Positive Reinforcement**

The CPP paradigm was originally designed for investigating drug reinforcement and has been used to test the rewarding quality of several addictive drugs.\(^8\) This is relevant to pain research because opioids as the most potent class of analgesics have reward and addiction side effects that limit their use. Given the potent antihyperalgesia mediated by use of sEH inhibitors, the CPP paradigm was used to also investigate possible positive reinforcement (reward) on t-TUCB administration. Importantly, there was no effect of the 10 mg/kg/d dose in nondiabetic control mice in the CPP assay. This is despite the plasma levels in control mice demonstrating a concentration 40\(^\times\) of the murine IC\(_{50}\) within 15 minutes after a subcutaneous injection of the inhibitor. Thus, there were no observable rewarding effects mediated by sEH inhibition in control mice. This result in controls, though remarkable, was expected because the sEH inhibitors have demonstrated a lack of activity in the absence of a painful state, even at high dose.\(^{16,36}\) It is also essential to note that the controls treated with 10 mg/kg t-TUCB also did not show an aversion to the drug-paired chamber. An aversion to the drug-paired chamber may indicate negative physiologic or sensory effects related to the treatment.\(^{25,36}\)

Overall, the outcome indicates that the sEH inhibitor is not active in the absence of a painful state and does not have effects that induce aversion in the CPP assay. The results in the sEH-knockout mice further support the absence of positive reinforcement related to t-TUCB.

The repeated administration of the inhibitor at the same 10 mg/kg dose in sEH-null mice did not produce a place preference. Therefore, there are also no observed rewarding effects of the small molecule inhibitor independent of sEH inhibition. When morphine was tested in the sEH-null mice as a positive control for reward, there was a robust place preference response. Thus, the inhibitor did not demonstrate positive reinforcement in mice lacking the target enzyme, and additionally these sEH-null mice respond appropriately to reward conditioning. This is the first test of potential off-target rewarding effects of an sEH inhibitor, though these inhibitors were previously known to have no hypoalgesic effects in control animals. Given the lack of positive reinforcing effects and high efficacy against neuropathy, we believe that inhibiting the sEH enzyme has potential as a novel strategy in treating chronic pain.

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**Supplementary Data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpain.2014.05.008.


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Supplemental Figures.

The CPP apparatus as used in the laboratory (Left). One chamber with isolation divider in place for conditioning (Right). A mouse in the apparatus, open to both sides for the testing paradigm (Below). The box is made of white acrylic walls set over a removable floor that has equal sides of wire mesh and metal rods (Med Associates Inc.). Videos are taken with a Sony Handycam positioned on above the apparatus as seen through antireflective glass. The video is analyzed by custom software that tracks the mouse and calculates the time spent on either side of the apparatus.