Murine Models of Inflammatory and Neuropathic Hypersensitivity, Morphine Tolerance, and Precipitated Withdrawal are Reduced after Intrathecal Injection of K-ATP Channel Prodrugs

A THESIS SUBMITTED TO THE FACULTY OF
THE UNIVERSITY OF MINNESOTA BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

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August 2022

Acknowledgements

I would like to acknowledge the many people who have helped me along the way. Thank you to my friends and family for their patience with my busy schedule.

Thank you to Dr. Peter Dosa for making the compounds used in my project. I am grateful to the faculty and staff of the department of Chemistry and Biochemistry for their continued support throughout my education at the University of Minnesota, Duluth. I would like to thank my committee for taking the time to help me on this journey and providing your expertise to ensure my success.

Thank you, Kayla, for training me and all your help throughout my time in the Klein Lab. To the Klein Lab members, thank you for your help planning and completing experiments, as well as keeping me entertained on long days. I will cherish these memories forever. Sunna, Shelby, and Elena: thank you for helping scoring mouse behavioral data. I would also like to thank Heidi Aldrich and Ian Aldrich for caring for the animals used in my experiments.

Finally, I would like to send my sincere gratitude to my advisor, Dr. Klein. Thank you for welcoming me into your lab and providing me with a variety of experiences. Thank you for being an excellent advisor.

Abstract

Opioids are commonly used for the treatment of chronic pain, but long-term opioid use can lead to tolerance. The analgesic effect of opioids is attenuated over time, leading to dosage escalation to accomplish the same level of analgesia, which can potentially lead to substance dependence. While opioids are incredibly effective therapeutics for chronic pain, it is necessary to find therapeutics to reduce the need for opioid analgesics, alleviate opioid tolerance development, and decrease symptoms of withdrawal to help fight the opioid epidemic in the United States. The goal of this project is to develop therapeutics for chronic pain treatment to combat the overuse of opioids. The body produces similar physiological changes in its response to chronic pain and opioid therapy, including the loss of activity of potassium channels in the nervous system. Previous studies show ATP-sensitive potassium (K_{ATP}) channel agonists can counteract the decreased antinociceptive effects seen with long term use of opioids. Many agonists of K_{ATP} channels are not soluble in physiologically relevant vehicles, requiring adaptation for clinical use. Novel K_{ATP} channel targeting prodrugs, CKLP1, CKLP2, and CF3-CKLP1 were developed, as they are cleaved by endogenous alkaline phosphatase enzymes present in the nervous system. Analgesic capabilities of intrathecally injected prodrugs were tested in a rodent model of chronic neuropathic and inflammatory pain. The reduction of opioid tolerance and opioid-induced hypersensitivity in mice treated chronically with morphine was also evaluated. Prodrug cleavage in vivo was confirmed by HPLC analysis. The studies may aid in the further development of K_{ATP} channel prodrugs for use in treatments of chronic pain, opioid tolerance, and withdrawal.

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List of Abbreviations

AC	Adenylyl cyclase
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
Ca_v	Voltage gated calcium channel
cAMP	Cyclic adenosine monophosphate
CF3-CKLP1	Trifluorocarbonated cromakalim prodrug one
CFA	Complete Freund's Adjuvant
CKLP1	Cromakalim prodrug 1
CKLP2	Cromakalim prodrug 2
Contra	Contralateral
GIRK	G-protein gated inwardly rectifying potassium channel
HCN	Hyperpolarization-activated, cyclic nucleotide-gated channel
HPLC	High performance liquid chromatography
i.t.	Intrathecal
Ipsi	Ipsilateral
K_{ATP} Channel	ATP sensitive potassium channel
$K_{ir}6.1$	Inwardly rectifying potassium subunit 6.1 (*Kcnj8*)
$K_{ir}6.2$	Inwardly rectifying potassium subunit 6.2 (*Kcnj11*)
L4	Lumbar vertebrae 4
L5	Lumbar vertebrae 5
MOR	Mu opioid receptor
Na_v	Voltage gated sodium channel

OIH	Opioid induced hyperalgesia
OMP	Oxymethylphosphate
PGA	Polyglycolic acid
PKA	Protein kinase A
S1	Sacral vertebrae 1
s.c.	Subcutaneous
SNL	Spinal nerve ligation
SUR1	Sulfonylurea receptor 1 (*Abcc8*)
SUR2	Sulfonylurea receptor 2 (*Abcc9*)
TRPM	Transient receptor potential ion channel melastatin 3
TRPV1	Transient receptor potential cation channel subfamily V member 1

Introduction

Opioids are commonly used for treating chronic pain. Chronic pain is defined as pain persisting longer than normal healing time lasting more than 3 months (Alexander 1954). Chronic pain can be caused by nerve injuries, chronic diseases such as diabetes, inflammation, cancer, and many other conditions. Long term use of opioids leads to tolerance, a progressive decrease in analgesic effect of the same dose, resulting in dosage escalation to maintain the same level of analgesia. Dose escalation can even lead to opioid-induced hyperalgesia, a sensitization of nociception to stimuli not previously painful. After long term opioid use, the cessation or even tapering down of opioids can have negative physical and psychological consequences. Opioid withdrawal symptoms include anxiety, irritability, nausea, vomiting, diarrhea, and abdominal pain. The use of opioid receptor antagonist treatments such as methadone and buprenorphine-naloxone aid in reducing withdrawal affects in treatment, but prevention of tolerance is necessary to contain the opioid crisis in the United States (Srivastava, Kahan et al. 2017).

The use of opioids in high doses can lead to an opioid overdose causing life threatening respiratory depression characterized by the loss of spontaneous breathing and eventually hypoxia. From April 2020 to April 2021, an estimated 75,673 people died from opioid overdoses in the United States (National Vital Statistics Reports. 2021). Opioid receptor antagonists, such as naloxone, are used for treating opioid overdoses. Naloxone is a competitive mu-opioid receptor antagonist (Zuurmond, Meert et al. 2002). The antagonist's key function includes the ability to occupy the active site while not activating the downstream signaling cascade. This allows the neuron function to return to normal and prevents opioids from binding to the receptor. In situations of respiratory

distress, a small dose of naloxone will cause spontaneous respiration to return. When naloxone is given in a greater dose than necessary or given when breathing is not compromised in individuals with recent opioid use, withdrawal is induced (Heishman, Stitzer et al. 1989, Kosten, Jacobsen et al. 1989).

Under naïve conditions, opioid medications dampen neuron excitability, neurotransmitter release and decrease function of the nervous system (Reshef, Sperling et al. 1998). Neurons communicate with electrical activity, called action potentials, and these signals are sent from one neuron to the next. Action potentials are modulated by the activity of various proteins and ion channels. Acute treatment with opioids activates mu-opioid receptors (MORs) inducing multiple intracellular changes: decreased adenylyl cyclase activity, decreased calcium channel activity, and opening of ATP-sensitive potassium channels (Figure 1) (Williams, Ingram et al. 2013). Over time, chronic opioid stimulation results in loss of downstream MOR signaling, resulting in lessened analgesia, tolerance, and withdrawal.

Opening of potassium channels change the polarization state of the neuron, preventing the propagation of action potentials and neurotransmitter release. While ATP-sensitive potassium channels (K_{ATP} channels) are inhibited by the presence of ATP, the activation of the channel via an agonist opens the inner pore allowing potassium ions to flow into the cell, creating hyperpolarization of the neuron and preventing electrical activity of the cell. K_{ATP} channel agonists are proven to be effective for chronic pain modulation in several rodent models (Niu, Saloman et al. 2011, Afify, Khedr et al. 2013).

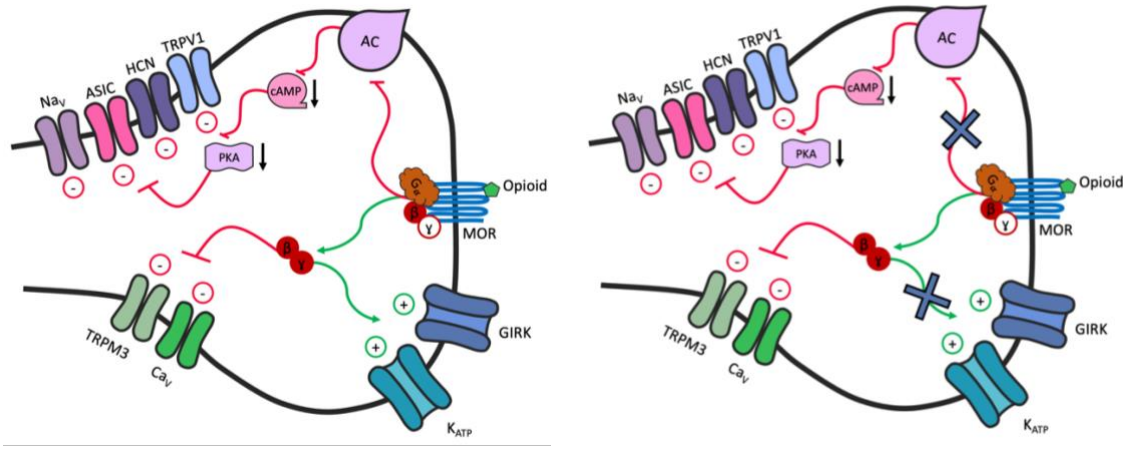


Figure 1. Opioid binding activates downstream signaling cascade inhibiting the flux of negative charges across the membrane. Opioid signaling under naïve conditions (left). An opioid molecule binds to the Mu Opioid Receptor (MOR) initiating the signaling cascade by dissociation of subunits. The alpha subunit inhibits adenylyl cyclase (AC), cyclic AMP (cAMP) formation, and protein kinase A (PKA) activity. This ultimately blocks ion channels. Channels blocked include voltage gated sodium (Na_v), acid sensing ion channels (ASIC), Hyperpolarization-activated, cyclic nucleotide-gated channels (HCN), and transient receptor potential cation channel subfamily V member 1, capsaicin (TRPV1). The Gβγ subunits inhibit phosphoinositide-dependent ion channel (TRPM3) and voltage gated calcium channel (Ca_v) and activate voltage gated potassium (K_{ATP}) and G protein gated inwardly rectifying potassium (GIRK) channels. These actions work together to decrease neuron excitability. Chronic opioid receptor activation decreases signaling, (right). Inhibition of adenylyl cyclase is stopped, resuming the activity of Na_v, ASIC, HCN, and TRPV1. The activation of K_{ATP} and GIRK is ceased. Figure modified from (Machelska and Celik 2018).

K_{ATP} channels are inwardly rectifying potassium channels and are composed of sulfonylurea receptors (SUR) and inwardly rectifying potassium (K_{ir}) inner subunits. K_{ir}6.x and SURx units are closely associated to form heterotetramers (Figure 2) expressed in the peripheral and central nervous system (Thomzig, Wenzel et al. 2001). In addition to being expressed in the nervous system, SUR1/2 and K_{ir}6.1/6.2 are expressed elsewhere in the body (Fagerberg, Hallström et al. 2014). *Abcc8* (SUR1) is expressed in the endocrine tissues, and pancreas. *Abcc9* (SUR2) has high expression in the heart, pancreas, digestive tract, and muscle tissues. *Kcnj8* (K_{ir}6.1) has high expression in the endocrine tissues, respiratory system, liver, gallbladder pancreas, muscle tissues, and skin tissues. *Kcnj11* (K_{ir}6.2) has greatest expression in the digestive tract and muscle tissues. The expression and activity of K_{ATP} channels are decreased under opioid tolerance conditions in rodents (Cao, Dai et al. 2016). In particular, mice lacking the SUR1 subtype of K_{ATP} channels have increased sensitivity to mechanical pain (Luu, Bjork et al. 2019) and loss of morphine induced antinociception (Fisher, Johnson et al. 2019, Sakamaki, Johnson et al. 2021).

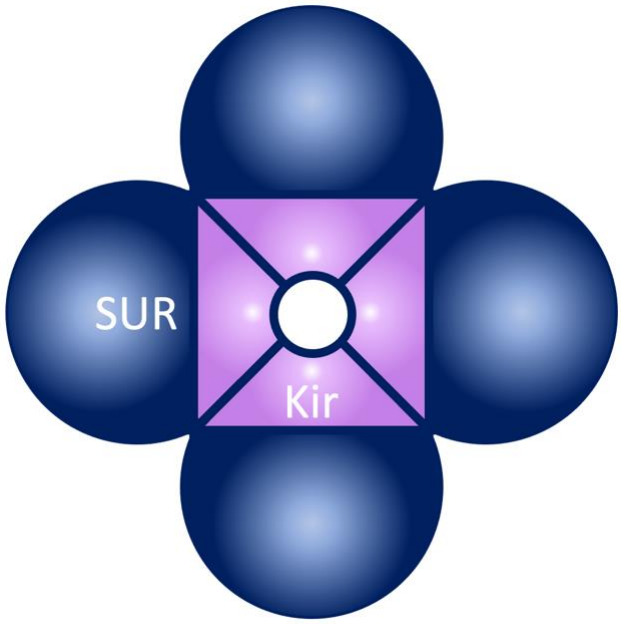


Figure 2. Representation of K_{ATP} channel complex top view molecular subunits SUR and K_{ir}. K_{ir} subunits create the pore of the channel. SUR subunits are regulatory and contain binding site for cromakalim. Figure adapted from (Frances 2005).

Most K_{ATP} channel agonists work by binding to the SUR subunits, causing the pore to open and hyperpolarize the cell, decreasing neuron activity (Vivaudou, Moreau et al. 2009). Many potassium channel openers, including cromakalim (EC_{50}= 170 nM - 1.6 uM), diazoxide (EC_{50}= 3.42 - 34.2 µM), and pinacidil (EC_{50}= 1.29 - 1.9 µM) fall into the classification of benzopyrans (Russ, Metzger et al. 1997, Plujà, Yokoshiki et al. 1998, Pataricza, Hõhn et al. 2000, Suzuki, Li et al. 2001, Wang, Murakami et al. 2018). The K_{ATP} channel agonist pinacidil acts on the SUR2 subtype, and diazoxide acts on the SUR1subtype (Moreau, Jacquet et al. 2000). Cromakalim acts on the sulfonylurea receptor with high specificity to the SUR2 subtype over SUR1 (Tricarico, Mele et al. 2006). Opening of K_{ATP} channels with cromakalim creates an analgesic response in mice when delivered intrathecally (10 to 100 µg dose) or intracerebrovascularly (0.1 to 10 µg) (Nakao, Takahashi et al. 1996, Asano, Dohi et al. 2000). Cromakalim delivered intrathecally (10 to 100 µM, 10µL) also causes a reduction in opioid induced hyperalgesia and opioid tolerance in mice (Wu, Liu et al. 2011).

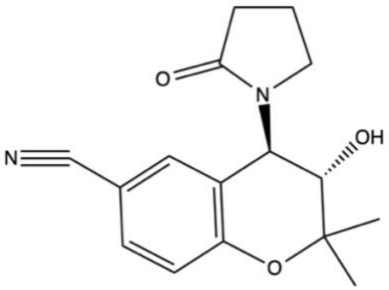
Further development of compounds designed to increase K_{ATP} channel activity would prove beneficial for use in the clinical setting. Cromakalim, and other K_{ATP} channel agonists are not optimal for use in preclinical and clinical studies due to low solubility in aqueous solutions. Use of detergents and solvents increase drug solubility, but make systemic administration limited to intraperitoneal and subcutaneous delivery in rodent preclinical models, in relatively low doses. Increasing aqueous solubility of cromakalim would allow for further testing of analgesic properties *in vivo*. Modification to the cromakalim compound to optimize the drug delivery options (e.g., intrathecal, intravenous, dissolving tablet) would be beneficial for *in vivo* experiments.

Prodrugs are a class of drugs that enter the body in an inactive form and later activated by a biological process. This process of drug activation is essential for development of new medications and optimizing drug delivery. Formulation of prodrugs for increasing solubility can be achieved by the addition of charged functional groups to improve the solubility of a molecule. For example, the addition of a phosphate ester or a phosphate group with oxymethyl linker (oxymethylphosphate, OMP) significantly increases the solubility of several compounds (Rautio, Kumpulainen et al. 2008). OMP prodrugs, are activated through dephosphorylation by the endogenously expressed enzyme alkaline phosphatase by the cleaving of the phosphate group (Wiemer and Wiemer 2015). The enzyme alkaline phosphatase cleaves off phosphate groups, activating the prodrug to its parent structure. Levels of this enzyme, alkaline phosphatase are shown to increase under injury when metabolism of ATP is increased. Specifically, concentrations increase in the spinal column under nerve injury state in humans (Citak, Grasmücke et al. 2016, Vimalraj 2020). Due to the increased expression of alkaline phosphatase, conversion of OMP prodrugs is expected to increase under painful syndromes.

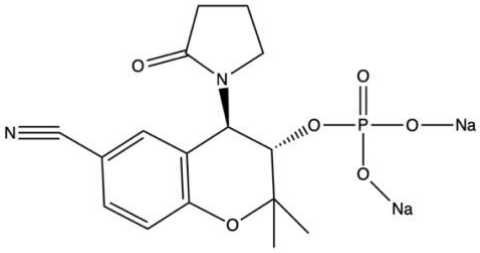
Prodrugs of cromakalim, previously designed for treatment of glaucoma, have shown to reduce intraocular pressure (1 mM to 20 mM in 50µL) when applied topically (Chowdhury, Rinkoski et al. 2017). A recently developed cromakalim prodrug, which acts as a K_{ATP} channel agonist, has high aqueous solubility allowing for *in vivo* opening of K_{ATP} channels. Three cromakalim prodrugs CKLP1, CKLP2, and CF3-CKLP1 (Figure 3) were designed by replacing the alcohol group with a phosphate ester group (Roy Chowdhury, Bahler et al. 2015). The compound CF3-CKLP1 is similar to CKLP1, where

the nitrile group is replaced with a trifluorocarbon increasing metabolic stability. The increased solubility of cromakalim via these prodrugs would allow for drug delivery through intravenous, intrathecal, and subcutaneous routes.

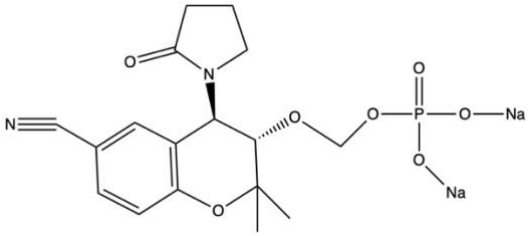
(a) Cromakalim



(b) CKLP1



(c) CKLP2



(d) CF3-CKLP1

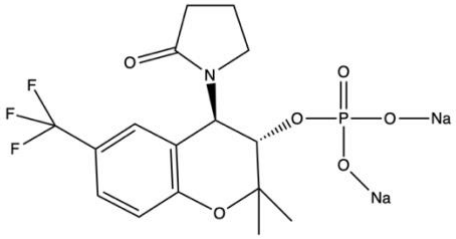


Figure 3. Structures of cromakalim and cromakalim prodrugs. The cromakalim parent structure (a) is the precursor compound to the prodrugs CKLP1 (b), CKLP2 (c), and CF3-CKLP1(d).

K_{ATP} channels are involved in antinociception, opioid signaling, and opioid analgesia. Here cromakalim prodrugs were tested to reduce mechanical hypersensitivity because the parent structure, cromakalim, has already demonstrated analgesic capabilities, similar to other K_{ATP} channel agonists. The increase in dephosphorylation and activation of the cromakalim prodrugs under injury conditions, due to elevated alkaline phosphatase activity, would make this compound more available during nerve injury and/or inflammatory conditions. The pharmacokinetic properties and conversion of the cromakalim prodrugs systemically *in vivo* are relatively unknown. It's predicted compounds with greater phosphate group cleaving will have higher analgesic efficacy.

In order to test these hypotheses, mechanical paw withdrawal testing was utilized to evaluate analgesic responses to prodrug compounds in a mouse model. The Von Frey method of mechanical paw withdrawal testing measures the force applied to the hind paw required to illicit a nocifensive behavior. Nocifensive responses may be shown during stimuli, such as paw withdrawal, or immediately following licking, or shaking of the paw. An increase in the force administered to provoke a response compared to baseline measurements is consistent with analgesia. Alternatively, a decrease in mechanical force is seen in hyperalgesia which is common in rodent models of neuropathic or inflammatory pain. In this study, a spinal nerve ligation (SNL) model of neuropathic pain and Complete Freund's Adjuvant (CFA) as a model of inflammatory pain were used to investigate intrathecal administration of cromakalim prodrugs as analgesics through mechanical paw withdrawal thresholds. The effect of cromakalim prodrugs on opioid tolerance and withdrawal were tested using daily morphine administration followed by naloxone precipitated withdrawal. Mice in opioid withdrawal typically have escapism

behaviors or display the animal is not content in their surroundings. These include behaviors of jumping, burrowing, and rearing (El-kadi and Sharif 1994). Recording and manual scoring of behaviors were used to quantify the intensity of opioid withdrawal following a period of opioid tolerance. Phosphatase cleaving of prodrug into the parent compound within the spinal column were confirmed by analysis with reverse phase high performance liquid chromatography (HPLC). Molecules in the spinal cord samples were separated based on their polarity and solubility in aqueous and non-aqueous solutions. The mice displaying the greatest analgesic effect after treatment with prodrug are predicted to have the greatest concentration of cleaved compound present in the spinal cord 24-hours after injection.

Methods

Animals

All experimental procedures involving animals were approved and performed in accordance with the University of Minnesota Institutional Animal Care and Use Committee guidelines. Adult male and female C57Bl/6N mice were obtained from Charles River (Raleigh, NC) at five to six weeks old weighing 18.1-24.2 g. Mice were acclimated to the facility on a 14-hour light/10-hour dark cycle, and individual testing apparatuses prior to behavioral testing. Animals were randomly assigned to treatment groups each group containing 5 males and 5 females. Mice were euthanized with carbon dioxide followed by decapitation at the end of the study.

Mechanical Paw Withdrawal Measurements

Animals were placed in testing apparatus for acclimation in two separate occasions lasting 45-60 minutes. The testing apparatus is individual acrylic chambers on a mesh floor to allow access to paws for testing. Mechanical paw withdrawal thresholds were measured using electronic von Frey testing equipment (Electric von Frey Anesthesiometer, 2390, Almemo® 2450, IITC Life Science, Woodland Hills, CA). Hind paw plantar surfaces were gently pressed with the von Frey probe until a nocifencive response was elicited. A nocifensive response included either paw lifting or jumping. Measurements were collected five times per hind paw and averaged for baseline mechanical paw withdrawal thresholds. Single measurements were used for post-drug SNL time-course experiments. An average of 5 measurements per paw were used for mechanical testing in the post drug CFA model, and 3 measurements per paw were used under opioid tolerance tests.

K_{ATP} Channel Prodrug Delivery

Intrathecal injections into L5 intrathecal space were performed via Hamilton syringe, PE-10 tubing, and 30 G dental needle set up. Prodrugs were obtained from collaborators (Peter Dosa, University of Minnesota, Department of Medicinal Chemistry) and synthesized as described (Chowdhury, Viker et al. 2016). Prodrugs were delivered intrathecally as 60 µg or 30 µg doses in 10 µl normal saline (Fairbanks 2003).

Morphine Tolerance and Withdrawal

Morphine tolerance was established in naïve mice via twice daily injections of morphine for a total of 5 days (~0800 hours and 1700 hours). Morphine (Sigma Chemical, St. Louis, MO) was administered through a 100 µl subcutaneous injection in saline (15 mg/kg) (Liang, Li et al. 2011). Animals received intrathecal injection of prodrug (60 µg in 10 µl saline) or saline control 30 min prior to receiving subcutaneous morphine. Mechanical paw withdrawal testing was performed before administration of morphine as well as 30 minutes after morphine delivery.

Opioid withdrawal was induced using naloxone hydrochloride (Alfa Aesar, Ward Hill, MA). Morphine tolerance was established in naïve male mice by administration of cromakalim prodrug CKLP1, CKLP2, CF3-CKLP1, or saline control (60 µg in 10 µl, i.t.) and morphine (15 mg/kg in 100 µl saline s.c.) twice daily for five days. On day 6, mice were given final subcutaneous dose of morphine (15 mg/kg in 100 µl saline). Animals were placed in 4-inch by 6-inch Plexiglas chambers. Animal behavior was video recorded for 15 minutes to determine baseline animal behavior, 30 minutes after morphine administration. 2 hours post morphine administration, intrathecal injection of CKLP1, CKLP2, CF3-CKLP1, or saline control (60 µg in 10 µl, i.t.) was administered.

Withdrawal was precipitated with intraperitoneal injection of naloxone (1 mg/kg in 100 µl saline). After naloxone administration, animal behavior was video recorded for an additional 15 minutes. After the 15 minutes of withdrawal, episodes of diarrhea were counted. The number of jumps, bouts of rearing and bouts of burrowing behaviors were counted in one-minute intervals for the entire 15 minutes of recorded animal behavior by two independent scorers, blinded to animal groups (El-kadi and Sharif 1994).

Spinal Nerve Ligation

A spinal nerve ligated model of neuropathic pain was used to determine the effective time-course of cromakalim prodrugs as analgesics in a chronic neuropathic pain mouse model. Spinal nerve ligation creates a neuropathic pain model by severing the L5 spinal nerve (Rigaud, Gemes et al. 2008). To create a mouse model of neuropathic pain, unilateral ligation of the L4 spinal nerve was completed. Mice were anesthetized with 1-3% isoflurane (Fluriso, Vetone, Boise, ID) in oxygen. The surgical site (L4 to S1) was shaved, followed by cleaning with povidone-iodine prep pads (Betadine, Dynarex, Orangeburg, NY) and 70% v/v ethanol. An incision was made from L4 to S1 using a sterile #15 surgical blade (Exelint International) to expose and resect the left L4 spinal nerve. A section of the nerve, approximately 2 mm long, was removed from the animal (Rigaud, Gemes et al. 2008, Ye, Savelieva et al. 2015). The surgical site was closed with two layers of 4-0 PGA absorbable sutures (GDT, Beer Sheva, Israel). Surgical supplies were sterilized by autoclave, and ethanol glass bead sterilization prior to use. All surgeries were completed by the same person.

After healing for 14 days, mechanical paw withdrawal threshold testing was completed on both hind paws including the side with SNL (ipsilateral) and the side not

receiving L4 spinal nerve ligation (contralateral). Mechanical paw withdrawal threshold measurements were taken 30 min, 1 hour, 1.5 hours, 2 hours, 4 hours, 6 hours, and 8 hours post intrathecal injection.

Complete Freund's Adjuvant

An inflammatory pain model was created using an intraplantar injection of Complete Freund's Adjuvant (Liang, Li et al. 2011). The efficacy of cromakalim prodrugs as analgesics in an inflammatory pain model mouse was determined by a unilateral intraplantar injection of CFA (10 µl, undiluted) (F5881, Sigma Chemical, St. Louis, MO). CFA injection is used to create a local inflammatory response, which increases mechanical sensitivity to the affected hind paw, and is a commonly used rodent model for inflammatory pain (Fehrenbacher, Vasko et al. 2012). The one-hour time point was chosen based on the experiments from the SNL model results. Mechanical paw withdrawal threshold measurements were taken prior to CFA injection, and one-hour post intrathecal injection CKLP1, CF3-CKLP1, CKLP2, and saline (30 µg and 60 µg in 10 µl). Further mechanical paw withdrawal threshold measurements were taken on days 2, 3 and 8 post CFA administration.

Morphine Dose Response

The effect of cromakalim prodrugs on acute morphine antinociception was tested. The effect of CKLP1 and CF3-CKLP1 on mechanical paw withdrawal thresholds with increasing doses of morphine was tested in naïve male mice. Baseline mechanical paw withdrawal threshold measurements were taken. Prodrugs CKLP1, CF3-CKLP1, and saline control (60 µg in 10 µl saline) were injected intrathecally and 30 minutes later morphine sulfate (in saline, 100 µl, s.c.) was delivered to the nape of the neck. Timelines

for cromakalim prodrug delivery were decided from the SNL data. As the activity of morphine peaks at 30-40 minutes, mechanical paw withdrawal threshold measurements were taken 30 minutes after morphine administration. Consecutive increasing doses were performed at 0, 5, 10, and 20 mg/kg 30 minutes apart.

Tissue Collection and Tissue Sample Preparation

Animals used in CFA as well as morphine tolerance and withdrawal were euthanized 24 hours after final injection of prodrug. Spinal cords of naïve animals were collected for extraction efficiency calculations. Euthanasia was completed with carbon dioxide followed by decapitation. Spinal cords were harvested from animals and immediately frozen at -20°C and stored until used for conversion analysis.

Spinal cord preparation methods were adapted from (Nakhi, Wong et al. 2021). Animal tissue was transferred to a clean microcentrifuge tube prechilled with 200 µl of mobile phase starting solution (2.5 mM ammonium formate in 5% acetonitrile pH 2.15). Tissue was homogenized on ice using 1.5 mL micro-tube sample pestles until no tissue remnants were visible. The pestle was rinsed with an additional 100 µl of mobile phase mixture. Homogenate was vortexed and placed in prechilled (4°C) centrifuge for 25 minutes at 16,100 rcf. Supernatant was transferred to a clean tube. 120 µl of homogenate was mixed with 5 µl of flavopiridol internal standard and placed into amber autosampler vial with 200 µl insert.

Liquid Chromatography

The LC system consisted of Thermo Scientific Ultimate 3000 UHPLC+ focused pump, autosampler and column compartment were used with Chromeleon 7.2 software. Waters XBridge BEH C18 130Å, 3.5 µm, 3 mm x 50 mm column with column Waters

XBridge BEH C18 3.5 µm, 2.1 mm x 5 mm guard column was maintained at 30°C. Biological samples were stored at 4°C when not injecting to suppress additional enzymatic activity prior to *ex vivo* analysis. Injection volumes of 2 µl were used. Mobile phase A consisted of 2.5 mM ammonium formate in water at a pH of 2.15. Mobile phase B was 2.5 mM ammonium formate in acetonitrile. The pH of 2.15 was chosen due to apparent phosphate group pKa around pH of 2.2, causing full protonation of phosphate oxygens. The column was preequilibrated from 10/90 Mobile A/B to 95/5 Mobile A/B over 2 minutes, then held at 95/5 Mobile A/B for one minute prior to injection. 95/5 Mobile A/B was held for 3 minutes after injection. The mobile phases were then ramped to 80% Mobile B over 10 minutes for elution of prodrug and cromakalim and held at 20/80 mobile A/B for 5 minutes. The method used a constant flow rate of 0.8mL/min while monitoring absorbance of 252 nm for prodrugs, cromakalim, flavopiridol internal standard and other products. All samples were analyzed with HPLC in triplicate.

Retention times were determined from standards in extraction buffer. Area of compound peak over area of internal standard peak was used to create five-point standard curves of CKLP1, CKLP2 and cromakalim (1.563 µM to 100 µM) in extraction buffer for quantification. Standard curves were used to calculate compound concentration in spinal cord homogenate sample as prepared. Extraction efficiency of cromakalim and the prodrugs was calculated to determine the percentage of compound that could be recovered from spinal cord sample tissues. Extracted spinal cords were spiked with compound, then homogenized as described above. A separate set of spinal cords were homogenized, then spiked with the same volume and concentration of compound. Calculated concentrations were compared (Equation 1).

$$\frac{\text{[Extracted spinal cord spiked with compound,then homogenized]}}{\text{[Homogenized spinal cord,then spiked with compound]}} \quad \text{[Equation 1]}$$

Data Analysis

Animal data was collected and analyzed by blinded personnel. T-tests, ANOVA (one-way and repeated measures) with Dunnett's post hoc analysis were used to determine significance for mechanical thresholds and withdrawal behaviors. GraphPad Prism version 9 (GraphPad Software, San Diego, CA) was used for statistical analysis. HPLC chromatograms were automatically integrated with Chromeleon 7.2, and manually checked for consistency.

Results

Determining prodrug efficacy as an analgesic for neuropathic pain.

To determine the efficacy of cromakalim prodrugs against neuropathic pain, animals were injected with 30 µg or 60 µg intrathecally and mechanical paw withdrawal thresholds were tested 14 days after SNL. A significant increase in mechanical withdrawal thresholds were found with the 60 µg dose of CKLP1 at 30 minutes after intrathecal administration when compared to saline treated animals (Figure 4a). There was no significant difference in the 60 µg dose when tested in the in contralateral hind paws (Figure 4b). There was a significant increase in the 30 µg dose in the contralateral hind paws when compared to saline.

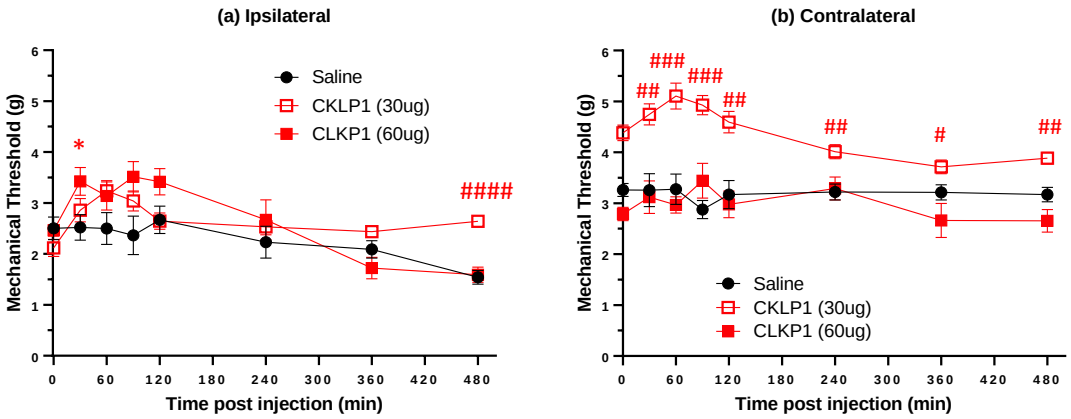


Figure 4. Intrathecal administration of cromakalim prodrug CKLP1 increased mechanical paw withdrawal thresholds 14 days after spinal nerve ligation in mice. Mechanical paw withdrawal thresholds on the ipsilateral (a) and contralateral (b) hind paws. CKLP1 at the 60 µg dose significantly increased paw withdrawal thresholds compared to saline treated animals at 30 minutes post injection on the ipsilateral hind paw (Repeated measures ANOVA, Time x Drug effect F(14, 189) = 2.867, P = 0.0006; $P_{adj,30}$ = 0.0472). The 30 µg dose was significantly greater than saline at 480 minutes in the ipsilateral hind paw (P_{adj} < 0.0001). All time points of the 30 µg dose were significantly greater than the saline treated animals when testing the contralateral hind paw. (Repeated measures ANOVA, Time x Drug effect F(14, 189) = 2.300, P = 0.0062; $P_{adj,0}$ = 0.0028, $P_{adj,30}$ <0.001, $P_{adj,60}$ < 0.001, $P_{adj,90}$ = 0.0015, $P_{adj,120}$ = 0.0026, $P_{adj,240}$ = 0.0417, $P_{adj,360}$ = 0.0417 and $P_{adj,480}$ = 0.020). Data is plotted as the treatment group average with SEM error bars, # indicates significance of 30 µg dose, * indicates significance of 60 µg dose. n=5 male and 5 female mice per group.

The efficacy of the cromakalim prodrug CKLP2 was also tested 14 days after SNL (Figure 5). Similar to the CKLP1 compound, the cromakalim prodrug CKLP2 increased the ipsilateral hind paw thresholds with the 60 µg dose at 60 minutes and 90 minutes post intrathecal injection for the ipsilateral hind paw compared to saline. The 30 µg dose of CKLP2 did not increase mechanical paw withdrawal force in the ipsilateral paw. In the contralateral hind paw, all points had increased mechanical thresholds with the 30 µg dose when compared to saline. The 60 µg dose significantly increased mechanical paw withdrawal thresholds at 30 minutes, 60 minutes, 90 minutes, 120 minutes, and 480 minutes in the contralateral hind paw when compared to the saline treated animals.

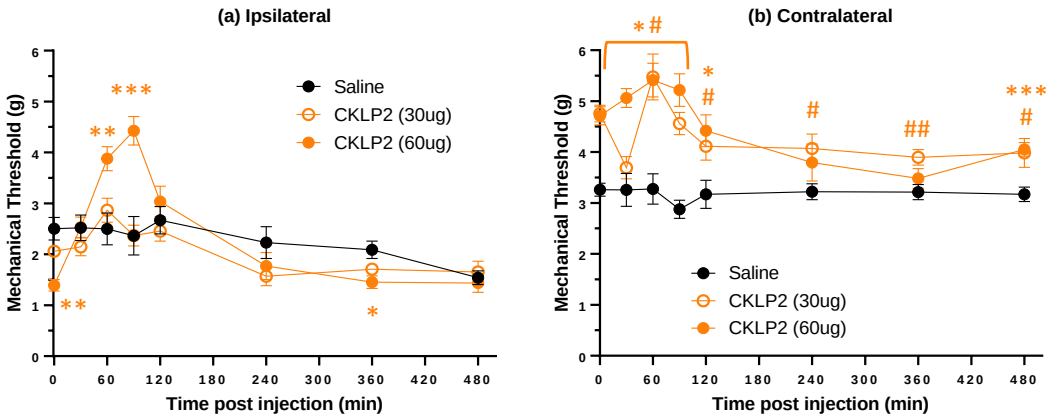


Figure 5. Intrathecal administration of cromakalim prodrug CKLP2 increased mechanical paw withdrawal thresholds 14 days after spinal nerve ligation in mice. Mechanical paw withdrawal thresholds on the ipsilateral (a) and contralateral (b) hind paws. CKLP2 at a 60 µg dose was significantly greater than saline at 60 and 90 minutes on the ipsilateral hind paw (repeated measures ANOVA, Time x Drug effect F(14, 189) = 6.366, $P < 0.0001$; $P_{adj,60} = 0.005$, $P_{adj,90} = 0.0008$). The contralateral hind paw was significantly greater in the 30 ug dose than saline at baseline, 60 minutes, 90 minutes, 120 minutes, 240 minutes, 360 minutes, and 480 minutes (repeated measures ANOVA, Time x Drug effect F(14, 189) = 3.526, $P < 0.0001$; $P_{adj,0} < 0.001$, $P_{adj,60} = 0.0018$, $P_{adj,90} < 0.001$, $P_{adj,120} = 0.0483$, $P_{adj,240} = 0.0365$, $P_{adj,360} = 0.0096$ and $P_{adj,480} = 0.0421$). The contralateral hind paw was significantly greater in the 60 µg dose than saline at baseline, 30 minutes, 60 minutes, 90 minutes, 120 minutes and at 480 minutes ($P_{adj,0} < 0.001$, $P_{adj,30} < 0.001$, $P_{adj,60} < 0.001$, $P_{adj,90} < 0.001$, $P_{adj,120} = 0.0151$, and $P_{adj,480} < 0.001$). Data is plotted as the treatment group average with SEM error bars, # indicates significance of 30 µg dose, * indicates significance of 60 µg dose. n=5 male and 5 female mice per group.

The efficacy of the cromakalim prodrug CF3-CKLP1 was tested 14 days after SNL (Figure 6). The cromakalim prodrug CF3-CKLP1 had an increase in the ipsilateral hind paw mechanical paw withdrawal thresholds. There was a significant increase in the mechanical paw thresholds for the 30 µg dose at 360 minutes and at 480 minutes post intrathecal injection. There was a significant increase in mechanical paw withdrawal measurements for the 30 µg dose in the contralateral hind paw. There was no significant difference in the 60 µg dose in ipsilateral or contralateral hind paw when compared to saline.

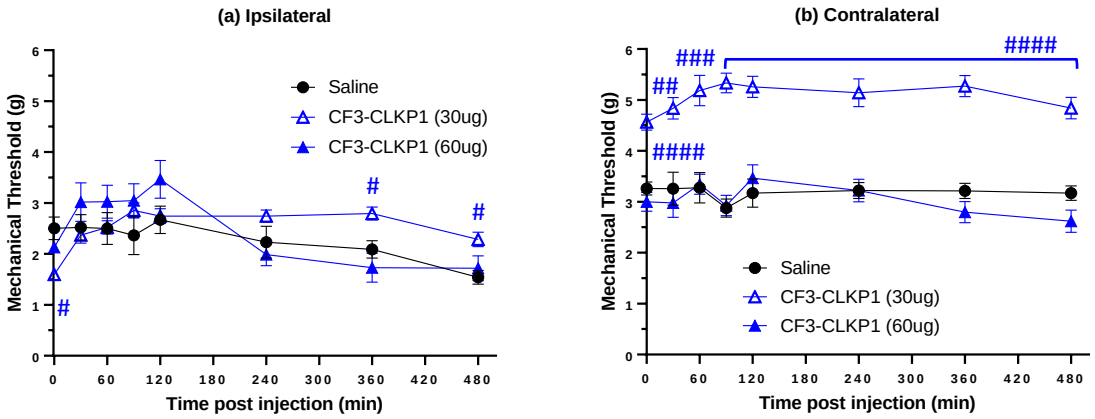


Figure 6. Intrathecal administration of cromakalim prodrug CF3-CKLP1 increased mechanical paw withdrawal thresholds 14 days after spinal nerve ligation in mice. Mechanical paw withdrawal thresholds on the ipsilateral (a) and contralateral (b) hind paws. CF3-CKLP1 at a 30 µg dose was significantly greater than saline at 360 minutes and at 480 minutes in the ipsilateral hind paw (repeated measures ANOVA, Time x Drug effect F(14, 189) = 2.995, P =0.0004; $P_{adj,360}$ = 0.0051, $P_{adj,480}$ = 0.0025). In the contralateral hind paw, the 60 µg dose had increased mechanical paw withdrawal thresholds at all time points (repeated measures ANOVA, Time x Drug effect F(14, 189) = 1.229 P = 0.2573; $P_{adj,0}$ < 0.0001, $P_{adj,30}$ = 0.0018, $P_{adj,60}$ = 0.0005, $P_{adj,90}$ < 0.0001, $P_{adj,120}$ < 0.0001, $P_{adj,240}$ < 0.0001, $P_{adj,360}$ < 0.0001 and $P_{adj,480}$ < 0.0001). Data is plotted as the treatment group average with SEM error bars, # indicates significance of 30 µg dose, * indicates significance of 60 µg dose. n=5 male and 5 female mice per group.

Table 1. Significance of intrathecal delivery of cromakalim prodrug efficacy in SNL. Values are adjusted P-values from repeated measures ANOVA prodrug animals compared to saline. CKLP1 ipsilateral F(14, 189)=2.867, contralateral F(14, 189)=2.300. CKLP2 ipsilateral F(14, 189) = 6.366, contralateral F(14, 189) = 3.526. CF3-CKLP1 ipsilateral F(14, 189) = 2.995, contralateral F(14, 189) = 1.229. # indicates significance of 30 µg dose, * indicates significance of 60 µg dose. **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001.

		0 Min	**30 Min**	**60 Min**	**90 Min**	**120 Min**	**240 Min**	**360 Min**	**480 Min**
CKLP1 (30 µg)	Ipsi	0.3116	0.5229	0.1106	0.2289	0.9964	0.6089	0.1851	#### <0.0001
CKLP1 (60 µg)	Ipsi	0.9824	* 0.0472	0.2427	0.0508	0.1062	0.5959	0.3233	0.9578
CKLP1 (30 µg)	Contra	#### <0.0001	## 0.0028	### 0.0004	#### <0.0001	## 0.0015	## 0.0026	# 0.0417	## 0.0020
CKLP1 (60 µg)	Contra	* 0.0349	0.9366	0.5753	0.2740	0.8348	0.9563	0.2641	0.1212
CKLP2 (30 µg)	Ipsi	0.1607	0.3869	0.5550	>0.9999	0.7515	0.1530	0.1308	0.8671
CKLP2 (60 µg)	Ipsi	** 0.0012	0.9991	** 0.0050	*** 0.0008	0.5715	0.4397	* 0.0149	0.8584
CKLP2 (30 µg)	Contra	#### <0.0001	0.4512	## 0.0018	#### <0.0001	# 0.0483	# 0.0365	## 0.0096	# 0.0421
CKLP2 (60 µg)	Contra	**** <0.0001	*** 0.0005	*** 0.0003	**** <0.0001	* 0.0151	0.2879	0.4558	*** 0.0005
CF3-CKLP1 (30 µg)	Ipsi	## 0.0051	0.8221	0.9969	0.4036	0.9583	0.2548	## 0.0082	## 0.0025
CF3-CKLP1 (60 µg)	Ipsi	0.2511	0.4573	0.4121	0.3147	0.1738	0.7501	0.4608	0.7563
CF3-CKLP1 (30 µg)	Contra	#### <0.0001	## 0.0018	### 0.005	#### <0.0001	#### <0.0001	#### <0.0001	#### <0.0001	#### <0.0001
CF3-CKLP1 (60 µg)	Contra	0.4209	0.7458	0.9740	0.9745	0.6664	>0.9999	0.2036	0.0882

The increase in mechanical paw withdrawal thresholds compared to saline indicates the prodrugs are effective as analgesics in a mouse model of neuropathic pain. The 60-minute post injection time point was chosen for further experiments due to the increase in mechanical paw withdrawal thresholds for multiple prodrug treatment groups and dosages.

Determining prodrug efficacy as an analgesic for inflammatory pain.

To test the efficacy of the cromakalim prodrugs during inflammatory pain, animals were injected with CFA in the left hind paw, and mechanical paw withdrawal testing was completed 30 minutes, as well as 24, 36, and 168 hours after CFA injection, corresponding with one hour after prodrug injection. The cromakalim prodrug CKLP1 increased mechanical paw withdrawal thresholds in both hind paws under inflammatory pain conditions (Figure 7). There was a significant increase in paw withdrawal thresholds for the 30 µg and 60 µg doses at 30 minutes, 24 hours, 48 hours, and 168 hours compared to saline.

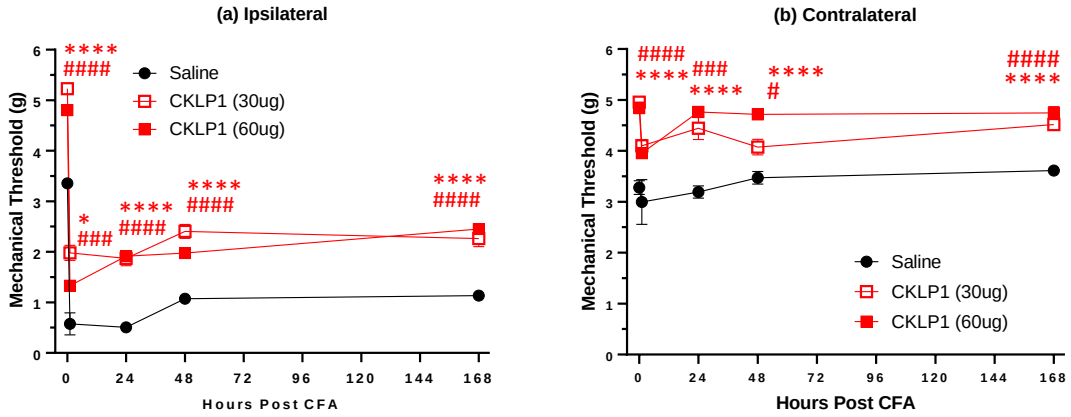


Figure 7. Intrathecal administration of cromakalim prodrug CKLP1 increased mechanical paw withdrawal thresholds in CFA inflammatory pain model mice. Mechanical paw withdrawal thresholds on ipsilateral (a) and contralateral (b) hind paws. In the ipsilateral hind paw, the 60 µg dose increased mechanical paw withdrawal thresholds on days 1, 2, 3 and 8 (Repeated measures ANOVA, Time x Drug effect F(8, 108) = 4.749, $P < 0.0001$; $P_{adj,0} < 0.0001$, $P_{adj,1} = 0.0140$, $P_{adj,2} < 0.0001$, $P_{adj,3} < 0.0001$, $P_{adj,8} < 0.0001$). The 30 µg dose increased mechanical paw withdrawal thresholds on days 1, 2, 3 and 8 in the ipsilateral hind paw ($P_{adj,0} < 0.0001$, $P_{adj,1} = 0.0002$, $P_{adj,2} < 0.0001$, $P_{adj,3} < 0.0001$, $P_{adj,8} < 0.0001$). In the contralateral hind paw, the 60 µg dose increased mechanical paw withdrawal thresholds on day 2, 3 and 8 (Repeated measures ANOVA, Time x Drug effect F(8, 108) = 2.252; $P = 0.0289$; $P_{adj,0} < 0.0001$, $P_{adj,2} < 0.0001$, $P_{adj,3} < 0.0001$, $P_{adj,8} < 0.0001$). The 30 µg dose increased mechanical paw withdrawal thresholds on days 2, 3 and 8 ($P_{adj,0} < 0.0001$, $P_{adj,2} = 0.0004$, $P_{adj,3} = 0.0135$, $P_{adj,8} < 0.0001$). Data is plotted as the treatment group average with SEM error bars, # indicates significance of 30 µg dose, * indicates significance of 60 µg dose. n=5 male and 5 female mice per group.

Administration of the cromakalim prodrug CKLP2 increased mechanical paw withdrawal thresholds in both hind paws under inflammatory pain conditions (Figure 8) when compared to saline. An increase in mechanical paw withdrawal thresholds is more evident in the 60 µg dose of CKLP2 at all time points in the ipsilateral paw, as well as at 24, 72 and 168 hours in the contralateral hind paw compared to saline. The 30 µg dose of CKLP2 increased mechanical paw withdrawal thresholds at 24 and 168 hours after CFA administration in the ipsilateral hind paw, and at 24 hours in the contralateral hind paw.

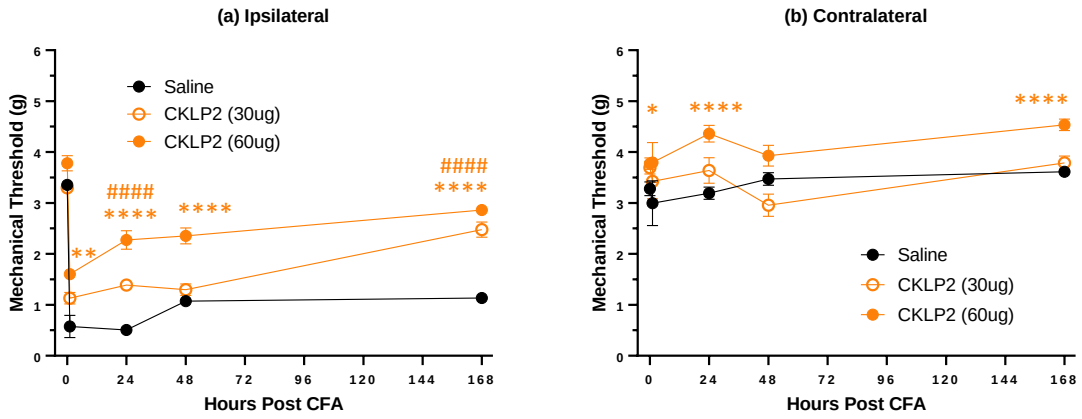


Figure 8. Intrathecal administration of cromakalim prodrug CKLP2 increased mechanical paw withdrawal thresholds in CFA inflammatory pain model mice. Mechanical paw withdrawal thresholds on ipsilateral (a) and contralateral (b) hind paws. In the ipsilateral hind paw, the 30 µg dose increased mechanical paw withdrawal thresholds on day 2 and day 8 (Repeated measures ANOVA, Time x Drug effect F(8, 108) = 9.555, $P < 0.0001$; $P_{adj,2} < 0.0001$, $P_{adj,8} < 0.0001$). The 60 µg dose increased mechanical paw withdrawal thresholds on days 1, 2, 3, and 8 ($P_{adj,1} = 0.0021$, $P_{adj,2} < 0.0001$, $P_{adj,3} < 0.0001$, $P_{adj,8} < 0.0001$). In the contralateral hind paw, the 60 µg dose increased mechanical paw withdrawal thresholds on day 2 and 8 (repeated measures ANOVA, Time x Drug effect F(8, 108) = 2.252; $P = 0.1895$; $P_{adj,0} = 0.0215$, $P_{adj,2} < 0.0001$, $P_{adj,8} < 0.0001$). Data is plotted as the treatment group average with SEM error bars, # indicates significance of 30 µg dose, * indicates significance of 60 µg dose. n=5 male and 5 female mice per group.

Administration of the cromakalim prodrug CF3-CKLP1 increased mechanical paw withdrawal thresholds in both hind paws under inflammatory pain conditions (Figure 9) when compared to saline. The 30 µg dose of CF3-CKLP1 increased mechanical paw withdrawal thresholds at all time points in the ipsilateral and contralateral hind paw when compared to saline. There is an increase in mechanical paw withdrawal thresholds measurements for the 60 µg dose of CF3-CKLP1 in the ipsilateral hind paw at 24, 48 and 168 hours when compared to saline. An increase in mechanical paw withdrawal thresholds were seen with the 60 µg dose of CF3-CKLP1 at all time points when compared to saline.

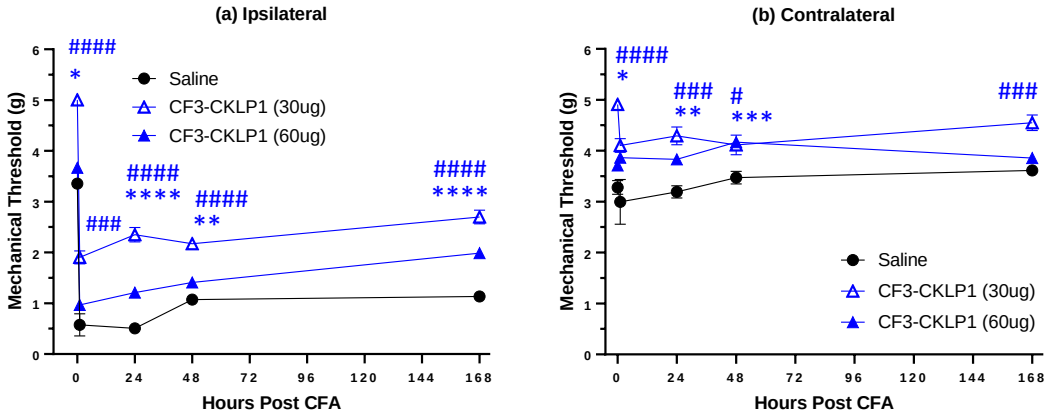


Figure 9. Intrathecal administration of cromakalim prodrug CF3-CKLP1 increased mechanical paw withdrawal thresholds in CFA inflammatory pain model mice. Mechanical paw withdrawal thresholds on ipsilateral (a) and contralateral (b) hind paws. In the ipsilateral hind paw, the 30 µg dose increased mechanical paw withdrawal thresholds on all days (Repeated measures ANOVA, Time x Drug effect F(8, 108)=3.684, P =0.0008; $P_{adj,0} < 0.0001$, $P_{adj,1} = 0.0002$, $P_{adj,2} < 0.0001$, $P_{adj,3} < 0.0001$, $P_{adj,8} < 0.0001$). The 60 µg dose increased mechanical paw withdrawal thresholds on days 2, 3 and 8 in the ipsilateral hind paw ($P_{adj,0} = 0.0313$, $P_{adj,2} < 0.0001$, $P_{adj,3} = 0.0035$, $P_{adj,8} < 0.0001$). In the contralateral hind paw, the 30 µg dose increased mechanical paw withdrawal thresholds on day 2, 3 and 8 (Repeated measures ANOVA, Time x Drug effect F(8, 108) = 2.755, P = 0.0083; $P_{adj,0} < 0.0001$, $P_{adj,2} = 0.0002$, $P_{adj,3} = 0.0251$, $P_{adj,8} = 0.0002$). The 60 µg dose increased mechanical paw withdrawal thresholds on day 2 and day 3 (Repeated measures ANOVA, F(8, 108) = 2.755; $P_{adj,0} = 0.0212$, $P_{adj,2} = 0.0010$, $P_{adj,3} = 0.0005$). Data is plotted as the treatment group average with SEM error bars, # indicates significance of 30 µg dose, * indicates significance of 60 µg dose. n=5 male and 5 female mice per group.

Table 2. Significance of intrathecal delivery of cromakalim prodrug efficacy in CFA. Values are adjusted P-values from repeated measures ANOVA prodrug animals compared to saline. CKLP1 ipsilateral F(8, 108) = 4.749, contralateral F(8, 108) = 2.252. CKLP2 ipsilateral F(8, 108) = 9.555, contralateral F(8, 108) = 1.437. CF3-CKLP1 ipsilateral F(8, 108) = 3.684, contralateral F(8, 108) = 2.755. # indicates significance of 30 µg dose, * indicates significance of 60 µg dose. *P < 0.05, **P <0.01, ***P <0.001, ****P <0.0001.

		Pre CFA	Day 1	Day 2	Day 3	Day 8
CKLP1 (30 µg)	Ipsi	#### <0.0001	### 0.0002	#### <0.0001	#### <0.0001	#### <0.0001
CKLP1 (60 µg)	Ipsi	**** <0.0001	* 0.0140	**** <0.0001	**** <0.0001	**** <0.0001
CKLP1 (30 µg)	Contra	#### <0.0001	0.0627	### 0.0004	# 0.0135	#### <0.0001
CKLP1 (60 µg)	Contra	**** <0.0001	0.1036	**** <0.0001	**** <0.0001	**** <0.0001
CKLP2 (30 µg)	Ipsi	0.8827	0.0750	#### <0.0001	0.1390	#### <0.0001
CKLP2 (60 µg)	Ipsi	0.0551	** 0.0021	**** <0.0001	**** <0.0001	**** <0.0001
CKLP2 (30 µg)	Contra	0.0636	0.6653	0.2249	0.1049	0.4946
CKLP2 (60 µg)	Contra	* 0.0215	0.3184	**** <0.0001	0.1323	**** <0.0001
CF3-CKLP1 (30 µg)	Ipsi	#### <0.0001	### 0.0002	#### <0.0001	#### <0.0001	#### <0.0001
CF3-CKLP1 (60 µg)	Ipsi	* 0.0313	0.1910	**** <0.0001	** 0.0035	**** <0.0001
CF3-CKLP1 (30 µg)	Contra	#### <0.0001	0.0633	### 0.0002	# 0.0251	### 0.0002
CF3-CKLP1 (60 µg)	Contra	* 0.0212	0.1384	** 0.0010	*** 0.0005	0.0749

Effect of cromakalim prodrugs on acute morphine antinociception.

Cromakalim prodrugs CKLP1 and CF3-CKLP1 did not have an effect on the acute antinociception of morphine (Figure 10). This was expected as the antinociceptive effect of morphine is much greater than the effect created by the prodrugs. The increase in mechanical paw withdrawal thresholds can be attributed to morphine with no additive or synergistic effects when paired with cromakalim prodrugs.

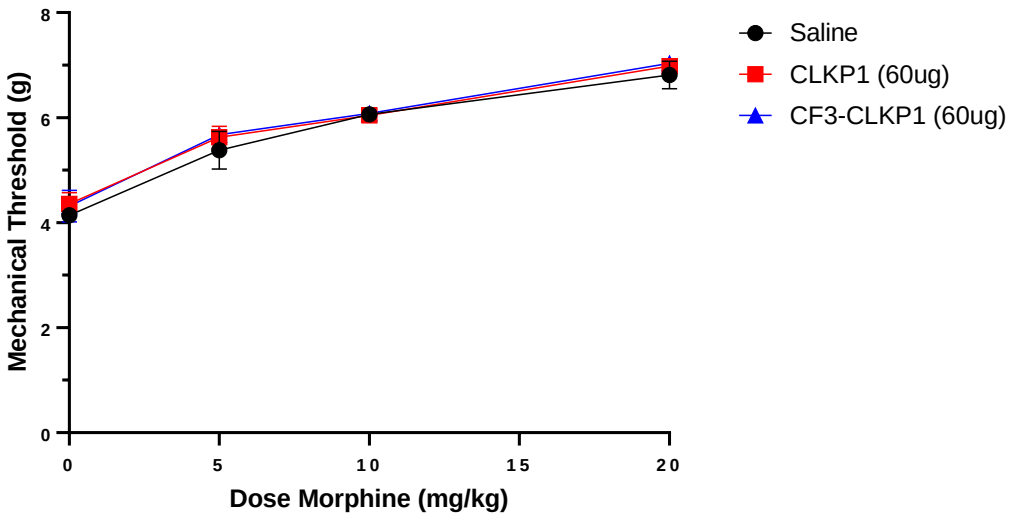


Figure 10. Intrathecal administration of cromakalim prodrugs does not affect morphine cumulative dose response curves. Mechanical paw withdrawal thresholds on both hind paws. Data is plotted as the treatment group average with SEM error bars, n=5 males per group.

Effect of cromakalim prodrugs on mechanical paw withdrawal thresholds under morphine tolerance.

In order to measure the effects of K_{ATP} channel prodrugs on opioid induced hyperalgesia (OIH) and morphine tolerance, we measured mechanical paw withdrawal thresholds pre and post chronic morphine delivery, respectively. Morphine tolerance was established with morphine injections (15 mg/kg) accompanied with prodrug injections (60 µg in 10 µl saline) one-hour prior to morphine injection twice daily for five days. The cromakalim prodrugs increased mechanical paw withdrawal thresholds pre-morphine (Figure 11a) when compared to saline. Cromakalim prodrugs also increased mechanical paw withdrawal thresholds post-morphine (Figure 11b) compared to saline.

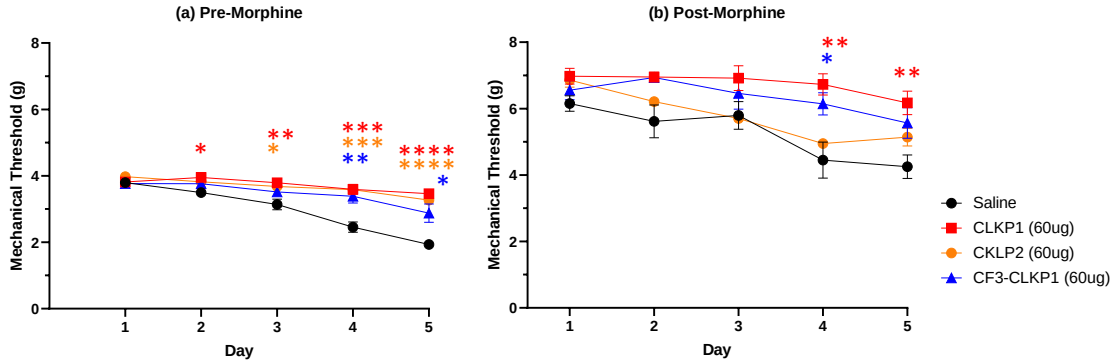


Figure 11. Mechanical paw withdrawal thresholds under opioid induced hyperalgesia and opioid tolerance with intrathecal cromakalim prodrug administration. Mechanical thresholds are expressed as an average force in grams on hind paws (a) before morphine administration and (b) after morphine. Cromakalim prodrugs attenuate opioid induced hypersensitivity (a) (Repeated measures ANOVA, $F(12,140) = 5.588$, $P_{adj,2,CKLP1} = 0.0315$, $P_{adj,3,CKLP1} = 0.0080$, $P_{adj,3,CKLP2} = 0.0226$, $P_{adj,4,CKLP1} = 0.0002$, $P_{adj,3,CKLP2} = 0.0001$, $P_{adj,3,CF3\text{-}CKLP1} = 0.0058$, $P_{adj,8,CKLP1} < 0.0001$, $P_{adj,8,CKLP2} = 0.0232$, $P_{adj,3,CF3\text{-}CKLP1} < 0.0001$). Cromakalim prodrugs attenuate morphine tolerance (b) (Repeated measures ANOVA, $F(12,140) = 2.742$, $P_{adj,4,CF3\text{-}CKLP1} = 0.0489$, $P_{adj,4,CKLP1} = 0.0080$, $P_{adj,5,CKLP1} = 0.0036$). Data is plotted as the treatment group average with SEM error bars, *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001. n=5 male and 5 female mice per group.

Table 3. Significance of cromakalim prodrug efficacy in opioid induced hyperalgesia and opioid tolerance with intrathecal cromakalim prodrug administration. Values are adjusted P-values from repeated measures ANOVA prodrug animals compared to saline. Pre-Morphine F(12, 140) = 5.588. Post-Morphine F(12, 140) = 2.742. # indicates significance of 30 µg dose, * indicates significance of 60 µg dose. *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001.

		Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
CKLP1 (60 µg)	Pre-Morphine	0.9984	* 0.0315	** 0.0080	*** 0.0002	**** <0.0001
CKLP1 (60 µg)	Post-Morphine	0.0615	0.0694	0.1440	** 0.0083	** 0.0036
CKLP2 (60 µg)	Pre-Morphine	0.2846	0.2901	* 0.0226	*** 0.0001	* 0.0232
CKLP2 (60 µg)	Post-Morphine	0.0958	0.5303	0.9921	0.7107	0.1445
CF3-CKLP1 (60 µg)	Pre-Morphine	0.9899	0.3094	0.2040	** 0.0058	**** <0.0001
CF3-CKLP1 (60 µg)	Post-Morphine	0.5647	0.0706	0.5929	* 0.0489	0.0900

Effect of prodrugs on withdrawal behaviors after precipitated withdrawal after chronic morphine.

To measure the effects of K_{ATP} channel prodrugs on opioid withdrawal in mice, withdrawal was induced with naloxone following five days of twice daily morphine and prodrug injection. Animal behaviors were independently scored for the first 15 minutes following naloxone injection. During baseline animal activity, no significant withdrawal behaviors were found (data not shown). Animal jumping behavior peaked at minute four of the withdrawal period (Figure 12). The first 10 minutes of activity were further analyzed based on the initial bout of jumping activity decreasing significantly after this time period. The total number of jumps in the first five minutes of withdrawal was scored for each animal and averaged within the treatment group (Figure 13). The prodrug CKLP2 significantly decreased in jumps when compared to the saline treated group (Figure 13).

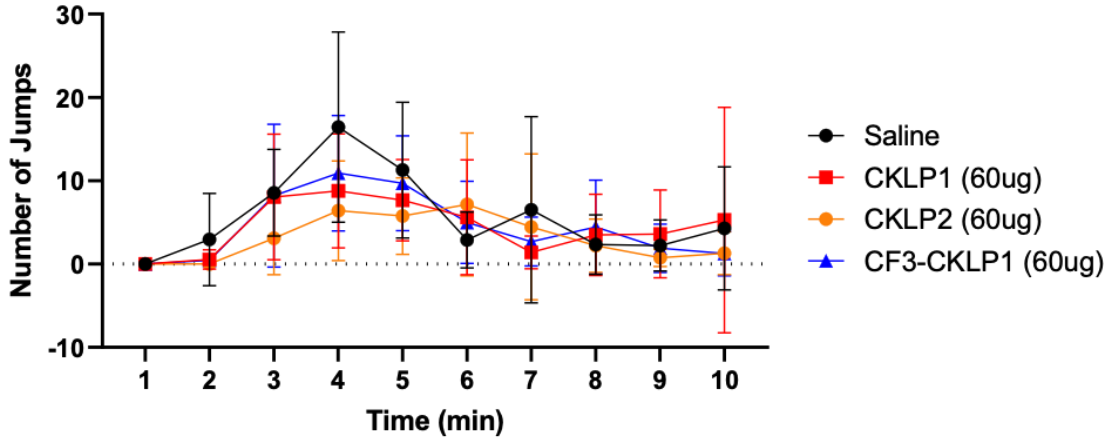


Figure 12. Number of jumps per minute in naloxone precipitated opioid withdrawal after morphine tolerance and intrathecal administration of cromakalim prodrugs. Prodrug had no effect on the number of jumps in withdrawal (Two-Way ANOVA, Drug effect $F(3,34) = 2.590$, $P = 0.0689$; Time x Drug effect $F(27, 306) = 1.160$, $P = 0.2705$). Data is expressed as the number of jumps per animal in each one-minute bin averaged for each treatment group. (n= 5 males and 5 females per group).

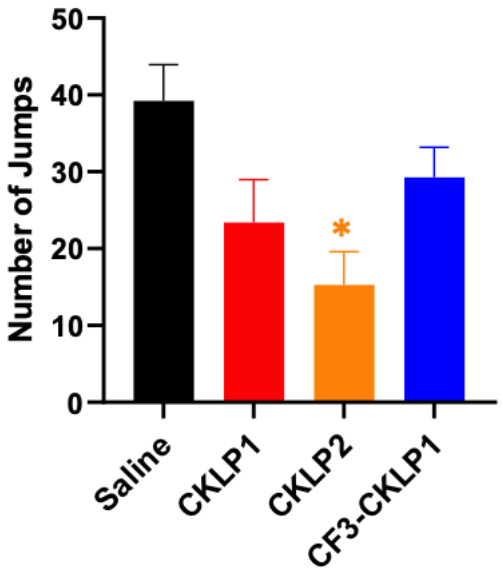


Figure 13. Total number of jumps in the first 5 minutes of naloxone precipitated opioid withdrawal after morphine tolerance and intrathecal administration of cromakalim prodrugs. CKLP2 decreased withdrawal jumps compared to saline (One-way ANOVA, F(3, 34) = 4.455, P_{CKLP1} = 0.1253, P_{CKLP2} = 0.0028, $P_{CF3\text{-}CKLP1}$ = 0.3174). CF3-CKLP1 has no effect on the withdrawal symptom of jumping when compared to saline. Data is expressed as the number of jumps per animal in the first 5 minutes or recorded behaviors averaged for each treatment group. *P <0.05, n= 5 males and 5 females per group.

Table 4. Time-course jumping in withdrawal behaviors two-way ANOVA adjusted P-values compared to saline. Time x Drug effect F(27, 306)= 1.160, *P*= 0.2705

	CKLP1	**CKLP2**	**CF3-CKLP1**
1 min	--	--	--
2 min	0.4961	0.3190	0.4395
3 min	>0.9999	0.0644	0.9990
4 min	0.2414	0.0871	0.4670
5 min	0.5414	0.2200	0.9283
6 min	0.5920	0.3647	0.5642
7 min	0.4309	0.9438	0.6440
8 min	0.8876	0.9995	0.6375
9 min	0.8322	0.4146	0.9905
10 min	0.9944	0.5422	0.5322

Bouts of rearing were present only in the first 10 minutes of the withdrawal period, with majority within the initial 5 minutes of withdrawal (Figure 14). Saline treated animals had the greatest score with an average of ~15 bouts of rearing in the first minute of withdrawal. Animals in the CKLP2 treated group had the lowest rearing score with an average of ~4.5 bouts in the minute of withdrawal. Total bearing bouts in the first five minutes of withdrawal were scored for each animal and averaged for each treatment group. Animals treated with CKLP1 and CF3-CKLP1 had no significant difference in the number of rearing bouts in the first five minutes of withdrawal when compared to the saline treated animals. CKLP2 treated animals had a significantly decreased number of rearing bouts when compared to saline for the first five minutes of withdrawal.

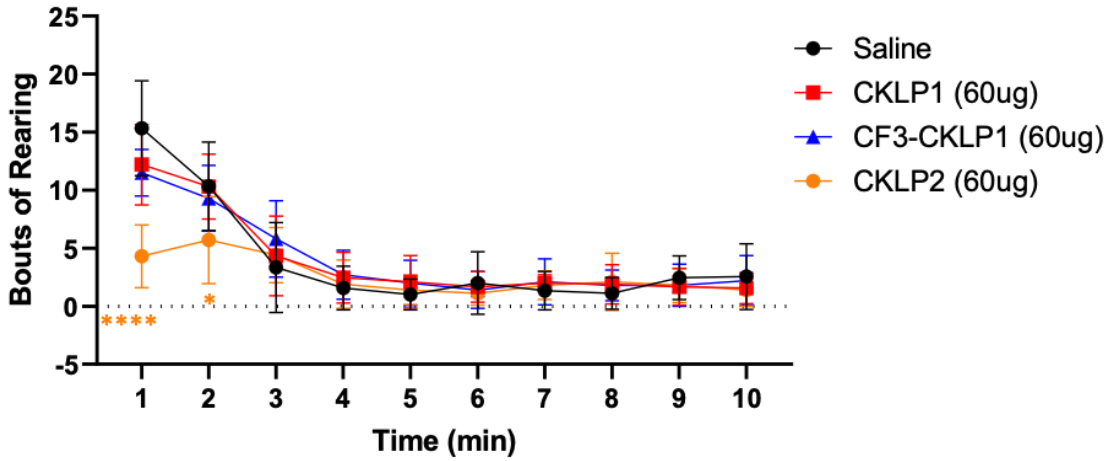


Figure 14. Number of rearing bouts per minute in naloxone precipitated opioid withdrawal after morphine tolerance and intrathecal administration of cromakalim prodrugs. Prodrugs decreased number of rearing bouts (Two-Way ANOVA, Drug effect $F(3,34) = 3.141$, $P = 0.0378$; Time x Drug effect $F(27, 306)= 5.822$, $P < 0.0001$, $P_{CKLP2,1} < 0.0001$, $P_{CKLP2,2} = 0.0383$). Data is expressed as the number of jumps per animal in each one-minute bin averaged for each treatment group. *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001. n= 5 males and 5 females per group.

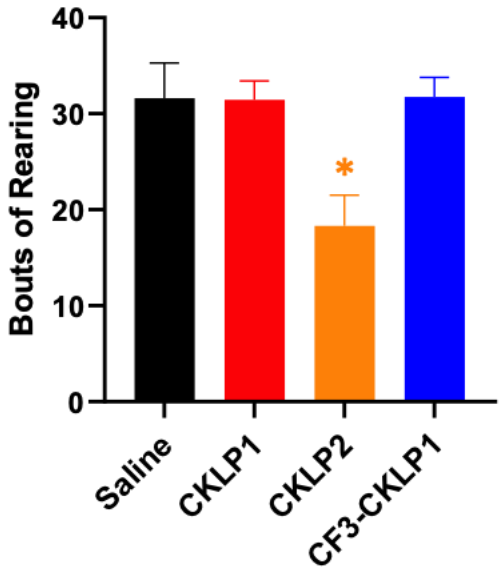


Figure 15. Total bouts of rearing in the first 5 minutes of naloxone precipitated opioid withdrawal after morphine tolerance and intrathecal administration of cromakalim prodrugs. CKLP2 decreased withdrawal rearing compared to saline (One-Way ANOVA, $F(3, 34) = 4.455$, $P_{CKLP1} = 0.9986$, $P_{CKLP2} = 0.0059$, $P_{CF3\text{-}CKLP1} > 0.9999$). CKLP1 and CF3-CKLP1 had no effect on the withdrawal symptom of jumping when compared to saline. Data is expressed as the total number of rearing bouts per animal in the first 5 minutes or recorded behaviors averaged for each treatment group. $^*P < 0.05$, n= 5 males and 5 females per group.

Table 5. Time-course burrowing bouts in withdrawal behaviors two-way ANOVA adjusted P-values compared to saline. Time x Drug effect F(27, 306) = 5.822, *P* < 0.0001.

	CKLP1	**CKLP2**	**CF3-CKLP1**
1 min	0.2720	**** <0.0001	0.0625
2 min	0.9551	* 0.0383	0.9726
3 min	0.8986	0.7429	0.3585
4 min	0.6681	0.7924	0.1536
5 min	0.4824	>0.9999	0.5249
6 min	0.9984	0.9296	0.9410
7 min	0.4292	0.8466	0.6670
8 min	0.6037	0.6670	0.6446
9 min	0.9405	0.9590	0.8308
10 min	0.8631	0.5488	0.9697

HPLC Analysis

HPLC was used to separate cromakalim, CKLP1, CKLP2 and flavopiridol (Figure 16). Compound concentrations were calculated from standard curves plotted as peak area of interest compared to internal standard peak area versus the compound concentration. Extraction efficiency was calculated for CKLP1, CKLP2 and cromakalim (Equation 1). Cromakalim, CKLP1 and CKLP2 were extracted from doped mouse spinal cords with extraction efficiencies ranging from 54% to 70% (Table 6).

We were unable to determine detectable levels of CKLP1 or CKLP2 in spinal cord samples from animals receiving intrathecal injection of either compound. Cromakalim was detected in three of the 30 animals treated with CKLP1. The average concentration of cromakalim was 2.499± 0.449 µM in animals intrathecally injected with 60 µg of CKLP1. Cromakalim was detected in 11 of the 30 animals treated with CKLP2. The average concentration of cromakalim was 2.218± 0.618 µM in animals injected with 60 µg intrathecally and 1.171± 0.079 µM in animals given 30 µg CKLP2 intrathecally. CF3-CKLP1 was not able to be quantified with reverse phase HPLC because the compound eluted in the solvent front. The CF3-CKLP1 treated animals did not have any cromakalim detected within the spinal cord homogenate samples. No compounds were detected in saline treated animals.

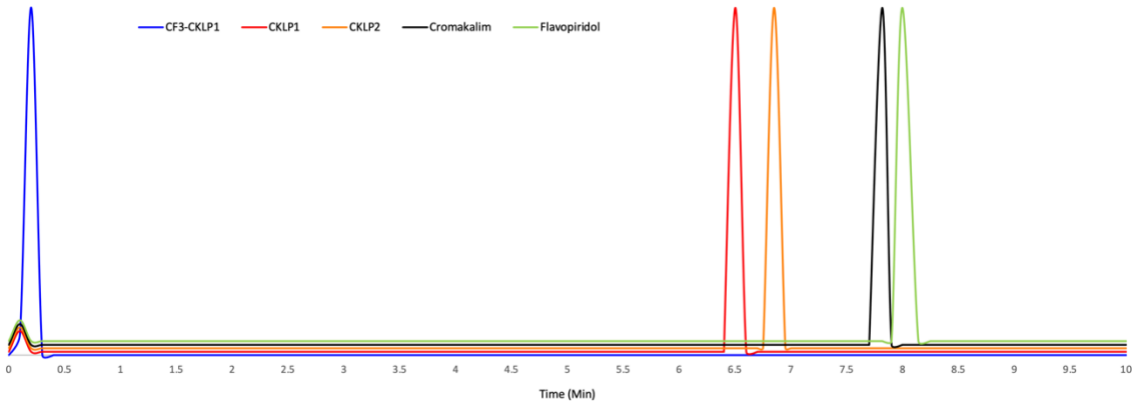


Figure 16. Representative chromatograms of cromakalim prodrugs, cromakalim, and flavopiridol. Average retention time of standards include CF3-CKLP1 in solvent front, CKLP1 at 6.502 min, CKLP2 at 6.849 min, Cromakalim at 7.817 min, and flavopiridol at 7.996 min.

Table 6. HPLC cromakalim, prodrugs and internal standard retention time and extraction efficiency. Retention times of all standards were averaged, with standard error listed. Extraction efficiency comparison of concentrations from extracted compound from doped spinal cord and spiked blank homogenate.

Compound	**Retention Time**	**Extraction Efficiency**
CF3-CKLP1	Solvent Front	Unable to quantify
CKLP1	6.502 ± 0.003 min	54.02 ± 11.91%
CKLP2	6.849 ± 0.001 min	69.93 ± 4.71%
Cromakalim	7.817 ± 0.002 min	66.71 ± 3.25%
Flavopiridol	7.996 ± 0.002 min	N/A

Discussion

Cromakalim prodrugs effectively increased the mechanical paw withdrawal thresholds in mouse models of chronic neuropathic and inflammatory pain. The prodrugs tested increased mechanical paw withdraw thresholds under chronic morphine pre- and post- morphine, as well as decreased opioid withdrawal behaviors. Cromakalim was detected in some of the prodrug treated animals.

Determining prodrug efficacy as an analgesic for neuropathic and inflammatory pain.

Cromakalim prodrug CKLP1 was effective at increasing mechanical paw withdrawal in a sciatic nerve ligation model of chronic neuropathic pain and a CFA model of inflammatory pain in mice. Data is consistent with analgesia on the ipsilateral hind paw, and antinociception on the contralateral hind paw at both doses of CKLP1 under neuropathic pain (Figure 4). Cromakalim prodrug CKLP2 also effectively increased mechanical paw withdrawal thresholds in a sciatic nerve ligation model of chronic neuropathic pain and a CFA model of inflammatory pain. Both doses of CKLP1 and CKLP2 increased mechanical paw withdrawal thresholds under inflammatory pain conditions (Figure 7). Collectively, CKLP1 or CKPL2 could be good candidates to use as analgesics in future pain studies. In contrast, cromakalim Prodrug CF3-CKLP1 increased mechanical paw withdrawal thresholds of only on the ipsilateral hind paw in the CFA mouse model of inflammatory pain (Figure 9). There was no effect on the mechanical paw withdrawal measurements on the ipsilateral hind paw for the sciatic nerve ligation model of chronic neuropathic pain (Figure 6). CF3-CKLP1 could be used in treatment of inflammatory pain, however it is not the most effective compound used in this study for creating an analgesic response.

Intrathecal administration of the cromakalim prodrugs all demonstrated an analgesic effect in an inflammatory pain model, as expected based on potassium channel opener antinociception studies (Afify, Khedr et al. 2013, Fisher, Johnson et al. 2019). CKLP1 and CKLP2 also demonstrated an analgesic effect in the SNL model. The time frame from injection to the maximum paw withdrawal latency during the SNL model is likely due to the time of alkaline phosphatase dephosphorylation of prodrugs, and the time for the parent structure, cromakalim, to act on the potassium channel. It has been suggested that potassium channel openers, such as cromakalim, have anti-inflammatory properties due to their vasodilation capability (Inagaki, Gonoi et al. 1996, Plachinta, de Klaver et al. 2004). Decreasing ischemia and increasing vasodilation in the inflammatory process is beneficial for increasing blood flow to damaged cells, providing immune defense, and removing waste from the area (Mizumura, Nithipatikom et al. 1995). This could explain why all three cromakalim prodrugs would increase the mechanical paw withdrawal thresholds in the inflammatory pain model, but not in the neuropathic pain model.

Cromakalim prodrug efficacy in inflammatory pain models could also be attributed to a difference in alkaline phosphatase expression or activity under different injury models, however no direct comparison between inflammatory and neuropathic pain and alkaline phosphatase has been done. Expression of alkaline phosphatase increases in times of cellular stress and under nerve injury (Brichacek and Brown 2019). Studies have also shown an increase of alkaline phosphatase correlating with chronic inflammation (Park, Lee et al. 2020) and protection in gastrointestinal inflammation (Fawley and Gourlay 2016). It is possible the expression of alkaline phosphatase is

increased under inflammation as a method of cellular protection and dephosphorylation of ATP in times of increased metabolism where transport of nutrients and waste is increased. Increased levels of alkaline phosphatase would increase the dephosphorylation of cromakalim prodrugs in the inflammatory pain model, contributing to the overall increase in antinociception of cromakalim prodrug treated animals. It is possible the expression of phosphatases under inflammatory pain is greater than neuropathic pain, leading to an increase in the total dephosphorylation of the cromakalim prodrugs tested in this experiment.

The structural difference of CKLP1 and CF3-CKLP1 is the replacement of the nitrile functional group with trifluorocarbon. This is commonly done to increase metabolic stability and the elimination half-life of a compound. The addition of a bulkier functional group may cause interference and prevent binding to the sulfonylurea receptor, decreasing the effect of CF3-CKLP1 compared to the other prodrugs investigated. The overall success of CKLP2 in all experiments is likely attributed to the additional carbon and oxygen linking the phosphate group to cromakalim. The extra atoms linking a phosphate group to cromakalim likely provide bond rotation and space for alkaline phosphatase active site to access and cleave the phosphate group.

Determining prodrug efficacy in reducing opioid tolerance and symptoms of withdrawal.

Our data indicate cromakalim prodrugs decrease opioid induced hyperalgesia in a mouse model of morphine tolerance. CKLP1 and CKLP2 had the greatest effect at reducing hyperalgesia, increasing mechanical paw withdrawal threshold values over subsequent days prior to morphine administration on days four and five of chronic

morphine exposure. CF3-CKLP1 also decreased opioid induced hyperalgesia, but to a smaller extent. The pairing of cromakalim prodrugs with morphine also reduced opioid tolerance. This was demonstrated through the mechanical thresholds having maintained a high level after morphine administration compared to the saline treated animals. CKLP1 and CF3-CKLP1 were the most effective at maintaining higher mechanical paw withdrawal threshold measurements after morphine administration in days two through five. CKLP2 had a small increase in mechanical paw withdrawal threshold forces on day five of morphine tolerance.

A large decrease in withdrawal behaviors was seen in CKPL2 treated mice, with the greatest decrease of opioid withdrawal symptoms, decreasing the bouts of rearing from 15.05 in saline treated animals to 4.5 bouts in CKLP2 treated animals in the first minute of withdrawal. Withdrawal jumping decreased in the CKLP1 and CKLP2 treated mice compared to saline treated mice, with an average of ~39 jumps in the first five minutes of withdrawal compared to ~23 and ~15 jumps for CKLP1 and CKLP2, respectively. Withdrawal jumping symptoms were unchanged in CF3-CKLP1 treated mice with ~29 jumps compared to saline treated animals average of ~39 jumps.

The decrease in withdrawal symptoms might be due to the opening of K_{ATP} channels independently of opioid signaling, decreasing overall neuron excitability (Yang, Wu et al. 2014). The intraperitoneal delivery of a K_{ATP} channel openers, including cromakalim, decreased symptoms of withdrawal, while the K_{ATP} channel blocker increased withdrawal symptoms (Robles, Barrios et al. 1994, Seth, Ahmad et al. 2010). Symptoms of withdrawal intensify under increased concentrations of ATP in the central nervous system (Burma, Bonin et al. 2017). The increased concentration of ATP inhibits

K_{ATP} channels, counteracting this with an agonist opens the channel to decrease withdrawal symptoms. Potassium channels are a potential target for decreasing opioid withdrawal symptoms due to these phenomena.

Cromakalim Prodrug Conversion.

HPLC data shows cromakalim prodrug phosphate group is cleaved after intrathecal injection, converting the compound into the cromakalim parent compound with no prodrug left in detectable levels. Animals treated with CKLP1 and CKLP2 had similar concentrations of cromakalim present in the spinal cord 24-hours after injection, confirming dephosphorylation of prodrug took place in the spinal column of the animal used in behavior experiments. The presence of cromakalim in these animals likely correlates to the antinociceptive response seen in behavior data of CKLP1 and CKLP2 treated animals. Cromakalim was able to be quantified in more animals treated with CKLP2 than in the other experimental animals in this study. CF3-CKLP1 was not able to be separated from the solvent front. This is likely due to the trifluorocarbon group replacing the nitrile group on CKLP1 affecting interaction with the stationary phase of the column, preventing CF3-CKLP1 from being retained.

HPLC analysis showed some peaks with retention times similar to cromakalim and the prodrugs. These peaks were excluded from analysis due to varying from the respective standard peak more than three times the standard error of the peak's average retention time, without a shift in internal standard retention time. Other peaks not seen in blanks or saline treated animals were also noted. These peaks could be the compounds of interest, with small structural changes having taken place *in vivo*. The identification of the compounds eluted remain unknown due to UV-detector limitations. Use of mass

spectrometry detection could allow for determining the identity of the peaks eluting at times other than seen in standards.

Cromakalim and CKLP1 in other body systems.

Opening of K_{ATP} channels, such as with cromakalim, in the aqueous humor leads to vasodilation of endothelial cells, decreasing blood pressure in the eye (Chowdhury, Bahler et al. 2015). The cromakalim prodrug CKLP1 is an effective treatment for glaucoma due to the drug's ability to decrease intraocular pressure by opening K_{ATP} channels in mice and rabbits (Chowdhury, Rinkoski et al. 2017, Chowdhury, Kudgus et al. 2020) and recently in nonhuman primates (Chowdhury, Kudgus et al. 2021). The success of CKLP1 as a K_{ATP} channel opener at therapeutic levels in a variety of animals provides hope for the use of CKLP1 and CKLP2 as analgesics in other animal models.

Future Directions.

This study used intrathecal administration of cromakalim prodrugs. Intrathecal administration has advantages such as direct injection into the location of synapse from the peripheral to central nervous system. This target stops the transduction of pain signals early in transduction preventing communication to the brain. It is, however, not ideal in a clinical setting to treat chronic pain only via intrathecal delivery. Intrathecal delivery of a drug to patients with chronic pain is invasive, expensive, and can have many complications. An alternative to intrathecal delivery would be oral medications, increasing patient adherence with an easier pain management regimen.

Systemic delivery of cromakalim prodrugs will have its own challenges and side effects. Cromakalim binds to the sulfonylurea receptor with high specificity to the SUR2 subtype over SUR1. SUR2 specificity is also seen in the known K_{ATP} channel openers

pinacidil and nicorandil (Moreau, Jacquet et al. 2000). The SUR2 subtype (*Abcc9*) is expressed in multiple tissues throughout the body, the liver and heart being the most potentially problematic (Fagerberg, Hallström et al. 2014). Systemic effects of cromakalim prodrugs could lead to hypotension when opening cardiac potassium channels (Huang, Hu et al. 2019) or insulin secretion can be affected by potassium channel activation in the insulin secreting Beta cells of the pancreas (McTaggart, Clark et al. 2010). Future studies will look at the overall physiological response to cromakalim prodrug administration, such as the effect of blood pressure and blood sugar after systemic delivery.

It would be worth investigating prodrugs of other K_{ATP} channel agonists for the purpose of analgesia. Other K_{ATP} channel agonists more specific to SUR1 (*Abcc8*) subtypes most highly expressed in the nervous system, such as diazoxide would be worth investigating (Moreau, Jacquet et al. 2000, Fagerberg, Hallström et al. 2014). The K_{ATP} channel opener pinacidil is more specific to SUR1 subtypes, and already shows antinociception capabilities, but faces the solubility difficulties as cromakalim (Moreau, Jacquet et al. 2000, Luu, Bjork et al. 2019).

Determining the *in vivo* dephosphorylation time or pharmacokinetics would be beneficial in future experiments. Similar to the neuropathic pain model, investigating the ratio of prodrug conversion after injection at various time points by HPLC analysis on injected tissues could be used in comparison to analgesic response SNL data to determine peak time of drug activity. It would also be worth investigating the conversion under different injury states when levels of alkaline phosphatase are increased due to injury.

Due to the overall efficacy of CKLP2 as an analgesic in neuropathic and inflammatory pain, as well as decreasing opioid tolerance and withdrawal symptoms, it would be beneficial to move forward with investigating CKLP2 or investigating similar analogues for other K_{ATP} channel agonists.

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