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Kratom alkaloids, natural and semi-synthetic, show less physical dependence and ameliorate opioid withdrawal

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Abstract

Chronic administration of opioids produces physical dependence and opioid-induced hyperalgesia. Users claim the Thai traditional tea “Kratom” and component alkaloid mitragynine ameliorate opioid withdrawal without increased sensitivity to pain. Testing these claims, we assessed the combined Kratom alkaloid extract (KAE) and two individual alkaloids, mitragynine (MG) and the analog mitragynine pseudoindoxyl (MP), evaluating their ability to produce physical dependence and induce hyperalgesia after chronic administration, and as treatments for withdrawal in morphine-dependent subjects. C57BL/6J mice (n=10/drug) were administered repeated saline, or graded, escalating doses of morphine (intraperitoneal; i.p.), Kratom alkaloid extract (orally, p.o.), mitragynine (p.o.) or MP (subcutaneously, s.c.) for five days. Mice treated chronically with morphine, KAE or mitragynine demonstrated significant drug-induced hyperalgesia by day 5 in a 48 °C warm-water tail-withdrawal test. Mice were then administered naloxone (10 mg/kg, s.c.)

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Ethics approval: All animal studies were preapproved by the University of Florida (Gainesville, FL, USA) Institutional Animal Care and Use Committee, in accordance with the 2011 National Institute of Health Guide for the Care and Use of Laboratory Animals. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010). Initial sample sizes were approximated by power analysis, with animals assigned to groups randomly. Drug treatment experiments were conducted in a blinded fashion.

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and tested for opioid withdrawal signs. Kratom alkaloid extract and the two individual alkaloids demonstrated significantly fewer naloxone-precipitated withdrawal signs than morphine-treated mice. Additional C57BL/6J mice made physically-dependent on morphine were then used to test the therapeutic potential of combined KAE, mitragynine or MP given twice daily over the next three days at either a fixed dose or in graded, tapering descending doses. When administered naloxone, mice treated with KAE, mitragynine or MP under either regimen demonstrated significantly fewer signs of precipitated withdrawal than control mice that continued to receive morphine. In conclusion, while retaining some liabilities, Kratom, mitragynine and mitragynine pseudoindoxyl produced significantly less physical dependence and ameliorated precipitated withdrawal in morphine-dependent animals, suggesting some clinical value.

Keywords

Opioid; Physical Dependence; Kratom; Mitragynine; Withdrawal

Introduction

While opioid agonists of the mu-opioid receptor (MOR) have proven therapeutic advantages in the treatment of pain, MOR activation also produces challenging side-effects (Minami & Satoh, 1995; Li & Zhang, 2012). For example, repetitive administration of opioids induces hyperalgesia (Hutchinson et al., 2011; Xin et al., 2011), paradoxically increasing sensitivity to pain. Moreover, continual exposure to opioid agonists results in changes of physical homeostatic mechanisms such as adenylyl cyclase activity, factors collectively thought to contribute to undesired effects such as tolerance and physical dependence. (Koob et al., 1997) These neuroadaptive mechanisms are theorized to be the body's defense against life-threatening effects following chronic use of drugs (Koob et al., 1997). Interruption of opioid intake results in clearance of the drug following metabolism (Shafer & Varvel, 1991), but adaptations to homeostasis do not reverse rapidly. The absence of the drug after chronic use leads to physical withdrawal symptoms (Hutcheson et al., 2001). Symptoms related to opioid-induced withdrawal include body aches, headache, anxiety, seizures, and/or influenza-like conditions (Khor et al., 2011). Avoidance of opioid withdrawal symptoms is the leading cause of opioid seeking and relapse in abstinent subjects (Khor et al., 2011). The MOR agonist methadone and buprenorphine (a multifunctional MOR partial agonist, KOR/DOR antagonist and NOP weak agonist) are used to treat opioid use disorder and prevent opioid withdrawal related symptoms in the clinic (Kosten & O'Connor, 2003). However, these opioid agonists potentially produce liabilities of their own, including respiratory depression and substance abuse (Kreek et al., 2002). Thus, identifying new, safer treatments for the symptoms of opioid withdrawal would also potentially help address the urgent problem of opioid substance abuse disorder.

Mitragyna speciosa, or Kratom, a natural plant product native to Southeast Asia, has recently gained popularity in regards for its use in self-treatment of opioid addiction and withdrawal syndrome (Boyer et al., 2008; Kruegel & Grundmann, 2018; Singh et al., 2019; Chakraborty and Majumdar, 2020). Mitragynine, the main active alkaloid in Kratom, has been found to make up approximately 66% of the extract content within the plant (Adkins et al. 2011;

Hassan et al. 2013). An initial study reported that a single administration of mitragynine reduced behavioral signs of withdrawal in morphine-dependent rats (Hassan et al., 2020). Although a mechanism was not investigated, previous studies with mitragynine show that most of its physiological effects are via the MOR subtype (Yusoff et al. 2017; Matsumoto et al., 1996a; Kruegel et al., 2019). Similarly, the semi-synthetic analog mitragynine pseudoindoxyl (MP) demonstrated potent antinociception with reduced liabilities attributed to its multifunctional MOR biased agonism and DOR antagonism (Váradi et al., 2016). Recent *in vitro* studies also suggest that mitragynine pseudoindoxyl is a metabolite of mitragynine (Kamble et al., 2020). Lyophilized kratom tea itself has been shown to act via the opioid system (Yusoff et al. 2017; Wilson et al., 2020), mitigate opioid withdrawal syndrome in zebra fish (Khor et al. 2011), and substitute for morphine in rodent discrimination studies (Harun et al. 2015).

From this and the limited clinical reports, we hypothesize that Kratom alkaloid extract (KAE) tested presently or its major constituents may be therapeutic candidates for the treatment of opioid withdrawal syndrome. Accordingly, the aim of this study was to assess the ability of KAE, the major alkaloid mitragynine and the semi-synthetic analog mitragynine pseudoindoxyl for the amelioration of naloxone-precipitated opioid withdrawal syndrome in mice, first comparing them to morphine for their potential to induce opioid induced hyperalgesia and physical dependence directly after chronic treatment.

Methods

Male C57BL/6J mice (25–35 mg) were used (Jackson Laboratories Bar Harbor, Maine, USA). The mice were housed five per cage on a 12:12h light/dark cycle (lights off at 7:00 P.M. and on at 7:00 A.M.) with *ad libitum* access to food and water. All animals were habituated to the testing room for a minimum of 1 hour prior to testing. All animal studies were approved and conducted in agreement with the Institutional Animal Care and Use Committees at the University of Florida, in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010).

Chemicals

The kratom alkaloid extract (KAE) used for assays was prepared as described by Sabetghadam et al., 2010; Váradi et al., 2016; Gutridge et al., 2020 and Uprety et al. (submitted) (see also below) from Kratom “Red Indonesian Micro Powder,” purchased from Moon Kratom (Austin, TX). Mitragynine pseudoindoxyl was synthesized from mitragynine as previously described (Váradi et al., 2016; Uprety et al. (submitted)). Mitragynine itself was extracted from the powdered leaves of *Mitragyna speciosa* (kratom) by following our previously described method (Váradi et al., 2016). Briefly, Kratom powder (500 g) was heated to reflux in MeOH 700 mL for 40 min. The suspension was filtered and the methanolic extraction process was repeated three times (3 × 500 mL). The solvent of combined methanolic extract was evaporated under reduced pressure and the content was dried using high vacuum. The dry residue was suspended in 20% acetic acid solution (1 L) and washed with petroleum ether (4 × 500 mL). The aqueous layer was then cooled on an

ice bath and basified (pH ~9–10) with aqueous NaOH solution (3.5M. ~1L) slowly. Alkaloids were extracted in EtOAc (4 × 400 mL) from the aqueous layer. The combined EtOAc part was washed with brine 300 mL and dried over anhydrous Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure, and the residue was dried under high vacuum to obtain kratom extract (11 g). Then, 200 mg of kratom extract was subjected to silica gel column chromatography; using 0–40% EtOAc in hexanes to isolate mitragynine (97.2 mg); paynantheine (11.6 mg), speciogynine (7 mg), and 0–15% MeOH in DCM to isolate speciociliatine (27.4 mg). This correspond to approximately 48.6% mitragynine, 5.8% paynantheine, 3.5% speciogynine and 13.7% speciociliatine in the kratom extract along with other minor alkaloids which are below 0.1%.

All drugs and chemicals otherwise used were purchased from Sigma-Aldrich (St. Louis, MO, USA). For experiments, sterile isotonic saline (0.9%) was used to dissolve drugs to desired concentrations for testing.

Hyperalgesic Testing (48 °C Warm-Water Tail-Withdrawal Assay)

The nociceptive stimulus was 48 °C water, with the latency to withdraw the tail taken as the end point (Journigan et al., 2014). The water temperature of 48 °C was selected for this work to ensure a moderate tail-withdrawal response, with a measurable decrease in withdrawal time possible but also a significant temperature for hyperalgesic testing. If the mouse failed to display a tail-flick in 30 s, the tail was removed from the water to minimize tissue damage. Animals showing an initial baseline latency fewer than 4 s or more than 30 s were removed from the study; no mice were so excluded. After determining control latencies on day 1, mice received chronic vehicle (saline), morphine, morphine + acute clonidine on day 5, Kratom alkaloid extract, mitragynine or mitragynine pseudoindoxyl pretreatment for four days as described below. Dosing of these compounds was based on demonstration of equianalgesic efficacy drawn from earlier antinociceptive dose response and time course studies (Sabetghadam et al., 2010; Váradi et al., 2016; Kruegel et al., 2019; Wilson et al., 2020; Uprety et al., submitted; see below). Mice were then tested prior to treatment on day 5 for evidence of induced hyperalgesia. Experimentally induced decreases in tail-withdrawal latencies in this assay indicate hyperalgesic effects (Crain and Shen, 2001 and 2007; Journigan et al., 2014). Tail withdrawal baselines were measured on day 5 prior to opioid or Kratom alkaloid administration and subsequent administration and assessment of naloxone precipitated opioid withdrawal symptoms that are describe below in experiment design 1.

Naloxone Precipitated Opioid Withdrawal Assay

Mice were randomly divided into 14 groups: six groups to assess potential physical dependence (Experimental design 1, n=10/group) and eight therapeutic challenge groups (Experimental design 2, n=9–10/group) utilizing a modified dosing regimen (as described below, and in Table 1 and 2, respectively). Kratom alkaloid extract, mitragynine, or mitragynine pseudoindoxyl (MP) were used to challenge morphine withdrawal. Dosing of these compounds was based on earlier antinociceptive dose response studies (Sabetghadam et al., 2010; Váradi et al., 2016; Kruegel et al., 2019; Wilson et al., 2020; Uprety et al., submitted). All daily repeated administrations occurred at 9:00 A.M. and 7:00 P.M., as

previously reported (Kamei and Ohsawa, 1997; Wilson et al., 2020). On the last day, withdrawal was precipitated with 10 mg/kg naloxone (s.c.) in all groups.

Experimental design 1: Test of direct physical dependence

Animals were randomly assigned to one of the following groups: saline (i.p.), morphine (10–75 mg/kg, i.p.), morphine (10–75 mg/kg, i.p.) + acute clonidine (5 mg/kg i.p., on day 5), Kratom alkaloid extract (30–125 mg/kg, p.o.), mitragynine (10–75 mg/kg, p.o.), or mitragynine pseudoindoxyl (1–35 mg/kg, s.c.). Dosing for experimental design 1 was performed on a 5-day schedule, with an escalating dose of the compound given twice daily for 4 days, with a final dose administered on the 5th day (see table 1). Two hours post-treatment on the last day, all mice were administered naloxone (10 mg/kg, s.c.) to precipitate opioid withdrawal symptoms (Kamei and Ohsawa, 1997; Özdoğan et al., 2003; Wilson et al., 2020; see also below). For the morphine + clonidine group, clonidine was administered 90 minutes after the last morphine treatment and 30 min preceding naloxone treatment on day 5. (Özdoğan et al., 2003)

Experimental Design 2: Test of therapeutic potential to ameliorate precipitated withdrawal

Mice were randomly placed into eight groups and treated repeatedly with: saline (i.p.), or morphine (10–75 mg/kg, i.p.) for five days (see Table 2). For the next four days, groups were administered twice daily fixed doses of saline (i.p.), morphine (80 mg/kg, i.p.), tapering doses of Kratom alkaloid extract (100–30 mg/kg, p.o.), fixed doses of mitragynine (80 mg/kg, p.o.), tapering doses of mitragynine (80–25 mg/kg, p.o.), fixed doses of MP (30 mg/kg, s.c.), or tapering doses of MP (30–1 mg/kg, s.c.). Two hours post-injection on the last day of testing, all mice were injected with naloxone (10 mg/kg, s.c.) to precipitate opioid withdrawal symptoms.

Measurement of Opioid Withdrawal Behavior Signs

During evaluation of opioid withdrawal, animals were observed individually while moving freely in a Plexiglas cylinder for 15 min after naloxone administration using established methods (Way et al., 1969, Shaw-Luthman et al., 2002, Wilson et al., 2020). In vivo activity was digitally recorded (Noldus EthoVision software), then later evaluated for withdrawal behaviors. Evaluators were blind to the treatment of mice they were scoring. Each animal was assessed for the number of times they demonstrated the following behaviors: forepaw tremors, wet dog shakes, jumping, rearing, and teeth chattering frequencies. Jumping was defined as an attempt to bound and/or leap off the surface. Forepaw tremor was defined as a rapid up/down motion with the front paws that did not touching of the head or ears. Rearing was defined as repetitive standing on hind paws with front paws not touching any parts of the observation container. Forepaw licking was defined as the mouse licking both of its forepaws at least once without touching head or ears in a grooming way. Wet dog shakes were defined as a spontaneous and quick shaking of the entire head and upper body of the mouse. Straightening was defined as the number times each mouse stretched or elongated their body with all four paws on the touching the bottom of the enclosure. Teeth chattering was defined as repetitive moving up and down of the lower lip portion of the mouth in a

"gum chewing" manner. Diarrhea frequency was defined as the number of soft and/or wet excreta pellets.

Statistical Analysis

All data are plotted as mean ± S.E.M. and were analyzed using Prism 8.0 software (GraphPad Software, La Jolla, California, USA). Normality and equal variance were confirmed statistically and justified using parametric analysis. Significant differences in behavioral data were analyzed by ANOVA (one- or two-way with or without repeated measures as appropriate) with significant results further analyzed with Sidak or Tukey *post hoc* tests for significant pairwise comparisons within and between groups. All significance was set at $p \leq 0.05$.

Results

Assessment of treatment-induced hyperalgesia.

Mice (n=10/treatment) were treated twice daily for four days with escalating doses of saline (i.p.), morphine (i.p.), the combined Kratom alkaloid extract (p.o.), mitragynine (p.o.) or mitragynine psuedoindoxyl (s.c.) as described in the methods. Tail-withdrawal latencies were collected both prior to the start of treatment (on day 1), and again on the morning of day 5. Each group of mice showed equivalent initial responses on day 1 ($F_{(4,45)}$=1.12; p=0.995; one-way ANOVA; Fig 1). Treatment significantly changed this response on day 5 ($F_{(1,45)}$=56.3; p<0.0001; two-way RM ANOVA w/Sidak's post-hoc test), with mice treated with morphine (p<0.0001), combined Kratom alkaloid extract (p=0.002) and mitragynine (p=0.002) displaying reduced latencies indicative of induced hyperalgesia (Fig 1). Neither saline (p=0.68) or mitragynine pseudoindoxyl (p=0.10) displayed significant differences from their initial responses, or each other (p=0.27, Student's t-test), suggesting the mitragynine pseudoindoxyl treatment regimen did not induce hyperalgesia.

Assessment of direct treatment-induced physical dependence.

Following chronic twice-daily administration with either saline (i.p.), escalating doses of morphine (10–75 mg/kg, i.p.), or escalaing doses of the test compounds, mice on day 5 were administered naloxone (10 mg/kg, s.c.) and precipitated opioid withdrawal signs were assessed. Alone, morphine adminstration significantly reduced forepaw licking ($F_{(5, 54)}$ = 14.03, p<0.0001; One-way ANOVA with Tukey's test; Fig 2b) and rearing ($F_{(5, 54)}$ = 30.29, p<0.0001; One-way ANOVA with Tukey's test; Fig 2c). Naloxone administered to mice chronically treated with morphine also precipitated increased occurances of diarrhea ($F_{(5, 54)}$ = 10.74, p< 0.01; One-way ANOVA with Tukey's test; Fig 2e), teeth chattering ($F_{(5, 54)}$ = 14.28, p< 0.01; One-way ANOVA with Tukey's test; Fig 2g), and jumping ($F_{(5, 54)}$ = 43.5, p< 0.0001; One-way ANOVA with Tukey's test; Fig 2h) after the administration of naloxone. No significant effects were observed with assessments of mouse straightening ($F_{(5, 54)}$ = 3.69, p= 0.77; One-way ANOVA with Tukey's test; Fig 2c), forepaw tremor ($F_{(5, 54)}$ = 5.6, p= 0.99; One-way ANOVA with Tukey's test; Fig 2f), or wet dog shakes ($F_{(5, 54)}$ = 6.7, p> 0.99; One-way ANOVA with Tukey's test; Fig 2h).

The α_2-adrenoreceptor agonist clonidine has been shown to ameliorate some sequelae of opioid withdrawal (Jasinski et al., 1985). As a positive control, a set of mice treated chronically 5 days with morphine (10–75 mg/kg, i.p.) received an acute dose of clonidine (5 mg/kg, i.p.) 30 min prior to naloxone administration. Clonidine treatment was unable to attenuate naloxone-precipitated reductions in forepaw licking ($p<0.0001$; Fig 2b), body straightening ($p=0.008$; Fig 2c), and rearing ($p=0.003$; Fig 2d) when compared to the saline control groups. However, compared to the group treated with only morphine, clonidine significantly reduced instances of naloxone-precipitated diarrhea ($p<0.0001$, Fig 2d), and teeth chattering ($p<0.0001$, Fig 2g). Acute administration of clonidine did not reduce significant naloxone-precipitated jumping compared to the saline control group ($p<0.0001$; Fig. 2h), although it should be noted that the mice treated with chronic morphine and acute clonidine displayed immobility and persistent full body trembling behaviors between jumping that was not observed in any other groups. There were no sigificant effects seen in forepaw tremor (Fig. 2f) and wet dog shakes (Fig. 2i) compared to either the saline or morphine-only treated groups.

Chronic escalating treatment with the Kratom alkaloid extract (30–125 mg/kg, p.o.) or mitragynine (10–75 mg/kg, p.o.) produced minor withdrawal effects in comparison (Fig 2). Combined Kratom alkaloid extract ($p<0.0001$) and mitragynine ($p<0.0001$) each produced a significant decrease in forepaw licking (Fig 2b), mouse straightening ($p=0.01$ each, Fig 2b), and rearing ($p=0.005$ and $p=0.0001$, respectively; Fig 2d). The decrease in rearing, however, was also significantly higher in the combined Kratom alkaloid extract ($p<0.0001$) and mitragynine ($p=0.0003$) treated groups than the effects of the morphine positive control. Unlike the combined Kratom alkaloid extract, the administration of mitragynine alone demonstrated an increase in the occurance of diarrhea ($p=0.02$, Fig 2e) and forepaw tremor ($p=0.008$, Fig 2f). Otherwise, chronic treatment with the combined Kratom alkaloid extract and mitragynine resulted in no other significant signs of opioid withdrawal (teeth chattering (Fig 2g), jumping (Fig 2h) or frequency of wet dog shakes (Fig 2i).

Mitragynine pseudoindoxyl (MP), the semi-synthetic Kratom alkaloid, demonstrated significant signs of opioid physical dependence after chronic twice-daily administration (1–35 mg/kg, s.c.; Fig 2). Although forepaw tremors, teeth chattering, and straightening responses remained unchanged (Fig 2f, 2g, and 2c, respectively), mitragynine pseudoindoxyl treated mice displayed significant decreases in forepaw licking ($p<0.0001$; Fig 2b) and increases in “wet dog shakes” ($p=0.001$; Fig 2i). Moreover, mitragynine pseudoindoxyl treatment showed significant increases in the presence of diarrhea ($p=0.003$; Fig 2e), and jumping ($p<0.01$; Fig 2i), as well as a decrease in rearing frequency ($p<0.0001$; Fig 2d). Collectively, these signs suggest mitragynine pseudoindoxyl displayed a greater magnitude of opioid physical dependence as compared to mitragynine, but far less than morphine under this treatment regimen.

Evaluation of alkaloid’s ability to ameliorate withdrawal in subjects physically dependent on morphine.

After four days of treatment twice daily with escalating doses of morphine (10–75 mg/kg, i.p.), mice were then treated twice daily for three more days with a fixed high dose of either

morphine (80 mg/kg, i.p.), mitragynine (80 mg/kg, p.o.), or MP (30 mg/kg, s.c.) prior to a single final treatment on day 8 and the subsequent administration of naloxone (Fig. 3A). Mice treated with the fixed dose of mitragynine still demonstrated a significant naloxone-precipitated increase in the frequency of rearing $F_{(3, 34)} = 6.9$, p=0.003, One-way ANOVA with Tukey's test; Fig 3d). Fixed doses of both mitragynine and MP demonstrated a significant decrease in the frequency of naloxone-precipitated diarrhea ($F_{(3, 33)} = 60.2$, p<0.0001 and p=0.04 respectively, One-way ANOVA with Tukey's test; Fig 3e) compared to the morphine control, although MP-treated mice still experienced increased incidence of diarrhea (p<0.0001) compared to the saline control. Compared to morphine, both mitragynine and MP significantly decreased naloxone-precipitated teeth chattering ($F_{(3, 32)} = 10.8$, p<0.0002 and p=0.0005 respectively, One-way ANOVA with Tukey's test; Fig 3g) and jumping ($F_{(3, 34)} = 64.9$, p<0.0001 each, One-way ANOVA with Tukey's test; Fig 3i), while showing no significant changes in forepaw licking (Fig. 3b), straightening (Fig. 3c), forepaw tremor (Fig 3f), or wet dog shakes (Fig. 3h).

When tested for its abilities to mitigate naloxone-precipitated withdrawal in morphine dependent mice, four days' treatment with tapering doses of the combined Kratom alkaloid extract (100 down to 40 mg/kg, p.o.; Fig 4A) successfully modulated most precipitated withdrawal effects when compared to mice receiving only additional morphine (80 mg/kg, i.p). Mice treated with tapering doses of Kratom alkaloid extract or mitragynine displayed significant decreases in the frequency of naloxone-precipitated diarrhea ($F_{(4, 42)} = 33.4$, p <0.0001 each, One-way ANOVA with Tukey's test; Fig 4e), while MP-treated mice demonstrated an increased frequency of diarrhea (p <0.0001) as compared to the saline control. Kratom alkaloid extract, mitragynine and MP treated mice each showed significant reductions in naloxone-precipitated teeth chattering ($F_{(4, 42)} = 11.0$, p <0.0001 each; Fig 4g) and jumping ($F_{(4, 42)} = 115.6$, p <0.0001; Fig 4i) compared to the morphine control group. Curiously, mice treated with the combined Kratom alkaloid extract displayed significantly increased forepaw tremor ($F_{(4, 42)} = 2.7$, p=0.04; Fig 4f) and both Kratom alkaloid extract and mitragynine treated mice demonstrated increased rearing frequency ($F_{(4, 42)} = 11$, p <0.0001; Fig 4d) compared to the morphine only group. MP-treated mice, however, demonstrated decreased rearing (p=0.03) following naloxone administration when compared to the saline treated group. Mice treated with Kratom alkaloid extract or MP demonstrated no significant changes in forepaw licking following naloxone administration (Fig 4b); however, there was a significant increase observed in the group administering tapering doses of mitragynine as compared to responses of both the saline and morphine treated groups ($F_{(4, 42)} = 4.04$, p=0.01, One-way ANOVA with Tukey's test). Only mice administered tapering doses of MP presented a significant increase in the frequency of naloxone-precipitated wet dog shakes compared to both the saline and morphine controls ($F_{(4, 42)} = 4.7$, p=0.004 and p=0.02 respectively, One-way ANOVA with Tukey's test; Fig 4h). No tapering treatment regimen produced significant changes in quantitated straightening behavior after naloxone administration ($F_{(4, 42)} = 1.6$, p=0.19, One-way ANOVA with Tukey's test; Fig 4c).

Discussion

This study found that Kratom alkaloid extract and two component alkaloids mitragynine and mitragynine pseudoindoxyl ameliorated varying sequelae of naloxone-precipitated opioid withdrawal syndrome in mice physically dependent on morphine. Moreover, similar to morphine, chronic administration of mitragynine and KAE resulted in significant hyperalgesic effects not produced by mitragynine pseudoindoxyl under the chronic treatment regimen utilized.

Opioid-induced hyperalgesia (OIH) is an increase of sensitivity to pain that develops with repeated exposure to opioids such as morphine (Hutchinson et al., 2011). A significant clinical complication of opioid use to treat chronic pain, OIH been demonstrated in patients experiencing both spontaneous and precipitated withdrawal after acute- or chronic opioid administration (Lipman and Blumenkopf, 1989; Devulder et al., 1996; Sun, 1998). Studies have also shown the development of hyperalgesia in persons with substance abuse disorder after sudden decreases in the dosage of opioids (Miser et al., 1986; Lipman and Blumenkopf, 1989). Previous studies have also demonstrated opioid withdrawal-induced hyperalgesia in rodents (Célèrier et al., 2000; Li et al., 2001; Laulin et al., 2002; Balter and Dykstra, 2013). The current study shows that the combined Kratom Alkaloid extract and its main component, mitragynine, induced hyperalgesia equivalent to that of morphine. In contrast, the semi-synthetic alkaloid, mitragynine pseudoindoxyl, did not demonstrate significant hyperalgesia under the current treatment conditions. Interestingly, mitragynine pseudoindoxyl was previously shown to retain antinociceptive activity with minimal tolerance under a range of chronic administration conditions (Váradi et al., 2016). The development of opioid-induced hyperalgesia is not well understood, but thought to stem from induced neuroplasticity that enhances the release of excitatory neurotransmitters such as substance P and glutamate from primary afferent fibers in the spinal cord (Jhamandas et al., 1996), potentially as part of the physiological adjustment in homeostasis in response to chronic opioid exposure. Consistent with this, prolonged exposure to MOR agonists is shown to lead to sensitization of the nociceptive system (King et al., 2005). Sufka et al. (1991) demonstrated that opioid-induced hyperalgesia occurs primarily via MOR, although the same study indicated that kappa opioid receptor (KOR) antagonism may attenuate these effects at high doses. Notably, these results are supported by present observations, as mitragynine pseudoindoxyl was characterized earlier to possess both MOR agonism and DOR antagonism (Váradi et al., 2016), and hyperalgesic effects were not evident in mice treated with MP under the present conditions. However, while the influence of this pharmacology on the development of opioid-induced hyperalgesia remains a topic for further study, additional testing of MP at varying higher doses for prolonged periods is warranted in future investigations.

Opioid withdrawal symptoms were precipitated with naloxone in the morphine treated groups as compared to the saline-treated group. Similar effects were seen with escalating doses of mitragynine pseudoindoxyl, but minimal effects were seen with the Kratom alkaloids extract and mitragynine. While mitragynine pseudoindoxyl (Váradi et al., 2016), the combined Kratom alkaloid extract, and mitragynine (Kruegel et al., 2019; Uprety et al., submitted) each have been shown to produce MOR agonism, reports suggest alternative

mechanisms of action between them that may contribute to the differences in opioid withdrawal observed presently. Given that mitragynine pseudoindoxyl is a potent MOR agonist (Váradi et al., 2016), it was not surprising that it demonstrated withdrawal symptoms consistent with (if less than) those produced by the morphine-treated mice when administered naloxone. In contrast, significantly reduced withdrawal effects were seen with the mice chronically treated with combined Kratom alkaloid extract or mitragynine alone, despite evidence that these samples produce much of their pharmacological effects via MOR agonism (Gold et al., 1978; Matsumoto et al., 1996b; Boyer et al., 2008; Gowing et al., 2014; Hiranita et al., 2019; Kruegel et al., 2019; Uprety et al., submitted). The minimal withdrawal symptoms demonstrated by mitragynine-treated mice confirm the finding of Meepong and Sooksawate (2019), which reported the absence of withdrawal behaviors after treatment with either acute or chronic low doses. It has been speculated that mitragynine's reduced opioid withdrawal syndrome may be due to intrinsic properties of this alkaloid, as intracellular signaling of mitragynine has been shown to be G-protein biased over β-arrestin (Kruegel et al., 2016; Váradi et al., 2016; Gutridge et al., 2020). Substitution with a G-protein-biased MOR agonist was reported to prevent withdrawal in mice made physically dependent on morphine (Grim et al., 2020), but it is possible that higher doses of the compounds than tested here may result in more activation of the β-arrestin pathway and the emergence of physical dependence, although this remains to be examined.

In morphine-dependent animals, treatment with either the combined Kratom alkaloid extract or mitragynine alone successfully ameliorated naloxone-precipitated withdrawal symptoms, whereas treatment with mitragynine pseudoindoxyl was less efficacious. These results are consistent with recent demonstrations that treatment with lyophilized kratom tea ameliorated naloxone-precipitated withdrawal in morphine-dependent mice (Wilson et al., 2020), whereas treatment with mitragynine attenuated acute withdrawal signs in morphine dependent rats undergoing spontaneous morphine abstinence over a period of 28 days (Hassan et al., 2020). These findings are logical, as similar to buprenorphine, mitragynine is a partial agonist at MOR (Kruegel et al., 2016; Váradi et al., 2016), thought to minimize the physiological changes arising during cessation of morphine treatment. However, both the Kratom natural product and mitragynine alkaloid have been shown to act as weak agonists of the α2-adrenergic system (Boyer et al., 2008, Hiranita et al., 2019; Obeng et al., 2020), whereas mitragynine pseudoindoxyl does not (Váradi et al., 2016). Studies have indicated that α2-adrenoreceptor agonists such as clonidine reduce some (primarily noradrenergic-mediated) withdrawal effects in opioid-dependent subjects (Jasinski, 1985; Katz, 1986; Gowing et al., 2014), a result confirmed presently and that supports an additional possible mechanism by which the Kratom alkaloid extract or mitragynine may ameliorate naloxone-precipitated withdrawal in morphine-dependent subjects. In contrast, mitragynine pseudoindoxyl-mediated antinociception was not attenuated by the α2-adrenoreceptor antagonist yohimbine (Váradi et al., 2016), potentially accounting for its significant physical dependence alone and reduced ability presently to mitigate morphine-induced withdrawal effects. Detailed evaluation in human subject of the Kratom alkaloid extract or mitragynine itself against opioid withdrawal symptoms attributed to non-noradrenergic-mediation, such as disruption of sleep or subjective discomfort would be of potential value in further assessing their utility in the treatment of opioid dependence and withdrawal.

Inpatient treatment with methadone or buprenorphine to ameliorate opioid withdrawal is typically started at higher doses that are tapered to lower doses over 5–7 days (Kleber et al., 2007), but emergent withdrawal symptoms often complicate this transition, sometimes resulting in prolonged use of a maintenance dose (Srivastava et al., 2020). Accordingly, we compared the therapeutic efficacy of a tapering dose regimen in the current study. Of interest, tapering doses of the combined Kratom alkaloid extract or mitragynine over four days were as effective as fixed high doses of each treatment in ameliorating naloxone-precipitated withdrawal in morphine-physically dependent subjects. The application of pharmaceuticals to reduce and wean opioid-dependent subjects has been an ongoing goal of managing clinical pain and physical dependence (Srivastava et al., 2020). The full MOR agonist methadone has been widely used for this purpose for decades (Kreek et al., 2010), but retains a significant risk of misuse and overdose (Kosten et al., 2019), while also potentially requiring a much longer taper than other treatment options (Srivastava et al., 2020). While some animal studies (Pinelli et al., 1997) and human case studies suggest the feasibility of this approach with tapering doses of the serotonin receptor antagonist ondansetron (Wakim, 2012), larger studies reported ondansetron was unsuccessful in either preventing or reducing withdrawal in patients physically dependent on opioid analgesics (Chu et al., 2017 and 2018). Of interest, the α2-adenergic receptor agonist lofexidine was recently FDA-approved for the treatment of opioid withdrawal (Doughty et al., 2019), and although showing efficacy as compared to placebo, failed to completely suppress withdrawal symptoms (Fishman et al., 2019), much as did clonidine in earlier studies (Jasinski et al., 1985). More widely used is the multifunctional, partial MOR agonist buprenorphine, sometimes in tapering doses (Srivastava et al., 2020). While successful in lessening the severity of symptoms from naltrexone-precipitated opioid withdrawal (Umbricht et al., 1999; Srivastava et al., 2020; Wilson et al., 2020) and potentially safer than methadone treatment, it is notable that buprenorphine still carries some risk of respiratory depression (Dahan et al., 2005) and may itself produce physical dependence (Dum et al., 1981) and reinforcement (Canestrelli et al., 2014). The present demonstration that the Kratom alkaloid extract and the alkaloid mitragynine effectively ameliorated morphine withdrawal provides a controlled animal study to add to emerging evidence of therapeutic efficacy (Boyer et al., 2008; Toce et al., 2018, Hassan et al., 2020). Currently, the demonstrated results with mitragynine further expand the findings of Hassan et al (2020), showing that even higher doses of mitragynine produce few opioid related liabilities on its own, while still modulating opioid induced withdrawal in dependent mice. The currently studies also demonstrates mitragynine's ability to modulate opioid withdrawal with decreasing doses, which is lacking currently within the literature. However, broader therapeutic acceptance of these indole alkaloids will require further evaluation for safety and efficacy in randomized controlled trials with defined, pharmaceutical grade products (as used here).

In conclusion, administration of combined Kratom alkaloid extract or mitragynine each demonstrated minimal symptoms of withdrawal alone, suggesting these agents produce less physical dependence than the full MOR agonist morphine. Substitution of the combined Kratom alkaloid extract and mitragynine in morphine-dependent mice was able to ameliorate naloxone precipitated withdrawal symptoms as was mitragynine pseudoindoxyl to a lesser degree. Similar to morphine, mitragynine and the combined Kratom Alkaloid

extract demonstrasted significant hyperalgesic effects that were not produced by chronic treatment with mitragynine pseudoindoxyl. Taken together, this data suggests that while the combined Kratom alkaloid extract and mitragynine have confirmed promise for the treatment of opioid physical dependence and withdrawal, they are not without liabilities. Further evaluation of these extracts is warranted to evaluate their usefulness in the clinic.

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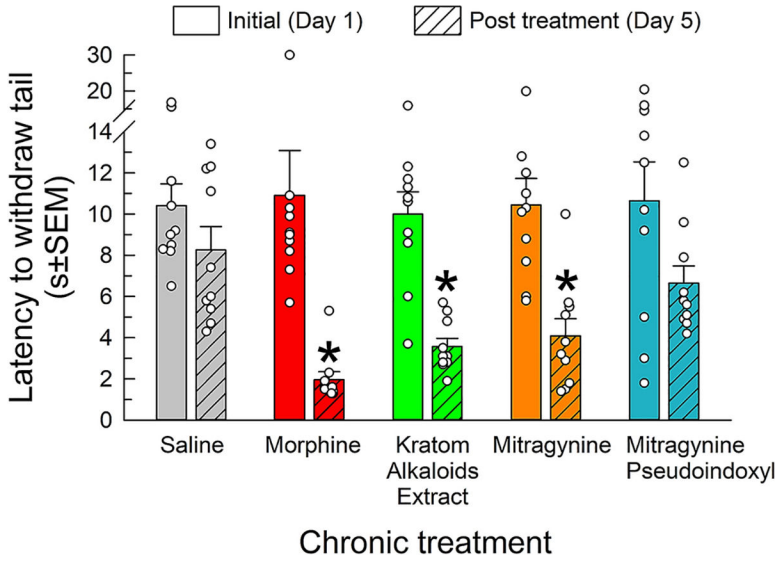

Figure 1. Assessment of treatment-induced hyperalgesia using the 48°C warm-water tail-withdrawal test.

Mice were first tested on day 1 to establish a baseline reading prior to any treatment. Mice were then subjected to a dosing regimen with saline (i.p.), morphine (10–75 mg/kg, i.p.), Kratom alkaloid extract (30–125 mg/kg, p.o.), mitragynine (10–75 mg/kg, p,o.), or mitragynine pseudoindoxyl (1–35 mg/kg, s.c.) for 4 days. On day 5, prior to final dosing and withdrawal testing, mice were evaluated a final time in the warm-water tail withdrawal assay. Data is shown as mean ± SEM of pre and post dosing testing. * $p<0.05$ versus baseline, # $p<0.05$ versus morphine, Two-Way RM ANOVA with Sidak's multiple comparisons post-hoc test. n=10 mice/treatment.

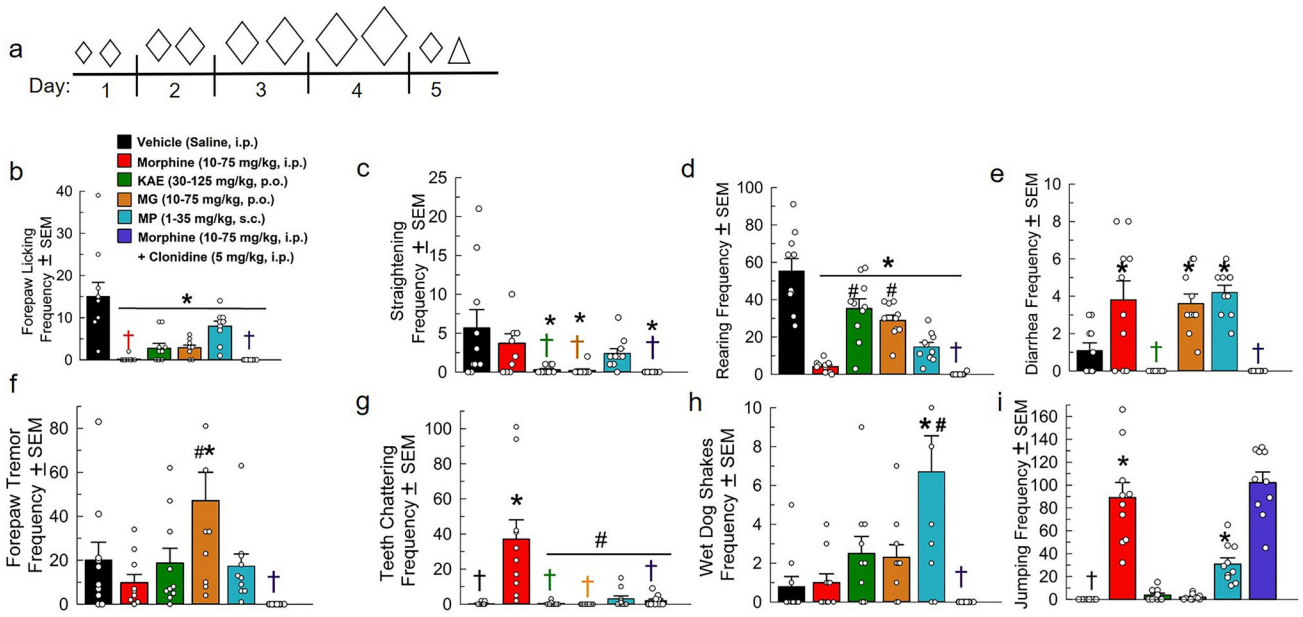

Figure 2. Assessment of direct Kratom alkaloid extract, mitragynine, and mitragynine pseudoindoxyl treatment-induced physical dependence.

(a) Dosing schematic for treatment groups. Increasing diamond (◊) size indicates increased dosage of morphine. The triangle (Δ) indicates naloxone treatment. **Days 1–4**: Treated with (a.m. and p.m.): **Group 1**: (n=10) Saline, i.p. x 2/day. **Group 2**: (n=10), Morphine x2/day. Day 1: 10 + 15 mg/kg, i.p. Day 2: 20 + 30 mg/kg, i.p. Day 3: 50 + 60 mg/kg, i.p. Day 4: 70 + 75 mg/kg, i.p. **Group 3**: (n=10) Kratom alkaloid extract (KAE) x2/day: Day 1: 30 + 35 mg/kg, p.o. Day 2: 45 + 60 mg/kg, p.o. Day 3: 100 + 100 mg/kg, p.o. Day 4: 125 + 125 mg/kg, p.o. **Group 4**: (n=10) Mitragynine (MG) x2/day Day 1: 10 + 15 mg/kg, p.o. Day 2: 20 + 30 mg/kg, p.o. Day 3: 50 + 60 mg/kg, p.o. Day 4: 70 + 75 mg/kg, p.o. **Group 5**: (n= 10) Mitragynine Pseudoindoxyl (MP) x2/day: Day 1: 1 + 3 mg/kg, s.c. Day 2: 3 + 10 mg/kg, s.c. Day 3: 15 + 20 mg/kg, s.c. Day 4: 30 + 35 mg/kg, s.c. **Group 6**: (n=10), Morphine x2/ day. Day 1: 10 + 15 mg/kg, i.p. Day 2: 20 + 30 mg/kg, i.p. Day 3: 50 + 60 mg/kg, i.p. Day 4: 70 + 75 mg/kg, i.p. **Day 5: Group 1**: (n=10) Saline, i.p.; **Group 2**: Morphine (25 mg/kg, i.p.); **Group 3**: KAE (25 mg/kg, p.o.); **Group 4**: MG (25 mg/kg, p.o.); **Group 5**: MP (1 mg/kg, s.c.); **Group 6**: Morphine (25 mg/kg, i.p.). 90 mins post-morphine treatment on day 5 group 6 was additionally administered an acute dose of clonidine (5 mg/kg, i.p.). All groups were administered naloxone (10 mg/kg, s.c.) 120 min post respective day 5 am treatment then observed for withdrawal behaviors (i.e. forepaw licking (**b**), mouse straightening (**c**), rearing (**d**), frequency of diarrhea (**e**), forepaw tremor (**f**), teeth chattering (**g**), jumping (**h**), and wet dog shakes (**i**)) for 15 min. * $p<0.05$ versus vehicle control, # $p<0.05$ versus morphine, One-Way RM ANOVA with Tukey's multiple comparisons post-hoc test. †Mean and SEM lower than 1.

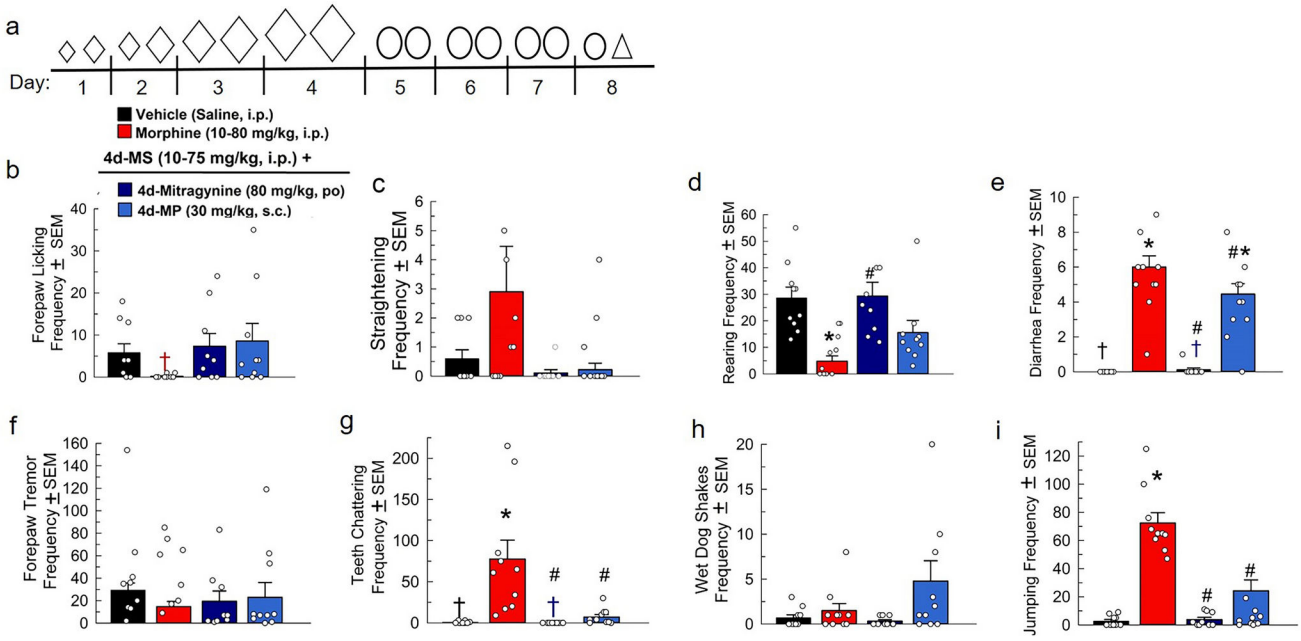

Figure 3. Evaluation of fixed doses of mitragynine's and mitragynine pseduoindoxyl's ability to ameliorate naloxone precipitated opioid withdrawal symptoms in morphine dependent mice.

(a) Dosing schematic for treatment groups. Increasing diamond (◊) size indicates increased dosage of morphine. The circles (○) represent fixed doses of either mitragynine or MP. The triangle (Δ) indicates naloxone treatment. **Days 1–4**: Treated with (a.m. and p.m.): **Group 1**: (n=10) Saline, i.p. x 2/day. **Groups 2–4**: (n=9–10/group), Morphine x2/day. Day 1: 10 + 15 mg/kg, i.p. Day 2: 20 + 30 mg/kg, i.p. Day 3: 50 + 60 mg/kg, i.p. Day 4: 70 + 75 mg/kg, i.p. **Days 5–7:** Treated with (a.m. and p.m.) **Group 1**: Saline, i.p. x 2/day; **Group 2**: Morphine x2/day: 80mg/kg, i.p. **Group 3**: Mitragynine (MG) x2/day: 80mg/kg, p.o. **Group 4**: Mitragynine Pseudoindoxyl (MP) x2/day: 30 mg/kg,s.c. **Day 8**: **Group 1**: (n=10) Saline, i.p.; **Group 2**: Morphine (25 mg/kg, i.p.); **Group 3**: MG (25 mg/kg, p.o.); **Group 4**: MP (3 mg/kg, s.c.). All groups were administered naloxone (10 mg/kg, s.c.) 120 min post respective day 5 am treatment then observed for withdrawal behaviors (i.e. forepaw licking (**b**), mouse straightening (**c**), rearing (**d**), frequency of diarrhea (**e**), forepaw tremor (**f**), teeth chattering (**g**), jumping (**h**), and wet dog shakes (**i**))120 min post respective day 5 am treatment then observed for withdrawal behaviors (i.e. forepaw licking (**b**), mouse straightening (**c**), rearing (**d**), frequency of diarrhea (**e**), forepaw tremor (**f**), teeth chattering (**g**), jumping (**h**), and wet dog shakes (**i**)) for 15 min. * $p<0.05$ versus vehicle control, # $p<0.05$ versus morphine, One-Way RM ANOVA with Tukey's multiple comparisons post-hoc test. †Mean and SEM lower than 1.

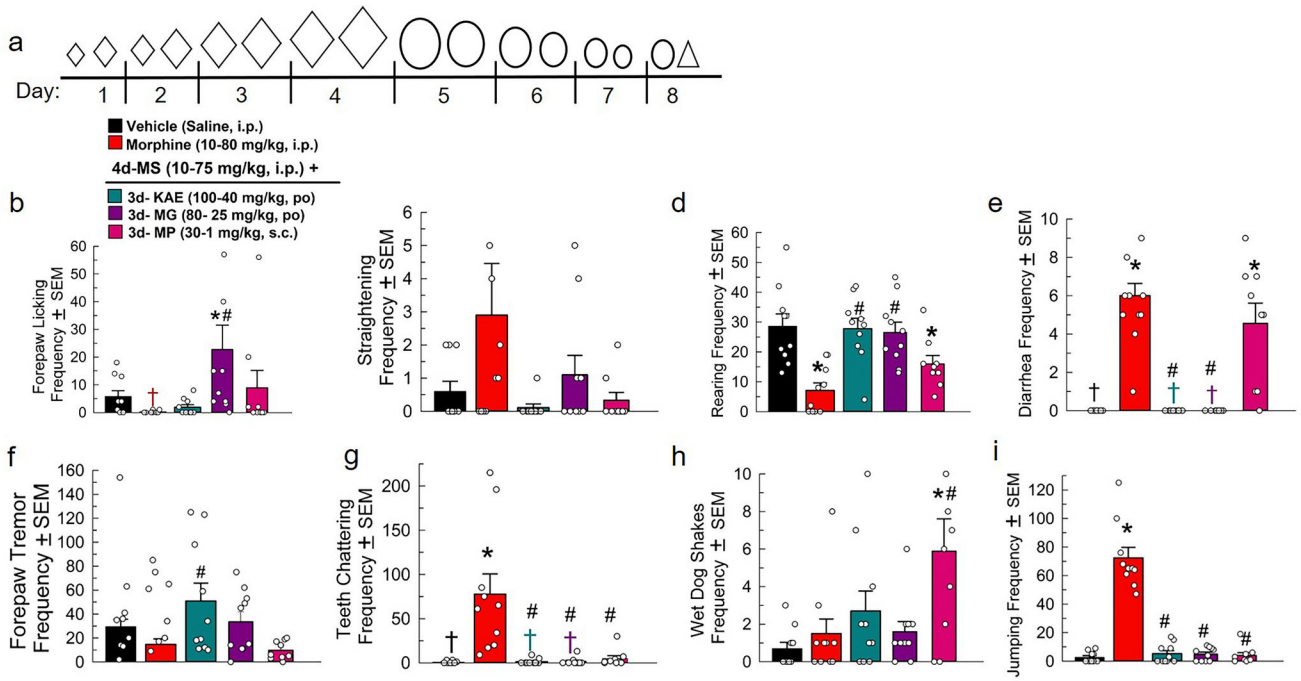

Figure 4. Evaluation of tapering doses of Kratom alkaloid extract, mitragyine, and mitragynine pseudoindoxyl's ability to reduce naloxone precipitated opioid withdrawal symptoms in morphine dependent mice.

(a) Dosing schematic for treatment groups. Increasing diamond (◊) size indicates increased dosage of morphine. The decreasing size of the circles (○) represent tapering doses of either kratom alkaloid extract, mitragynine, or MP. The triangle (Δ) indicates naloxone treatment. **Days 1–4**: Treated with (a.m. and p.m.): **Group 1**: (n=10) Saline, i.p. x 2/day. **Groups 2–5**: (n=9–10/group), Morphine x2/day. Day 1: 10 + 15 mg/kg, i.p. Day 2: 20 + 30 mg/kg, i.p. Day 3: 50 + 60 mg/kg, i.p. Day 4: 70 + 75 mg/kg, i.p. **Days 5–7:** Treated with (a.m. and p.m.) **Group 1**: Saline, i.p. x 2/day; **Group 2**: Morphine x2/day: 80mg/kg, i.p. **Group 3**: Kratom alkaloid extract (KAE) x2/day: Day 5: 100 mg/kg, p.o. Day 6: 80 + 70 mg/kg, p.o. Day 7: 60 + 50 mg/kg, p.o. **Group 4**: Mitragynine (MG) x2/day: Day 5: 80 mg/kg, p.o. Day 6: 70 + 60 mg/kg, p.o. Day 7: 50 + 40 mg/kg, p.o. **Group 5**: Mitragynine Pseudoindoxyl (MP) x2/day: Day 5: 30 + 20 mg/kg, s.c. Day 6: 15 + 10 mg/kg, s.c. Day 7: 3 + 1 mg/kg, s.c. **Day 8**: **Group 1**: (n=10) Saline, i.p.; **Group 2**: Morphine (25 mg/kg, i.p.); Group 3: KAE (40 mg/kg, p.o.); **Group 4**: MG (25 mg/kg, p.o.); **Group 5**: MP (1 mg/kg, s.c.). All groups were administered naloxone (10 mg/kg, i.p.) 120 min post respective day 5 am treatment then observed for withdrawal behaviors (i.e. forepaw licking (**b**), mouse straightening (**c**), rearing (**d**), frequency of diarrhea (**e**), forepaw tremor (**f**), teeth chattering (**g**), jumping (**h**), and wet dog shakes (**i**))120 min post respective day 5 am treatment then observed for withdrawal behaviors (i.e. forepaw licking (**b**), mouse straightening (**c**), rearing (**d**), frequency of diarrhea (**e**), forepaw tremor (**f**), teeth chattering (**g**), jumping (**h**), and wet dog shakes (**i**))* $p<0.05$ versus vehicle control, # $p<0.05$ versus morphine, One-Way RM ANOVA with Tukey's multiple comparisons post-hoc test. n=9–10 mice/treatment. †Mean and SEM lower than 1.

Table 1

Design of Experiment 1: Dosing over five days to assess potential physical dependence.

	Treatment					
Treatment Day	**Saline (i.p.)**	**Morphine (10– 75 mg/kg, i.p.)**	**Morphine (10–75 mg/kg, i.p.) + Clonidine (5 mg/kg, i.p.)**	**Kratom Alkaloid Extract (KAE) (30–125 mg/kg, p.o.)**	**Mitragynine (MG) (10–75 mg/kg, p.o.)**	**Mitragynine Pseudoindoxyl (MP) (1–35 mg/kg, s.c.)**
Day 1 am	+	10 mg/kg	10 mg/kg	30 mg/kg KAE	10 mg/kg MG	1 mg/kg MP
pm	+	15 mg/kg	15 mg/kg	35 mg/kg KAE	15 mg/kg MG	3 mg/kg MP
Day 2 am	+	20 mg/kg	20 mg/kg	45 mg/kg KAE	20 mg/kg MG	3 mg/kg MP
pm	+	30 mg/kg	30 mg/kg	60 mg/kg KAE	30 mg/kg MG	10 mg/kg MP
Day 3 am	+	50 mg/kg	50 mg/kg	100 mg/kg KAE	50 mg/kg MG	15 mg/kg MP
pm	+	60 mg/kg	60 mg/kg	100 mg/kg KAE	60 mg/kg MG	20 mg/kg MP
Day 4 am	+	70 mg/kg	70 mg/kg	125 mg/kg KAE	70 mg/kg MG	30 mg/kg MP
pm	+	75 mg/kg	75 mg/kg	125 mg/kg KAE	75 mg/kg MG	35 mg/kg MP
Day 5 am	+	25 mg/kg	25 mg/kg morphine + 5 mg/kg Clonidine	25 mg/kg KAE	25 mg/kg MG	1 mg/kg MP
pm	–	–	–	–	–	–

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Methods Table 2

Design of Experiment 2: Fixed or tapering dosing to test alkaloid potential to ameliorate precipitated opioid withdrawal.

	Treatment						
Treatment Day	**Saline (i.p.)**	**Morphine (i.p.)**	**Morphine (d 1–4), then Tapering Doses of Kratom Alkaloid Extract (KAE) (100–40 mg/kg, p.o.)**	**Morphine (d 1–4), then Fixed Mitragynine (MG) (80 mg/kg, p.o.)**	**Morphine (d 1–4), then Tapering Mitragynine (MG) (80–25 mg/kg, p.o.)**	**Morphine (d 1–4), then Fixed MP (30 mg/kg, s.c.)**	**Morphine (d 1–4), then Tapering MP (30–1 mg/kg, s.c.)**
Day 1 am	+	*10 mg/kg*	*10 mg/kg, i.p.*	*10 mg/kg, i.p.*	*10 mg/kg, i.p.*	*10 mg/kg, i.p.*	*10 mg/kg, i.p.*
pm	+	*15 mg/kg*	*15 mg/kg, i.p.*	*15 mg/kg, i.p.*	*15 mg/kg, i.p.*	*15 mg/kg, i.p.*	*15 mg/kg, i.p.*
Day 2 am	+	*20 mg/kg*	*20 mg/kg, i.p.*	*20 mg/kg, i.p.*	*20 mg/kg, i.p.*	*20 mg/kg, i.p.*	*20 mg/kg, i.p.*
pm	+	*30 mg/kg*	*30 mg/kg, i.p.*	*30 mg/kg, i.p.*	*30 mg/kg, i.p.*	*30 mg/kg, i.p.*	*30 mg/kg, i.p.*
Day 3 am	+	*50 mg/kg*	*50 mg/kg, i.p.*	*50 mg/kg, i.p.*	*50 mg/kg, i.p.*	*50 mg/kg, i.p.*	*50 mg/kg, i.p.*
pm	+	*60 mg/kg*	*60 mg/kg, i.p.*	*60 mg/kg, i.p.*	*60 mg/kg, i.p.*	*60 mg/kg, i.p.*	*60 mg/kg, i.p.*
Day 4 am	+	*70 mg/kg*	*70 mg/kg, i.p.*	*70 mg/kg, i.p.*	*70 mg/kg, i.p.*	*70 mg/kg, i.p.*	*70 mg/kg, i.p.*
pm	+	*75 mg/kg*	*75 mg/kg, i.p.*	*75 mg/kg, i.p.*	*75 mg/kg, i.p.*	*75 mg/kg, i.p.*	*75 mg/kg, i.p.*
Day 5 am	+	*80 mg/kg*	100 mg/kg KAE	80 mg/kg MG	80 mg/kg MG	30 mg/kg MP	30 mg/kg MP
pm	+	*80 mg/kg*	100 mg/kg KAE	80 mg/kg MG	80 mg/kg MG	30 mg/kg MP	20 mg/kg MP
Day 6 am	+	*80 mg/kg*	80 mg/kg KAE	80 mg/kg MG	70 mg/kg MG	30 mg/kg MP	15 mg/kg MP
pm	+	*80 mg/kg*	70 mg/kg KAE	80 mg/kg MG	60 mg/kg MG	30 mg/kg MP	10 mg/kg MP
Day 7 am	+	*80 mg/kg*	60 mg/kg KAE	80 mg/kg MG	50 mg/kg MG	30 mg/kg MP	3 mg/kg MP
pm	+	*80 mg/kg*	50 mg/kg KAE	80 mg/kg MG	40 mg/kg MG	30 mg/kg MP	1 mg/kg MP
Day 8 am	+	*25 mg/kg*	40 mg/kg KAE	25 mg/kg MG	25 mg/kg MG	3 mg/kg MP	1 mg/kg MP
pm	–	–	–	–	–	–	–

*Italic text indicates morphine dosing regimen