

## Antinociceptive profile of salvinorin A, a structurally unique kappa opioid receptor agonist

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### Abstract

Salvinorin A, is a structurally unique, non-nitrogenous, kappa opioid receptor (KOP) agonist. Given the role of KOPs in analgesic processes, we set out to determine whether salvinorin A has antinociceptive activity in thermal and chemo-nociceptive assays. The tail-flick assay was employed to investigate 1) salvinorin A's (0.5, 1.0, 2.0, and 4.0 mg/kg) dose–response and time-course (10, 20, and 30 min) effects in a thermal nociceptive assay, and 2) the ability for the KOP antagonist norBNI (10.0 mg/kg) to prevent salvinorin A antinociception. The hotplate assay was utilized as a second thermal nociceptive measure to test salvinorin A's dose–response effects. The acetic acid abdominal constriction assay was used to study salvinorin A's dose–response and time-course (over 30 min) effects in a chemo-nociceptive assay. Together, these studies revealed that salvinorin A produces a dose-dependent antinociception that peaked at 10 min post-injection but rapidly returned to baseline. Additionally, pretreatment with the KOP antagonist norbinaltorphimine (norBNI) reversed salvinorin A-induced antinociception. These findings demonstrate that salvinorin A produces a KOP mediated antinociceptive effect with a short duration of action.

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**Keywords:** Salvinorin A; Kappa opioid receptor agonist; Tail-flick; Hotplate; Acetic acid writhing; Mouse; Antinociception

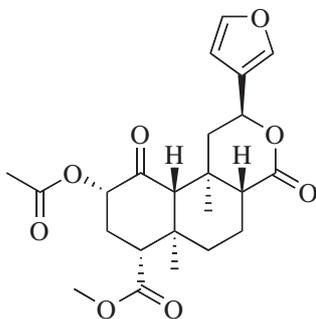
### 1. Introduction

Salvinorin A (Fig. 1) is a potent and highly selective kappa opioid receptor (KOP) agonist as demonstrated in in vitro assays (Roth et al., 2002; Chavkin et al., 2004). A compound lacking nitrogen with such high selectivity for KOP and virtually no affinity for many other psychoactive drug targets (Roth et al., 2002) has not previously been reported. *Salvia divinorum*, the plant from which salvinorin A is isolated, has been used in ritualistic and spiritual practices of the Mazateca curanderos (healers) for many years for its ability to produce lucid and inward thought (Ortega et al., 1982). Interestingly, in smaller doses, when administered orally, *Salvia divinorum* has

been utilized by the curanderos to treat various ailments such as providing relief from headaches, rheumatism, and gastrointestinal movement disorders (Valdes et al., 1983). These uses are not surprising given the well-documented role KOPs play in spinal-mediated pain processing, the GI tract, as well as the bladder. Throughout its use, salvinorin A or more specifically, extracts from the plant, have not shown any addictive potential (Valdes et al., 1983; Zhang et al., 2005) and therefore, could serve as a template for non-addictive opioid analgesics if one could delineate the analgesic effects from the psychotropic effects. Salvinorin A has also been shown to produce kappa opioid agonist-like discriminative effects in rhesus monkeys (Butelman et al., 2004), slight aversive properties in mice (Zhang et al., 2005), sedative effects in mice (Fantegrossi et al., 2005) and antidepressant effects in one human case report (Hanes, 2001). Collectively, these studies suggest a range of effects quite similar to known kappa opioid agonists. However, reports of analgesia from salvinorin A have been limited. In

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**SALVINORIN A**

Fig. 1. Structure of salvininorin A.

fact, one report demonstrated that salvininorin A, even at high doses, lacked antinociceptive effects in the abdominal constriction test (Wang et al., 2005). While we were preparing this manuscript, a study by Harding et al. (2005) was published indicating salvininorin A indeed demonstrated analgesic effects in two different nociceptive assays. Although details were not given on time-course or doses used ED<sub>50</sub> values were presented.

Traditional folk literature reports suggest the use of leaf material from *Salvia divinorum* for analgesic purposes (Valdes et al., 1983). It is reasonable to predict salvininorin A would be the analgesic component of the leaf material, given the well-documented role kappa opioid receptors play in spinal-mediated pain processing (Pasternak, 1993). The purpose of the present research is to characterize the antinociceptive properties of purified salvininorin A in thermal and chemonociceptive assays, establishment of a peak effect time for salvininorin A for antinociceptive activity and a reversal study with norBNI demonstrating KOP selectivity in vivo.

## 2. Materials and methods

### 2.1. Subjects

For all experiments, male Swiss mice (23–30 g, Harlan, Indianapolis, IN, USA) were group housed at a population density of  $n=2-3$  in polycarbonate cages (20 × 35 × 12 cm). Food (Purina 5001 Laboratory Rodent Chow, St. Louis, MO, USA) and water were available ad libitum. Room temperature was maintained at 22 ± 1 °C and overhead fluorescent illumination was maintained on a 12-h light–dark cycle.

### 2.2. Drugs

Salvinorin A was obtained by reported extraction and purification methods (Munro and Rizzacasa, 2003) from *Salvia divinorum* leaves harvested from plants (Theatrum Botanicum, Laytonville, CA, USA) propagated at the University of Mississippi. Purified, crystalline salvininorin A agreed with published characterization data (Ortega et al., 1982). Salvininorin A was dissolved in a vehicle consisting of 10% DMSO and 90% propylene glycol. This vehicle was utilized based on

previous work with other lactones in our laboratory that were freely soluble in propylene glycol. Salvininorin A was not soluble in 100% propylene glycol to our surprise. However, solubility increased in propylene glycol by adding increasing concentrations of DMSO. A 9:1 mixture ended up being suitable for our studies and did not interfere with analgesia on its own. For the kappa antagonist challenge study, norBNI (Tocris, Ellisville, MO) was dissolved in 0.9% physiological saline. All drugs were delivered IP.

### 2.3. Tail-flick studies

The tail-flick test was used to characterize 1) the dose–response and time-course effects of salvininorin A on thermal nociception and 2) the KOR antagonist effects of norBNI on salvininorin A antinociception. Thermal nociception was quantified using a tail-flick apparatus that integrated both a thermal nociceptive stimulus and an automated response timer (Columbus Instruments, Model #0104-300M; intensity setting 4). For tests, mice were lightly restrained in a soft cloth with their tail positioned in a groove above an aperture that presented the onset of the thermal stimulus with the start of the timer. The average of two trials, taken 20 to 30 s apart, served as the dependent measure for each subject. A cut-off score of 10 s was utilized to minimize the risk of tissue damage. Baseline measures were taken 10 min prior to administration of drug probes; no statistical differences were detected in this measure across assigned groups and these data are not presented herein.

In the dose–response/time-course experiment, mice received vehicle or 0.5, 1.0, 2.0, or 4.0 mg/kg salvininorin A and tested 10, 20 and 30 min post-injection. In the norBNI challenge experiment, mice were given 10 mg/kg norBNI 1 h before administration of 2.0 mg/kg salvininorin A; tail-flick tests were conducted 10 min after the salvininorin A injections. These dose selections and injection-to-test intervals were selected from pilot studies and from previously published reports (Endoh et al., 1992). Sample sizes were  $n=8-10$ .

### 2.4. Hotplate study

The hotplate was used to characterize the dose–response effects of salvininorin A on thermal nociception. Although this assay is generally considered to be less sensitive to KOP analgesics, it does assess involvement of both spinal and supraspinal nociceptive processing. Prior to testing, mice received one apparatus habituation trial (2 min) in which they were placed inside the acrylic enclosure with the hotplate (Harvard, Model #52-8570) surface temperature maintained at 40 °C. Nociceptive tests (hotplate temperature=52 °C) were conducted 10 min after injections of vehicle or 0.5, 1.0 or 2.0 mg/kg salvininorin A (higher doses were found to produce motor side effects that confounded the hotplate response measure). A timer was manually started when all four paws made contact with the apparatus floor. Latency to flutter or lick a hindpaw or perform an escape response (i.e., jumping or scurrying) served as the nociceptive measure (45 s cut-off score). Sample sizes were  $n=8-10$  for all groups.

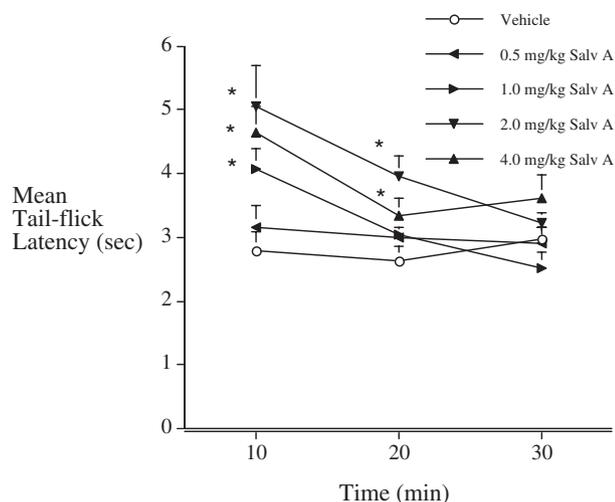


Fig. 2. Dose–response and time-course effects of salvinorin A (0.5, 1.0, 2.0, and 4.0 mg/kg) on tail-flick response latency. Sample sizes=8–9 per group. \*Indicates significant antinociception ( $p < .05$ ).

### 2.5. Acetic acid abdominal constriction study

For the abdominal constriction assay, mice received 25 min of apparatus habituation in a 15 cm diameter acrylic observation enclosure. Salvinorin A (vehicle, 0.5, 1.0 and 2.0 mg/kg) was delivered 5 min before an IP injection of 0.9% acetic acid (1 ml/0.1 kg). The number of writhing responses over the course of a 30 min observation period (in six 5 min blocks) served as the dependent measure. In this assay, it is typical for 10–20% of vehicle treated animals to be non-responders (Mogil et al., 2001). In the present study, exactly 2 of 12 subjects from each group (i.e., 17%) did not respond to acetic acid injections and were omitted prior to data analyses.

### 2.6. Statistical analyses

For each experiment, data were examined for homogeneity of variance. Subject scores greater than two standard deviations from the mean were omitted from analyses. Data were then analyzed using analysis of variance (ANOVA). Post hoc analyses were performed using Fisher's LSD test.

These procedures were conducted under the ethical standards of the International Association for the Study of Pain and in Accordance with the principles of laboratory animal care as detailed in the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and were approved by the University of Mississippi IACUC (Protocol # 04-027).

## 3. Results

### 3.1. Tail-flick studies

The dose–response and time-course effects of salvinorin A on thermal nociception are summarized in Fig. 1. Mice receiving salvinorin A exhibited a dose- and time-dependent antinociceptive effect that was evidenced by increased response

latencies at 10–15 min following drug administration. Thereafter, thermal nociceptive measures displayed a rapid return to baseline levels. A two-way ANOVA of these data revealed a significant main effect for Dose [ $F(4,117)=4.76$ ,  $p < .005$ ], a significant main effect for Time [ $F(3,117)=12.52$ ,  $p < .0001$ ], and a significant Dose  $\times$  Time interaction [ $F(12, 117)=2.59$ ,  $p < .005$ ]. Simple effects analyses at each time point revealed significant ANOVAs at the 10 min [ $F(4,39)=4.77$ ,  $p < .005$ ] and 20 min [ $F(4,39)=4.24$ ,  $p < .01$ ] test intervals. Post hoc analyses at the 10 min test interval demonstrated significant increases in tail-flick latencies in groups receiving 1.0 ( $p = .05$ ), 2.0 ( $p < .001$ ), and 4.0 ( $p < .01$ ) mg/kg salvinorin A. Post hoc analyses at the 20 min test interval demonstrated significant increases in tail-flick latencies in groups receiving 2.0 ( $p < .001$ ) and 4.0 ( $p < .05$ ) mg/kg salvinorin A. All other relevant comparisons failed to reach statistical significance.

The effects of the norBNI challenge on salvinorin A antinociception are summarized in Fig. 2. Salvinorin A produced a robust antinociceptive effect that was reversed by pretreatment with the kappa opioid receptor antagonist norBNI. A two-way ANOVA of baseline tail-flick latencies did not reveal any significant treatment effects. However, a two-way ANOVA of tail-flick latencies following administration of drug probes revealed a significant main effect for salvinorin A [ $F(1,32)=5.17$ ,  $p < .05$ ], a marginally significant main effect for norBNI [ $F(1,32)=3.52$ ,  $p = .07$ ], and a significant salvinorin A  $\times$  norBNI interaction term [ $F(1,32)=4.27$ ,  $p < .005$ ]. Post hoc analyses demonstrated that mice receiving saline/salvinorin A had significantly longer tail-flick latencies than mice receiving saline/vehicle ( $p = .01$ ). Furthermore, mice receiving norBNI/salvinorin A had significantly lower tail-flick latencies than saline/salvinorin A ( $p < .01$ ) (Fig. 3).

### 3.2. Hotplate studies

The dose–response effects of salvinorin A on a second measure of thermal nociception are summarized in Fig. 4. Mice

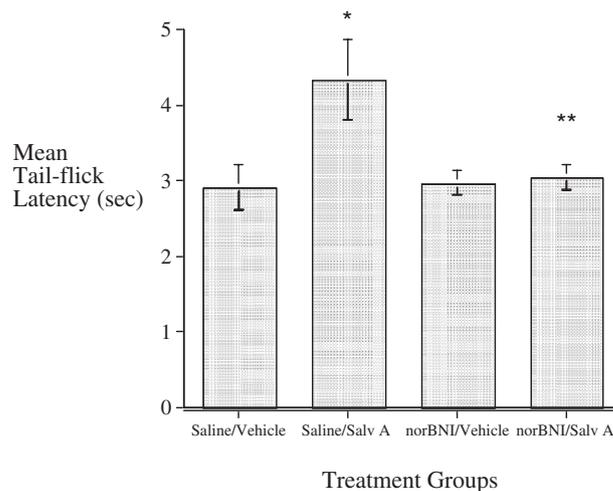


Fig. 3. Effects of physiological saline or norBNI (10.0 mg/kg) pretreatment and vehicle or salvinorin A (2.0 mg/kg) on tail-flick response latency. Sample sizes=9–10 per group. \*Indicates significant antinociception ( $p < .01$ ). \*\* Indicates a significant attenuation of thermal antinociception. ( $p < .05$ ).

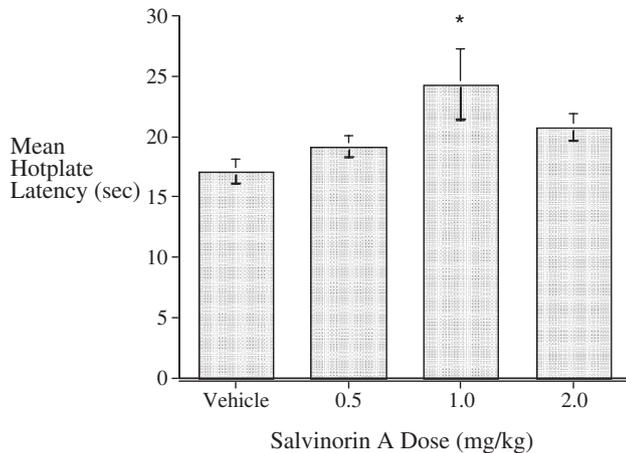


Fig. 4. Dose–response effects of salvinorin A (0.5, 1.0 and 2.0 mg/kg) on hotplate response latency. Sample sizes=8–10 per group. \*Indicates significant antinociception ( $p < .01$ ).

receiving 1.0 mg/kg salvinorin A exhibited an antinociceptive effect that was evidenced by increased response latencies. A one-way ANOVA of these data revealed a marginally significant main effect for drug dose [ $F(3, 34) = 2.69$ ,  $p = .06$ ]. Post hoc analyses demonstrated that mice receiving the 1.0 mg/kg doses of salvinorin A had significantly longer hotplate latencies than vehicle treated mice,  $p < .01$ .

### 3.3. Acetic acid abdominal constriction study

The dose–response and time-course effects of salvinorin A on the acetic acid abdominal constriction assay are summarized in Fig. 5. In vehicle treated mice, acetic acid produced writhing responses that peaked in the 10 min block, declined by approximately 25% in the 15 min block and remained stable throughout the remainder of the test session, a nociceptive pattern consistent with earlier studies in mice (Mogil et al., 2001). Salvinorin A exhibited a robust dose-dependent antinociceptive effect, as evidenced by a decrease in number of writhes, at the initial portions of the test session. Consistent with these observations, a two-way ANOVA revealed a significant main effect for Dose [ $F(3, 170) = 3.60$ ,  $p < .05$ ], a significant main effect for Time [ $F(5, 170) = 22.56$ ,  $p < .0001$ ] and a significant Dose  $\times$  Time interaction term [ $F(15, 170) = 3.80$ ,  $p < .0001$ ]. Simple effects analyses for Dose were conducted at each time block with post hoc analyses revealing that 1) the 1.0 and 2.0 mg/kg salvinorin A doses significantly reduced writhes at the 5 min block and 2) all three dose of salvinorin A significantly reduced writhes at the 10 and 15 min blocks (all  $p^s < .05$ ).

## 4. Discussion

KOP agonists have been of therapeutic interest in the treatment of pain (Delvaux, 2001; Binder et al., 2001), drug addiction (Nagase et al., 1999; Schlechtingen and Goodman, 1999), eating disorders (Leighton et al., 1988; Marrazzi et al., 1990; Gulati et al., 1991; Bodnar, 1998), depression (Ukai et al., 2002; Hanes, 2001) and even human immunodeficiency

virus (HIV) infections (Lokensgard et al., 2002; Smith and Darlington, 2000).

Salvinorin A is an interesting KOP agonist as it does not agree with any of the currently accepted pharmacophores of KOP ligands, or opioid pharmacophores in general, and demonstrates a new structural class of KOP ligand. Salvinorin A is a fast and short acting potent KOP analgesic when delivered by systemic administration. The short duration of action seen in mice is consistent with the recent report of Schmidt et al. (2005) where salvinorin A was demonstrated to have a rapid half-life in non-human primates. Salvinorin A produced significant and short acting antinociceptive effects in the murine tail-flick, hotplate, and abdominal constriction assays with peak effects being demonstrated at the 10-min time point in each assay (not all time courses shown). A previous report indicated that salvinorin A produced no effect in the abdominal constriction assay (Wang et al., 2005). In that study, salvinorin A was administered 20 min before acetic acid challenge. Our data clearly shows the analgesic effect of salvinorin A is no longer present 20 min after administration. Although salvinorin A has a short duration of action, the antinociceptive activity is consistent with other known KOP agonists and adds further validity to the recent report of salvinorin A analgesia by Harding et al. (2005).

Furthermore, the action of the KOP selective antagonist norBNI, fully reversed salvinorin A antinociception. While salvinorin A has been reported to be a potent and selective KOP agonist in vitro, other studies in primates have suggested that KOP selective antagonists are unable to fully antagonize the effects of salvinorin A (Butelman et al., 2004). In our study with earlier administration times (30 min) of norBNI we were unable to fully reverse the antinociceptive effects of salvinorin A. It has been documented previously that norBNI has an inherent agonist effect at early time points of administration but has long lasting selective kappa opioid receptor antagonism after at least 1 h post-administration (Endoh et al., 1992).

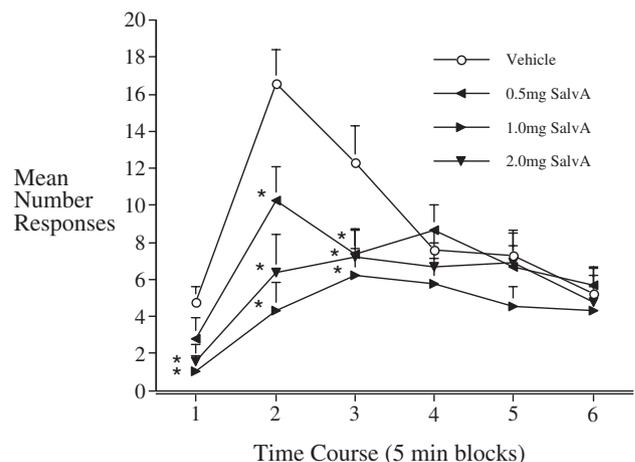


Fig. 5. Dose–response and time-course effects of salvinorin A (0.5, 1.0 and 2.0 mg/kg) on the acetic acid abdominal constriction assay. Sample sizes=8–9 per group. \*Indicates significant antinociception within a given time block ( $p < .05$ ).

The utility of centrally acting kappa opioid receptor agonists as analgesics has met with limited success due to the psychoactive effects produced. Indeed, the antinociceptive effects reported here are in a similar dosage range that has been reported for hallucinogenic activity in humans (Siebert, 1994), indicating the potential for salvinorin A, itself, to serve as an analgesic are limited. Moreover, the short duration of action makes salvinorin A even less attractive as a clinically useful agent and may even limit potential use as a pharmacological probe for in vivo study of the KOP system. However, structurally related analogs may have longer lasting analgesic properties, devoid of psychotropic activity (ie peripherally restricted), and should be investigated for potential therapeutic utility in the treatment of pain or other medical conditions. In this regard, the recent discovery of a mu opioid receptor (MOP) agonist derived from the structural scaffold of salvinorin A (Harding et al., 2005), indicate this template can be manipulated to produce novel opioid receptor ligands with the potential to treat pain and drug addictions.

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