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TITLE PAGE

N-methylacetamide analogue of salvinorin A: a highly potent and selective kappa opioid receptor agonist with oral efficacy

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Salvinorin A analogues: selectivity, metabolism, and potency

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d.) non-standard abbreviations:

3FLB: diethyl-2,4-di-[3-fluorophenyl]-3,7-dimethyl-3,7-diazabicyclo[3.3.1]nonane-9-one-1,5-dicarboxylate

DADLE: Tyr-D-Ala-Gly-Phe-D-Leu

DAMGO: Tyr-D-Ala-Gly-*N*-methyl-Phe-Gly-ol

DMSO: dimethylsulfoxide

EE: salvinorin B ethyl ether

FST: forced swim test

[^{35}S]GTPγS: guanosine 5'-*O*-(3-[^{35}S]thio)triphosphate

HPLC: high-performance liquid chromatography

ICSS: intracranial self-stimulation

IPA: *N*-(2-propyl)-2-amido salvinorin B (using IUPAC functional replacement nomenclature)

MPA: mobile phase A

MPB: mobile phase B

NADPH: nicotinamide adenine dinucleotide phosphate

NMA: *N*-acetyl-*N*-methyl-2-amido salvinorin B (using IUPAC functional replacement nomenclature)

NMP: *N*-propionyl-*N*-methyl-2-amido salvinorin B (using IUPAC functional replacement nomenclature)

NMR: nuclear magnetic resonance

SalvA: salvinorin A

SAR: structure-activity relationship

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TRK820: 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl) acrylamido]morphinan hydrochloride

U50,488: (1*S*,2*S*)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide hydrochloride

U69,593: (+)-(5*R*,7*S*,8*S*)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzenacetamide

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BEHAVIORAL PHARMACOLOGY

ABSTRACT

Several preclinical studies indicate that selective kappa opioid receptor (KOR) antagonists have antidepressant-like effects whereas KOR agonists have opposite effects, suggesting that each might be useful in the treatment of mood abnormalities. Salvinorin A (salvA) is a valuable KOR agonist for further study due to its high potency and receptor selectivity. However, it has short lasting effects in vivo and limited oral bioavailability, likely due to acetate metabolism. We compared the in vitro receptor binding selectivity of salvA and four analogues containing an ethyl ether (EE), isopropylamine (IPA), *N*-methylacetamide (NMA), or *N*-methylpropionamide (NMP) at C-2. All compounds showed high binding affinity for the KOR (K_i = 0.11–6.3 nM), although only salvA, EE, and NMA exhibited KOR selectivity. In a liver microsomal assay, salvA was least stable, whereas NMA and IPA displayed slower metabolic transformations. Intraperitoneal (IP) administration of salvA, NMA, and NMP dose-dependently elevated brain reward thresholds in the intracranial self-administration (ICSS) test, consistent with prodepressive-like KOR agonist effects. NMA and NMP were equipotent to salvA but displayed longer lasting effects (6- and 10-fold, respectively). A dose of salvA with prominent effects in the ICSS test after IP administration (2.0 mg/kg) was inactive after oral administration, whereas the same oral dose of NMA elevated ICSS thresholds. These studies suggest that although salvA and NMA are similar in potency and selectivity as KOR agonists in vitro, NMA has improved stability and longer lasting actions that might make it more useful for studies of KOR agonist effects in animals and humans.

INTRODUCTION

Brain kappa opioid receptors (KORs) have been implicated in many functions including regulation of mood (Mague et al., 2003; Todtenkopf et al., 2004; Cohen and Murphy, 2007), responses to drugs of abuse (Shippenberg and Rea, 1997), sense of space and time (Dortch-Carnes and Potter, 2005; Pfeiffer et al., 1986; Walsh et al., 2001), and pain perception (Barber and Gottschlich, 1997; Dortch-Carnes and Potter, 2005). Selective KOR ligands may, therefore, enable a more detailed understanding of the molecular mechanisms that regulate complex brain function and possess the potential for clinical applications.

A small number of synthetic KOR agonists with high selectivity for the KOR have been developed. The most specific of these, U50,488 and U69,593 and their congeners spiradoline and enadoline, have limited efficacy when administered orally (Endoh et al., 1999). Salvinorin A (salvA) is a natural substance that is the major component of *Salvia divinorum*, a plant used in shamanistic rituals in Mexico. Recent work has established that salvA is a potent and highly selective KOR agonist (Roth et al., 2002; Butelman et al., 2004; Vortherms and Roth, 2006). SalvA appears to bind to KOR via both conserved and non-conserved amino acid residues (Yan et al., 2005; Vortherms et al., 2007), contributing to its extraordinary selectivity. In vitro, salvA has properties that are unique among KOR agonists. For example, in a specific assay designed to monitor the KOR-mediated activation of potassium (Kir3 channel) currents, salvA was more efficacious than U50,488, U69,593, or dynorphin A, the endogenous peptide neurotransmitter for the KOR (Chavkin et al., 2004). SalvA also caused less human KOR (hKOR) internalization

and down-regulation than TRK-820 or 3FLB (Wang et al., 2005). It is not known if these properties are maintained in vivo or whether they contribute to unique behavioral and cognitive effects of salvA.

Most in vivo studies of salvA have focused on its effects on pain (Ansonoff et al., 2006; John et al., 2006; McCurdy et al., 2006; Wang et al., 2005) and behaviors that reflect motivation (Carlezon et al., 2006; Zhang et al., 2005). One recent report suggested that salvA might reduce cocaine self-administration (Prisinzano et al., 2005), whereas another indicated that salvA induced rewarding effects at low doses and aversive effects at high doses in place conditioning tests involving zebra fish (Braida et al., 2007). SalvA has dose-dependent analgesic effects in the mouse tail flick, hot plate, and acetic acid abdominal constriction tests after intraperitonal (IP), intracerebroventricular, or intrathecal administration (Ansonoff et al., 2006; John et al., 2006; McCurdy et al., 2006). The time course of salvA effects was consistently brief across these analgesia studies: it produced maximum antinociceptive effects 10–15 min post-administration, and these effects had waned by 30–60 min. The behavioral and neurochemical effects of the drug appear somewhat more sustained in other tests, however (Carlezon et al., 2006; Zhang et al., 2005).

As part of an effort to identify novel treatments for affective disorders, we have characterized the effects of selective KOR ligands in animal models often used to study mood (Mague et al., 2003; Todtenkopf et al., 2004; Carlezon et al., 2006). SalvA increased immobility behavior in the forced swim test (FST) and elevated intracranial

self-stimulation (ICSS) thresholds (Carlezon et al., 2006), putative indicators of reduced motivation and depressive-like effects. These observations raised the possibility that selective KOR agonists might relieve symptoms of mania, a disorder characterized by dysregulated motivation.

While these preclinical studies suggested potential therapeutic applications for salvA, they also confirmed its relatively short duration of behavioral effects. A longer lasting salvA-like agent that is orally available may offer some advantages in the study and treatment of mood and other disorders. The relatively short duration of action of salvA and its low activity when taken orally (Siebert, 1994) is reportedly due to rapid hydrolysis of salvA acetate to form the weak KOR agonist salvinorin B (Schmidt et al., 2005a; Schmidt et al., 2005b). We modified the functionality of the C-2 substituent of salvA with the goal of increasing metabolic stability. Structure-activity relationship (SAR) studies led to the identification of chemical alterations compatible with maintaining KOR recognition. Using an in vitro [^{35}S]GTPγS assay, we found that the salvinorin analogues (Fig. 1) featuring ethyl ether (EE), isopropylamine (IPA), *N*-methylacetamide (NMA), or *N*-methylpropionamide (NMP) moieties at C-2 were potent KOR agonists (Béguin et al., 2005; Béguin et al., 2006). To characterize the pharmacodynamic and pharmacokinetic properties of these new derivatives, we examined their effects in assays that quantify their receptor binding selectivity, microsomal stability, and effects on motivated behavior.

METHODS

Materials: Dried *S. divinorum* leaves were purchased from Bouncing Bear Botanicals (Lawrence, KS). SalvA (Fig. 1, **1**) was extracted, isolated, and purified using published methods (Munro and Rizzacasa, 2003; Lee et al., 2005). The C-2 modified salvinorin analogues EE (Fig. 1, **2**) and IPA (Fig. 1, **3**) were synthesized from salvA as previously described (see Béguin et al., 2005 for EE; see Béguin et al., 2006 for IPA). NMA (Fig. 1, **3**) and NMP (Fig. 1, **4**) were prepared as previously described (Béguin et al., 2006), omitting purification of the intermediate 2-*epi*-salvinorin B to improve yields. The final products were purified by column chromatography (SiO_2, NMA: 0–10% MeOH/Et_2O; NMP: 5% MeOH/45% CH_2Cl_2/50% hexanes) to give NMA in 49% yield and NMP in 51% yield, over 5 steps from salvinorin B. ^{1}H NMR spectroscopy showed only trace impurities (residual solvents, which cannot account for the in vitro and in vivo properties of the compound). Solutions of each test compound in 75% dimethylsulfoxide (DMSO)-25% distilled water were administered by intraperitoneal (IP) injection or oral (PO) gavage in a volume of 1 mL/kg. Human and rat liver microsomes were purchased from BD Biosciences (San Jose, CA). (1*S*,2*S*)-U50,488, U69,593, nicotinamide adenine dinucleotide phosphate (reduced, NADPH), potassium phosphate, and magnesium chloride were purchased from Sigma-Aldrich (St Louis, MO).

In vitro radioligand binding studies: Radioligand-binding assays at human cloned GPCRs, ion channels, and transporters were performed as described in the literature (Shapiro et al., 2004; Rothman et al., 2000) by using the resources of the National

Institute of Mental Health Psychoactive Drug Screening Program (NIMH-PDSP). Specifically, KOR radioligand-binding assays were performed using cloned rat KOR (rKOR) and [^{3}H]U69,593 as the radioligand. Delta opioid receptor (DOR) binding affinities were determined using cloned human DOR (hDOR) and [^{3}H]DADLE as the radioligand. Finally, the affinity of the test compounds for mu opioid receptors (MOR) were obtained using cloned human MOR (hMOR) and [^{3}H]DAMGO as the radioligand. Detailed on-line protocols are available for all assays at the NIMH-PDSP website (http://pdsp.med.unc.edu). Initial screening assays were performed in quadruplicate using 10 µM test compound, and the percent inhibition of specific binding was determined. Where 10 µM of the test compound inhibited >50% of specific binding, K_i determinations were performed by using six concentrations of unlabeled ligand spanning a 10,000-fold dose range. K_i values were calculated by using GRAPHPAD PRISM and represent the mean ± SEM of quadruplicate determinations.

In vitro metabolism experiments and Cl_{int} calculations: The in vitro intrinsic clearances, CL_{int}, of the test compounds were determined in incubations with human and rat liver microsomes. Control compounds verapamil, dextromethorphan and diclofenac were included as indicators of microsomal CYP450 activity, specifically CYP3A4, CYP2D6 and CYP2C9 in human liver microsomes. The 400 µL incubations contained 0.25 mg/mL of microsomal protein, 1 mM NADPH, 2 mM $MgCl_2$, and 50 mM potassium phosphate buffer (pH 7.4). To begin the reactions, test compounds were added to the pre-warmed (37 °C) incubation mixtures at the final concentration of 1 µM. At 0, 10, 20, 30, and 40 min following addition of the test compound, aliquots of incubation mixture

(35 µL) were collected into an equal volume of acetonitrile and internal standard (1 µM tolbutamide). For the controls without NADPH, compounds were incubated as above excluding NADPH, and samples were collected at 0, 20, and 40 min. The samples were centrifuged at 3000 g for 15 min and analyzed on a liquid chromatography tandem mass spectrometry system consisting of 2 Shimadzu LC-10AD HPLC pumps and a DGU-14A degasser (Shimadzu, Columbia, MD), a CTC PAL autoinjector (Leap Technologies, Carrboro, NC) and a API3000 LC-MSMS system. Chromatography was conducted on a Sprite Armor C18 (20 × 2.1 mm, 10 µm) analytical column (Analytical Sales and Products, Pompton Plains, NJ) with a 0.5 µm PEEK guard filter, using the following mobile phase gradient program: MPA = H_20 with 0.1% formic acid; MPB = acetonitrile with 0.1% formic acid; 0 min = 98% MPA, 2% MPB; 0.3 min = 98% MPA, 2% MPB; 0.7 min = 5% MPA, 95% MPB; 1.3 min = 5% MPA, 95% MPB; 1.4 min = 98% MPA, 2% MPB; 1.7 min = end of run; approximately 2 min between sample injections. For each compound, peak areas for the 10, 20, 30, and 40 min samples were converted to the natural log of the % remaining relative to the 0 min samples (Obach, 1999). The resulting slope (k) of these values relative to time was used to calculate in vitro $T_{1/2}$ where $T_{1/2} = -0.693/k$. CL_{int} was calculated using the following equation: $CL_{int} = (0.693/T_{1/2}) \times (1/0.25)$.

Rats: A total of 22 male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) were used in these studies. Rats were housed individually and weighed 350–400 g at the time of stereotaxic surgery. All rats were maintained on a 12 h light (0700–1900 h)-12 h dark cycle with free access to food and water except during testing. Experiments were

conducted in accordance with the 1996 Guide for the Care and Use of Laboratory Animals (NIH), as well as McLean Hospital and National Institute on Drug Abuse-Intramural Research Program (NIDA-IRP) policies.

Intracranial Self-Stimulation (ICSS): ICSS experiments were conducted as described previously (Carlezon et al., 2006). Each of 22 rats was anesthetized with Nembutal (sodium pentobarbital, 50 mg/kg, IP; Henry Schein, Port Washington, NY), and given subcutaneous (SC) atropine sulfate (0.25 mg/kg, Sigma, St Louis, MO) to minimize bronchial secretions. Each rat was then implanted with a monopolar, stainless steel electrode (0.25 mm diameter; Plastics One, Roanoke, VA) aimed at the left medial forebrain bundle (MFB), at the level of the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral from the midsaggital suture and 7.8 mm below the dura; Paxinos and Watson, 1986). The electrodes were coated with polyamide insulation except at the flattened tip. Skull screws (one of which served as the ground) and the electrode were secured to the skull with dental acrylic.

After one week recovery, the rats were trained on a continuous reinforcement schedule (FR1) to respond to brain stimulation (Todtenkopf et al., 2004; Carlezon et al., 2006). Each lever-press earned a 0.5 s train of square-wave cathodal pulses (0.1 ms pulse duration) at a set frequency of 141 Hz. Responses during the 0.5 s stimulation period did not earn additional stimulation. The stimulation current (100–250 μA) was adjusted gradually to the lowest value that would sustain a reliable rate of responding (at least 30

rewards per min). Once an appropriate current was found for each rat, it was held constant for the remainder of the experiment.

Each rat was then adapted to brief tests with each of a descending series of 15 stimulation frequencies (126–25 Hz). Each series comprised 1 min test trials at each frequency. For each frequency tested, there was an initial 5 s "priming" phase during which non-contingent stimulation was given, followed by a 50 s test phase during which the number of responses was counted. Following the test phase, there was a 5 s time out period during which no stimulation was available. The stimulation frequency was then lowered by approximately 10% (0.05 $\log_{10}$ units), and another trial was started. After responding had been evaluated at each of the 15 frequencies, the procedure was repeated such that each rat was given 6 such series per day (90 min of training). During the training procedure, the stimulation current was adjusted for each rat so that only the highest 6–8 frequencies would sustain responding. To characterize the functions relating response strength to reward magnitude, a least-squares line of best fit was plotted across the frequencies that sustained responding at 20, 30, 40, 50, and 60% of the maximum rate using customized analysis software. ICSS threshold was defined as the frequency at which the line intersected the x-axis (theta-0 (θ_0); see Carlezon et al., 2006). Drug testing started when mean ICSS thresholds varied by less than 10% over 3 consecutive sessions.

We performed three consecutive experiments to determine (a) the dose-response curves and time course (0–90 min) after IP administered salvA, NMA, and NMP; (b) the time course (3–24 h) of effects of NMA and NMP in the ICSS test after IP injection; (c) the

ICSS effect of orally administered salvA and NMA. For each experiment, three rate-frequency functions ("curves") were determined for each rat immediately prior to drug treatment. The first curve served as a warm-up period and was discarded because it tended to be unreliable. The second and third curves were averaged to obtain the baseline parameters for threshold and maximal response rates.

For the dose-response curves and 0–90 min time course experiment, each rat then received an IP injection of drug or vehicle, and six more 15 min rate-frequency curves were obtained (90 min of testing). Eight rats received the same daily treatments in a standardized order: saline, vehicle (75% DMSO), and salvA at 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 mg/kg (IP). Six rats received the same daily treatments in a standardized order: saline, vehicle (75% DMSO), and NMA at 0.25, 0.5, 1.0, and 2.0 mg/kg (IP). Eight rats received the same daily treatments in a standardized order: saline, vehicle (75% DMSO), and NMP at 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/kg (IP). To test whether drug treatment induces tolerance or desensitization, the doses were given in ascending and then descending order, such that each rat received vehicle and each dose of the drug twice. To determine if there were differences between the first and second test with each treatment, the effects of saline, vehicle (75% DMSO), salvA, NMA, and NMP on ICSS thresholds and maximal response rates over the test period were evaluated in separate two-way analyses of variance (ANOVAs) (drug dose × test number) with repeated measures. Data from the first and last tests with saline were used. Since there was no significant effect of dose order, as is typical of ICSS (see Carlezon et al., 2001), the first and second tests at each dose were then combined into single means, and the drug effects on thresholds and maximum rates were evaluated with separate one-way ANOVAs. Significant effects

were analyzed further using *post-hoc* Dunnett's tests. Since the strongest effects of salvA ICSS thresholds occurred within the first 15 minutes post-administration, dose-response curve thresholds were determined using the first 15 min rate-frequency curve. The ED_{50} values were determined using GRAPHPAD PRISM.

When determining the time course of drug actions, the effects from the first 90 min of testing were obtained from the dose-response curves data set. For the determination of the ICSS effects at the other time points (3, 10, 24 h), each rat (N = 8) received an IP injection of drug (NMA, 2 mg/kg or NMP, 1 mg/kg) or vehicle (75% DMSO) immediately after the warm up and the two baseline rate-frequency curves. One 15 min rate-frequency curve was obtained immediately after injection to confirm the expected drug effect. Then, three sets of three rate-frequency curves (three 45 min sessions) were collected 2.5, 9.5, and 23.5 h post-injection. The first and last rate-frequency curve of each set was discarded, and the second one was used to obtain the thresholds at 3, 10, and 24 h time points. As always, the average of the second and third 15-min rate-frequency functions obtained immediately before drug administration served as baseline thresholds.

For the studies of oral drug administration, a small number of rats (N = 4) received vehicle or 2 mg/kg drug (salvA or NMA) by gavage, and six 15 min rate-frequency curves were recorded (90 min of testing).

For the time course and oral administration experiments, the effects of the test compound on ICSS thresholds and maximum response rates were evaluated in separate two-way

analyses of variance with repeated measures. At each time point, significant effects were further analyzed by *post-hoc* Dunnett's tests.

Rats were drug-free for at least one week between each experiment. In addition, on alternate days rats were tested after IP injections of saline, to ensure that they had recovered from prior treatment and to minimize the possibility of conditioned drug effects.

Histology: Each rat was overdosed with pentobarbital (130 mg/kg, IP) and perfused with 4% paraformaldehyde. The fixed brains were sliced in 40-μm sections for cresyl violet staining to confirm electrode placements.

RESULTS

In vitro radioligand binding studies: SalvA, EE, IPA, NMA, and NMP were screened against 39 receptors and transporters ($5HT_{1a}$, $5HT_{1b}$, $5HT_{1d}$, $5HT_{1e}$, $5HT_{2a}$, $5HT_{2b}$, $5HT_{2c}$, $5HT_3$, $5HT_{5a}$, $5HT_6$, $5HT_7$, α_{1a}, α_{1b}, α_{2a}, α_{2b}, α_{2c}, β_1, D1, D2, D3, D4, D5, DAT, DOR, H1, H2, H3, H4, KOR, M1, M2, M3, M4, M5, MOR, NET, SERT, σ_1, σ_2) using the NIMH-PDSP comprehensive radioligand binding assay protocol. The most potent isomer of U50,488 ((1*S*,2*S*) isomer) and U69,593 were tested under the same conditions for comparison. The binding affinities for the receptors at which at least one of the test compounds (10 μM) showed >50% displacement of radioligand are summarized in Table 1. SalvA, EE, IPA, NMA, and NMP did not bind to any of the non-opioid receptors.

Consistent with previous findings (Roth et al., 2002), salvA was a potent and selective rKOR agonist. As expected from previous studies (Béguin et al., 2005; Béguin et al., 2006), the four C-2 analogues (EE, IPA, NMA, and NMP) showed high rKOR binding affinity (K_i = 0.11–6.3 nM). The C-2 substituent did not have a strong effect on the interaction of the molecule with the hDOR: only NMP showed moderate affinity for the hDOR (K_i = 366 nM). Furthermore, with the exception of EE (K_i = 6,938 nM), modifications at C-2 led to an increase in hMOR affinity: the *N*-containing salvinorin analogues showed weak to high affinity for the hMOR (K_i = 15–135 nM). Under the same experimental conditions, the synthetic KOR agonists (1*S*,2*S*)-U50,488 and U69,593 displayed high rKOR/hDOR and rKOR/hMOR selectivity (K_i ratios > 1,000). However the selectivity of (1*S*,2*S*)-U50,488 for the KOR is diminished by its affinity for the α_{2c} (K_i = 118 nM) and σ_2 (K_i = 401 nM) receptors.

SalvA, EE, IPA, NMA, and NMP were also screened against the mDOR and rMOR at Temple University (see supplemental data). The mDOR and rMOR K_i values were compared with the previously reported hKOR K_i values (Béguin et al., 2005; Béguin et al., 2006) to estimate receptor selectivity. In general, the binding data obtained for EE, IPA, NMA, and NMP were comparable to the ones obtained using the PDSP resource and suggested similar KOR selectivity profiles.

In vitro metabolism experiments: Each test compound was incubated in the presence of human or rat liver microsomes with or without the P 450 cofactor NADPH. Table 2 shows the in vitro intrinsic clearance values (CL_{int}): a lower CL_{int} indicates greater in

vitro stability. SalvA was the least stable substrate in the presence of NADPH (rat CL_{int} = 226 µL.min^{-1}.mg^{-1}, human CL_{int} = 233 µL.min^{-1}.mg^{-1}). In comparison, C-2 modified salvinorin analogues EE, IPA, NMA, and NMP were more stable in both species (rat CL_{int} = 24–140 µL.min^{-1}.mg^{-1}, human CL_{int} = 5–17 µL.min^{-1}.mg^{-1}). SalvA also displayed significant NADPH-independent metabolism (rat CL_{int} = 109 µL.min^{-1}.mg^{-1}, human CL_{int} = 168 µL.min^{-1}.mg^{-1}), whereas the other four compounds did not. This may be indicative of esterase-dependent clearance of salvA in the liver and, by implication, blood, in vivo and in vitro. Overall, the rank order of metabolic stability in rat liver microsomes was NMA > IPA > NMP > EE > salvA, and in human liver microsomes NMA ~ IPA > EE > NMP > salvA. Verapamil, dextromethorphan and diclofenac were used as control substances to verify assay performance.

Intracranial Self-Stimulation (ICSS): In pilot ICSS studies designed to identify dose ranges of the salvA derivatives, EE had no effect on ICSS thresholds at doses up to 8.0 mg/kg, IP (data not shown). Given the lack of solubility of EE at higher doses in 75% DMSO and its lower in vitro binding affinity, when compared to salvA, IPA, NMA, and NMP, we did not further evaluate EE in the ICSS test. IPA, NMA, and NMP showed elevations of ICSS thresholds at 1, 0.5, and 0.25 mg/kg, respectively (data not shown). We did not characterize the dose-response curve of IPA because of its reduced in vitro KOR selectivity (Table 1, MOR/KOR K_i ratio = 25) compared to salvA, NMA, and NMP. In a newly trained group of rats, salvA, NMA, and NMP dose dependently increased ICSS thresholds (Fig. 2a, c, e) and the effect depended on treatment (salvA: $F_{7.63} = 9.14$, $P < 0.01$; NMA: $F_{5.35} = 7.26$, $P < 0.01$; salvA: $F_{6.55} = 7.25$, $P < 0.01$). The

dose response curve data (Fig. 2a, c, e) suggest that salvA, NMA, and NMP were equipotent. There was a trend for decreases in ICSS maximum rates at the highest doses tested (Fig. 2b, d, f) but none reached significance. Vehicle (75% DMSO) had negligible effects on thresholds and maximum response rates. Figure 3 shows representative response-frequency functions for vehicle and NMA (2 mg/kg, IP).

Using doses that produced maximal elevations of ICSS thresholds (Fig. 2, ~140%), we next examined the effects of vehicle, NMA (2 mg/kg, IP), and NMP (1 mg/kg, IP) at 3 h, 10 h, and 24 h post-injection. There was a significant interaction between time and drug treatment (Fig. 4, $F_{16.136} = 4.61$, $P < 0.01$). The data suggested that salvA showed maximum elevation of ICSS thresholds within the first 15 min post-injection ($P < 0.01$, Dunnett's test) and that the effect decreased to non-significant levels 30 min post-injection. In comparison, the effects of NMA peaked within the first 15 min post-injection ($P < 0.01$) and remained stable until 1.5 h post-injection ($P < 0.01$) before returning to baseline. Similar to NMA, NMP displayed long lasting effects, with significant effects still observed 3 h post-injection ($P < 0.01$).

Taken together, these experiments suggested that while IPA and NMP showed reduced in vitro KOR selectivity, EE lacked in vivo efficacy. NMA was therefore the most attractive candidate for preliminary oral efficacy studies. To compare the ICSS effects of NMA and salvA after oral administration, rats (N = 4) were administered vehicle (75% DMSO), NMA, or salvA by oral gavage. There was a significant interaction between time and drug treatment (Fig. 5, $F_{10.45} = 2.05$, $P < 0.05$). When rats were administered

vehicle (75% DMSO, PO) or salvA (2 mg/kg, PO), their ICSS thresholds remained unchanged. In comparison, NMA (2 mg/kg, PO) caused a significant elevation of ICSS thresholds (Fig. 5, $P < 0.01$). Vehicle (75% DMSO) seems to decrease response rates but there was no significant difference between vehicle and each drug treatment (Fig. 5).

DISCUSSION

We have previously reported the SAR of C-2 modified salvinorin derivatives and found that 3–4 atom long substituents led to high KOR agonist potency (Béguin et al., 2005; Béguin et al., 2006). In particular, four C-2 modified salvinorin analogues designed to have increased metabolic stability (EE, IPA, NMA, and NMP) were identified as potent KOR agonists. In this study, we further characterized these compounds by first determining their KOR binding selectivity. SalvA, EE, IPA, NMA, and NMP were screened against common CNS targets (39 receptors and transporters) using the PDSP screening facility (Table 1). As previously observed using hKOR (see supplemental data, Béguin et al., 2005; Béguin et al., 2006), EE, IPA, NMA, and NMP exhibited high binding affinity for the rKOR (Table 1, K_i = 0.11–6.3 nM). The rank order of KOR selectivity was salvA > NMA ~ EE > NMP > IPA. For comparison, we tested the standard KOR agonists U50,488 and U69,593 against the same 39 receptors or transporters. The synthetic KOR agonist U50,488 can exist in four diastereoisomeric forms. The racemic mixture of the two trans isomers is most commonly used in pharmacological studies. Previous investigations have shown that the trans isomer (1*S*,2*S*)-U50,488 was the most potent isomer with subnanomolar K_i value at the KOR (Rothman et al., 1989). Consistent with this findings, (1*S*,2*S*)-U50,488 showed high

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rKOR binding affinity (Table 1). As previously reported (Clark et al., 1988), it also had excellent KOR selectivity over DOR and MOR. However, its affinity for the α_{2C} and σ_2 receptors led to reduced KOR selectivity. In comparison, U69,593 was highly selective for the KOR in the PDSP screening assay. Taken together, these data indicate that, from the subset of compounds selected in this study, U69,593, salvA, NMA, and EE are the most selective KOR ligands.

To determine if C-2 chemical modifications led to increased metabolic stability, we incubated salvA, EE, IPA, NMA, and NMP with rat and human liver microsomes. The observed intrinsic clearances demonstrate that salvA was the least stable compound. As expected, our modifications at C-2 (EE, IPA, NMA, NMP) led to increased resistance to metabolism, with NMA and IPA being the most stable derivatives in vitro. It was anticipated that the improved metabolic stability of EE, IPA, NMA, and NMP in vitro should translate to improved half-life and bioavailability in vivo.

We then determined the potencies of salvA, EE, IPA, NMA, and NMP in vivo. We used ICSS since it is sensitive to KOR agonist effects (Todtenkopf et al., 2004) and can provide information about the time course of drug actions. In pilot ICSS studies, the order of potency was NMP > NMA ~ salvA > IPA > EE. As predicted from in vitro results (using hKOR or rKOR binding assays), EE was the least potent analogue and thus not further characterized in vivo. Similarly, the dose-response curve of IPA was not determined due to its reduced in vitro KOR selectivity. We had previously evaluated salvA (0.125–2 mg/kg, IP) in the ICSS assay (Carlezon et al., 2006). Here we tested a

more extensive dose range of salvA to confirm its prodepressant-like effects and facilitate direct comparisons among the derivatives. We found that salvA, NMA, and NMP dose-dependently increased ICSS thresholds without significantly affecting maximum response rates, which can reflect treatment-induced alterations in motor capabilities. The effects were consistent with our previous findings using salvA (Carlezon et al., 2006) and U69,593 (Todtenkopf et al., 2004). In addition, salvA, NMA, and NMP were equipotent and produced similar maximal effects in this behavioral assay.

In vitro, NMP showed reduced KOR over MOR selectivity when compared to salvA and NMA. However, the three test compounds induced comparable elevations in ICSS thresholds. It is conceivable that ICSS does not allow us to distinguish highly selective (salvA, NMA) from less selective (NMP) KOR agents, or the doses used were too low to detect any MOR-mediated effects. Alternatively, the prodepressant effects of KOR stimulation may mask the rewarding actions of MOR stimulation (Devine and Wise, 1994).

The purpose of our SAR study at C-2 was to design salvinorin analogues with increased in vivo stability. We compared the time courses of salvA, NMA, and NMP on ICSS. The time course of salvA effects on ICSS was consistent with its short lived effects observed in antinociceptive assays (Ansonoff et al., 2006; John et al., 2006; McCurdy et al., 2006): it produced maximal threshold-elevating effects within the first 15 min post-administration, and these effects had waned by 30–60 min. NMA and NMP produced longer lasting prodepressant-like effects, 6-fold and 10-fold, respectively. These data

suggest that the increase in duration of action of the C-2 modified salvinorin analogues may be due, at least in part, to their enhanced metabolic stability. Other C-2 modified salvinorin derivatives may also display longer lasting in vivo effects (Liu-Chen, personal communication).

A common characteristic of currently available selective KOR agonists is limited oral bioavailability. Using a mouse acetic acid-induced abdominal constriction test, Endoh et al. (1999) reported a 10- fold or higher difference between the ED_{50} obtained after PO and IP administrations of several KOR agonists (TRK-820, U50,488, CI-977, ICI-199441, and PD-117302), suggesting a limited oral absorption of these drugs. In our study, after oral administration, NMA (2 mg/kg) retained in vivo activity, whereas a similar dose of salvA was inactive. The slower onset of action of NMA after PO compared to IP administration was consistent with a slower rate of absorption. Vehicle (75% DMSO, PO) had no effects on ICSS thresholds.

In conclusion, this study confirmed that salvA and U69,593 are potent and selective KOR ligands. We found that NMA had a similar highly selective in vitro pharmacological profile, while (1*S*,2*S*)-U50,488 had lesser KOR selectivity due to its moderate affinity for the adrenergic α_{2C} receptor. We confirmed that salvA had short lasting in vivo behavioral effects, most likely due to metabolic lability. In contrast, we found that NMA produced long lasting prodepressant-like effects in the ICSS test, and preliminary data indicate that oral availability is improved by reducing metabolic lability. NMA may be a new pharmacological tool in experiments that require oral availability and long lasting KOR

activation. More exhaustive pharmacokinetics and toxicity studies are needed to determine if NMA might have clinical applications.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1 Chemical structures of salvA, C-2 modified analogues (EE, IPA, NMA, and NMP), U50,488, and U69,593 (see abbreviation section for full names).

Fig. 2 Effect of salvA, NMA, and NMP on ICSS after IP administration. (**a**) vehicle (75% DMSO) had no significant effect on ICSS thresholds (mean ± SEM) over the 15 min test session. SalvA dose-dependently increased ICSS thresholds. *, $P < 0.05$; **, $P < 0.01$, compared with vehicle, Dunnett test, eight rats per group. (**b**) in comparison with vehicle, none of the doses of salvA affected response capabilities (maximal rates). (**c**) NMA dose-dependently increased ICSS thresholds during the 15 min session. *, $P < 0.05$; **, $P < 0.01$, compared with vehicle, Dunnett test, six rats per group. (**d**) in comparison with vehicle, none of the doses of NMA affected response capabilities (maximal rates). (**e**) NMP dose-dependently increased ICSS thresholds during the 15 min session. **, $P < 0.01$, compared with vehicle, Dunnett test, eight rats per group. (**f**) in comparison with vehicle, none of the doses of NMP affected response capabilities (maximal rates).

Fig. 3 (**a**) Vehicle had no effect on rate-frequency curves when compared to baseline. (**b**) NMA (2 mg/kg, IP) caused rightward shifts in rate-frequency functions when compared to baseline. Data are from representative rats.

Fig. 4 Time course of effect of vehicle, salvA, and NMA on ICSS after IP administration. Vehicle (75% DMSO, IP) had no significant effect on ICSS

thresholds (mean ± SEM) over 24 h. In comparison, salvA (2 mg/kg, IP) increased ICSS thresholds 15 min post-injection. $$, $P < 0.01$, compared with vehicle, Dunnett test, eight rats per group. NMA (2 mg/kg, IP) increased ICSS thresholds up to 1.5 h post-injection. ##, $P < 0.01$, compared with vehicle, Dunnett test, six rats per group. NMP (1 mg/kg, IP) increased ICSS thresholds up to 3 h post-injection. **, $P < 0.01$, compared with vehicle, Dunnett test, eight rats per group.

Fig. 5 Effect of vehicle, salvA, and NMA on ICSS after PO administration. (**a**) vehicle (75% DMSO, PO) and salvA (2 mg/kg, PO) had no significant effect on ICSS thresholds (mean ± SEM) over the 90 min test session. In comparison, NMA (2 mg/kg, PO) increased ICSS thresholds. *, $P < 0.05$; **, $P < 0.01$, compared with vehicle, Dunnett test, eight rats per group. (**b**) the average percent baseline thresholds over the 1.5 h test session showed similar effects. **, $P < 0.01$, compared with vehicle, Dunnett test, eight rats per group.

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Table 1. Binding affinities (K_i) of salvA, C-2 modified analogues, U50,488, and U69,593.

	K_i, nM					K_i ratio	
Compd	α_{2C}[a]	σ_2[a]	DOR[a,b]	KOR[c]	MOR[a,d]	DOR/KOR	MOR/KOR
1, SalvA	–[e]	–[e]	6,404 ± 1,080	0.28 ± 0.22	4,879 ± 5,055	>10,000	>10,000
2, EE	–[e]	–[e]	–[e]	6.3 ± 3.6	6,938 ± 2,216	>529	1,101
3, IPA	–[e]	–[e]	–[e]	4.5 ± 2.0	111 ± 49	>740	25
4, NMA	–[e]	–[e]	1,690 ± 285	0.37 ± 0.30	135 ± 4	4,568	365
5, NMP	–[e]	–[e]	366 ± 38	0.11 ± 0.10	15 ± 3	3,327	136
U50,488	118 ± 20	401± 33	1,772 ± 249	0.42 ± 0.22	1,095 ± 186	4,219	2,607
U69,593	–[e]	–[e]	–[e]	2.5 ± 0.3	5,286 ± 2,117	>1,333	2,114

[a]Using cloned human receptors expressed in human embryonic kidney-293 (HEK-293) cells.

[b]Using [^{3}H]DADLE as the radioligand.

[c]Using cloned rat opioid receptors and and [^{3}H]U69,593 as the radioligand.

[d]Using [^{3}H]DAMGO as the radioligand.

[e]Test ligand (10 μM) caused <50% inhibition of radioligand binding.

Data represent mean ± SEM of computer-derived estimates of K_i and pK_i values for N > 3 separate experiments. Each compound was screened against 39 receptors and transporters. We included in the table only the receptors at which any test ligand (at 10 μM) showed >50% inhibition of radioligand binding. SalvA, EE, NMA, NMP, U50,488, and U69,593 are highly selective for the KOR over DOR and MOR.

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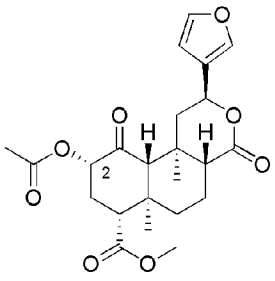
Table 2. Intrinsic clearances (CL_{int}) of salvA, C-2 modified analogues, verapamil, dextromethorphan, and diclofenac.

	CL_{int} ($\mu L.min^{-1}.mg^{-1}$) ± S.D.			
	Rat		Human	
Compd	+ NADPH	- NADPH	+ NADPH	- NADPH
1, SalvA	226 ± 3	109 ± 20	233 ± 4	168 ± 5
2, EE	140 ± 5	<5	9.70 ± 6.18	<5
3, IPA	41.8 ± 6.7	<5	<5	<5
4, NMA	24.2 ± 4.3	<5	<5	<5
5, NMP	77.2 ± 5.1	<5	17.2 ± 4.3	<5
verapamil	273 ± 10	<5	166 ± 10	<5
dextromethorphan	247 ± 5	<5	32 ± 6	<5
diclofenac	254 ± 4	<5	362 ± 22	<5

Data represent the intrinsic clearances obtained by incubating each test compound with rat or human liver microsomes, in the presence or absence of the P450 cofactor NADPH. Verapamil, dextromethorphan, and diclofenac served as standard compounds. The order of in vitro metabolic stability in rat microsomes was NMA > IPA > NMP > EE > salvA. The order of in vitro metabolic stability in human microsomes was NMA ~ IPA > EE > NMP >salvA.

Figure 1



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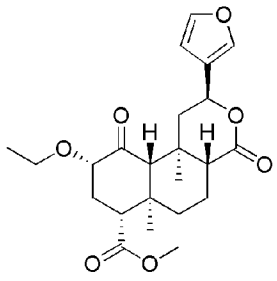

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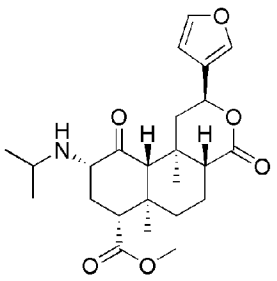

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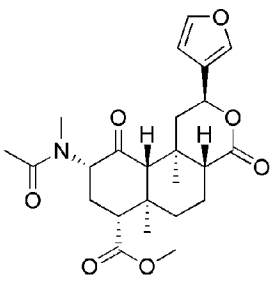

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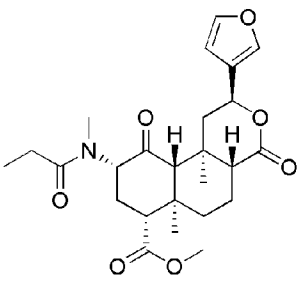

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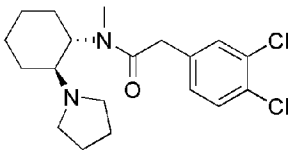
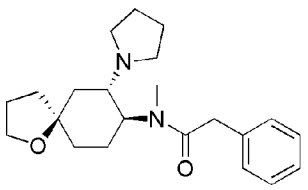
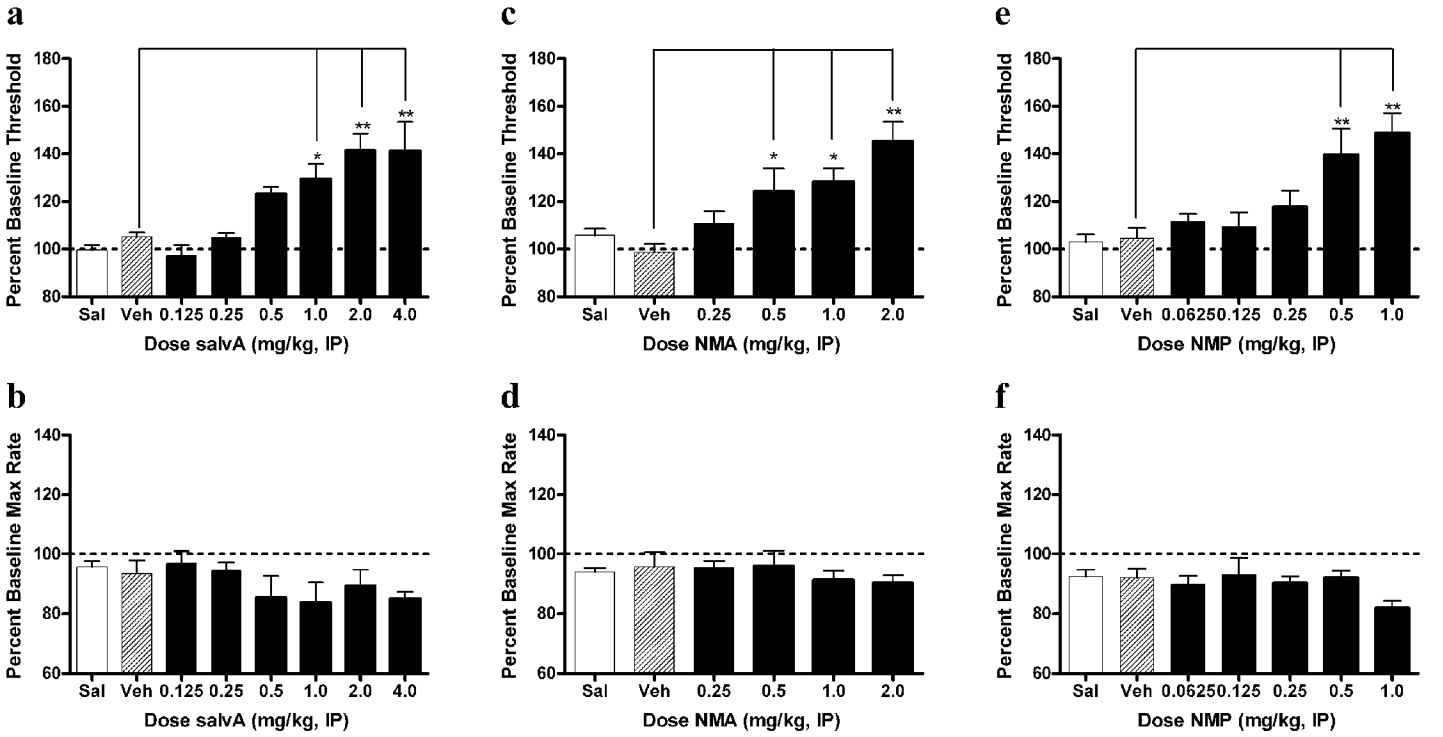


6, U50,488



7, U69,593

Figure 2


a
Percent Baseline Threshold
180
160
140
120
100
80
Sal
Veh
0.125
0.25
0.5
1.0
2.0
4.0
Dose salvA (mg/kg, IP)
b
Percent Baseline Max Rate
140
120
100
80
60
Sal
Veh
0.125
0.25
0.5
1.0
2.0
4.0
Dose salvA (mg/kg, IP)
c
Percent Baseline Threshold
180
160
140
120
100
80
Sal
Veh
0.25
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2.0
Dose NMA (mg/kg, IP)
d
Percent Baseline Max Rate
140
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1.0
2.0
Dose NMA (mg/kg, IP)
e
Percent Baseline Threshold
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Dose NMP (mg/kg, IP)
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Percent Baseline Max Rate
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Sal
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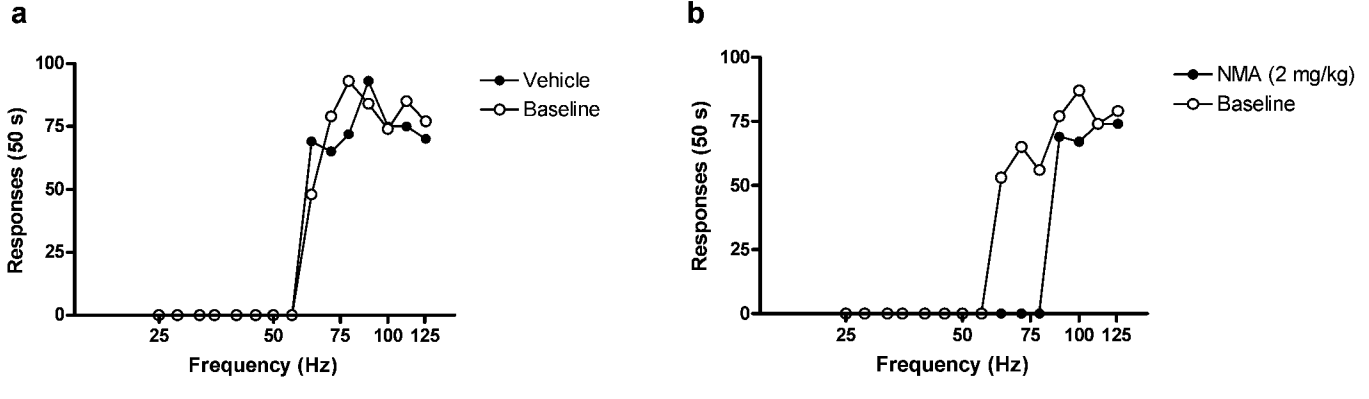
Dose NMP (mg/kg, IP)

Figure 3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Frequency (Hz)
Vehicle
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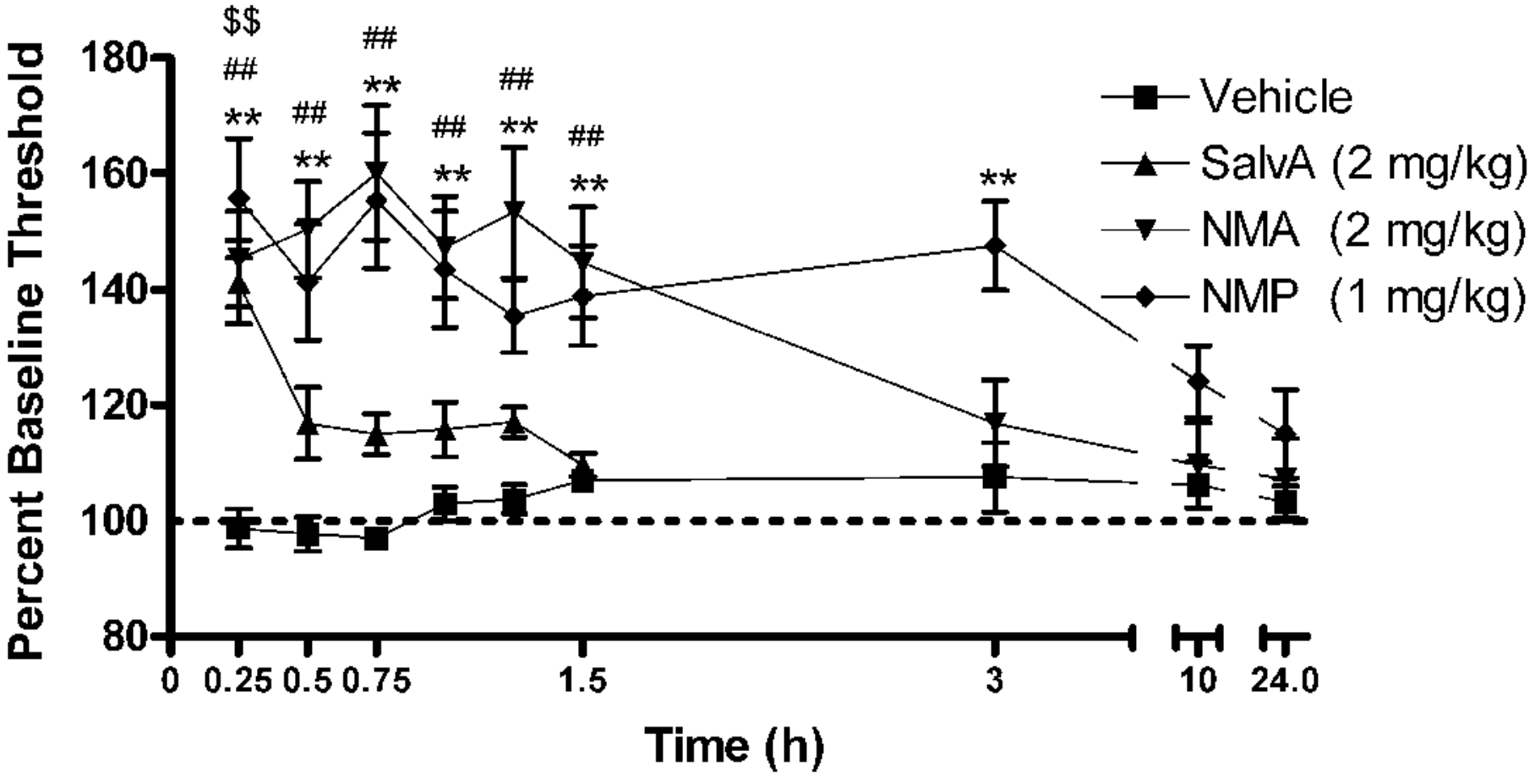
NMA (2 mg/kg)
Baseline

Figure 4


Percent Baseline Threshold
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24.0
Time (h)
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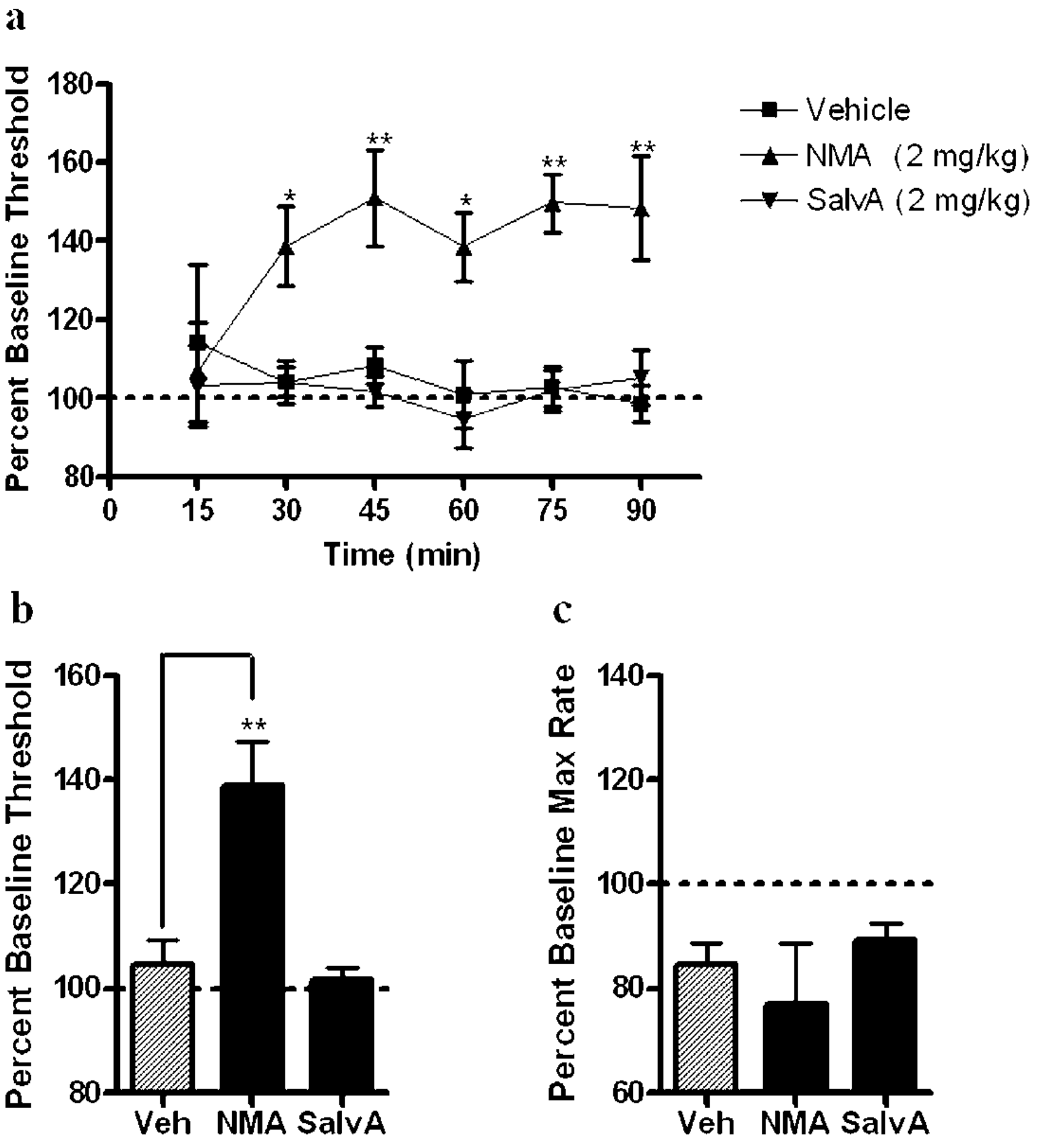
##
**
Vehicle
SalvA (2 mg/kg)
NMA (2 mg/kg)
NMP (1 mg/kg)

Figure 5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Percent Baseline Threshold
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15
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Time (min)
Vehicle
NMA (2 mg/kg)
SalvA (2 mg/kg)
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b
Percent Baseline Threshold
160
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