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Research paper

Evaluation of the transport, *in vitro* metabolism and pharmacokinetics of Salvinorin A, a potent hallucinogen

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ABSTRACT

Salvinorin A is an unregulated potent hallucinogen isolated from the leaves of Salvia divinorum. It is the only known non-nitrogenous kappa-opioid selective agonist, and rivals synthetic lysergic acid diethylamide (LSD) in potency. The objective of this study was to characterize the *in vitro* transport, *in vitro* metabolism, and pharmacokinetic properties of Salvinorin A. The transport characteristics of Salvinorin A were assessed using MDCK-MDR1 cell monolayers. The P-glycoprotein (P-gp) affinity status was assessed by the P-gp ATPase assay. *In vitro* metabolism studies were performed with various specific human CYP450 isoforms and UGT2B7 to assess the metabolic characteristics of Salvinorin A. Cohorts ($n = 3$) of male Sprague Dawley rats were used to evaluate the pharmacokinetics and brain distribution of Salvinorin A (10 mg/kg, intraperitoneal (i.p.) over a 240-min period. A validated UV-HPLC and LC/MS/MS method was used to quantify the hallucinogen concentrations obtained from the *in vitro* and *in vivo* studies, respectively. Salvinorin A displayed a high secretory transport in the MDCK-MDR1 cells ($4.07 \pm 1.34 \times 10^{-5}$ cm/s). Salvinorin A also stimulated the P-gp ATPase activity in a concentration (5 and 10 μM)-dependent manner, suggesting that it may be a substrate of (P-gp). A significant decrease in Salvinorin A concentration ranging from 14.7 ± 0.80% to 31.1 ± 1.20% was observed after incubation with CYP2D6, CYP1A1, CYP2C18, and CYP2E1, respectively. A significant decrease was also observed after incubation with UGT2B7. These results suggest that Salvinorin A maybe a substrate of UGT2B7, CYP2D6, CYP1A1, CYP2E1, and CYP2C18. The *in vivo* pharmacokinetic study showed a relatively fast elimination with a half-life ($t_{1/2}$) of 75 min and a clearance (Cl/F) of 26 L/h/kg. The distribution was extensive (Vd of 47.1 L/kg); however, the brain to plasma ratio was 0.050. Accordingly, the brain half-life was relatively short, 36 min. Salvinorin A is rapidly eliminated after i.p. dosing, in accordance with its fast onset and short duration of action. Further, it appears to be a substrate for various oxidative enzymes and multi-drug resistant protein, P-gp.

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1. Introduction

Psychotropic natural products are widely available through a variety of commercial sources; however, they represent a class of agents that are understudied and possibly toxic and possess pharmacologic properties consistent with drug abuse liability. Salvinorin A (Fig. 1), active component of Salvia divinorum, is a potent hallucinogen whose use is associated with "altered consciousness", and its status in this country and abroad has been under review. This substance has been banned in five states (Delaware, Louisiana, Missouri, Oklahoma, and Tennessee), and two states (New Jersey and New York) are currently formulating legislature on Salvia divinorum. Other countries such as Australia, Denmark, Finland, Italy, and Sweden have recently classified Salvia divinorum as a controlled substance [1–5]. Reports have predicted that its use will most likely reach the levels associated with similar hallucinogenic agents such as 3,4-methylenedioxy methamphetamine (MDMA), phencyclidine (PCP), and lysergic acid diethylamide (LSD) in the next 5–10 years [6,7]. This is evidenced by a sharp increase in its consumption by college students and young adults over the last few years [3,6–10].

Salvia divinorum is typically consumed by smoking a quantity of dried leaves, although buccal absorption by chewing dried leaves or ingesting a tincture is also used. The onset of action is

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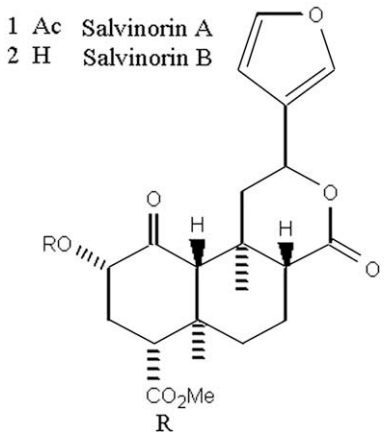

Fig. 1. Chemical structures of Salvinorin A and B.

relatively rapid, on the order of 30 s for smoking and 5–10 min for buccal absorption after ingestion [2,11]. Salvinorin A is an extremely potent naturally occurring hallucinogen, with an effective dose, when smoked, of 200–500 μg [2,12]. It induces an intense, short-lived hallucinogenic experience in humans appearing in less than 1 min and lasting for 15 min or less [13]. Its effect is reported to be qualitatively distinct from that induced by the classical hallucinogens such as LSD, psilocybin and mescaline. Salvinorin A is a neoclerodane diterpene (Fig. 1), and chemically, it lacks a basic nitrogen structure which is an uncommon feature associated with psychoactive hallucinogenic agents; however, it does have a number of carbon atoms that enhance its lipophilicity.

Pharmacologically, Salvinorin A does not act at the molecular target responsible for the actions of classic hallucinogens, the serotonin 5-HT2A receptor [14,15]. Salvinorin A is selective for the κ opioid receptor (KOR) [16,17], and produces KOR-like discriminative effects in rhesus monkeys [18], low dopamine levels in the mouse caudate putamen [19], and rat nucleus acumbens [20] through activation of KORs. Presently, limited research has been conducted to characterize Salvinorin A. It is suggested that Salvinorin B (Fig. 1) is an inactive metabolite of Salvinorin A [21,22] and that it possibly shares metabolic pathway(s) with cocaine, heroin, tetrahydrocannabinol (THC) and MDMA and is metabolized by esterase in the blood [23]. In addition, a pharmacokinetic study in rhesus monkeys found that its elimination half-life is rapid (56.6 ± 24.8 min), [24] which corresponds with its short duration of action. Recently, positron emission tomography (PET) studies performed in baboons indicated extremely rapid brain uptake. [^{11}C]-Salvinorin A was distributed throughout the brain with the highest concentration in the cerebellum and a notable concentration in the visual cortex perhaps accounting for its physiological effects when smoked [25].

To date, there are several investigations underway to evaluate Salvinorin A's pharmacologic properties; however, its blood–brain barrier (BBB) transport, metabolism, and pharmacokinetics have not been described well. As such, the rate and extent of its distribution across the BBB into the central nervous system (CNS) responsible for producing hallucinogenic effects are unknown as are those dispositional properties that mediate its duration of action. To investigate these, the following three objectives were pursued: (1) assessment of the *in vitro* transport mechanism of Salvinorin A across MDCK-MDR1 cell monolayers, (2) characterization of the *in vitro* metabolism of Salvinorin A using recombinant human CYP450 (InVitroSomes™) and UGT2B7 enzyme (Supersomes™ membrane fractions from insects cells expressing UDP-glucurosyltransferases (UGT) isoform), and (3) evaluation of the single-dose pharmacokinetics of Salvinorin A in male Sprague Dawley rats.

2. Materials and methods

2.1. Materials

Salvinorin A was provided by Dr. Thomas Prisinzano (Iowa University, Iowa). 4-Chlorobenzotropine (BZT) was synthesized and provided by Dr. Amy H. Newman (NIH, Baltimore, MD). The purities of Salvinorin A and BZT were >98%. All chemicals and solvents were of American Chemical Society analytical grade or HPLC grade. InVitroSomes™, human recombinant cytochrome P450 enzymes, were purchased from InVitro Technologies (Baltimore, MD). Human UGT2B7 Supersomes™ enzymes were purchased from BD Biosciences Discovery Labware (Woburn, MA). MDCK-MDR1 cells were provided by Dr. Peter W. Swaan (University of Maryland). DMEM, phosphate buffered saline, non-essential amino acid, fetal bovine serum (FBS), L-glutamine, penicillin G-streptomycin sulfate antibiotic mixture and trypsin (0.25%)–EDTA (1 mM) were purchased from Invitrogen Laboratories (Carlsbad, CA). Polymyxin, amphotericin, heparin, and dextran were purchased from the Sigma Chemical Co. (St. Louis, MO). Twelve-well transport plates (cell culture treated) were purchased from Corning Costar (Cambridge, MA).

2.2. Salvinorin A-stimulated P-gp ATPase activity

In order to assess whether Salvinorin A was a P-gp substrate, we determined its ability to stimulate ATPase activity. Salvinorin A-stimulated P-gp ATPase activity was estimated by Pgp-GIO assay system (Promega, Madison, WI). This method relies on the ATP dependence of the light-generating reaction of firefly luciferase where ATP consumption is detected as a decrease in luminescence. In a 96-well plate, recombinant human P-gp was incubated with P-gp-GIO assay buffer™ (20 μL), verapamil (200 μM), sodium orthovanadate (100 μM), and Salvinorin A (2.5–100 μM). Each compound was loaded into four individual wells. Verapamil served as a positive control, while sodium orthovanadate was used as a P-gp ATPase inhibitor. In the presence of sodium orthovanadate, ATP consumption by P-gp is negligible, and without sodium orthovanadate, P-gp consumes ATP to a greater or lesser extent than the control, which is dependent on the effect of the test compounds. The reaction was initiated by the addition of MgATP (10 mM), stopped 40 min later by the addition of 50 μL of firefly luciferase reaction mixture (ATP detection reagent) that initiated an ATP-dependent luminescence reaction. Signals were measured 100 min later by Lmax® luminometer (Molecular Devices Corporation, Sunnyvale, CA), and were converted to ATP concentrations by interpolation from a luminescent ATP standard curve. The rate of ATP consumption (pmol/min/μg protein) was determined as the difference between the amount of ATP in the absence and presence of sodium orthovanadate. Salvinorin A-stimulated P-gp ATPase activity was reported also as fold-stimulation relative to the basal P-gp ATPase activity in the absence of the compound (control).

2.3. MDCK-MDR1 cells

MDCK-MDR1 cells were cultured in Dulbecco's modified eagle serum (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL of penicillin and streptomycin. The cells were plated onto 12-well Costar Transwell® inserts (0.4 μM pore size, 1 cm^2 surface area) at a density of 425,000 cells/cm^2. The cells were cultured and maintained in DMEM supplemented with 10% FBS, 2% L-glutamine, 1% non-essential amino acid, 1% penicillin-streptomycin under standard conditions of 5% CO_2, 37 ± 0.5 °C and 95% humidity until confluence was reached on day four. The medium was changed every day after seeding, and confluent monolayers were used for transport studies outlined below. Monolayer integrity was checked by measuring the transepithelial resistance (TEER), and

[^{14}C]mannitol permeability. P-gp functional confirmation was determined by [^{14}C]paclitaxel efflux values.

2.4. Transport study

MDCK-MDR1 transport studies were performed as previously described in our laboratory [26]. All transport experiments were performed at 37 °C in phosphate buffered saline (PBS). Salvinorin A (5 μM), radiolabeled marker ([^{14}C]mannitol, [^{14}C]paclitaxel) or blank buffer was added to either the apical or basolateral side. Cell monolayers were continuously agitated on a plate shaker during transport experiments (60–70 rpm). For examination of transport in the apical to basolateral (A → B) direction, the transwell inserts were moved to wells with fresh PBS. At time $t = 0$, 0.5 mL of Salvinorin A solution was added to the apical side of the monolayer. Inserts were moved to new wells at times between 10 and 120 minutes. For examination in the basolateral to apical (B → A) direction, Salvinorin A (1.5 mL) was initially added to the basolateral side at time $t = 0$. At various time intervals between 10 and 120 min, samples were collected from the apical side and were replaced with fresh, pre-warmed PBS. Samples were analyzed by an UV-HPLC method for Salvinorin A. Apparent permeability coefficients were determined for each transport study. Radioactive compounds were analyzed by scintillation counter (Beckman Coulter LS 6500).

2.5. In vitro metabolism screening with various human CYP450 isoforms and UGT2B7

A screening phenotyping approach was used to identify the isozyme(s) responsible for its metabolism using various cytochrome (CYP) isoforms including CYP2D6, CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2E1, CYP3A4, and CYP3A5. InVitroSomes™ used in this study are single enzyme systems that express a specific CYP isoform (e.g., CYP 2D6) and yeast CYP-reductase co-expressed in Saccharomyces cerevisiae. InVitroSomes™ are ideal for use in drug metabolism studies [27], particularly in specific CYP pathway identification (reaction phenotyping) and CYP inhibition screening. InVitroSomes™ expressing the aforementioned isoforms were incubated with Salvinorin A (0, 50 μm, or 5 μm) and TE (500 mM Tris, 10 mM EDTA) reaction buffer. TSE (500 mM Tris, 2 M NaCl, 10 mM EDTA) reaction buffer was used for CYP2B6, CYP3A4, and CYP3A5 instead of TE buffer. This solution was incubated with shaking for 5 min at 37 °C. At the end of this period, 20 μL of NADPH (5 mg/mL) was added to each sample to start the reaction. This was followed by one hour incubation at 37 °C. At the end of this second incubation period, 100 μL of cold acetonitrile was added to stop the reaction. All samples were analyzed for Salvinorin A using our validated UV-HPLC method. Preliminary studies were performed to find the optimal incubation time which was used in subsequent studies. Salvinorin A (50 μM) was incubated with CYP2D6 at different incubation times (10, 30, and 60 min) at 37 °C. Significant decrease was observed at 60 min, and this incubation time period was used for subsequent studies.

In vitro metabolism studies were also conducted to evaluate the role of UGT in Salvinorin A's metabolism. Supersomes™ were used for possible glucuronidation activity. The reaction mixture (0.2 mL) containing 1 mg/mL protein (UGT2B7), 1 mM uridine diphosphoglucuronic acid (UDPGA), 10 mM magnesium chloride, 0.025 mg/mL alamethicin and 0, 10, or 50 μM of Salvinorin A in 50 mM Tris (pH 7.5) was incubated at 37 °C for 1 h. After the incubation period, the reaction was stopped by the addition of 100 μL acetonitrile, and the reaction mixture was centrifuged (10,000 rpm) for 15 min. All the samples were analyzed using an UV-HPLC method.

2.6. Pharmacokinetic studies

2.6.1. Animals

Male Sprague Dawley rats weighing 275–300 g were purchased from Harlan Laboratories (Indianapolis, IN). The animals were housed in an AAALAC-accredited facility run on a 12-h light and dark cycle. The animals were allowed unrestricted access to food and water. The protocol for the animal studies was approved by the School of Pharmacy, University of Maryland IACUC.

2.6.2. Dosing and sampling

To evaluate the *in vivo* pharmacokinetic and brain distribution of Salvinorin A, a single dose study was performed in adult male Sprague Dawley rats. Salvinorin A was administered as a single i.p. dose of 10 mg/kg (in Cremophor EL:ethanol, 70%:30%). A destructive sampling study design was followed where cohorts of three animals were euthanized by CO_2 asphyxiation at pre-dose and at the following time points post dosing: 5, 10, 15, 30, 60, 90, 120, and 240 min. Blood samples were collected by cardiac puncture using pre-heparinized syringes and were immediately transferred into tubes containing acetonitrile to inhibit possible esterase metabolism. Blood samples were centrifuged at 3500 rpm for 10 min and plasma was separated. Brain tissue was immediately excised, blotted dry, and weighed. All samples were stored at −80 °C until analyzed.

2.7. Quantification of Salvinorin A

2.7.1. UV-HPLC systems to quantify Salvinorin A in in vitro samples

An UV-HPLC method was used to quantify the Salvinorin A concentrations in the transport and *in vitro* metabolism studies. The chromatographic conditions consisted of a Waters Symmetry (C_{18} 5 μm, 150 × 4.6 mm) column plus Supelguard 5 μm LC-18, 2 cm guard column. The mobile phase (acetonitrile:water, 55:45 v/v) was filtered through a 0.45-μm nylon filter and was degassed under ultrasound and vacuum for 15 min and pumped at a flow rate 1 mL/min over a 20-min period. The injection volume was 200 μL. Salvinorin A was quantified at an UV wavelength of 210 nm, and its retention time was 12 min. The sensitivity limit for Salvinorin A using this method was 100 ng/mL.

2.7.2. LC/MS/MS analytical method to quantify Salvinorin A in biological matrix

An LC/MS/MS analytical method was used to quantify Salvinorin A in plasma and brain. Four milliliters of hexane were added to 0.4 mL of plasma, vortexed (1 min) and centrifuged at 10,000 rpm for 10 min. The supernatant was evaporated to dryness at 40 °C under a gentle stream of nitrogen and was reconstituted with 100 μL of mobile phase. Brain tissue was homogenized, and diluted with an equal volume of PBS. Four milliliters of hexane were added to the brain homogenate (0.8 mL), vortexed for 2 min and centrifuged at 10,000 rpm for 10 min. The organic phase was transferred to a clean test tube, evaporated under nitrogen and reconstituted with 100 μL of mobile phase. The supernatant was transferred to a microvial, and 30 μL was injected onto the LC/MS/MS system.

The LC/MS/MS system consisted of a quattro micro triple quadrupole mass spectrometer (Micromass-Waters, Millford, Boston) operated in the positive ion mode with an ESI-probe. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. The source was operated at 140 degree, and nitrogen was used as the nebulizer gas and argon was used as the collision gas set at 10 psi. The cone voltage was 45 V, capillary voltage was 3.5 V, and the entrance and exit voltages were −5 and 1, respectively. The HPLC system consisted of a Waters 2695 quaternary system, Xterra MS C_{18} (2.5 μm, 2.1 × 50 mm) column, and the mobile phase was composed of acetonitrile and water (55:45, v/v).

The mobile phase was pumped at a flow rate of 0.2 mL/min, and the injection volume was 30 μL. Following HPLC separation, the Salvinorin A peak area corresponding to 433.5–373 parent–daughter transition and the internal standard peak (BZT) peak area corresponding to 342.5–201.1 parent–daughter transition were quantitated. The retention times for Salvinorin A were 2.8 min and 1.6 min for the internal standard. The total run time was five minutes. The plasma and brain calibration curves were linear in the range of 7.5–500 ng/mL ($r^2 \geqslant 0.999$) and 7.5–200 ng/mL ($r^2 \geqslant 0.997$) for plasma and brain, respectively.

2.8. Data analysis

2.8.1. ATPase assay

Basal P-gp activity, test compound stimulated P-gp activity and fold stimulation by a test compound were calculated according to the following equations:

$$\text{Basal Pgp activity (pmole ATP consumed/μg Pgp/minute)} = \frac{\text{ATP}_{\text{vanadate}} - \text{ATP}_{\text{control}}}{25_{\mu\text{gPgp}} \times 40_{\text{min}}} \quad (1)$$

$$\text{Test compound stimulated Pgp activity (pmole ATP consumed/μg Pgp/minute)} = \frac{\text{ATP}_{\text{vanadate}} - \text{ATP}_{\text{compound}}}{25_{\mu\text{gPgp}} \times 40_{\text{min}}} \quad (2)$$

where $\text{ATP}_{\text{vanadate}}$ is the number of non-consumed (total) pmoles of ATP in the presence of sodium orthovanadate, $\text{ATP}_{\text{control}}$ is the number of non-consumed pmoles of ATP in the presence of the assay buffer, and $\text{ATP}_{\text{compound}}$ is the number of non-consumed pmoles of ATP in the presence of a test compound.

$$\text{Fold stimulation by a test compound} = \frac{\text{Test compound stimulated Pgp activity}}{\text{Basal Pgp activity}} \quad (3)$$

2.8.2. Permeability calculations and cell culture data analysis

The calculation of apparent permeability (P_{app}) for transport studies across cell monolayers was determined from the following equation:

$$P_{\text{app}} = \frac{V_r^{dC_r}/dt}{AC_d} \quad (4)$$

where P_{app} is the permeability, V_r is the receiver compartment volume, dC_r is the receiver compartment concentration at the end of the interval, dt is the time of the interval, A is the area of the filter, and C_d is the donor compartment concentration at the start of the interval. All experiments were performed in triplicate, and data from the transport experiments are presented as mean ± standard deviation (SD). The efflux ratio (R) was calculated according to the following equation:

$$R = \frac{P_{\text{app}}(B \rightarrow A)}{P_{\text{app}}(A \rightarrow B)} \quad (5)$$

2.8.3. In vitro metabolism data analysis

In vitro metabolism data were converted to the percent remaining of Salvinorin A as below:

$$\%\ remaining = \frac{C_{\text{treated}} * 100}{C_{\text{initial}}} \quad (6)$$

where C_{treated} is the enzyme-treated Salvinorin A concentration, C_{initial} is the initial Salvinorin A concentration. All metabolism experiments for each enzyme were done in triplicate, and data are presented as mean ± SD. Data were statistically compared by Student's t-test, and significance was set at $p < 0.05$.

2.8.4. Pharmacokinetic analysis

Non-compartmental modeling was used to estimate Salvinorin A pharmacokinetics parameters after single-dose administration. The Salvinorin A plasma or brain concentration–time data were evaluated using the nonlinear regression program WinNonlin™ (Pharsight Corp., Mountainview, CA; version 4.1). Pharmacokinetic parameters C_{max} (maximum Salvinorin A concentration), t_{max} (the time when C_{max} occurred), AUC_{0-240} (area under the plasma concentration time curve from 0 up to 240 min), $\text{AUC}_{0\text{-inf}}$ (area under the plasma concentration time curve from 0 up to infinity), k_{el} (elimination rate constant), $t_{1/2}$ (half-life), Vd/F (volume of distribution), and Cl/F (clearance) were determined using non-compartmental analysis. Brain distribution of Salvinorin A was determined by calculating brain to plasma partition coefficient of Salvinorin A (R_{i}). The $\text{AUC}_{0\text{-inf}}$ for both plasma and brain was determined by non-compartmental methods utilizing the linear trapezoidal rule. R_{iAUC} was calculated according to the following equation using the brain and plasma $\text{AUC}_{0\text{-inf}}$ values:

$$R_{\text{iAUC}} = \frac{\text{AUC}_{0\text{-inf(brain)}}}{\text{AUC}_{0\text{-inf(plasma)}}} \quad (7)$$

It should be noted that this was a destructive sampling study design, and hence variability associated with the pharmacokinetic parameters could not be determined.

3. Results

3.1. Salvinorin A-stimulated P-gp ATPase activity

Various concentrations of Salvinorin A (2, 5, 10 μM) were examined for their effects on P-gp ATPase activity. Each Salvinorin A concentration together with a known excess of ATP was incubated with recombinant human P-gp for 100 min. ATP consumption was detected as a decrease in luminescence, i.e. the higher the stimulation of the P-gp ATPase activity, the lower the luminescence signal. As seen in Table 2, concentrations of Salvinorin A of 5 μM and 10 μM, and the rate of ATP consumption (47.48 ± 3.36 and 41.14 ± 5.86 pmol/μg P-gp/min) were significantly different ($p < 0.05$) from the control (21 ± 4.21 pmol/μg P-gp/min). The known P-gp substrate, verapamil (200 μM), stimulated the rate of ATP consumption by 103.01 ± 5.86 pmol/μg P-gp/min ($p < 0.05$). The P-gp ATPase assay indicated that the tested Salvinorin A concentrations stimulated P-gp ATPase activity in a concentration-dependent manner (5 μM, 10 μM), indicating that the hallucinogen is most likely a P-gp substrate.

3.2. Salvinorin A transport across MDCK-MDR1

The objective of this study was to determine if this hallucinogen is highly permeable across cell lines expressing P-gp. In MDCK-MDR1 cells, the secretory transport ($4.07 \pm 1.34 \times 10^{-5}$ cm/s) of Salvinorin A was higher than the absorptive transport which could not be determined. It should be noted that the limit of detection of the analytical method was 100 ng/mL for the UV-HPLC method, and was 7.5 ng/mL for the LC/MS/MS method. In MDCK-MDR1 cells, TEER values above 900 Ω cm^2 were used in the study. Mannitol permeability was $3.23 \pm 1.16 \times 10^{-6}$ cm/s, and the paclitaxel efflux was 15.2.

3.3. In vitro metabolism of Salvinorin A

Salvinorin A's chemical structure suggests that it may be a substrate of CYP450 (oxidative metabolism), UGT (hydrolysis) or carboxylesterases (ChEs) (hydrolysis). A recent study has provided

evidence for Salvinorin A's metabolism by blood ChEs [23]. However, CYP450 and UGT enzymes that contribute to its metabolism have not been elucidated. Salvinorin A displays a short duration of action after ingestion, suggesting that it is cleared rapidly and that metabolism most likely plays a significant role in the dissipation of its effect. Metabolism studies using a series of specific human CYP450 isoforms and UGT2B7 enzyme were performed. After 1 h, there was a significant decrease ($p < 0.05$) of 10(±1.2)%, 5.3(±2)%, 6(±1.2)%, and 6.4(±1.6)% when Salvinorin A (50 μM) was incubated with CYP2D6, CYP1A1, CYP2C18, and CYP2E1, respectively, as seen in Fig. 2(a). Further, when Salvinorin A was incubated at a concentration of 5 μM with CYP2D6, CYP1A1, CYP2C18, and CYP2E1, there was also a statistically significant ($p < 0.01$) reduction of 14.7(±0.80)%, 31.1(±1.20)%, 20.6(±1.00)%, and 22(±0.80)%, respectively (Fig. 2b). Salvinorin A (50 μM) percent reductions for CYP1A1, CPY2C18, and CYP2E1 when compared to the 5 μM concentrations were found to be lower. These results suggest that the metabolism of Salvinorin A in these CYP450 isoforms follows Michaelis–Menten kinetics.

In vitro metabolism studies were also conducted to evaluate the role of UGT in Salvinorin A's metabolism. Based on its structure, it was postulated that it would also undergo glucuronidation. Among the UGTs identified in humans, UGT2B7 is the major enzyme involved in glucuronidation of most drugs [28–30]. Because of this, UGT2B7 was used to examine the possible subsequent glucuronidation of Salvinorin A using a baculovirus expression system (Supersomes™). UGT2B7 was incubated with Salvinorin A at 0, 5, 10, and 50 μM. A decrease of 7(±5.60)% ($p < 0.05$), 18.1(±5.20)% ($p < 0.05$), and 51(±4.00)% ($p < 0.01$) was observed for the 50, 10, and 5 μM Salvinorin A concentration vs. the control as illustrated in Fig. 3. In addition, as observed for CYP1A1, CPY2C18 and CYP2E1, Salvinorin A metabolism via UGTB27 appears to be saturable at higher concentrations.

3.4. Pharmacokinetic evaluation

The mean plasma and brain concentration versus time profiles for Salvinorin A after a single i.p. dose of Salvinorin A are presented in Fig. 4(a) and (b), respectively. Brain levels were detected over 1 h. It should be noted that the limit of detection of LS/MS/MS method was 7.5 ng/mL, and recovery was >92%. The pharmacokinetic parameters were calculated using non-compartmental analysis method using WinNonlin™, and pharmacokinetic parameters are summarized in Table 2. Due to the study design (destructive sampling), the variability associated with the pharmacokinetic parameters could not be determined. The plasma profile and brain uptake of Salvinorin A were found to be rapid with an apparent t_{max} occurring at 10–15 min after i.p. administration. $AUC_{0\text{-inf}}$ in plasma and brain was 410 μg h/L and 20.6 μg h/L, respectively. The elimination of Salvinorin A was relatively fast with a $t_{1/2}$ of 75 min and a clearance (Cl/F) of 26 L/h/kg. The distribution was extensive (Vd of 47.1 L/kg); however, the brain to plasma ratio was very low ranging from 0.092 to 0.074 over a 60-min period (Fig. 4). Accordingly, the brain half-life was relatively short, 36 min, and the brain/plasma partitioning was 0.050.

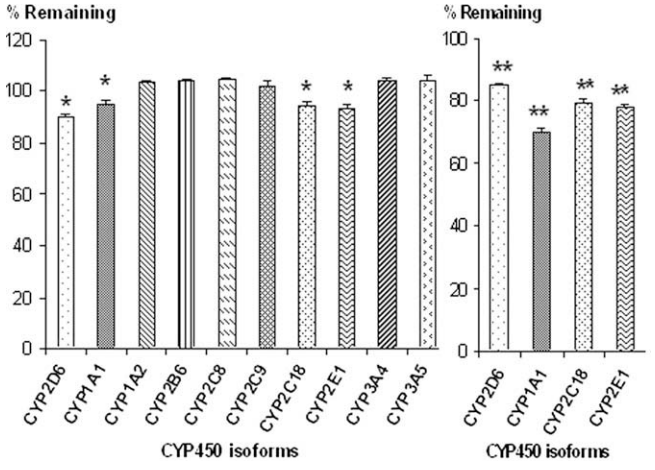

Fig. 2. Salvinorin A *in vitro* metabolism screening with various CYP450 isoforms. Salvinorin was incubated with CYP450 enzymes at a concentration of 50 μM (a) or 5 μM (b) as indicated. The values show the percent reduction in the initial concentration (mean ± SD, $n = 3$). Different from the initial concentration $^{*}p < 0.05$ and $^{**}p < 0.01$.

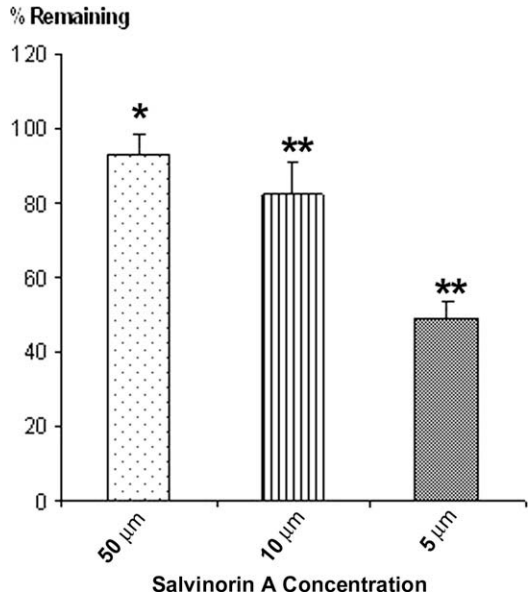

Fig. 3. Salvinorin A *in vitro* metabolism screening with UGT2B7 ($n = 3$). Salvinorin was incubated with UGT2B7 at a concentration of 10 and 50 μM (a), or 5 μM (b) as indicated. The values show the percent reduction in the initial concentration (mean ± SD, $n = 3$). Different from the initial concentration $^{*}p < 0.05$ and $^{**}p < 0.01$.

4. Discussion

Salvinorin A displays a fast onset of its pharmacological action with a relatively short duration of action. After ingestion through smoking, it is transported across the BBB with relative ease and accumulates into the CNS. To better understand the psychotropic activity of Salvinorin A from a pharmacokinetic perspective, we investigated its transport and pharmacokinetic characteristics, potential metabolism pathways, plasma to brain distribution, and the rate and extent of its distribution across the BBB.

The P-gp affinity status of Salvinorin A was evaluated using efflux studies and by assessing its ATPase activity. P-gp is an ATP-dependent efflux pump that is important in multi-drug resistance and certain drug–drug interactions. The MDCK-MDR1 cell line has been used as model of transepithelial transport due to its formation of highly confluent monolayer and the expression of the multi-drug resistance gene (MDR1) encoding high levels of P-gp [31]. In addition, these cell monolayers have been used as an *in vitro* model to predict brain uptake potential and the brain uptake potential of compounds that interact with P-gp [32]. We observed a secretory transport for Salvinorin A ($4.07 \pm 1.34 \times 10^{-5}$ cm/s) which was higher than the absorptive transport (not detected), suggesting P-gp affinity status for this hallucinogen.

The ATPase activity of Salvinorin A was evaluated by assessing P-gp-dependent decrease in luminescence, an indication of ATP

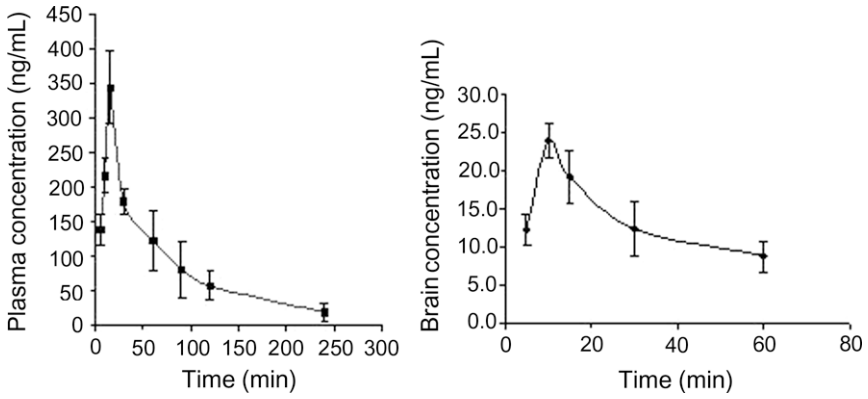

Fig. 4. Salvinorin A (a) plasma concentration versus time profile; (b) brain concentration versus time profile, after a 10 mg/kg i.p. dose to Sprague Dawley rats (n = 3/pt) (mean ± SD).

consumption by P-gp. The results obtained suggest that Salvinorin A (5 and 10 μM) is a substrate of P-gp based on comparison to non-treated cells. We observed a trend toward a decrease in P-gp stimulation with increasing Salvinorin A concentrations, however, the observed fold stimulation range of 1.74 –2.39 (Table 1) is within the range which indicates P-gp stimulation and ATP consumption. The value of 21.31 pmol/μg/P-gp/min for the untreated group is well below the range (37.08–57.08 pmol/μg/P-gp/min) observed for Salvinorin A concentrations (2.5–25 μM) evaluated, suggesting that it is not a P-gp inhibitor. In addition, statistical differences were observed for the Salvinorin A concentrations (5 and 10 μM) vs. the non-treated group, but no statistical differences were observed among the P-gp stimulations for the Salvinorin A concentrations observed.

Even though these results suggest that Salvinorin A is a substrate of P-gp, the implications of these results have to be evaluated within the context of its known *in vivo* potency and brain uptake. Salvinorin A is very lipophilic ($X\log P$ = 2.3), displays an extremely rapid onset of activity *in vivo,* and is extremely potent (dose of 200–500 μg) [2,3,10,13,18,21,24]. Indicated in our pharmacokinetic studies and a recent PET imaging study, Salvinorin A crosses the BBB rapidly after administration [25]. These findings would suggest that even though Salvinorin A appears to be a P-gp substrate, its high lipophilicity, potency and passive permeability offset the impact of P-gp-mediated efflux on the CNS levels achieved.

The chemical structure of Salvinorin A suggests that it may be a substrate of CYP450, UGTs or ChEs. As illustrated in Fig. 1, Salvinorin A has an ester group which serves as a potential site for hydrolytic metabolism by ChEs or conjugative (glucuronidation) metabolism by UGTs enzymes. To determine potential biotransformation pathways of Salvinorin A, we performed *in vitro* metabolism studies by screening with various human CYP450 isoforms and human UGT2B7. We observed significant decreases of 14.7(±0.80), 31.1(±1.20), 20.6(±1.00), and 22(±0.80)% ($p < 0.01$) in the concentration of Salvinorin A (5 μM) upon incubation with CYP2D6, CYP1A1, CYP2C18, and CYP2E1, respectively (Fig. 2). These enzymes therefore may be involved in the metabolism of Salvinorin A *in vivo*. We also observed a significant decrease of 51.0 (±4.00)% ($p < 0.01$) in Salvinorin A (5 μM) concentration when incubated with UGT2B7 (Fig. 3). These results suggest that Salvinorin A is a substrate of UGT2B7. It should be noted that there were lower rates of metabolism for CYP1A1, CPY2C18 CYP2E1, and UGT2B7 at 50 μM vs. 5 μM, suggesting saturable metabolism.

Sharing metabolic pathways as well as having the potential to be used concurrently, clearly suggests the potential for drug interactions with certain abused drugs and Salvinorin A [33]. It appears that Salvinorin A may share pathways of metabolism with drugs of abuse, and this may result in drug–drug interactions. It should be noted that a number of "drugs of abuse" [heroin, codeine and morphine (UGT2B7), oxycodone (CYP2D6, CYP3A4), MDMA (CYP2D6, CYP3A4, CYP1A2), THC (CYP3A4), ketamine (CYP2B6), cocaine (ChEs)] share one or more of the aforementioned metabolic pathways with Salvinorin A [28,29,34–37].

Non-compartmental modeling was used to estimate Salvinorin A pharmacokinetics parameters after single i.p. dose administration. Salvinorin A has been reported to have a rapid onset of action and a short duration [1,2]. Accordingly, the absorption and brain uptake of Salvinorin A were found to be rapid with an apparent t_{max} occurring at 10–15 min after i.p. administration. These data support the immediate effect of Salvinorin A reported in a recent PET imaging study using female baboons [25]. This study observed extremely rapid brain uptake of Salvinorin A reaching a peak 40 s after dosing. In our study, the maximum plasma concentration was found to be 345 ng/mL. Salvinorin A was cleared quickly from the plasma with a Cl/F of 26 L/h/kg and a $t_{1/2}$ of 75.4 min (Table 2). A pharmacokinetic analysis of Salvinorin A was performed after a single dose in non-human primates (n = 4), and plasma $t_{1/2}$ was reported to be 56.6 min [24]. Salvinorin A enters the CNS after

Table 1
Salvinorin A enhancement of P-gp ATPase activity in drug-treated membranes compared with non-treated membranes.

Drug	No. of wells	pmol/μg P-gp/min	Fold stimulation
Non-treated	4	21.31 ± 4.21	1.0
Verapamil (200 μM)	4	103.01 ± 1.99[a]	4.83
Salvinorin A (2.5 μM)	3	50.94 ± 17.87	2.39
Salvinorin A (5 μM)	3	47.78 ± 3.36[a]	2.24
Salvinorin A (10 μM)	3	41.14 ± 5.86[b]	1.93
Salvinorin A (25 μM)	3	37.08 ± 21.18	1.74

[a] Significant difference from the non-treated membranes at $p < 0.01$.
[b] Significant difference from the non-treated membranes at $p < 0.05$.

Table 2
Non-compartmental pharmacokinetic parameters for Salvinorin A after single i.p. dose of 10 mg/kg to male Sprague Dawley rats.

Pharmacokinetic parameter	Plasma	Brain
AUC_{0-240} (μg h /L)	348	13.0
$AUC_{0\text{-inf}}$ (μg h/L)	410	20.6
C_{max} (ng/mL)	345	23.9
t_{max} (min)	15	10
λ_z (h^{-1})	0.552	0.115
$t_{1/2}$ (min)	75.4	36.1
Vd/F (L/kg)	47.1	
Cl/F (L/h/kg)	26.0	

reaching the systemic circulation via buccal absorption, oral ingestion or smoking. Recent findings have revealed exceptionally rapid brain uptake for Salvinorin A when smoked [25]. The major determinants of permeation of drugs across the BBB have long been thought to be lipophilicity and molecular weight [38]. Salvinorin A has a molecular weight of 432 Da, and is highly lipophilic which may explain its rapid uptake in the CNS and it would appear to penetrate the BBB easily. However, our *in vivo* brain uptake results show that although it does rapidly enter the brain, the levels achieved are extremely low as compared to plasma concentrations. Also its short duration of action suggests that its clearance from the systemic circulation is relatively high.

5. Conclusion

In conclusion, the BBB transport, metabolism and pharmacokinetics of Salvinorin A contribute significantly to its rapid onset and short duration of action. Salvinorin A is likely a P-gp substrate; however, this does not appear to significantly minimize the rate at which Salvinorin A enters the brain. Further, its major metabolic pathway is glucuronidation, and as such it may share pathways with abused agents producing botanical/drug interactions. Studies are underway to better understand the fundamental pharmacologic properties of Salvinorin A, and the studies performed herein provide an insight into those BBB transport, pharmacokinetic and metabolic properties of this agent that drive its hallucinogen effect.

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