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In vitro studies on metabolism of salvinorin A

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Abstract
Microbial transformation of natural products is a well established model for mammalian metabolism. Salvinorin A, a diterpenoid isolated from the hallucinogenic mint Salvia divinorum Epling & Jávica-M (Lamiaceae), is a potent non-nitrogenous κ-opioid receptor agonist. The metabolism of salvinorin A has still not yet been well established. Thirty fungal species were screened for the ability to metabolize salvinorin A. We observed that salvinorin A undergoes fast hydrolysis of the acetate group at carbon atom C2, resulting in formation of the pharmacologically inactive product, salvinorin B. Ex vivo experiments were also performed using organelle fractions isolated from rat liver and brain. Crude tissue homogenate and individual organelles show that the primary route of salvinorin A metabolism is hydrolysis to salvinorin B. No metabolic transformation of salvinorin B was observed in these studies.

Keywords: In vitro metabolism; microbial transformation; Salvia divinorum; salvinorin A

Introduction
The diterpenoid salvinorin A is a natural product with hallucinogenic properties (Valdes et al., 1983). It has been isolated from Mexican sage Salvia divinorum Epling & Jávica-M (Labiatae), the only known hallucinogenic species of the Salvia genus. Salvinorin A attracted interest after Roth and co-workers (2002) reported that it is a selective and highly potent naturally occurring κ-opioid receptor agonist. It has become a lead compound in searches for new drugs to combat psychiatric disorders such as schizophrenia, or Alzheimer’s or other dementias, in which κ-opioid receptors are believed to be involved (Sheffler & Roth, 2003). In experiments with monkeys addicted to cocaine, κ-opioid agonists reduce cocaine craving (Mello & Negus, 2000). Recent results show that salvinorin A, when given to rats along with cocaine, attenuates cocaine-induced locomotor activity (Chartoff et al., 2008).

The in vivo metabolism of salvinorin A is not fully understood (Babu et al., 2008). Although studies with isolated body fluids from primates and humans incubated with salvinorin A revealed salvinorin B as a metabolite (Schmidt et al., 2005b), in vivo pharmacokinetic experiments with nonhuman primates failed to detect salvinorin B or any other metabolite in the bloodstream (Schmidt et al., 2005a). Prompted by these conflicting observations, we undertook studies of the fungal transformation and ex vivo metabolism with salvinorin A.

Smith and Rosazza were the first to propose microbes (fungi) as a model for mammalian metabolism (Sebek, 1982). Metabolites from fermentation are similar to those released in phase I (functionalization), or more rarely phase II (conjugation), of xenobiotic modification by mammalian enzymes (Abourashed et al., 1999; Hanson, 1992). Microbial transformation of organic compounds is also a convenient method of obtaining structurally novel leads for drug discovery. The classes of chemicals being enzymatically modified range from simple monoterpensoids (Miyazawa et al., 2003), sesquiterpenoids (artemisinin) (Parshikov et al., 2006), and

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Materials and methods

General experimental procedures

Mass spectra of crude extracts were generated by liquid chromatography-mass spectrometry (LC-MS) (Bruker Daltonik microTOF; Leipzig, Germany). Analysis was performed using a 150 × 4.6 mm C8 column (Phenomenex Luna; Torrance, CA) with 3 µm particle size and a linear gradient ranging from 20% of MeCN to 100% over 20 min. The spectrometer was furnished with a photodiode array detector (Agilent 1100; Palo Alto, CA). Positive ionization mode was used in high-resolution electrospray ionization (HR-ESI)-MS analysis. Nuclear magnetic resonance (NMR) spectra were recorded on a 600 MHz Varian Innova instrument with a 3 mm Nalorac dual broadband probe. The NMR sample of salvinorin B was dissolved in acetonitrile-d₆. Chemical shifts were calibrated to residual solvent resonances. NMR resonance assignments of salvinorin B were according to Giner et al. (2007). Thin layer chromatography (TLC) of transformation products was performed with Merck HF₂₄₅₃ silica gel plates, developed using a mobile phase composed of hexanes and ethyl acetate 1:1, v/v, and visualized by dipping in a 10% solution of vanillin-sulfuric acid reagent (1 g of vanillin in 40 mL of EtOH and 0.5 mL of conc. H₂SO₄), followed by heating at 120°C.

Chemicals

All chemicals were purchased from Fisher Scientific except for Difco potato dextrose broth (Becton, Dickinson and Co., Sparks, MD). Salvia divinorum was purchased from the Salvia divinorum Research Center, Malibu, CA, USA, and deposited in the Department of Pharmacognosy plant repository (voucher specimen number FH4-408-07). Crude plant extract was obtained through exhaustive extraction of 500 g of dry plant material with 6000 mL of acetone (3×2000 mL). Solvent was removed in vacuo at 35°C and reconstituted in ethanol for subsequent crystallization. After 24 h, crystals were filtered and washed with hexane to yield 95% pure salvinorin A. Salvinorin B was prepared as described elsewhere (Chavkin et al., 2004). Briefly, 1 g of salvinorin A was dissolved in 500 mL of MeOH, 1 g of K₂CO₃ was added, and the mixture was stirred for 36 h at room temperature. The precipitate of 95% pure salvinorin B was then filtered and dried under vacuum for further use.

Chromatographic conditions

Residues obtained from liquid extraction of microbial cultures were dissolved in acetonitrile (MeCN) and filtered through a 0.2 µm high-performance liquid chromatography (HPLC) filter (Millipore®-GN; Bedford, MA) prior to analysis. Analytical profiling of extracts was performed using HPLC (Waters Alliance; Waters Corp., Milford, MA) furnished with a Synergy Fusion-RP C18 column (150×10 mm, 4 µm particle size) (Phenomenex). The mobile phase composition consisted of phase A – MeCN, and phase B – water. The gradient started at 20% of phase A and increased to 80% over 30 min, and further to 100% of MeCN in the next 10 min, followed by 10 min of wash with 100% MeCN. Salvinorin A retention time was 20.7 min and salvinorin B 15.6 min. Isolation of metabolites was performed also by HPLC, where the crude extract was loaded onto a Phenomenex C8 (250×10 mm, 5 µm particle size) column connected to an HPLC (Delta Prep 4000; Waters Corp.) system equipped with a dual wavelength detector (Model 2487; Waters Corp.). The mobile phase composition consisted of an MeCN:H₂O (40:60) isocratic system. Retention time for salvinorin A was 11.3 min and salvinorin B 4.1 min. For detection, ultraviolet (UV) radiation, λ = 210 and 254 nm, was used. For analysis of extracts from ex vivo experiments, a Waters C18 XTerra column (100×7.8 mm, 5 µm particle size) was used, where salvinorin A and B retention times were
16.3 min and 12.9 min, respectively. The mobile phase composition was the same as for the analytical method presented above.

**Microorganisms**


**Microbial transformation screening and scale-up**

For all stages of fermentation the same medium containing: peptone, yeast extract, potassium phosphate, sodium chloride, and dextrose in weight ratio 1:1:1:1:4 dissolved in double deionized water was used, and the pH was adjusted to 7.2 by the addition of 0.1 M potassium hydroxide. Medium was autoclaved at 121°C for 15 min. Cultures were grown according to the standard two-stage procedure (Abourashed et al., 1999). Fermentation was inoculated to 125 mL conical Belco Delong steel capped flasks containing 25 mL of broth, where the initial stage was run for 72 h, followed by transfer to a fresh medium. After 1 day of culture, 5 mg of salvinorin A was added, with the final concentration 0.25 mg/mL in dimethylsulfoxide (DMSO) (stage II). The second stage was allowed to proceed for 14 days at 28°C on a rotary shaker (C24KC refrigerated shaker; New Brunswick Scientific, Edison, NJ) at 200 rpm. Control experiments with the same set of microorganisms containing vehicle only were conducted simultaneously. After 14 days of incubation the culture broth was filtered and extracted with ethyl acetate. The organic solvent was evaporated in vacuo at 35°C and samples were later re-dissolved in acetonitrile for analysis. Scale-up experiments were performed under the same experimental conditions with a 10-fold increase in broth volume and substrate content per selected microorganism. The kinetics of salvinorin A conversion was measured by sampling 1 mL of culture broth per day, followed by extraction with ethyl acetate. The organic extract was subsequently injected into an analytical HPLC apparatus and the peak area was measured with regard to standard curves of salvinorins A and B.

**Ex vivo assays**

Adult Sprague-Dawley rats (Harlan, 250–300 g), maintained in a 12 h light/dark cycle and fed ad libitum, were used for the study. The protocol for the study was approved by the University of Mississippi Institutional Animal Care and Use Committee (IACUC). Rat brain and liver were excised upon decapitation. After dissection, the tissues were weighed and immediately homogenized in ice-cold buffer (10 mM Tris and 0.25 M sucrose, pH 7.2). Homogenates were further fractionated by centrifugation at 1000 g for 10 min, 10,000 g for 20 min (Allegra 25R centrifuge; Beckmann Coulter, Fullerton, CA), and 100,000 g for 60 min (Optima Max ultracentrifuge; Beckmann Coulter) to obtain fractions of nuclei, mitochondria, and microsomes, respectively. Assays were carried out with homogenates of each organ or respective organelle fractions in 1 mL of 10 mM Tris buffer (150 mM NaCl, pH 7.2). Treatment was done in triplicate whereas controls were done in duplicate. Treatment samples were incubated with 4 μL (10 mmol) salvinorin A dissolved in DMSO (50 mM stock). Two types of controls were used in the study: the first comprised only buffer and salvinorin A to evaluate the stability of salvinorin A in blank Tris buffer, and the second contained the respective tissue/ organelle fraction and vehicle. The assay was carried out for 1 h at 37°C on a rotary shaker (Thermomixer® R; Eppendorf, Westbury, NY) at 800 rpm. The reaction was terminated by centrifugation at 4°C at 10,000 g for 5 min. The supernatant was then collected and stored at −20°C for further HPLC analysis.

**Salvinorin B**

Salvinorin B was isolated as a white solid, HR-MS m/z [M + H]+ 391.1518 (calcd for C21H26O7 390.1679); NMR spectroscopic data matched those previously published (Giner et al., 2007).

**Results and discussion**

**Microbial transformation of salvinorin A**

Microorganisms used for the experiment were previously reported capable of transforming diterpenoids in reactions such as hydroxylation, oxidation, reduction, or
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epoxidation (Hanson, 1992). A preliminary screening of salvinorin A (5 mg) showed that most of the microorganisms transformed the substrate into salvinorin B (Scheme 1). TLC monitoring of the transformation process revealed that some microbes were capable of transforming approximately 50% of the substrate within the first 24 h after addition of salvinorin A into a medium (data not shown). However, the conversion efficiency varied between organisms, so, for example, C. lunata, M. zonata, R. rhodochrous, M. plumbeus, A. niger, C. bainieri, and R. stolonifer were capable of complete hydrolysis of salvinorin A to salvinorin B within 14 days of incubation. Approximately 70% of salvinorin A was metabolized into salvinorin B by A. glauca, A. ochraceus, and B. bassiana. M. mucedo, M. ramannianus, and R. oryzae hydrolyzed approximately 50% of the substrate in this time period. By contrast, V. lecanii, C. lunata, and C. echinulata poorly transformed salvinorin A (only about 10–15% of conversion to salvinorin B) over 14 days of incubation.

Scale-up experiments involved four species and all were carried out for 14 days: C. bainieri, M. zonata, R. stolonifer, and A. glauca, which were chosen based on their TLC and analytical HPLC chromatographic profiles. LC-MS analysis was performed to confirm the presence of salvinorin B in fermentation extracts. HPLC profiles of extracts from scale-up experiments showed the exact rate of transformation of salvinorin A into salvinorin B. C. bainieri converted salvinorin A to salvinorin B in 100% yield (Figure 1), and was used later on to determine the kinetics of the conversion. The presented data (Figure 2) indicate that 50% of salvinorin A was hydrolyzed to salvinorin B within 48 h, and that it was almost completely converted 6 days after adding substrate to the culture. M. zonata, which also gave 100% conversion of salvinorin A to salvinorin B (~45 mg), was used to isolate and identify the metabolite. NMR spectra of the metabolite were in full agreement with published data, and confirmed that the obtained product was indeed salvinorin B (Giner et al., 2007). R. stolonifer produced salvinorin B in 90% yield, but no other products were detected, comparing with the control strains. The preparative yield of A. glauca did not correlate with that of the screening scale, with only 37% conversion of salvinorin A to salvinorin B.

To make sure that salvinorin B was indeed the final product of microbial metabolism, we performed another experiment where we incubated all 30 microorganisms that we used to biotransform salvinorin A with pure salvinorin B. Attempts to demonstrate further transformation of this compound were unsuccessful.

Scheme 1. Possible routes of hydrolytic transformations of salvinorin A.
The screening experiment revealed that none of the 30 species was able to further metabolize salvinorin B. HPLC analysis of additional scale-up experiments involving *C. echinulata* and *A. glauca* confirmed these results. Both salvinorin A and salvinorin B proved to be stable in the medium with the absence of microorganisms (substrate control), ruling out any possibility of medium-influenced hydrolysis or decomposition of the substrate.

There has been only one report indicating that salvinorin A is rapidly hydrolyzed *in vitro* to its inactive metabolite, salvinorin B. Schmidt and co-workers (2005a) described the formation of salvinorin B within monkey plasma incubated with salvinorin A at 37°C. The authors reported that within the first 15 min of incubation, approximately 20% of salvinorin A was converted to salvinorin B, where blood esterases were mainly responsible for that conversion. They did not observe any other metabolites (Schmidt et al., 2005a). Such a short presence of salvinorin A under *in vitro* conditions was also confirmed by *in vivo* experiments conducted by Pichini and co-workers (2005). The average recovery of salvinorin A from saliva, 1 h post-treatment, was about 18.0 ng/mL, and about 6.7 ng/mL from urine (*n* = 2). The total amount of salvinorin A extracted from urine after that time was about 0.8% of the administered dose (0.5 mg). Blood samples were not analyzed in that study (Pichini et al., 2005). An *in vivo* experiment performed by Schmidt et al. (2005b) also showed rapid elimination of salvinorin A in the rhesus monkey, which varied with gender. For the male rhesus monkey, the elimination half-life was 37.9 ± 5.6 min, whereas for the female it was much slower, 80.0 ± 13.1 min. In none of these *in vivo* experiments, however, did the authors observe the formation of salvinorin B, or any other metabolites of salvinorin A. These should have been detectable in the analyzed matrices (Pichini et al., 2005; Schmidt et al., 2005b).

In our experiments we focused on the microbial model as that which could potentially reflect a wider variety of the phase I enzymes, resembling the situation present in more specific organs such as the liver. Although the use of a variety of microbial species was aimed to assess possible diverse enzymatic catalysis of salvinorin A into more oxidized products than salvinorin B, we were unable to show such transformations. At this point we conclude that rapid decay of the hallucinogenic effect...
of salvinorin A, after administration, is most probably due to a fast and non-specific hydrolysis of the acetate group at C-2 to form salvinorin B. Recent experiments with 2-methoxymethyl salvinorin B, which has an ether instead of an ester bond, shows that its metabolism is greatly slowed by the presence of a hydrolysis-resistant group at C-2 (Wang et al., 2008).

**Ex vivo metabolism of salvinorin A**

We carried out ex vivo metabolism studies using rat liver crude homogenate. Incubation of salvinorin A with liver crude homogenate resulted in complete hydrolysis of substrate to salvinorin B. Further fractionation of the homogenate to nuclei, mitochondria, and microsomes confirmed the same reaction, with 100% conversion in all fractions. Incubation of salvinorin A with cytosol also gave a similar result. The ubiquity of hydrolases in liver tissue gave the expected deacetylation product of salvinorin A. While the liver is the primary site of drug metabolism in many organisms, we were expecting to observe additional routes of metabolism, such as oxidation by cytochrome P450, or conjugation with glucuronic acid. However, none of these reactions were detected. Brain tissue is also a place of drug metabolism, but unlike the liver, it has a different enzyme composition. In our experiment, enzymes from brain homogenate metabolized salvinorin A as efficiently as those from the liver. The incubation of individual organelles with salvinorin A resulted in full hydrolysis to salvinorin B. Incubation with the nuclear and mitochondrial fractions as well as with microsomes and cytosol gave the same result. Although the cytosol is rich in phase II enzymes, we did not observe any transformation product of salvinorin A, characteristic of phase II metabolism.

There is no noticeable difference in metabolism of salvinorin A between brain and liver, two functionally distinct tissues. Although both the liver and the brain contain enzymes of phase I of drug metabolism (cytochrome P450, epoxidehydrolases, dehydrogenases, various types of oxidases, and oxidoreductases), the brain has an effective way of removing drugs from its space, for example by using the P-glycoprotein efflux pump (Allt & Lawrenson, 2000; Cordon-Cardo et al., 1989). Whether there is any deposition of salvinorin B in vivo in any of the analyzed tissues remains unknown.

**Conclusions**

In conclusion, we are reporting here that the biotransformation of salvinorin A, using two independent models of mammalian metabolism (fungi/rat liver and brain), yielded salvinorin B as the same metabolite. The metabolite concentration, however, varied between the models. While the substrate is more available to free enzyme(s) in ex vivo experiments, fungi, on the other hand, vary in mechanisms of substrate uptake and enzyme composition, which may have affected the rate of salvinorin B production. Attempts to demonstrate metabolism of salvinorin B did not yield any further detectable metabolites in either model. Although microbial transformation in general is a good method of derivatization of natural products, it was of limited utility in this case. There is a need for appropriately radiolabeled salvinorin A, which would be a highly valuable tool for tracking metabolites in in vivo models.

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