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TITLE PAGE

SALVINORIN A: ALLOSTERIC INTERACTIONS AT THE MU OPIOID RECEPTOR

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Running title: Salvinorin A allosterically modulates mu opioid receptors

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Non-standard abbreviations: CHO cells (chinese hamster ovary cells); hMOR-CHO (CHO cells expressing the cloned human μ opioid receptor) DAMGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol); Herkinorin, ((2*S*,4a*R*,6a*R*,7*R*,9*S*,10a*S*,10b*R*)-9-(Benzoyloxy)-2-(3-furanyl)dodecahydro-6a,10b-dimethyl-4,10-dioxo-2*H*-naphtho-[2,1-*c*]pyran-7-carboxylic Acid Methyl Ester); [^{35}S]GTP-γ-S (guanosine 5'-*O*-(3-[^{35}S]thio)triphosphate); cAMP (adenosine 3',5'-cyclic monophosphate); U40,488H ((±)-trans-3,4-dichloro-N-methyl-N-[2-

(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide); U69,593 (5α,7α,8β-(-)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-phenyl-benzeneacetamide).

Abstract

Salvinorin A is a hallucinogenic kappa opioid receptor agonist that lacks the usual basic nitrogen atom present in other known opioid ligands. Our first published studies indicated that Salvinorin A weakly inhibited mu receptor binding and subsequent experiments revealed that Salvinorin A partially inhibited mu receptor binding. We therefore hypothesized that Salvinorin A allosterically modulates mu receptor binding. To test this hypothesis, we used CHO cells expressing the cloned human opioid receptor. Salvinorin A partially inhibited [^{3}H]DAMGO (0.5, 2.0 and 8.0 nM) binding with E_{MAX} values of 78.6%, 72.1% and 45.7%, respectively and EC_{50} values of 955, 1124 and 4527 nM, respectively. Salvinorin A also partially inhibited [^{3}H]diprenorphine (0.02, 0.1 and 0.5 nM) binding with E_{MAX} values of 86.2%, 64%, and 33.6%, respectively and EC_{50} values of 1231, 866, 3078 nM, respectively. Saturation binding studies with [^{3}H]DAMGO showed that Salvinorin A (10 and 30 μM) decreased the mu receptor Bmax and increased the Kd in a dose-dependent non-linear manner. Saturation binding studies with [^{3}H]diprenorphine showed that Salvinorin A (10 and 40 μM) decreased the mu receptor Bmax and increased the Kd in a dose-dependent non-linear manner. Similar findings were observed in rat brain with [^{3}H]DAMGO. Kinetic experiments demonstrated that Salvinorin A altered the dissociation kinetics of both [^{3}H]DAMGO and [^{3}H]diprenorphine binding to mu receptors. Additionally, Salvinorin A acted as an uncompetitive inhibitor of DAMGO-stimulated [^{35}S]-GTP-γ-S binding. Viewed collectively, these data support the hypothesis that Salvinorin A allosterically modulates the mu opioid receptor.

Introduction

S. divinorum is a plant from the Sage family that has been used in the traditional spiritual practices by the Mazatec Indians of Oaxaca, Mexico, to produce “mystical” or hallucinogenic experiences. The active ingredient isolated from the leaves of *S. divinorum* is salvinorin A, a neoclerodane diterpene. Current evidence suggests that Salvinorin A-induced hallucinogenic effects are mediated by activation of kappa opioid receptors (for review see (Sheffler and Roth, 2003)).

Salvinorin A, a kappa opioid receptor agonist (Roth et al., 2002), is a unique opioid receptor ligand. It bears little structural similarity to other structural classes of non-peptidic opioid receptor ligands, including kappa agonists such as U50,488H and U69,593 (Harding et al., 2005). The common structural motif among all of these compounds is the presence of a basic amino group. Until recently, it had been assumed that the presence of a positively charged nitrogen atom in opioid compounds represented an absolute requirement for their interaction with opioid receptors (Rees and Hunter, 1990). The general assumption was that this cationic amino charge on the opioid ligand would interact with the side chain carboxyl group of an aspartate residue located in TM III of the opioid receptor (Surratt et al., 1994; Eguchi, 2004). Given the lack of a basic nitrogen in Salvinorin A, this interaction is not an absolute requirement.

The pharmacology of Salvinorin A differs from that of other kappa agonists (Wang et al., 2005). Although Salvinorin A and U50,488H stimulated [^{35}S]-GTP-γ-S binding with similar potency in Chinese Hamster ovary cells (CHO) expressing the cloned human kappa receptor, salvinorin A was about 40-fold less potent than U50,488H in promoting receptor internalization. As observed with other kappa agonists (Devine et al., 1993), Salvinorin A produces decreases in extracellular DA in both mouse caudate (Zhang et al., 2005) and rat n. accumbens (Carlezon et al., 2006).

Our initial binding studies showed that Salvinorin A weakly inhibited mu and delta opioid receptor binding (Roth et al., 2002), a finding replicated by others (Wang et al., 2005). In a subsequent report (Harding et al., 2005), using the radioligand, [^{125}I]IOXY, we generated more detailed Salvinorin A inhibition curves and observed that Salvinorin A, and certain other Salvinorin A analogs, partially inhibited [^{125}I]IOXY binding to the cloned human mu receptor expressed in CHO cells (hMOR-CHO cells). In the present study, we characterized the interaction of Salvinorin A with mu opioid receptors. We report evidence that Salvinorin A allosterically modulates mu receptor binding.

Methods

Cell culture and membrane preparation.

The recombinant CHO cells (hMOR-CHO) were produced by stable transfection with the human opioid receptor cDNA, and provided by Dr. Larry Toll (SRI International, CA). The cells were grown on plastic flasks in DMEM/ F-12 (50%/ 50%) medium (hMOR-CHO) containing 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and G-418 (0.20-0.25 mg/ml) under 95% air/5% CO_2 at 37° C. Cell monolayers were harvested and homogenized using a polytron in 50 mM Tris-HCl, pH 7.4, containing 4 µg/mL leupeptin, 2 µg/mL chymostatin, 10 µg/mL bestatin and 100 µg/mL bacitracin, The homogenate was centrifuged at 15,000 rpm for 10 min at 4° C, and the supernatant discarded. The membrane pellets were resuspended in binding buffer and used for [^{35}S]GTP-γ-S binding assays. For drug pretreatment experiments, the medium was changed, and then cells were incubated with various test drugs for 20 hr. Cells were washed three times with phosphate-buffered saline (PBS, pH 7.4), and cell membranes were prepared as described above. This treatment produces tolerance to opioid drugs (Xu et al., 2003).

[^{35}S]-GTP-γ-S binding assays.

[^{35}S]-GTP-γ-S binding was determined as described previously (Xu et al., 2001). Briefly, test tubes received the following additions: 50 µl buffer A (50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA), 50 µl GDP in buffer A (final concentration = 50 µM), 50 µl drug in buffer A/0.1% BSA, 50 µl [^{35}S]-GTP-γ-S in buffer A (final concentration = 50 pM), and 300 µl of cell membranes (50 µg of protein) in buffer B. The final concentrations of reagents in the [^{35}S]-GTP-γ-S binding assays were: 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT

and 0.1 % BSA. Incubations proceeded for 2 hrs at 25 ° C. Nonspecific binding was determined using GTP-γ-S (40 μM). Bound and free [^{35}S]-GTP-γ-S were separated by vacuum filtration through GF/B filters. The filters were punched into 24-well plates to which was added 0.6 ml LSC-cocktail (Cytoscint). Samples were counted, after an overnight extraction, in a Trilux liquid scintillation counter at 60% efficiency.

Opioid binding assays.

We used [^{3}H][D-Ala^{2}-MePhe^{4},Gly-ol^{5}]enkephalin-([^{3}H]DAMGO) (SA = 46 Ci/mmol), [^{3}H]diprenorphine (SA = 54.9 Ci/mmol) and [^{125}I]IOXY (SA=2200 Ci/mmol) to label mu binding sites. All assays took place in 50 mM Tris-HCl, pH 7.4, with a protease inhibitor cocktail [bacitracin (100 μg/ml), bestatin (10 μg/ml), leupeptin (4 μg/ml) and chymostatin (2 μg/ml)], in a final assay volume of 0.5 ml. Nonspecific binding was determined using 20 μM levallorphan. Triplicate samples were filtered with Brandell Cell Harvesters (Biomedical Research & Development Inc., Gaithersburg, MD), over Whatman GF/B filters, after a 2-3 hr incubation at 25° C. For the [^{125}I]IOXY experiments, the filters were punched into 12 x 75 mm glass test tubes and counted in a Micromedic gamma counter at 80% efficiency. For the [^{3}H]ligand binding assays, the filters were punched into 24-well plates to which was added 0.6 ml LSC-cocktail (Cytoscint). Samples were counted, after an overnight extraction, in a Trilux liquid scintillation counter at 44% efficiency. Opioid binding assays using membranes prepared from hMOR-CHO cells had ~30 μg protein per assay tube.

Inhibition curves were generated by displacing a single concentration of radioligand by 10 concentrations of drug. For binding surface experiments (Rothman, 1986; Rothman et al., 1991) two different concentrations of radioligand were each

displaced by ten concentrations of non-radioactive ligand agents in the absence or presence of various blockers.

Cyclic AMP assays.

Functional coupling of the cloned μ opioid receptor to adenylate cyclase was determined by measuring changes in the levels of cellular cAMP. The assay procedures followed the protocol provided by Molecular Devices, CatchPoint Cyclic–AMP Fluorecent Assay Kit (a horseradish-peroxidase based competitive immunoassay kit). For acute studies, hMOR-CHO cells were grown to 80-90% confluence in 96 well black-walled, clear bottom plates that had been treated with poly-L-lysine. After aspirating the medium, cells were washed with 300 μl/well Krebs-Ringer Bicarbonate Buffer with glucose (KRBG, pH 7.4). KRBG containing 0.75 mM 3-isobutyl-1-methylxanthine and 1 mg/ml bovine serum albumin (KIB) and appropriate agonists were added to each well (90 μl). After 30 min incubation at 37ºC, 100 μM forskolin in KIB was added to each well in a volume of 10 μl. Cyclic AMP production was terminated 40 min later by the addition of 50 μl of a cell lysing solution (Molecular Devices Corporation, Sunnyvale, CA). For chronic studies, cells were grown to 80% confluence in 96 well black-walled, clear bottom plates (Corning Incorporated, Corning, NY #3603) that had been treated with poly-L-lysine. After treatment with medium or 10 μM drug for 20 hr, cells were rinsed three times with 300 μl/well Krebs-Ringer Bicarbonate Buffer with glucose (KRBG, pH 7.4) and assayed as described above. This assay was sensitive between 0.1 and 10 pmoles cAMP in a 40 μl sample volume. A FlexStation II (Molecular Devices) was used to read and quantitate fluorescence intensity of the plate. Data from three experiments

were analyzed using the program Prism (Version 3.0, GraphPad Software, San Diego, CA). Results are presented as the mean ± S.E.M.

Stimulation of p42/p44 MAPK phosphorylation

The assay procedures followed the protocol provided by Cell Signaling, PhosphoPlus p44/42 MAP Kinase (Thr202/Tyr204) Antibody Kit (Beverly, MA). Briefly, cells were grown to 80-90% confluence in 6-well plates. The assay started by the addition of any agonists and stopped after 5 min by rinsing the cells with ice-cold 1 X PBS. Cells were lysed by adding SDS sample buffer (100 µl) and immediately scraped to a microcentrifuge tube on ice, sonicated for 10-15 sec, boiled for 5 min. Samples (20 µl) were loaded onto SDS-PAGE gel as described previously (Xu et al., 2005). Western blots were digitized and quantified using densitometric analysis (NIH Image software). Results from at least 3 experiments were analyzed using the program Prism.

Data Analysis and Statistics

For [^{35}S]-GTP-γ-S binding experiments, the percent stimulation of [^{35}S]-GTP-γ-S binding was calculated according to the following formula: (S – B)/B x 100, where B is the basal level of [^{35}S]-GTP-γ-S binding and S is the stimulated level of [^{35}S]-GTP-γ-S binding (Xu et al., 2004). EC_{50} values (the concentration that produces fifty percent maximal stimulation of [^{35}S]-GTP-γ-S binding) and E_{max} (% of maximal stimulation in the [^{35}S]-GTP-γ-S binding) were determined using the program MLAB-PC (Civilized Software, Bethesda, MD).

The amount of cAMP in the samples was measured using a cAMP standard curve. Forskolin (100 µM) stimulated cAMP formation in the absence of agonist was defined as 100%. The EC_{50} (the concentration of agonist that produces fifty percent

inhibition of forskolin stimulated cAMP formation) and E_{max} (% of maximal inhibition of forskolin stimulated cAMP) were calculated using the program Prism.

In receptor binding experiments, for drugs that produced inhibition curves without apparent plateaus, the data were fit to the two-parameter logistic equation for the best-fit estimates of the IC_{50} and N values (Nightingale et al., 2005). For curves with apparent plateaus, the data were transformed to "percent inhibition" and fit to two parameter dose-response curve model: $Y= E_{MAX} \times ([D]/([D] + EC_{50})$ for the best fit estimates of the E_{MAX} and EC_{50} using either KaleidaGraph version 3.6.4 or MLAB-PC (Nightingale et al., 2005). Radioligand binding surfaces generated with [^{3}H]DAMGO or [^{3}H]diprenorphine were fit to one site binding models using MLAB-PC as described elsewhere (Rothman et al., 1991). Statistical significance among binding parameters was determined using the F-test (Rothman et al., 1991). Dissociation experiments were conducted with minor modification of published procedures, with the data being fit to a two-component dissociation model (Rothman et al., 1991). Statistical significance among kinetic model parameters was determined using the Students t-test.

Sources.

[^{35}S]GTP-γ-S (SA = 1,250 Ci/mmol) was obtained from DuPont NEN (Boston, MA). Various opioid peptides were provided by Multiple Peptide System via the Research Technology Branch, NIDA. [^{125}I]IOXY was prepared as described (de Costa et al., 1992; Ni et al., 1993). The sources of other agents are published (Xu et al., 2004). Salvinorin A and herkinorin were synthesized as described (Harding et al., 2005). For experiments using Salvinorin A or herkinorin, drug dilution curves were made up from freshly prepared 10 mM stock solutions in DMSO. As is our standard operating procedure, all drug dilution curves used buffer with 1 mg/ml bovine serum albumin.

Results

Ligand binding experiments.

Our initial experiments demonstrated that Salvinorin A, whose structure is shown in Fig. 1, partially inhibited [^{125}I]IOXY (0.9 nM) binding to membranes prepared from hMOR-CHO cells (Fig. 1) with an EC_{50} value of 1728 nM and an E_{MAX} value of 48%. Similar results were observed in rat brain membranes, using [^{3}H]DAMGO to label mu receptors (Fig. 2). In this case, Salvinorin A partially inhibited mu receptor binding with an EC_{50} value of 2322 nM and an E_{MAX} value of 60%.

To further characterize Salvinorin A-mediated partial inhibition of mu receptor binding, we generated Salvinorin A inhibition curves using three concentrations of [^{3}H]DAMGO (0.5, 2.0 and 8.0 nM) designed to produce varying levels of mu receptor occupation (the Kd is about 2 nM), using membranes prepared from hMOR-CHO cells. As reported in Fig. 3, Salvinorin A partially inhibited mu receptor binding at all three [^{3}H]DAMGO concentrations. The EC_{50} and E_{MAX} values are reported in Table 1. These results show that the Salvinorin A inhibition curve observed with 8.0 nM [^{3}H]DAMGO resulted in a significantly lower E_{MAX} and higher EC_{50} values as compared to the two lower [^{3}H]DAMGO concentrations.

Similarly, we generated Salvinorin A inhibition curves using three concentrations of [^{3}H]diprenorphine (0.02, 0.1 and 0.5 nM) designed to produce varying levels of mu receptor occupation (the Kd is about 0.7 nM), using membranes prepared from hMOR-CHO cells. As reported in Fig. 4A, Salvinorin A partially inhibited mu receptor binding at all three [^{3}H]diprenorphine concentrations. The EC_{50} and E_{MAX} values are reported in Table 1. These results show that the Salvinorin A inhibition curve observed with 0.5 nM [^{3}H]diprenorphine resulted in a significantly lower E_{MAX} and higher EC_{50} values as compared to the two lower [^{3}H]diprenorphine concentrations. Its apparent from these

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inhibition curves that Salvinorin A is correctly identified as being “inactive”, or having less than 50% inhibition of mu receptor binding at a concentration of 10 μM, when higher [^{3}H]ligand concentrations are used (Roth et al., 2002). In contrast to the results observed for Salvinorin A, both (-)-U50,488, a kappa agonist, and naloxone, a mu receptor antagonist, fully inhibited [^{3}H]diprenorphine binding, producing classical inhibition curves consistent with simple competitive inhibition (Fig. 4B, Table 1).

Using the method of binding surface analysis, we determined the effect of fixed concentrations of Salvinorin A on the Kd and Bmax of [^{3}H]DAMGO binding to membranes prepared from both MOR-CHO cells and rat brain. As reported in Table 2, Salvinorin A had complex actions on the Kd and Bmax values in hMOR-CHO cells. Salvinorin A increased the Kd value in a dose-dependent manner, producing a maximum increase to about 8.9 nM. After increasing the Bmax value at a concentration of 6400 nM, Salvinorin A proceeded to decrease the Bmax value at higher concentrations. These data, normalized as percent changes are reported in Figs. 5A and 5B. The data clearly show that Salvinorin A increased the Kd in dose-dependent non-linear manner with an EC_{50} value of 1730 nM and an E_{MAX} value of 248%. In contrast, a competitive inhibitor increases the Kd in a strictly linear manner. In rat brain, 1000 nM Salvinorin A increased the Kd without changing the Bmax. A higher concentration of Salvinorin A (5000 nM) substantially reduced the Bmax by 48% while increasing the Kd to a smaller extent than 1000 nM Salvinorin A.

We also determined the effect of Salvinorin A on the Kd and Bmax of [^{3}H]diprenorphine binding to hMOR-CHO cells. As reported in Table 3, both 10,000 and 40,000 nM Salvinorin A decreased the Bmax value by about 34% and increased the Kd by about 2-fold. Consistent with the plateau reported in Fig. 4, increasing the Salvinorin A concentration 4-fold from 10,000 to 40,000 nM had no additional effect on

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[^{3}H]diprenorphine binding. Naloxone (10 nM), on the other hand, acted as a competitive inhibitor of [^{3}H]diprenorphine binding to hMOR-CHO cells (Table 3).

Kinetic experiments.

To determine if Salvinorin A altered the rate of [^{3}H]DAMGO dissociation from the mu opioid receptor, membranes prepared from hMOR-CHO cells were incubated with 1 nM [^{3}H]DAMGO for 120 min at 25^o C. At this point, defined as time 0, baseline samples were filtered, and then drugs were added into paired samples to generate the following conditions: control (no addition), DAMGO (10 μM), Salvinorin A (30 μM), DAMGO (10 μM) + SA (30 μM). Samples were then filtered at the indicated time points. As reported in Fig. 6, the addition of Salvinorin A to DAMGO appeared to slightly speed up the dissociation [^{3}H]DAMGO binding, whereas the addition of Salvinorin A appeared to slow the dissociation [^{3}H]DAMGO binding. Quantitative analysis of these data revealed that a two-component dissociation model fit the data much better than a one-component model ($p<1E-10$) (Table 4), and that the addition of Salvinorin A to the DAMGO condition significantly increased the dissociation rate constant (K2) of the faster-dissociating component. Salvinorin A significantly decreased (1.5-fold) the dissociation rate constant (K1) of the slower-dissociating component, accounting for the apparent slower dissociation rate observed in this condition.

Hoping to conduct a dissociation experiment under conditions of a one component dissociation model, we repeated this experiment using the antagonist, [^{3}H]diprenorphine. These experiments were conducted at 37^o C, since [^{3}H]diprenorphine dissociation was too slow at 25^o C. As reported in Fig. 7 and Table 5, the addition of 10 μM diprenorphine resulted in a fairly rapid dissociation that was best described by a two component dissociation model. Salvinorin A alone (30 μM) resulted in a much slower dissociation of [^{3}H]diprenorphine, an observation mainly accounted for by a decreased

value of K1 from 0.021 min^{-1} to 0.0025 min^{-1} (Table 5). Interestingly, the addition of an approximately IC_{50} concentration (-)-U50,488 (1 μM) produced a dissociation curve not significantly different than Salvinorin A. Importantly, the diprenorphine+Salvinorin A condition resulted in statistically significant changes in the kinetic parameters as compared to the diprenorphine condition: decreased A1, decreased K1 and increased A2.

Functional experiments.

Using the [^{35}S]-GTP-γ-S binding assay, we assessed the effect of Salvinorin A on measures of mu receptor function. As reported in Fig 8A and Table 6, Salvinorin A weakly stimulated [^{35}S]-GTP-γ-S binding with an EC_{50} of about 65,000 nM and an extrapolated E_{MAX} value (202%) about 40% lower than that of DAMGO. Interestingly, 10 nM naloxone reduced Salvinorin A-stimulated [^{35}S]-GTP-γ-S binding in a non-competitive manner, significantly reducing the E_{MAX} value without changing the ED_{50} value.

Consistent with the partial agonist profile described above, Salvinorin A significantly reduced the E_{MAX} value of DAMGO-stimulated [^{35}S]-GTP-γ-S binding (Fig. 8B, Table 6) by 31% and 42% at 10 μM and 50 μM, respectively. Salvinorin A also increased the DAMGO ED_{50} values. At a concentration of 10 μM, Salvinorin A increased the ED_{50} for DAMGO from 39 nM to 192 nM, resulting in a calculated Ke (antagonist Ki value) of 2549 nM. If simple competitive antagonism of Salvinorin A at the mu receptor were responsible for this 4.9-fold increase in the DAMGO ED_{50}, then one would predict that a 5-fold increase in the Salvinorin A concentration to 50 μM should further increase the DAMGO ED_{50} to 804 nM, such that the same Ke value would result. However, 50 μM Salvinorin A increased the DAMGO ED_{50} only an additional 1.13-fold to 218 nM. In

contrast, 10 nM naloxone, a competitive antagonist, increased the DAMGO ED_{50} more than predicted on the basis of its Ke determined with a 2.5 nM dose.

We determined the effect of Salvinorin A (50 µM) and DAMGO (10 µM) on basal and forskolin-stimulated cAMP levels. As reported in Fig. 9A, DAMGO and Salvinorin A did not alter basal cAMP levels. As expected, DAMGO almost completely, inhibited forskolin-stimulated cAMP accumulation. Salvinorin A inhibited forskolin-stimulated cAMP accumulation by 44%. We compared the effect of naloxone (a competitive inhibitor) and Salvinorin A on DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation in the hMOR-CHO cells. As reported in Table 7, 50 µM Salvinorin A significantly increased the ED_{50} value. The calculated apparent Ke was 27 µM. Salvinorin A also decreased the E_{MAX} value by ~9%. In contrast, naloxone increased the ED_{50} without decreasing the E_{MAX}. Thus, in the cAMP assay, Salvinorin A demonstrated partial agonist activity.

The cellular adaptations produced by chronic opioids are generally accepted as signs of opioid dependence. We next determined the effect of Salvinorin A (50 µM) on the cellular adaptations produced by chronic treatment of cells with DAMGO (10 µM) and the novel mu opioid agonist, herkinorin (10 µM) (Harding et al., 2005). Cells were treated for 20 hr with DAMGO or herkinorin, in the absence and presence of Salvinorin A. We measured two endpoints: forskolin-stimulated cAMP, which detects cAMP super-activation, and naloxone-stimulated cAMP in the presence of forskolin, which detects the presence of constitutively active receptors. As reported in Fig. 9B, chronic DAMGO treatment produced cAMP super-activation without a naloxone overshoot. Chronic herkinorin treatment reduced forskolin-stimulated cAMP, but the addition of naloxone revealed the occurrence of cAMP super-activation and the presence of constitutively

active receptors. Salvinorin A did not change the cellular response to either treatment. However, chronic Salvinorin A treatment produced signs of cAMP super-activation.

In contrast to the activity of Salvinorin A in functional assays that measured changes in the level of cellular cAMP, Salvinorin A was inactive in the MAP kinase assay, which is activated by the Gβγ subunit. As reported in Fig. 10, DAMGO stimulated the phosphorylation of MAP kinase. Salvinorin A alone had no effect and did not alter the effect of DAMGO in this assay.

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Discussion

As described in a recent review (Christopoulos and Kenakin, 2002), allosteric modulators of G protein-coupled receptors (GPCR) may be of interest as potential targets for medication development. There are few reports of allosteric modulators of opioid receptors that we are aware of. In 1987, Vaysse et al. (Vaysse et al., 1987) reported that cannabidiol non-competitively inhibited radioligand binding to mu and delta opioid receptors, a finding consistent with allosteric modulation. Subsequent work by another laboratory provided additional evidence for this hypothesis by showing that cannabidiol accelerated the dissociation of [^{3}H]DAMGO and [^{3}H]naltrindole from rat brain mu and delta receptors, respectively (Kathmann et al., 2006). We reported in 1991 that pre-treating rat brain membranes with (+)-cis-methylfentanyl increased the dissociation rate of mu receptors labeled with [^{3}H]ohmfentanyl (Xu et al., 1991).

In our recent paper we briefly noted that Salvinorin A, a potent kappa opioid receptor agonist (Roth et al., 2002), partially inhibited [^{125}I]IOXY binding to mu opioid receptors (see Table 1 in (Harding et al., 2005)). In the present study, we tested the hypothesis that Salvinorin A allosterically modulates mu opioid receptors. Several lines of evidence support this hypothesis.

First, Salvinorin A partially inhibits mu receptor binding, using both the cloned human mu receptor expressed in CHO cells, as well as the native mu receptor present in rat brain membranes. We observed a partial inhibition pattern with three radioligands: [^{3}H]DAMGO, [^{3}H]diprenorphine and [^{125}I]IOXY. As reported in Figs. 3 and 4, the presence of a plateau is most readily observed using radioligand concentrations sufficient to occupy a substantial fraction of mu receptors, such as 0.9 nM [^{125}I]IOXY (Fig. 1), 8 nM [^{3}H]DAMGO (Fig. 3) or 0.5 nM [^{3}H]diprenorphine (Fig. 4). The partial inhibition pattern we observed, where the inhibition curve shifts to the right with a lower

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E_{MAX} value, as the radioligand concentration is increased, is consistent with the theoretical predictions made by Ehlert in 1988 for negative allosteric modulators (Ehlert, 1988). This partial inhibition pattern is unique to Salvinorin A, since (-)-U50,488, a potent kappa agonist, and naloxone, a competitive mu antagonist, produce "normal" inhibition curves without any evidence of a plateau.

Second, Salvinorin A affects the Kd and Bmax of the mu receptor in a manner inconsistent with competitive binding. Using [^{3}H]DAMGO and hMOR-CHO cells, Salvinorin A first increases the mu receptor Bmax, followed by highly significant decreases in the Bmax at higher concentrations. Importantly, Salvinorin A increases the Kd of the mu receptor in a dose-dependent manner (Fig. 5A), rather than a linear manner, as would observed with a competitive inhibitor (Ehlert, 1988). Thus, the ability of Salvinorin A to increase the Kd reaches a ceiling at about 200% of control. Salvinorin A-mediated uncompetitive inhibition of mu receptor binding is also observed in rat brain (Table 2) and in hMOR-CHO cells using [^{3}H]diprenorphine (Table 3).

Third, Salvinorin A alters the kinetics of radioligand dissociation. It is well known that allosteric modulators can alter the rate of radioligand dissociation (Kostenis and Mohr, 1996). As reported in Table 4, [^{3}H]DAMGO dissociation from mu receptors expressed in hMOR-CHO cells was bi-exponential, with readily measurable slower (K1 = 0.006 min^{-1}) and faster (K2 = 0.10 min^{-1}) components. The addition of Salvinorin A to DAMGO increased the faster dissociation rate by 160% to 0.16 min^{-1} and decreased the A2 value by 24% to 26%. Interestingly, the addition of Salvinorin A alone decreased K1 by 33% to 0.004 min^{-1}. When the mu receptors were labeled with an antagonist ([^{3}H]diprenorphine), the addition of Salvinorin A alone substantially slowed [^{3}H]diprenorphine dissociation (Fig. 7) mainly by decreasing K1 by over 10-fold. Viewed in context with the other findings, such as the partial inhibition pattern, these data are

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consistent with an allosteric effect. However, the addition of a non-alllosteric compound, (-)-U50,488, at an approximately IC_{50} concentration, resulted in a similarly slowed dissociation of [^{3}H]diprenorphine, making it more difficult to interpret the slowed [^{3}H]diprenorphine dissociation produced by Salvinorin A. However, the concurrent addition of Salvinorin A and diprenorphine decreased the A1 value by 31%, decreased the K1 value by 43% and increased the K2 value by 162%, providing direct evidence for an allosteric effect of Salvinorin A.

Four, Salvinorin A acts as an uncompetitive inhibitor of DAMGO-stimulated [^{35}S]-GTP-γ-S binding (Fig. 8B). As reported in Table 6, Salvinorin A produced a dose-dependent decrease in the E_{MAX} and failed to increase the ED_{50} value significantly when the Salvinorin A concentration was increased from 10 μM to 50 μM, as was observed for a competitive inhibitor, naloxone. We believe that these four lines of data support the hypothesis that Salvinorin A allosterically modulates mu receptor binding and function.

The effects of Salvinorin A in the functional assays deserve additional study. For example, Salvinorin A stimulates [^{35}S]-GTP-γ-S binding with low potency and an extrapolated E_{MAX} value about 42% that of DAMGO. The simplest explanation of these data is that Salvinorin A is a weak partial mu agonist. This finding is supported by the ability of Salvinorin A to decrease forskolin-stimulated cAMP accumulation and to induce cAMP superactivation (Fig. 9). However, naloxone non-competitively inhibits Salvinorin A-stimulated [^{35}S]-GTP-γ-S binding, suggesting that Salvinorin A acts at a site on the mu receptor distinct from that of typical mu ligands. This viewpoint is supported by point four elaborated on in the previous paragraph as well as the fact that Salvinorin A, unlike DAMGO, had no activity in the MAP kinase assay (Fig. 10). Viewed collectively, the functional data indicate that Salvinorin A may have some partial agonist activity at mu receptors in addition to the allosteric effects described above. Assuming

that Salvinorin A has partial agonist activity at mu receptors, it might be possible to detect mu-mediated antinociception following administration of Salvinorin A. However, Salvinorin A-induced antinociception is not observed in kappa receptor knock out mice (Ansonoff et al., 2006), suggesting that the potency of Salvinorin A as a partial mu agonist is probably too low to produce detectable antinociception.

The ultimate significance of this work remains to be seen. However, the immediate significance of our findings is the clear demonstration that the mu opioid receptor possesses an allosteric modulator site. A major challenge of subsequent work will be to identify more potent ligands for the allosteric site. Towards this end, we note that certain analogs of Salvinorin A also partially inhibit mu opioid receptors (Tidgewell et al., 2006) as well as delta opioid receptors (unpublished data). Thus, we anticipate that the Salvinorin A structural template will yield a number of allosteric modulators of opioid receptors. A more complete structure-activity profile of the allosteric site will be used to design more potent allosteric ligands. Once these are available, it will be possible to determine the in vivo effects and potential therapeutic application of allosteric modulators of mu opioid receptors. It is possible, as observed with other classes of medications that work via allosteric mechanisms (benzodiazepines), that allosteric modulators of opioid receptors will have therapeutic value. Since a positive allosteric modulator will enhance the action of endogenous ligands acting via mu receptors, such a drug could produce analgesia with fewer adverse effects than produced by direct mu receptor agonists. Additionally, it is unfortunate that we do not yet know the molecular basis of the allosteric actions of Salvinorin A at the mu opioid receptor. Site-directed mutagenesis studies, such as those that delineated the interaction of Salvinorin A at the kappa opioid receptor (Yan et al., 2005), will be necessary to definitively prove that the Salvinorin-A induced allosteric effects reported

here are mediated via a binding site on the mu receptor distinct from the binding site of other mu ligands.

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Legends for Figures

Legend to Figure 1. Inhibition of [^{125}I]IOXY binding to hMOR-CHO cell membranes by Salvinorin A. [^{125}I]IOXY (0.9 nM) was displaced by ten concentrations of Salvinorin A. The data of three experiments, expressed as percent inhibition, were combined and analyzed for the best-fit estimates of the E_{MAX} and EC_{50} (±SD) as described in methods. Each point is the mean±SD (n=3).

Legend to Figure 2. Inhibition of [^{3}H]DAMGO binding to rat brain membranes by Salvinorin A. [^{3}H]DAMGO (0.8 nM) was displaced by fourteen concentrations of Salvinorin A. The data of two experiments, expressed as percent inhibition, were combined and analyzed for the best-fit estimates of the E_{MAX} and EC_{50} (±SD) as described in methods. Each point is the mean±SD (n=2).

Legend to Figure 3. Inhibition of [^{3}H]DAMGO binding to hMOR-CHO membranes by Salvinorin A. Three concentrations of [^{3}H]DAMGO were each displaced by ten concentrations of Salvinorin A. The data, expressed as percent inhibition, were combined and analyzed for the best-fit estimates of the E_{MAX} and EC_{50} (See Table 1) as described in methods. Each point is the mean±SEM (n=3-7).

Legend to Figure 4. Inhibition of [^{3}H]diprenorphine binding to hMOR-CHO membranes by Salvinorin A. Three concentrations of [^{3}H] diprenorphine were each displaced by ten concentrations of Salvinorin A (Panel A) or (-)-U50,488 (Panel B). The data of three experiments, expressed as percent inhibition, were combined and analyzed for the best-

fit estimates of the E_{MAX} and EC_{50} (See Table 1) as described in methods. Each point is the mean±SEM (n=3).

Legend to Figure 5. Salvinorin A has complex effects on the Bmax and Kd of mu receptors labeled by [^{3}H]DAMGO in hMOR-CHO cell membranes. As described in the legend to Table 2, DAMGO binding surfaces were generated by displacing two concentrations of [^{3}H]DAMGO (0.5 and 2.5 nM) by 9 concentrations of DAMGO in the absence and presence of the indicated concentrations of Salvinorin A. **Panel A.** Salvinorin A increases the [^{3}H]DAMGO Kd value in a dose-dependent manner. All Salvinorin A-induced Kd changes were statistically significant. Each value is the ±SD (n=3-4). **Panel B.** Salvinorin A decreases the [^{3}H]DAMGO Bmax value in at 10 µM and 30 µM. Each value is the ±SD (n=3-4). *p<0.01 when compared to control (F-test).

Legend to Figure 6. Salvinorin A alters the dissociation of [^{3}H]DAMGO binding from membranes prepared from hMOR-CHO cells. Membranes were incubated with 1 nM [^{3}H]DAMGO for 120 min at 25^o C. At this point, defined as time 0, baseline samples were filtered, and then drugs were added into paired samples to generate the following conditions: control (no addition), DAMGO (10 µM), Salvinorin A (30 µM), DAMGO (10 µM) + Salvinorin A (30 µM). Samples were then filtered at the indicated time points. The percent of control was the binding observed in the control condition. Each point is ±SD (n=8-12).

Legend to Figure 7. Salvinorin A alters the dissociation of [^{3}H]diprenorphine binding from membranes prepared from hMOR-CHO cells. Membranes were incubated with 0.1 nM [^{3}H]diprenorphine for 120 min at 37^o C. At this point, defined as time 0, baseline

samples were filtered, and then drugs were added into paired samples to generate the following conditions: control (no addition), diprenorphine (10 μM), Salvinorin A (30 μM), diprenorphine (10 μM) + Salvinorin A (30 μM), (-)-U50,488 (1 μM), (-)-U50,488 (1 μM) + diprenorphine (10 μM). Samples were then filtered at the indicated time points. The percent of control was the binding observed in the control condition. Each point is ±SD (n=4).

Legend to Figure 8. **Panel A.** Salvinorin A-stimulated [^{35}S]-GTP-γ-S binding in hMOR-CHO cells. Using membranes prepared from hMOR-CHO cells, Salvinorin A dose-response curves were generated in the absence and presence of 10 nM naloxone The data were pooled and analyzed for the best-fit estimates of the E_{MAX} and ED_{50} (see Table 6). Each value is ±SD (n=3). **Panel B.** Salvinorin A antagonizes DAMGO-stimulated [^{35}S]-GTP-γ-S binding. Using membranes prepared from hMOR-CHO cells, DAMGO dose-response curves were generated in the absence and presence of various concentrations of Salvinorin A and naloxone. The data were pooled and analyzed for the best-fit estimates of the E_{MAX} and ED_{50} (see Table 6). Each value is ±SD (n=3).

Legend to Figure 9. Effects of Salvinorin A on the cAMP.

Panel A: Comparison of Salvinorin A (50 μM) and DAMGO (10 μM) on basal and forskolin-stimulated cAMP accumulation. Each value is ± SEM (n=3). *p < 0.01 when compared with basal, #p < 0.01 when compared with forskolin group.

Panel B. Comparison of the effects of naloxone (10 μM) on forskolin (100 μM)-stimulated cAMP accumulation in the control or pretreated hMOR-CHO cells. Results are presented as mean ± SEM (n=3). *P < 0.05 when compared with control cells (two-

tailed or one-tailed Students t-test); #P < 0.01 when compared with no addition group (two-tailed Students t-test).

Legend to Figure 10. Agonist-stimulated p42/p44 MAP kinase phosphorylation in the hMOR-CHO cells. The assay was started by the addition of test agents and stopped after 5 min. Western blotting was performed as described in the Methods section. Results are presented as mean ± SEM (n=4). Representative blots of phosphorylated MAP kinase (A) and total MAP kinase (B) are shown. The concentration of Salvinorin A (SA) was 50 μM. *p < 0.01 when compared with no addition group (two-tailed Students t-test).

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Table 1

Summary of Results for Inhibition Curves in hMOR-CHO Cells

[^{3}H]DAMGO	Plateau (E_{MAX}) Value (%±SD)	EC_{50} Value (nM±SD)
Salvinorin A		
0.5 nM (n=7)	78.6±2.0	955±112
2.0 nM (n=7)	72.1±2.2	1124±152
8.0 nM (n=3)	45.7±3.3*	4527±1108*
Salvinorin A		
[^{3}H]DIPRENORPHINE		
0.02 nM (n=4)	68.2±3.1	1231±241
0.1 nM (n=4)	64.0±3.1	866±192
0.5 nM (n=4)	33.6±4.6*	3078±830*
(-)-U50,488		
0.02 nM (n=3)	100±5.2	1166±138
0.1 nM (n=3)	89.0±5.6	729±145*
0.5 nM (n=3)	94.5±3.1	2605±228*
Naloxone		
0.02 nM (n=4)	97.5±2.7	2.15±0.28
0.1 nM (n=4)	97.3±2.2	2.46±0.25
0.5 nM (n=4)	103±4.3	11.7±1.6*

Drug inhibition curves were generated using membranes prepared from hMOR-CHO cells using the indication concentrations of either [^{3}H]DAMGO or [^{3}H]diprenorphine. The data of 3-7 experiments were combined and analyzed for the best-fit estimates of the E_{MAX} and EC_{50} (±SD) as described in methods. *$p<0.05$ when compared to 0.02 nM [^{3}H]DIP or 0.5 nM [^{3}H]DAMGO (Students t test).

Table 2

[^{3}H]DAMGO Binding Surfaces

Salvinorin A (nM)	Bmax (fmol/mg protein±SD)	Kd (nM±SD)
Experiment 1 (n=4) **hMOR-CHO cells**		
0	2275±125	2.0±0.1
1600	2225±150	3.9±0.3**
6400	2950±225*	7.0±0.5*
Experiment 2 (n=3) **hMOR-CHO cells**		
0	2725±125	2.8±0.1
10000	2025±175*	8.3±0.6**
30000	1750±200**	8.9±1.0**
Experiment 3 (n=3) **Rat brain**		
0	111±5	1.9±0.1
5000	97±11	6.4±0.64**
10000	53±8**	3.2±0.5*

DAMGO binding surfaces were generated by displacing two concentrations of [^{3}H]DAMGO (0.5 and 2.5 nM) by 9 concentrations of DAMGO in the absence and presence of the indicated concentrations of Salvinorin A. Experiment 1 was done 4 times, generating a total of 160 data points per experimental condition. Experiments 2

and were done 3 times, generating 120 data points per experimental condition. The combined data of each condition was fit to the one site binding model using MLAB-PC for the best-fit estimates (±SD) of the Kd and Bmax values. *p<0.01, **p<0.001 (F-test).

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Table 3

[^{3}H]Diprenorphine Binding Surfaces in hMOR-CHO Cells

Test Drug	Bmax (fmol/mg protein±SD)	Kd (nM±SD)
Salvinorin A (nM) (n=4)		
0	288±18	0.73±0.04
10000	186±18*	1.43±0.11*
40000	190±14*	1.56±0.12*
Naloxone		
0	515±25	0.97±0.05
10 nM	445±47	2.64±0.25*

[^{3}H]Diprenorphine binding surfaces were generated by displacing two concentrations of [^{3}H]diprenorphine (0.08 and 0.47 nM) by 9 concentrations of diprenorphine in the absence and presence of the indicated concentrations of either Salvinorin A or naloxone, generating 20 data points per experimental condition. The combined data of each condition (160 points for the Salvinorin A experiments, 120 points for the naloxone experiments) were fit to the one site binding model using MLAB-PC for the best-fit estimates (±SD) of the Kd and Bmax values. *$p<0.001$ (F-test).

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Table 4

Best-Fit Parameter Estimates for [^{3}H]DAMGO Dissociation

Condition	A1 (%±SD)	K1 min^{-1}±SD	A2 (%±SD)	K2 min^{-1}±SD	N (data points)
DAMGO (10 µM)	64±5	0.006±0.0001	34±5	0.10±0.03	12 (88)
DAMGO (10 µM)+ Salvinorin A (30 µM)	67±3	0.007±0.001	26±4*	0.16±0.06*	8 (61)
Salvinorin A (30 µM)	67±5	0.004±0.001*	37±5	0.08±0.02	12 (84)

[^{3}H]DAMGO (1.0 nM) dissociation curves were generated as described in Methods at 37^o C. The data of each experimental condition, generated with hMOR-CHO cell membranes, were pooled and fit, using MLAB-PC, to the two-component exponential decay model for the best-fit estimates reported above. Each value is the mean±SD (n=8-12). *p<0.05 when compared to the DAMGO condition (Students t-test).

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Table 5

Best-Fit Parameter Estimates for [^{3}H]Diprenorphine Dissociation

Condition	A1 (%±SD)	K1 min^{-1}±SD	A2 (%±SD)	K2 min^{-1}±SD	N (data points)
Diprenorphine (10 μM)	66.8±8.2	0.021±0.003	33.1±8.6	0.22±0.16	4 (28)
Salvinorin A (30 μM)	49.1±3.7*	0.0025±0.0006*	50.5±4.3*	0.093±0.017	4 (28)
Diprenorphine (10 μM) +Salvinorin A (30 μM)	46.0±9.6*	0.012±0.003*	53.6±9.8*	0.10±0.03	4 (28)
(-)-U50,488 (1 μM)	51.8±4.2*	0.0015±0.0006*	47.7±5.4*	0.10±0.03	4 (28)
Diprenorphine (10 μM) + (-)-U50,488 (1 μM)	61.2±11.3	0.021±0.004	38.7±11.6	0.18±0.09	4 (28)

[^{3}H]Diprenorphine (0.1 nM) dissociation curves were generated as described in Methods at 37^o C. The data of each experimental condition, generated with hMOR-CHO cell membranes, were averaged and fit, using KaleidaGraph 3.5, to the two-component exponential decay model for the best-fit estimates reported above. Each value is the

mean±SD (n=4). *p<0.05 when compared to the diprenorphine condition (Students t-test).

Table 6

Effect of Salvinorin A on [^{35}S]-GTP-γ-S Binding

	E_{MAX} (% Increase ±SD)	ED_{50} (nM±SD)	Apparent Ke (nM)
A. Salvinorin A-Stimulated [^{35}S]-GTP-γ-S Binding			
Salvinorin A	202±22	65260±17060	
Salvinorin A + 10 nM naloxone	148±22*	56580±19920	
B. DAMGO-Stimulated [^{35}S]-GTP-γ-S Binding			
DAMGO	349 ± 11	39 ± 4	
DAMGO + 1 uM Salvinorin A	381 ± 16	47 ± 7	
DAMGO + 10 uM Salvinorin A	241 ± 7*	192 ± 14*	2549
DAMGO + 50 uM Salvinorin A	203 ± 9*	218 ± 23*	1089
DAMGO + 2.5 nM naloxone	274 ± 15*	57 ± 12	5.4
DAMGO + 10 nM naloxone	295 ± 10*	146 ± 20*	3.6

DAMGO-stimulated [^{35}S]-GTP-γ-S binding dose response curves were generated using membranes prepared from hMOR-CHO cells as described in methods. The data of

three experiments were pooled and the best-fit estimates of the ED_{50} and E_{MAX} determined using MLAB-PC. Each value is ± SD (n=3). *$p<0.05$ when compared to control E_{MAX} (Students t-test).

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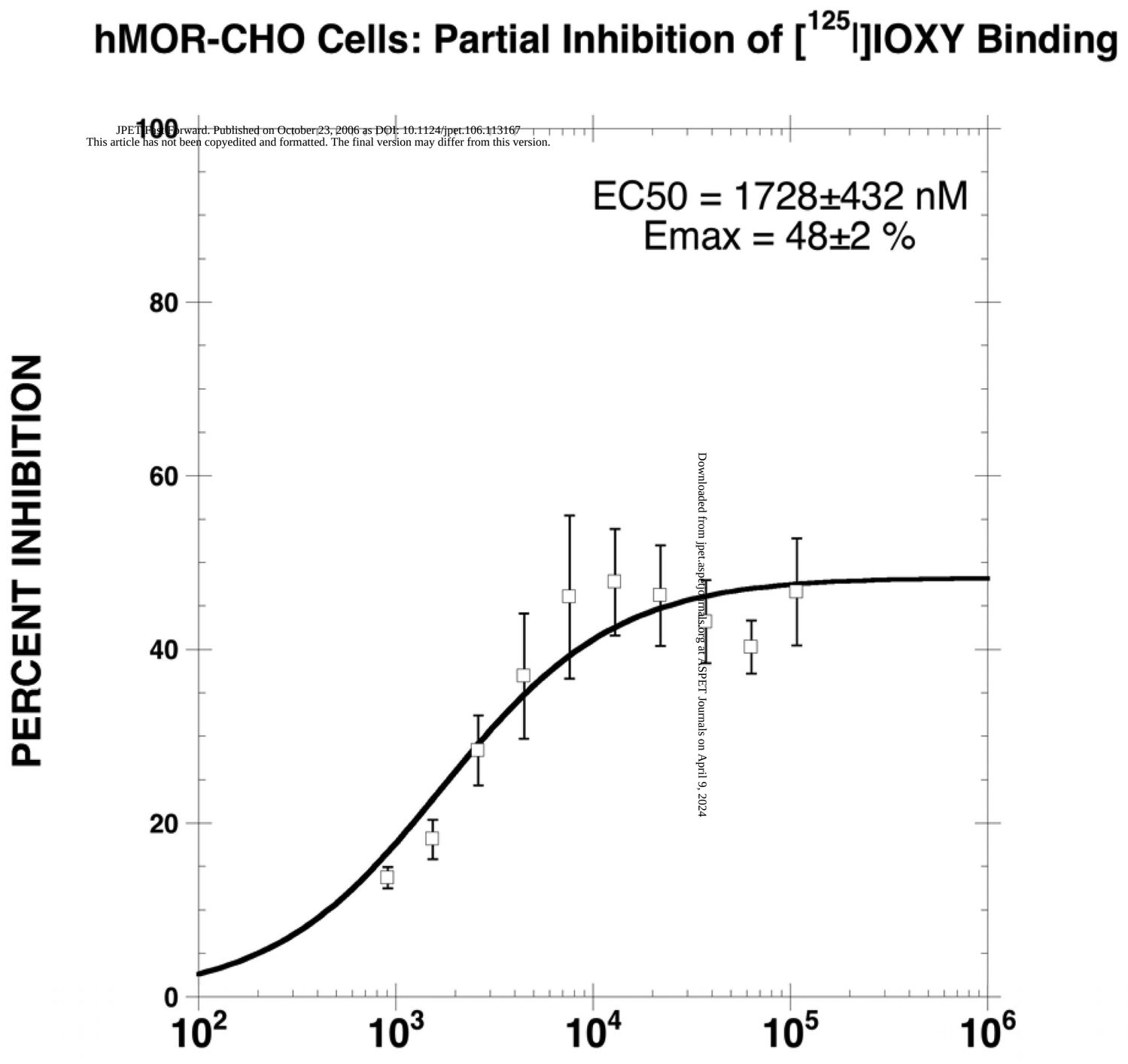
Table 7

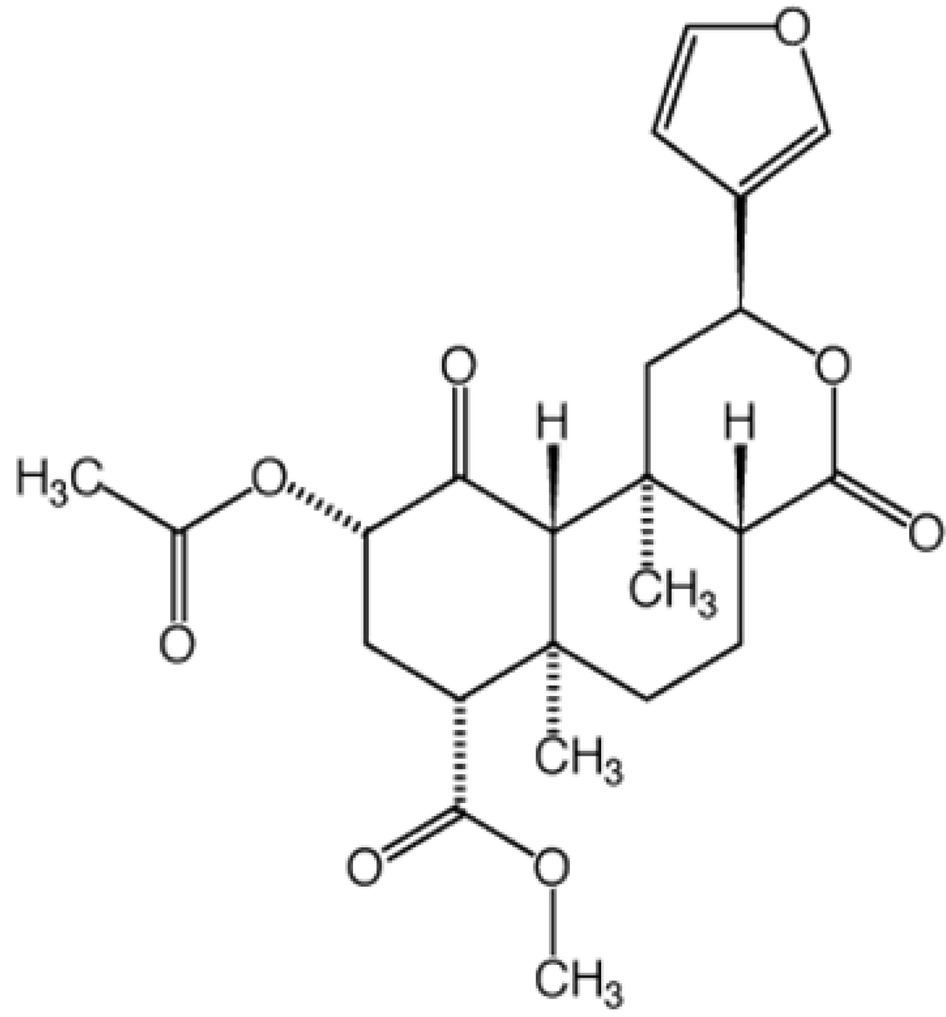
Effect of Salvinorin A on DAMGO-Mediated Inhibition of Forskolin-Stimulated cAMP Accumulation

	ED_{50} (nM±SD)	E_{MAX} (% Maximal Inhibition ±SD)	Apparent Ke
DAMGO	4.0 ± 0.7	85.6 ± 1.0	
DAMGO + 10 µM Salvinorin A	8.7 ± 2.4	81.8 ± 4.0	8.6 µM
DAMGO + 50 µM Salvinorin A	11.6 ± 0.3**	78.7 ± 1.9*	27 µM
DAMGO + 10 nM naloxone	18.9 ± 3.0**	84.1 ± 2.0	2.7 nM

Dose response curves for DAMGO-mediated inhibition of forskolin-stimulated cAMP accumulation were generated as described in Methods. EC_{50} and E_{max} were determined using the program Prism. Each value is the mean ± SEM (n=3). * $p < 0.05$, ** $p < 0.01$.

Figure 1





Salvinorin A

Figure 2

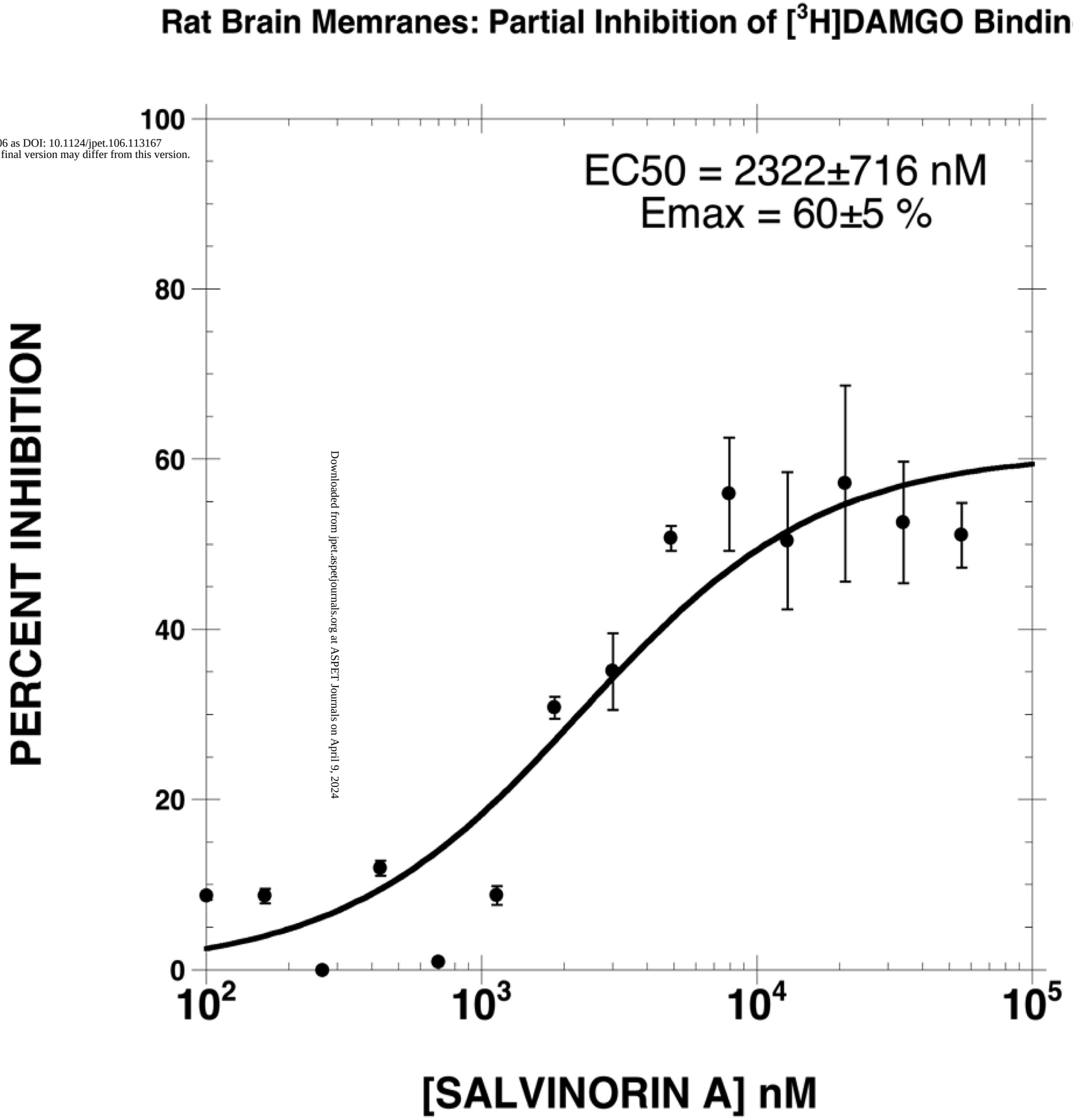


Figure 3

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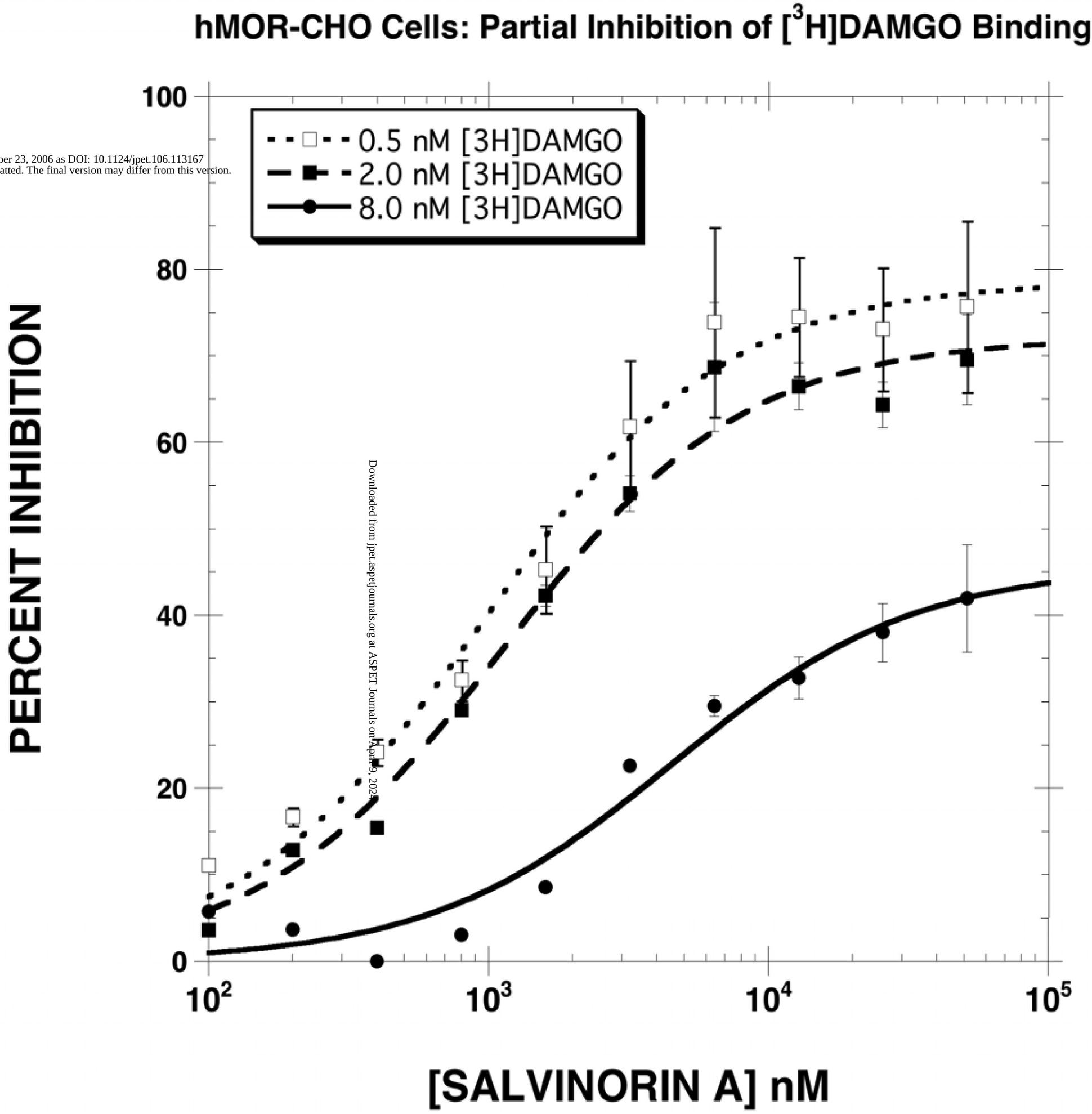
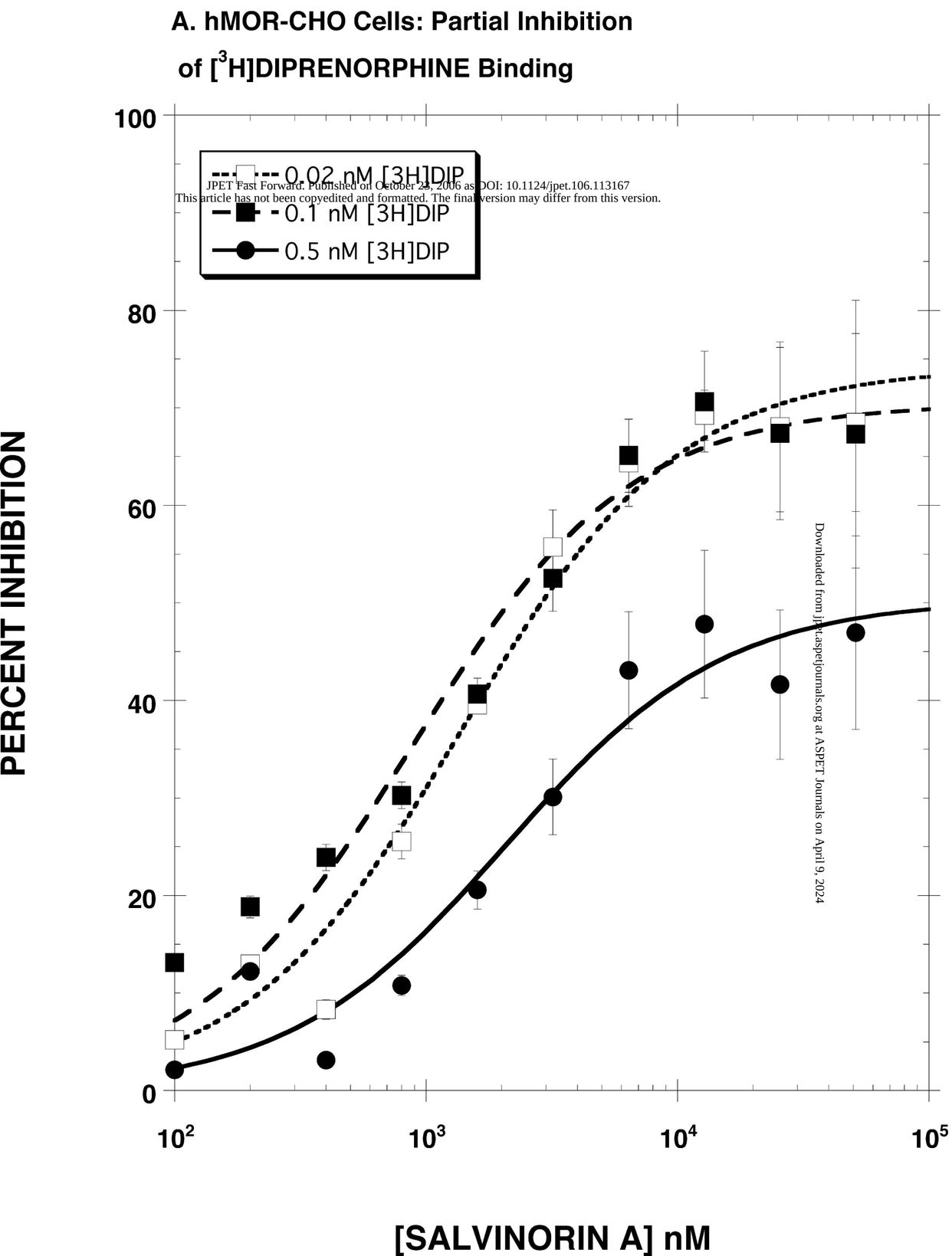


FIGURE 4



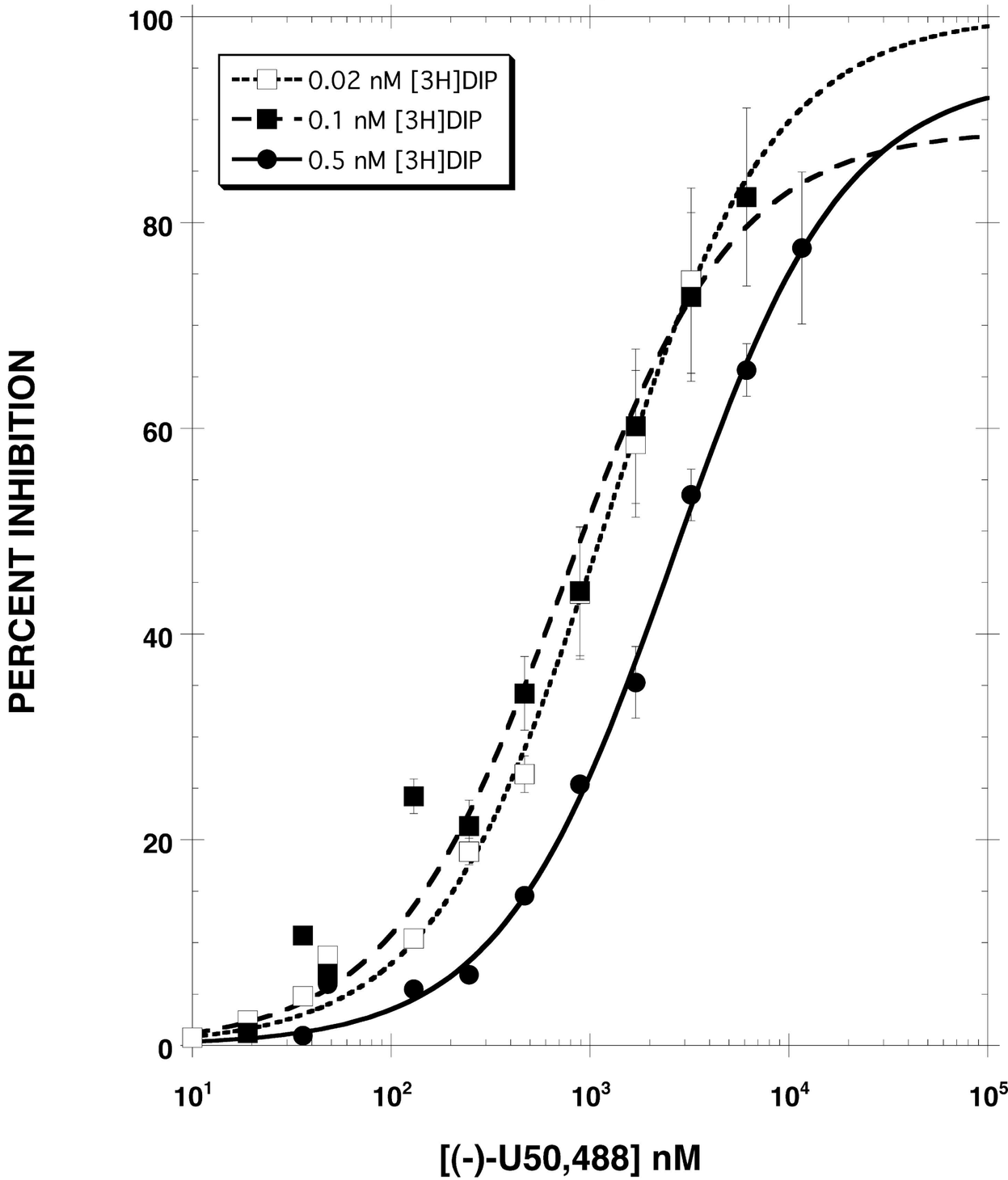
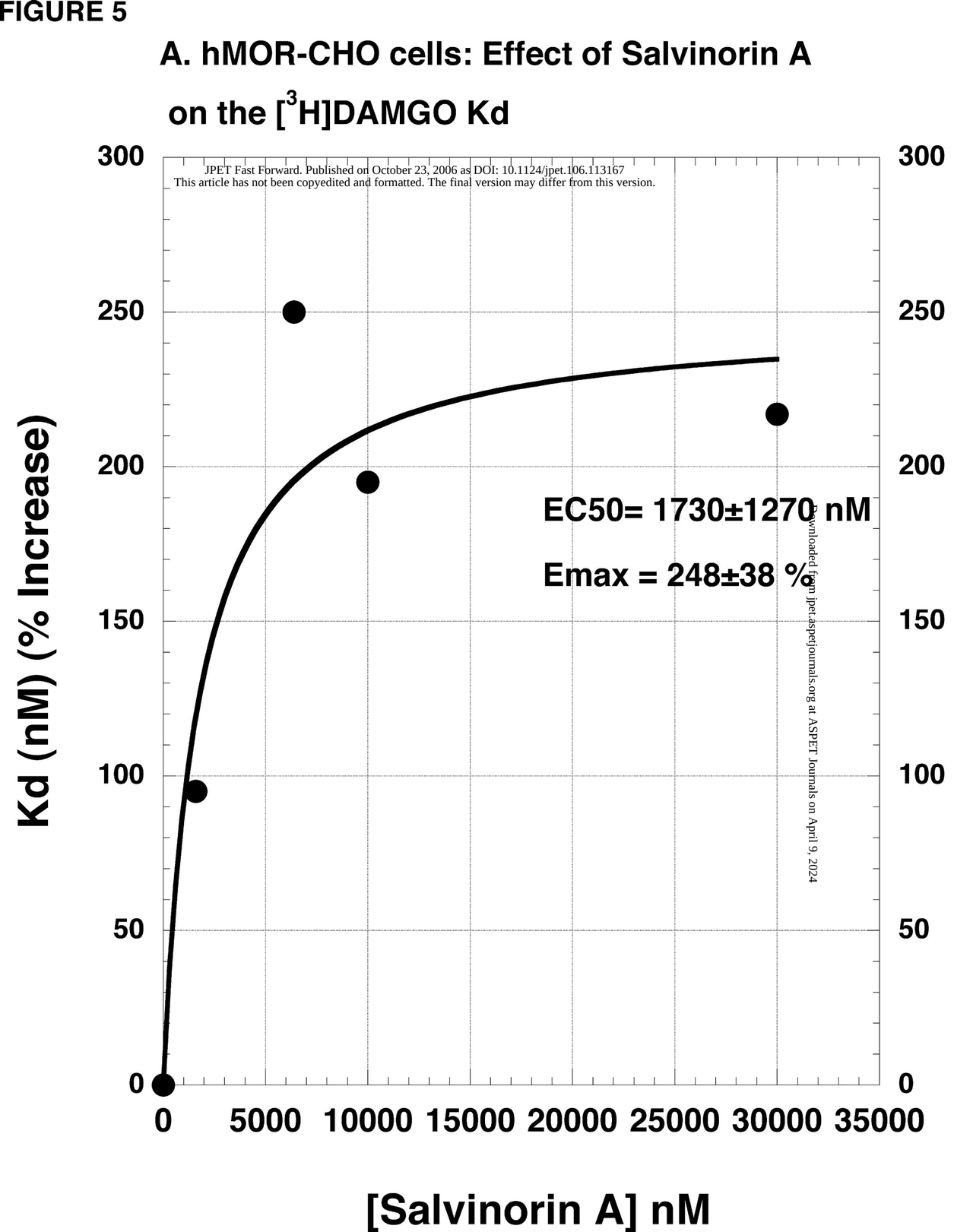


FIGURE 5



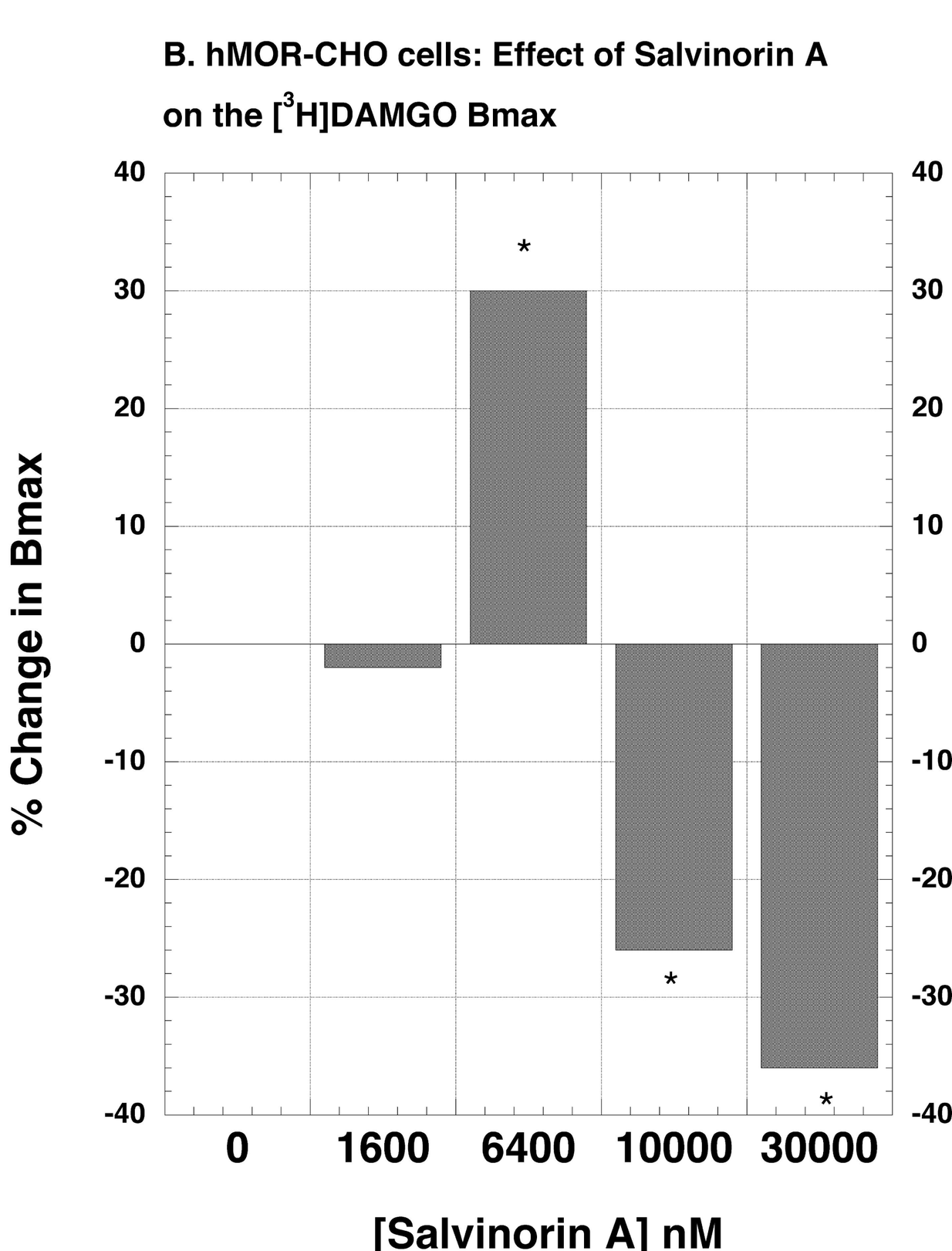


Figure 6

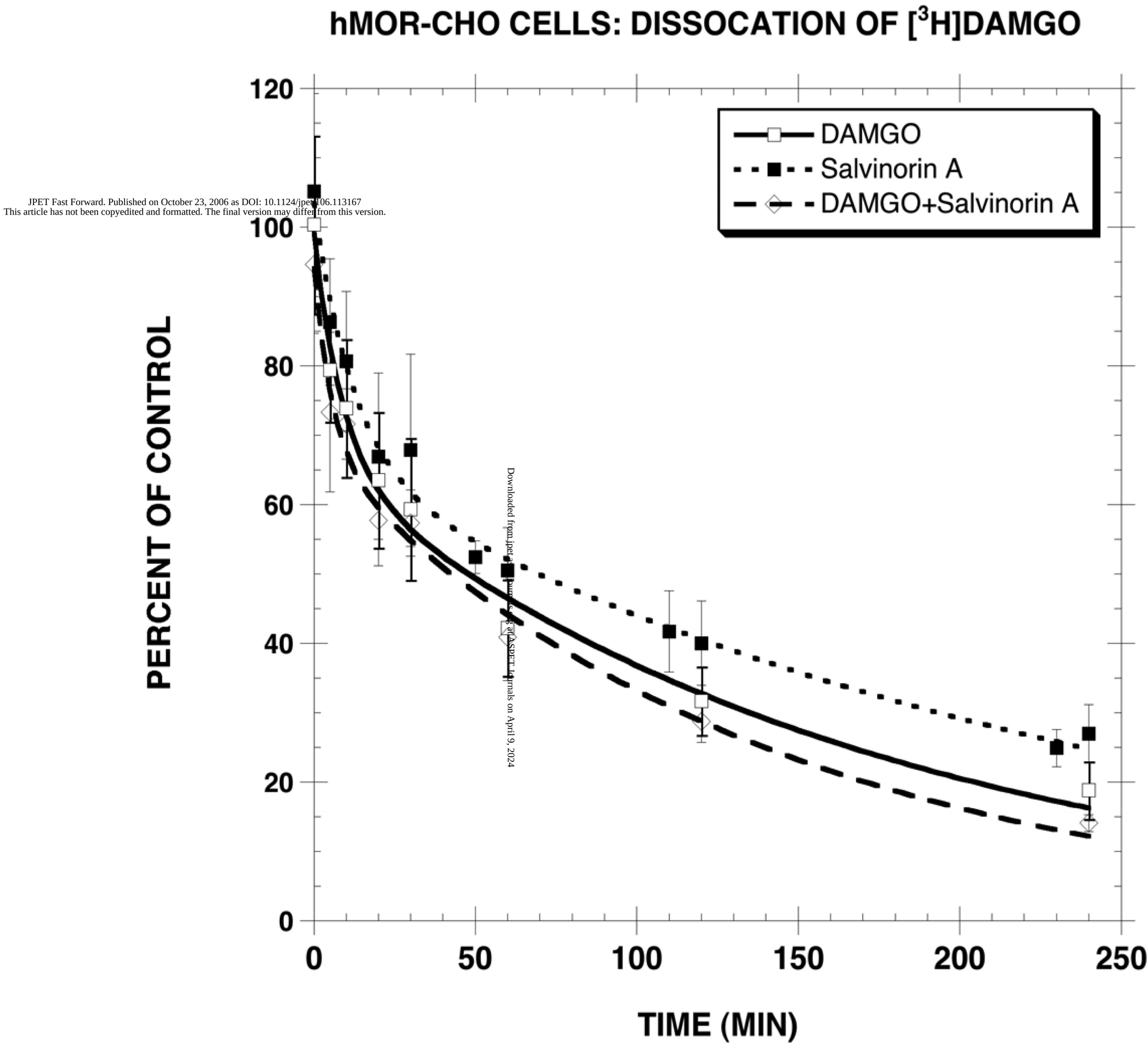


Figure 7

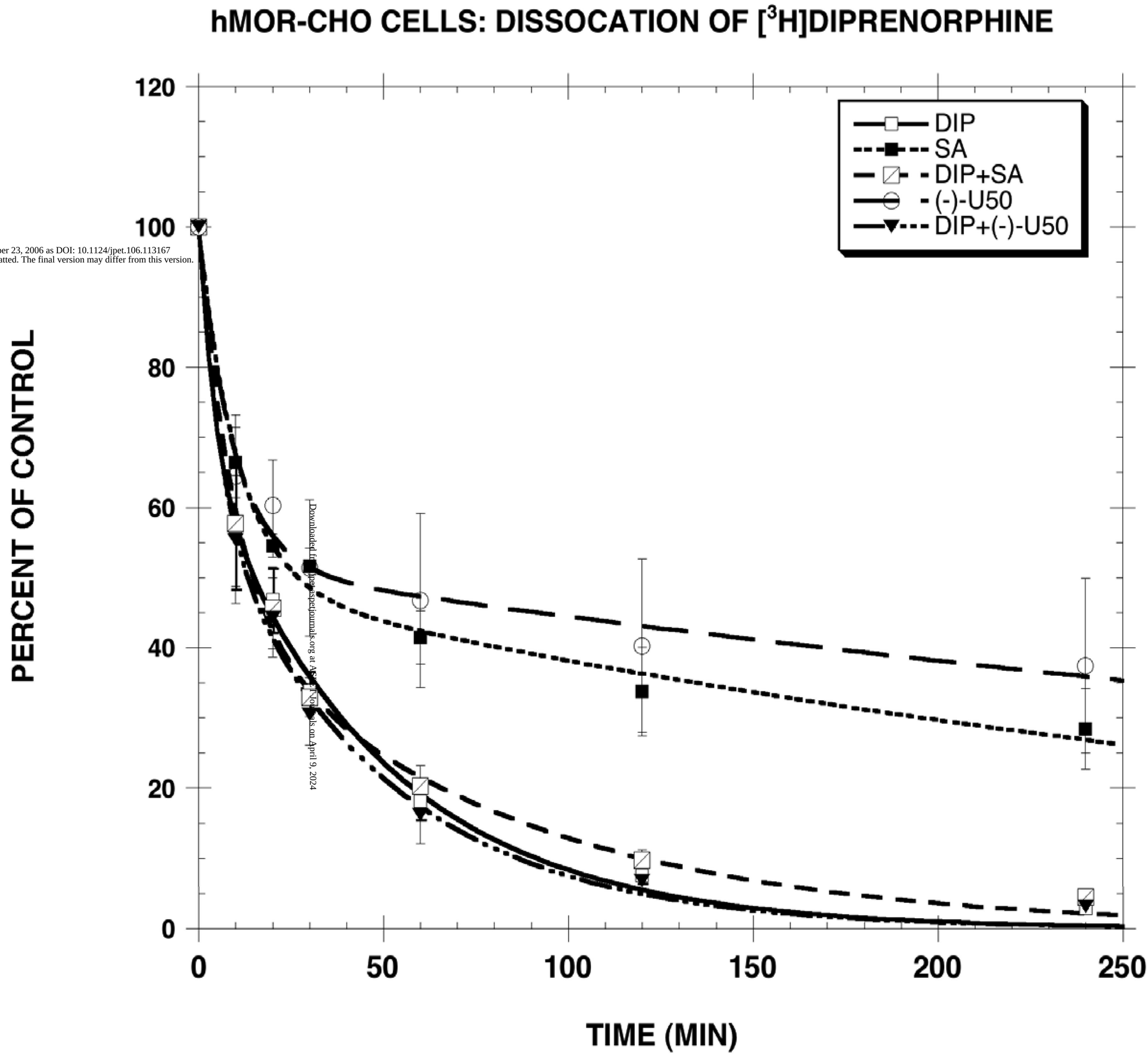


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FIGURE 8

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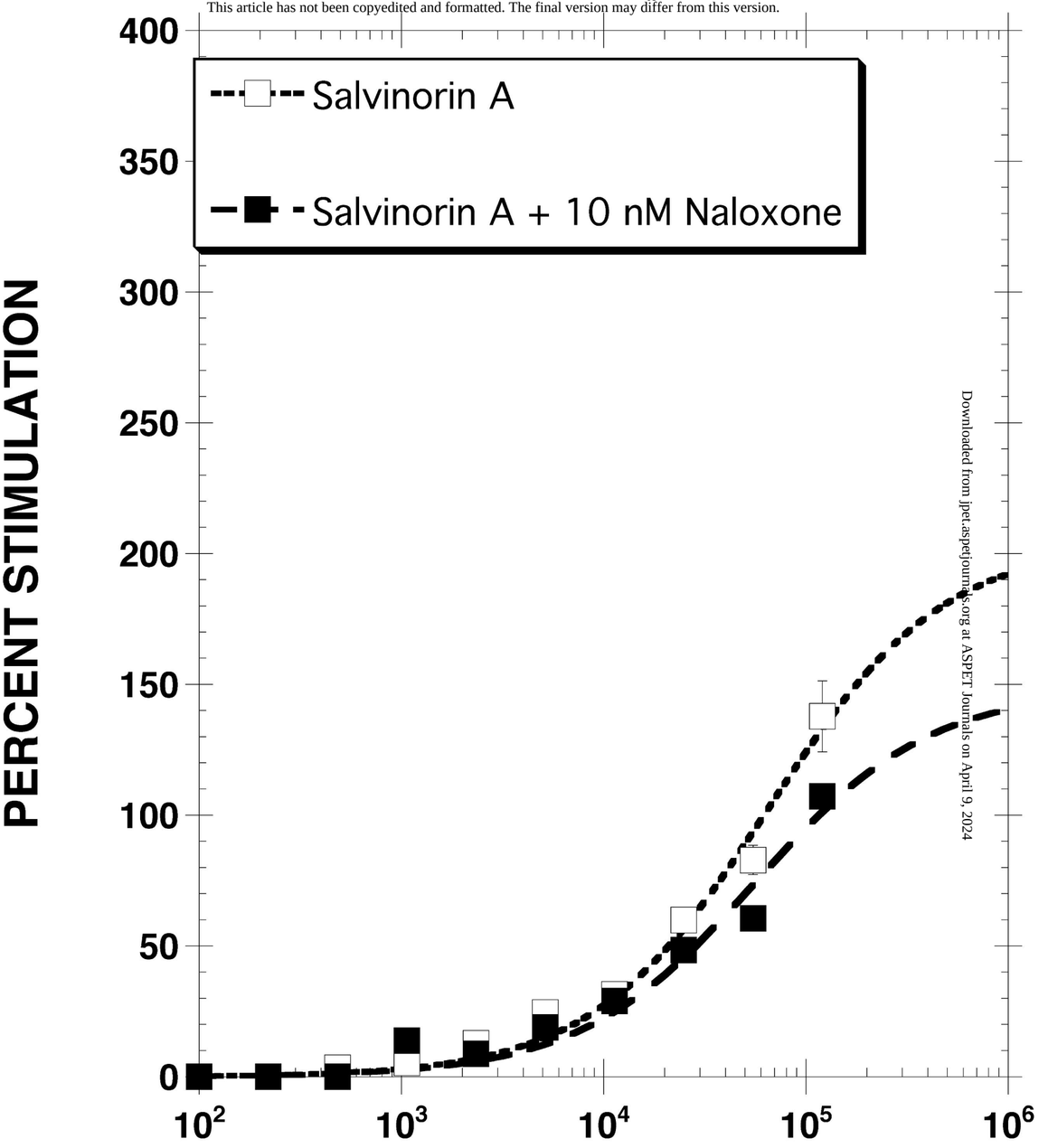

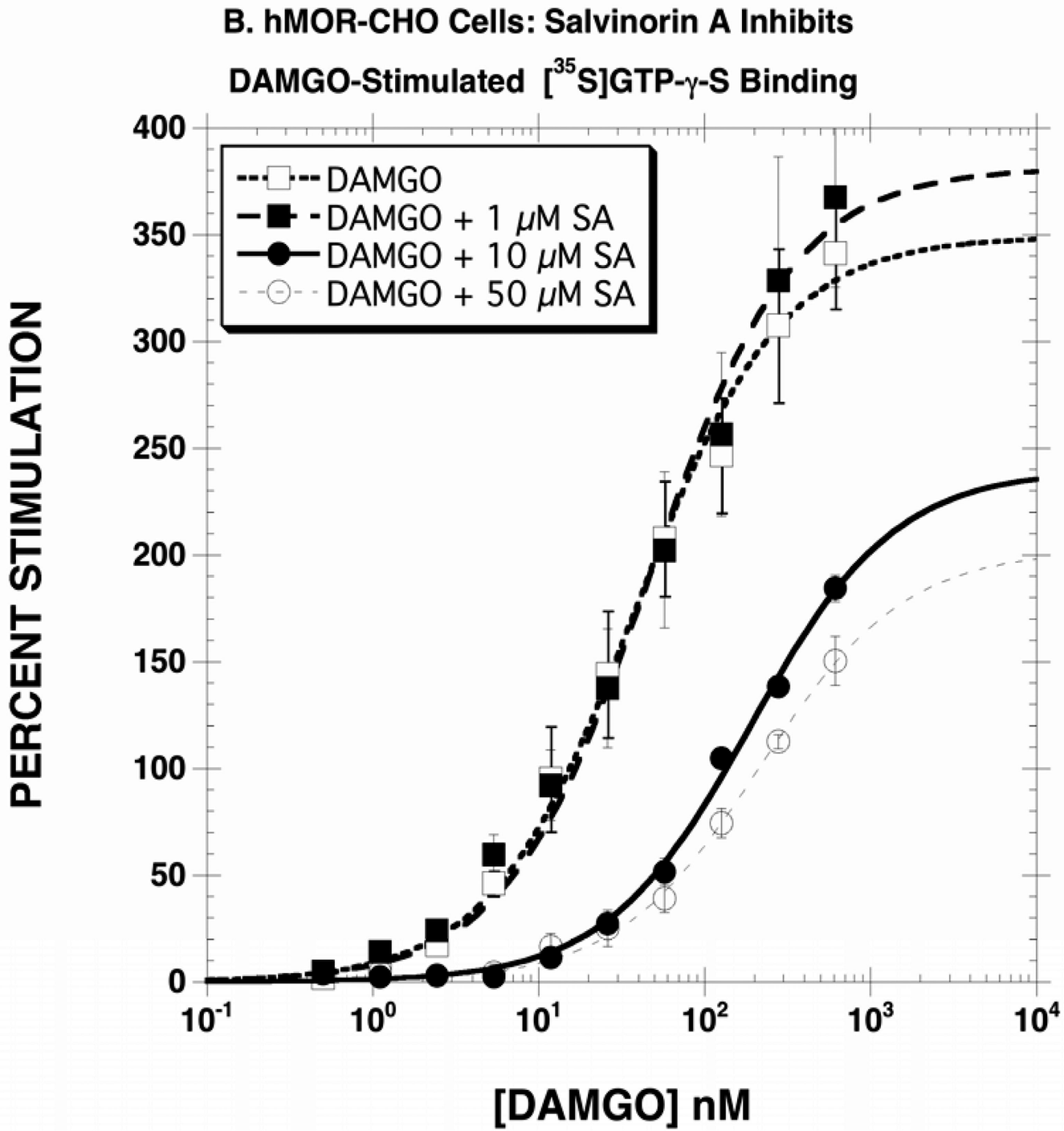

Figure 9

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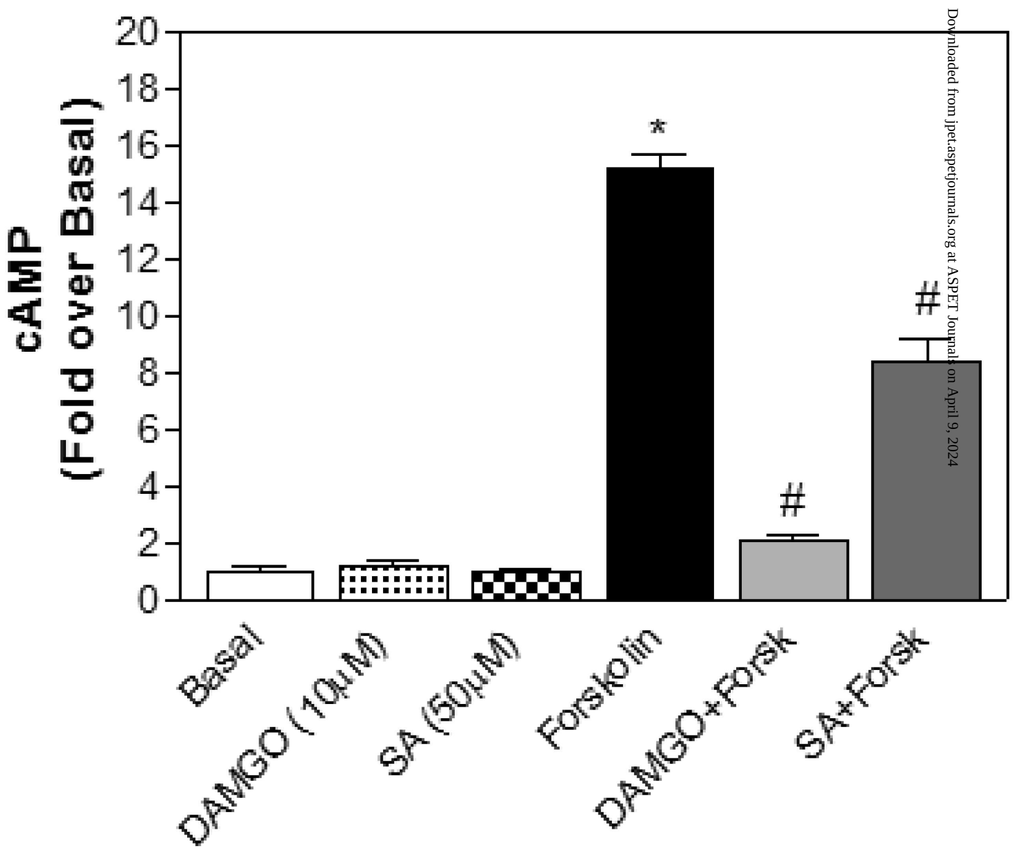
A. Acute Study



B. Chronic Study

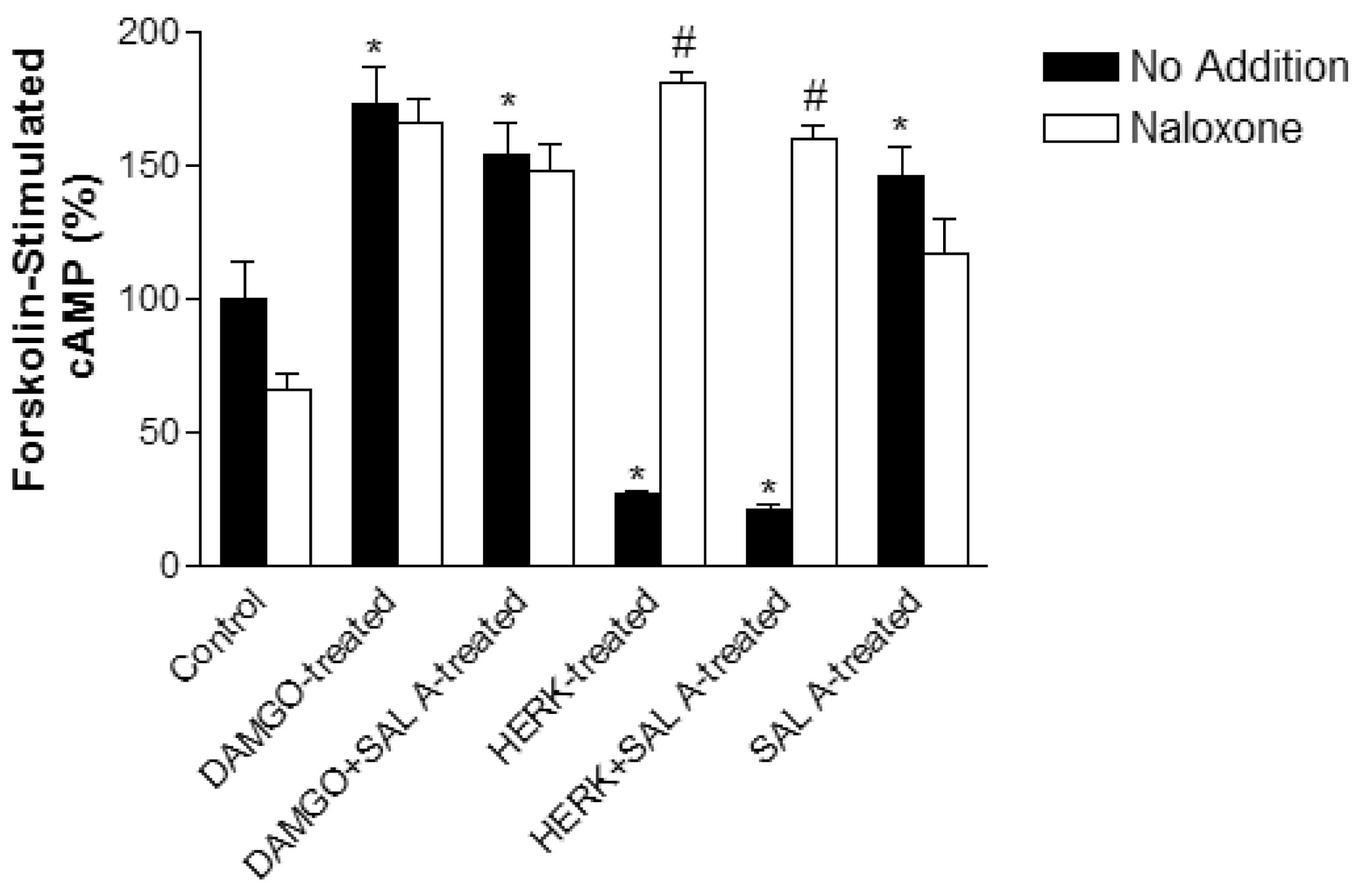


Figure 10

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