Alleviation of Cocaine Withdrawal and Pertinent Interactions between Salvinorin-based Antagonists and Kappa Opioid Receptor

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ABSTRACT: The kappa opioid receptor (KOR) is involved in the regulation of both the reward and mood processes. Recent reports find that the use of drugs of abuse increases the production of dynorphin and overall activation of KOR. Long-acting KOR antagonists, such as norbinaltorphimine (nor-BNI), JDTic, and 5'-guanidinonaltrindole (GNTI), have been shown to stop depressive and anxiety-related disorders, which are the common side effects of withdrawal that can lead to the relapse in drug use. Unfortunately, these prototypical KOR antagonists are known to induce selective KOR antagonism that is delayed by hours and extremely prolonged, and their use in humans comes with serious safety concerns because they possess a large window for potential drug-drug interactions. Furthermore, their persistent pharmacodynamic activities can hinder the ability to reverse unanticipated side effects immediately. Herein we report our studies of the lead selective, salvinorin-based KOR antagonist (1) as well as nor-BNI on C57BL/6N mice for spontaneous cocaine withdrawal. Assessment of pharmacokinetics showed that 1 is a short-acting compound with an average half-life of 3.75 h across different compartments (brain, spinal cord, liver, and plasma). Both 1 (5 mg/kg) and nor-BNI (5 mg/kg) were shown to reduce spontaneous withdrawal behavior in mice, with 1 producing additional anti-anxiety-like behavior in a light-dark transition test (however, no mood-related effects of 1 or nor-BNI were observed at the current dosing in an elevated plus maze or a tail suspension test). Our results support the study of selective, short-acting KOR antagonists for the treatment of psychostimulant withdrawal and the associated negative mood states that contribute to relapse. Furthermore, we identified pertinent interactions between 1 and KOR via computational studies, including induced-fit docking, mutagenesis, and molecular dynamics simulations, to gain insight into the design of future selective, potent, and short-acting salvinorin-based antagonists.

Keywords: Kappa opioid receptor, short-acting antagonists, salvinorin-based compounds, cocaine withdrawal, cocaine use disorder

1. INTRODUCTION

The current drug crises in America are primarily focused on opioids. However, cocaine overdose is increasing exponentially in the U.S.1-4 From 2018 to 2019, cocaine overdose deaths increased by nearly 9%.5 In 2019, cocaine was responsible for 16,000 American deaths (19% of all overdose deaths in the United States).5 In 2020, over 5 million Americans (almost 2% of the population) self-reported current cocaine use;5 the actual number of cocaine users is likely to be much higher. There are no current FDA-approved treatments for cocaine use disorders. Unlike the case of opioid/heroin use disorder, which has some therapeutic recourse (albeit, limited),6-8 cognitive behavioral therapy is the only effective clinical option for cocaine abuse.9,10 Research on cocaine dependence has been widely carried out, but has not yet produced effective medications.11 A cocaine epidemic is imminent; thus, the need for new therapeutics for cocaine use disorder is urgent.12

Preclinical models have shown that the kappa opioid receptor (KOR) is a central player in the regulation of reward and mood processes.13,14 KOR contributes to the regulation of the
dopamine mesolimbic pathway, which is composed of dopaminergic neurons from the ventral tegmental area of the midbrain (VTA) that project to limbic structures of the forebrain, including the nucleus accumbens (NAc) and the prefrontal cortex (PFC) (Figure 1). KOR signaling is implicated in the rewarding properties of drugs of abuse, natural stimuli, and the neurobiological effects of stressful experiences. Studies have shown that the use of drugs of abuse increases the production of dynorphin and overall activation of KOR. In a recent study, co-administration of buprenorphine and naltrexone, intended to produce the effects of a pure KOR antagonist, resulted in an improvement in the outcome of opioid dependence in terms of treatment retention, negative urinalyses, reduced dysphoria, improved mood, and reduced craving. Another study showed that blocking KOR within the VTA prior to forced-swim stress in mice prevented the reinstatement of cocaine-seeking. KOR antagonism was also recently shown to reverse heroin withdrawal-induced hyperalgesia in male and female rats. These results suggested that antagonizing KOR may halt some depressive and anxiety-related symptoms that are associated with withdrawal, along with motivational withdrawal symptoms, to ultimately reduce drug use relapse.

Long-acting KOR antagonists, such as norbinaltorphimine (nor-BNI), JDTic, and 5'-guanidinonaltrindole (GNTI), have been used as prototypical therapeutic agents for various neuropsychiatric conditions, including depression, anxiety, and substance abuse disorders. Unfortunately, these prototypical KOR antagonists are known to induce selective KOR antagonism that is delayed by hours and extremely prolonged. A study in mice showed that brain uptake of these compounds was very slow, and their presence in the brain was persistent and still detectable at one week. Their pharmacodynamic activities can persist long after the compounds were eliminated from the body. A single injection of nor-BNI into humans can block the effects of KOR agonists for months. Therefore, while these long-acting KOR antagonists do not chemically alter KOR, they are often considered KOR inactivators due to their long-term effects. The use of these receptor-inactivating KOR antagonists in humans raises serious safety concerns because they possess a large window for potential drug-drug interactions. These compounds also promote desensitization in drug abuse treatment, potentially promoting tolerance, and complicate preclinical evaluations in paradigms that require multiple administrations (such as self-administration). Additionally, their persistent pharmacodynamic activities can hinder the ability to instantly reverse unanticipated side effects; thus, these receptor-inactivating KOR antagonists can lead to detrimental results when serious side effects occur. JDTic, for example, advanced to phase I human clinical trials for the treatment of cocaine abuse, but its development was halted due to adverse events such as ventricular tachycardia that could not be reversed instantly. The reasons for the extraordinarily long time course of these KOR antagonists are yet to be understood.

![Figure 1. Dynorphinergic CNS Circuitry.](image)

A simplified scheme of pre- and post-synaptic kappa opioid receptor (KOR) expression and dynorphinergic (or other) projection neurons in the nucleus accumbens (NAcc), ventral tegmental area (VTA), medial prefrontal cortex (mPFC), dorsal raphe nucleus (DRN), and hypothalamus (Hyp). Withdrawal dysregulates dynorphin/dopamine signaling, and blocking dynorphin signaling at KOR is hypothesized to reduce psychostimulant withdrawal symptoms and related anxiety/depression, and thus the vulnerability to relapse.

KOR antagonists that are shorter-acting than nor-BNI, JDTic, or GNTI are currently being
developed and studied as they possess more favorable drug profiles. These compounds, such as CERC-501 (a.k.a. aticaprant), PF-4455242, and AZ-MTAB, have already shown promising results for depression and substance use disorders in preclinical models.\textsuperscript{14,24} For example, CERC-501 has a biological half-life ($t_{1/2}$) in humans (plasma) of 38.5 hours\textsuperscript{14} and reversed the analgesic effects induced by U-69593, a potent KOR agonist, for up to a week.\textsuperscript{21} It advanced to phase II clinical trials as an augmentation of antidepressant therapy for treatment-resistant depression; unfortunately, the clinical trials were later terminated due to slow enrollment.\textsuperscript{25}

Salvinorin A is the main active ingredient of the hallucinogenic plant Salvia divinorum that has been safely used by the Mazatec people for centuries in religious rituals.\textsuperscript{26} It is one of the most potent, naturally occurring opioid agonists, with high selectivity and affinity for KOR. It has the potential to be beneficial in treatment therapies for various central nervous system (CNS) disorders. atai Life Sciences has been developing salvinorin A for treatment-resistant depression, substance use disorder, and pain, with clinical trials expected to begin in the second half of 2022.\textsuperscript{27} Additionally, salvinorin A has been used as an important prototype for the development of related drug candidates.\textsuperscript{28–38} In particular, there are only six salvinorin-based compounds in the literature that have demonstrated antagonism against any of the opioid receptors (1–6, Figure 2);\textsuperscript{38} all other reported salvinorin-based compounds are agonists. Compounds 1–5 are antagonists at KOR, $\mu$-opioid receptor (MOR), and $\delta$-opioid receptor (DOR), with 1$\alpha$-hydroxysalvinorin A (1) being the most selective for KOR.\textsuperscript{38} Meanwhile, compound 6 is an antagonist at MOR and DOR, but a partial agonist at KOR.\textsuperscript{38}

![Figure 2. Structures of salvinorin A and known salvinorin-based opioid receptor antagonists 1–6](image)

The common feature among these opioid receptor antagonists is a small structural modification at C1, replacing the ketone with alcohol, alkene, or methylene functional group. We recognized that although the structural modification at C1 was small, it significantly altered the overall spatial arrangement of tricyclic rings within the salvinorin scaffold and produced major functional differences for the opioid receptors; however, no salvinorin antagonists have ever been evaluated in animal models to gauge neurobehavioral attributes. In previous studies, salvinorin C2-esters were identified as readily hydrolysable to an inactive metabolite, salvinorin B, and displayed short-acting activities.\textsuperscript{39–44} Herein we report our studies of 1$\alpha$-hydroxysalvinorin A (1) on C57BL/6N mice for spontaneous cocaine withdrawal and compared
with the standard KOR antagonist nor-BNI. We also performed pharmacokinetic (PK) studies to establish the tissue-specific distribution and other PK parameters. In-depth computational studies rationalized the unique and pertinent interactions associated with 1 to KOR antagonism.

2. RESULTS AND DISCUSSION

2.1 Synthesis of 1α-hydroxysalvinorin A (1)

We started the synthesis of 1 by reducing the C1 ketone in salvinorin A to alcohol using NaBH₄. However, deacetylation at C2 and epimerization at C8 also occurred during this reaction, possibly due to elevated temperature. The deacetylated diol 7a was obtained with a 57% yield (Scheme 1). The C8-epimer of 7a (which is 7b) was obtained in a 40% yield. Reactylation at C2 of 7a using acetic anhydride in pyridine at room temperature provided the desired product, compound 1, in a 91% yield. Overall, 1 was obtained in a 52% yield after 2 steps.

To establish the premise of our earlier hypothesis on short-acting KOR antagonism of the C2-salvinorin ester 1, metabolism and distribution of 1 were carried out. A single ip dose of 1 (5 mg/kg) was injected into C57BL/6N mice (n=3/group), and their brain, dorsal spinal cord, liver, and plasma were collected at 5, 15, 30, 60, 120, and 360 min after injection. Circulating, central, and liver contents were assessed via LC-MS/MS. Based on concentration vs. time curves (Figures 3A–D), we project that 1 is present in the brain for up to ~26–31 h, spinal cord ~19–22 h, liver ~13–15 h, and plasma ~18–22 h. The half-life t₁/₂ of 1 in the brain (5.2 h) was longer than in other biological matrices (spinal cord 3.7, liver 2.5, plasma 3.6 h). The apparent Tₘₐₓ (the time to maximum concentration) was observed at 30 minutes in the brain, spinal cord, and plasma samples and 60 minutes in the liver. Compound 1 exhibited the shortest half-life, but the highest Tₘₐₓ in the liver. These values suggested that 1 may be metabolized in the liver and excreted in bile, similar to the parent compound, salvinorin A.⁴⁵ The Riₐₐᵋₐ (calculating experimental matrix to plasma partition coefficient of 1) was 0.9–1.0.⁴⁶ Overall, results indicate 1 to be the first short-acting, salvinorin-based KOR antagonist.

2.3 Evaluation of 1 on spontaneous motor, anxiety-like, and depression-like behaviors associated with cocaine withdrawal

To assess the effects of compound 1 on the motoric and affective withdrawal associated with cocaine, C57BL/6N mice (n=7–13/group) were administered saline or an escalating regimen of cocaine over three days (5, 10, and 20 mg/kg) to produce psychostimulation and sensitization when administered sequentially.⁴⁰,⁴¹ On the final day, mice were co-administered either vehicle, nor-BNI, or compound 1 (both 5 mg/kg; Figure 4). Mice were allowed to undergo 48 h of withdrawal.

A previous study has shown that spontaneous motor behaviors indicate cocaine withdrawal;⁴⁷ therefore, we first assessed the motor phenotype of mice that had received saline or escalating cocaine doses in an open field test. The results showed that cocaine exposure significantly influenced the distance [F(5,48) = 4.93, p < 0.05] (Figure 5A), the speed of travel [F(5,48) = 4.87, p < 0.05] (Figure 5B), and the number of rears made by mice in an open field [F(5,48) = 3.44, p < 0.05] (Figure 5C). Consistent with the behaviors of cocaine withdrawal, mice in the cocaine-treated control group traveled significantly greater distances (p = 0.003; Figure 5A) at higher speeds (p = 0.004; Figure 5B) and demonstrated more frequent rearing behavior (p =
significantly attenuated this effect \( p = 0.03; \) Table 1). Significant differences were not observed in the time spent in the light or dark chambers (Table 1). Similarly, no differences were observed in the amount of time spent on the open or closed arms of an elevated plus maze; however, nor-BNI did increase the total number of arm entries when combined with cocaine \( [F(5,48) = 2.76, p < 0.05] \) (Table 1). Significant differences were not observed in the amount of time spent immobile in the tail suspension test (Table 1).

We also assessed the capacity of 1 to alter the rewarding effects of cocaine or the psychomotor effects of cocaine when 1 was on-board. No effects were observed for 1 to alter cocaine-reward or the psychostimulatory capacity of cocaine (Figure S1).

Overall, our evaluation of 1 on spontaneous motor, anxiety-like, and depression-like behaviors associated with cocaine withdrawal has shown that 1 reduced the motoric and affective complications of cocaine withdrawal. These primary therapeutic actions of 1 may be due in part to its longer residence in the brain than in other compartments.

**Figure 3.** PK properties of compound 1 including half-life \( t_{1/2} \), max. concentration \( C_{\text{max}} \), and time to \( C_{\text{max}} \) \( T_{\text{max}} \) in (A) brain, (B) spinal cord, (C) liver, and (D) plasma \( n=3 \) independent observations per timepoint
Figure 4. Dosing schedule and timeline for motor and affective withdrawal assays

Figure 5. Mice (n=7-13) were administered saline or an escalating regimen of cocaine with nor-BNI (5 mg/kg) or 1 (5 mg/kg) as depicted in the Figure 3 timeline. 48 h after cessation of cocaine, all mice were assessed in an open field for the (A) distance, (B) velocity traveled, and (C) frequency of rearing. * significantly differs from saline/vehicle control. † significantly differs from cocaine/vehicle-treatment, p < 0.05.

Table 1. Motor and affective behavioral measures. *significantly differs from saline/vehicle; †significantly differs from escalated cocaine/vehicle; ‡significantly differs from saline/nor-BNI, p < 0.05.

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2.4 Docking and computational site-directed mutagenesis on KOR, MOR, and DOR

In the two-state receptor model, a full agonist binds only to the active state of the G protein-coupled receptors (GPCRs), whereas an antagonist binds equally well to both the active and inactive states. 48 The aspartic acid residue on the third helix of all aminergic GPCRs, D138, plays a significant role in the binding of protonated compounds. 49 Specifically, in the case of opioid receptors, before the discovery of the molecular target of salvinorin A, it was hypothesized that a basic amine on the structure of the ligand was required to bind to opioid receptors, based on fentanyl and morphine derivatives. 49 When KOR was identified as the molecular target of salvinorin A, the hypothesis above was contested since salvinorin A is nonnitrogenous. 49 Interestingly, with other small molecules, mutagenesis and computational studies indicated that an extended hydrogen bond network between the conserved aspartic acid residue on the third helix of KOR, D138, and water might be sufficient to modulate KOR activity. However, in the case of salvinorin A, this interaction is suboptimal, and the D138A mutation decreased the cost of desolvation, thus increasing the binding affinity to KOR. 50 Since the co-crystal structure of KOR with salvinorin A has not yet been obtained, many researchers have hypothesized the putative binding modes of salvinorin A to KOR via in vitro mutagenesis and computational studies. 50–52 The initial recognition pose of salvinorin A in the inactive-state X-ray crystal structure of KOR (PDB: 4DJI)52 and the putative binding mode of salvinorin A in the active-state X-ray crystal structure of KOR (PDB: 6B73) were proposed. 53 To the best of our knowledge, similar studies have not been carried out for salvinorin-based KOR antagonists.

To study the pertinent interactions between salvinorin-based antagonists and KOR, we docked compounds 1, 2, 6, and salvinorin A into the active-state (PDB: 6B73) and inactive-state (PDB: 4DJI) X-ray crystal structures of KOR using Glide. Although our work focused mainly on compound 1, additional computational studies were carried out for compounds 2 (also a KOR antagonist, but less potent than 1) and 6 (a partial KOR agonist) to probe the potential impact of small structural variations (at C1 and C10 positions) on the selectivity and functionality of the compounds toward KOR. The importance of each ligand-protein interaction between 1 and 2 to KOR was inferred from computational site-directed mutagenesis and successive binding free energy measurements using Prime MM-GBSA in Maestro. 54–56 Our results showed that salvinorin A, 1, and 2 displayed similar docking poses and GlideScores in the active state of KOR. While the C1 ketone of salvinorin A showed a hydrogen bonding interaction with Q115 and the C1 hydroxyl of 1 displayed a hydrogen bonding interaction with D138, 2 did not show any hydrogen bonding interaction to the residues in that area (Figure 6A and Table S2). The docking results also predicted that the binding interactions between salvinorin A and the active state of KOR (to Y139, C210, and Y312) were similar to the previously reported results by the Roth group (Figure 6B).

Our docking of salvinorin A in the inactive state of KOR resulted in a binding mode that matched the previously reported one done by Roth and coworkers (Figure 6C). 50 This binding mode of salvinorin A has been suggested to be the recognition pose of salvinorin A in KOR before activation of the receptor (salvinorin A then rearranges into the binding mode in the active state). 50 Meanwhile, 1 and 2 displayed similar binding modes in both the active state and the inactive state of KOR, reflecting their nature as antagonists to have equal binding to both the active and inactive states of KOR (Figure 6D). A D138A computational mutation of D138 resulted in a total loss of the original predicted binding modes for both 1 and 2 within the inactive state of KOR, presumably due to the smaller size of the neutral alanine amino acid (Table S3). To further support our assumption, another computational mutation (D138L) was tested. This mutation revealed a similar binding mode as compared to the wild type, and it experienced no loss in GlideScore or predicted binding affinity (Table S3). We recognized that changing from D to A, or D to L, also changed the polarity besides size. However, the fact that 2 (which did not show
any hydrogen bonding interaction to the residues in that area, as mentioned above) did not change its binding mode in the mutation compared to the wild type led us to believe that size in this area is more important for binding affinity.

Both 1 and 2 exhibited a strong hydrogen bonding interaction with K227$^{5.52}$ and hydrophobic interactions with Y139$^{3.43}$ and M142$^{3.46}$ in the inactive state of KOR (Figures 6D–E). These three residues are known to be important for the function of KOR.$^{50}$ A K227A mutation of K227$^{5.52}$ resulted in a decrease in the predicted binding affinity to the inactive state of KOR for both 1 and 2 (Table S3). Meanwhile, a Y139A mutation of Y139$^{3.43}$ and an M142A mutation of M142$^{3.46}$ resulted in a decrease in the predicted binding affinity to the inactive state of

Figure 6. A) Overlay of the binding modes of 1, 2, and salvinorin A in the active state of KOR; B) Salvinorin A within the active state of the KOR; C) Salvinorin A within the inactive state of KOR; D) Hydrogen bonding interactions of 1 and 2 with D138 and K227 within the inactive state of KOR; E) Hydrophobic interactions of 1 and 2 with Y139 and M142 in the inactive state of KOR; F) Hydrophobic interactions of 1 and 2 with I294, Y312, and V118 in the inactive state of KOR. Compound 1 is in blue; 2 is in green; salvinorin A is in orange; the active state of KOR (PDB: 6B73) is in yellow cartoon; the inactive state of KOR (PDB: 4DJH) is in purple cartoon; hydrogen bonding interactions are depicted in green dotted lines; and hydrophobic interactions are depicted by orange dotted lines; hydrogen atoms were omitted for clarity purposes.
KOR for 1, but not for 2 (Table S3). In particular, 1 and 2 also showed hydrophobic interactions with I294, Y312, and V118 in the inactive state of KOR, three of the four residues that were previously recognized as important for KOR selectivity (V108 is the other residue) (Figure 6F).

**Structural insights for binding to KOR:**

Similar docking and computational mutagenesis studies were carried out for 1, 2, and 6 in the inactive states of MOR (PDB: 4DKL) and DOR (PDB: 4N6H) (Tables S4–7 and Figures S2–5). Our results reinforced the earlier premise on the importance of I294, Y312, and V118 for the selectivity of ligands, such as 1, to KOR.

Although V118 and Y312 were observed to interact well with 1 in KOR, the corresponding N127 and K108 in MOR (of V118), and W318 and L301 in MOR and DOR, respectively, did not show any interactions with 1. Meanwhile, 2 and 6 possessed alternate binding modes and different interactions within MOR and DOR (Figure S2). As mentioned above, D138 does not play a role in the affinity or functional (agonistic) activity of salvinorin A toward KOR. Even though compound 1 lacks a basic nitrogen atom, hydrogen-bonding interactions with D138 in KOR, D147 in MOR, and D128 in DOR (Figure S3) were observed. Therefore, our results have further suggested that the selectivity of salvinorin-based compounds, such as 1, to KOR did not likely involve D138, but rather all other residues mentioned above, such as I6, V2, and Y7.

A previous study done by Roth and coworkers suggested that non-charged ligands, such as salvinorin A, were highly sensitive to mutation within the binding pocket. As mentioned earlier, this study identified the recognition pose of salvinorin A in the inactive state of KOR before the activation of the receptor (salvinorin A then rearranged into the binding mode in the active state). This study also carried out in vitro site-directed mutagenesis for Q115, I116, V118, Y139, Y312, Y313, and Y320, and suggested that all the mutations resulted in the reduction of the affinity or potency of salvinorin A to KOR. Y139, M149, K227, H291, and Y320 were shown to specifically affect KOR activation through modulations of P238, I146, and F283. In addition, small rotameric changes of the PIF motif (TM5 Pro residue, TM3 Ile, and TM6 Phe), which connects the transmembrane helix 3 (TM3), transmembrane helix 5 (TM5), and transmembrane helix 6 (TM6), upon ligand binding led to a large-scale movement of these helices during activation. Our docking data have suggested that the binding of a salvinorin-based KOR antagonist would alter the rotameric change of this PIF motif, blocking the large-scale movement of these helices, thus preventing the activation of the receptor.

As mentioned above, compounds 1 and 2 exhibited similar predicted binding modes in the active and inactive states of KOR, reflecting their nature as antagonists to have equal binding to both the active and inactive states. Compound 1 exhibited a hydrogen-bonding interaction between C1-hydroxyl and D138 and hydrophobic interactions between C4-ester and V118, rings C and D and I294, ring A and Y312, and C2 acetoxy and Y320. Meanwhile, compound 2 (which does not have C1-hydroxyl) possessed similar hydrophobic interactions, but lacked the critical hydrogen-bonding interaction with D138. Therefore, we hypothesized that the hydrogen bonding interaction between C1-hydroxyl of 1 and D138 helped anchor 1 to its putative binding mode and improved the interactions known to aid in the binding, selectivity, and agonistic functionality to KOR.

**Structural insights for binding to MOR:**

Studies have shown that ligands that bind to MOR would commonly interact with two crucial residues, D147 and Y148, and antagonists generally display more polar interactions with Q124, Y128, N150, K233, E229, and W318 residues than do agonists. As previously reported by the Prisinzano lab, 2 and 6 exhibited higher binding affinities to MOR than that of 1 (K values for 1, 2, and 6 are 2300, 200, and 170 nM, respectively). The authors reported these as K values, but we believe they meant K values—the dissociation constant in the inhibition
of [35S]GTP-γ-S binding). Therefore, we completed docking and computational site-directed mutagenesis studies on MOR (similar to our calculations for KOR) with these salvinorin-based antagonists to identify the important residues for the selectivity and functionality of MOR. The results indicated that 1 displayed a slightly different binding mode to MOR than 2 and 6 (Figure S2, Tables S4–5). Small differences among these binding modes were observed, most likely due to the C1-hydroxyl group on 1 showing a hydrogen bonding interaction with D14732 while 2 and 6 did not (Figures S4B–D). Compound 2 exhibited hydrogen bonding with Y14833, K23358, and W31835, as well as hydrophobic interactions with other residues (Figure S4C). Compound 6 exhibited hydrogen bonding with K23358 and W31835, as well as π-stacking interactions with Y326742 and W293648 (Figure S4D). The results suggested that these additional hydrogen bonding and hydrophobic interactions may also be responsible for the slightly different binding modes and the higher binding affinities of 2 and 6 to MOR, compared to 1.

We then performed computational site-directed mutagenesis studies to determine the importance of these polar and nonpolar interactions in the binding of 1, 2, and 6 to MOR (Tables S4–5). A K233A mutation resulted in a precipitous loss in predicted binding affinities of 2 and 6, whereas that of 1 was minimally affected (Table S5). Meanwhile, a Y147A mutation also lowered predicted binding affinities of 1, 2, and 6, but the effects were not as drastic as those of the K233A mutation (Table S5). These results suggested that interaction with Y147733 in MOR may contribute to the functionality of ligands in MOR, but would have a limited effect on their binding affinities. Furthermore, a W318G mutation resulted in a loss of expected binding affinities for all three compounds (the loss of binding affinity for 2 was greater than for 6, which was greater than for 1) (Table S5). As mentioned earlier, 1 possessed additional hydrogen-bonding interactions with D14732 and K23358 that 2 did not have, and 6 displayed additional π-stacking interactions with Y326742 and W293648 that 2 did not possess; therefore, these K233A, Y147A, and W318G mutations affected 2 to a much greater degree than they did 1 and 6.

Mutations of Y128264, V236543, W293648, I296651, H297652, K305658, and I322738 to alanine produced a loss in predicted binding affinities for both 2 and 6, but not for 1 (Table S5). These mutations resulted in a small loss or gain of predicted binding affinity of 1. Meanwhile, mutations of I144327 and Y326742 to alanine resulted in a loss in predicted binding affinities for all three compounds (Table S5).

Overall, 1 exhibited a hydrogen bonding interaction with D14732 in MOR (the conserved aspartic acid residue within all aminergic GPCRs) while 2 and 6 did not. Compounds 2 and 6 possessed additional hydrogen bonding and hydrophobic interactions with other residues, such as Y14833, K23358, and W318735 (for 2), and K23358, W318735, Y326742, and W293648 (for 6). These additional interactions are likely responsible for the better binding affinities of 2 and 6 to MOR than that of 1.

**Structural insights for binding to DOR:**

A previous study has suggested that the conserved aspartic acid residue D128332 in DOR contributed to stabilizing the ligands, but was not required for their selectivities to the receptor.58 Similar to KOR and MOR, our docking studies in DOR indicated the hydrogen bonding interaction between D128332 and C1-hydroxyl of 1 (Figure S5B). Meanwhile, compounds 2 and 6 showed a hydrogen-bonding interaction with K108263, while 1 did not (Figures S5C–D). Further computational site-directed mutagenesis studies on DOR (similar to those for KOR and MOR) facilitated the identification of critical residues necessary for the selectivity and functionality of this receptor (Tables S6–7).

Previous in vitro mutagenesis studies have indicated that K108263, F225547, W274648, and Y308742 were important for ligand binding to DOR.42 Our computational mutagenesis studies indicated that a K108A mutation would lead to a loss of the original binding modes of 2 and 6 (Table S7), suggesting that K108 was vital for the binding of these compounds to DOR. While F225547 displayed no hydrophobic interactions
with 1, 2, or 6, we studied its mutation to determine if it contributed to the binding of these compounds to DOR. The results showed that its mutation had minimal effects on the binding of 1, but produced a loss of the original binding mode of 2 and a loss in the predicted binding affinity of 6. These results suggested that F222<sup>5.47</sup> played an important role in the binding of the ligand to DOR and contributed to the optimal binding of 2 and 6 (Table S7).

Compound 1 exhibited a π-stacking interaction with Y308<sup>7.42</sup>, while compounds 2 and 6 did not; instead, these two analogs exhibited hydrophobic interactions with other residues (Figure S5). A Y308A mutation lowered the predicted binding affinities or produced an overall loss of the original binding modes for all three compounds towards DOR (Table S7).

These results suggested that Y308<sup>7.42</sup> played a pivotal role in stabilizing the original binding modes of these compounds in DOR. In addition, mutations of Y109<sup>2.64</sup>, L217<sup>5.43</sup>, V281<sup>6.55</sup>, L300<sup>7.34</sup>, and I304<sup>7.38</sup> to alanine resulted in lower predicted binding affinities or loss of the original binding modes for 2 and 6 (but not for 1) (Table S7). These results suggested that Y109<sup>2.64</sup>, L217<sup>5.43</sup>, V281<sup>6.55</sup>, L300<sup>7.34</sup>, and I304<sup>7.38</sup> contributed to the binding of 2 and 6 to DOR. Notably, 1 also interacted with the residues above; however, their mutations had limited effects on the predicted binding affinity of 1 to DOR and displayed a different binding mode to DOR than those of 2 and 6 (Figure S5). These results suggested that the residues mentioned above helped, but were not expressly vital, to binding 1 to DOR.

![Figure 7](image-url)

**Figure 7.** Molecular dynamics simulation frame snapshots and interaction histograms of compound 1 and salvinorin A within KOR during a 200 ns simulation. A) The binding mode of 1 in the inactive state of KOR (PDB: 4DJH); B) The binding mode of salvinorin A in the active state of KOR (PDB: 6B73); C) Interaction histogram of 1 in the inactive state of KOR; D) Interaction histogram of salvinorin A in the active state of KOR. Compound 1 is in blue; salvinorin A is in orange; hydrophobic interactions are denoted as orange dotted lines; and hydrogen bonding interactions are denoted as green dotted lines.
2.5 Molecular dynamics simulations of 1 and salvinorin A within KOR

We also carried out molecular dynamics (MD) simulations of 1 (an antagonist) and of salvinorin A (an agonist) in both the active and inactive states of KOR to further understand their dynamics and the vital interactions for the functionality of the ligands in this receptor. 200 nanosecond (ns) MD simulations were performed using Desmond\textsuperscript{40}. The root-mean-square deviation (RMSD) analysis of C-α atoms indicated that KOR reached equilibrium after \(~50\) ns and remained on a plateau throughout the rest of the 200 ns simulations (Figure S6). The RMSD values varied only within a 0.4–1.4 Å range, suggesting the stable equilibrium structures of the receptor–ligand complexes, which were not altered greatly from the starting structures. The active-state KOR is in yellow cartoon and the inactive-state KOR is in purple cartoon.

Figure 8. A) Overlay of the transducer regions that are involved in the activation of KOR. Conformational changes between active-state KOR (ball and stick model, blue sticks) and inactive-state KOR (ball and stick model, grey sticks) are highlighted for (B) K227\textsuperscript{5.40}/E297\textsuperscript{5.40}/Y139\textsuperscript{3.33} motif, (C) P238\textsuperscript{5.50}/I146\textsuperscript{3.40}/F283\textsuperscript{6.44} motif and toggle switch residue W287\textsuperscript{6.48}, and (D) Y66\textsuperscript{1.39}/Y119\textsuperscript{2.64}/Y320\textsuperscript{7.42}/D138\textsuperscript{3.32} putative motif. Hydrogen bonding interactions are denoted as green dotted lines and π-stacking interactions are denoted as blue dotted lines.

200 ns simulations (Figure S6). The RMSD values varied only within a 0.4–1.4 Å range, suggesting the stable equilibrium structures of the receptor–ligand complexes, which were not altered greatly from the starting structures. The RMSD and Prime MMGBSA binding free energy data suggested that compound 1 (an antagonist) stably interacted with both the active (\(\Delta G = -67.844 \pm 5.575\) kcal/mol) and inactive states of KOR (\(\Delta G = -59.132 \pm 8.091\) kcal/mol), with a slight preference towards the active state. Compound 1 was observed to exhibit interactions with D138\textsuperscript{3.32} (water bridge and hydrogen bonding), Y139\textsuperscript{3.33} (water bridge and
Our results on the MD simulation of salvinorin A in KOR matched with previously reported results (Figures 7B and 7D). Previous studies on the D138N and D138A mutations elucidated the important role of D138 in the binding affinity and potency of salvinorin A to KOR, possibly through the removal of an unfavorable desolvation cost. The water-mediated interaction with D138 in KOR was observed to increase from ~25% in the case of salvinorin A within the active state (with the C1-ketone of salvinorin A, an agonist) to 100% in the case of I within the inactive state (with the C1-hydroxyl of I, an antagonist). These results suggested that a water-mediated interaction with D138 in the inactive state may be required to stabilize the inactive-state receptor–ligand complex, as seen with the hydrogen bonding interaction between the C1-hydroxyl of the antagonist I and D138 in the inactive state of KOR. On the other hand, the limited interaction of salvinorin A with D138 provides further evidence that the extended hydrogen-bonding network between D138, water, and salvinorin A is unfavorable, likely due to the overall cost of desolvation. Furthermore, these data suggest that incorporating a hydrogen-bond donor or removing the hydrogen-bond acceptor can influence the functionality of salvinorin-based compounds, as seen with compounds 1, 2, and 6.

Interestingly, when we changed the stereochemistry of C1 in 1 (which bears the C1-hydroxyl group) from (S) to (R), the docking pose of I in the inactive state of KOR flipped to maintain the interaction with D138 (Figure S7). This result strengthened the argument that the hydrogen-bonding interaction of the correct stereochemistry at C1 with D138 plays a critical role in maintaining the binding affinity and functionality of salvinorin ligands to KOR.

Previous studies suggested that all other residues mentioned above that were important for binding of the ligands to KOR (see Section 2.4) showed a reduction in the functionality of the agonists. Our MD simulations showed limited interactions between these residues and I in the inactive state of KOR (Figure 7C). These include Y139 (~100% interaction within the 200 ns simulation), M142 (~60%), K227 (~5%), and H291 (<5%). Only two of these residues, Y139 and M142, interacted 50% or more with I in the inactive state of KOR. In particular, while our docking studies suggested a good interaction between I and K227 in the inactive state of KOR, the MD simulation indicated a limited interaction between them (<5%). Instead, our MD simulation had a significant water-mediated interaction with Y139 (~100%). Alternatively, within the active-state KOR, compound I interacted with the aforementioned residues in an alternative manner: Y139 (~40%) M142 (~5%), K227 (~1%), and H291 (~55%) (Figure 9A).

Unsurprisingly, the interactions are markedly different from each other between states. With this knowledge in hand, we further examined these residues and known motifs important for activating the KOR. Our results suggested that the lactone–Y139 interaction between I (an antagonist) and KOR can significantly alter the normal water bridge formed between Y139 and K227, thus preventing the activation of the receptor (Figure 8B). Furthermore, H291 lies near the P238, I146, F283 motif and the rotameric toggle switch residue W287, two structural features known to be important for the activation of the receptor. Interestingly, we found that within the inactive state of KOR (grey sticks), a pi-stacking network formed between F231, F235, W287, and H291, potentially contributing to the stabilization of the inactive state of the receptor (Figure 8C). Furthermore, a water molecule was trapped between the P238, I146, and F283 residues within the inactive state of KOR, but not in the active state of KOR, matching prior mutagenesis and computational studies (Figure 8C and Figure S8). We also investigated other transducer binding sites that are known to be important for receptor activation, including the sodium pocket, the NPxxY motif, and the DRY motif (Figure S9). In general, these sites showed similar movement upon activation reported in the X-ray crystal structure of KOR and the selective...
KOR agonist MP1104\textsuperscript{53} (Figure S9). Interestingly, the X-ray crystal structure of KOR and the KOR antagonist JDTic showed a hydrogen-bonding interaction between T111\textsuperscript{2.56} and Y320\textsuperscript{7.42} (Figure S9A), which was suspected to contribute to stabilization of the inactive state of the receptor.\textsuperscript{53} Previously, depending on the ligands, Y320\textsuperscript{7.42} was observed to form a hydrogen bonding interaction with D138\textsuperscript{3.32}, which affected the movement of transmembrane helix 7 (TM7) during the activation of KOR. In our studies, Y320\textsuperscript{7.42} was seen to form a π-stacking network with Y66\textsuperscript{1.39} and Y119\textsuperscript{2.64} within the inactive-state KOR–compound 1 complex (Figure 8D).

![Figure 9. A) Interaction histogram of compound 1 in the active state of KOR after 200 ns MD simulations. (B) Water-mediated H-bonding of W287 with compound 1 and H-bonding and π-stacking with L212 and Y320, respectively. C) and D) Overlaid view of conformational differences between active state KOR–compound 1 complex (ball and stick model, light green sticks) and active state KOR–salvinorin A complex (ball and stick model, dark green sticks) in the (C) Y320\textsuperscript{7.42}/D138\textsuperscript{3.32} region and (D) Y139\textsuperscript{3.33}/K227\textsuperscript{5.40}/H291\textsuperscript{6.52} motif.](image)

Our MD simulation of compound 1 (an antagonist) in the active state of KOR showed extensive contacts with Q115\textsuperscript{2.60} (water bridge/hydrogen bonding), D138\textsuperscript{3.32} (water mediated/H-bond), Y139\textsuperscript{3.33} (hydrophobic/water bridge), L212\textsuperscript{ECL2} (H-bond), W287\textsuperscript{6.48} (hydrophobic/water-bridge), I290\textsuperscript{6.51} (hydrophobic), H291\textsuperscript{6.52} (water bridge), I294\textsuperscript{6.55} (hydrophobic) and Y320\textsuperscript{7.42} (hydrophobic/water bridge) (Figure 9A). Interestingly, compound 1 also exhibited a high incidence of water-mediated interaction, but within the active state of KOR, it predominantly participated in hydrogen bonding interaction with
D138$^{3,32}$ (Figure 9A). Compound 1 also exhibited extensive hydrophobic interactions with Y320$^{7,42}$ (Figure 9A-B). Additionally, Y320$^{7,42}$ formed a hydrogen-bonding interaction with D138$^{3,32}$ in the active-state KOR–salvinorin A complex (Figure 9C). The distances between D138$^{3,32}$ and Y320$^{7,42}$ (from the C=O in D138$^{3,32}$ to the OH in Y320$^{7,42}$), within the active-state KOR–compound 1 and the active-state KOR–salvinorin A complexes, were found to be 4.5 Å and 1.5 Å, respectively (Figure 9C). Since the interaction between D138$^{3,32}$ and Y320$^{7,42}$ contributes to the stability of the active-state KOR, the cooperativity of the interactions between different parts of 1 and those aforementioned residues probably disrupted this interaction between D138$^{3,32}$ and Y320$^{7,42}$, thus destabilizing the active state of KOR and contributing to the antagonistic functionality of 1 (Figure 9B). Compound 1 also interacted with Y139$^{3,33}$, predominantly through a hydrophobic interaction (Figure 9A), while salvinorin A exhibited extensive hydrogen bonding with the ring C lactone. Our MD simulation suggested that compound 1 may have altered the position of K227$^{5,40}$ and allowed Y139$^{3,33}$ to form a water-mediated interaction with H291$^{6,52}$ (Figure 9D). Our studies strongly suggested that all of the interactions mentioned above contributed to the deactivation of the receptor. Furthermore, the data suggested that while D138$^{3,32}$ may not directly affect the binding or functionality of the ligands, this residue provided an anchor point to which both compound 1 (an antagonist) and salvinorin A (an agonist) bind within the inactive state and the active state of KOR, respectively. Subsequent interactions that occurred as a result of compound binding greatly affected the stabilization of compound–receptor complexes. Altogether, our studies suggested that compound 1 possessed improved interactions with D$^{3,32}$ compared to those of salvinorin A, disrupting the interaction between the residue pairs involved in receptor activation (Y139$^{3,33}$ and K227$^{5,40}$, D138$^{3,32}$ and Y320$^{7,42}$, and M142$^{3,36}$ and H291$^{6,52}$), thus destabilizing the active-state KOR. Furthermore, the transducer regions were observed to have significant differences between the inactive and active states of KOR. These differences matched those of previous reports on KOR X-ray crystallography and molecular dynamics studies. For the first time, we reported a π-stacking network between Y66$^{1,39}$, Y119$^{2,64}$, and Y320$^{7,42}$ in response to the binding of compound 1 within the inactive state of KOR. Our studies also indicated that this YYY motif might aid in the stabilization of the inactive state of KOR through the prevention of specific intrahelical interactions that were known to be important for signal transduction and receptor activation.

3. CONCLUSION

We have reported, for the first time, pharmacokinetic and in vivo studies of a salvinorin-based antagonist. Administration of the KOR antagonist 1 reduced spontaneous cocaine-withdrawal behaviors, comparable to nor-BNI. Moreover, 1 produced anti-anxiety-like behavior in the light-dark transition test that was not observed with nor-BNI. The pharmacokinetic profile of 1 is promising with accumulation in the central compartment evident after systemic administration. Unlike nor-BNI, 1 appeared to be a short-acting compound (average half-life = 3.75 h) with an ideal t$_{1/2}$ in the brain (5.2 h, indicative of complete elimination within ~26 h, which is consistent with once daily dosing). T$_{max}$ occurred at 30 min in the brain, spinal cord, and plasma, and at 60 min in the liver. These results support the notion that modulation of KOR via selective, short-acting antagonism may reduce psychostimulant withdrawal and the associated negative affective symptoms that can contribute to relapse. As such, this class of compounds may hold promise for improving recovery outcomes. Furthermore, we also carried out in-depth computational studies, including induced-fit docking, computational mutagenesis, and molecular dynamics simulations, to gain insight into why compound 1 acts as an antagonist to KOR, as well as its selectivity towards KOR. Our computational modeling studies indicated that this compound binds to KOR, MOR, and DOR through a conserved interaction with C1-OH–D$^{3,32}$ through hydrogen bonding or water-mediated interaction, likely anchoring the compound in its putative binding mode. As a result, interactions with previously identified residues important for function and affinity to the KOR occur. Our MD
simulations revealed compound 1 fit well in the orthosteric pocket of KOR. In particular, 1 displayed strong interactions with the key residues D138, Y139, M142, and V230. Compound 1 also stabilized specific motifs known to aid in receptor activation via π-stacking (YYY and PIF motif), hydrogen bonding (KEY and DRY motif), or water-mediated interactions (NPxxY motif) within the 1–inactive-state KOR complex. Compound 1 also caused significant changes to the aforementioned motifs in the 1–active state KOR complex. In terms of drug discovery, the data suggested that incorporating a hydrogen bond donor (as in compound 1) or removing the hydrogen bond acceptor (as in compounds 2 and 6) can influence the functionality of salvinorin-based compounds. Therefore, alteration at C1 and C10 or concurrent modification at C2 could provide a novel strategy for the future design and development of selective short-acting KOR antagonists.

4. MATERIALS AND METHODS

4.1 Synthesis of 1

Salvinorin A was purchased from Apple Pharms Ingredients Inc. (Bakersville, NC). All other chemicals were purchased from Sigma-Aldrich or Fisher Scientific and used as received unless specified. All syntheses were conducted under anhydrous conditions under an argon atmosphere, using flame-dried glassware and employing standard techniques for handling air-sensitive materials unless otherwise noted. Before use, all solvents were distilled and stored under an argon or nitrogen atmosphere. 1H NMR and 13C NMR spectra were recorded on a Bruker-400 or a Bruker-500 spectrometer using CDCl3 as the solvent. Chemical shifts (δ) were recorded in parts per million and referenced to CDCl3 (7.24 ppm for 1H NMR and 77.23 ppm for 13C NMR). Coupling constants (J) are in Hz. The following abbreviations were used to designate the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad. Melting points were measured using an OptiMelt automated melting point system. LCMS were measured using an ACQUITY-Waters micromass (ESCl) system. High-resolution mass spectra (HRMS) were measured using a Waters Synapt XS HRMS. Compound 1 was purified via column chromatography (1:2, ethyl acetate:hexanes) and further by HPLC if necessary (7.8×30 mm, 7 µm, C18, gradient water in acetonitrile, flow rate 2 mL/min) until their purities were higher than 95% before being evaluated in in vitro and in vivo assays; purities were measured using a Waters 2695 analytical HPLC system.

Methyl (2S,4aR,6aR,7R,9S,10R,10aS,10bR)-2-(furan-3-yl)-9,10-dihydroxy-6a,10b-dimethyl-4-oxododecahydro-2H-benzo[f]isochromene-7-carboxylate, 7a

Salvinorin A (21.6 mg, 0.05 mmol, 1 eq) was dissolved in 4 mL of ethanol, and NaBH4 (9.5 mg, 0.25 mmol, 5 eq) was added at room temperature. The solution was then heated and stirred at 40 °C for 1 hour. Upon completion of the reaction as determined by TLC, the solution was cooled to 0 °C and acidified to a pH 3–4. The mixture was then diluted with water (10 mL), extracted with ethyl acetate (3×10 mL), washed with brine (10 mL), dried with sodium sulfate, and dried in vacuo. The crude was separated by column chromatography (2:1 ethyl acetate:hexanes) to produce 7a (57% yield) and 7b (40% yield). Mp (7a) 228–232 °C. 1H NMR (400 MHz, CDCl3) δ 7.49 – 7.42 (m, 2H), 6.43 (dd, J = 1.9, 0.9 Hz, 1H), 5.59 (dd, J = 11.5, 5.4 Hz, 1H), 4.23 (s, 1H), 3.70 (s, 3H), 3.64 – 3.58 (m, 1H), 2.50 (dd, J = 13.1, 5.4 Hz, 1H), 2.28 – 2.09 (m, 5H), 1.79 – 1.73 (m, 3H), 1.65 (d, J = 24.6 Hz, 2H), 1.49 (s, 3H), 1.42 – 1.38 (m, 3H), 0.94 (d, J = 1.8 Hz, 1H). 13C NMR (101 MHz, CDCl3) δ 172.97, 172.09, 139.33, 125.79, 108.42, 71.90, 71.80, 68.54, 55.73, 54.92, 52.56, 51.54, 44.23, 40.46, 37.71, 36.61, 28.20, 18.62, 17.76, 16.86. HRMS m/z calcd for C21H18O4 [M+H]+ 393.1913, found 393.1916. Purity after HPLC: 94.64%.

Methyl (2S,4aS,6aR,7R,9S,10R,10aS,10bR)-2-(furan-3-yl)-9,10-dihydroxy-6a,10b-dimethyl-4-oxododecahydro-2H-benzo[f]isochromene-7-carboxylate, 7b

Mp (7b) 233–235 °C. 1H NMR (400 MHz, CDCl3) δ 7.50 (dt, J = 1.6, 0.8 Hz, 1H), 7.44 (t, J = 1.7 Hz, 1H), 6.43 (dd, J = 1.9, 0.9 Hz, 1H), 5.30 (dd, J =
11.9, 1.5 Hz, 1H), 4.08 (d, J = 2.9 Hz, 1H), 3.56 (ddd, J = 11.4, 4.8, 3.2 Hz, 1H), 2.47 (d, J = 4.8 Hz, 1H), 2.28 – 2.11 (m, 5H), 1.96 – 1.86 (m, 3H), 1.72 (d, J = 6.7 Hz, 2H), 1.68 (s, 4H), 1.58 – 1.43 (m, 2H), 1.33 (s, 3H), 0.91 (d, J = 1.6 Hz, 1H).

\[^{13}\text{C}\] NMR (126 MHz, CDCl\(_3\)) \(\delta 172.58, 171.95, 169.80, 143.83, 139.41, 125.66, 108.46, 74.47, 71.78, 67.21, 55.62, 54.85, 52.46, 51.57, 44.15, 40.51, 37.75, 36.85, 24.75, 21.15, 18.59, 17.89, 16.77\). HRMS m/z calcd for \(\text{C}_{23}\text{H}_{31}\text{O}_8\) [M+H]\(^+\) 435.2047, found 435.2047. Purity after HPLC: 96.24%.

**Methyl (2S,4aR,6aR,7R,9S,10R,10aS,10bR)-9-acetoxy-2-(furan-3-yl)-10-hydroxy-6a,10b-dimethyl-4-oxododecahydro-2H-benzo[f]isochromene-7-carboxylate, 1**

The diol 7a (25 mg, 0.06 mmol, 1 eq) was dissolved in 7 mL of dry pyridine, 1 mL of acetic anhydride was added, and the mixture was stirred for 5 hours. As determined by TLC, the reaction was terminated by adding 1 mL of MeOH upon completion. The mixture was then poured into ice water (50 mL), its pH was adjusted to ~10 with NH\(_4\)OH, and the resulting mixture was extracted with CHCl\(_3\) (2 x 60 mL). The combined organic layers were washed with 10% HCl and 25 mL of water, dried over anhydrous sodium sulfate, and evaporated in vacuo. The crude residue was purified by column chromatography (1:1 ethyl acetate:hexanes) to yield compound 1 in 91%. Mp 110-112 °C. \[^{1}\]H NMR (500 MHz, CDCl\(_3\)) \(\delta 7.48 – 7.41\) (m, 2H), 6.42 (d, J = 1.9 Hz, 1H), 5.55 (dd, J = 11.4, 5.4 Hz, 1H), 4.70 (ddd, J = 12.1, 4.8, 3.1 Hz, 1H), 4.32 – 4.29 (m, 1H), 3.69 (s, 3H), 2.42 (dd, J = 13.2, 5.4 Hz, 1H), 2.32 (q, J = 12.6 Hz, 1H), 2.21 (dd, J = 13.2, 2.6 Hz, 1H), 2.14 – 2.09 (m, 5H), 1.85 – 1.62 (m, 5H), 1.46 (s, 3H), 1.39 (s, 3H), 1.01 (d, J = 1.8 Hz, 1H). \[^{13}\]C NMR (126 MHz, CDCl\(_3\)) \(\delta 172.58, 171.95, 169.80, 143.83, 139.41, 125.66, 108.46, 74.47, 71.78, 67.21, 55.62, 54.85, 52.46, 51.57, 44.15, 40.51, 37.75, 36.85, 24.75, 21.15, 18.59, 17.89, 16.77\). HRMS m/z calcd for \(\text{C}_{23}\text{H}_{32}\text{O}_7\) [M+H]\(^+\) 439.1913, found 439.1922. Purity after HPLC: 96.36%.

**4.2 In vivo studies**

All procedures were preapproved by the Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi. Experiments were carried out in accordance with ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85-23).

**4.2.1 Subjects and housing**

Male, adult, C57BL/6N mice (n = 55) were bred in the vivarium at the University of Mississippi (University, MS, USA). Mice were housed 2–5 per cage and kept in a temperature- and humidity-controlled environment on a 12:12 h light:dark cycle (lights off at 09:00 h) with *ad libitum* access to food and water.

**4.2.2 Chemicals**

Norbinaltorphimine (nor-BNI) and cocaine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA) and diluted to concentration in sterile saline (0.9%). Compound 1 was synthesized as described above and diluted to concentration in a vehicle (Solutol HS 30% in sterile 0.9% saline).

**4.2.3 Pharmacokinetic assessment of 1**

Mice received an i.p. injection of 1 and underwent anesthetized, transcardial perfusion with PBS either 5, 15, 30, 60, 120, or 360 min later (n=3/time-point) as described. Brain, liver, dorsal spinal cord, and plasma were collected at each time-point as described below.

**4.2.3.1 Tissue collection and extraction**

At each time point, mice (C57BL/6N) were anesthetized using isoflurane (4%). The heart was exposed, an incision was made in the right atrium, and whole blood was collected and kept on wet ice in tubes containing 3.8% sodium citrate (10% of blood volume). After transcardial perfusion with 20 mL of PBS, the liver, brain, and dorsal spinal cord were grossly dissected. Immediately after each dissection, tissue wet weights were collected, and tissues were rinsed and homogenized in PBS (0.4 mL), and diluted with an equal volume of PBS. Plasma was collected from whole blood via centrifugation (10,000 rpm, 10 min). Four mL of hexanes were added to plasma (0.4 mL) and vortexed (1 min) or to tissue homogenates (0.8 mL) and vortexed (2 min) and centrifuged (10,000 rpm, 10 min).
supernatant was collected and evaluated by UV-HPLC as described in Section 4.2.3.2.

### 4.2.3.2 Analytical UV-HPLC systems to quantify 1 in the biological matrices

A previously published UV-HPLC method was used to quantify concentrations of 1 in transport and in vitro metabolism studies. An Agilent 1260 Infinity II (Santa Clara, CA) high-performance liquid chromatography (HPLC) with a photodiode array detector (PDA) was used. Thirteen different concentrations of 1 between 0.056 and 230 μM were analyzed using a reversed-phase C18 column (Agilent Poroshell 120 column, C18, 2.7 μm, 50 × 3.0 mm; 0.5 mL/min; 40% CH3CN in H2O for 20 min). The isocratic mobile phase was filtered through a nylon filter (0.45 μm) and degassed under ultrasound and vacuum for 20 min. The PDA detector on the isocratic mobile phase was operated by Agilent Chemstation Software. The injection volume was 90 μL. Compound 1 was quantified at a UV wavelength of 195 nm and its retention time was 6.1 min. The quantification curve was linear in the range of 0.225 to 230.15 μM (r2 ≥ 0.9997) (Figure S36).

The organic phase extracted from each brain, liver, spinal cord, and plasma was transferred to a clean test tube, evaporated under nitrogen, and reconstituted with 1 mL of acetonitrile. The samples were vortexed for 2 minutes and were filtered through a nylon filter (0.45 μm). The samples were analyzed with the same system as before. After HPLC separation, the peak area of compound 1 was quantitated and calculated using the PK solver.

### 4.2.4 Behavioral assays

**Open field test.** The open-field test was used to assess motor and exploratory behavior. Briefly, mice were placed in the corner of a square Plexiglas box (40 × 40 × 35 cm; Stoelting Co., Wood Dale, IL, USA) and allowed to behave for 5 min. Their mean velocity (meters/sec) and total distance traveled (meters) were used as indices of motor behavior. The frequency and time spent rearing were also assessed as an index of motor/exploratory behavior.

**Light-dark transition test.** The light-dark transition test was used to assess anxiety-like behavior. Briefly, mice were placed in the brightly lit corner of a square Plexiglas box (40 × 40 × 35 cm; Stoelting Co.) that was divided into two compartments (one brightly lit and one enclosed and dark; 20 × 20 cm ea.). Mice were allowed to explore for 5 min. The latency to enter the dark compartment and the time spent in the light chamber were considered indices of anxiety-like behavior. The number of transitions between compartments was used as an index of motor behavior.

**Elevated plus maze test.** The elevated plus maze test was used to assess anxiety-like behavior. Briefly, a plus-shaped elevated maze (37.5 cm from the floor) consisted of two open and two enclosed arms (61 × 5 cm ea.) connected by a central area (5 × 5 cm; Stoelting Co.). Mice were placed in the central area and allowed to freely explore for 5 min. Shorter latencies to enter the open arms and greater time spent on the open arms were considered indices of anti-anxiety-like behavior. The total number of arm entries was recorded as an index of motor behavior.

The tail suspension test was carried out as previously described. Briefly, mice were suspended vertically (18 in. from the floor) with their tails secured by laboratory tape to a horizontal surface. A small clean plastic cup was placed over the tails to prevent tail climbing. Behavior was video recorded for 6 min (with the initial 2 min discarded for acclimation), and the time spent immobile was scored. Greater immobility time was considered an index of increased depression-like behavior.

**Biased conditioned place preference method.** Conditioned place preference (CPP) to cocaine (10 mg/kg, ip, diluted to concentration in sterile 0.9% saline; Sigma-Aldrich, St. Louis, MO, USA) was evaluated in a biased paradigm as previously described. Briefly, the behavior was recorded and digitally encoded by an ANY-maze behavioral tracking system (Stoelting Co., Wood Dale, IL, USA). Locomotor behavior was assessed during conditioning days. The CPP apparatus (#64101; Stoelting Co.) consisted of
two black conditioning chambers (18 × 20 × 35 cm), each visually distinguished by white circles or horizontal stripes on the walls of the chamber, as well as ∼30 lux difference in ambient lighting. The conditioning chambers were connected by a start box/transition chamber (10 × 20 cm). On day 1, mice freely explored the apparatus for 15 min to establish an initial chamber preference (no significant side preferences were observed across groups). On days 2 and 3, mice were pretreated with vehicle or 1 (5 mg/kg, i.p.) and underwent one cycle of cocaine-conditioning per day consisting of an i.p. saline injection paired with confinement to the preferred chamber for 30 min, followed 4 h later by an i.p. cocaine (10 mg/kg) injection paired with confinement to the less preferred chamber for 30 min. On day 4, mice freely explored the apparatus to assess their final chamber preference. The amount of time that mice spent in the chambers or the start/transition box, as well as the distance traveled, was recorded on each day. CPP was quantified as a difference score: CPP d-score = (time spent in the cocaine-paired chamber) – (time spent in the saline-paired chamber).74

4.2.5 Behavioral procedure

To assess the motor and affective responses to spontaneous cocaine withdrawal, mice were administered vehicle or a short series of escalating doses of cocaine over 3 days (5, 10, 20 mg/kg, i.p.; Figure 4). This dosing range is demonstrated to produce psychostimulation in mice and sensitization when administered sequentially.75 After receiving the third and final dose of vehicle or cocaine, mice were administered vehicle, compound 1 (5 mg/kg, i.p.), or nor-BNI (5 mg/kg, i.p.) immediately following the final cocaine dose. Forty-eight hours after cocaine cessation, mice were evaluated in a behavioral battery to assess their spontaneous motor behavior (open field) as an index of withdrawal, their anxiety-like behavior (light-dark transition test, elevated plus maze), and their depression-like behavior (tail suspension test).

To assess the effects of 1 on the rewarding properties of cocaine, mice were assessed in a biased cocaine-CPP paradigm, as described above. Mice received vehicle or 1 (5 mg/kg) on conditioning days immediately prior to each saline-cocaine conditioning cycle, as described above.

For all behavioral testing, mice were acclimated to the testing room for 30 min in their homecage prior to evaluation.

4.2.6 Statistical analyses

Motor and affective behavioral data were analyzed using separate one-way analyses of variance (ANOVA). Following main effects, group differences were determined using Fisher’s Protected Least Significant Difference (PLSD) post hoc tests with α corrected for family-wise error. A priori comparisons were made between manipulated groups and cocaine-exposed mice administered vehicle. Cocaine-CPP was analyzed via repeated measures ANOVA with main effects assessed via Fisher’s PLSD. No interactions were detected. All analyses were considered significant when \( p < 0.05 \).

4.3 Computational studies

4.3.1 Ligand preparation

Two-dimensional (2D) structures of salvinorin-based antagonists 1, 2, 6, and salvinorin A were sketched in the 2D sketcher module of Maestro and energy minimized using the LigPrep protocol of the Schrödinger suite 2019-1.58,76 All calculations unless otherwise mentioned were completed using Optimized Potentials for Liquid Simulations 3e (OPLS3e) force field in the gas phase.55

4.3.2 Opioid receptors and generation of receptor grid

X-ray crystal structures of agonist-bound KOR (PDB: 6B73), antagonist-bound KOR (PDB: 4DJH), antagonist-bound MOR (PDB: 4DKL), and antagonist-bound DOR (PDB: 4N6H) were used in the docking studies. Protein structures were prepared using the Schrödinger Small Molecule Drug Discovery Suite. All water molecules from the X-ray crystal structures were removed, and mutated residues in the X-ray crystal structures were changed back to the wild
type. We added missing side chains and removed any extraneous ligands from the active and inactive-state X-ray crystal structures of the opioid receptors. Grid generation was centered on the co-crystallized ligand within the active or inactive-state X-ray crystal structure and defined a 20 x 20 x 20 Å³ box around the ligand. The rotatable residues within 3 Å of the ligand were defined, and the grid was generated.

4.3.3 Native Docking

The prepared protein complexes of the opioid receptors were used to conduct native docking. The native ligand was docked back into the protein structure with flexible ligand sampling using Glide SP (Schrödinger 2019-1). The docking results were evaluated by comparing the best-docked pose determined by the Emodel score and the cocry stallized pose by the root-mean-square deviation (RMSD).

4.3.4. Molecular Docking and Scoring

The standard precision (SP) docking method was applied considering flexible ligand sampling using the Glide software. A total of five distinct binding poses (with RMSD ≥ 0.5 Å relative to the other poses) were generated for each ligand whenever possible. The selection of the best pose for each ligand was based on the computed values of the Emodel score and GlideScore and a thorough visual inspection of the predicted binding modes in which favorable interactions with key residues were considered as previously reported.

4.3.5. Binding Free Energy Calculations

The Prime Molecular Mechanics/Generalized Born Surface Area (MM-GBSA) method was used to calculate the receptor–ligand binding free energies using Prime energy, molecular mechanics (force fields), and the continuum (implicit) solvation energy function (kcal/mol). These were done using the OPLS3e force field in the gas phase and considering the protein side-chain flexibility that was limited to amino acids within a region of 5 Å around the bound ligand.

4.3.6. Molecular Dynamics Simulations

The MD simulations were carried out using the Desmond module of Schrödinger suite 2019. To further assess the stability of the complexes, salvinorin A bound inactive state KOR, salvinorin A bound active-state KOR, 1 bound inactive-state KOR, and 1 bound active-state KOR were embedded in a POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) bilayer and solvated with an 11 Å TIP3P water buffer using the OPLS_2005 (optimized potentials for liquid simulations) force field implemented in Desmond. The system was neutralized by adding chloride ions as needed, and 0.15 M NaCl was added. The system was equilibrated using the previously published protocol with slight modification. In brief, the system was simulated for 1 ns using Brownian dynamics in the NVT ensemble at 10 K with the restraint of 50 kcal/mol on solute heavy atoms. Secondly, a 300 ps simulation was run in the NVT ensemble using the Berendsen thermostat (10 K) while retaining the restraint on solute heavy atoms. Thirdly, a 300 ps simulation was run in the NPT ensemble using the Berendsen thermostat (10 K) and barostat (1 atm) while restraints were retained. Over the next 300 ps, the system was gradually heated to 300 K. A final 5 ns simulation was performed in which all restraints were removed. The NPT ensemble with a temperature of 300 K and a pressure of 1 bar was applied in all the simulations. The simulation length of the production run was 200 ns. The OPLS_2005 force field parameters were used in all simulations. The long-range electrostatic interactions were calculated using the particle mesh Ewald method. The cutoff radius for Coulomb interactions was 9.0 Å. The Langevin coupling schemes were used for the pressure and temperature controls for the 200 ns production run. Nonbonded forces were calculated using the RESPA integrator, and the trajectories were saved at 13.3 ps intervals for analysis. The dynamical behavior and interactions between the ligand and protein were analyzed using the Simulation Interaction Diagram tool implemented in the Desmond MD package. The stability of the MD simulations was monitored by looking at the RMSD of the ligand and protein atom positions in time and by RMSF. Following MD simulations of the complexes, the binding free energies of 1 and
salvinorin A were computed on frames extracted from the trajectory at an interval of 4 ns using the thermal_mmgbsa script by Schrödinger.

ASSOCIATED CONTENT

Supporting Information

Assessment of 1 in conditioned place preference; quantification curve of 1 for pharmacokinetic assays; computational studies, including induced-fit docking, mutagenesis, and molecular dynamics simulations; and NMR spectra. The Supporting Information is available free of charge at: https://pubs.acs.org/

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N.S.A. synthesized compound 1 and carried out computational studies, including induced-fit docking and mutagenesis. P.P. carried out the molecular dynamics simulation studies. S.F.M., E.M., C.I.W., and J.J.P. carried out the behavioral assessment. F.M., S.J.K., and J.J.P. carried out the pharmacokinetics studies. M.I.H.K. and M.M.K. contributed to the synthesis of 1. A.G.C. and R.J.D. advised and contributed to the computational studies. H.V.L. designed the project and supervised the overall coordination of the research. N.S.A., P.P., S.J.K., J.J.P., and H.V.L. wrote the manuscript. All authors approved the final version.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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Brain

$t_{1/2} = 5.2 \text{ h}$

$C_{\text{max}} = 17.4 \mu\text{M}$

$T_{\text{max}} = 30 \text{ min}$