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Identification of short-acting κ-opioid receptor antagonists with anxiolytic-like activity

Matthew F. Peters *, Anna Zacco, John Gordon, Carla M. Maciag, Linda C. Litwin, Carolann Thompson, Patricia Schroeder, Linda A. Sygowski, Timothy M. Piser, Todd A. Brugel

AstraZeneca Pharmaceuticals, 1800 Concord Pike, Wilmington, DE 19850, United States
AstraZeneca Pharmaceuticals, Rm B110, 1800 Concord Pike, Wilmington, DE 19803, United States

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ABSTRACT

The κ-opioid receptor plays a central role in mediating the response to stressful life events. Inhibiting κ-opioid receptor signaling is proposed as a mechanism for treating stress-related conditions such as depression and anxiety. Preclinical testing consistently confirms that disruption of κ-opioid signaling is efficacious in animal models of mood disorders. However, concerns about the feasibility of developing antagonists into drugs stem from an unusual pharmacodynamic property of prototypic κ-opioid receptor-selective antagonists; they inhibit receptor signaling for weeks to months after a single dose. Several fundamental questions include — is it possible to identify short-acting antagonists; is long-lasting inhibition necessary for efficacy; and is it safe to develop long-acting antagonists in the clinic. Here, we test representative compounds (AZ-ECPC, AZ-MTAB, and LY-DMPF) from three new chemical series of κ-opioid receptor ligands for long-lasting inhibition. Each compound dose-dependently reversed κ-opioid agonist-induced diuresis. However, unlike the prototypic antagonist, nBNI, which fully inhibited evoked diuresis for at least four weeks, the new compounds showed no inhibition after one week. The two compounds with greater potency and selectivity were tested in prenatally-stressed rats on the elevated plus maze, an exploration-based model of anxiety. Spontaneous exploration of open arms in the elevated plus maze was suppressed by prenatal stress and restored with both compounds. These findings indicate that persistent inhibition is not an inherent property of κ-opioid-selective antagonists and that post-stress dosing with transient inhibitors can be effective in a mood disorder model. This further supports κ-opioid receptor as a promising target for developing novel psychiatric medications.

1. Introduction

Major depressive disorder, or depression, is a debilitating disease with a lifetime prevalence of about 16% in the general population (Kessler et al., 2003). The current first-line drug treatments primarily target the serotonin and norepinepherine pathways. Responses to these drugs are variable and remission is achieved in only 22–40% of patients (Trivedi et al., 2006). Thus, there remain a significant unmet medical need and a growing recognition that new treatments targeting alternative signaling pathways are required.

Depression and anxiety can be triggered or exacerbated by stressful life events (van Praag, 2004). Accumulating evidence indicates that the κ-opioid receptor signaling pathway is required to mediate the negative effects of stress on mood. The endogenous κ-opioid receptor agonist, Dynorphin A, is up-regulated in response to stress in brain regions associated with mood (Shirayama et al., 2004). This up-regulated κ-opioid receptor activity is predicted to be prodepressive since numerous exogenous κ-opioid receptor agonists are profoundly dysphoric/depressive in humans and animal models (Knoll and Carlezon, 2010; Pfeiffer et al., 1986). Conversely, disruption of κ-opioid receptor signaling by genetic or pharmacologic means has antidepressant- and anxiolytic-like effects in animal models. While it remains to be determined if the antidepressant-like effects of antagonists will translate to humans, expectations are high based on the remarkable diversity in the animal models that confirm efficacy. Specifically, κ-opioid receptor inhibition prevents stress-induced behavioral deficits in elevated plus maze, forced-swim, learned-helplessness, social-defeat stress, fear-potentiated startle, intercranial self-stimulation, light-dark box, and open field (reviewed in Knoll and Carlezon, 2010).

While the preclinical validation is remarkable, concerns about the feasibility of developing κ-opioid receptor antagonists into drugs and subsequent scope of potential therapeutic applications have been raised by the unusual pharmacodynamics of the prototypic ligands. Each of the well-established, κ-opioid receptor-selective antagonists persistently inactivates κ-opioid receptor signaling (Bruchas et al., 2007; Horan et al., 1992), an effect that can last up to 20 weeks after a single dose (Ko et al., 2003). This long-lasting inhibition could impede or perhaps even prohibit clinical development due to the risk of

* Corresponding author at: AstraZeneca Pharmaceuticals, Rm B110, 1800 Concord Pike, Wilmington, DE 19803, United States. Tel.: +1 302 886 2567; fax: +1 302 886 2341.
E-mail address: matt.peters@astrazeneca.com (M.F. Peters).

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patients having adverse events with pseudo-irreversible kinetics. Thus, for the first-in-class κ-opioid receptor antagonists there is a clear need for short-lasting inhibition. However, no short-lasting compounds have been identified and there is no evidence that transient inhibition will be effective in treat mood disorders. Rather, the validation studies have been limited to chronic inhibition — using either long-lasting antagonists (nBNI, JDTic, GNTI), an irreversible ligand (DIPPA), or genetic deletion (κ-opioid receptor, dynorphin) (reviewed in Knoll and Carlezon, 2010). Furthermore, the existing κ-opioid receptor antagonists have poor CNS penetration, which typically limits the window for dosing prior to or immediately following the stressful event in order to achieve sufficient exposure at testing. Taken together, the constraints of the available tools raise fundamental questions regarding the therapeutic potential: Is chronic inhibition required? Does κ-opioid receptor inhibition need to occur prior to the stressful event? In order to identify compounds that lack long-lasting inhibition and to begin to test these critical questions, we examine representative compounds from three new chemical series of κ-opioid receptor antagonists for duration of activity and effects on models of stress-induced behavioral deficits.

2. Material and methods

2.1. Materials

3-((1*R*,3r,5*S*)-8-((5-MethylThiophen-2-yl)Methyl)-8-Azabicyclo [3.2.1]octan-3-yloxy)Benzamide (AZ-MTAB, Brugel et al., 2010a,b), Ethyl 4-(3-Carbamoylbenzyl)-1-(Cyclohexylmethyl)Piperidine-4-Carboxylate (AZ-ECPC, Fig. 1A), (*S*)-4-(4-((2-(3,5-Dimethylphenyl) Pyrrolidin-1-yl)Methyl)Phenoxy)-3-Fluorobenzamide (LY-DMPF, Buezo et al., 2009), and JDTic were synthesized at AstraZeneca. nBNI, U50488, and SNC-80 were purchased from Tocris Bioscience (Ellisville, MO). Dynorphin A (1–13), naloxone, naltrexone, and naltrindole were purchased from Sigma (St. Louis, MO). DAMGO was purchased from Bachem (Torrance, CA).

2.2. GTPγS

Opiate receptor [^{35}S]GTPγS antagonist mode assays for kappa, delta, and mu opiate subtypes were individually co-optimized for stimulus potency, signal to noise, and low agonist activity for well-known *in vivo* antagonists. All assay buffers included 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 4 mM GDP, and 0.2 mg/ml BSA. For κ-opioid receptor, the buffer included 225 mM NaCl and 50 μg/ml saponin; the stimulus was 15 nM Dynorphin A (1–13), and specific binding was defined with 0.3 μM nBNI. For the δ-opioid receptor, the buffer included 150 mM NaCl, 200 μg/ml saponin, and 0.1 mM DTT; the stimulus was 89 nM SNC-80, and specific binding was defined with 0.3 μM naltrindole. For μ-opioid receptor, the buffer included 75 mM NaCl and 50 μg/ml saponin; the stimulus was 300 nM DAMGO, and specific binding was defined with 3 μM naloxone. The same general protocol was used for each subtype. Membranes (0.05–0.1 mg/ml) from HEK cells stably transfected with kappa, delta, or mu human opiate receptor subtypes were incubated with 8.5 mg/ml WGA-SPA beads (Perkin-Elmer) in their respective assay buffers for 30 min at room temperature on a plate rocker just prior to use. First, 1 μl of 100× concentration of test antagonist in neat DMSO was spotted to each well of white OptiPlate-96 polystyrene plates. Next, 59 μl of membrane/bead suspension was added, and the plates were gently agitated for 20 min at room temperature. Then, 40 μl of buffer containing the stimulus and 0.5 nM [^{35}S]GTPγS were added; plates were sealed and agitated vigorously for 2 h at room temperature. Plates were centrifuged 10 min at 1400 RPM and counted in a TopCount NXT HTS (Perkin-Elmer). GTPγS antagonist IC_{50}s were determined versus an EC_{80} of the stimulus (subtype selective full agonist) using 10-point concentration-effect curves on duplicate plates.

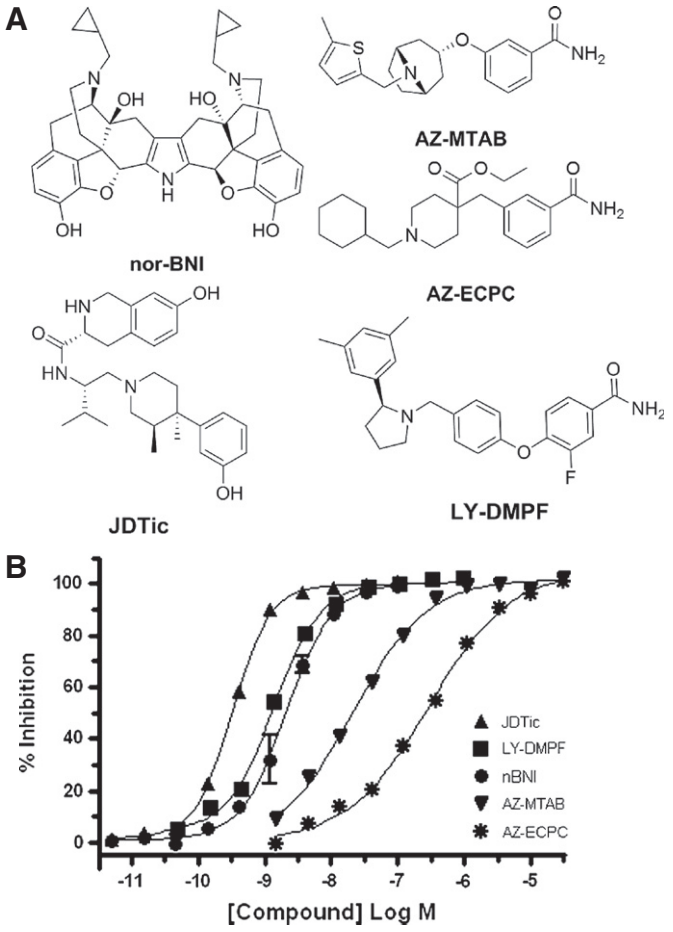

Fig. 1. Inhibition of κ-opioid receptor activity. A. Structures of κ-opioid receptor-selective antagonists. B. Potency and efficacy of inhibition of Dynorphin A-stimulated κ-opioid receptor in a GTPγS assay was determined. Data for JDTic (▲), LY-DMPF (■), nBNI (●), AZ-MTAB (▼), AZ-ECPC (✱), are the average ± standard deviation from three representative experiments.

2.3. κ-opioid receptor binding

Test antagonists were incubated with 10 μg human κ-opioid receptor-HEK293s membranes and 0.6 nM [^{3}H]-U69593 (Perkin Elmer) in 200 μl of 50 mM Tris HCl/3 mM $MgCl_2$/1 mg/ml BSA in 96-well polypropylene plates for 75 min at room temperature. Reactions were filtered over Unifilter GF/B plates with a cell harvester and washed with cold 50 mM Tris HCl/3 mM $MgCl_2$. Plates were dried, after addition of 40 μl Microscint-40/well, and counted in a TopCount NXT HTS. Nonspecific binding was defined with 10 μM naloxone. Antagonists IC_{50}s were determined using 11-point concentration-effect curves on duplicate plates.

2.4. Diuresis

All animal experiments were carried out in accordance with the National Institute of Health Guide for the care and use of laboratory animals. Male Sprague Dawley (SD) rats (Charles River) were housed two per cage for one week prior to the study and were allowed water and chow *ad libitum*. Animals weighed 200–260 g on the day of the initial study and were habituated to the test room for at least 1 h prior to study. Rats were dosed subcutaneously (s.c.) with (−)-U50488 hydrochloride or vehicle. For antagonist studies, the antagonist was administered immediately following agonist injection on the other side of the body. Rats were placed in metabolism cages that funneled urine into graduated cylinders. Cumulative urine output was measured at 5 h or at indicated times. Animals were returned to the vivarium and, after the indicated

number of weeks, retested with agonist alone. Increases in body mass are associated with increased urine output (Craft et al., 2000). Therefore, to more clearly compare dose-related effects over four repeat trails, the nBNI time-course was expressed as a percent of control (vehicle/U50488-treated for the same date). Effects of nBNI in the absence of U50488 stimulation were tested in manually hydrated rats. Briefly, hydration procedures were adapted from Huidobro-Toro and Parada (1984) and rats received an oral administration of 2 ml/100 g of H_2O and subcutaneous administration of nBNI 30 μmol/kg or saline (vehicle). One hour later rats received an oral administration of 2 ml/100 g of 0.9% saline.

2.5. Determination of free compound concentrations in plasma and brain

The fraction of each compound that was free (unbound to protein) in plasma and brain homogenate was determined using a 96-well equilibrium dialysis system as previously described (Banker et al., 2003). Briefly, brain homogenate, or plasma, was spiked with test compound such that the final concentration was 1 μM and dialyzed on a shaking device for 18 h at 37 °C prior to sample analysis by LC–MS/MS. The unbound fraction in brain homogenate and plasma were calculated as described by Barre et al. (1985).

The concentrations of all compounds tested were determined by a Waters Ultima LC–MS/MS operated in MRM mode with an electrospray ionization source. For nBNI, the targeted precursor ion was doubly charged with a m/z of 331.60, which upon collisional dissociation, resulted in a monitored product ion of 590.0 m/z. For LY-DMPF (MW 418.51) the targeted precursor ion was singly charged with a m/z of 419.22, which upon collisional dissociation, resulted in a monitored product ion of 244.12 m/z. For AZ-MTAB (MW 356.49), the targeted precursor ion was singly charged with a m/z of 355.15, which upon collisional dissociation resulted in a monitored product ion of 108.78 m/z. AZ-ECPC (MW 386.53) the targeted precursor ion was singly charged with a m/z of 387.17, which upon collisional dissociation resulted in a monitored product ion of 313.13 m/z.

The free plasma concentration used for comparison with GTPγS potencies was determined by averaging plasma concentrations over the five hour diuresis assay. Plasma area under the curve (AUC_{0-5h}) was calculated by the log-linear trapezoidal method in WinNonlin version 5.2 (Pharsight, Mountain View, CA). Estimates of averaged plasma levels during the diuresis test were made by dividing the dose-normalized AUC_{0-5h} by 5 h, the duration of the test interval. Unbound concentrations in the interstitial fluid were estimated to be equivalent to unbound plasma levels in the absence of active transport at the blood brain barrier.

2.6. Microdialysis

Due to the poor CNS penetration of nBNI, the ratio of unbound concentrations in the brain to plasma was estimated from an ultra-slow quantitative microdialysis study performed by *Brains On-Line* (Groningen, Netherlands). Methods have been previously described (Cremers et al., 2009). Briefly, a PES MetaQuant probe was positioned on the medial prefrontal cortex of male Wistar rats. Dialysis experiments on freely moving animals were performed with an artificial cerebrospinal fluid delivered through the central inlet (0.1 μl/min) and ultra-purified water delivered as a carrier (0.9 μl/min) through the left inlet. Samples were collected at 30 min time intervals for 8 h.

2.7. Prenatal stress

Timed pregnant female SD rats (Charles River Laboratories, Wilmington, MA) arrived at gestation day 6 (E6) and were single-housed in standard rat cages. After a 6-day acclimation period (E7–E13), the pregnant dams were assigned to either the control group (non-prenatal stress) in which the dams were left alone in the animal housing room or the prenatal stress group in which the dams were exposed to a novel, variable stress paradigm during the third week of gestation (E14–E20). The pregnant dams were exposed to a different stressor each of the seven days. This particular stress model consisted of the following stressors: (1) The motion stressor involved placing the rat in an empty, clear, $11.5' \times 11.5' \times 7.5'$ container attached to a shaker that toggled the container back and forth; the session lasted 30 min. (2) Forced-swim stressor entailed a thick, clear, plastic cylinder, 15′ in height, 6.5′ in diameter, about 3/4 full (~1.5 l) with water at room temperature (~23 °C). The pregnant dam was then placed in the cylinder for a period of 10 min. (3) The rat was put in a restraint apparatus for 60 min. (4) Wet bedding stressor involved 1.5–1.7 l of water added to the rat's bedding in which the rat remained overnight. Cages with wet bedding were replaced with clean cages the following day. (5) The rats were kept in a novel room for 36-hours of darkness. (6) Pregnant dams were placed on a rack and brought outside a closed room that contained barking dogs for six hours. (7) The rat received intermittent puffs of air using a hair dryer set on cool (three 5-minute sessions). Once the pups were born, approximately prenatal day 22, each litter was culled and sexed to four females and six males. The dams and pups were left undisturbed for three weeks, at which point the pups were weaned and group-housed on postnatal day (pnd) 21. Behavioral measures were assessed in adult offspring after pnd 70. The effects of prenatal stress on exploratory behaviors were evaluated using the elevated plus maze.

2.8. Elevated plus maze test

The elevated plus maze (Hamilton Kinder, Poway, CA) consisted of 4 arms at right angles elevated 85 cm above the ground. Two of the arms were black and enclosed by black panels 40 cm high (closed arms), whereas the other 2 white arms were open to the room (open arms). Motor Monitor recorded animal activity with a microprocessor and infrared photobeam technology; beam-breaks were recorded by a microprocessor in the control chassis; and the collected data were transmitted *via* serial communication to a host PC. The maze was lit by 2 light bulbs shining the length of each open arm so that the illumination was 10 and 15 Lux in the center and end of each open arm, respectively, <1 Lux in the closed arms, and 3 Lux in the center of the maze. Rats were placed in the center of the maze facing an open arm and behavior was recorded for exactly 5 min. The percent time spent on the open arms, the percent entries onto the open and closed arms, and the total number of entries onto the open and closed arms were recorded. The rats were dosed sc with either vehicle or compound prior to testing in the elevated plus maze.

2.9. Statistical analysis

GTPγS and binding data curve fitting was performed using interactive, nonlinear, least-squares curve-fitting programs in which inhibition curves were fit to their own maximum effect. GTPγS confidence intervals (95%) for fold difference of duplicate IC_{50}s are 1.8 for kappa, 1.6 for delta, and 2.1 for mu. K_i values were calculated using the Cheng–Prusoff equation with a K_d of 0.51 nM. Binding confidence interval (95%) for duplicate IC_{50}s is 1.6-fold. Diuresis data was assessed for significance versus control by one-way ANOVA followed by Dunnett's test. The effects of drug treatment in elevated plus maze were assessed using a one-way ANOVA followed by Dunnett's multiple comparison. The effect of stress in the vehicle-treated animals was assessed with a one-tailed *t*-test.

3. Results

3.1. In vitro pharmacology

In an effort to identify κ-opioid receptor antagonist(s) lacking long-lasting inhibition, representative compounds from three new

structural classes were selected (Fig. 1A). These new ligands, along with well-established reference compounds, were profiled for affinity and potency at the κ-opioid receptor (Fig. 1B and Table 1). In a κ-opioid receptor GTPγS assay, the ligands completely inhibited Dynorphin A responses with a range of potencies.

Achieving selectivity versus the μ-opioid receptor has been a key hurdle for κ-opioid receptor antagonist development. In GTPγS assays, the new compounds showed either a κ-opioid receptor preference (4.5-fold, AZ-EPCP) or κ-opioid receptor selectivity (AZ-MTAB and LY-DMPF) that exceeded an initial 30-fold target for candidate drugs (Table 1).

3.2. Diuresis assay

Antagonist reversal of agonist-evoked diuresis was selected as the *in vivo* model. Consistent with the literature data, we found that the κ-opioid receptor-selective agonist, U50488, induced a robust increase urine output (Fig. 2A, B). Agonist-evoked diuresis was dose-dependently reversed by co-administering the κ-opioid receptor-selective antagonist, nBNI (Fig. 2C). This antagonist reversal was judged to be κ-opioid receptor-specific since, in the absence of U50488 stimulation, nBNI did not affect either basal urine output (~2 ml, data not shown) nor did it inhibit urine output elevated by forced hydration (~8 ml, Fig. 2D and Craft and McNiel, 2003).

3.3. Long-lasting inhibition

To confirm that our diuresis model was sensitive to long-lasting inhibition, animals were tracked for the recovery of U50488-induced diuresis after receiving a single administration of nBNI. Each of the doses tested in Fig. 2C was included in order to evaluate the sensitivity and consistency of the assay. At the lowest dose (0.45 μmol/kg) statistically significant inhibition lasted for one 1 week. Inhibition lasted progressively longer as doses were elevated. At the highest doses (15 and 45 μmol/kg) significant inhibition lasted at least four weeks.

For the purpose of identifying the presence/absence of long-lasting inhibition, only the one week follow-up was needed. AZ-EPCP, AZ-MTAB, and LY-DMPF were evaluated along with JDTic — a positive control for long-lasting inhibition (Carroll et al., 2004). Each compound dose-dependently inhibited U50488-evoked diuresis on the initial test (Fig. 4 left panel). However, only JDTic sustained inhibition for one week (Fig. 4 right panel). The lack of persistent inhibition for the new antagonists was not due to insufficient dosing since urine output was inhibited to near basal levels for each compound on day 0.

Table 1
In vitro profiles of κ-opioid receptor ligands. New κ-opioid receptor ligands and relevant reference compounds were assessed for κ-opioid receptor affinity and functional activity. κ-, μ-, δ-opioid receptor potency data were determined in GTPγS assay stimulated with Dynorphin A, DAMGO, and SNC-80, respectively. Statics on the precision of each assay are discussed in the Methods section.

Name	MW	Ki κ-OR (nM)	GTPγS IC_{50} κ-OR (nM)	μ-OR fold selectivity (GTPγS)	δ-OR fold selectivity (GTPγS)
Naloxone	327	1.6	84	0.17	0.01
Naltrexone	341	0.17	21	0.22	0.1
nBNI	662	0.87	2.1	102	4.3
JDTic	466	0.32	0.35	87	4040
AZ-ECPC	387	53	83	4.5	35
AZ-MTAB	356	32	19	37	440
LY-DMPF	419	1.8	1.2	40	490

3.4. In vitro/in vivo correlation

It is important to understand the relationship between the potencies observed *in vivo* (diuresis) and *in vitro* (GTPγS). Since the diuresis response is mediated by receptors located primarily behind the blood–brain barrier (Brooks et al., 1993; Gottlieb et al., 2005), drug levels in the CNS rather than plasma are most relevant. CNS penetration was evaluated by comparing the concentrations of compound in brain homogenates with levels in plasma. This method of evaluating CNS exposure is quantitative down to brain levels of 5% of plasma because whole brain extracts contain ~3–5% plasma from microvasculature. The brain levels of AZ-ECPC, AZ-MTAB, and LY-DMPF were indistinguishable from plasma thereby suggesting unrestricted CNS penetration. By contrast, brain exposure of nBNI and JDTic was not detectable (<5% of plasma). The CNS exposure of nBNI was further evaluated by quantitative microdialysis after several different doses. At the diuresis ID_{50}, the nBNI concentration was estimated to be 6.2 nM, which is comparable to the *in vitro* potency (GTPγS IC_{50} = 2.1 nM). The *in vivo* and *in vitro* potency values were also similar for AZ-ECPC (~80 versus 83 nM) and AZ-MTAB (~40 versus 19 nM). Unexpectedly, LY-DMPF was much more potent *in vivo* than *in vitro* (~0.01 versus 1.2 nM). The mechanism(s) underlying the discrepancy for LY-DMPF are unknown.

3.5. Anxiolytic-like activity in prenatal stress rat in elevated plus maze

Accumulating evidence indicates that the effects of early-life stress can persist into adulthood and lead to mood disorders (Pechtel and Pizzagalli, 2011). Prenatal stress in animals has been used to model anxiety and depression (Secoli and Teixeira, 1998, reviewed in Willner, 2005). For κ-opioid receptor antagonists, prenatal stress offers a valuable method for separating the stressful event used to induce anxiety-like behaviors from the antagonist dosing and response testing. Here, we established a prenatal stress model similar to Koenig et al. (2005) whereby pregnant rats were stressed daily for the seven days that comprise the third trimester. The offspring were raised to adulthood before testing anxiety-like behavior in the elevated plus maze. Prenatal stress suppressed spontaneous exploration of open arms in the elevated plus maze. AZ-MTAB and LY-DMPF were selected for testing based on superior potency and selectivity. Both compounds restored spontaneous exploration to normal levels in prenatally stressed animals (Fig. 5).

4. Discussion

Extensive preclinical evidence indicates that κ-opioid receptor antagonists may offer a novel mechanism for treating mood disorders. Despite the strength of the data, testing the hypothesis in humans and developing compounds into drugs may be impeded if not prohibited by the safety risks — the inhibitory effects of κ-opioid receptor antagonists cannot be reversed for weeks. Since each of the available κ-opioid-selective ligands exhibits long-lasting inhibition, we sought to identify short-acting compounds. Three antagonists, AZ-ECPC, AZ-MTAB, and LY-DMPF, from different chemical series each lacked long-lasting inhibition in a diuresis assay (Fig. 3). The diuresis assay did detect robust, long-lasting inhibition with nBNI and JDTic. While we cannot eliminate the possibility that these compounds have some low level of receptor inactivation, they are dramatically different from the existing κ-opioid receptor-selective antagonists.

A second major difference between the new κ-opioid antagonists and prototypic ligands is CNS exposure (Table 2). Improved brain penetration is likely to have a significant impact. Previously, in order to achieve sufficient brain exposure during behavioral testing, the majority of studies dosed antagonists prior to or immediately following the stressful insult (reviewed in Knoll and Carlezon, 2010). This pre-stress dosing resembles preventative therapy whereas a primary therapeutic

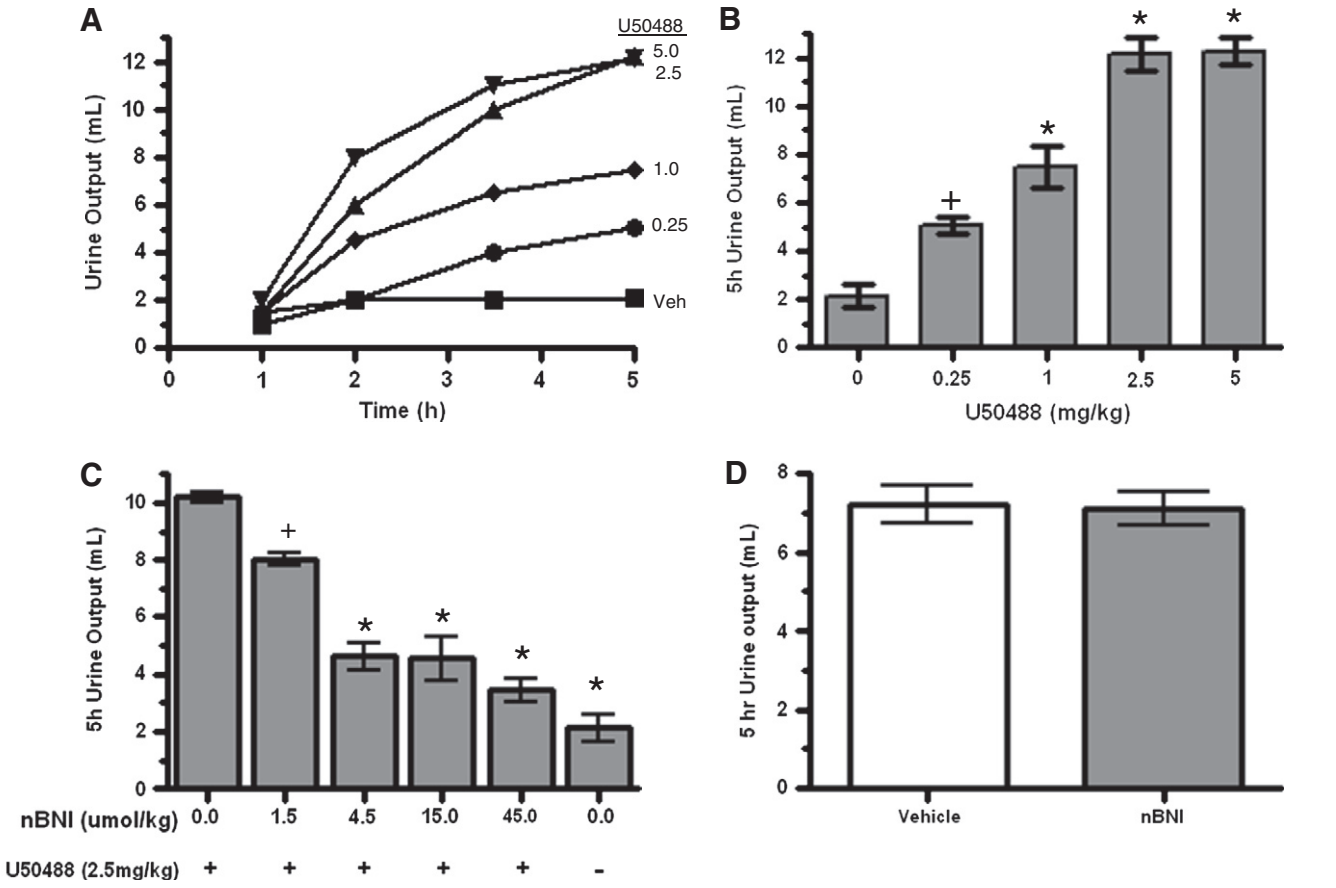

Fig. 2. κ-opioid receptor agonist-induced diuresis. A. The time course of urine output was evaluated for individual rats dosed with either vehicle (□) or with the selective κ-opioid receptor agonist U50488 (● 0.25, ■ 1.0, ▲ 2.5, ▼ 5.0 mg/ml). B. The dose–response relationship was evaluated for U50488-induced diuresis (cumulative urine output at 5 h). C. U50488-induced diuresis was specifically inhibited by nBNI (ID_{50} 3.0 μmol/kg). D. nBNI did not inhibit urine output elevated by forced hydration. Data are the average ± standard deviation for four rats. Significance versus control was assessed by one-way ANOVA followed by Dunnett's test, + $P<0.05$, *$P<0.01$.

aim is post-stress treatment to reverse mood disorders. Thus, the improved CNS exposure of the new compounds will expand the capacity to develop therapeutically-aligned preclinical models.

Our finding that AZ-MTAB and LY-DMPF reverse prenatal stress-induced behavior deficits in the elevated-plus maze (Fig. 5) has important implications for the feasibility of developing κ-opioid antagonists as drugs. Previous studies were unable to address the possibility that long-lasting inhibition and/or pre-stress dosing might be required for a mood disorder efficacy (reviewed in Knoll and Carlezon, 2010). Here, we offer an initial test of both questions by using short-acting antagonists in an assay where drug is administered 70 days after the stressful insult. This data in the elevated-plus maze is consistent with anxiolytic-like activity. Buezo et al. (2009) found that LY-DMPF (now recognized as short-acting) is active in the forced-swim test consistent with antidepressant-like activity. Additional studies will be needed to determine if these findings are confirmed in other models and extend to different mood disorders.

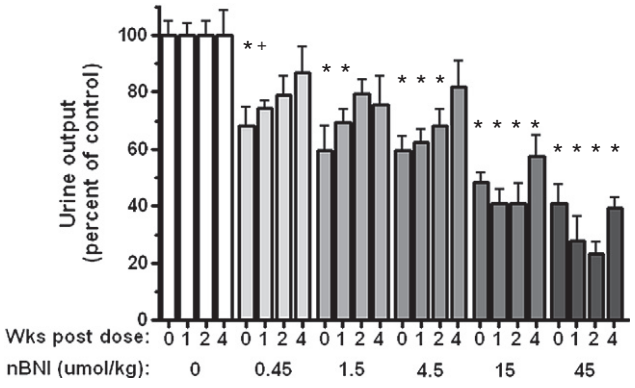

Fig. 3. nBNI shows long-lasting inhibition of κ-opioid receptor agonist-induced diuresis. Each dose of nBNI was administered to a group of rats (n = 4). Groups were monitored for inhibition of U50488-induced diuresis. After the initial 5 h test (week 0), animals were housed for indicated number of weeks and retested for recovery of U50488-induced diuresis. No additional antagonist was administered. ID_{50}s for nBNI on weeks 0, 1, 2, 4 were 3.7, 3.7, 5.7, and 15.5 μmol/kg, respectively. Data are the average ± standard deviation for four rats. Significance versus same day control was assessed by one-way ANOVA followed by Dunnett's test, + $P<0.05$, *$P<0.01$.

Table 2
CNS exposure/potency relationship. Potency (ID_{50}) for inhibiting U50488-induced urine output was determined. Unbound plasma concentrations were extrapolated from satellite pharmacokinetic studies assuming dose–exposure linearity over the dose interval tested.

Name	Diuresis ID_{50} (μmol/kg)	Unbound plasma conc. at ID_{50}(nM)	Unbound brain/ plasma ratio	Unbound brain conc. at ID_{50} (nM)	GTPγS IC_{50} (nM)	Unbound brain conc./ GTPγS IC_{50}
nBNI	3.3	>2000	<0.05[a]	13[b]	2.1	6.2
JDTic	17	100	<0.05[a]	n/d	0.35	n/d
AZ-ECPC	13	80	~1	~80	83	~1
AZ-MTAB	6.0	40	~1	~40	19	~2
LY-DMPF	0.028	0.01	~1	~0.01	1.2	0.008

[a] Free brain concentration was estimated equal to unbound plasma.
[b] Free brain concentration estimated from quantitative microdialysis.

The evidence accumulated here and in the literature indicate that the diuresis assay is well-suited as a pharmacodynamic assay for CNS drug discovery. The response is mediated by receptors located primarily in the CNS (Brooks et al., 1993; Gottlieb et al., 2005) and doses required to achieve efficacy generally require CNS exposure similar to the GTPγS potency (Table 2). The assay yields highly reproducible results with a minimal number of animals (Figs. 3, 4 and Ur et al., 1997). The data is sufficiently sensitive to reveal trends in response recovery after long-lasting inhibition. Specifically, at low doses of nBNI, a progressive recovery trend was observable in as little as one week (Fig. 3, and Carroll et al., 2004) whereas after higher doses, recovery was delayed for weeks (Fig. 3, and Ko et al., 2003). Thus, the diuresis assay exhibits an impressive combination of features for a pharmacodynamic assay: straightforward execution,

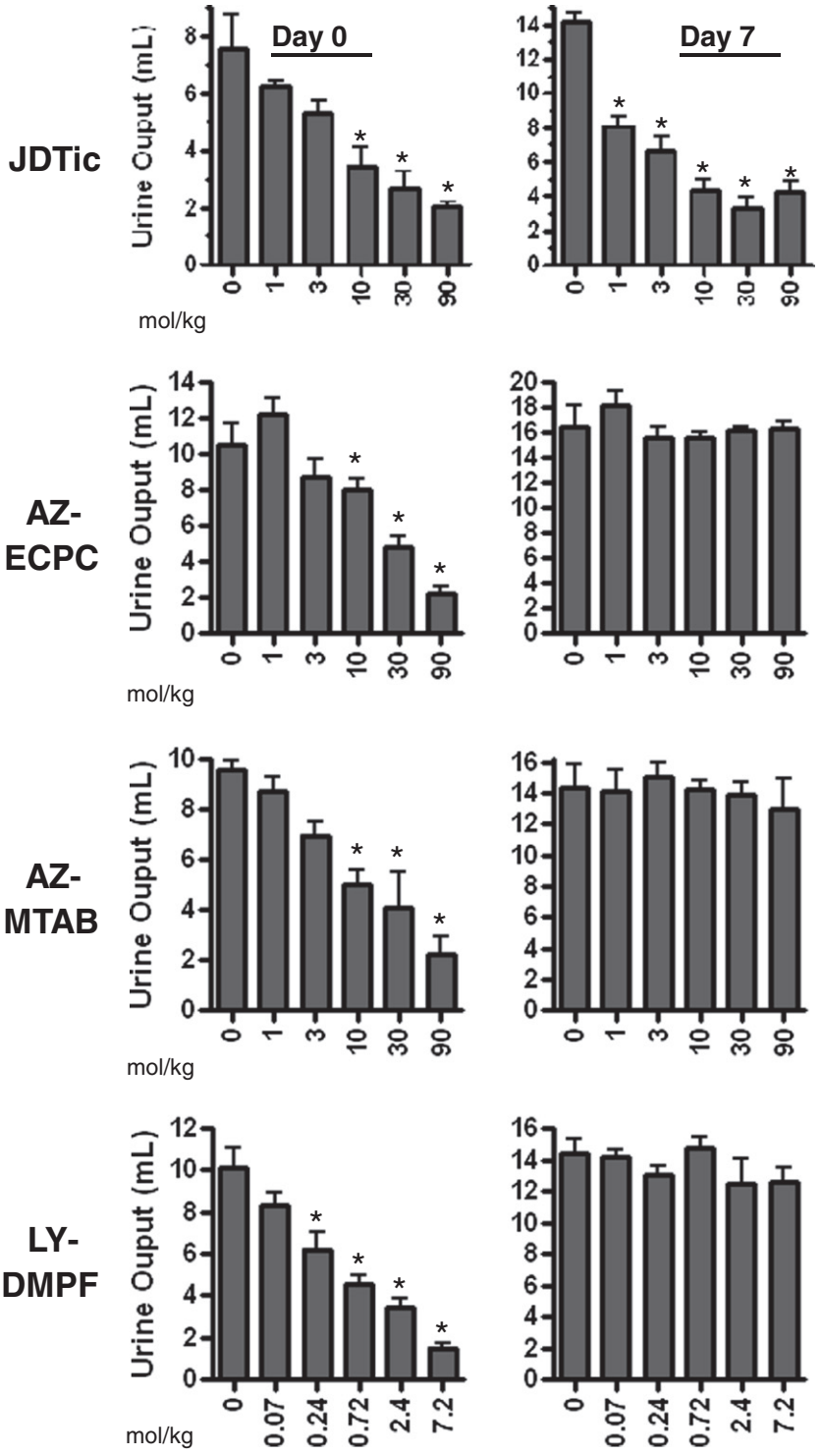

Fig. 4. JDTic and representatives of three new chemical series of κ-opioid receptor antagonists were compared for persistent inhibition of κ-opioid receptor agonist-induced diuresis one week after dosing. For each indicated dose of antagonist, four rats were treated. U50488 (2.5 mg/kg s.c.) was dosed with antagonist on day 0 and alone on day 7. Cumulative urine output was measured 5 h after U50488. Data are the average ± standard deviation for four rats. Significance versus same day control was assessed by one-way ANOVA followed by Dunnett's test, *$P<0.01$.

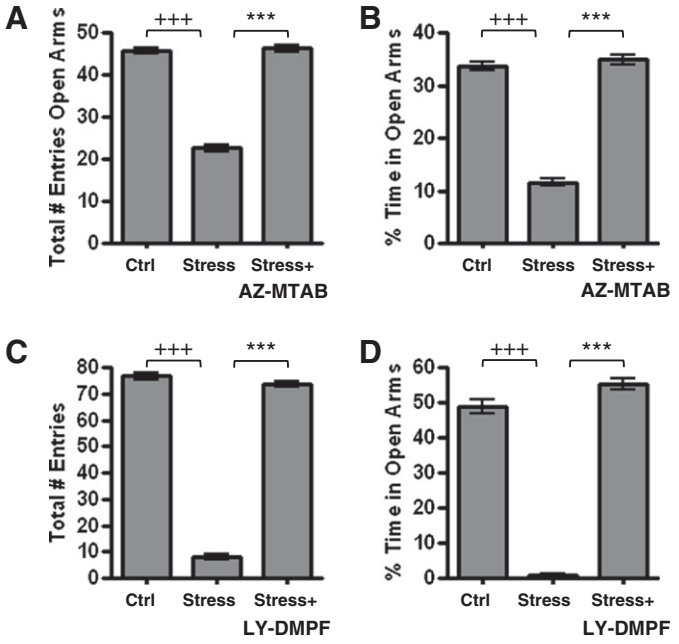

Fig. 5. Effects of κ-opioid receptor antagonists in prenatal stress adult rats on elevated plus maze. Prenatal stress suppresses spontaneous exploration of open arms in the elevated plus maze. Rats were dosed (s.c.) with κ-opioid receptor antagonists 2 h prior to testing elevated plus maze. AZ-MTAB (30 μmol/kg) and LY-DMPF (24 μmol/kg) restored the spontaneous exploration of open arms suppressed by prenatal stress. Different cohorts of stressed animals were used for A, B (64 rats) and C, D (53 rats). Data are average ± S.E.M. $^{+++}$ $P<0.001$, vehicle vs. vehicle prenatal stress (one-tailed *t*-test), *** $P<0.001$, vehicle prenatal stress vs. drug prenatal stress (two-tailed *t*-test).

minimal animal requirements, robust data, CNS-mediation, sensitivity to long-lasting inhibition, and conservation across species ranging from rodents to primates (Carroll et al., 2004; Ko et al., 2003).

Although the current studies do not directly address the mechanism of long-lasting inhibition, these compounds should provide missing tools for important experiments. Work by Chavkin, Bruchas, and colleagues indicate that functional selectivity underlies long-lasting inhibition (Bruchas et al., 2007; Bruchas and Chavkin, 2010; Melief et al., 2010). κ-opioid receptor antagonists including nBNI, JDTic, and GNTI, bind to the receptor and behave as antagonists with respect to the $G\alpha_i$ pathway while simultaneously acting as agonists via the JNK pathway. Non-selective opioid antagonists do not exhibit either the functional selectivity for JNK or long-lasting activity, thereby suggesting that JNK activation mediates long-lasting inhibition. Consistent with the hypothesis, genetic deletion of JNK1 or pretreatment with a JNK inhibitor prevented long-lasting inhibition with κ-opioid receptor-selective ligands (Bruchas et al., 2007; Melief et al., 2010). Thus, based on this evidence, one would predict that the short-lasting compounds described here would lack JNK activation. Testing these compounds in a JNK assay would be an important evaluation of the hypothesis and could validate JNK activity as an *in vitro* surrogate for long-lasting activity. A validated *in vitro* assay is needed to develop the structure-activity relationship for long-lasting inhibition.

5. Conclusions

The present findings establish a new set of κ-opioid receptor-selective antagonists with more drug-like pharmacodynamic and pharmacokinetic properties. These compounds should dramatically expand the capacity to explore the relationship between stressful events and the timing of κ-opioid receptor inhibition required to achieve efficacy in preclinical models of mood disorder models. Data with these compounds in forced-swim test (Buezo et al., 2009) and prenatal stress elevated plus maze (Fig. 5) are consistent with the conclusion that transient κ-opioid receptor inhibitors retain antidepressant-like and anxiolytic-like efficacy. These data address fundamental concerns about κ-opioid receptor antagonist druggability and strengthen the case for κ-opioid receptor as a promising target for developing novel psychiatric medications.

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