Behavioral/Systems/Cognitive

3-(4-Chloro-2-Morpholin-4-yl-Thiazol-5-yl)-8-(1-Ethylpropyl)-2,6-Dimethyl-Imidazo[1,2-*b*]Pyridazine: A Novel Brain-Penetrant, Orally Available Corticotropin-Releasing Factor Receptor 1 Antagonist with Efficacy in Animal Models of Alcoholism

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We describe a novel corticotropin-releasing factor receptor 1 (CRF_1) antagonist with advantageous properties for clinical development, and its *in vivo* activity in preclinical alcoholism models. 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-*b*]pyridazine (MTIP) inhibited ^{125}I-sauvagine binding to rat pituitary membranes and cloned human CRF_1 with subnanomolar affinities, with no detectable activity at the CRF_2 receptor or other common drug targets. After oral administration to rats, MTIP inhibited ^{125}I-sauvagine binding to rat cerebellar membranes *ex vivo* with an ED_{50} of ~1.3 mg/kg and an oral bioavailability of 91.1%. Compared with R121919 (2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylamino-pyrazolo[1,5-*a*]pyrimidine) and CP154526 (*N*-butyl-*N*-ethyl-4,9-dimethyl-7-(2,4,6-trimethylphenyl)-3,5,7-triazabicyclo[4.3.0]nona-2,4,8,10-tetraen-2-amine), MTIP had a markedly reduced volume of distribution and clearance. Neither open-field activity nor baseline exploration of an elevated plus-maze was affected by MTIP (1–10 mg/kg). In contrast, MTIP dose-dependently reversed anxiogenic effects of withdrawal from a 3 g/kg alcohol dose. Similarly, MTIP blocked excessive alcohol self-administration in Wistar rats with a history of dependence, and in a genetic model of high alcohol preference, the msP rat, at doses that had no effect in nondependent Wistar rats. Also, MTIP blocked reinstatement of stress-induced alcohol seeking both in postdependent and in genetically selected msP animals, again at doses that were ineffective in nondependent Wistar rats. Based on these findings, MTIP is a promising candidate for treatment of alcohol dependence.

Key words: **alcoholism; drug seeking; self-administration; relapse; stress; CRF**

Introduction

Extrahypothalamic corticotropin-releasing factor (CRF) systems mediate behavioral stress responses (Heinrichs and Koob, 2004), primarily through CRF_1 receptors (Contarino et al., 1999; Muller and Wurst, 2004). Similar to other potent stressors, acute alcohol withdrawal induces anxiety-like responses that are correlated with increased CRF levels in the central nucleus of the amygdala and in the bed nucleus of the stria terminalis (Olive et al., 2002), sites mediating behavioral stress responses. Accordingly, anxiogenic effects of alcohol withdrawal are reversed by CRF antagonism (Baldwin et al., 1991).

CRF has been proposed to play a major role in drug and alcohol addiction (Sarnyai et al., 2001; Valdez and Koob, 2004). Recent findings suggest that neuroadaptative changes triggered by a prolonged history of alcohol exposure lead to a chronically upregulated CRF system activity. Rats withdrawn from prolonged alcohol exposure initially exhibited reduced CRF content in the amygdala, but this was followed by a progressive increase, culminating in elevated levels 6 weeks after withdrawal (Zorrilla et al., 2001). Consistent with this, a history of prolonged alcohol exposure renders animals more sensitive to stress, as well as to anxiolytic effects of CRF antagonists (Valdez et al., 2003; Breese et al., 2005b). Repeated cycles of intoxication and withdrawal may be

Received Nov. 16, 2006; revised Dec. 23, 2006; accepted Jan. 21, 2007.

This research has been performed under a standard U.S. Government Collaborative Research and Development Agreement between the National Institute on Alcohol Abuse and Alcoholism and Eli Lilly Research Laboratories. Authors so listed are employees of Eli Lilly and Co. A.D.L. has received contract funding from Eli Lilly Research Laboratories.

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DOI:10.1523/JNEUROSCI.4985-06.2007

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particularly potent in triggering the underlying neuroadaptive processes, and based on this, a "kindling/stress" hypothesis of affect dysregulation in alcohol dependence has been proposed (Breese et al., 2005a). Thus, CRF antagonists offer an attractive modality for treatment of alcohol-induced negative affective states.

Most importantly, upregulated CRF signaling may underlie core symptoms of alcohol dependence such as excessive alcohol self-administration, and stress-induced relapse to alcohol seeking. An allostatic shift to excessive alcohol self-administration is found after a history of prolonged alcohol exposure (Roberts et al., 2000; Rimondini et al., 2002) and is reversed by CRF_1 antagonists (Funk et al., 2007). Excessive alcohol self-administration sensitive to CRF_1 antagonism is also found in genetically selected high-preferring msP rats (Hansson et al., 2006), whereas CRF antagonists selectively attenuate stress-induced relapse to alcohol seeking (Le et al., 2000; Liu and Weiss, 2002; Hansson et al., 2006) through extrahypothalamic mechanisms (Le et al., 2000, 2002). Finally, genetic variation at the human *Crhr1* locus encoding the CRF_1 receptor has recently been reported to be associated with alcohol use disorders, demonstrating a clinical relevance of the animal findings (Treutlein et al., 2006).

These and other data suggest CRF_1 antagonism to be a promising novel principle for treatment of alcohol dependence (Heilig and Egli, 2006). Translation of these findings into clinical use has been limited by difficulties in developing orally available, brain-penetrant CRF_1 antagonists. To date, only data from a single clinical study have been published with a compound in this class, 2,5-dimethyl-3-(6-dimethyl-4-methylpyridin-3-yl)-7-dipropylamino-pyrazolo[1,5-*a*]pyrimidine (R121919) (Zobel et al., 2000), and the clinical development of this molecule has been discontinued. Here, we report on the discovery of 3-(4-chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-*b*]pyridazine (MTIP), an orally available, highly brain-penetrant CRF_1 antagonist with advantageous properties for clinical development, and demonstrate its *in vivo* activity in preclinical alcoholism models.

Materials and Methods

Animals. Male Sprague Dawley rats (190–250 g; Harlan Sprague Dawley, Indianapolis, IN) were used for the pharmacokinetic and binding studies, except in one experiment performed to determine equivalence of exposure between oral and intraperitoneal dosing, performed in Wistar rats to parallel the behavioral experiments. Behavioral experiments were performed in male Wistar rats (Charles River, Calco, Italy) or in male Marchegian Sardinian (msP) rats, a Wistar-derived line genetically selected for high alcohol preference (Ciccocioppo et al., 2006). msP rats were bred at the University of Camerino (Marche, Italy) for 51 generations from Sardinian alcohol-preferring (sP) rats of the 13th generation, provided by the University of Cagliari (Colombo et al., 2006). The rats were housed in a temperature (20−22°C) and humidity (45–55%) controlled vivarium. For behavioral studies, animals were kept on a reverse 12 h light/dark cycle (lights off at 8:00 A.M.) and tested during the dark phase. All procedures were conducted in adherence with the *ECC Directive for Care and Use of Laboratory Animals* and with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

^{125}I-Sauvagine binding to rat pituitary and human CRF_1- and human CRF_2-expressing cells. ^{125}I-Sauvagine is a nonselective, high-affinity radioligand for the CRF_1 and CRF_2 receptors. It was used for *in vitro* studies because of its superior binding properties compared with other available radioligands. Assay selectivity was obtained by using transfected cells expressing only CRF_1 or CRF_2 receptors, or rat pituitary, a tissue that contains predominantly the CRF_1 receptor subtype. Rat pituitary was selected because it contained a high density of CRF_1 receptors and allowed the evaluation of receptor affinity in tissue from the species evaluated in the subsequent *in vivo* studies. For pituitary binding, male Sprague Dawley rats were decapitated and pituitaries were removed, frozen on dry ice, and stored at –70°C. On the day of the assay, tissue was thawed, homogenized in buffer (50 mM Tris HCl, 2 mM EGTA, 10 mM $MgCl_2$), and incubated at 37°C for 1 h. For the binding assay, homogenate corresponding to ~20 μg of protein was combined with a final concentration of 0.175 nM ^{125}I-Tyr^0-sauvagine (PerkinElmer, Boston, MA) and various concentrations of test compound in assay buffer containing 0.1% bovine serum albumin, 0.1% bacitracin, and 100 kU/ml aprotinin. Nonspecific binding was determined by the addition of 1 μM ovine CRF (American Peptide Company, Sunnyvale, CA). After incubation at room temperature for 120 min, the assay was terminated by centrifugation and binding assessed using a gamma counter. K_i values were calculated using Excel (Microsoft, Redmond, WA) and Prism (GraphPad, San Diego, CA). Human CRF_1 ($hCRF_1$) binding was conducted using an identical protocol with the exception of substitution of membranes from the $hCRF_1$-expressing cell line (see below) for the rat pituitary membranes. $hCRF_2$ binding was evaluated in a similar manner with the substitution of membranes from the $hCRF_2$-expressing cell lines, and the incubations were terminated by filtration. To assess the *in vitro* selectivity of MTIP, R121919, and 4-(2-chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl) ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-amine hydrochloride (SSR125543A) at a broad number of non-CRF-related targets, the compounds were tested at a concentration of 10 μM in a panel of 74 receptors and channels (see Table 2) in assays optimized for each target (Cerep, Paris, France) according to published standard protocols available at www.cerep.fr.

Inhibition of CRF-induced cAMP activity in hCRF1/HEK293 cells. $hCRF_1$ and $hCRF_2$ were expressed in HEK293 cells by retroviral transfection. CRF_1/HEK293 cells were incubated with MTIP for 15 min before stimulation with 5.0 nM CRF (EC_{50}, 2.0 nM) in the presence of 500 μM IBMX. After 30 min, reactions were terminated and cAMP levels determined using the AlphaScreen cAMP kit (PerkinElmer) according to the manufacturer's recommendations. Because no significant interactions with CRF_2 were detected in radioligand binding studies, evaluation of functional activity was not performed at this receptor.

Ex vivo *binding.* The method for CRF_1 binding *ex vivo* has been described previously using rat cerebellum (Gehlert et al., 2005). Rat cerebellum was selected because it had been previously shown to contain predominantly CRF_1 receptors at a density suitable to perform binding assays. In addition, this tissue is within the blood–brain barrier and allowed us to estimate central occupancy by the compound. Rats were gavaged with vehicle (2 ml/kg; 3% DMSO, 20% Emulphor in water) or compound in vehicle. In a subsequent study, rats were injected intraperitoneally with vehicle (2 ml/kg; 10% Tween 80 in distilled water) or MTIP in vehicle. After 1 h, rats were decapitated and cerebella were removed, frozen on dry ice, and stored at –70°C. On the day of the assay, tissue was thawed, homogenized in buffer (50 mM Tris HCl, 2 mM EGTA, 10 mM $MgCl_2$), and incubated at 37°C for 1 h. For the binding assay, ~200 μg of prepared homogenate was combined with a final concentration of 0.175 nM ^{125}I-Tyr^0-sauvagine (PerkinElmer) in assay buffer with 0.1% bovine serum albumin, 0.1% bacitracin, and 100 kU/ml aprotinin. Nonspecific binding was determined by addition of 1 μM ovine CRF (American Peptide Company). After incubation at room temperature for 120 min, the assay was terminated by centrifugation, and binding was assessed using a gamma counter. ED_{50} values were calculated in GraphPad Prism using the four-parameter sigmoidal dose–response model.

Pharmacokinetics. Compounds were administered as either a single 1 or 3 mg/kg intravenous dose or a 10 mg/kg oral gavage [per os (p.o.)] dose. Dose solutions (final concentration, 1.5 or 0.5 mg/ml for i.v. and 1 mg/ml for p.o.) were prepared in 20% solutol:ethanol (1:1)/80% dextrose (5%) in purified water for intravenous administration and 1% sodium carboxymethyl cellulose, 0.5% sodium lauryl sulfate in water for per os administration. Blood samples were collected into tubes containing sodium heparin via jugular cannulas from three rats per treatment. The time points collected were 0 (per os dose only), 0.08 (intravenous dose only), 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h. Plasma was separated by centrifugation and frozen at −70°C until assayed.

The plasma concentrations of MTIP, R121919, and SSR125543A were determined by positive turbo ionspray liquid chromatography/mass spectrometry/mass spectrometry using a Sciex 4000 (Sciex, Concord, Ontario, Canada). Samples were prepared by methanol precipitation and centrifuged to remove particulate matter. An aliquot of the supernatant was transferred and diluted with 80% water/20% methanol. The lower limit of quantitation was 1 ng/ml. Pharmacokinetic parameters were calculated by noncompartmental analysis, using the trapezoidal rule for area under the curve (AUC) calculation using a validated internally developed pharmacokinetic calculation program called WinPTK.

Administration of MTIP for behavioral experiments. For all behavioral experiments, vehicle (10% Tween 80 in distilled water) or MTIP in vehicle were administered intraperitoneally 30 min before testing. Intraperitoneal administration was used in the behavioral experiments, because preliminary studies showed that the carboxymethyl-cellulose-based vehicle for per os administration was not compatible with oral alcohol intake. To bridge between the intraperitoneal dosing of the behavioral experiments and the pharmacokinetic data obtained with oral treatment, equivalence of exposure was separately established.

Locomotor activity and open field. Locomotor activity was measured in sound-attenuated behavioral chambers equipped with an open field (43 × 43 cm) containing infrared beam detectors (Med Associates, St. Albans, VT). Activity was examined under nonhabituated conditions, at ambient light level of 190–210 lux. Three sets of 16 infrared beams were used to automatically track both horizontal and vertical movements. In addition to overall activity and rest time, the automated system also gave perimeter activity and central area activity. The perimeter activity was defined as the activity measured by the outer six (three plus three) sets of infrared beams in the *x* and *y* direction. The central area was defined as beam-breaks made at the remaining central 10 beam-pairs. Each animal ($n = 8$ per group) was only tested once.

Elevated plus-maze. The elevated plus-maze studies were conducted as described previously (Thorsell et al., 2000). Testing was performed under low light conditions, red light/15 lux, and each animal was used only once. Percent open time and entries [(open arms)/(open arms + time closed arms) × 100%] were used as measures of anxiety-like behavior. The total number of entries onto any arm (open plus closed) was used as an indicator of general activity. When plus-maze was run under alcohol withdrawal conditions, alcohol (3 g/kg) was administered intraperitoneally 12 h before the animal being put on the plus-maze.

For baseline (no alcohol withdrawal) plus-maze testing, the entire experiment could be performed within a single day. *N* was 15 for the control group, and 12 for both the 3 and 10 mg/kg dose groups. Because of limits on assay capacity, the MTIP dose–response experiment for reversal of alcohol-withdrawal anxiety was split across 3 test days. On each day, a separate control group was run, yielding $n = 25$ for vehicle-treated controls, and $n = 11$–15 for the respective treatment groups. A lack of baseline differences between test days was tested for and excluded, after which the control groups were pooled.

Alcohol self-administration and stress-induced reinstatement of alcohol seeking. Operant self-administration of 10% alcohol was established using a saccharin fading procedure and daily 30 min fixed-ratio 1 sessions, in which each operant response resulted in the administration of 0.1 ml of the alcohol solution. Self-administration and reinstatement were studied as described previously (Ciccocioppo et al., 2002). Self-administration and reinstatement of alcohol seeking were examined in Wistar and msP rats without a history of forced alcohol exposure and imposed abstinence ("nondependent"), as well as in Wistar rats with such a history ("postdependent").

All subjects were given 15 d self-administration to establish a stable baseline. For nondependent Wistar and msP rats, drug testing was initiated at this point (see below). For the postdependent Wistar group, self-administration training was first completed. At this point, intoxication was imposed by gastric gavage with 25% alcohol or vehicle (6% w/v sucrose solution supplemented with milk powder enriched in minerals and vitamins) for 6 consecutive days, as described previously (Majchrowicz, 1975). Briefly, day 1 animals received 12 g/kg alcohol or its vehicle in 16 h (3 g/kg, two times, followed by 2.0 g/kg, three times; administrations every 4 h). From day 2, rats received 9 g/kg alcohol or vehicle (3 g/kg, one time, followed by 2.0 g/kg, three times; administrations every 4 h). Blood alcohol levels (BALs) were monitored on days 1, 2, and 5, 1 h after the first and the third daily injection. BALs (mean ± SEM; milligrams/deciliter) after the first and third injection, respectively, were as follows: day 1, 161.0 ± 11.9 (range, 134–224) and 188.7 ± 12.9 (range, 165–259 mg/dl); day 2, 163.6 ± 14.27 (range, 123–214) and 229.8 ± 12.7 (range, 165–259l); day 5, 161 ± 11.9 (range, 134–224 mg/dl) and 188.7 ± 12.9 (range, 183–261 mg/dl). Animals were not injected during the light phase (8:00 P.M. to 8:00 A.M.), during which blood alcohol fell to nondetectable levels. After 1 week recovery, subjects were returned to daily self-administration sessions for 10 d, followed by three cycles of 5 d deprivations interspersed with 5 d periods of self-administration. Drug testing was initiated at this point.

To examine effects of MTIP on alcohol self-administration, a within-subjects design was used. Each subject received 0 (vehicle), 3, and 10 mg/kg MTIP in a Latin square counterbalanced design. Treatment was given 30 min before self-administration sessions, and experiments were every 4 d, with untreated daily self-administration sessions on intervening days.

To assess the effects of MTIP on reinstatement of alcohol seeking, a between-subjects design was used. Self-administration was established as described above, and extinguished over 15 d. Tests began 1 d after the last extinction session. To evaluate the ability of MTIP to block shock-induced reinstatement, groups of nonselected Wistar rats without or with a history of dependence as described above, or msP rats were treated with vehicle or MTIP (3 or 10 mg/kg) and 30 min later reinstatement of alcohol seeking induced by 15 min intermittent footshock stress (0.6 mA; train length, 0.5 s) administered via the grid floor of the chamber under a variable-interval 40 s schedule (interval range, 10–70 s).

Stress-induced corticosterone response. Animals ($n = 5$ per group) were subjected to unpredictable footshock of the same intensity as that used for stress-induced reinstatement (0.6 mA) for 10 min (shock duration, 12–60 s; intershock interval, 10–60 s). Blood was collected by tail bleed at 0, 15, and 60 min after stress. Plasma was separated by centrifugation (15 min × 3000 rpm; 4°C) and frozen at −80°C until assaying using ^{125}I radioimmunoassay kits (MP Biomedicals, Irvine, CA). Standards and plasma samples from each experiment were run in duplicate in single assays.

Loss of righting reflex and blood alcohol levels. Loss of righting reflex (LORR) was assessed as described previously (Radcliffe et al., 2004). Blood was collected at time of return of righting reflex and also at 30 min after injection (for peak levels). Blood alcohol levels were determined using gas chromatography.

Statistics. Data were assessed for homogeneity of variance and did not violate this assumption. ANOVA was performed using the general linear model of Statistica (StatSoft, Tulsa, OK). Factors, their within- or between-subjects nature, and their respective degrees of freedom are given for the respective experiment. For individual group contrasts, Tukey's honestly significant difference (HSD) *post hoc* test was used.

Results

MTIP (Fig. 1) was discovered as the result of a directed structure–activity relationship effort to obtain high CRF_1 potency while optimizing the pharmacokinetic properties of the molecule. The *in vitro* binding potency of the CRF_1 antagonists were evaluated using $hCRF_1$ receptors and rat pituitary. In the pituitary binding assay, MTIP inhibited binding with a K_i of 0.39 nM, whereas R121919 inhibited with a K_i of 0.22 nM, SSR125543A inhibited with a K_i of 0.37 nM, and *N*-butyl-*N*-ethyl-4,9-dimethyl-7-(2,4,6-trimethylphenyl)-3,5,7-triazabicyclo[4.3.0]nona-2,4,8,10-tetraen-2-amine (CP154526) with a K_i of 0.77 nM (Fig. 2, Table 1). MTIP exhibited similar potency when cell membranes from HEK cells expressing $hCRF_1$ receptors were used (Table 1). To assess the functional potency, the ability of the compounds to inhibit CRF-induced cAMP formation in HEK293 cells expressing $hCRF_1$ receptors was evaluated. In this assay, MTIP had a K_b of 5.35 nM, whereas R121919 inhibited with a K_b of 7.75 nM,

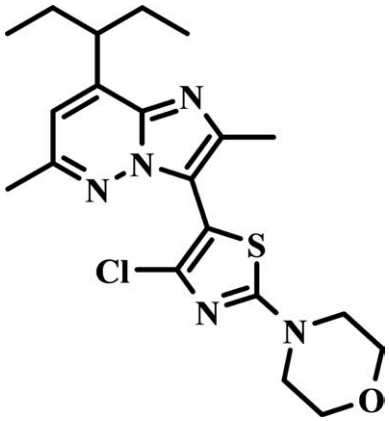

Figure 1. Chemical structure of MTIP.

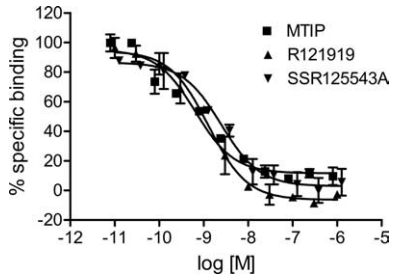

Figure 2. Inhibition of ^{125}I-sauvagine binding to rat pituitary membranes by MTIP, R121919, and SSR125543A. Error bars indicate SEM.

Table 1. *In vitro* affinity of CRF_1 antagonists in rat pituitary, $hCRF_1$ and $hCRF_2$ binding assays, and functional potencies in HEK/$hCRF_1$ cyclase assays

	Binding K_i (nM)			Cyclase K_b (nM)
Compound	Rat pituitary	hCRF1	hCRF2	hCRF1
MTIP	0.39 ± 0.33	0.22 ± 0.03	>1000	5.35 ± 1.25 (10)
R121919	0.22 ± 0.16	0.24 ± 0.16	>1000	7.75 ± 1.54 (4)
SSR125543A	0.37 ± 0.27	0.44 ± 0.16	>1000	9.55 (2)
CP154526	0.76 ± 0.33	0.44 (1)	>1000	2.04 (2)

Compounds were evaluated in binding and cyclase assays as described in Materials and Methods. Data are expressed as mean ± SEM. For the binding data, $n = 4$. For the cyclase data, n is indicated in parentheses.

SSR125543A inhibited with a K_b of 9.55 nM, and CP154526 with a K_b of 2.04 nM (Table 1). None of the three compounds inhibited ^{125}I-sauvagine binding to the CRF_2 receptor at concentrations up to 10 μM. Higher concentrations were not examined because of limited solubility of the compounds at concentrations >10 μM under the assay conditions. The selectivity of the CRF_1 antagonists was tested in a panel of 74 receptors and ion channels (Table 2). In these studies, MTIP exhibited no inhibition >50%. R121919 produced >50% inhibition at the hA_3 receptor (67%), α2 adrenoceptor (78%), AT_1 (64%), hCGRP (75%), KOP (74%), σ (86%), Na^+ channel (63%), and Cl^- channel (59%). SSR125543A exhibited activity at 10 μM in the hA_3 (91%), peripheral benzodiazepine (59%), σ (51%), and Na^+ channel (53%).

To assess the antagonism of CRF_1 receptors *in vivo*, the technique of *ex vivo* binding was used. After oral administration, MTIP produced a statistically significant inhibition of ^{125}I-sauvagine binding to rat cerebellar homogenates *ex vivo* at doses of 1, 3, 10, and 30 mg/kg, resulting in a calculated ED_{50} of 1.3 mg/kg (Fig. 3). Using identical conditions, R121919 inhibited binding with an ED_{50} of 0.74 mg/kg, SSR125543A inhibited with an ED_{50} of 1.0 mg/kg, and CP154526 with an ED50 of 17.7 mg/kg. To bridge between the *ex vivo* binding studies and the behavioral studies, MTIP was dosed by the per os and intraperitoneal routes to Wistar rats using the 10% Tween vehicle. MTIP was also effective in Wistar rats by the per os (ED_{50}, 1.5 mg/kg) and intraperitoneal (ED_{50}, 0.60 mg/kg) routes. The pharmacokinetic properties of these compounds were assessed using per os and intravenous administration to intravenously cannulated rats, and are summarized in Table 3. In these studies, MTIP had a half-life

Table 2. Panel of receptors and ion channels used to evaluate CRH_1 antagonist selectivity

Class	Receptor/ion channel/transporter
Adenosine	A_1, A_{2A}, A_3
Adrenergic	α1, α2, β1, β2, NET
Angiotensin	AT_1, AT_2
Benzodiazepine	Central, peripheral
Bombesin	BB (nonselective)
Bradykinin	B^2
CGRP	$CGRP_1$
Canabinoid	CB_1
Cholecystokinin	CCK_1, CCK_2
Dopamine	D_1, D_{2S}, D_3, $D_{4.4}$, D_5, DAT
Endothelin	ET_A, ET_B
GABA	Nonselective
Galanin	GAL1, GAL2
PDGF	PDGF
IL-8	CXCR2
TNF-α	TNF-α
CCR1	CCR1
Histamine	H_1, H_2
Melanocortin	MC_4
Muscarinic	M_1, M_2, M_3
Tachykinin	NK_1, NK_2, NK_3
Neuropeptide Y	Y_1, Y_2
Neurotensin	NT_1
Opioid	MOP, DOP, KOP, NOP
PACAP	PAC_1
PCP	PCP
TXA_2/PGH_2	TXA_2/PGH_2
Purine	P2X, P2Y
Serotonin	5-HT_{1a}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_{5A}, 5-HT_6, 5-HT_7
σ	Nonselective
Somatostatin	Nonselective
VIP	VIP_1
Vasopressin	V_{1a}
Ca^{2+} channel	L type
K^+ channel	K^+_V channel
SK^+_{Ca} channel	SK^+_{Ca} channel
Na^+ channel	Site 2
Cl^- channel	Cl^- channel

At a 10 μM concentration, MTIP did not induce a 50% inhibition at any of the targets listed.

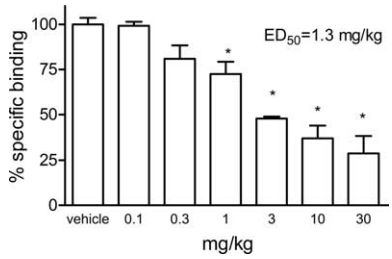

Figure 3. *Ex vivo* ^{125}I-sauvagine binding to rat cerebellar membranes 1 h after oral administration of increasing MTIP doses, indicating half-maximal brain receptor occupancy at an oral dose of 1.3 mg/kg. Error bars indicate SEM.

Table 3. Pharmacokinetic parameters for MTIP, CP154526, SSR125543A, and R121919

	Compound/route/dose							
	MTIP		CP154526		SSR125543A		R121919	
	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
	1 mg/kg	10 mg/kg	3 mg/kg	10 mg/kg	3 mg/kg	10 mg/kg	3 mg/kg	10 mg/kg
AUC_{0-t} (ng · h/ml)	3407 ± 277	27,035 ± 3848	568 ± 12	355 ± 44	11,634 ± 2948	9116 ± 1892	154 ± 1	378 ± 25
$AUC_{0-\infty\,h}$ (ng · h/ml)	3435 ± 275	31,290 (2)	605 ± 16	369 ± 39	12,370 ± 3160	10,118 (2)	164 ± 4	381 ± 26
$t_{½}$ (h)	3.9 ± 0.3	3.3 (2)	15.1 ± 2.7	8.2 ± 1.4	10.53 ± 1.84	5.88 (2)	2.8 ± 0.2	1.52 ± 0.07
C_{max} (ng/ml)		2773 ± 148		66.5 ± 3.9		1603 ± 387		77.8 ± 11.1
C_0 (ng/ml)	4714 ± 1952		1274 ± 104		25,744 ± 8468		261 ± 11	
t_{max} (h)		2.2 ± 1.0		4 ± 0		4.7 ± 1.8		2 ± 0
Clearance (ml · min^{-1} · kg^{-1})	4.9 ± 0.4		82.7 ± 2.1		4.6 ± 1.1		306 ± 7	
V_d, β (L/kg)	1.7 ± 0.2		114 ± 16		4.2 ± 0.8		75.7 ± 7.2	
% Oral bioavailability		91.1		18.3		24.5		69.7

$n = 3$, except where indicated otherwise in parentheses.

of 3.9 h by the intravenous route compared with 2.8 h for R121919, 15.2 h for CP154526, and 10.53 for SSR125543. MTIP exhibited a substantially higher plasma C_{max} and area under the plasma exposure curve after oral administration than the comparator compounds and had a markedly lower volume of distribution (V_d). Oral bioavailability of MTIP was greater than observed with the comparators at 91.1%. Studies performed using the 10% Tween 80 vehicle by the intraperitoneal route in Wistar rat produced very similar MTIP plasma exposure curves to that observed in the oral studies (data not shown).

Table 4. Nonstressed, spontaneous behaviors unaffected by MTIP treatment

Behavior	Vehicle	3 mg/kg	10 mg/kg
Locomotion			
Distance traveled (cm)	3036 ± 395	2530 ± 195	2472 ± 322
Ambulatory counts (*n*)	997 ± 160	785 ± 79	813 ± 122
Stereotypy counts (*n*)	4549 ± 444	4780 ± 295	4316 ± 325
Open field			
Center time (s)	730 ± 177	773 ± 207	641 ± 131
Perimeter time (s)	2870 ± 177	2827 ± 207	2959 ± 131
Center distance (cm)	1810 ± 292	1403 ± 134	1427 ± 233
Perimeter distance (cm)	1229 ± 137	1127 ± 124	1044 ± 121
Plus maze			
% Open arm time	23.1 ± 6.2	20.9 ± 3.2	21.1 ± 6.0
% Open arm entries	32.1 ± 6.7	33.0 ± 3.7	36.5 ± 5.7
Closed arm entries (*n*)	6.3 ± 0.4	6.3 ± 0.5	5.8 ± 0.6

In contrast to what was observed during acute alcohol withdrawal or after a history of dependence, spontaneous behaviors under nonstressed conditions were unaffected by MTIP. Data are means ± SEM; $p > 0.10$ in all cases.

In initial studies in alcohol-naive Wistar rats under unstressed baseline conditions, MTIP (3–10 mg/kg) affected neither total locomotion nor the pattern of exploration in an open field ($p > 0.10$ in all cases). Exploration on an elevated plus-maze under these conditions was equally unaffected (Table 4). Because it has been reported that alcohol withdrawal-induced anxiety is blocked by central administration of a CRF antagonist (Rassnick et al., 1993), effects of MTIP on anxiety were next examined during acute withdrawal from a single large dose of alcohol. It was first established that a dose-dependent suppression of open arm exploration is achieved on the elevated plus-maze 12 h after a single alcohol dose of 1–3 g/kg (percentage open time, $F_{(3,39)} = 3.7$, $p = 0.02$; percentage open entries, $F_{(3,39)} = 4.1$, $p = 0.01$), with *post hoc* analysis (Tukey's HSD) demonstrating a robust effect of the 3 g/kg dose on both parameters. This anxiogenic-like effect of withdrawal was not accompanied by general locomotor suppressing effects as measured by enclosed arm entries (Fig. 4, top). In a separate experiment, the potent anxiogenic-like effect of the 3 g/kg alcohol dose was dose-dependently reversed by MTIP at 0.1–10 mg/kg (percentage open time, $F_{(5,84)} = 6.8$, $p = 0.00002$; percentage open entries, $F_{(5,84)} = 7.9$, $p = 0.000004$), with *post hoc* analysis demonstrating significant effects at 1.0, 3.0 ($p < 0.01$), and 10.0 mg/kg ($p < 0.001$) for percentage open time, and at 3.0 ($p < 0.01$) and 10.0 mg/kg ($p < 0.001$) for percentage open entries. Closed arm entries, a measure of locomotor activity, did not differ from control levels at any of the MTIP doses ($p > 0.4$ for all doses).

Alcohol self-administration data are summarized in Figure 5. A history of dependence (i.e., imposed intoxication followed by cycles of forced deprivations) led to markedly elevated alcohol self-administration compared with animals without such a history (two-way ANOVA with history as between-subjects factor, and MTIP dose as within-subjects factor: main effect of history, $F_{(1,13)} = 7.2$, $p = 0.018$; Tukey's HSD *post hoc* analysis of vehicle-treated group with history of dependence vs vehicle-treated group without such a history, $p < 0.05$).

A robust, dose-dependent suppression of self-administration rates by MTIP was found (main effect of MTIP treatment: $F_{(2,26)} = 9.7$, $p < 0.001$). Among the Wistar rats, the effect of MTIP was confined to the postdependent group, as shown by *post hoc* analysis. This showed that the 10 mg/kg postdependent group differed ($p = 0.001$) from its corresponding vehicle control group, whereas neither 3 mg/kg ($p = 1.00$) nor 10 mg/kg treated ($p = 0.80$) subjects without a history of dependence differed from theirs. Similar to the effect observed in postdependent Wistar rats, a robust suppression of self-administration rates by MTIP was also seen in msP rats (main effect of treatment: $F_{(3,18)} = 5.3$, $p = 0.009$; both 10 and 20 mg/kg dose significantly different from vehicle control on *post hoc* analysis at $p < 0.05$). Responses on the nonactive control lever were not affected in any of the groups examined (data not shown).

Stress-induced reinstatement data are summarized in Figure 6. For all three main groups examined (i.e., nondependent and postdependent Wistar, as well as msP rats), footshock induced robust reinstatement of responding on the previously alcohol-associated lever. A robust suppression was found in postdependent Wistar animals (main effect of treatment: $F_{(2,17)} = 6.2$, $p =$

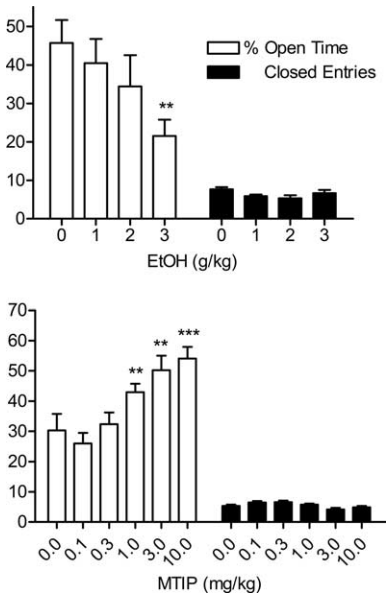

Figure 4. Top, "Hangover anxiety" 12 h after a single large alcohol dose; $p < 0.01$ for 3 g/kg versus vehicle control ($n = 9–16$). Bottom, Dose-dependent reversal of hangover anxiety observed 12 h after a 3 g/kg alcohol dose by MTIP (main effect of treatment on percentage open time: $p = 0.00002$; $n = 25$ for the control group, 11–15 for each dose group). Differences on *post hoc* analysis versus the respective control group at $p < 0.01$ or 0.001 are indicated by asterisks (** and ***). For detailed statistics, see Results.

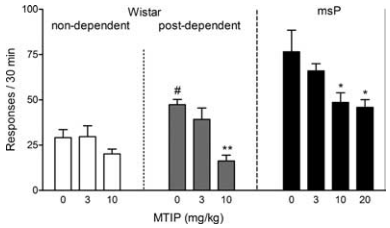

Figure 5. Operant self-administration of alcohol and modulation of this behavior by MTIP. Each lever press resulted in the delivery of 0.1 ml of 10% alcohol solution. No effect was found in genetically nonselected Wistar rats without a history of dependence (left group; $n = 7$). In contrast, a dose-dependent suppression (main treatment effect, $p < 0.001$) was seen in nonselected Wistar animals with a history of dependence (middle group; $n = 8$). A similar dose-dependent suppression was also observed in genetically selected, alcohol-preferring msP rats (right group; main treatment effect, $p = 0.009$; $n = 7$). Significant ($p < 0.05$) increase of self-administration in postdependent, vehicle-treated controls versus nondependent vehicle-treated subjects on *post hoc* comparison is indicated by the number sign (#). Significant suppression of self-administration by MTIP ($p < 0.05$ and $p < 0.001$, respectively, for individual groups) on *post hoc* comparison versus corresponding controls is indicated by asterisks (* and **). For detailed statistics, see Results. Error bars indicate SEM.

0.014), with *post hoc* analysis demonstrating a significant effect of the 10 mg/kg dose ($p = 0.012$), and a trend-level effect of the 3 mg/kg dose ($p = 0.08$). No significant effect of MTIP to suppress the reinstatement response was seen in Wistar rats without a history of dependence ($F_{(2,27)} = 0.19$; $p = 0.82$).

An even more potent effect of MTIP treatment to suppress reinstatement responding was found in msP rats ($F_{(2,19)} = 7.14$; $p = 0.005$), in which *post hoc* analysis demonstrated a significant effect both of the 3 mg/kg ($p = 0.025$) and the 10 mg/kg ($p = 0.005$) dose. Also, in the reinstatement experiment, responding at the inactive control lever was very low and was not affected by stress exposure or drug treatment (data not shown).

After intermittent footshock at the intensity used to induce reinstatement (0.6 mA), there was a significant change of corticosterone over time (main effect of time as within-subjects factor: $F_{(2,27)} = 9.1$, $p < 0.001$). MTIP treatment at doses used in the behavioral experiments did not affect this response (main effect of treatment as between-subjects factor: $F_{(2,27)} = 0.58$; $p = 0.57$; treatment by time interaction: $F_{(4,27)} = 1.3$, $p = 0.29$) (Table 5). Furthermore, MTIP at the highest behaviorally active dose, 10 mg/kg, did not affect alcohol sensitivity as measured by LORR time, or alcohol elimination as assessed by decline of BAL over time after a single challenge dose of 3.5 g/kg (data not shown).

Discussion

We show that the novel compound MTIP is a potent, selective, and brain-penetrant CRF_1 antagonist. In standard preclinical models, MTIP effectively blocks alcoholism-related behavioral pathology, irrespectively of its neuroadaptive or genetic origin. Effects of MTIP are behaviorally specific, with several baseline behaviors and alcohol metabolism unaffected. Behavioral MTIP actions are likely mediated through extrahypothalamic CRF systems, because the corticosterone response to footshock stress was unaffected at behaviorally active MTIP doses.

Data suggesting efficacy of CRF_1 antagonism in alcoholism models have previously been provided using the CRF_1 antagonists antalarmin, its structural analog CP154526, MJL-1-109-2 (pyrazolol [1,5-a]-1,3,5-triazin-4-amine,8-[-4-(bromo)-2-chlorphenyl]-*N*,*N*-bis(2-methoxyethyl)-2,7-dimethyl-(9Cl)), and R121919 (Le et al., 2000; Liu and Weiss, 2002; Funk et al., 2006, 2007; Hansson et al., 2006). Among these compounds, R121919 had previously reached human trials for depression, but its development was subsequently discontinued (Zobel et al., 2000). An additional compound of interest is SSR125543A (Griebel et al., 2002; Gully et al., 2002). Although no alcohol-related data are available with this compound, it points to some interesting commonalities with the present data. Based on an extensive behavioral characterization, it was concluded that SSR125543A lacked activity in anxiety models based on spontaneous exploration, but reversed the anxiogenic effects of inescapable stressors (Griebel et al., 2002). Here, we find a similar lack of activity in open field and basal plus-maze exploration with MTIP, but potent activity to reverse anxiogenic effects of acute alcohol withdrawal. The doses to accomplish this were the same as the doses required to obtain brain CRF_1 receptor occupancy using *ex vivo* binding. Collectively, these data suggest that central CRF systems are quiescent under baseline conditions and activated only during high levels of stress, promising an attractive tolerability profile for compounds targeting this system.

Reduction of ongoing excessive alcohol self-administration and prevention of relapse after abstinence are two key objectives for any novel alcohol dependence treatment (Heilig and Egli, 2006). Both are effectively achieved by MTIP in standard preclinical models. Our data confirm recent suggestions that alcohol self-administration is a compound phenomenon, and that a fundamental distinction exists between basal and excessive alcohol self-administration. Robust responding for alcohol can be obtained in nonselected Wistar rats without a history of dependence, but pharmacological modulation of this behavior is of questionable relevance for predicting clinical activity in alcoholism (Egli, 2005; Heilig and Egli, 2006). This basal self-administration component is independent of the CRF system, as shown both by the present MTIP data, and previous reports using other CRF antagonists (Valdez et al., 2002; Hansson et al., 2006; Funk et al., 2007). In contrast, excessive self-administration rates are seen after a history of dependence (Roberts et al., 2000; Rimondini et al., 2002). The self-administration component added through a history of dependence is fully antagonized by

MTIP, also in agreement with published data for other CRF antagonists (Valdez et al., 2002; Funk et al., 2007). Similarly, excessive self-administration is seen in msP rats because of preexisting genetic susceptibility, and is also highly sensitive to MTIP, in agreement with a previous study using the CRF_1 antagonist antalarmin (Hansson et al., 2006).

Differential effects of MTIP on self-administration in postdependent and msP rats versus animals without a history of dependence could be related to the higher response rates in the former two groups, making them more susceptible to effects of MTIP. However, we believe such an explanation unlikely given the reinstatement data (Fig. 5). Here, vehicle controls of all groups had the same reinstatement responding rates, yet MTIP was only effective in blocking reinstatement in postdependent and msP rats. Together, these findings suggest that excessive, but not basal alcohol self-administration can be selectively blocked by CRF_1 antagonism. This may be related to recent observations that expression of CRF_1 receptor mRNA is upregulated in msP (Hansson et al., 2006) and postdependent rats (Sommer et al., 2007).

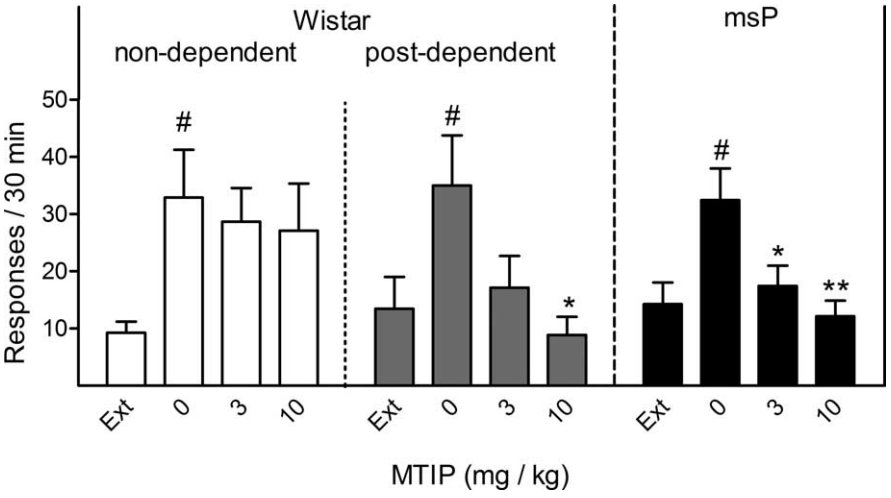

Figure 6. Reinstatement of alcohol seeking (i.e., responding on a previously alcohol-reinforced lever), induced by 0.6 mA footshock stress, in Wistar rats trained to self-administer alcohol, and then extinguished over 15 nonreinforced sessions, and modulation of this behavior by MTIP. No effect was found in genetically nonselected Wistar rats without a history of dependence (left group; $n = 9–11$ per dose). In contrast, a dose-dependent suppression (main treatment effect, $p = 0.014$) was seen in nonselected Wistars with a history of dependence (middle group; $n = 7$ per dose). A similar dose-dependent suppression was also observed in genetically selected, alcohol-preferring msP rats (right group; main treatment effect, $p = 0.004$; $n = 7$ per dose). Significant ($p < 0.05$) reinstatement after footshock stress versus extinguished responding (Ext) is indicated by the number sign (#). Significant suppression of stress-induced responding by MTIP ($p < 0.05$ and $p < 0.001$, respectively, on *post hoc* comparison) versus respective controls is indicated by asterisks (* and **). For detailed statistics, see Results. Error bars indicate SEM.

Effects of CRF_1 antagonism on stress-induced relapse-like behavior follow a similar pattern. Both in postdependent and in msP animals, MTIP abolished reinstatement responding induced by footshock. This is in agreement with a previous report in which the nonselective peptide CRF antagonist D-Phe-$CRF1_{2–41}$ abolished reinstatement of alcohol seeking induced by footshock in postdependent Wistar rats (Liu and Weiss, 2002). In contrast, in our present study, doses of MTIP that abolished reinstatement in postdependent animals and in the msP line were ineffective in genetically heterogeneous animals without a history of dependence. It has been reported (Le et al., 2000) that, at higher doses, both D-Phe-$CRF1_{2–41}$ and the CRF_1 selective antagonist CP154526 are able to block reinstatement in Wistar rats without a history of dependence. Thus, a recruitment of the CRF system caused by a postdependent state or preexisting genetic susceptibility renders animals preferentially sensitive to blockade of relapse-like behavior by CRF_1 antagonism. This offers the promise that therapeutic effects of MTIP in alcoholism will be possible to achieve with doses that do not affect normal behavior.

Stressors constitute one of the main categories of stimuli capable of inducing reinstatement of alcohol seeking (Le et al., 1998, 2005). Another clinically relevant category of relapse-inducing stimuli is composed of alcohol-associated cues, the effects of which are additive with those of stress. In contrast to stress-induced relapse-like behavior, triggered by CRF signaling, effects of alcohol-associated cues are mediated through opioid systems, and blocked by the opioid antagonist naltrexone (Liu and Weiss, 2002). Although naltrexone is available for clinical use, no treatments are presently available to block stress-induced relapse in humans. A CRF_1 antagonist may therefore be an attractive therapeutic modality to combine with naltrexone.

Both MTIP, SSR122543A, and R121919 exhibited high affinity for the $hCRF_1$ receptor in binding and functional assays. In rat pituitary, a tissue containing a high density of predominantly

Table 5. Corticosterone levels after 10 min exposure to intermittent footshock (0.6 mA) delivered under conditions identical with those used for footshock-induced reinstatement of alcohol seeking

	0 min	15 min	60 min
Control	248 ± 32	361 ± 45	216 ± 29
MTIP (3 mg/kg)	259 ± 32	292 ± 44	217 ± 43
MTIP (10 mg/kg)	314 ± 39	304 ± 24	221 ± 31

Data are means ± SEM (nanograms/milliliter). MTIP did not affect the corticosterone response to the stressor at any of the doses used for the behavioral experiments. For statistical analysis, see Results.

CRF_1 receptors, all three compounds exhibited similar affinity compared with hCRF1. Furthermore, all three compounds had 1000× selectivity for CRF_1 versus CRF_2 receptors. To assess potential non-CRF_1 activity, the three compounds were evaluated in a panel of assays for a number of receptors, ion channels, and transporters. MTIP did not produce an inhibition >50% at a concentration of 10 μM in any of these assays, making is unlikely that its *in vivo* effects are produced through any non-CRF_1 actions. To estimate receptor occupancy *in vivo*, MTIP was evaluated using *ex vivo* binding to rat cerebellum, a tissue that contains predominantly the CRF_1 receptor and is located within the blood–brain barrier (Gehlert et al., 2005). MTIP inhibited *ex vivo* binding with an estimated ED_{50} that compares well with the potency observed in the alcohol withdrawal-induced anxiety model. Collectively, these results establish that the behavioral effects reported in the present study are the result of CRF_1 receptor occupancy *in vivo*.

One of the challenges in the discovery and development of CRF_1 antagonists has been the physicochemical properties of the compounds. Historically, high lipophilicity has been required to optimize *in vitro* affinity of compounds for the CRF_1 receptor. Basic lipophilic drugs are characterized by extensive accumulation in tissues, resulting in a high apparent V_d in pharmacokinetic studies (Bickel et al., 1983). Early efforts to discover CRF_1 antagonists yielded highly lipophilic compounds with very high V_d (Hsin et al., 2002). This may lead to a number of development

issues including accumulation of the compound on repeated dosing, accumulation of high concentrations of the compound in target organs for toxicity, and/or limited brain exposure. For example, the potent CRF_1 antagonist CP154526 was highly lipophilic, had a V_d of 105 L/kg, and had a very long half-life (51 h) in rats (Keller et al., 2002). In the present study, we observed similar pharmacokinetic parameters for CP154526, with a V_d of 114 L/kg and a half-life of 15.1 h after intravenous administration.

More recently, compounds such as R121919 (Chen et al., 2004) were discovered that had improved V_d and maintained adequate receptor affinity. Improvements in solubility and V_d often come at the expense of substantially increased clearance, as observed with R121919. Our efforts were directed toward reducing V_d while maintaining a plasma half-life that would be suitable for once or twice daily dosing (P. A. Hipskind, J. L. Bishop, Q. Chen, E. A. Collins, D. J. Garmene, S. J. Green, C. Hamdouchi, E. J. Hembre, S. Jia, R. D. Johnston, K. Lobb, J. Lu, J. K. Myers, K. A. Savin, K. Somoza, T. Takakuwa, R. C. Thompson, J. L. Toth, and D. R. Gehlert, unpublished observations). In the present study, we assessed the pharmacokinetic properties of MTIP and compared it to R121919, CP154526, and SSR125543A. Assayed under identical conditions in rats, MTIP had a V_d of 1.7 L/kg compared with the very high values obtained with R121919 (75.7 L/kg) and CP154526 (114 L/kg) and the more moderate value obtained for SSR125543A (4.2 L/kg). Despite the improvement in V_d, the clearance of MTIP was lower than that observed with R121919 and CP154526. Plasma half-life of MTIP was 3.3 h after oral administration, intermediate between the comparators. The improved V_d and reduced clearance of MTIP should be advantageous for the drug development process.

In summary, upregulated activity of the CRF system confers susceptibility for excessive self-administration of alcohol, and for relapse into alcohol seeking after abstinence. MTIP is a novel, orally available, and brain-penetrant CRF_1 antagonist. Its activity in animal models predicts efficacy in alcohol dependence. Based on these findings, MTIP should be developed for clinical use in alcoholism.

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