JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711

JPET#81711

Anxiolytic- and antidepressant-like profile of ATC0065 and ATC0175: Nonpeptidic and orally active melanin-concentrating hormone receptor 1 antagonists

Shigeyuki Chaki, Takeo Funakoshi, Shiho Hirota-Okuno, Mariko Nishiguchi, Toshiharu Shimazaki, Michihiko Iijima, Andrew J. Grottick, Kosuke Kanuma, Katsunori Omodera, Yoshinori Sekiguchi, Shigeru Okuyama, Thuy-Anh Tran, Graeme Semple, William Thomsen

Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-cho, Kita-ku, Saitama, Saitama 331-9530, Japan (S.C., T.F., S.H.O., M.N., T.S., M.I., K.K., K.O., Y.S., S.O.)

Arena Pharmaceuticals Inc., 6166 Nancy Ridge Drive, San Diego, CA 92121, USA (A.J.G., T.A.T., G.S., W.T.)

Running title:

Psychopharmacological profiles of MCHR1 antagonists

Address for corrspondence:

Shigeyuki Chaki, Ph.D.

Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-cho, Kita-ku Saitama, Saitama 331-9530, Japan

TEL: +81-48-669-3028

FAX: +81-48-652-7254

E-mail: s.chaki@po.rd.taisho.co.jp

Number of text pages: 27

Number of Tables: 1

Number of Figures: 10

Number of references: 43

Number of words in the abstract: 208 words

Number of words in the introduction: 624 words

Number of words in the discussion: 1,313 words

List of non-standard abbreviations:

*N*2-[*cis*-4-({2-[4-bromo-2-(trifluoromethoxy)phenyl]ethyl}amino)cyclohexyl]-*N*4,*N*4-dimethylquinazoline-2,4-diamine dihydrochloride (ATC0065) and *N*-(*cis*-4-{[4-(dimethylamino)quinazolin-2-yl]amino}cyclohexyl)-3,4-difluorobenzamide hydrochloride (ATC0175)

Recommended section: Neuropharmacology

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Melanin-concentrating hormone (MCH) is a cyclic peptide produced in the lateral hypothalamus. It has been implicated in a number of physiological processes including feeding behavior, energy balance and the regulation of emotional states. Here we report *in vitro* and *in vivo* profiles of *N*2-[*cis*-4-({2-[4-bromo-2-(trifluoromethoxy)phenyl]ethyl}amino)cyclohexyl]-*N*4,*N*4-dimethylquinazoline-2,4-diamine dihydrochloride (ATC0065) and *N*-(*cis*-4-{[4-(dimethylamino)quinazolin-2-yl]amino}cyclohexyl)-3,4-difluorobenzamide hydrochloride (ATC0175), newly synthesized MCH receptor 1 (MCHR1) antagonists. Both ATC0065 and ATC0175 had high affinities for human MCHR1 with IC_{50} values of 15.7 ± 1.95 and 7.23 ± 0.59 nM, respectively. Both ATC0065 (IC_{50}=21.4 ± 1.57 nM) and ATC0175 (IC_{50}=13.5 ± 0.78 nM) showed potent antagonist activities at MCHR1, as assessed by MCH-increased [^{35}S]GTPγS binding to human MCHR1. Oral administration of ATC0065 (3-30 mg/kg) or ATC0175 (1–10 mg/kg) significantly reduced immobility time in the forced swimming test in rats, indicating antidepressant-like effects. Both ATC0065 and ATC0175 significantly reversed swim stress-induced anxiety in the elevated plus-maze test in rats and stress-induced hyperthermia in mice. ATC0175 significantly increased social interaction between

unfamiliar rats, and reduced separation-induced vocalizations in guinea pig pups, indicating anxiolytic potential. In contrast, ATC0065 and ATC0175 did not affect spontaneous locomotor activity or rotarod performance in rats. These findings indicate that ATC0065 and ATC0175 are potent and orally active MCHR1 antagonists with anxiolytic- and antidepressant activity in rodents.

INTRODUCTION

Melanin-concentrating hormone (MCH) is a cyclic neuropeptide originally isolated from salmon pituitary (Kawaguchi et al., 1983). In mammals, MCH is produced predominantly by neurons in the lateral hypothalamus and zona incerta with extensive projections throughout the brain (Bittencourt et al., 1992). This expression pattern supports a role for MCH in numerous physiological processes including motivated behavior, stress responses, regulation of neuroendocrine function and feeding.

Several groups independently identified a G protein-coupled receptor, SLC-1/GPR24, as an MCH receptor (MCHR1) (Bachner et al., 1999; Chambers et al., 1999; Lembo et al., 1999; Shimomura et al., 1999; Saito et al., 1999), and MCHR2 was identified subsequently on the basis of the sequence homology to MCHR1 (An et al., 2001; Hill et al., 2001; Mori et al., 2001; Sailer et al., 2001). Potential physiological functions of MCHR2 have not been elucidated due to the species-specific expression of the receptor (Tan et al., 2002), and therefore current research has focused on MCHR1.

There are several lines of evidence implicating MCHR1 in feeding and energy homeostasis. MCHR1 mRNA is increased by fasting or genetic leptin deficiency (ob/ob mice) (Kokkotou et al., 2001). It has been reported that mice lacking MCHR1 were lean and resistant to diet-induced obesity, and had reduced fat mass (Chen et al., 2002;

Marsh et al., 2002), similar to the phenotype of MCH peptide deficient mice. In addition, MCH-induced body weight gain and hyperphagia are absent in MCHR1 null mice (Marsh et al., 2002), indicating the importance of this receptor in mediating the orexigenic and metabolic effects of MCH. Importantly, non-peptide small molecule MCHR1 antagonists attenuate food intake stimulated by MCH (Borowsky et al., 2002; Takekawa et al., 2002), and reduce both food intake and body weight gain in diet-induced obese rats (Borowsky et al., 2002).

In addition to a well documented role in the regulation of feeding and energy expenditure, an emerging body of evidence suggests that MCHR1 plays an important role in the regulation of mood and stress. Within the central nervous system, MCHR1 mRNA and protein are distributed in various hypothalamic nuclei including the paraventricular nucleus (PVN), and several limbic structures including hippocampus, septum, amygdala, locus coeruleus and dorsal raphe nucleus, all of which are implicated in the regulation of emotion and stress (Hervieu et al., 2000; Saito et al., 2001). In addition, dense labeling is detected in the nucleus accumbens shell (Borowsky et al., 2002; Saito et al., 2001). Injection of MCH directly into the PVN has been found to increase plasma adrenocorticotropic hormone (ACTH) (Kennedy et al., 2003). MCH also induces corticotropin-releasing factor (CRF) release from hypothalamic explants,

an effect which is sensitive to blockade by an MCHR1 antagonist (Kennedy et al., 2003), and increases in plasma ACTH by intracerebroventricular injection of MCH are prevented by an anti-CRF antibody (Jezova et al., 1992). It thus seems likely that stimulation of MCHR1 causes activation of the hypothalamus-pituitary-adrenal (HPA) axis through increases in CRF excretion. Regarding the role of MCH in emotional states, it has been reported that administration of MCH into the medial preoptic area induces anxiety (Gonzalez et al., 1996), although there are contradictory results reporting anxiolytic-like effects of MCH injection (Kela et al., 2003). Injection of MCH into the nucleus accumbens shell, in which MCHR1 is abundant, increased immobility in a forced swim test in rats, suggesting increased depressive behavior (Sears et al., 2004). Moreover, Borowsky et al (2002) reported that the MCHR1 antagonist SNAP-7941, exhibited antidepressant and anxiolytic-like effects in three rodent tests, supporting a role for MCHR1 in depression and anxiety.

Recently, we have synthesized novel nonpeptide MCHR1 antagonists, *N*2-[*cis*-4-({2-[4-bromo-2-(trifluoromethoxy)phenyl]ethyl}amino)cyclohexyl]-*N*4,*N*4-dimethylquinazoline-2,4-diamine dihydrochloride (ATC0065) and *N*-(*cis*-4-{[4-(dimethylamino)quinazolin-2-yl]amino}cyclohexyl)-3,4-difluorobenzamid

e hydrochloride (ATC0175). We now report *in vitro* and *in vivo* profiles of ATC0065 and ATC0175 in various rodent tests predictive of anxiolytic and antidepressant activity.

Materials and Methods

Animals. Male ICR mice (24 - 33 g, Charles River, Yokohama, Japan) were housed 8-10 per cage, and were used for the marble burying test and stress-induced hyperthermia. Male Sprague-Dawley rats (190 - 250 g, Charles River, Yokohama, Japan) were housed 4 per cage, and were used for the elevated plus-maze task, the forced swimming test, locomotor activity and the rotarod test, and Male Sprague-Dawley rats (250 – 330 g, Charles River, Yokohama, Japan) were used for the social interaction test. Pregnant guinea pigs were obtained from SLC (Hamamatsu, Japan). All these animals were maintained under a 12-h light/dark cycle (light on 7:00 AM) in a temperature- and humidity-controlled holding room. Food and water were available *ad libitum* except during testing.

Ethics. All studies were reviewed by the Taisho Pharmaceutical Co., Ltd. Animal Care Committee and met the Japanese Experimental Animal Research Association standards, as defined in the Guidelines for Animal Experiments (1987).

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

Chemicals.

*N*2-[*cis*-4-({2-[4-bromo-2-(trifluoromethoxy)phenyl]ethyl}amino)cyclohexyl]-*N*4,*N*4-dimethylquinazoline-2,4-diamine dihydrochloride (ATC0065) and *N*-(*cis*-4-{[4-(dimethylamino)quinazolin-2-yl]amino}cyclohexyl)-3,4-difluorobenzamide hydrochloride (ATC0175) (Fig. 1), and (-)-N-[6-(dimethylamino)-methyl]-5,6,7,8-tetrahydro-2-naphthalenyl]-4'-fluoro[1,1'-biphenyl]-4-carboxamide (T-226296) were synthesized in Taisho Research Laboratories. [^{125}I][Phe^{13},Tyr^{19}]Melanin concentrating hormone (MCH) (specific radioactivity: 81.4 TBq/mmol) and [^{3}H]8-OH-DPAT (specific radioactivity: 7.99TBq/mmol) were purchased from Amersham Biosciences UK, Ltd (Buckinghamshire, UK), and [^{125}I]Lyserigic acid diethylamide (LSD) (specific radioactivity: 81.4 TBq/mmol), [^{35}S]GTPγS (specific radioactivity: 46.25 TBq/mmol) and human 5-HT$_{1A}$ receptor-expressing CHO cell membranes were purchased from PerkinElmer Life Sciences Inc. (Boston, MA). MCH was purchased from Sigma-Aldrich (St. Louis, MO). Human MCHR1-expressing CHO cell membranes and human 5-HT$_{2B}$ receptor-expressing CHO cell membranes were purchased from Euroscreen (Brussels, Belgium). For the *in vitro* studies, ATC0065 and ATC0175 were dissolved in 0.1 % dimethyl sulfoxide, and dimethyl sulfoxide (0.1 %) did not affect ligand bindings. For *in*

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

vivo studies, ATC0065 and ATC0175 were dissolved (or suspended) in 22.5 % hydroxypropyl-β-cyclodextrin.

[^{125}I][Phe^{13},Tyr^{19}]MCH Binding. The membranes of CHO-K1 cells expressing the human MCHR1 were suspended in 25 mM HEPES buffer (pH 7.4) containing 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride and 0.2 % bovine serum albumin at a protein concentration of 10 μg/ml. Membranes were incubated with [^{125}I][Phe^{13},Tyr^{19}]MCH (0.1 nM) for 120 min at 25 °C. The reaction was terminated by rapid filtration under vacuum through a UniFilter GF/C microplate (PerkinElmer Life Sciences, Boston, MA) presoaked with 0.3 % polyethyleneimine, after which the filters were washed three times with 0.3 ml of phosphate buffered saline containing 0.5 M NaCl, using a UniFliter96 harvester (Packard Instruments, Meriden, CT). Filter-bound activity was counted in a TopCount NXT Microplate Scintillation and Luminescence Counter C384V01J (Packard Instruments, Meriden, CT). Nonspecific binding was determined in the presence of 10 μM T-226296.

[^{35}S]GTPγS Binding. The membranes of CHO cells expressing human MCHR1 were suspended in assay buffer (20 mM HEPES buffer containing 100 mM NaCl, 10 mM $MgCl_2$, 1 μM GDP and 0.2 % bovine serum albumin (pH 7.4)) to give a protein concentration of 2 μg/assay. Membranes were pre-incubated with various

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

concentrations of test compounds and 4 nM MCH for 20 min at 30 °C. [^{35}S]GTPγS (0.1 nM) was then added, and incubated for 30 min at 30 °C. The reaction was terminated by rapid filtration under vacuum through a UniFilter GF/C microplate (PerkinElmer Life Sciences, Boston, MA) presoaked with assay buffer, after which the filters were washed three times with 0.3 ml of ice-cold assay buffer, using a UniFliter96 harvester (Packard Instruments, Meriden, CT). Filter-bound activity was counted in a TopCount NXT Microplate Scintillation and Luminescence Counter C384V01J (Packard Instruments, Meriden, CT).

MCHR2 Calcium Assay. Stable HEK293 cells expressing human MCHR2 were seeded (55,000 cells/100µl of complete culture media Dulbecco's modified Eagle medium with 10% Fetal Bovine Serum, 2mM L-glutamine, 1mM sodium pyruvate)/well into poly-D-lysine pretreated black/clear bottom plates 24 hours before the assay. Prior to the assay, media was removed and HEK293 cells were loaded with 4 µM Fluo-4 calcium-sensitive dye (Molecular Probes, Eugene, OR) in complete culture media also containing 0.04% pluronic acid and 2.5mM probenecid for one hour at 37^{o}C in 5% CO_2 followed by washing of dye loaded cells four times with 100µl of wash buffer (Hank's balanced salt solution supplemented with 2.5mM probenecid, 20mM Hepes, pH 7.4, and 0.5% BSA). Cells were then resuspended in wash buffer and fluorescence emission

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

caused by increases in intracellular calcium mobilization elicited by 75nM MCH was measured with FLIPR (Molecular Devises, Sunnyvale, CA). The maximum increase in fluorescence was determined and compared for each well. Antagonist activity of ATC0065 and ATC0175 was evaluated by preincubation of cells with compound for 30 min in 5% CO_2 followed by addition of MCH.

5-HT_{1A} and 5-HT_{2B} Receptor Assays. Membranes of CHO cells expressing human 5-HT_{1A} receptor were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 25 mM $MgCl_2$ and 0.1 % bovine serum albumin, and incubated with [^{3}H]8-OH-DPAT (0.5 nM) at for 30 min 37 °C. In case of 5-HT_{2B} receptor binding, CHO cell membranes expressing human 5-HT_{2B} receptor were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 4 mM $CaCl_2$, 0.1 % ascorbic acid and 0.2 % bovine serum albumin, and incubated with [^{125}I]LSD (0.75 nM) at for 30 min 37 °C. The reaction was terminated by rapid filtration under vacuum through a UniFilter GF/C microplate (PerkinElmer Life Sciences, Boston, MA) presoaked with 0.3 % polyethyleneimine, after which the filters were washed three times with 0.3 ml of 50 mM Tris-HCl buffer (pH 7.4) (for [^{3}H]8-OH-DPAT binding) or 50 mM Tris-HCl buffer (pH 7.4) containing 4 mM $CaCl_2$ and 0.1 % ascorbic acid (for [^{125}I]LSD binding), using a UniFliter96 harvester (Packard Instruments, Meriden, CT). Filter-bound activity was

counted in a TopCount NXT Microplate Scintillation and Luminescence Counter C384V01J (Packard Instruments, Meriden, CT). Nonspecific binding was determined in the presence of 10 μM NAN-190 (for [^{3}H]8-OH-DPAT binding) or 10 μM clozapine (for [^{125}I]LSD binding).

Stress-Induced Anxiety in Rats. Rats were placed in a 40-cm tall, 20-cm wide cylindrical plastic container containing 25 cm of water maintaining at 25 ± 1 ºC for two min. Subjects were then removed from the tank and allowed to recover for a further 5 min before the elevated plus-maze test was performed. The apparatus consisted of a plus-shaped maze, elevated 50 cm from the floor, with two opposite open arms, 50 x 10 cm, crossed at right angles by two arms of the same dimensions, but enclosed by 40-cm-high walls with an open roof. In addition, a 1-cm-high edge made of Plexiglas surrounded the open arms to prevent falls. Illumination measured at the center of the maze was 40 lx. Each rat was placed in the center of the plus-maze facing one enclosed arm. The amount of time spent in open arms of the maze was recorded with a video tracking system (video tracking system CompACT VAS for windows plus-maze ver.3.05, Muromachi Kikai, Tokyo, Japan). Rats were naïve to the apparatus. Our pharmacokinetic study showed that ATC0065 reached a maximal plasma concentration more slowly than ATC0175 following oral administration (Tmax values, 4 hr for

ATC0065 and 3 hr for ATC0175). ATC0065 and ATC0175 were administered p.o. 3 and 2 hr prior to swim stress, respectively. Moreover, in a preliminary study, both compounds exhibited reasonable plasma concentrations (Cmax value, 78.42 ng/ml for ATC0065, 121.94 ng/ml for ATC0175) following oral administration of ATC0065 or ATC0175 at 10 mg/g.

Social Interaction Test in Rats. This test was done in an open-field apparatus placed in an isolated chamber. The apparatus was an open-topped perspex box (45 x 27 x 30 cm) with a solid floor. Tests were conducted in an illuminated room (300 radiometric lx). A camera was mounted above the arena. The rats were allocated to a test pair on the basis of weight. Both members of a pair were given the same drug treatment. For the two days before the test, the rats were allowed to explore the apparatus individually for 4 min per day. By doing this, the rats are able to familiarize themselves with the apparatus but not with their partner. In this test, rats were placed in the test arena for a 10-min trial, and the following behaviors were scored as social interaction: sniffing, following, social grooming, crawling under and over. ATC0175 was administered p.o. 2 hr prior to the test. Chlordiazepoxide was suspended in 0.4 % carboxymethylcellulose, and administered p.o. 1 hr prior to the test.

Stress-Induced Hyperthermia in Mice. The test was performed according to the

method previously reported (Spooren et al., 2002). In this test, rectal temperature is recorded in singly housed mice at two consecutive time points (T_1 and T_2), which are interspaced by a defined time-interval. Since the value at the second temperature-recording exceeds the value of the initial measure (which mirrors the stress reaction), it is the difference between these two core-temperatures which reflects stress-induced hyperthermia. Body temperature was measured in each mouse twice, at t=0 min (T_1) and t= +15 min (T_2). The difference in temperature (T_2-T_1) was considered to reflect the stress-induced hyperthermia. Rectal temperature was measured by a thermometer (BWT-100, Bio Research Center, Japan) via a lubricated thermistor probe for mice inserted 20 mm into the rectum while the mouse was hand-held near the base of the tail. The probe was left in place until steady readings were obtained. A comparison between T_1 in vehicle-treated mice and in animals treated with a given dose of a test-compound was used to measure whether a test compound per se affect body temperature. ATC0065, ATC0175 and diazepam were administered p.o. 1 hr prior to the test.

Separation-Induced Vocalization in Guinea Pig Pups. The test was conducted according to the method reported by Molewijk et al. (1996). Pregnant females were housed in individual cages until parturition and thereafter with their pups throughout the

study. The test cage consisted of a sound-proof box with a white interior and white illumination, and the microphone was mounted in the ceiling of the test cage. During the test, this box was closed. From 7 days of age pups entered a pretest in which their vocalization were recorded for 5 min. Only animals that vocalized for longer than 200 sec were used for drug evaluation. Pups that did not reach the criterion underwent a second pretest. On test days, ATC0175 or vehicle was given i.p., and pups were immediately returned to the home cage with its mother and littermates for 60 min before testing. Fluvoxamine was administered i.p. 30 min prior to the test. The same subjects were retested on five test days with 2-day intervals for wash-out of drug treatment for up to 3 weeks according to a Latin square design, providing their baseline vocalization response remained at > 200 sec.

Marble-Burying Behavior in Mice. Mice were individually placed in transparent, polycarbonate cages (22 x 32 x 13.5 cm) containing a 5-cm layer of sawdust and 24 glass marbles (1.5 cm in diameter) were evenly placed on the sawdust of the cage. Thirty min later the animals were removed from the cages and the number of marbles at least two-thirds buried in the sawdust was recorded. ATC0065 and ATC0175 were administered p.o. 3 and 2 hr prior to the test, respectively. Fluvoxamine was administered s.c. 30 min prior to the test.

Forced Swimming Test in Rats. A forced swimming test was performed according to the method previously reported (Chaki et al., 2004), and the effect of the compound was evaluated by measuring the period of immobility and by a time-sampling technique. A time sampling technique was used to score several types of behavior (immobility, swimming, climbing) as described by Detke et al. (1995). Swimming sessions were conducted by placing rats in cylinders containing 25 ºC water 30 cm deep, so that rats could not support themselves by touching the bottom with their feet. Two sessions were conducted between 10:00 AM and 4:00 PM, an initial 15 min pretest followed 24 hr later by a 5 min test. ATC0065 and ATC0175 were administered p.o. during the period between these two sessions (24 and 3 hr for ATC0065, 24 and 2 hr for ATC0175 prior to the test). Following swimming sessions, the rats were removed from the cylinders, placed in a heated cage for 15 min, then returned to their home cages. Test sessions were videotaped from the front of the cylinders for later scoring. The water in the cylinders was changed after every trial. A time-sampling technique was used to score behavior during a single viewing. At the end of each 5-s period during the test session, the scorer, who was unaware of the drug treatment, rated the rat's behavior as one of the following three behaviors: 1) immobility-floating in the water without struggling, and making only movements necessary to keep its head above water; 2) swimming-making

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

active swimming motions between quadrants of the cylinder, more than necessary to merely keep the head above water, moving around in the cylinder; and 3) climbing-movements with forepaws in and out of the water, usually directed against walls of the cylinder.

Spontaneous Locomotor Activity in Rats. Animals were housed individually in transparent acrylic cages (47 x 28.5 x 29.5 cm), and spontaneous locomotor activity was recorded every 5 min for 60 min, using a SCANET apparatus (Neuroscience Inc, Japan) placed in a sound-proof box. ATC0065 and ATC0175 were administered p.o. 3 and 2 hr prior to the test, respectively.

Rotarod Performance in Rats. The rotarod (Campdem Instruments, UK), consisted of a gritted plastic roller (3 cm in diameter, 9 cm long) flanked by two large round plates to prevent the animals from escaping and was run at 10 rpm. All animals were given control trials prior to the test. A rat was placed on the roller, and the length of time it remained there was measured. A maximum of 2 min was allowed for each animal. ATC0065 and ATC0175 were administered p.o. 3 and 2 hr prior to the test, respectively.

Statistical Analysis. The concentration of the test compound that caused 50% inhibition of specific binding (IC_{50} value) was determined from each concentration-response curve. IC_{50} values were determined by the Marquardt-Levenberg nonlinear least-squares

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

curve-fitting procedure, using the MicroCal ORIGIN program (MicroCal, Northampton, MA). Data from *in vivo* experiment were analyzed by student-t test or one-way ANOVA and significant differences between groups were determined, using Dunnett's test.

Results

***In vitro* Receptor Profiles of ATC0065 and ATC0175.** Both ATC0065 and ATC0175 inhibited [^{125}I][Phe^{13},Tyr^{19}]MCH binding to recombinant human MCHR1 with IC_{50} values of 15.7 ± 1.95 nM and 7.23 ± 0.59 nM, respectively (Fig. 2a, Table 1). Scatchard plot analysis of saturation curve of [^{125}I][Phe^{13},Tyr^{19}]MCH binding showed that 5 nM ATC0175 reduced a Bmax value (5.50 ± 1.53 pmol/mg protein and 3.47 ± 0.75 pmol/mg protein in the absence and presence of ATC0175, respectively) without causing a significant changes in a Kd value (0.58 ± 0.13 nM and 0.60 ± 0.09 nM in the absence and presence of ATC0175, respectively), indicating that ATC0175 inhibited [^{125}I][Phe^{13},Tyr^{19}]MCH binding in a noncompetitive manner (Fig. 3). MCH increased [^{35}S]GTPγS binding to recombinant human MCHR1, and both ATC0065 and ATC0175 concentration-dependently inhibited MCH (4 nM)-induced increase in [^{35}S]GTPγS binding with IC_{50} values of 21.4 ± 1.57 nM and 13.5 ± 0.78 nM, respectively (Fig. 2b, Table 1), thereby indicating that ATC0065 and ATC0175 act as an antagonist at the

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

MCHR1. Both ATC0065 and ATC0175 did not affect basal [^{35}S]GTPγS binding (data not shown). In contrast, neither ATC0065 nor ATC0175 exhibited significant activity for MCHR2, as assessed by MCH-induced [Ca^{2+}]i assay (Table 1). ATC0065 and ATC0175 also exhibited high to moderate affinity for 5-HT_{2B} receptors (ATC0065, IC_{50}=266 ± 91.8 nM; ATC0175, IC_{50}=9.66 ± 1.58 nM) and for 5-HT_{1A} receptors (ATC0065, IC_{50}=62.9 ± 11.8 nM; ATC0175, IC_{50}=16.9 ± 1.56 nM) (Table 1). In a preliminary study, ATC0175 did not show agonist activity even at 10 μM in a [^{3}H]phosphoinositide accumulation assay, suggesting that ATC0175 act as an antagonist at 5-HT_{2B} receptor (data not shown).

Effect on Stress-Induced Anxiogenic-Like Behavior in Rats. Exposure to swim stress for 2 min significantly reduced the time spent in open arms in the elevated plus-maze task, showing that rats become more anxious (Fig. 4a,b). Both ATC0065 [$F(3,28)=3.56$, $p<0.05$] and ATC0175 [$F(3,28)=4.99$, $p<0.01$] dose-dependently reversed anxiety induced by swim stress with lowest effective doses of 10 and 1 mg/kg, respectively (Fig. 4a,b). We have demonstrated previously that diazepam (3 mg/kg, p.o.) reversed stress-induced changes under identical condition (data not shown).

Effect in the Social Interaction Test in Rats. Chlordiazepoxide (4 mg/kg, p.o.) significantly prolonged a social interaction period [$F(1,11)=48.67$, $p<0.01$] (Fig. 5a).

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

Likewise, ATC0175 significantly and dose-dependently increased social interaction [$F(4,35)=5.78$, $p<0.01$] (Fig. 5b).

Effect in Stress-Induced Hyperthermia in Mice. Diazepam (1 mg/kg, p.o.) attenuated stress-induced hyperthermia ([$F(1,14)=16.08$, $p<0.01$ for Fig. 6a; [$F(1,14)=18.16$, $p<0.01$] for Fig. 6b). Both ATC0065 [$F(3,28)=3.07$, $p<0.05$] and ATC0175 [$F(3,28)=3.75$, $p<0.05$] significantly reduced stress-induced hyperthermia at a dose of 30 mg/kg, p.o. (Fig. 6a,b). In contrast to diazepam which decreased baseline temperature, neither ATC0065 nor ATC0175 had any significant effect on T_1 at any of the tested doses (ATC0065: vehicle, 36.6±0.14 ºC; 3 mg/kg, 36.9±0.25 ºC; 10 mg/kg, 37.0±0.14 ºC, 30 mg/kg, 36.5±0.23 ºC; ATC0175: vehicle, 36.9±0.11 ºC; 3 mg/kg, 36.8±0.16 ºC; 10 mg/kg, 36.8±0.11 ºC; 30 mg/kg, 36.7±0.10 ºC).

Separation-Induced Vocalization in Guinea Pig Pups. Intraperitoneal administration of ATC0175 significantly reduced both duration [$F(3,28)=14.17$, $p<0.01$] and number of vocalizations [$F(3,28)=14.8$, $p<0.01$] induced by separation from their mother and littermates in guinea pig pups (Fig. 7a,b). Fluvoxamine also significantly reduced both duration [$F(1,14)=17.11$, $p<0.01$] and number of vocalizations [$F(1,14)=23.62$, $p<0.01$] (Fig. 7a,b)

Effect in the Marble Burying Test in Mice. Oral administration of ATC0065

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

[*F*(3,26)=1.45, p=0.2503] and ATC0175 [*F*(3,36)=1.19, p=0.3267] did not alter marble burying behavior up to 30 mg/kg in mice (Fig. 8b,c), while fluvoxamine significantly reduced the number of marbles buried [*F*(3,36)=25.14, p<0.01] (Fig. 8a).

Effect in the Forced Swimming Test in Rats. Both ATC0065 [*F*(3,28)=5.41, p<0.01] and ATC0175 [*F*(3,28)=8.07, p<0.01] significantly and dose-dependently reduced immobility time in the forced swimming test in rats (Fig. 9a). When assessed according to the behavioral scoring method reported by Detke et al. (1995), both compounds significantly increased swimming behavior without altering climbing behavior (Fig. 9b). We have previously demonstrated a similar profile with the serotonin specific reuptake inhibitor (SSRI) fluvoxamine (3 mg/kg, p.o.) (data not shown).

Effect on General Behaviors in Rats. ATC0065 and ATC0175 did not affect either spontaneous locomotor activity [*F*(3,20)=0.97, p=0.4280 (ATC0065); *F*(3,28)=0.45, p=0.7178 (ATC0175)] (Fig. 10a) or rotarod performance when tested in rats [*F*(3,28)=0.12, p=0.9451 (ATC0065); *F*(3,28)=0.83, p=0.4886 (ATC0175)] (Fig. 10b).

Discussion

In the present study, we demonstrate that ATC0065 and ATC0175 are potent MCHR1 antagonists, which exhibit anxiolytic- and antidepressant-like effects in rodents

after oral administration.

Both ATC0065 and ATC0175, structurally similar compounds, potently inhibited [^{125}I][Phe^{13},Tyr^{19}]MCH binding to human MCHR1, and prevented MCH-induced increase in [^{35}S]GTPγS binding, indicating that both ATC0065 and ATC0175 are potent antagonists for MCHR1. In contrast, both compounds failed to inhibit MCH-induced increase in [Ca^{2+}]i in MCHR2-expressing cell lines, showing that both compounds are highly selective for MCHR1 among MCH receptors. Scatchard plot analysis revealed that ATC0175 reduced the Bmax value of [^{125}I][Phe^{13},Tyr^{19}]MCH binding to MCHR1 without altering its affinity (Kd value). Thus, ATC0175 antagonizes the responses of MCH at MCHR1 in a noncompetitive manner, different from a previously reported MCHR1 antagonist, SNAP-7941, which is a competitive antagonist in a [^{3}H]phosphoinositide accumulation assay (Borowsky et al., 2002). Since ATC0065 has a similar chemical structure as ATC0175, ATC0065 may be a noncompetitive antagonist for MCHR1 as well.

In addition to high affinity for MCHR1, ATC0175 showed a moderate to high affinity for both 5-HT_{2B} and 5-HT_{1A} receptors, and ATC0065 showed a moderate affinity for 5-HT_{1A} receptors. In a preliminary study, ATC0175 acted as an antagonist at 5-HT_{2B} receptors in a [^{3}H]phosphoinositide accumulation assay, and both compounds

acted as partial agonists at 5-HT_{1A} receptors in a [^{35}S]GTPγS binding assay (data not shown). Based on these *in vitro* assays, ATC0175 is a MCHR1 and 5-HT_{2B} receptor antagonist with partial agonism at 5-HT_{1A} receptor, and ATC0065 is a MCHR1 antagonist and 5-HT_{1A} receptor partial agonist.

The anxiolytic potential of ATC0065 and ATC0175 was assessed in two tests involving exposure to stress. Exposure to swim stress markedly shortened exploratory behavior in the elevated plus-maze task, indicating increased anxiety. Both ATC0065 and ATC0175 significantly attenuated this stress-induced behavior in rats. The anti-stress profile of ATC0065 and ATC0175 was confirmed by a significant reduction of stress-induced hyperthermia in mice. In both paradigms, anxiolytic activity has been reported previously with CRF_1 antagonists (Heinrichs et al., 2002; Griebel et al., 2002; Okuyama et al., 1999), an effect which has been related to an attenuation of stress-induced increases in ACTH (Heinrichs et al., 2002; Spooren et al., 2002). It has been suggested that stimulation of MCHR1 activates the HPA axis through increases in CRF release from the hypothalamus (Jezova et al., 1992; Kennedy et al., 2003). Therefore, it is conceivable that ATC0065 and ATC0175 may exert their anxiolytic and anti-stress effects by attenuating stress-induced increases in HPA function. It is of note

that hyperthermia induced by anticipatory anxiety is a well described phenomenon in humans (Reeves et al., 1985), and that autonomic hyperactivity is one of the items in the diagnosis of generalized anxiety disorders mentioned in DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition). Therefore, MCHR1 antagonists may ameliorate not only emotional aspects but also somatic symptoms observed in patients with anxiety disorders.

The anxiolytic potential of ATC0065 and ATC0175 was further evaluated in two tests of anxiety (isolation-induced vocalizations and marble burying) in which not only benzodiazepines but also antidepressants such as SSRIs and tricyclic antidepressants are effective (Borsini et al., 2002). ATC0175 significantly reduced separation-induced vocalization in guinea pig pups, whereas ATC0065 and ATC0175 failed to display significant anxiolytic activity in the marble burying test. In both paradigms, both SSRIs and 5-HT_{1A} agonists show potent anxiolytic effects (Borsini et al., 2002). This suggests that the anxiolytic activity of ATC0175 observed in distress vocalization may be mediated through MCHR1 antagonism, although the involvement of 5-HT_{1A} partial agonism in the action of ATC0175 cannot be ruled out.

ATC0175 was additionally anxiolytic in the rat social interaction test. This is consistent with effects observed in other anxiety models in which anxiety was induced

by stressful conditions. In this paradigm an unfamiliar pair of rats is introduced to a neutral territory, which induces increase in plasma ACTH (File and Seth, 2003), and CRF_1 antagonists, whose anxiolytic effects are more active in highly stressful conditions, effectively increase social interaction in this paradigm (Millan et al., 2001). It has also been reported that acute administration of another MCHR1 antagonist, SNAP-7941, is active in this paradigm (Borowsky et al., 2002).

MCHR1 is highly expressed in the nucleus accumbens shell, an area involved in motivation and reward (Borowsky et al., 2002; Hervieu et al., 2000; Saito et al., 2001). Given that clinical depression is marked by anhedonia, it has been suggested that dysfunction within brain reward circuitry contributes to the pathophysiology of depression (Nestler et al., 2002; Willner et al., 1992). Indeed, it has been reported that inhibition of reward pathways via increased expression of cAMP response element-binding protein (CREB) and brain derived neurotrophic factor (BDNF) in the nucleus accumbens induces a depressive-like behavior in rodents (Eisch et al., 2003; Newton et al., 2002). Interestingly, these manipulations additionally lead to increases in dynorphin expression in the nucleus accumbens. It has been reported that MCHR1 is co-expressed in dynorphin positive medium spiny neurons, and that administration of

MCH to the nucleus accumbens shell causes depressive behavior (Sears et al., 2004). In the present study, both ATC0065 and ATC0175 significantly shortened immobility time in the rat forced swimming test, a behavioral despair model predictive of antidepressant activity. When assessed by a behavioral sampling method, both compounds increased swimming behavior without altering climbing behavior. This is in agreement with results using another MCHR1 antagonist (Borowsky et al., 2002), and similar to that of agents which enhance serotonergic transmission (Detke et al., 1995). Given that MCHR1 is expressed in the dorsal raphe nucleus (Borowsky et al., 2002), it is possible that augmentation of serotonergic transmission is, at least in part, involved in the antidepressant-like activity of MCHR1 antagonists.

In the present study, ATC0065 and ATC0175 did not affect either spontaneous locomotor activity or rotarod performance in rats at doses much higher than those in the pharmacologically effective range. Thus, the anxiolytic and antidepressant-like effects of ATC0065 and ATC0175 observed in the present work can not be ascribed to altered locomotor activity or motor function. Therefore ATC0065 and ATC0175 may be without the unwanted central nervous system side effects sometimes observed with certain antidepressant and anxiolytic medications.

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

In addition to potent antagonistic activity at MCHR1, ATC0175 showed a high affinity for the 5-HT_{2B} receptor, where they act as antagonists. The contribution of 5-HT_{2B} receptor antagonism to the actions of ATC0175 is not clear. However, the 5-HT_{2B} receptor is located principally in the periphery, and only sparsely in the central nervous system in rats (Pompeiano et al., 1994). In addition, whilst it has been reported that a selective 5-HT_{2B} receptor agonist exhibited anxiolytic effects in rodents, a selective 5-HT_{2B} receptor antagonist was without effect (Kennett et al.,1996, 1998). Lastly, anxiolytic activity of a mixed 5-$HT_{2C/2B}$ receptor antagonist has been ascribed to activity at the 5-HT_{2C} but not 5-HT_{2B} receptor (Bromidge et al., 2000). Therefore, it is unlikely that a 5-HT_{2B} receptor antagonist has anxiolytic-like activity, and thus that 5-HT_{2B} receptor antagonism is involved in the actions of ATC0175. ATC0065 and ATC0175 also displayed moderate to weak affinities for some monoamine receptors including α_1, α_{2A} and 5-HT_{2C} receptors. However, our pharmacokinetic data demonstrates that brain concentrations of the compounds at doses effective in behavioral tests do not exceed the IC_{50} values for each receptor. Therefore, we can attribute the anxiolytic- and antidepressant-like effects of ATC0065 and ATC0175 to MCHR1 antagonism. It should be mentioned that T-226296, another MCHR1 antagonist

(Takekawa et al., 2002), exhibited a similar *in vivo* profile to that produced by ATC0065 and ATC0175 (unpublished data).

In conclusion, the present studies demonstrate that ATC0065 and ATC0175 are potent and orally active MCHR1 antagonists which exhibit anxiolytic- and antidepressant-like effects in various rodent tests without causing sedation or impairment of motor coordination. Taken together, both ATC0065 and ATC0175 may have potential in the treatment of subjects with depression and anxiety-related disorders without showing unwanted central nervous system side effects.

References

An S, Cutler G, Zhao JJ, Huang SG, Tian H, Li W, Liang L, Rich M, Bakleh A, Du J, Chen JL and Dai K (2001) Identification and characterization of a melanin-concentrating hormone receptor. *Proc Natl Acad Sci USA* 98:7576-7581.

Bachner D, Kreienkamp H, Weise C, Buck F and Richter D (1999) Identification of melanin concentrating hormone (MCH) as the natural ligand for the orphan somatostatin-like receptor 1 (SLC-1). *FEBS Lett* 457:522-524.

Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, Vale W and Sawchenko PE (1992) The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. *J Comp Neurol* 319:218-245.

Borowsky B, Durkin MM, Ogozalek K, Marzabadi MR, DeLeon J, Lagu B, Heurich R, Lichtblau H, Shaposhnik Z, Daniewska I, Blackburn TP, Branchek TA, Gerald C, Vaysse PJ and Forray C (2002) Antidepressant, anxiolytic and anorectic effects of a melanin-concentrating hormone-1 receptor antagonist. *Nat Med* 8:825-830.

Borsini F, Podhorna J and Marazziti D (2002) Do animal models of anxiety predict anxiolytic-like effects of antidepressants? *Psychopharmacology (Berl)* 163:121-141.

Bromidge SM, Dabbs S, Davies DT, Davies S, Duckworth DM, Forbes IT, Gaster LM, Ham P, Jones GE, King FD, Mulholland KR, Saunders DV, Wyman PA, Blaney FE, Clarke SE, Blackburn TP, Holland V, Kennett GA, Lightowler S, Middlemiss DN, Trail B, Riley GJ and Wood MD (2000) Biarylcarbamoylindolines are novel and selective 5-HT(2C) receptor inverse agonists: identification of 5-methyl-1-[[2-[(2-methyl-3-pyridyl)oxy]-5-pyridyl]carbamoyl]-6-trifluoromethylindoline (SB-243213) as a potential antidepressant/anxiolytic agent. *J Med Chem* 43:1123-1134.

Chaki S, Yoshikawa R, Hirota S, Shimazaki T, Maeda M, Kawashima N, Yoshimizu T, Yasuhara A, Sakagami K, Okuyama S, Nakanishi S and Nakazato A (2004) MGS0039: a potent and selective group II metabotropic glutamate receptor antagonist with antidepressant-like activity. *Neuropharmacology* 46:457-467.

Chambers J, Ames RS, Bergsma D, Muir A, Fitzgerald LR, Hervieu G, Dytko GM, Foley JJ, Martin J, Liu WS, Park J, Ellis C, Ganguly S, Konchar S, Cluderay J, Leslie R, Wilson S and Sarau HM (1999) Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. *Nature* 400:261-265.

Chen Y, Hu C, Hsu CK, Zhang Q, Bi C, Asnicar M, Hsiung HM, Fox N, Slieker LJ, Yang DD, Heiman ML and Shi Y (2002) Targeted disruption of the melanin-concentrating hormone receptor-1 results in hyperphagia and resistance to diet-induced obesity. *Endocrinology* 143:2469-2477.

Detke MJ, Rickels M and Lucki I (1995) Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology* 121:66-72.

Eisch AJ, Bolanos CA, de Wit J, Simonak RD, Pudiak CM, Barrot M, Verhaagen J and Nestler EJ (2003) Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. *Biol Psychiatry* 54:994-1005.

File SE and Seth P (2003) A review of 25 years of the social interaction test. *Eur J Pharmacol* 463:35-53.

Gonzalez MI, Vaziri S and Wilson CA (1996) Behavioral effects of alpha-MSH and MCH after central administration in the female rat. *Peptides* 17:171-177.

Griebel G, Simiand J, Steinberg R, Jung M, Gully D, Roger P, Geslin M, Scatton B, Maffrand JP and Soubrie P (2002) 4-(2-Chloro-4-methoxy-5-methylphenyl)-N-[(1S)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-N-(2-propynyl)-1, 3-thiazol-2-amine hydrochloride (SSR125543A), a potent and selective corticotrophin-releasing factor(1) receptor antagonist. II. Characterization in rodent models of stress-related disorders. *J Pharmacol Exp Ther* 301:333-345.

Heinrichs SC, De Souza EB, Schulteis G, Lapsansky JL and Grigoriadis DE (2002) Brain penetrance, receptor occupancy and antistress *in vivo* efficacy of a small molecule corticotropin releasing factor type I receptor selective antagonist. *Neuropsychopharmacology* 27:194-202.

Hervieu GJ, Cluderay JE, Harrison D, Meakin J, Maycox P, Nasir S and Leslie RA (2000) The distribution of the mRNA and protein products of the melanin-concentrating hormone (MCH) receptor gene, slc-1, in the central nervous system of the rat. *Eur J Neurosci* 12:1194-1216.

Hill J, Duckworth M, Murdock P, Rennie G, Sabido-David C, Ames RS, Szekeres P, Wilson S, Bergsma DJ, Gloger IS, Levy DS, Chambers JK and Muir AI (2001) Molecular cloning and functional characterization of MCH2, a novel human MCH receptor. *J Biol Chem* 276:20125-20129.

Jezova D, Bartanusz V, Westergren I, Johansson BB, Rivier J, Vale W and Rivier C (1992) Rat melanin-concentrating hormone stimulates adrenocorticotropin secretion: evidence for a site of action in brain regions protected by the blood-brain barrier. *Endocrinology* 130:1024-1029.

Kawaguchi H, Kawazoe I, Tsubokawa M, Kishida M and Baker BI (1983) Characterization of melanin-concentrating hormone in chum salmon pituitaries. *Nature*

305:321-323.

Kela J, Salmi P, Rimondini-Giorgini R, Heilig M and Wahlestedt C (2003) Behavioural analysis of melanin-concentrating hormone in rats: evidence for orexigenic and anxiolytic properties. *Regul Pept* 114:109-114.

Kennedy AR, Todd JF, Dhillo WS, Seal LJ, Ghatei MA, O'Toole CP, Jones M, Witty D, Winborne K, Riley G, Hervieu G, Wilson S and Bloom SR (2003) Effect of direct injection of melanin-concentrating hormone into the paraventricular nucleus: further evidence for a stimulatory role in the adrenal axis via SLC-1. *J Neuroendocrinol* 15:268-272.

Kennett GA, Bright F, Trail B, Baxter GS and Blackburn TP (1996) Effects of the 5-HT2B receptor agonist, BW 723C86, on three rat models of anxiety. *Br J Pharmacol* 117:1443-1448.

Kennett GA, Trail B and Bright F (1998) Anxiolytic-like actions of BW 723C86 in the rat Vogel conflict test are 5-HT2B receptor mediated. *Neuropharmacology*

37:1603-1610.

Kokkotou EG, Tritos NA, Mastaitis JW, Slieker L and Maratos-Flier E (2001) Melanin-concentrating hormone receptor is a target of leptin action in the mouse brain. *Endocrinology* 142:680-686.

Lembo PM, Grazzini E, Cao J, Hubatsch DA, Pelletier M, Hoffert C, St-Onge S, Pou C, Labrecque J, Groblewski T, O'Donnell D, Payza K, Ahmad S and Walker P (1999) The receptor for the orexigenic peptide melanin-concentrating hormone is a G-protein-coupled receptor. *Nat Cell Biol* 1:267-271.

Marsh DJ, Weingarth DT, Novi DE, Chen HY, Trumbauer ME, Chen AS, Guan XM, Jiang MM, Feng Y, Camacho RE, Shen Z, Frazier EG, Yu H, Metzger JM, Kuca SJ, Shearman LP, Gopal-Truter S, MacNeil DJ, Strack AM, MacIntyre DE, Van der Ploeg LH and Qian S (2002) Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism. *Proc Natl Acad Sci USA* 99:3240-3245.

Millan MJ, Brocco M, Gobert A, Dorey G, Casara P and Dekeyne A (2001) Anxiolytic properties of the selective, non-peptidergic CRF(1) antagonists, CP154,526 and DMP695: a comparison to other classes of anxiolytic agent. *Neuropsychopharmacology* 25:585-600.

Molewijk HE, Hartog K, van der Poel AM, Mos J and Olivier B (1996) Reduction of guinea pig pup isolation calls by anxiolytic and antidepressant drugs. *Psychopharmacology (Berl)* 128:31-38.

Mori M, Harada M, Terao Y, Sugo T, Watanabe T, Shimomura Y, Abe M, Shintani Y, Onda H, Nishimura O and Fujino M (2001) Cloning of a novel G protein-coupled receptor, SLT, a subtype of the melanin-concentrating hormone receptor. *Biochem Biophys Res Commun* 283:1013-1018.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ and Monteggia LM (2002) Neurobiology of depression. *Neuron* 34:13-25.

Newton SS, Thome J, Wallace TL, Shirayama Y, Schlesinger L, Sakai N, Chen J, Neve

R, Nestler EJ and Duman RS (2002) Inhibition of cAMP response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. *J Neurosci* 22:10883-10890.

Okuyama S, Chaki S, Kawashima N, Suzuki Y, Ogawa S, Nakazato A, Kumagai T, Okubo T and Tomisawa K (1999) Receptor binding, behavioral, and electrophysiological profiles of nonpeptide corticotropin-releasing factor subtype 1 receptor antagonists CRA1000 and CRA1001. *J Pharmacol Exp Ther* 289:926-935.

Pompeiano M, Palacios JM and Mengod G. (1994) Distribution of the serotonin 5-HT2 receptor family mRNAs: comparison between 5-HT2A and 5-HT2C receptors. *Brain Res Mol Brain Res* 23:163-178.

Reeves DL, Levinson DM, Justesen DR and Lubin B (1985) Endogenous hyperthermia in normal human subjects: experimental study of emotional states (II) *Int J Psychosom* 32:18-23.

Sailer AW, Sano H, Zeng Z, McDonald TP, Pan J, Pong SS, Feighner SD, Tan CP,

Fukami T, Iwaasa H, Hreniuk DL, Morin NR, Sadowski SJ, Ito M, Ito M, Bansal A, Ky B, Figueroa DJ, Jiang Q, Austin CP, MacNeil DJ, Ishihara A, Ihara M, Kanatani A, Van der Ploeg LH, Howard AD and Liu Q (2001) Identification and characterization of a second melanin-concentrating hormone receptor, MCH-2R. *Proc Natl Acad Sci USA* 98:7564-7569.

Saito Y, Cheng M, Leslie FM and Civelli O (2001) Expression of the melanin-concentrating hormone (MCH) receptor mRNA in the rat brain. *J Comp Neurol* 435:26-40.

Saito Y, Nothacker HP, Wang Z, Lin SH, Leslie F and Civelli O (1999) Molecular characterization of the melanin-concentrating-hormone receptor. *Nature* 400:265-269.

Sears RM, Georgescu D, Maratos-Flier E, Marsh DJ, Nestler EJ and DiLeone RJ (2004) The neuropeptide MCH controls feeding and mood via action on the nucleus accumbens shell. *Soc Neurosci Abstr* 763.9

Shimomura Y, Mori M, Sugo T, Ishibashi Y, Abe M, Kurokawa T, Onda H, Nishimura O,

Sumino Y and Fujino M (1999) Isolation and identification of melanin-concentrating hormone as the endogenous ligand of the SLC-1 receptor. *Biochem Biophys Res Commun* 261:622-626.

Spooren WPJM, Schoeffer P, Gasparini F, Kuhn R and Gentsch C (2002) Pharmacological and endocrinological characterization of stress-induced hyperthermia in singly housed mice using classical and candidate anxiolytics (LY314582, MPEP and NKP608). *Eur J Pharmacol* 435:161-170.

Takekawa S, Asami A, Ishihara Y, Terauchi J, Kato K, Shimomura Y, Mori M, Murakoshi H, Kato K, Suzuki N, Nishimura O and Fujino M (2002) T-226296: a novel, orally active and selective melanin-concentrating hormone receptor antagonist. *Eur J Pharmacol* 438:129-135.

Tan CP, Sano H, Iwaasa H, Pan J, Sailer AW, Hreniuk DL, Feighner SD, Palyha OC, Pong SS, Figueroa DJ, Austin CP, Jiang MM, Yu H, Ito J, Ito M, Ito M, Guan XM, MacNeil DJ, Kanatani A, Van der Ploeg LH and Howard AD (2002) Melanin-concentrating hormone receptor subtypes 1 and 2: species-specific gene

expression. *Genomics* 79:785-792.

Willner P, Muscat R and Papp M (1992) Chronic mild stress-induced anhedonia: a realistic animal model of depression. *Neurosci Biobehav Rev*16:525-534.

JPET Fast Forward. Published on January 26, 2005 as DOI: 10.1124/jpet.104.081711
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Fig. 1 Chemical structures of ATC0065 and ATC0175.

Fig. 2 Affinity and antagonist activity of ATC0065 and ATC0175 for human MCHR1.

Affinity for MCHR1 was determined by [^{125}I][Phe^{13},Tyr^{19}]MCH binding to recombinant human MCHR1. Antagonist activity was determined by [^{35}S]GTPγS binding induced by 4 nM MCH. Data represent mean value obtained from 3-5 separate experiments.

Fig. 3 Scatchard plot analysis of [^{125}I][Phe^{13},Tyr^{19}]MCH binding in the presence of 5 nM ATC0175.

[^{125}I][Phe^{13},Tyr^{19}]MCH binding was conducted in increasing concentration of [^{125}I][Phe^{13},Tyr^{19}]MCH in the presence or absence of 5 nM ATC0175. Data represent mean value obtained from 3 separate experiments.

Fig. 4 Effect of ATC0065 and ATC0175 on swim stress-induced reduction in time spent in open arms in the elevated plus-maze task in rats.

Data represent mean ± SE (n=8). *p<0.05, **p<0.01 versus nonstress vehicle (Dunnett's test); #p<0.05, ##p<0.01 versus stress vehicle (Dunnett's test).

Fig. 5 Effect of ATC0175 in the social interaction test in rats.

Data represent mean ± SE (n=8). CDP: chlordiazepoxide. *p<0.05, **p<0.01 versus vehicle (Student-t test or Dunnett's test).

Fig. 6 Effect of ATC0065 and ATC0175 on stress-induced hyperthermia in mice.

Data represent mean ± SE (n=8). *p<0.05, **p<0.01 versus vehicle (Student-t test or Dunnett's test).

Fig. 7 Effect of ATC0175 on separation-induced vocalization in guinea pig pups.

Data represent mean ± SE (n=8). FLV: fluvoxamine. *p<0.05, **p<0.01 versus vehicle (Student-t test or Dunnett's test).

Fig. 8 Effect of ATC0065 and ATC0175 on marble-burying behavior in mice.

Data represent mean ± SE (n=7-10). *p<0.05, **p<0.01 versus vehicle (Dunnett's test).

Fig. 9 Effect of ATC0065 and ATC0175 in the forced swimming test in rats.

The effects of the compounds were evaluated by both the method duration of immobility (a) and the time-sampling technique (b) described by Detke et al. (1995).

Data represent mean ± SE (n=8). *p<0.05, **p<0.01 versus vehicle (Dunnett's test).

Fig. 10 Effect of ATC0065 and ATC0175 on general behavior in rats.

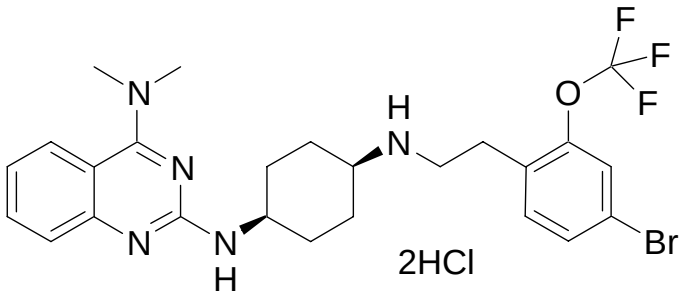
Effects on spontaneous locomotor activity (a) and rotarod performance (b) were evaluated.

Data represent mean ± SE (n=6-8).

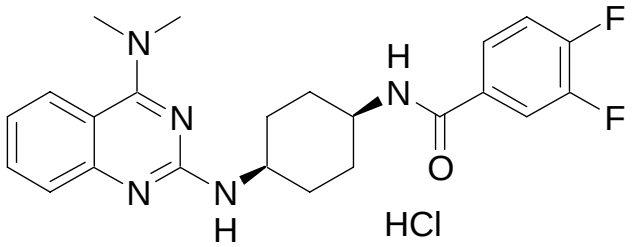
Table 1 Receptor profiles of ATC0065 and ATC0175

	MCHR1		MCHR2	5-HT2B	5-HT1A
	Affinity	Antagonist activity	Antagonist activity	Affinity	Affinity
			IC_{50} (nM)		
ATC0065	15.7±1.95 (4)	21.4±1.57 (3)	1510±140 (3)	266±91.8 (3)	62.9±11.8 (5)
ATC0175	7.23±0.59 (3)	13.5±0.78 (3)	>10,000 (3)	9.66±1.58 (5)	16.9±1.56 (5)

Affinity for each receptor was evaluated by receptor binding assays for each receptor as described in Materials and Methods. Antagonistic activity was evaluated by [^{35}S]GTPγS binding (MCHR1) or [Ca^{2+}]i (MCHR2).
Data represents mean ± SE obtained from 3-5 separate experiments, each done in duplicate.
( )=N



ATC0065



ATC0175

Fig. 1

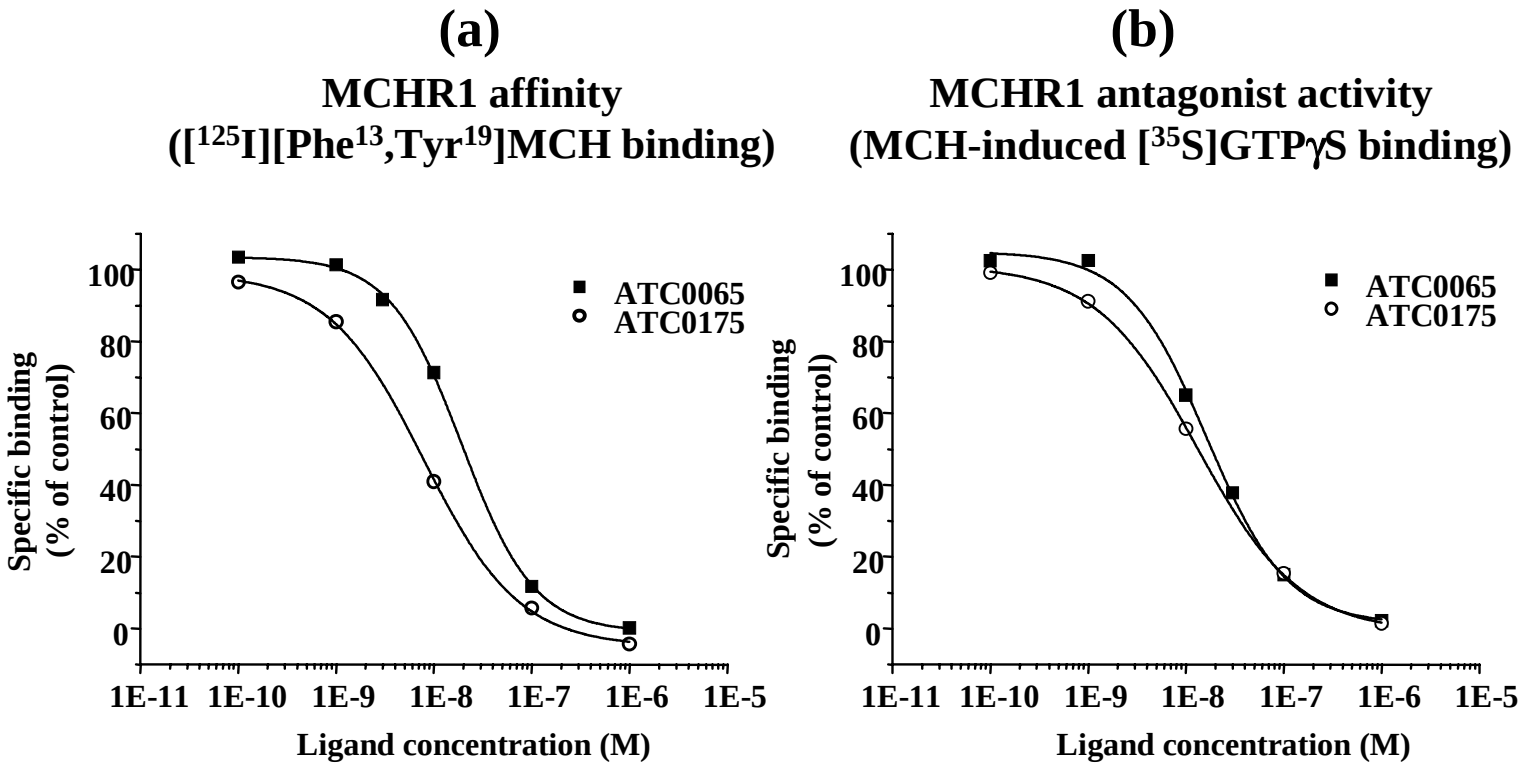


Fig. 2

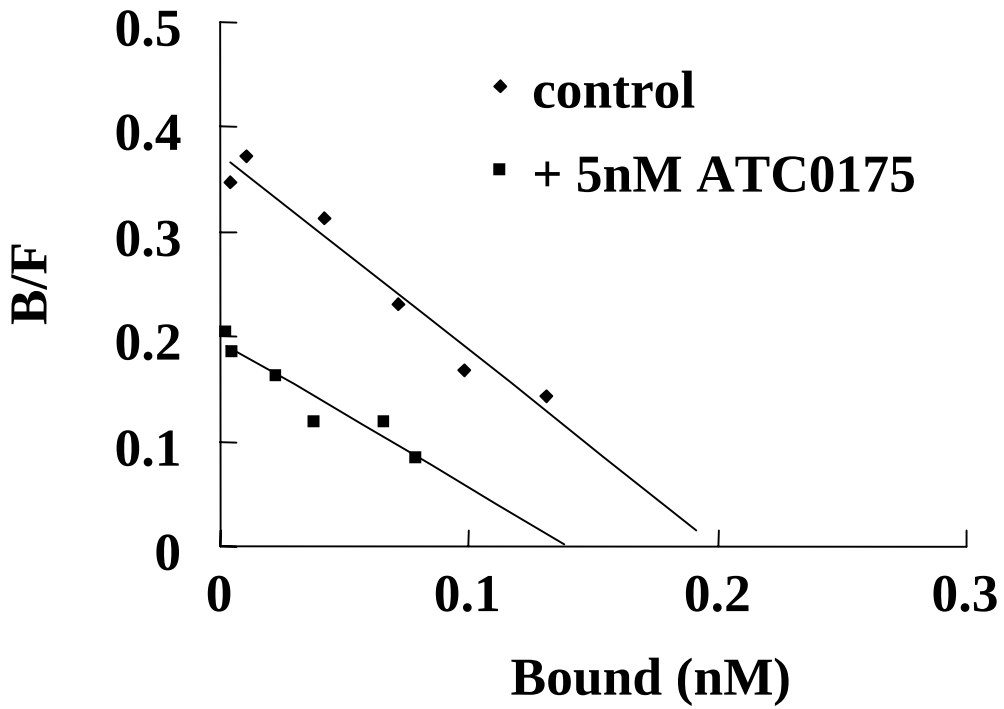


Fig. 3

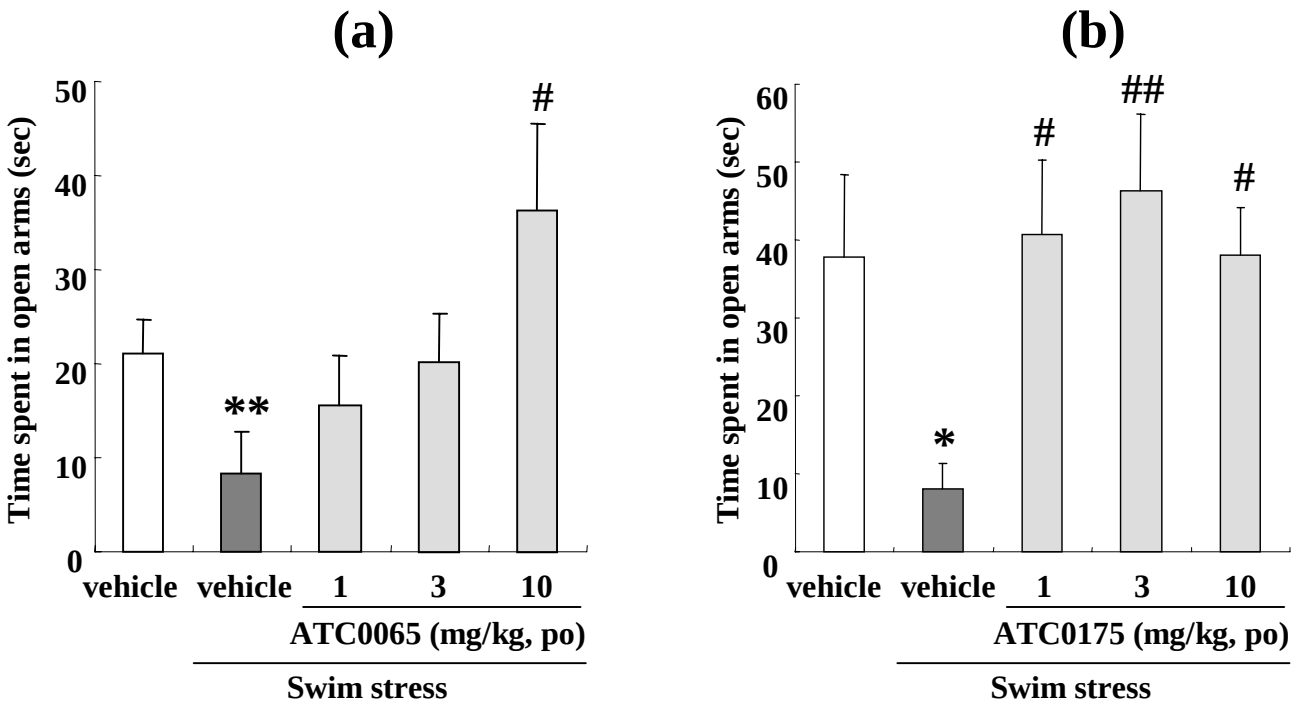


Fig. 4

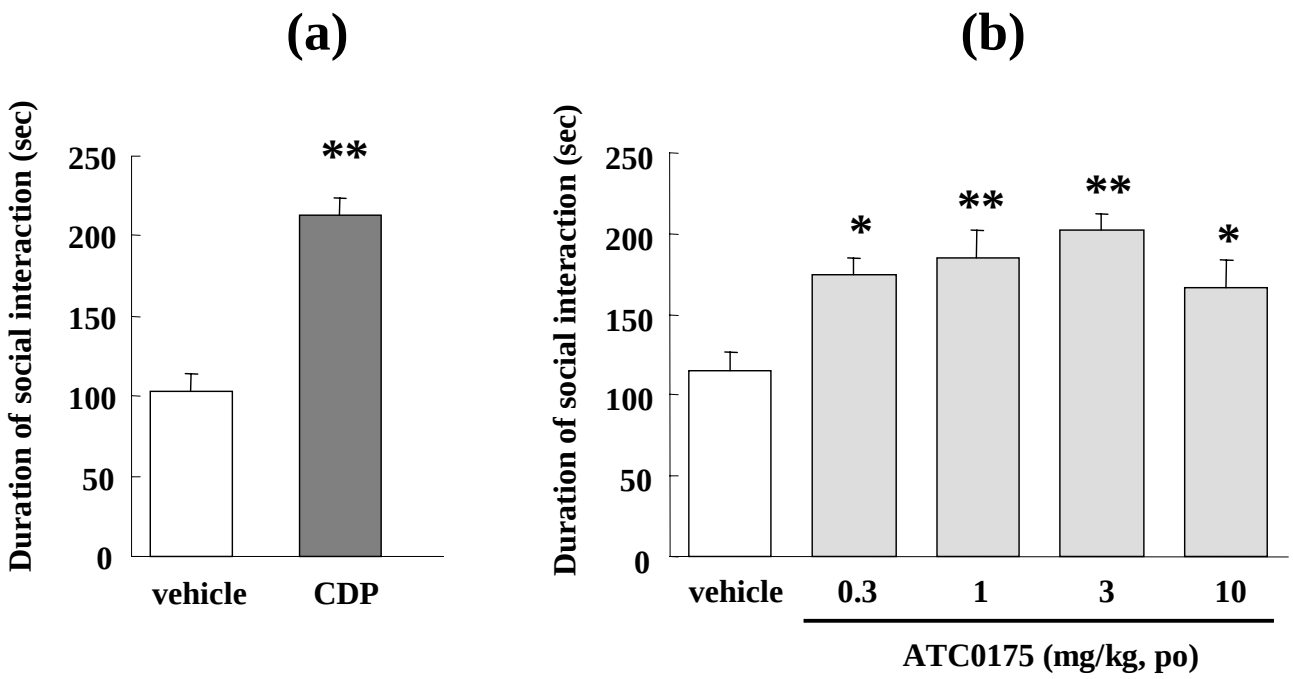


Fig. 5

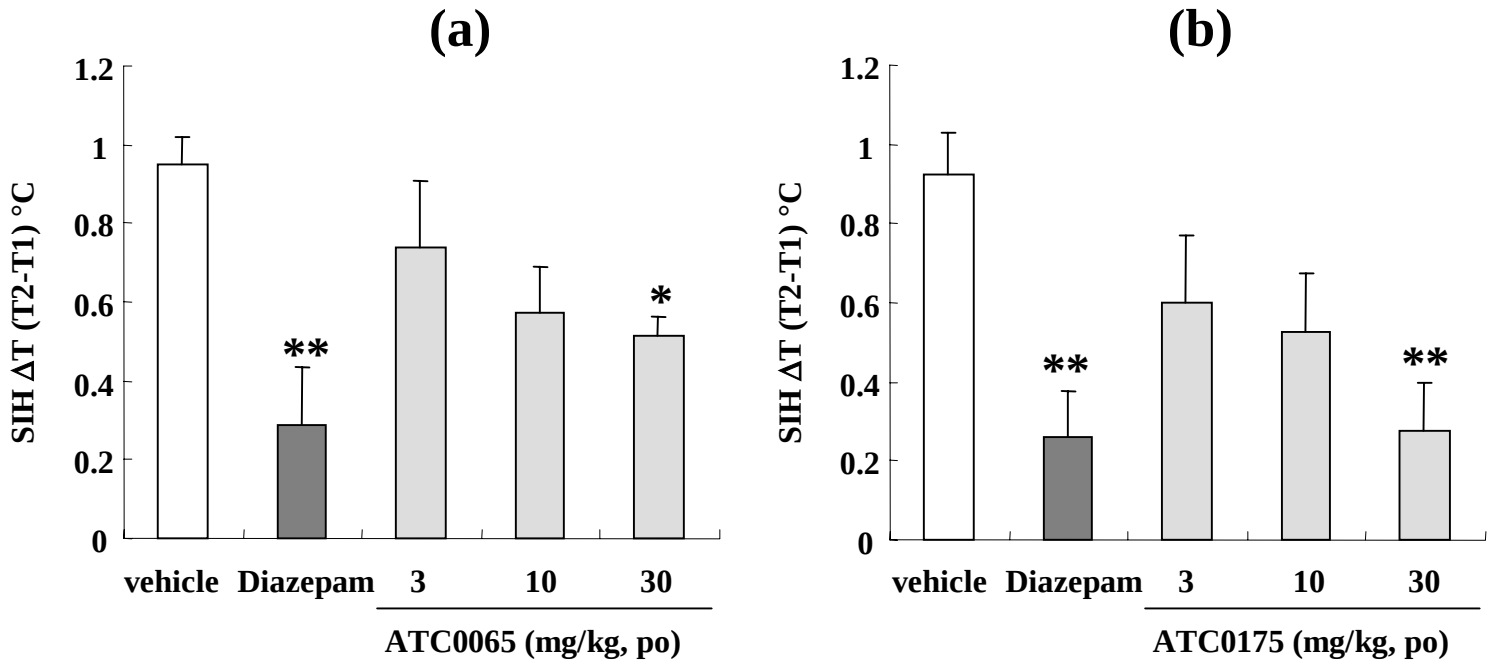


Fig. 6

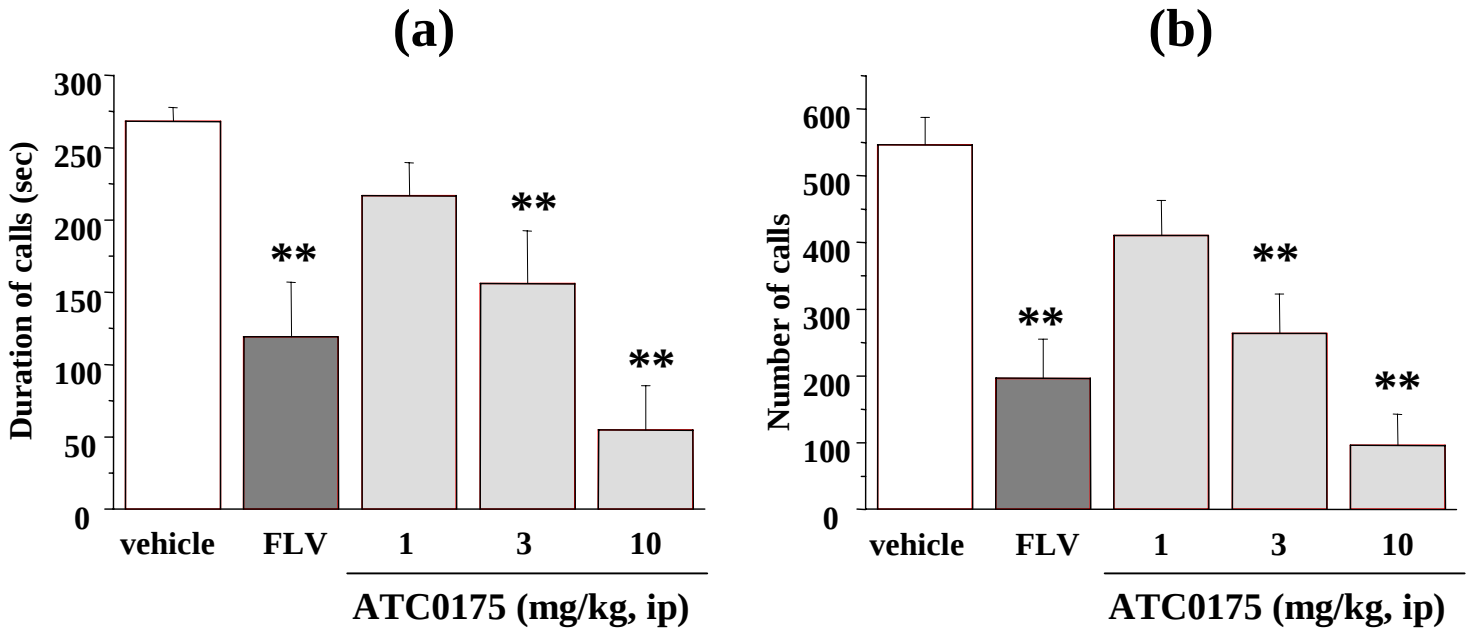


Fig. 7

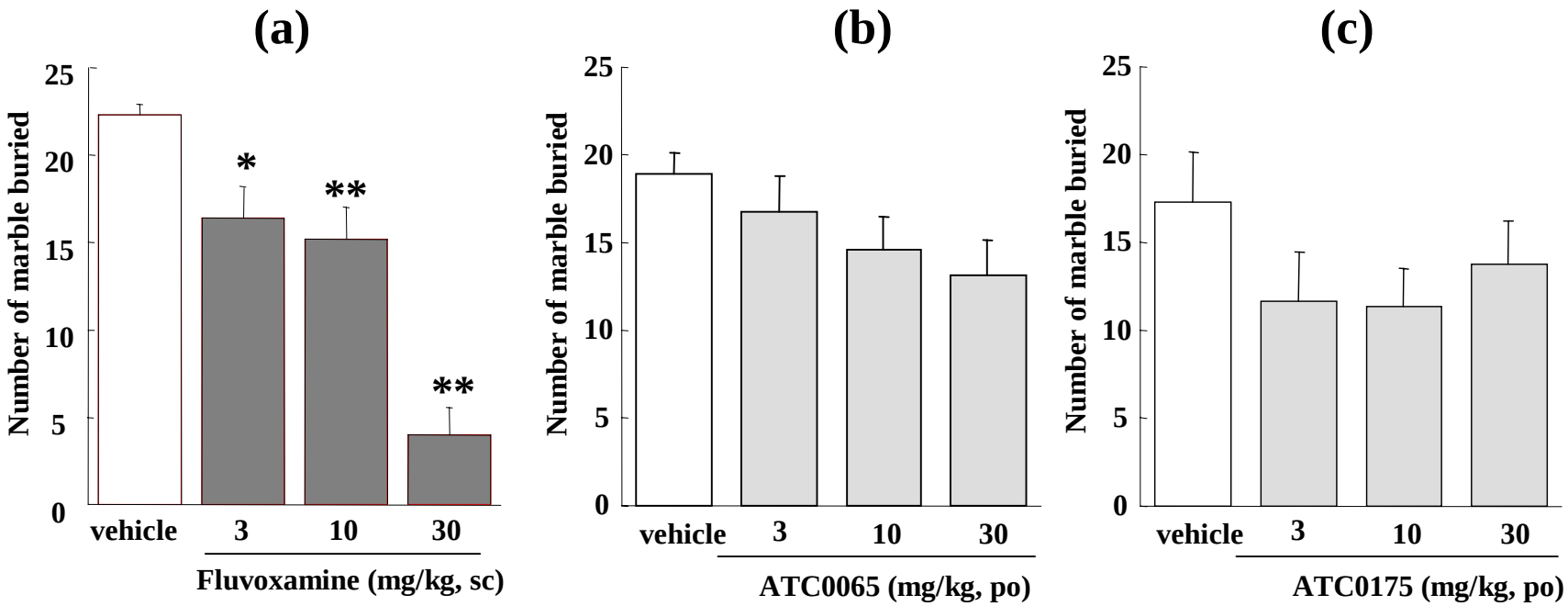


Fig. 8

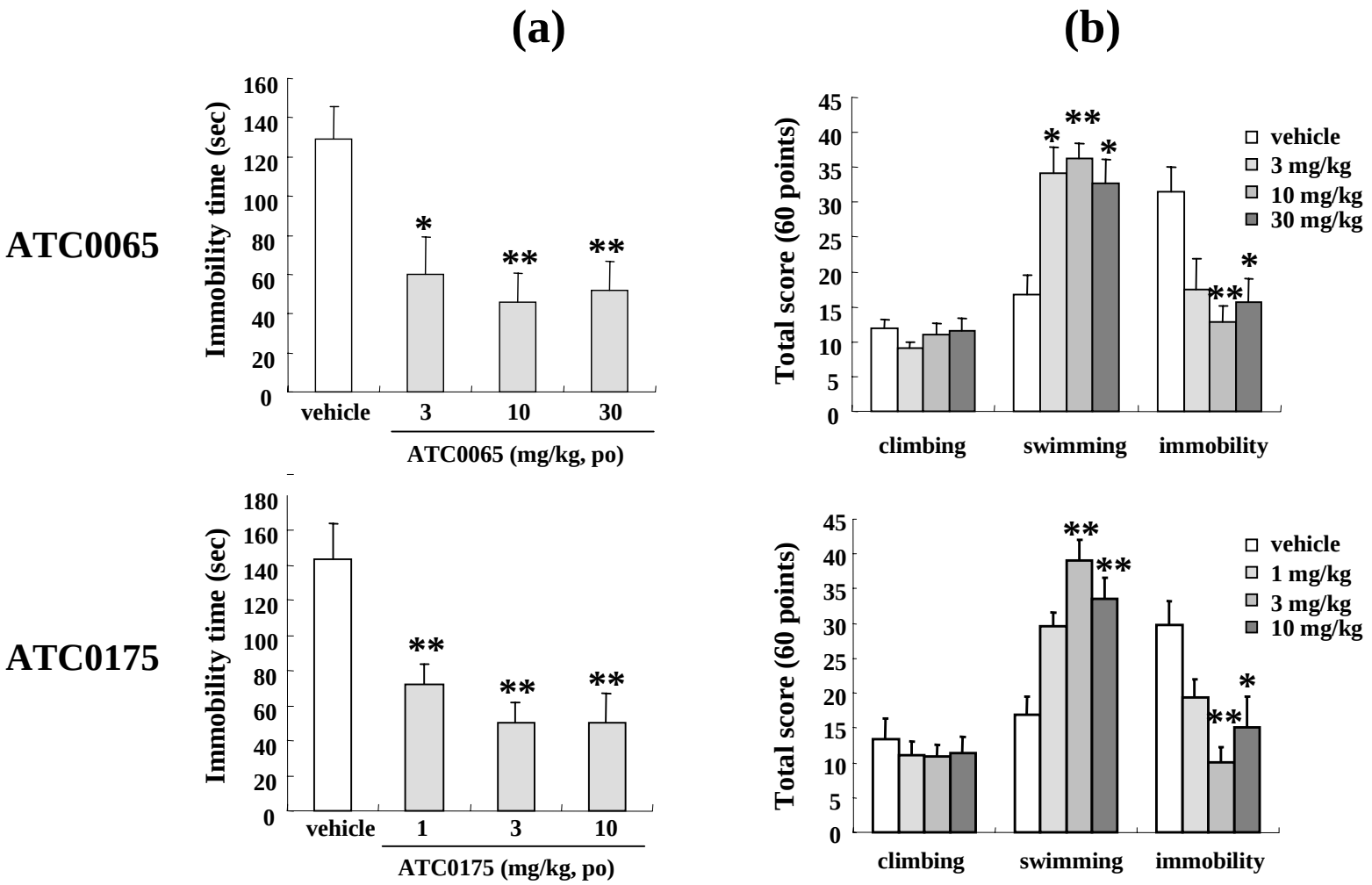


Fig. 9

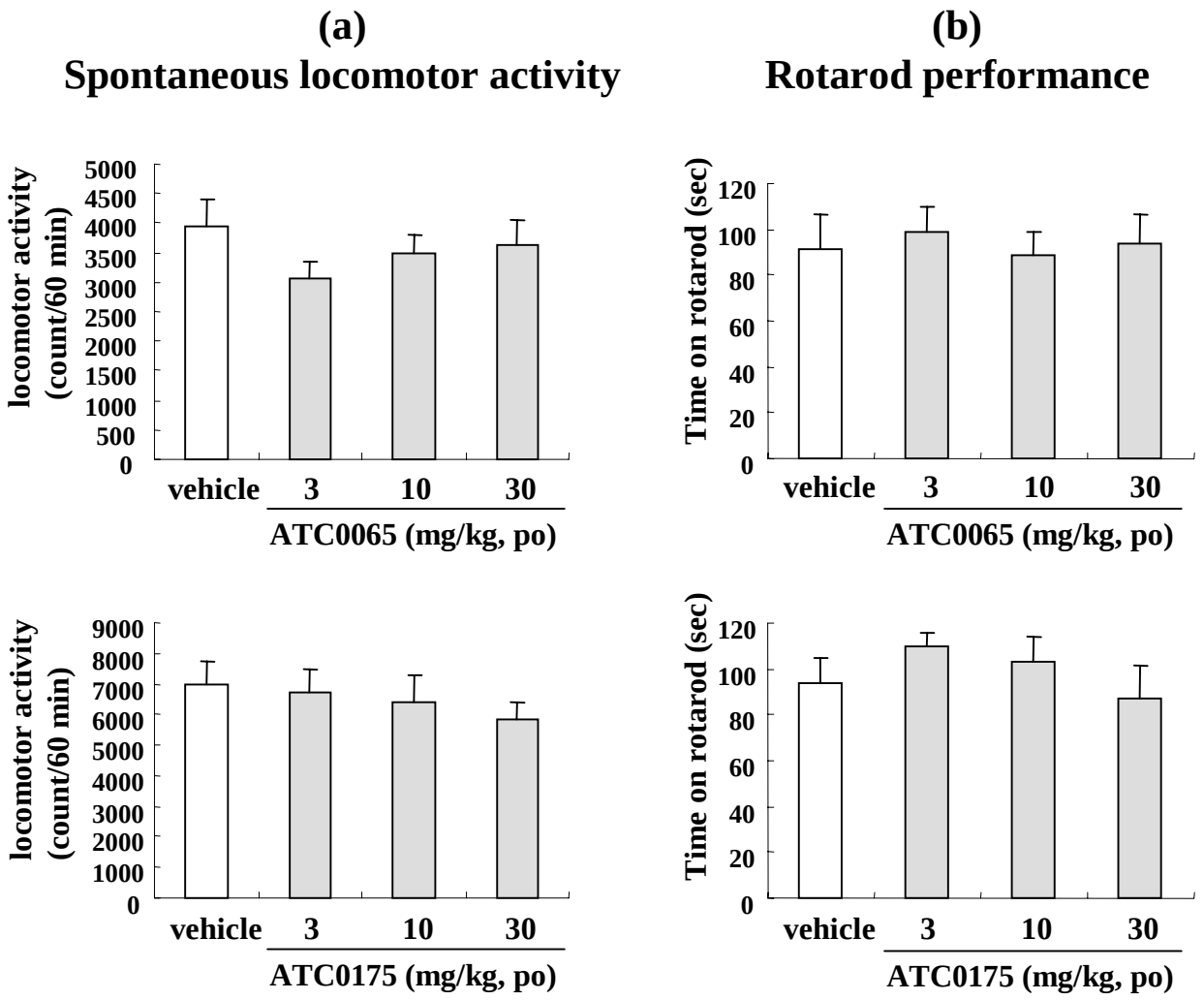


Fig. 10