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T-226296: a novel, orally active and selective melanin-concentrating hormone receptor antagonist

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Abstract

Through the screening of our in-house chemical compound library, we found a novel melanin-concentrating hormone (MCH) receptor antagonist, T-226296, a (−) enantiomer of *N*-[6-(dimethylamino)-methyl]-5,6,7,8-tetrahydro-2-naphthalenyl]-4′ -fluoro[1,1′ -biphenyl]-4-carboxamide. T-226296 exhibited high affinity for cloned human and rat MCH receptors (SLC-1) in receptor binding assays ($IC_{50} = 5.5 \pm 0.12$ nM for human SLC-1; 8.6 ± 0.32 nM for rat SLC-1). T-226296 had high selectivity over other receptors, including the second subtype of the MCH receptor, SLT (MCH2), transporters and ion channels. In Chinese hamster ovary (CHO) cells expressing human SLC-1, T-226296 reversed the MCH-mediated inhibition of forskolin-stimulated cAMP accumulation, inhibited MCH-induced intracellular Ca^{2+} increase, and also inhibited MCH-stimulated arachidonic acid release. In rats, oral administration of T-226296 (30 mg/kg) almost completely suppressed the food intake induced by intracerebroventricular injection of MCH. These results clearly indicate that T-226296 is a novel, orally active and selective MCH receptor antagonist that will be promising for further exploring the physiology and pathophysiology of MCH–SLC-1 signaling. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: (MCH) melanin-concentrating hormone; SLC-1 receptor; Receptor antagonist; Food intake; Obesity

1. Introduction

Melanin-concentrating hormone (MCH) is a cyclic peptide first isolated from the salmon pituitary as a hormone responsible for skin color change (Kawauchi et al., 1983), and subsequently found to be present in mammals (Vaughan et al., 1989). Human MCH is a cyclic 19-amino-acid peptide and identical to rat MCH (Presse et al., 1990). In mammals, MCH-producing neurons are located mainly in the lateral hypothalamus and zona incerta, and project to a wide variety of brain regions (Bittencourt et al., 1992). This extensive terminal distribution suggests that MCH plays many physiological functions in the central nervous system, such as feeding behavior, anxiety, stress response, learning and reproduction. In the peripheral tissues of rodents, MCH is expressed in the testis, stomach and intestine (Hervieu and Nahon, 1995), and thought to be involved in the secretion of insulin (Tadayyon et al., 2000) and leptin (Bradley et al., 2000).

Recently, MCH research has actively focused on its roles in the regulation of feeding behavior and energy balance. MCH acutely stimulates food intake in rats when injected intracerebroventricularly (Rossi et al., 1997). MCH mRNA levels are increased by fasting in genetically obese *ob*/*ob* mice (Qu et al., 1996), and in Ay/a (agouti) mice (Hanada et al., 2000). Generations of genetically engineered mice also reveal the importance of MCH in feeding behavior. MCH knockout mice have reduced body weight due to hypophagia and an increase in metabolic rate (Shimada et al., 1998). In contrast, transgenic mice overexpressing MCH in the lateral hypothalamus show obesity and insulin resistance (Ludwig et al., 2001).

Our group (Shimomura et al., 1999) and four others (Chambers et al., 1999; Saito et al., 1999; Lembo et al.,

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1999; Bächner et al., 1999) identified MCH as the cognate ligand for the orphan G-protein-coupled receptor, SLC-1. This receptor shows significant homology to somatostatin receptors (Kolakowski et al., 1996; Lakaye et al., 1998) and couples to multiple G proteins including G_i, G_o, and G_q (Hawes et al., 2000). SLC-1 is widely expressed in the central nervous system (Hervieu et al., 2000) and its expression is also found to be upregulated in *ob*/*ob* mice and by fasting (Kokkotou et al., 2001). These results suggest that the MCH–SLC-1 axis is a central target of leptin action in the mammalian brain and may play a crucial role in feeding behavior and energy balance. Although the pathophysiological roles of the MCH–SLC-1 axis in humans are presently unknown, the blockade of this signal is an attractive pharmaceutical target for the treatment of obesity.

Here we describe for the first time a novel and orally active MCH receptor antagonist, T-226296. This compound shows functional antagonistic activities in Chinese hamster ovary (CHO) cells expressing the human SLC-1 receptor and inhibits MCH-induced food intake in rats.

2. Materials and methods

2.1. Materials

An in-house chemical compound library specially synthesized for $G_{i/o}$-protein-coupled receptors was screened in a [^{35}S]GTPγS binding assay with the membrane fractions from CHO cells expressing human SLC-1. The chemical modification of a lead compound resulted in the discovery of T-226296, a (−) enantiomer of *N*-[6-(dimethylamino)-methyl]-5,6,7,8-tetrahydro-2-naphthalenyl]-4′-fluoro[1,1′-biphenyl]-4-carboxamide (Fig. 1). For evaluating its antagonistic effect on the SLC-1 receptor, T-226296 was synthesized by Takeda Chemical Industries. MCH, MCH-(4–19), leupeptin, pepstatin, and phosphoramidon were obtained from Peptide Institutes. [^{125}I] (monoiodinated) Bolton–Hunter reagent, [^{35}S]GTPγS, and [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid were purchased from NEN Lifescience Products. A cyclic AMP enzyme immunoassay (EIA) system was obtained from Amersham Pharmacia Biotech. Dulbecco's Modified Eagle Medium (DMEM) and α-Minimum Essential Medium (α-MEM) were purchased from GIBCO BRL. Dialyzed fetal calf serum was obtained from Hyclone. Phosphate-buffered saline (PBS), EDTA, sodium carbonate, $MgCl_2$, NaCl and polyethylenimine were purchased from Wako. Phenylmethylsulfonyl fluoride, 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS), and bovine serum albumin were obtained from Sigma.

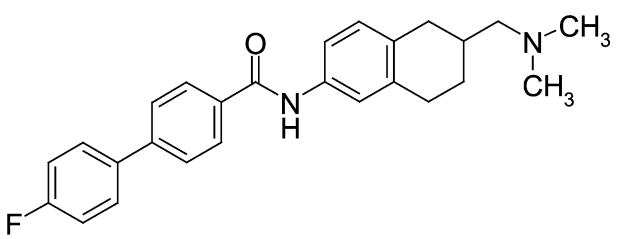

Fig. 1. Chemical structure of T-226296.

2.2. Membrane preparation from CHO cells expressing the SLC-1 receptor

CHO cells expressing the human and rat SLC-1 receptors were established as described previously (Shimomura et al., 1999), and grown in α-MEM medium supplemented with 10% dialyzed fetal calf serum. Cells were dispersed using PBS, pH 7.4 containing 0.2 mM EDTA and suspended in 10 mM sodium carbonate buffer containing 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml phosphoramidon, and 1 μg/ml pepstatin. The cells were then homogenized with a Polytron homogenizer and centrifuged at $1000 \times g$ for 10 min. The supernatants were ultracentrifuged twice at $100{,}000 \times g$ for 60 min. The pellets were then suspended in an assay buffer (25 mM Tris–HCl, pH 7.4 containing 0.1% bovine serum albumin, 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml phosphoramidon, and 1 μg/ml pepstatin) and used as membrane fractions.

2.3. Human SLC-1 GTPγS binding assay

The human SLC-1 membrane fraction (4 μg protein for each assay) was suspended in the GTPγS assay buffer (50 mM Tris–HCl, pH 7.5 containing 0.1% bovine serum albumin, 1 mM EDTA, 10 mM $MgCl_2$, 100 mM NaCl, 1 μM GDP, 0.25 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml phosphoramidon, and 1 μg/ml pepstatin) and incubated with 0.33 nM [^{35}S]GTPγS and 0.3 nM MCH in the presence of the test compound at various concentrations. The mixture was incubated for 60 min at room temperature and then filtered onto GF/C plates. After washing three times with 300 μl of 50 mM Tris–HCl buffer, pH 7.4, the plates were dried for 1 h at 37 °C. The radioactivity retained in the plates was determined with a Topcount scintillation counter (Packard). The concentrations of T-226296 causing 50% inhibition of the specific binding (IC_{50} value) were derived by fitting the data into a pseudo-Hill equation: $\log [\%SB/(100 - \%SB)] = n[\log (C) - \log (IC_{50})]$, where %SB is specific binding, n is a pseudo-Hill constant, and C is the concentration of the test compound.

2.4. Preparation of [^{125}I]MCH-(4–19)

We have developed a novel MCH radioligand, [^{125}I] Bolton–Hunter–MCH-(4–19), for investigating the binding affinity of T-226296 to SLC-1. The preparation of [^{125}I]MCH-(4–19) and its characterization will be described elsewhere. Briefly, MCH-(4–19) was reacted with [^{125}I] (monoiodinated) Bolton–Hunter reagent in 50 mM borate

bufffer (pH 8) for 2 h at 37 °C. The reaction mixture was separated by reverse-phase high performance liquid chromatography with a TSK gel ODS-80T_M column (4.6 × 250 mm, Tosoh) using a gradient of acetonitrile from 20% to 60% for 35 min at a flow rate of 1 ml/min. The major iodinated peak was collected and diluted with the binding assay buffer containing 0.5% CHAPS. The dissociation constant of [^{125}I]MCH-(4–19) for the membranous human SLC-1 was found to be 20.6 ± 0.6 pM.

2.5. MCH receptor-binding assays

Binding assays were performed in 96-well plates. The membrane fraction (0.2 μg protein for each assay) dissolved in the assay buffer containing 0.0625% CHAPS was incubated with 25 pM [^{125}I]MCH-(4–19) and various concentrations of T-226296 at room temperature for 60 min. Nonspecific binding was defined as [^{125}I]MCH-(4–19) binding in the presence of 1 μM MCH. The binding reaction was terminated by rapid filtration through GF/C glass filter plates pre-soaked in 0.2% polyethylenimine and 0.02% bovine serum albumin, followed by washing three times with 300 μl of 50 mM Tris–HCl, pH 7.5. The radioactivity retained in the filters was determined with a Topcount scintillation counter. IC_{50} values calculated as described above are expressed as the means ± S.E.M. of three independent determinations.

The receptor-binding assay for human SLT, the second subtype of the MCH receptor identified very recently (Mori et al., 2001; Hill et al., 2001; Sailer et al., 2001; An et al., 2001), was performed in a similar manner. To further examine the selectivity of T-226296 for SLC-1 receptors, binding assays for 83 other receptors, 11 ion channels and 6 transporters were carried out with standard protocols (MDS Pharma Services, Taiwan).

2.6. Measurement of arachidonic acid release

To examine the functional antagonistic activity, a cell-based assay for arachidonic acid release was carried out using CHO cells expressing the human SLC-1 receptor. CHO cells expressing the human SLC-1 receptor were plated in 24-well plates at a density of 50,000 cells/well and cultured for 1 day. The cells were incubated with [^{3}H]arachidonic acid (0.2 μCi/well) for 16 h and washed twice with 500 μl of DMEM supplemented with 20 mM HEPES (pH 7.4) and 0.2% bovine serum albumin. The cells were then pre-incubated with T-226296 at various concentrations at 37 °C for 30 min and the reaction was started by the addition of MCH. After incubation for 45 min, the radioactivity in the medium was measured with a liquid scintillation counter.

To measure the potency of the antagonist, the pA_2 values were determined using the equation: pA_2 = log (concentration ratio − 1) − log [B], where concentration ratio is the ratio of the EC_{50} values in the absence and the presence of the antagonist and [B] is the concentration of the antagonist. The pA_2 value is expressed as the means ± S.E.M. of three independent determinations.

2.7. Measurement of intracellular cAMP accumulation

CHO cells expressing the human SLC-1 receptor were plated in 24-well plates at a density of 100,000 cells/well and cultured for 1 day. The cells were washed with reaction buffer (Hanks' balanced salt solution supplemented with 20 mM HEPES, 0.2% bovine serum albumin, and 0.2 mM 3-isobutyl-1-methylxanthine) and pre-incubated with the test compound for 1 h. The cells were then incubated with 0.3 nM MCH and 3 μM forskolin at 37 °C for 30 min, followed by the addition of 20% perchloric acid on ice to stop the reaction. The amount of extracted intracellular cAMP was measured using an enzyme immunoassay kit. The IC_{50} value is expressed as the means ± S.E.M. of four independent determinations.

2.8. Food intake studies

Male Sprague–Dawley rats (10 weeks) were housed in individual cages under controlled temperature (22 ± 2 °C), humidity (55 ± 5%), and a 12-h light/dark cycle. Food and water were available ad libitum. Rats were anesthetized with pentobarbital and a stainless-steel guide cannula was implanted into the right lateral ventricle. The rats were allowed to recover for at least 1 week, and then adapted to the injection procedure.

To investigate the MCH-induced food intake in the rats, MCH (5 μg/5 μl) dissolved in distilled water was injected intracerebroventricularly at 9:30 am. The control rats were injected the same volume of distilled water. Immediately after the MCH injection, pre-weighed food pellets were placed in the cage. The weight of the remaining food pellets was measured 4 h after the feeding and subtracted from the initial weight to calculate the food intake.

The antagonistic activity of T-226296 was evaluated as follows. T-226296 prepared as a 2 mg/ml suspension in 0.5% methylcellulose was orally administered at a dose of 30 mg/kg at 8:30 am. The same amount of methylcellulose was administered to the control rats. MCH dissolved in distilled water (5 μg/5 μl) was injected intracerebroventricularly at 9:30 am and the food intake 4 h after the feeding was measured as described above.

All study procedures were performed in accordance with the institutional guidelines for animal care at Takeda Chemical Industries.

3. Results

3.1. Effects of T-226296 on MCH-stimulated GTPγS binding to the human SLC-1 receptor

In the human SLC-1 GTPγS binding assay, MCH caused a concentration-dependent increase in [^{35}S]GTPγS binding,

with an EC_{50} value of 0.1 nM. T-226296 showed inhibitory activity on 0.3 nM MCH-stimulated [^{35}S]GTPγS binding, with an IC_{50} value of 19 nM. T-226296 had no effect on basal [^{35}S]GTPγS binding at concentrations up to 10 μM, suggesting that it functioned as an antagonist rather than an agonist.

3.2. Effects of T-226296 on [^{125}I]MCH-(4–19) binding to SLC-1

In the binding assay, MCH showed a high affinity for the membrane fractions of human SLC-1, while T-226296 completely displaced the binding of [^{125}I]MCH-(4–19) to human SLC-1, with an IC_{50} value of 5.5 ± 0.12 nM (Fig. 2). Similarly, T-226296 showed a high affinity for the rat SLC-1 receptor, with an IC_{50} value of 8.6 ± 0.32 nM.

To verify the selectivity of T-226296 for the SLC-1 receptor, the compound was examined in other binding assays. T-226296 at 1 μM did not display an appreciable affinity for human SLT, somatostatin (sst_1–sst_5), urotensin II (GPR14), or opioid and opioid-like (Delta opioid peptide, Kappa opioid peptide, Mu opioid peptide and N/OFQ peptide) receptors which exhibit a high degree of homology with SLC-1, or to neuropeptide Y_1, galanin GAL1 or melanocortin (MC_3, MC_4) receptors which are thought to be involved in feeding behavior. T-226296 also did not bind to other G-protein-coupled receptors, ion channels or transporters, with IC_{50} values of less than 0.1 μM in those binding assays.

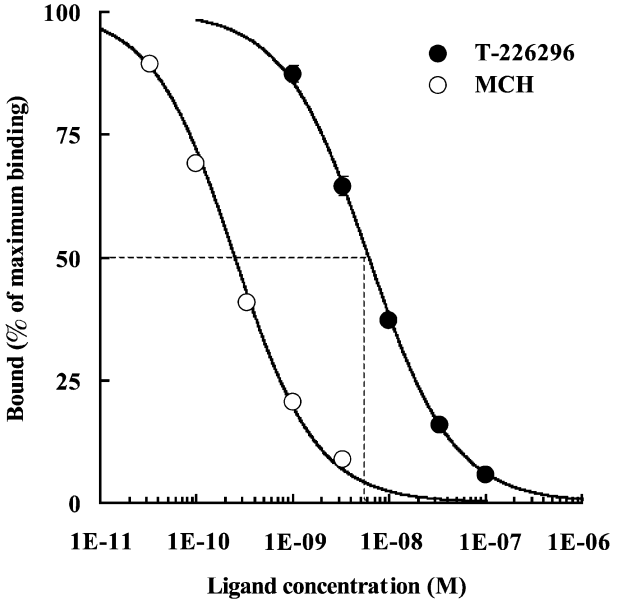

Fig. 2. Effects of T-226296 and MCH on [^{125}I] MCH-(4–19) binding to human SLC-1. The membrane fractions from CHO cells stably expressing human SLC-1 were incubated at room temperature for 1 h with the radiolabeled ligand, [^{125}I] MCH-(4–19), in the presence of various concentrations of T-226296 or MCH. The data shown are representative of three separate experiments with similar results.

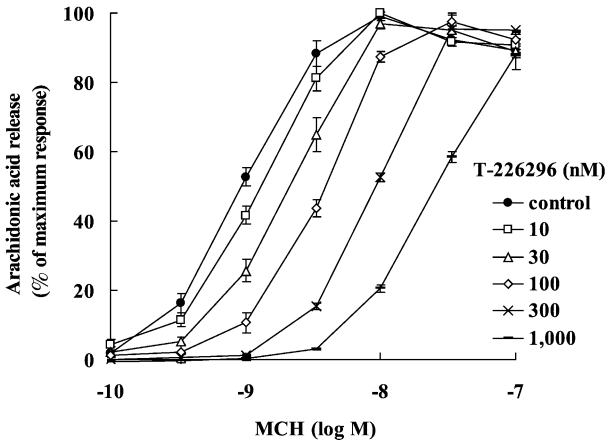

Fig. 3. Inhibition of MCH-stimulated arachidonic acid release by T-226296 in CHO cells expressing human SLC-1. CHO cells expressing human SLC-1 were incubated with [^{3}H]arachidonic acid for 16 h. The cells were washed with serum-free medium and incubated with increasing concentrations of MCH in the presence of T-226296 at 37 °C for 45 min. Radioactivity in the medium was measured with a liquid scintillation counter. The data shown are representative of three separate experiments with similar results. Values are expressed as mean ± S.E.M. (n = 3).

3.3. Effects of T-226296 on arachidonic acid release, cAMP accumulation, and intracellular Ca^{2+}

MCH stimulated the release of arachidonic acid in CHO cells expressing human SLC-1, with an EC_{50} value of 3 nM. T-226296 did not change the basal release rate but significantly inhibited the MCH-induced increase in the release of

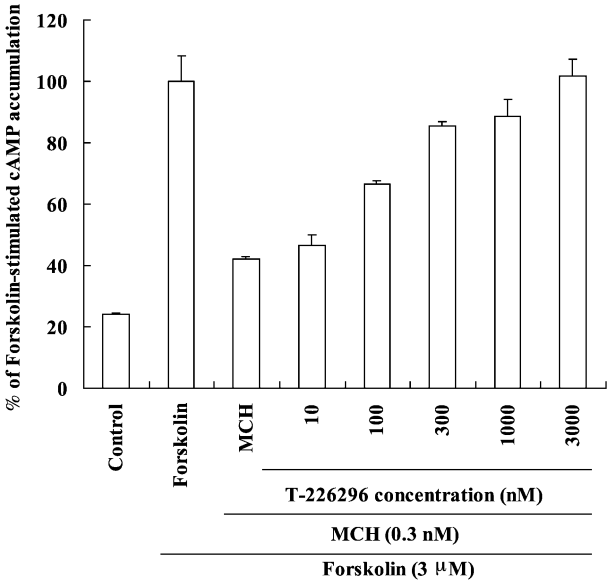

Fig. 4. Inhibition of forskolin-stimulated cAMP accumulation by MCH and its reversal by T-226296 in CHO cells expressing human SLC-1. CHO cells expressing human SLC-1 were incubated at 37 °C for 30 min with 3 μM forskolin, 0.3 nM MCH, and various concentrations of T-226296. Intracellular cAMP was then extracted with 20% perchloric acid and quantified using an enzyme immunoassay kit. The data shown are representative of four separate experiments with similar results. Values are expressed as mean ± S.E.M. (n = 3).

arachidonic acid. The addition of increasing concentrations of T-226296 caused a progressive shift of the MCH concentration–response curve to the right without any effect on maximum response. A Shild plot yielded a pA_2 value of 7.5 ± 0.029 and slope factor of 1.04 ± 0.0025, suggesting that T-226296 behaved as a competitive antagonist against MCH (Fig. 3).

MCH dose-dependently inhibited 3 μM forskolin-stimulated intracellular cAMP accumulation, with an EC_{50} value of 0.2 nM. T-226296 effectively reversed the MCH (0.3 nM)-mediated inhibition in a concentration-dependent manner, with an IC_{50} value of 160 ± 38 nM (Fig. 4). T-226296 at 1 μM did not directly affect either the basal cAMP level or the forskolin-stimulated cAMP accumulation (data not shown).

MCH dose-dependently induced an increase in intracellular Ca^{2+} concentration, with an EC_{50} value of 0.8 nM, while T-226296 inhibited the MCH (1 nM)-induced intracellular Ca^{2+} increase, with an IC_{50} value of approximately 100 nM (data not shown).

3.4. In vivo antagonistic activity of T-226296

Injection of MCH (5 μg) into the lateral ventricle of male Sprague–Dawley rats stimulated food intake about 2.5-fold during 4 h of the early light phase (Fig. 5A). To investigate the effects of T-226296 on MCH-stimulated food intake, T-226296 at a dose of 30 mg/kg was orally administered 1 h before the MCH injection. T-226296 suppressed the MCH-stimulated food intake by more than 90% (Fig. 5A,B) compared with the basal level of food intake in control rats.

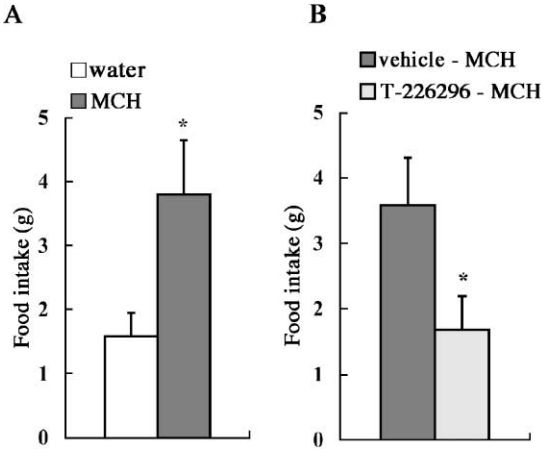

Fig. 5. (A) Effect of intracerebroventricular injection of MCH on food intake. MCH was dissolved in distilled water and injected into the lateral ventricle of Sprague–Dawley rats at 5 μg/5 μl. Food intake was measured for 4 h after injection. Each column represents the mean of nine animals with the S.E.M. $*p < 0.05$ compared with water-injected rats. (B) Effect of T-226296 on MCH-induced food intake. T-226296 (30 mg/kg) was orally administered 1 h before the injection of MCH (5 μg) into the lateral ventricle. After injection, the cumulative food intake was measured for 4 h. Each column represents the mean of eight animals with the S.E.M. $*p < 0.05$ compared with vehicle-administered rats.

4. Discussion

Recently, novel endogenous ligands for the orphan G-protein-coupled receptors, such as prolactin releasing peptide (PrRP), apelin, and galanin-like peptide (GALP), have been identified by applying the 'orphan receptor' strategy (Civelli et al., 2001). The development of specific receptor agonists/antagonists for these receptors provides useful tools for revealing the precise functions of the receptors and may further provide attractive candidates for therapeutic research if the receptor systems are found to be involved in pathological conditions.

Our group and others recently identified MCH as the ligand for the orphan G-protein-coupled receptor, SLC-1. Several lines of evidence suggest that MCH plays an important function in feeding behavior and energy balance and that MCH receptor antagonists could be major potential targets for the treatment of obesity. Although peptide analogs of MCH with moderate antagonistic potency ($K_B = 0.1–0.2$ μM) have been reported (Audinot et al., 2001), a small molecule with good oral bioavailability and brain penetrability has not yet been found.

In the present study, T-226296 effectively inhibited the MCH-induced [^{35}S]GTPγS binding to the membrane fractions from CHO cells expressing the human SLC-1 receptor. In our investigation of the binding affinity of T-226296 to SLC-1, our novel MCH radioligand, [^{125}I]Bolton–Hunter–MCH-(4–19), made it possible to gain a wide range of specific binding to SLC-1 without interacting with other peptides such as rat atrial natriuretic peptide (ANP) (Drozdz et al., 1995). In receptor-binding assays, T-226296 completely inhibited [^{125}I]MCH-(4–19) binding to human SLC-1 and exhibited a similar affinity for rat SLC-1. In our tests for specificity, T-226296 displayed no affinity for any of the other peptide receptors homologous to SLC-1 or those involved in feeding behavior. Furthermore, T-226296 did not bind with IC_{50} values of less than 0.1 μM to other G-protein-coupled receptors, ion channels or transporters, indicating that T-226296 is a potent and selective non-peptide ligand for the SLC-1 receptor.

In SLC-1-transfected CHO cells, MCH was found to inhibit intracellular cAMP accumulation and induce an increase in intracellular Ca^{2+}, suggesting that SLC-1 receptors are coupled to both G_i and G_q proteins (Chambers et al., 1999). Consistent with previous reports, MCH reduced the intracellular cAMP accumulation, with an EC_{50} of 0.2 nM, induced an increase of intracellular Ca^{2+}, with an EC_{50} of 0.8 nM, and also induced arachidonic acid release, with an EC_{50} of 3 nM, in our cell lines. The difference in the EC_{50} values suggests that SLC-1 may couple to G_i several times more efficiently than G_q. T-226296 directly affected neither the basal levels of cAMP and intracellular Ca^{2+} concentrations nor the arachidonic acid release. But T-226296 reversed the MCH-mediated inhibition of intracellular cAMP accumulation, inhibited the MCH-induced intracellular Ca^{2+} increase and inhibited the MCH-induced arach-

idonic acid release, indicating that T-226296 inhibits both G_i- and G_q-mediated signal pathways. These results indicate that T-226296 is a functional antagonist for the SLC-1 receptor and inhibits the multiple intracellular signaling pathways of the MCH–SLC-1 axis.

Oral administration of T-226296 almost completely suppressed the MCH-induced increase in food intake. In our pharmacokinetic study, T-226296 showed good oral bioavailability and brain penetrability, suggesting that orally administered T-226296 directly antagonized the SLC-1 receptor in the rat brain and consequently suppressed food intake. We cannot, however, rule out the possibility that a non-specific anorectic effect of T-226296 makes an additional contribution to the suppression of food intake. Further investigations, especially those in SLC-1-knockout animals, are expected to reveal the specificity of the anorectic effects of T-226296 that were observed in this study.

Quite recently, our group (Mori et al., 2001) and three others (Hill et al., 2001; Sailer et al., 2001; An et al., 2001) identified a second subtype of the human MCH receptor, SLT that exhibits a high degree of homology with the SLC-1 receptor (31.5% identity). The distribution of SLT in the brain was found to nearly overlap that of SLC-1 in humans, although the expression of SLT in the hypothalamus was lower than that of SLC-1. In the present study, T-226296 at 1 μM did not affect the binding of [^{125}I]MCH-(4–19) to SLT, indicating that T-226296 is an antagonist specific to SLC-1. The development of an SLT-specific antagonist will further elucidate the involvement of SLC-1 and SLT in feeding behavior.

Among orexigenic peptides in the hypothalamus such as neuropeptide Y, galanin and orexin, MCH is the only known peptide whose ablation results in leanness (Beck, 2001). Previous reports indicate that the specific antagonists of the orexigenic peptide receptors, such as neuropeptide Y_1, Y_5, and orexin-1 antagonists, significantly inhibit food intake in rodents. However, in contrast to the MCH knockout model, none of the knockout models of these peptides changed the feeding pattern and body weight. Therefore, it may be possible that a specific MCH receptor antagonist like T-226296 is more effective than the other orexigenic peptide receptor antagonists in inhibiting food intake and the treatment of obesity.

In conclusion, we have described for the first time a novel, orally active and selective MCH receptor antagonist, T-226296, that will provide a useful tool for further exploring the physiology of SLC-1 and its therapeutic possibilities.

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