Pharmacological Characterization and Feeding-Suppressive Property of FMS586 [3-(5,6,7,8-Tetrahydro-9-isopropyl-carbazol-3-yl)-1-methyl-1-(2-pyridin-4-yl-ethyl)-urea Hydrochloride], a Novel, Selective, and Orally Active Antagonist for Neuropeptide Y Y5 Receptor

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ABSTRACT

We evaluated the pharmacological profiles of FMS586 [3-(5,6,7,8-tetrahydro-9-isopropyl-carbazol-3-yl)-1-methyl-1-(2-pyridin-4-yl-ethyl)-urea hydrochloride], a novel tetrahydro-carbazole derivative as a neuropeptide Y (NPY) Y5 receptor antagonist. This compound showed a highly selective in vitro affinity for Y5 (IC_{50} = 4.3 ± 0.4 nM) relative to other NPY receptor subtypes like Y1 or Y2. Its binding to Y5 was found to be fully antagonistic from cyclic AMP accumulation assays in human embryonic kidney 293 cells. Pharmacokinetic analysis revealed sufficient oral availability and brain permeability of this compound accompanied with clear dose relation. We attempted to assess the selectivity of FMS586 and, thereby, to infer the physiological role of Y5 in the following feeding experiments in normal rats. An intracerebroventricular injection of NPY and Y5-selective agonist peptide induced acute and robust feeding responses in satiated rats, and prior administration of FMS586 at the doses from 25 to 100 mg/kg clearly inhibited these responses by approximately 55 and 90%, respectively. This compound also showed dose-dependent but transient suppression in natural feeding models of both overnight fasting-induced hyperphagia and spontaneous daily intake. FMS586 did not modulate food intake induced by the topical injection of norepinephrine, galanin, or γ-aminobutyric acid receptor agonist muscimol to the paraventricular nucleus. In addition, we confirmed the Y5-specific activity profile of FMS586 by immunohistochemical analysis. Taken together, we propose not only that our compound potentially expresses specific blockade of central Y5 signals but also that Y5 receptor would certainly contribute to physiological regulation of food intake in normal rats, as suggested from its origin.

Neuropeptide Y (NPY), since its isolation from porcine brain (Tatemoto et al., 1982), has been one of the most primarily focused and extensively studied bioactive peptides. It is a 36-amino acid peptide present in high abundance throughout the mammalian central and peripheral nervous systems (Lundberg et al., 1982; Tatemoto et al., 1982).

Among a wide variety of physiological roles of this peptide is the control of food intake. NPY is the most powerful stimulator of food intake identified so far (Inui, 1999). In rodents, acute central injection of NPY induces a rapid and robust ingestive behavior (Clark et al., 1984; Levine and Morley, 1984; Stanley and Leibowitz, 1984). More importantly, chronic infusion of this peptide into the brain promotes unabated hyperphagia, body weight gain, hyperlipidemia, hyperinsulinemia, and insulin resistance (Beck et al., 1992;}

ABBREVIATIONS: NPY, neuropeptide Y; FMS586, 3-(5,6,7,8-tetrahydro-9-isopropyl-carbazol-3-yl)-1-methyl-1-(2-pyridin-4-yl-ethyl)-urea hydrochloride; L-152,804, 2-(3,3-dimethyl-1-oxo-4H-1H-xanthen-9-yl)-5,5-dimethyl-cyclohexane-1,3-dione; PYY, peptide YY; PVN, paraventricular nucleus; IPVN, intraPVN; PP, pancreatic polypeptide; hPP, [cPP1–7,NPY19–23,Ala31,Aib32,Gln34]-human pancreatic polypeptide; IBMX, 3-isobutyl-1-methyl-xanthine; [^25]IPYY, [^25]iodotyrosyl PYY; NE, norepinephrine; GAL, galanin; MUS, muscimol hydrobromide; SPA, scintillation proximity assay; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; HEK, human embryonic kidney; HTRF, homogeneous time-resolved fluorescence; CNS, central nervous system; aCSF, artificial cerebrospinal fluid.
Zarjevski et al., 1993), which virtually resemble the phenotype of genetically obese/diabetic animals, suggesting the involvement of central activation of NPY system in the pathogenesis of such a metabolic syndrome. One of the richest sources of NPY neurons is found in the arcuate nucleus of hypothalamus, which sends dense projections to the paraventricular nucleus (PVN) and dorsomedial hypothalamus (Bai et al., 1985; Sawchenko et al., 1985). NPY is believed to exert its impact on the control of feeding and energy expenditure via interaction with specific NPY receptors located in both PVN and dorsomedial hypothalamus (Tomaszuk et al., 1996). Thus, it must be adequate for many investigators to have great expectations of the significance of the blockade of NPY receptor signals at hypothalamic level from a standpoint of discovering novel appetite suppressants or drugs for the treatment of metabolic syndrome.

The NPY family of peptides, including peptide YY (PYY) and pancreatic polypeptide (PP) besides NPY, mediates the specificity of their actions through a family of G protein-coupled receptors (Tomaszuk et al., 1996). Five different subtypes, Y1, Y2, Y4, Y5, and Y6, have been cloned from several species (Blomqvist and Herzog, 1997). In particular, Y5 receptor, more recently cloned subtype from rat hypothalamus (Gerald et al., 1996), appears to be a long sought “feeding” receptor of NPY due to the evidence of parallelism in pharmacological study in which in vitro binding affinity for this receptor and feeding response in rats quite highly correlate among NPY agonist peptides (Wyss et al., 1998). Indeed, microinjection of Y5-selective agonist peptides into the brain causes more potent hyperphagic responses than NPY itself (Cabrele et al., 2000). The above findings have encouraged the pharmaceutical industry to develop Y5-selective antagonists as appetite suppressants and antiobesity drugs. Although a large number of reports on pharmacological profile of low-molecular weight Y5 antagonists have been published so far, no clear consensus exists regarding physiological significance of Y5 in natural feeding and exogenous NPY-induced feeding (Daniels et al., 2002; Turnbull et al., 2002; Della-Zuana et al., 2004). As for the effective compounds, there needs to be more intensive assessment of the specificity. At the same time, as for the ineffective compounds, those findings might prove the nature of the target (i.e., minor contribution of Y5 as NPY feeding receptor) or there may be some reasons for those compounds to be bioinactive (such as incomplete brain permeability or nonspecific binding to neuronal or glial proteins and resultant scarce availability at synaptic cleft levels, etc.).

Here, we report the pharmacological profile of the FMS586 tetrahydrocarbazole derivative we optimized in our drug-discovery process as a novel Y5 antagonist and also the selectivity of this compound. We investigated the effect of this compound comprehensively from in vitro studies (receptor binding, functional assay) to in vivo experiments, including exogenous NPY-, selective Y5 agonist-, or overnight fasting-induced hyperphagia and spontaneous daily intake. Moreover, we tried to thoroughly evaluate the selectivity of this compound in other feeding stimulant-induced models, and finally, we intended to settle it by immunohistochemical analysis. On the basis of our findings, we would refer to the validity of FMS586 as an ideal Y5 antagonist and the physiological role of Y5 receptor in the control of food intake.

Materials and Methods

Animals

Male Wistar rats (7 weeks) were purchased from Charles River Japan Inc. (Atsugi, Japan). They were housed on a 12-h light/dark cycle (lights on from 7:00 AM to 7:00 PM) schedule in a temperature- and humidity-controlled room and, 1 or 2 weeks later, used for the following experiments. They were provided with the standard laboratory chow (NMF pellet; Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum, unless otherwise stated. Animal care was performed according to protocols reviewed by the ethical committee for animal experiment in Meiji Seika Pharmaceutical Research Department.

Reagents

FMS586 (Fig. 1) and L-152,804 (Kanatani et al., 2000) were synthesized at Synthetic Organic Chemistry Laboratories of Fuji Photo Film (Kanagawa, Japan). For in vitro assays, FMS586 and L-152,804 were dissolved in dimethyl sulfoxide and further diluted in assay buffer. FMS586 was dissolved in distilled water and given rats in the following in vivo experiments. Human/rat neuropeptide Y (NPY), rat galanin (GAL), and leupeptin were obtained from Peptide Institute Inc. (Osaka, Japan). [cPP1–7,NPY19–23,Ala31,Aib32,Gln34]-human pancreatic polypeptide (hPP), 3-isobutyl-1-methyl-xanthine (IBMX), and bacitracin were from Nacalai Tesque (Kyoto, Japan). Human NPY, norepinephrine (NE), muscimol hydrobromide (MUS), and buprenorphine hydrochloride were from Sigma-Aldrich (St. Louis, MO). Porcine [125I]iodotyrosyl PYY (148 TBq/mmol) and wheat germ agglutinin-scintillation proximity assay (SPA) beads were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Dulbecco’s modified Eagle’s medium (DMEM), I-PL-41 medium, and Ham’s F12 medium were from Invitrogen (Carlsbad, CA). Homogeneous time-resolved fluorescence (HTRF) cAMP assay kit was from Nihon Schering K.K. (Tokyo, Japan). All of the other chemicals used were of analytical grade.

Cell Culture

SK-N-MC human neuroblastoma cell line, KAN-TS human neuroblastoma cell line, and HEK 293 cell line were obtained from American Tissue Culture Collection (Manassas, VA). High Five insect cells were cultured at 37°C with 95% air and 5% CO2 in Ham’s F12 medium and minimum essential medium with nonessential amino acid (1:1) containing 15% fetal bovine serum, 100 μ/ml penicillin, and 50 μg/ml streptomycin (Meiji Seika, Tokyo, Japan). KAN-TS cells were routinely maintained at 37°C with 95% air and 5% CO2 in Ham’s F12 medium and minimum essential medium with nonessential amino acid (1:1) containing 15% fetal bovine serum, 100 μ/ml penicillin, and 50 μg/ml streptomycin. HER 293 cells expressing recombinant human Y5 receptors, prepared by Meiji Seika, were routinely maintained at 37°C with 95% air and 5% CO2 in DMEM containing 10% fetal bovine serum, 100 μ/ml penicillin, and 50 μg/ml streptomycin. HER 293 cells expressing recombinant human Y5 receptors, prepared by Meiji Seika, were routinely maintained at 37°C in DMEM containing 10% fetal bovine serum, 100 μ/ml penicillin, 50 μ/ml streptomycin, and 1000 μg/ml G418 (Meiji Seika). High Five cells were routinely maintained at 27°C in I-PL-41 medium with 10% fetal bovine serum, 2.6 mg/ml tryptose phosphate broth, 0.1% Phloric F-68, 50 μ/ml penicillin, and 25 μg/ml streptomycin.

Fig. 1. Chemical structure of a novel Y5 antagonist, FMS586.
Binding Experiments for Y1, Y2, and Y5 Receptors with [125I]PYY

Each membrane preparation of SK-N-MC cells, KAN-TS cells, and High Five cells expressing recombinant human Y5 receptors was used as Y1, Y2, and Y5 receptors, respectively. Each cell line was homogenized in 50 mM HEPES buffer (pH 7.4) containing 1 mM MgCl₂, 2.5 mM CaCl₂, and 15% glycerol and centrifuged at 40,000g for 20 min at 4°C. The pellets were resuspended in the same buffer and stored at −80°C until use. Binding of [125I]PYY to membrane preparations was performed by SPA method. In brief, 10 μl of each samples, 50 μl of membrane preparation (0.4 mg of protein/ml for Y1 and Y2, 1.0 mg of protein/ml for Y5), 40 μl of [125I]PYY, and 100 μl of WGA-SPA beads (10 mg/ml) diluted in 50 mM HEPES buffer (pH 7.4) containing 1 mM MgCl₂, 2.5 mM CaCl₂, 0.25 mg/ml bacitracin, 10 μg/ml leupeptin, and 0.1% bovine serum albumin were mixed and incubated at room temperature overnight (Y1 and Y2) or for 5 h (Y5). After the incubation, the radioactivity on the plate was measured using a TopCount96 (PerkinElmer, Wellesley, MA). Specific binding was defined as the difference between total binding and nonspecific binding in the presence of 500 ng/ml PYY. IC50 values were expressed as mean ± S.E.M. of three independent experiments.

Measurement of Intracellular cAMP Concentration

HEK 293 cells expressing recombinant human Y5 receptors were harvested and plated at 1 × 10⁴ cells/well/50 μl on 96-well plate with DMEM containing 100 μM IBMX (DMEM/IBMX). Varying concentrations of FMS586 (12.5 μl in DMEM/IBMX) and either NPY in 12.5 μl of DMEM/IBMX (final 50 nM as an antagonist format) or 12.5 μl of DMEM/IBMX (as an agonist format) were added to each well and incubated for 10 min at 37°C. Forskolin in 25 μl of DMEM/IBMX (final 10 μM) then was added. After 10 min of incubation at 37°C, 100 μl of 1% Triton X-100 solution were added to each well. Fifty microliters of the cell lysate in each well was transferred to 96-well plate. The concentration of cAMP was measured using HTRF cAMP assay kit with RUBYstar (BMG Labtech, Offenburg, Germany) for 20 min at 4°C. The pellets were resuspended in the same buffer and stored at −80°C until the analysis. Once thawed, samples were homogenized in 3 ml of 1% Triton X-100 solution were added to each well. Fifty microliters of the cell lysate in each well was transferred to 96-well plate. The concentration of cAMP was measured using HTRF cAMP assay kit with RUBYstar (BMG Labtech, Offenburg, Germany). IC50 values were expressed as mean ± S.E.M. of three independent experiments.

Pharmacokinetic Analysis

Rats were fasted overnight and orally given FMS586 at the doses of 25 and 50 mg/kg. At 0.5, 1, 2, and 4 h after the injection, plasma and brain samples were prepared. Under pentobarbital (50 mg/kg i.p.; Dainippon, Osaka, Japan) anesthesia, heparinized blood was withdrawn from the inferior vena cava and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was extracted in 10 volumes of methanol. Immediately after the blood sampling, whole brain was withdrawn from inferior vena cava and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was extracted in 10 volumes of methanol. The amounts of FMS586 in plasma and brain were measured by liquid chromatography/mass spectrometry (Agilent Technology, Palo Alto, CA).

Acute Hyperphagia Models

Surgery. Rats were used for hyperphagic models induced by the administration of several orexigenic substances. In NPY- or Y5-selective agonist hPP-induced hyperphagia, these peptides were intracerebroventricularly injected. Rats were anesthetized with pentobarbital (50 mg/kg i.p.) and implanted with a permanent 25-gauge stainless steel guide cannula (Eicom, Kyoto, Japan). The stereotaxic coordinates were 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 7.8 mm ventral from the skull surface according to Paxinos and Watson (1986). After the surgery, all of these rats were housed individually, injected with ampicillin (50 mg/kg i.m.; Meiji Seika) and buprenorphine (0.15 mg/kg s.c.) as analgesics for successive two days, handled everyday, and allowed to recover for more than 7 days.

Experimental Protocols. The doses of individual feeding inducers were decided as follows: NPY (300 pmol i.c.v.), hPP (100 pmol i.c.v.), NE [40 nmol of intrapVN (iPVN)], GAL (1 nmol of iPVN), or MUS (500 pmol of iPVN). These were in the submaximal range for each substance (N. Kakui and J. Tanaka, unpublished data), and initial feeding responses at 1 h were set to be almost within 3 to 5 g per rat. Acute experiments were conducted in the light phase from 10:00 AM to 4:00 PM. On the day of the experiment, rats were orally given FMS586 at the doses of 25, 50, or 100 mg/kg or its vehicle (0.1 mM HCl as an acidity-matched control). One hour later, each feeding inducer or artificial cerebrospinal fluid (aCSF) was infused in a volume of 10 (i.c.v.) or 2 μl (iPVN) with a Hamilton gas-tight syringe (Renon, NE) and a microinfusion pump (Eicom) over 1 min to ensure an accurate and constant volume of delivery. Immediately after the infusion, rats were returned to their home cages and given preweighed amounts of food pellets. Pooled intakes were monitored at 1, 2, and 4 h after the infusion. After the overall experiments, cannula positions were assessed by the injection of 0.5% Evans blue dye, and the animals showing precise placement were included in individual data.

Fasting-Induced Hyperphagic Model. Intact Wistar rats were fasted overnight and given FMS586 at the doses of 25, 50, or 100 mg/kg or its vehicle. One hour later, they were provided with preweighed food pellets, and cumulative feeding responses were measured at 1, 2, and 4 h after refeeding.

Chronic Administration Experiment. Intact Wistar rats were evenly divided into four groups based on their basal daily food intake and body weight monitored for 3 days beforehand. Rats were orally given FMS586 at the doses of 25, 50, or 100 mg/kg or its vehicle once a day at 5:00 PM for 3 days. Daily food intake and body weight gain were measured.

Immunohistochemistry

c-Fos and Y5 Immunohistochemistry. Rats were orally given FMS586 (50 mg/kg) or its vehicle and, 1 h later, given i.c.v. injection of hPP (100 pmol) or aCSF as described above. Their feeding responses were monitored 2 h postinjection. Rats then were sacrificed under pentobarbital (50 mg/kg i.p.) anesthesia. Whole brain was immediately perfused via aorta with ice-cold saline followed by ice-cold 4% paraformaldehyde in sodium phosphate buffer (pH 7.4). The brains were removed and postfixed in 4% paraformaldehyde buffer overnight. Equivalent 30-μm hypothalamic sections from each brain were cut on a vibratome (Series 1000; Technical Products International Inc., St. Louis, MO). Free-floating sections were washed three times in PBS and blocked for 1 h at room temperature with PBS containing 0.3% Triton X-100, 2% fetal calf serum, and 0.02% sodium azide. The sections were incubated with a sheep anti-c-Fos polyclonal IgG antibody (1:1000; α-Genosys, The Woodlands, TX) and a goat anti-Y5 polyclonal IgG antibody (anti-Y5R, N-20, 1:400; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2 h at 4°C. The sections were washed five times in PBS and stained with an Alexa 488-labeled donkey anti-sheep polyclonal IgG antibody (1:1000; Molecular Probes Inc., Eugene, OR) and an Alexa 555-labeled donkey anti-goat polyclonal IgG antibody (1:1000; Molecular Probes Inc.) for 5 h at room temperature in the dark. After several rinses in PBS, the sections were mounted on slides, coverslipped with Vectashield
mounting medium (Vector Laboratories Inc., Burlingame, CA), and stored in the dark.

**Confocal Laser Microscopy.** Confocal laser microscopy was used to analyze the double-label immunofluorescence images. LSM 510 confocal system (Carl Zeiss Corp., Oberkochen, Germany) consisted of an Axiovert 200M microscope equipped with an argon laser-producing light at 488 nm (for visualization of Alexa 488) and a HeNe laser-producing light at 543 nm (for visualization of Alexa 555). An objective (Plan Neofluar 20×) was used to scan the images. For each experiment, fluorescence signals were checked individually for bleed-through to the opposing detector. All of the bleed-through was eliminated by adjusting laser intensity and detector window width. A series of continuous optical sections at 3-µm intervals along the z-axis of the tissue section were scanned for each fluorescent signal. The signals were obtained for each fluorophore on one series of optical sections and stored separately as a series of 512 × 512 pixel images. The images were then processed with the LSM 5 Image Browser (Carl Zeiss Corp.). The confocal images were presented as projections of a stack of optical slices. The brightness and contrast of the images were adjusted in Photoshop (Adobe Systems Inc., San Jose, CA).

**Statistical Analysis.** All of the results were represented as mean ± S.E.M. Statistical significance of the differences among multiple groups was tested using a one-way analysis of variance followed by Dunnett’s multiple comparison test. A two-tailed Student’s t test was used to evaluate the difference between the two experimental groups. Values associated with a p value of <0.05 were considered significant.

**Results**

**Receptor Binding Assay.** In the binding assay, PYY showed a high affinity (1.0 ± 0.1 nM for [125I]PYY binding) for the membrane fractions expressing human Y5 receptors. FMS586 (Fig. 1) displaced the binding of [125I]PYY to Y5 receptors with an IC50 value of 4.3 ± 0.4 nM (Table 1). FMS586 did not show any inhibitory effects on the specific binding of [125I]PYY to Y1 and Y2 receptors at the concentration of 10 µM (Table 1). L-152,804, used only in receptor binding assay as a reference compound, also showed Y5-selective binding affinity (Table 1).

**Functional Assay.** Agonist/antagonist assay of FMS586 was performed by measuring cAMP concentration in HEK 293 cells expressing recombinant human Y5 receptors. NPY concentration-dependently (data showing only at the concentration of 50 nM) inhibited forskolin-induced (10 µM) intracellular cAMP accumulation through Gt-coupled Y5 receptor. In an agonist format, FMS586 by itself did not affect the forskolin-induced cAMP accumulation at the concentrations ranging from 3 to 1000 nM (Fig. 2). Meanwhile, FMS586 reversed NPY-dependent (50 nM) inhibition of cAMP accumulation in a concentration-dependent manner with an IC50 value of 65 ± 23 nM in an antagonist format (Fig. 2). These results suggest that FMS586 is a full antagonist for Y5 receptor.

**Pharmacokinetic Studies.** Pharmacokinetic analysis of FMS586 clearly indicated the property of sufficient gut absorption and blood-brain barrier permeability of this compound. At both of the doses, plasma and brain concentrations of FMS586 reached their maximal levels almost within 1 h in a dose-dependent manner and remained stable (50 mg/kg) or gradually declined (25 mg/kg) for up to 4 h (Fig. 3). Maximal concentrations were 10.76 ± 1.49 µg/ml in plasma and 1.57 ± 0.19 µg/g in brain at 25 mg/kg and 15.16 ± 1.78 µg/ml in plasma and 3.12 ± 0.62 µg/g in brain at 50 mg/kg (Fig. 3).

**Feeding Experiments.** We investigated the feeding-suppressive profile of FMS586 in a variety of experimental models. In the following series of feeding experiments, rats showed no abnormality in gross behaviors that might be linked to hypophagia-like sedation, hypothermia, or hypo/hyperlocomotion at the doses of up to 100 mg/kg FMS586 (data not shown). A basal intake measured for 1 h after the oral injection of three doses of FMS586 or its vehicle was comparable among all groups (approximately 0.6–0.8 g/rat; data not shown). An i.c.v. injection of NPY (300 pmol) caused a significant increase in food intake (at 1 h, 3.06 ± 0.40 g in vehicle group and 0.18 ± 0.16 g in aCSF group, respectively, p < 0.001; Fig. 4A). Treatment with FMS586 showed dose-dependent but partial suppression of NPY-induced food intake. The maximal inhibitory effects (approximately 55 to 60% of vehicle levels) were obtained at 1 h in both 50 and 100 mg/kg groups (p < 0.01; Fig. 4A). The cumulative inhibitory effect of FMS586 continued still at 2 h (at 100 mg/kg, p < 0.05; Fig. 4B), but it did not reach statistical significance at 4 h (Fig. 4C).

An i.c.v. injection of Y5-selective agonist hPP (100 pmol)
induced a significant and robust feeding response (4.92 ±
0.90 g at 1 h, p < 0.001 versus aCSF group) (Fig. 5A). Almost complete inhibition by FMS586 was observed for up to 4 h in all of the treated groups (p < 0.001 versus vehicle group; Fig. 5, A–C). In our pilot study, the suppressive effect of this compound on hPP-induced feeding decreased by approximately 50% at 12.5 mg/kg (data not shown).

In fasting-induced hyperphagia, FMS586 exhibited dose-dependent and transient inhibitory effect. Significant inhibition was observed only at 50 mg/kg (28.2% of vehicle group, p < 0.05) and 100 mg/kg (36.8% of vehicle group, p < 0.01) (Fig. 6A). After this time point, any significant differences of feeding responses among vehicle- and FMS586-treated groups were not detected (Fig. 6, B and C). At 4 h after refeeding, inhibitory effect of FMS586 disappeared (Fig. 6C).

Repeated injection of FMS586 to normal rats also caused dose-dependent inhibition of spontaneous daily food intake and body weight (Fig. 7). At 100 mg/kg, significant inhibition of cumulative food intake was detected at day 1 (29.3% of vehicle group, p < 0.001) and also at day 3 (11.2% of vehicle group, p < 0.05), but inhibitory effect was gradually attenuated (Fig. 7A). Inhibition of body weight gain was significant only at 100 mg/kg at day 1 (p < 0.05 versus vehicle group) (Fig. 7B).

To further evaluate the selectivity of in vivo actions of FMS586, we applied this compound to another set of specific...
inducer-dependent hyperphagia models. As shown in Fig. 8, the feeding responses stimulated by the topical injection of NE (40 nmol), GAL (1 nmol), or MUS, GABA\(_A\) agonist (500 pmol) into the PVN were not substantially affected by the treatment with 100 mg/kg FMS586 (irrespective of time after each inducer or dose of FMS586; data not shown), thus suggesting that this compound is a highly selective Y5 receptor antagonist.

**Immunohistochemical Analysis.** We also thoroughly examined the in vivo selectivity of FMS586 by immunohistochemical approach besides evaluation of ingestive behaviors. We analyzed simultaneously the immunofluorescence signals of both c-Fos expression (as a marker of neuronal activation) and Y5 localization in the PVN. Y5-positive cells were detected extensively from all slice samples (Fig. 9, A–D). An i.c.v. injection of hPP caused strong c-Fos expression (Fig. 9C) compared with aCSF group (Fig. 9A), and these c-Fos signals mostly overlapped with Y5-positive cells (Fig. 9C). On the other hand, FMS586 alone induced no apparent changes.

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**Pharmacological Profile of Novel Y5 Antagonist FMS586**

Fig. 5. Complete inhibitory effect of FMS586 on Y5 agonist peptide, hPP-induced hyperphagia, in satiated Wistar rats. Rats were orally given FMS586 at the doses of 25, 50, or 100 mg/kg or its vehicle. One hour later, rats were given hPP (100 pmol i.c.v.), and food intake was monitored at 1 (A), 2 (B), and 4 h after the i.c.v. injection. Time course of changes in feeding responses is shown in C. Data are represented as mean ± S.E.M. (n = 6–8). ###, p < 0.001 and ###, p < 0.001, significantly different from the values in aCSF group and vehicle group, respectively.

Fig. 6. Transient and modest inhibitory effect of FMS586 on fasting-induced hyperphagia in Wistar rats. Rats were fasted overnight and orally given FMS586 at the doses of 25, 50, or 100 mg/kg or its vehicle. One hour later, rats were refed and food intake was monitored at 1 (A), 2 (B), and 4 h after the refeeding. Time course of changes in feeding responses is shown in C. Data are represented as mean ± S.E.M. (n = 6). ##, p < 0.01 and #, p < 0.05, **, p < 0.01, significantly different from the values in nonfasted group and vehicle group, respectively.
in c-Fos expression (Fig. 9B), but it completely suppressed hPP-induced c-Fos signals shown in Y5-positive cells (Fig. 9D). The quantitative analysis showed the number of double-positive cells (c-Fos and Y5) counted in the lateral PVN area (approximately 0.4 mm²) from at least 10 slices of the animals (n = 3) significantly increased by the treatment of hPP (79.7 ± 6.2, \( p < 0.01 \)) compared with the aCSF group (14.0 ± 2.9). The number of these cells in aCSF-FMS586 (13.3 ± 3.1) and hPP-FMS586 (3.3 ± 0.9) group was almost the same as or below the levels in aCSF group.

**Discussion**

We evaluated the pharmacological profile of a novel tetrahydrocarbazole derivative, FMS586, as Y5 antagonist. FMS586 was shown to be a potent and selective Y5 antagonist from in vitro studies, and this was also confirmed in a series of in vivo hyperphagia models. This compound exhibited satisfactory brain permeability via the oral administration and clear suppressive effects on NPY- and hPP-induced feeding responses that appeared to be specific for Y5 antagonism. This compound also displayed inhibitory effects on natural feeding behaviors both in overnight fasting-induced feeding and spontaneous daily intake that were transient and reversible. Furthermore, by the immunohistochemical analysis, we found this compound showing quite a specific action for Y5-positive neurons in hypothalamus.

FMS586 (Fig. 1) is the novel and patented compound that we optimized through structure-activity relationship analysis of aminocarbazole lead by our high-throughput screening campaign. This compound showed a highly selective binding affinity to Y5 relative to Y1 and Y2 (Table 1) and proved to be a full antagonist from functional assays measuring cAMP levels (Fig. 2). One of our lead compounds, 3-aminocarbazole, has been known for the structure-dependent mutagenic ac-
tivity in the Ames test (LaVoie et al., 1981). During our optimization process, we sought to clear this problem and finally succeeded in finding out FMS586 by the partial reduction of the carbazole nucleus (Fig. 1). This compound was found to be selective in a panel assay against a wide range of receptors and enzymes, including feeding-related molecules like monoamines and neuropeptides at concentrations up to 10 \( \mu \)M (from MDS Pharma, Taipei, Taiwan and Cerep, Paris, France; data not shown). This compound was highly water-soluble (at up to 100 mg/ml), and its bioavailability was also sufficient (approximately 60% in-house; data not shown). Together with its brain permeability (Fig. 3), we consider this compound possessing an appropriate property as a central nervous system (CNS) drug.

FMS586 dose-dependently suppressed NPY-induced food intake with its maximal inhibition of approximately 50 to 60% of vehicle response (Fig. 4). This compound also exhibited almost complete inhibition of hyperphagia induced by hPP, a selective Y5 agonist peptide (Cabrele et al., 2000), at the dose of 25 mg/kg (Fig. 5). The Y1 and Y5 receptors have been implicated in mediating the appetite-stimulating activity of NPY (Inui, 1999; Raposoinho et al., 2004), but it has been very difficult to precisely establish the relative physiological contribution of each receptor. In some knockout mice studies, we should be aware of the biological counter-responses that probably may occur in the hypothalamus where extremely redundant neural networks exist (Beck, 2001). With reference to the specific Y5 antagonists, there is a variety of results on their effects on NPY-induced hyperphagia from positive (Crisicone et al., 1998; Daniels et al., 2002; Della-Zuana et al., 2004) to negative ones (Kanatani et al., 2000; Turnbull et al., 2002), for some of which the specificity of the compounds is doubtful (Crisicone et al., 1998; Della-Zuana et al., 2004). Our data showing partial inhibition in NPY model and complete inhibition in hPP model strongly suggest that orally administered FMS586 fulfills the prerequisite condition of central Y5 blockade. These features are common in some of our other tetrahydrocarbazole derivatives, so we interpret the effect of FMS586 as not peculiar to this compound but rather target-dependent (data not shown).

FMS586 also revealed dose-dependent and transient inhibition of fasting-induced hyperphagia (Fig. 6). Maximal inhibition (approximately 37% of vehicle group) was observed at 1 h, but the effect gradually disappeared within 4 h in spite of preservation of the drug concentration in brain (Fig. 3), even at this time. Repeated administration of this compound to normal rats also dose-dependently and transiently decreased their spontaneous daily food intake and body weight gain (Fig. 7). These results suggest that, under both fasted and nonfasted conditions, Y5-mediated component would have a certain contribution in natural feeding in normal rats, at least in early time, and the influence of Y5 blockade by the treatment with FMS586 would be gradually compensated by up-regulation of other orexigenic systems. Given that Y5 receptors are susceptible to such compensatory mechanisms, this interpretation will be compatible with a general understanding of quite a normal and rather late-onset obese phenotype of Y5 knockout mice (Marsh et al., 1998). The involvement of Y5 receptor in the regulation of natural feeding also does not appear to reach a general consensus. The repeated treatment of normal rats with antisense oligonucleotides for Y5 reduced cumulative spontaneous food intake by 32% and resultant hypothalamic Y5 protein expression by 50% without any modification of water intake levels or Y2 receptor expression (Campbell et al., 2001). According to Dube et al. (1994), central infusion of NPY antibodies to normal rats suppressed cumulative food intake by 40 to 60% relative to the control group. Other than NPY, there is a multitude of endogenous orexigenic substances like NE, GAL, melanin-concentrating hormone, GABA, opioids, or orexins (Kalra et al., 1999). Therefore, a compound having specific antagonistic activity for one NPY receptor subtype would not be expected to show suppression of natural feeding by more than 50% without any side effects (Crisicone et al., 1998; Bannon et al., 2000). From this quantitative point of view, it might be possible to consider our data reflecting the physiological role of Y5 receptor.

Furthermore, we investigated a sufficient condition for Y5 specificity of FMS586. We focused on several orexigenic substances, such as NE, GAL, and MUS, in terms of the following evidence regarding neuronal interactions with NPY system. NE-containing neurons projecting from brain stem to PVN are, at least in part, colocalized with NPY (Evert et al., 1984; Sawchenko et al., 1985), and possible antagonistic interaction between NE and NPY is also suggested (Kyrkouli et al., 1990). From morphological observation, GAL neurons are demonstrated to show synaptic contact with NPY neurons (Horvath et al., 1996). In a perfusion experiment of cultured hypothalamic neurons, GAL concentration-dependently stimulated NPY release (Bergonzelli et al., 2001). In addition, as for GABA, morphological study proved its existence in NPY-immunopositive axons and axon terminals within magnocellular and parvocellular PVN (Pu et al., 1999), and this study argued for an interaction between NPY and GAL in the stimulation of feeding. Therefore, we investigated the feeding responses induced by the topical injection of NE, GAL, or MUS into PVN and found out that FMS586 did not modulate these ingestive behaviors, even at the highest dose (100 mg/kg) (Fig. 8). This is strongly suggestive of the specificity of FMS586 in feeding suppression. In addition, taking into account that this compound does not bind receptors of NE, GAL, or GABA (MDS Pharma and Cerep Screen; data not shown), here we can raise the possibility of an independent relationship between Y5 and these feeding systems.

Immunohistochemical analysis by confocal laser microscopy also supported the Y5 specificity of our compound. FMS586 by itself did not induce any c-Fos expression (Fig. 9B) but completely inhibited hPP-induced c-Fos expression that was mainly detected in Y5-positive cells (Fig. 9, C” and D”). Other investigators referred to the necessity of assessment employing Y5 knockout mice to ascertain the specificity of any Y5 antagonists (Della-Zuana et al., 2004). There seems to be two aspects of interpretation in these genetic manipulation studies, e.g., clear assessment of the target dependence of test compounds (absolute merit) and unclear side effects of the compounds that might occur in response to the changes in background conditions of the animals (presumable demerit). At present, we do not possess any knockout mice data, mainly because of the problem of their availability. However, totally considering our series of data from in vitro to in vivo studies, we convincingly believe that FMS586 is a selective and bioactive Y5 antagonist.

Several critical roles of Y5 receptors in CNS, including not only regulation of food intake and energy metabolism (Hwa
et al., 1999; Henry et al., 2005) but also pituitary hormone secretion (Raposinho et al., 1999) and seizure/epilepsy (Marsh et al., 1999), have been widely recognized. So far, pharmacological profiles of Y5 antagonists as an antiobesity drug have actually shown disappointing results. However, to explore unknown Y5 functions, we are sure to need selective and biologically active Y5 antagonists with low-molecular weight as a tool of research. On that point, our compound showed specific blockade of CNS Y5 signals and thus proved to be an ideal Y5 antagonist.

In summary, we identified a novel tetrahydrocarbazole derivative, FMS586, and verified its pharmacological profile as a Y5 antagonist from diverse approaches. This compound showed specific blockade of CNS Y5 signals and thus proved to be an ideal Y5 antagonist.

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References


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