Pharmacological Evaluation of GPR40 Full Agonists in Metabolic Disease Models

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Pharmacological Evaluation of GPR40 Full Agonists in Metabolic Disease Models

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Abstract

GPR40/FFA1 receptor is a G-protein-coupled receptor expressed in the pancreatic islets and enteroendocrine cells, and its stimulation enhances insulin and incretin secretion. Since these hormones contribute to glycemic control and feeding regulation, GPR40 agonists have a potential to become a novel agent for the treatment of diabetes and obesity. Here, I report the pharmacological profiles of SCO-267, a newly-synthesized GPR40 full agonist. In addition, I introduce the contribution of afferent vagal nerves regarding GPR40 full agonism-derived feeding suppression, detailed mechanisms of which are still unknown.

In the first chapter, I conducted the *in vitro/in vivo* characterization of SCO-267. SCO-267 activated G_q signaling in both high- and low-*FFAR1*-expressing Chinese hamster ovary (CHO) cells, stimulated insulin secretion in MIN6 cells, and induced glucagon-like peptide-1 (GLP-1) release in GLUTag cells. When administered to normal rats, SCO-267 increased insulin, glucagon, GLP-1, glucose-dependent insulinotropic polypeptide, and peptide YY secretions. In single and 2-week dosing studies using diabetic N-STZ-1.5 rats, SCO-267 was highly effective in improving glucose tolerance with lower plasma exposure compared with fasiglifam, a partial agonist. Diet-induced obese (DIO) rats treated with SCO-267 for 2 weeks decreased food intake and body weight. These results show the full agonistic property of SCO-267 against GPR40 and suggest the therapeutic potential of SCO-267 for the treatment of diabetes and obesity.

In the second chapter, I tried a mechanism analysis of GPR40-mediated feeding suppression using T-3601386, another compound with potent full agonistic activity for GPR40. As was the case with SCO-267, T-3601386 showed Ca^{2+} mobilization in *FFAR1*-expressing CHO cells, *in vitro/in vivo* GLP-1 secretory capacity and body weight

reduction in DIO rats, indicating full agonistic properties of T-3601386 against GPR40. Immunohistochemical analysis demonstrated that T-3601386 increased the number of c-Fos positive cells in the nuclei of the solitary tract (NTS), which receives vagally-mediated signals. Incretin secretion, feeding suppression, weight loss and NTS activation induced by T-3601386 were completely abolished in *Ffar1*$^{-/-}$ mice. Two models with vagal nerve blockade counteracted the feeding suppression and weight loss induced by the administration of T-3601386. These results suggest that T-3601386-induced feeding suppression and NTS activation is dependent on GPR40, and that intact vagal afferents are required for the feeding suppression through GPR40.

In conclusion, SCO-267 is highly effective in improving diabetes and obesity and may induce similar favorable effects in patients with metabolic disease. Moreover, my novel findings raise the possibility that GPR40 full agonist can induce periphery-derived weight reduction, which may provide benefits such as less adverse effects in central nervous system compared to centrally-acting anti-obesity drugs.

Abbreviations

α-GI,	alpha-glucosidase inhibitor
ANOVA,	analysis of variance
AUC,	area under the curve
BSA,	bovine serum albumin
CCK,	cholecystokinin
CHO,	Chinese hamster ovary
CI,	confidence interval
C_{max},	maximum concentration
CNS,	central nervous system
DIO,	diet-induced obese
DPP-4,	dipeptidyl-peptidase 4
EC_{50},	half maximal effective concentration
ELISA,	enzyme-linked immunosorbent assay
E_{max},	maximum effect
FBS,	fetal bovine serum
FFAR1,	free fatty acid receptor 1
FLIPR,	fluorometric imaging plate reader
GIP,	glucose-dependent insulinotropic peptide
γ-LA,	gamma-linolenic acid
GLP-1,	glucagon-like peptide-1
GLP-1R,	glucagon-like peptide-1 receptor
GPCR,	G-protein-coupled receptor
HFD,	high fat diet

KO,	knock-out
KRB,	Krebs-Ringer bicarbonate
mAb,	monoclonal antibody
MC,	methylcellulose
NAFLD,	non-alcoholic fatty liver disease
NTS,	nuclei of the solitary tract
PBS,	phosphate-buffered saline
PYY,	peptide YY
RTX,	resiniferatoxin
SD,	Sprague-Dawley
S.D.,	standard deviation
S.E.M.,	standard error of the mean
SGLT2,	sodium/glucose cotransporter 2
STZ,	streptozotocin

General Introduction

Diabetes and obesity

Type 2 diabetes mellitus is characterized by elevated blood glucose concentration (hyperglycemia) mainly due to impaired insulin secretion from pancreatic β cells and peripheral insulin resistance **[1, 2]** (Fig. 1). Based on the global estimation by International Diabetes Federation, there are 451 million people with diabetes in the world and 9.9% of all-cause mortality in the world was attributable to diabetes in 2017 **[3]**. The number of people with diabetes will be expected to increase to 693 million by 2045. As well as diabetes, the people with obesity, which is defined as excessive fat accumulation that might impair health (body mass index ≥30 kg/m^2), has also increased worldwide. The mean prevalence of obesity in adults reaches 19.5% in the world although the percentage varies between countries (<6 % in Japan and >30 % in the United States) **[4]**. Obesity is deeply related to the progression of diabetes due to exacerbation of tissue insulin resistance and adaptive changes in pancreatic β cell mass/function, which lead to β cell failure **[5-7]**.

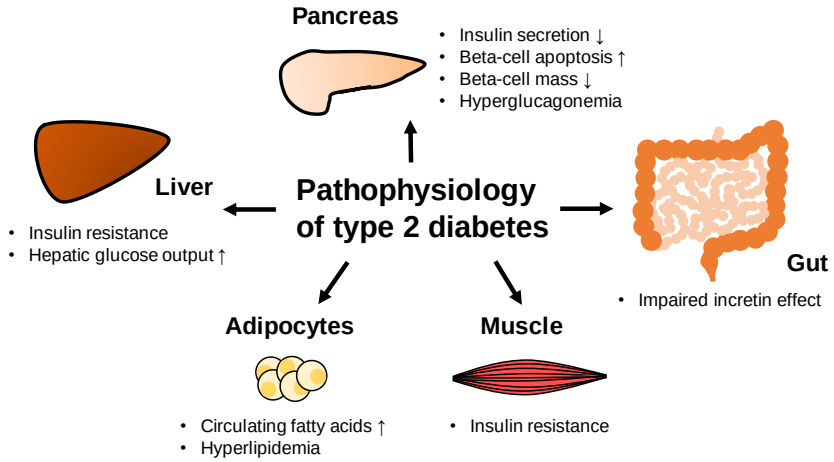


Figure 1. Pathophysiology of type 2 diabetes (originally drawn by referring to **[2]**).

Chronic hyperglycemia would lead to macrovascular- (such as coronary heart diseases and cerebral infarction) and microvascular- (retinopathy, nephropathy and neuropathy) related complications. Increased risks for cardiovascular events including death in people with diabetes have been demonstrated in some clinical evidence for a long time **[8-11]**. In addition, microvascular complications caused by poorly controlled glycemia result in blindness, dialysis, foot ulcers and amputation at later stages **[12]**. To prevent these diabetic complications, therefore, long term glycemic control is highly important.

Current medications for diabetes

In addition to some interventions of healthy diets and exercises, some medications are needed in patients with type 2 diabetes. To date, many glucose-lowering agents with various modes of action have been launched and used **[13]** (Fig. 2).

Sulphonylureas enhance insulin secretion by direct closure of K_{ATP} channel in pancreatic β cells and reduce blood glucose **[14]**. Biguanides (metformin) improve hyperglycemia by reducing hepatic glucose production and are recommended as an initial pharmacologic agent **[13, 15]**. Alpha-glucosidase inhibitors (α-GI) delay glucose absorption from intestine by suppressing carbohydrates digestion, which leads to the reduction of postprandial hyperglycemia **[16]**. Thiazolidinedione derivatives increase insulin sensitivity in the muscle, the adipose tissue and the liver by promoting glucose uptake and decreasing glucose production **[17]**. Dipeptidyl-peptidase 4 (DPP-4) inhibitors increase the plasma level of active glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) by inhibiting DPP-4 which degrades GLP-1 and GIP **[18]**. These hormones elicit glucose-dependent insulin secretion via their

receptors expressed in pancreatic β cells. Sodium/glucose cotransporter 2 (SGLT2) inhibitors suppress glucose re-absorption in the renal proximal tubule of the kidney and excretes glucose in the urine **[19]**.

Although these anti-diabetic agents show efficacy in patients with type 2 diabetes, these also have specific adverse effects based on each mechanism of action. For example, sulphonylureas induce hypoglycemia due to its glucose concentration-independent insulinotropic effect and thiazolidines gain weight because of its insulin-sensitizing effect **[14, 17]**. Therefore, oral medicine to treat diabetes in parallel with weight control with less adverse effects is desired.

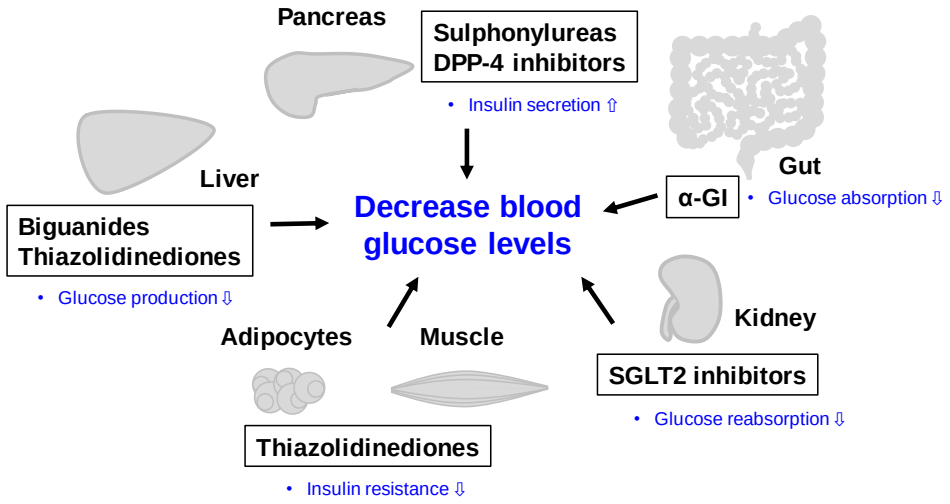


Figure 2. Current treatments for type 2 diabetes.

GPR40/FFAR1 as a potential target for diabetes treatment

GPR40/FFA1 receptor, a G-protein-coupled receptor (GPCR), couples predominantly with the $G_{q/11}$ protein, promoting phospholipase C-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-triphosphate **[20]**. This in turn increases intracellular Ca^{2+} levels **[21]**. Medium-to-long

chain fatty acids identified as endogenous ligands for GPR40 **[22, 23]** lead to elevations in intracellular Ca^{2+} levels followed by glucose-dependent insulin secretion in pancreatic β cells **[24, 25]**. Therefore, GPR40 activation is considered a novel option for treating diabetes with less hypoglycemic risk due to its glucose-dependent insulinotropic effect. To date, several synthetic compounds targeting GPR40 have been developed, and structure–activity relationships of these agonists have been investigated **[26]**. Among them, fasiglifam **[27-29]**, a GPR40 agonist, has been shown to significantly improve glycemic control in patients with type 2 diabetes **[30-32]**; however, fasiglifam testing was voluntarily terminated in phase 3 clinical trials because of possible adverse effects on the liver **[33]**. Proof of concept of a GPR40 agonist to treat diabetes has attracted considerable attention owing to its potential as a valuable drug target (Fig. 3).

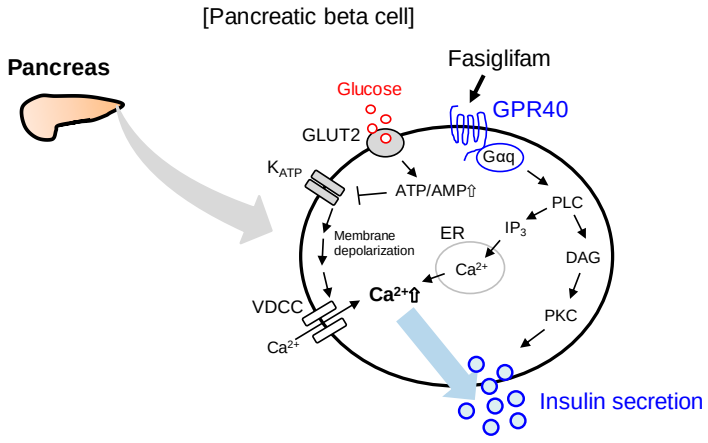


Figure 3. Insulin secreting machinery of fasiglifam, a GPR40 agonist (originally modified by referring to **[34]).** AMP, adenosine monophosphate; ATP, adenosine triphosphate; DAG, diacylglycerol; ER, endoplasmic reticulum; GLUT2, glucose transporter 2; IP_3, inositol 1,4,5-triphosphate; K_{ATP}, ATP-sensitive potassium channel; PKC, protein kinase C; PLC, phospholipase C; VDCC, voltage-dependent calcium channel.

A new generation GPR40 agonist

Concurrently, fasiglifam was demonstrated as a GPR40 partial agonist based on Ca^{2+} mobilization in GPR40-expressing cells **[35]**. Additional studies identified a novel series of GPR40 agonists with different profiles from partial agonists. A seminal study demonstrated that AM-1638 has full agonistic activity towards GPR40, and was more effective in stimulating insulin secretion and improving glucose-lowering efficacy *in vivo* than partial agonists **[36, 37]**. One of the unique profiles of GPR40 full agonist is to secrete intestinal hormones such as GLP-1 and GIP from endocrine cells as well as robust insulinotropic capacity **[37-40]**. GLP-1 and GIP are known to secrete insulin through their receptors expressed in β cells **[18]**. Moreover, as GLP-1 elicits an anorectic effect **[41, 42]**, GPR40 full agonists might represent a novel therapeutic agent for the treatment of both diabetes and obesity (Fig. 4).

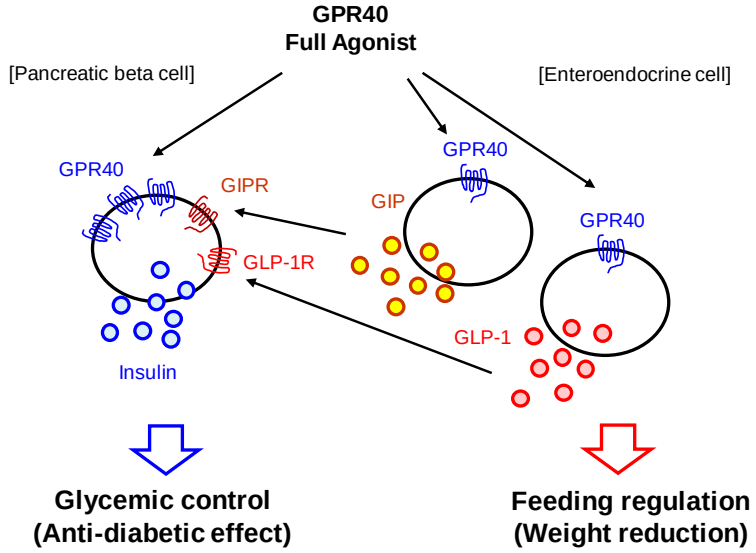


Figure 4. Putative mechanisms of action to regulate glycemia and food intake by GPR40 full agonist.

Objective of this research

In the first chapter, I characterized the *in vitro* / *in vivo* pharmacological profiles of SCO-267, a newly-synthesized potent GPR40 full agonist, in comparison with those of fasiglifam. In the second chapter, I tried to reveal the mechanism of feeding suppression derived from GPR40 full stimulation with respect to the involvement of afferent vagal nerves.

Chapter 1: SCO-267, a GPR40 full agonist, improves glycemic and body weight control in rat models of diabetes and obesity

Abstract

GPR40 full agonist may have a potential to treat diabetes and obesity due to its insulin- and incretin-secreting capacity. To demonstrate it in the first chapter, I examined the pharmacological profiles of SCO-267, a newly-synthesized full agonist of GPR40, in comparison with fasiglifam, a partial agonist of GPR40.

Treatment with SCO-267 mobilized intracellular Ca^{2+} in both high- and low-*FFAR1*-expressing CHO cells, stimulated insulin secretion in MIN6 cells, and induced GLP-1 release in GLUTag cells. SCO-267, but not fasiglifam, increased insulin, glucagon, GLP-1, GIP, and peptide YY (PYY) secretions when administered to non-fasting normal rats. These results show the full agonistic property of SCO-267 against GPR40. Hypoglycemia was not induced in SCO-267-treated rats during the fasting condition. In diabetic N-STZ-1.5 rats, SCO-267 was highly effective in improving glucose tolerance in single and 2-week dosing studies. Pharmacokinetic analysis revealed that lower plasma exposure of SCO-267 was sufficient to control glycemia compared with that of fasiglifam. SCO-267-treated DIO rats for 2 weeks showed elevated plasma GLP-1 and PYY levels, reduced food intake, and decreased body weight.

In conclusion, SCO-267 stimulated islet and gut hormone secretion, improved glycemic control in diabetic rats, and decreased body weight in obese rats. These data suggest the therapeutic potential of SCO-267 for the treatment of diabetes and obesity. Moreover, the lower plasma exposure in SCO-267 may be a safer advantage compared with that in fasiglifam.

Introduction

GPR40 full agonists can activate the enteroendocrine system while stimulating insulin secretion **[37]**. As insulin and incretin are pivotal for glycemic control **[18]**, these mechanisms seem highly valuable to developing a novel strategy for treating diabetes. Indeed, GLP-1 signaling activation has been proven to have beneficial effects, including improved long-term glucose control and decreased incidence of cardiovascular death, in patients with type 2 diabetes mellitus **[43]**. As GPR40 partial agonists improve glycemic control in patients with diabetes, GPR40 full agonists may provide superior efficacy and additional benefits in patients with metabolic diseases. To date, there has been no report on the clinical efficacy of GPR40 full agonist. Thus, preclinical efficacy studies including fasiglifam as a comparator would provide important information to develop novel GPR40 full agonists.

In this chapter, I characterized the pharmacological profiles and efficacy of SCO-267, a novel GPR40 full agonist **[44]**. Its *in-vitro* efficacy was evaluated using cell models, and its effect on islet, gut hormones and glycemic control was evaluated in rat models. SCO-267's contribution to body weight reduction was evaluated in obese rats. Finally, I compared some key effects induced by SCO-267 to fasiglifam **[45]**.

Materials and Methods

Materials

All reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Sigma-Aldrich (St Louis, MO), or Cayman Chemical (Ann Arbor, MI) unless otherwise indicated. SCO-267, fasiglifam, and AM-1638 were obtained from SCOHIA PHARMA, Inc. and Takeda Pharmaceutical Company Limited. Glimepiride was purchased from FUJIFILM Wako. For *in-vitro* studies, compounds were dissolved in dimethyl sulfoxide, and for *in-vivo* studies, compounds were suspended in 0.5 % methylcellulose (MC) solution (FUJIFILM Wako).

FFAR1-expressing CHO cell assay

Chinese hamster ovary (CHO) dhfr- cells (Clones #104 and #2) stably expressing human *FFAR1* **[35]** were cultured with minimum essential medium-alpha (FUJIFILM Wako) containing 10 % fetal bovine serum (FBS), 10 mmol/L HEPES (Thermo Fisher Scientific, Waltham, MA, USA), 100 IU/mL penicillin, and 100 µg/mL streptomycin in 5 % CO_2 at 37 °C. The cells (1×10^4 cells/well) were seeded in 384-well (black-walled clear-bottom) culture plates and incubated overnight in 5 % CO_2 at 37 °C. After removing the medium, the cells were incubated in 30 µL of loading buffer (Hank's Balanced Salt Solution, Thermo Fisher Scientific) containing 20 mmol/L HEPES, 0.1 % fatty acid-free bovine serum albumin (BSA), 0.08 % Pluronic F127 (#CSK-01F, Dojindo, Kumamoto, Japan), 2.5 mmol/L Probenecid (#CSK-03F, Dojindo), and 2.5 µg/mL Fluo4 (#F311, Dojindo) for 60 min in 5 % CO_2 at 37 °C. Test compounds of various concentrations were added to the cells and the increase in intracellular Ca^{2+} concentration was monitored for 180 s using the FLIPR Tetra system (Molecular Devices, Tokyo, Japan).

MIN6 cell assay

The pancreatic beta-cell line, MIN6, displays features of glucose metabolism and glucose-induced insulin secretion similar to those of normal islets **[46]**. MIN6 cells were seeded at 5×10^4 cells/well in 96-well plates and cultured as described previously **[46]**. After discarding the medium, the cells were pre-incubated for 2 h at 37 °C with 100 μL of Krebs-Ringer Bicarbonate–HEPES (KRBH) buffer (116 mmol/L NaCl, 4.7 mmol/L KCl, 1.17 mmol/L KH_2PO_4, 1.17 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 2.52 mmol/L $CaCl_2$, and 24 mmol/L HEPES) containing 0.2 % fatty acid-free BSA and 1 mmol/L glucose. After discarding the buffer, KRBH buffer containing 1 or 16 mmol/L glucose, 0.2 % fatty acid-free BSA and test materials at indicated concentrations was added and the plate was further incubated for 2 h at 37 °C. After incubation, supernatants were collected from all wells and the insulin concentrations were determined.

GLUTag cell assay

GLUTag cells are stable, immortalized, and relatively differentiated murine enteroendocrine cells **[47]** and in this study, these cells were seeded at 1×10^4 cells/well in 96-well poly-L-lysine-coated plates. Cells were cultured in Dulbecco's modified Eagle's medium with 10 % FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 25 mmol/L glucose. The culture medium was replaced with medium containing 10 % FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 5.5 mmol/L glucose prior to overnight incubation. After discarding the medium, KRBH buffer containing 10 mmol/L glucose, 0.2 % fatty acid-free BSA and the test materials at indicated concentrations was

added and the plate was further incubated for 2 h at 37 °C. The supernatants were collected from all wells and the GLP-1 concentrations were determined.

Animals

All animals were housed in a room with controlled temperature (23 °C), humidity (55 %), and lighting (lights on between 7:00 am and 7:00 pm). All animals were allowed free access to standard laboratory chow diet (CE-2, CLEA Japan, Inc.) and tap water. The care and use of animals and the experimental protocols were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company, Ltd. and SCOHIA PHARMA, Inc. All experiments were performed according to the guidelines and regulations of Takeda Pharmaceutical Company, Ltd., and Shonan Health Innovation Park. For animal studies, 0.5 % MC was used as vehicle. All blood samples used in the present study were obtained via the tail vein of animals.

Sprague-Dawley rat study

Male Sprague-Dawley (SD) rats were obtained from CLEA Japan, Inc (Tokyo, Japan). For the hormone secretion experiment in the non-fasted state, 6-week-old rats were randomized into groups (n = 6) based on body weight. To evaluate the compound's effect on fasting plasma glucose levels, 8-week-old rats were fasted overnight. Rats were randomized into groups (n = 6) based on body weight and plasma glucose. The test materials were orally administered at the indicated doses. Blood samples were collected at indicated time points, and plasma parameters were determined. A fasting plasma glucose range below 70 mg/dL was considered hypoglycemic **[48]**.

N-STZ-1.5 rat study

Male N-STZ-1.5 Wistar Kyoto rats (N-STZ-1.5 rats), which are diabetic, were developed via subcutaneous administration of 120 mg/kg streptozotocin (STZ) to Wistar Kyoto rats (RABICS, LTD. Kanagawa, Japan) at 1.5 days after birth. N-STZ rats were reported to display defects in insulin secretion and action which, in many ways, resemble those observed in human diabetic patients **[49]**. To evaluate the efficacy of single dosing, 25-week-old N-STZ-1.5 rats were subjected to overnight fasting and were randomized into groups (n = 6) based on fasting plasma glucose, triglyceride levels and body weight. The test materials were orally administered 60 min before oral glucose loading (1.5 g/kg). The effect of an oral dose of AM-1638 on glucose tolerance and its plasma concentration were compared to SCO-267 in a similarly designed experiment using male N-STZ-1.5 rats (32- and 18-week-old for glucose tolerance test (n = 6) and pharmacokinetic study (n = 3), respectively). Then, to evaluate the effects of repeated-dosing, 27-week-old N-STZ-1.5 rats (baseline body weight, 373 g) were randomized into groups (n = 6) based on glycosylated hemoglobin level, plasma glucose concentration, body weight, and food intake. Each group was treated with the indicated test materials once daily for 2 weeks. Following the 2-week treatment period, the rats were fasted overnight, and the test materials were orally administered at indicated doses 60 min before an oral glucose load (1.5 g/kg). Blood samples were collected at indicated time points and plasma parameters were determined. Following the oral glucose load test, a pharmacokinetic study was performed with the same non-fasted rats. A pharmacokinetic study of 0.3 mg/kg SCO-267 and 3 mg/kg fasiglifam was conducted under the same experimental conditions in 20-week-old male N-STZ-1.5 rats (n = 6).

Diet-induced obesity (DIO) rat study

Male F344 rats aged 29 weeks were obtained from CLEA Japan, Inc. and were fed a high-fat diet (D12451M, 45 kcal%, Research Diets, Inc., New Brunswick, NJ, USA) to induce obesity. At 49 weeks, the DIO rats (baseline body weight, 487 g) were randomized into groups (n = 6) based on body fat mass, daily food intake, and body weight. Each group was treated with the indicated test materials once daily for 2 weeks. Blood samples were collected at indicated time points and plasma parameters were determined. Body compositions were determined after 2 weeks of treatment. GLP-1 and PYY levels were measured 16 h after the 15^{th} dosing.

Measurements

Plasma metabolic parameters were measured with an Autoanalyzer 7180 (Hitachi, Tokyo, Japan). Insulin levels were measured using a radioimmunoassay kit (RI-13K, Merck Millipore, Burlington, MA, USA), ELISA kit (MS301, Takara, Shiga, Japan), or alphaLISA (PerkinElmer, Waltham, MA, USA). Plasma total GIP levels were determined using an ELISA kit (EZRMGIP-55K, Merck Millipore). Plasma glucagon was measured using a radioimmunoassay kit (GL-32K, Merck Millipore), or ELISA kit (10-1271-01, Mercodia, Uppsala, Sweden). Plasma total GLP-1 was determined by using an ELISA established by Takeda Pharmaceutical Company Limited or a commercially available ELISA kit (299-75501, FUJIFILM Wako). Plasma total PYY levels were determined using ELISA (291-73501, FUJIFILM Wako) or Rat/Mouse PYY RIA (RMPYY-68HK, Merck Millipore). Body composition was quantified by magnetic resonance imaging to directly measure total body fat mass and total body lean mass of rats that were not anaesthetized, at indicated ages (EchoMRI-900, Hitachi).

ELISA for GLP-1

Anti-GLP-1 monoclonal antibodies (mAbs, GLIT2-863.35-7 and GLIT4-1448.7) were generated from Balb/c mice immunized with GLP-1-BSA. These mAb pairs were screened for specificity to total GLP-1 in plasma and serum. Conventional sandwich ELISAs were established in a 96-well plate using those mAb pairs. GLP-1 (7-36) amide (PEPTIDE INSTITUTE, INC., Osaka, Japan) was used as a reference standard for the assay. The detection limit of this assay was 0.3-300 pmol/L; intra-assay and inter-assay variations were ≤ 15%.

Statistical analysis

Statistical significance was first analyzed using Bartlett's test for homogeneity of variances, followed by the Williams' test ($P > 0.05$) and Shirley–Williams test ($P \leq 0.05$) for dose-dependent studies, and Dunnett's test ($P > 0.05$) and Steel test ($P \leq 0.05$) for multiple comparisons. Alternatively, statistical significance was analyzed using the F test for homogeneity of variances, followed by Student's *t*-test ($P > 0.2$) or the Aspin–Welch test ($P \leq 0.2$). The Williams' and Shirley–Williams tests were conducted using a one-tailed significance level of 2.5 % (0.025). Other tests were conducted using a two-tailed significance level of 5 % (0.05). The half maximal (50 %) effective concentration (EC_{50}) values and maximum effect (E_{max}) were calculated using the 4-parameter logistic equation in Prism 7 software (GraphPad Software, San Diego, CA, USA). All data are presented as mean ± standard deviation (S.D.) (for *in-vivo* experiments) or mean ± standard error of the mean (S.E.M.) (for *in-vitro* experiments).

Results

SCO-267 is a GPR40 full agonist that stimulates insulin and GLP-1 secretions in cells

I identified SCO-267 as a new GPR40 agonist (Fig. 5A), and compound profiles were determined by *in-vitro* experiments. It has been previously reported that in Ca^{2+} mobilization assays in CHO cells with relatively high (clone #104) and low (clone #2) expression of human *FFAR1*, maximal response to the partial agonist fasiglifam dramatically decreases compared with an endogenous full agonist as expression levels of GPR40 decrease, indicating that partial and full agonists can be distinguished using this assay system **[35]**. Therefore, I used these two clones of CHO cells with human *FFAR1* (clone #104 and #2) to evaluate the agonistic activity of SCO-267 on Ca^{2+} response. SCO-267 showed EC_{50} values of 1.3 nmol/L (clone #104) and 12 nmol/L (clone #2), and E_{max} values (% γ-linolenic acid, an endogenous ligand [γ-LA]) of 125 % (clone #104) and 201 % (clone #2) (Fig. 5B, C, Table 1). AM-1638 **[36]**, a well-known GPR40 full agonist, had EC_{50} values of 7.1 nmol/L (clone #104) and 150 nmol/L (clone #2), and E_{max} values (% γ-LA) of 110 % (clone #104) and 182 % (clone #2) (Fig. 5B and C, Table 1). The insulinotropic effect of SCO-267 was examined in mouse insulinoma MIN6 cells. SCO-267 effectively stimulated insulin secretion under the high-glucose condition, unlike fasiglifam (EC_{50} = 0.85 nmol/L for SCO-267 and EC_{50} = 530 nmol/L for fasiglifam; E_{max} [% fasiglifam], 142 % for SCO-267) (Fig. 5D). When tested in mouse enteroendocrine GLUTag cells, SCO-267 stimulated GLP-1 secretion by more than 4-fold (EC_{50} = 8.5 nmol/L) the level observed upon treatment with dimethyl sulfoxide. SCO-267 was also more effective than 10 μmol/L fasiglifam (Fig. 5E). These data indicate that SCO-267 has full agonistic activity against GPR40.

SCO-267 stimulates secretion of islet and enteroendocrine hormones and does not induce hypoglycemia in normal rats

In the non-fasting condition, SCO-267 (0.3–10 mg/kg) administration stimulated the secretion of insulin, glucagon, GLP-1, GIP, and PYY (Fig. 6A-E). Fasiglifam (10 mg/kg) was not effective in inducing these hormonal changes (Fig. 6A-E). Administration of glimepiride, a sulfonylurea class antidiabetic drug **[50]**, at 10 mg/kg induced overt hypoglycemia, with plasma glucose concentrations below 70 mg/dL, in fasting rats. Although SCO-267 slightly decreased fasting plasma glucose levels, it did not induce hypoglycemia in normoglycemic rats (Fig. 6F).

SCO-267 improves glucose tolerance and its efficacy is durable in diabetic rats

A single oral administration of SCO-267 (0.1, 0.3, 1 and 3 mg/kg) increased plasma insulin and GLP-1 levels and improved glucose tolerance after an oral glucose load in N-STZ-1.5 rats in a dose-dependent manner (Fig. 7A-C). SCO-267 (0.3 mg/kg) and fasiglifam (3 mg/kg) had C_{max} values of 22.7 ng/mL and 6.17 μg/mL, respectively, in N-STZ-1.5 rats. The efficacy of SCO-267 was also compared to AM-1638 **[37]**, a well-studied GPR40 full agonist, in N-STZ-1.5 rats. A lower plasma exposure of SCO-267 was more effective in enhancing insulin secretion and improving glucose tolerance compared to those by AM-1638 in N-STZ-1.5 rats (C_{max} values of 176 and 543 ng/mL in SCO-267 and AM-1638, respectively) (Fig. 7D, E). To test the efficacy and durability, SCO-267 (1 mg/kg) was repeatedly administered to N-STZ-1.5 rats for 2 weeks. Consistent with the single dosing efficacy, SCO-267 increased insulin secretion and improved glucose tolerance in N-STZ-1.5 rats after 2 weeks of repeated dosing (Fig. 8A,

B). At the end of the study, SCO-267 (1 mg/kg) had a C_{max} of 139 ng/mL and $AUC_{0–24h}$ of 626 ng·h/mL, and fasiglifam (10 mg/kg) had a C_{max} of 39.8 μg/mL and $AUC_{0–24h}$ of 255 μg·h/mL in N-STZ-1.5 rats (Fig. 8C).

SCO-267 elevates circulating gut hormone and decreases body weight in DIO rats

The chronic effect of SCO-267 (0.3-3 mg/kg) was evaluated in obese rats that were fed a high-fat diet. After chronic dosing, GLP-1 and PYY levels were elevated after 16 h of SCO-267 dosing (Fig. 9A, B). During the experimental period, food intake levels were lower and total food intake was decreased in SCO-267-treated rats (Fig. 9C). Consistent with the reduction in food intake, body weight and fat mass were decreased in SCO-267-treated rats (Fig. 9D, E). The lean mass of SCO-267-treated DIO rats did not change.

Discussion

In this chapter, I demonstrated that SCO-267, a new GPR40 agonist, was highly effective in activating GPR40 and improving glucose control *in vivo*. The *in-vitro* experiments confirmed the stimulatory effect of SCO-267 on Ca^{2+} signaling, insulin release, and GLP-1 secretion in *FFAR1*-expressing CHO, MIN6, and GLUTag cells, respectively. When tested in normal rats, SCO-267 stimulated the secretion of hormones from the islet and gut. These results demonstrate the full agonistic property of SCO-267 against GPR40. Hypoglycemia was not induced in rats during the fasting condition. Studies in N-STZ-1.5 rats with diabetes confirmed the efficacy of SCO-267 in improving glucose control, and the durability of this effect. SCO-267 was also effective in decreasing body weight in DIO rats. The data suggest that SCO-267 has potential as a novel therapeutic agent for treating diabetes and obesity.

SCO-267 robustly stimulated insulin secretion in dysfunctional beta-cells in N-STZ-1.5 rats. In a single dosing study of N-STZ-1.5 rats, 0.3 mg/kg SCO-267 (C_{max}, 22.7 ng/mL) had a glucose-lowering efficacy comparable to that of 3 mg/kg fasiglifam (C_{max}, 6.17 μg/mL). This indicated that a substantially lower compound exposure is sufficient for inducing the efficacy of SCO-267. I also compared the efficacy of SCO-267 to AM-1638, a well-studied GPR40 full agonist. *In vitro* experiment showed that Ca^{2+} influx activity of SCO-267 was 5.5-12.5-fold more potent compared to AM-1638 in human *FFAR1*-expressing CHO cells. When each compound was orally dosed at the same dosage levels, SCO-267 was more effective in improving glucose tolerance compared to AM-1638 in N-STZ-1.5 rats. These results indicate that SCO-267 is a potent GPR40 full agonist. In addition, SCO-267 stimulated the secretion of GLP-1 and GIP, both of which are incretin hormones having insulinotropic action **[18]**. Taken together with the direct

insulinotropic effect on beta-cells expressing *Ffar1*, increased secretion of GLP-1 and GIP may have contributed to the enhanced insulin secretion observed in SCO-267-administered rats.

In a repeated-dosing study of N-STZ-1.5 rats, 1 mg/kg SCO-267 (C_{max}, 139 ng/mL; $AUC_{0–24h}$, 626 ng·h/mL) was more effective in improving glucose tolerance than 10 mg/kg fasiglifam (C_{max}, 39.8 μg/mL; $AUC_{0–24h}$, 255 μg·h/mL). Plasma protein binding of SCO-267 was similar across species (99.6–99.7%), and fasiglifam showed similar plasma protein binding across species (>99.4% for fasiglifam **[51]**). With the clinically effective exposure of 50 mg fasiglifam (C_{max}, 5.3 μg/mL; $AUC_{0–24h}$, 100.3 μg·h/mL **[52]**), SCO-267 may be effective in improving glucose control in patients with type 2 diabetes. Additionally, the lower plasma exposure to SCO-267 may be a safety advantage compared to exposure to fasiglifam, which was voluntarily terminated in phase 3 due to possible adverse effects on the liver. Considering the lower plasma exposure of SCO-267 in inducing efficacy and good pharmacokinetic profiles, SCO-267 is likely a good candidate for testing in clinical trials.

Food intake was diminished, and body weight was lowered in SCO-267-treated DIO rats. Plasma GLP-1 and PYY levels remained high 16 h after the final dose was administered to DIO rats. Considering the elevated levels of GLP-1 and PYY, both of which are physiological hormones regulating satiety and body weight **[53, 54]**, these hormones may have contributed to the body weight reduction observed in SCO-267-administered DIO rats. This property of SCO-267 may be beneficial in patients who are overweight or obese and have type 2 diabetes. I tried to elucidate the mechanism by which GPR40 full agonism regulates food intake in the next chapter

In conclusion, the GPR40 full agonist, SCO-267, stimulated insulin, glucagon, GLP-1, GIP, and PYY secretion. Furthermore, SCO-267 effectively improved glucose control and exerted strong efficacy in rats with diabetes. In addition, body weight loss was observed in obese rats. Thus, SCO-267 is effective in improving diabetes and obesity in rats and may induce similar favorable effects in patients with diabetes and obesity.

Table

Table 1. Ca^{2+} influx activity in human *FFAR1*-expressing CHO cells.

Test material	CHO cells with high *FFAR1* expression (clone #104)		CHO cells with low *FFAR1* expression (clone #2)	
	EC_{50} (nmol/L) [95% CI*]	E_{max} (% γ-linolenic acid)	EC_{50} (nmol/L) [95% CI*]	E_{max} (% γ-linolenic acid)
SCO-267	1.3 [0.97–1.7]	125	12 [11–14]	201
AM-1638	7.1 [5.6–9.2]	110	150 [120–180]	182
Fasiglifam	24 [16–37]	100	>1000	22
α-linolenic acid	>10000	110	>10000	93
Linoleic acid	>10000	109	>10000	102
Palmitic acid	>10000	113	>10000	78
γ-linolenic acid	>10000	100	>10000	100

*CI, confidence interval

Figures

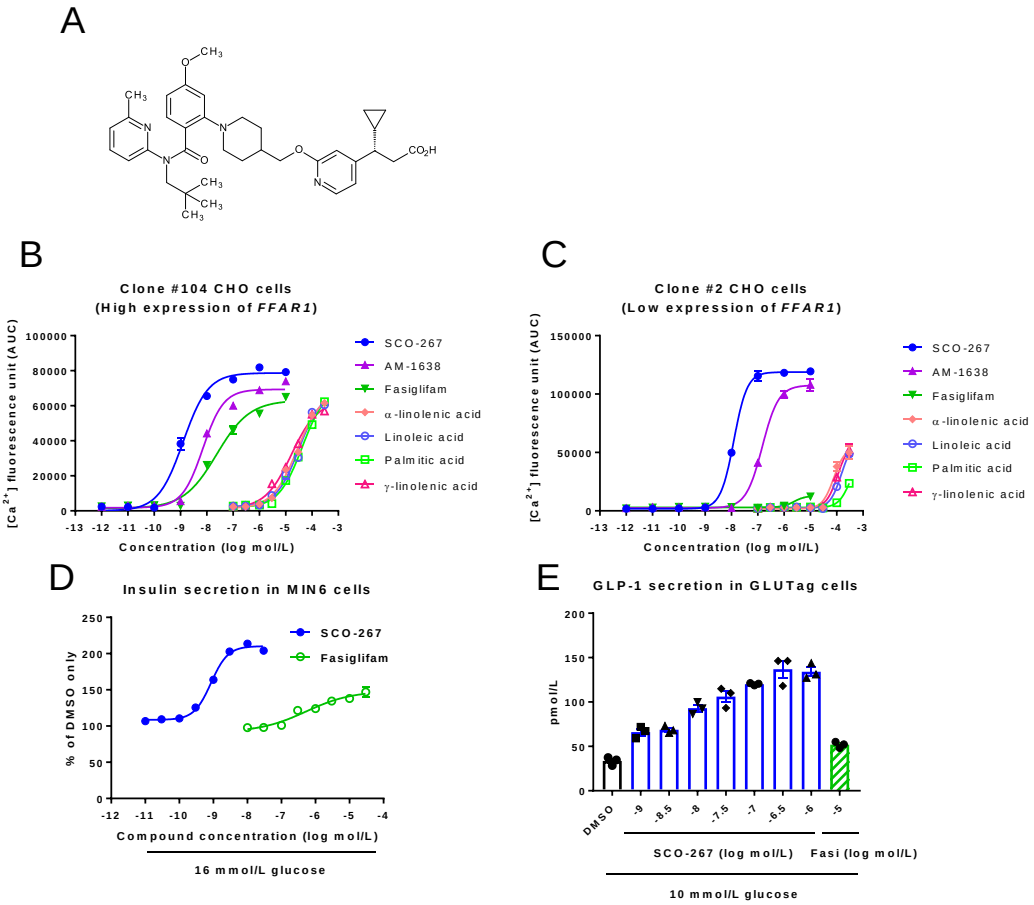


Figure 5. Effects of SCO-267 on Ca^{2+} concentration in *FFAR1*-expressing CHO cells, insulin secretion in MIN6 cells and GLP-1 secretion in GLUTag cells.

Chemical structure of SCO-267 (A). Intracellular Ca^{2+} responses against SCO-267 and other agonists in CHO cells expressing high (B) and low (C) levels of human *FFAR1* (mean ± S.E.M. of quadruplicate wells, similar results were obtained in an independent study). Concentration-dependent insulin secretion response of SCO-267 in MIN6 cells with 16 mmol/L glucose condition (mean ± S.E.M. of triplicate wells) (D). Stimulation of GLP-1 secretion in GLUTag cells with 10 mmol/L glucose condition (mean ± S.E.M. and individual data of triplicate wells) (E). Fasi, fasiglifam

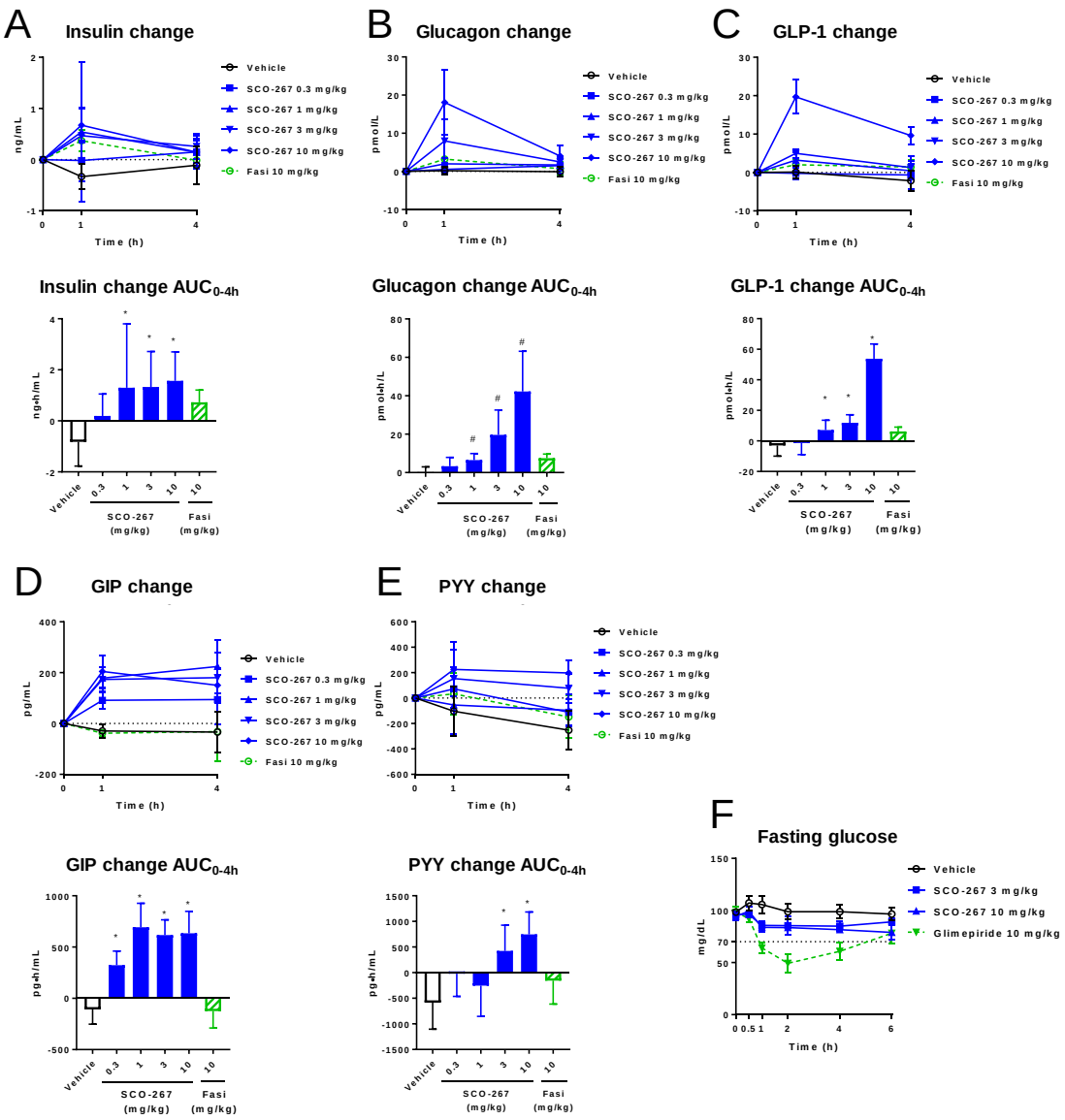


Figure 6. Effect of a single dosing of SCO-267 on hormone secretion and fasting glucose in normal rats.

Plasma levels of insulin (A), glucagon (B), GLP-1 (C), GIP (D), and PYY (E) in SCO-267-administered non-fasting normal rats. (F) Effects on fasting plasma glucose levels in overnight-fasted normal rats. Hypoglycemia was defined as plasma glucose levels <70 mg/dL in this experiment. * and # P<0.025 vs. vehicle by one-tailed Williams' test and

Shirley–Williams test, respectively. Values are presented as mean ± S.D. (n = 6). Fasi, fasiglifam

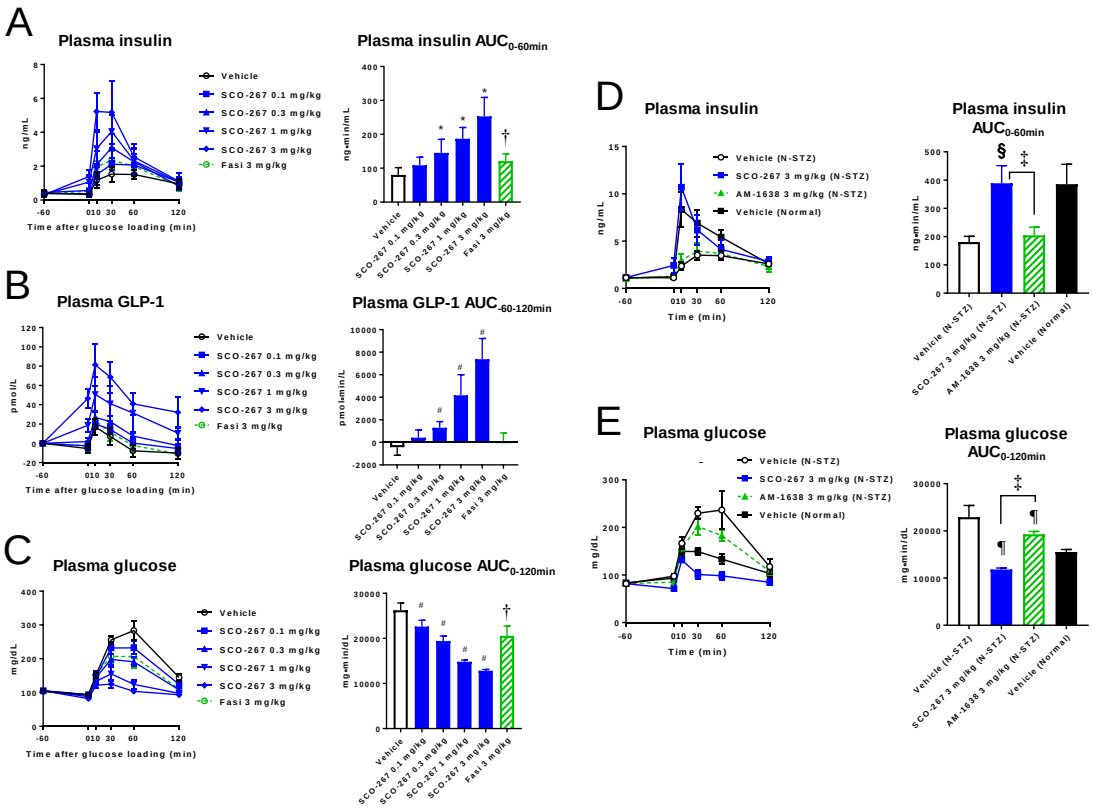
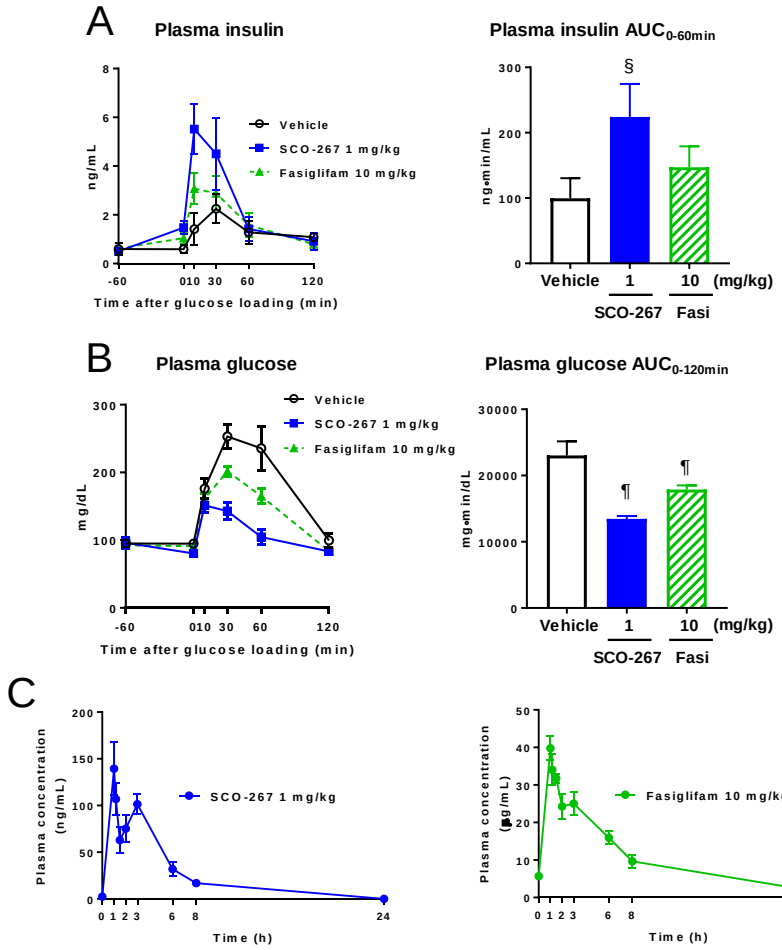


Figure 7. Effects of single administration of SCO-267 on hormone secretion and glucose control in diabetic N-STZ-1.5 rats.

Plasma insulin (A, D), GLP-1 (B), and glucose levels (C, E) after a single dosing of SCO-267 in comparison with fasiglifam (A-C) or AM-1638 (D, E) in N-STZ-1.5 rats. * and # $P<0.025$ vs. vehicle by one-tailed Williams' test and one-tailed Shirley–Williams test, respectively. † $P<0.05$ vs vehicle by Student's *t*-test. § and ¶ $P<0.05$ vs. vehicle (N-STZ) by Dunnett's test and Steel test, respectively. ‡ $P<0.05$ by Aspin–Welch test. Values are presented as mean ± S.D. (n = 6). Fasi, fasiglifam; Normal, Wistar Kyoto rats.



Pharmacokinetic parameters	SCO-267 1 mg/kg
C_{max} (ng/mL)	139 ± 28.2
T_{max} (h)	1.0 ± 0.0
$AUC_{0\text{-}24h}$ (ng·h/mL)	626 ± 32.2
MRT (h)	4.1 ± 0.4

Pharmacokinetic parameters	Fasiglifam 10 mg/kg
C_{max} (μg/mL)	39.8 ± 3.27
T_{max} (h)	1.0 ± 0.0
$AUC_{0\text{-}24h}$ (μg·h/mL)	255 ± 28.4
MRT (h)	5.7 ± 0.2

Figure 8. Effects of repeated administration of SCO-267 on glucose control in diabetic N-STZ-1.5 rats.

Plasma levels of insulin (A), glucose (B) and pharmacokinetic profiles (C) after 2 weeks of repeated-dosing with SCO-267 or fasiglifam in N-STZ-1.5 rats. § and ¶ $P<0.05$ vs. vehicle by Dunnett's test and Steel test, respectively. Values are presented as mean ± S.D. (n = 6). Fasi, fasiglifam; C_{max}, maximum concentration; T_{max}, time to reach maximum

concentration after dosing; $AUC_{0\text{-}24h}$, the area under the concentration-time curve from the time 0 to 24 hours; MRT, mean residence time.

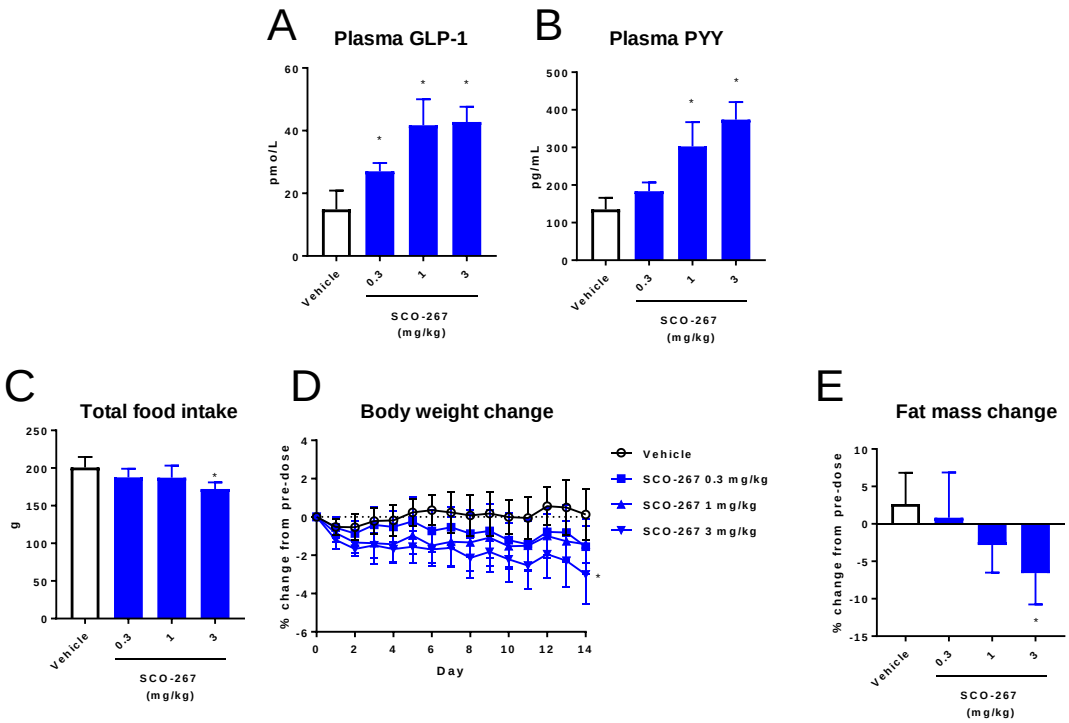


Figure 9. Effect of SCO-267 on gut hormone secretion and body weight control in DIO-rats.

Plasma levels of GLP-1 (A), PYY (B), total food intake (C), body weight change (D), and fat mass change (E) in DIO-rats. * P<0.025 vs. vehicle by one-tailed Williams' test. Values are presented as mean ± S.D. (n = 6)

Chapter 2: GPR40 full agonism exerts feeding suppression and weight loss through afferent vagal nerve

Abstract

Many previous studies related to GPR40 agonists have focused on their anti-diabetic actions and detailed mechanisms of feeding suppression are still unknown. In the second chapter, I conducted the *in vitro/in vivo* characterization of T-3601386, another compound with potent full agonistic activity for GPR40. I also evaluated feeding suppression and weight loss after the administration of T-3601386 and investigated the involvement of the vagal nerve in these effects.

T-3601386, but not a partial agonist fasiglifam, increased intracellular Ca^{2+} levels in CHO cells with low *FFAR1* expression, and single dosing of T-3601386 in DIO rats elevated plasma incretin levels, suggesting full agonistic properties of T-3601386 against GPR40. Multiple doses of T-3601386, but not fasiglifam, in DIO rats showed dose-dependent weight loss accompanied by feeding suppression and durable GLP-1 elevation, all of which were completely abolished in *Ffar1*$^{-/-}$ mice. Immunohistochemical analysis in the nuclei of the solitary tract demonstrated that T-3601386 increased the number of c-Fos positive cells, which also disappeared in *Ffar1*$^{-/-}$ mice. Surgical vagotomy and drug-induced deafferentation counteracted the feeding suppression and weight loss induced by the administration of T-3601386.

These results suggest that T-3601386 exerts incretin release and weight loss in a GPR40-dependent manner, and that afferent vagal nerves are important for the feeding suppression induced by GPR40 full agonism. These findings may indicate that GPR40 full agonism can provide periphery-derived weight reduction and does not require direct exposures to central nervous system.

Introduction

As shown in the chapter 1, GPR40 full agonist has a potential to treat diabetes and obesity. Unlike its glucose-lowering effect, however, the mechanisms of feeding suppression by GPR40 full agonists are poorly understood. Recently, Gorski JN. *et al.* reported GPR40 agonist-induced suppression of food intake and demonstrated that GLP-1 receptor (GLP-1R) as well as GPR40 contributed to the agonist-mediated feeding suppression **[55]**. Although this is considered reasonable because of the GLP-1-secreting capacity of GPR40 full agonists, detailed analysis of its action site has not been carried out.

Feeding behavior is regulated by direct action of peptides and hormones on the central nervous system (CNS) and/or periphery-derived signals **[56]**. To link peripheral signals to feeding behavior, the afferent vagal nerves and the nucleus of the solitary tract (NTS) located in the brain stem play important roles **[57, 58]**. Gastro-intestinal hormones such as GLP-1 and cholecystokinin (CCK) have been known to induce NTS activation and their anorectic effects were abolished in rodents with capsaicin-treated deafferentation or surgical vagotomy **[59-62]**, which suggests that food intake inhibition induced by these hormones requires intact vagal nerves and subsequent NTS activation. It is also possible that a GPR40 full stimulation evokes afferent vagal nerves and NTS activation by the release of endogenous GLP-1, and an eventual feeding suppression. However, the involvement of the vagal pathway in feeding behavior regulated by a GPR40 agonist has not been examined.

In this chapter, I first characterized 3-cyclopropyl-3-(3-((1-(2-(4,4-dimethylpentyl)-5-methoxyphenyl)piperidin-4-yl)methoxy)phenyl)propanoic acid (T-3601386), a racemic compound which clearly induces GPR40 full agonism **[63]**. Second,

I examined the anti-obesity effect of T-3601386 in a DIO model and conducted c-Fos immunohistochemistry in the NTS after T-3601386 administration, followed by the confirmation of their GPR40 dependence using *Ffar1*$^{-/-}$ mice. Finally, I investigated the contribution of the vagal pathway to T-3601386-induced feeding suppression using *in vivo* models with vagal nerve blockade **[64]**.

Materials and methods

Materials

T-3601386, AM-1638 **[37, 38]** and fasiglifam were synthesized at Takeda Pharmaceutical Company, Limited. Alpha-linolenic, linoleic, palmitic and γ-linolenic acid were purchased from FUJIFILM Wako, Cayman Chemical or Sigma. Sibutramine, liraglutide, and CCK-8 were purchased from Alexis Biochemicals (San Diego, CA), Novo Nordisk pharma (Tokyo, Japan), and Peptide Institute, Inc. (Osaka, Japan), respectively. Compounds were dissolved in dimethyl sulfoxide for the *in vitro* study and were suspended or dissolved in 0.5 % MC solution (FUJIFILM Wako) for oral dosing. Liraglutide and CCK-8 were dissolved in saline.

FFAR1-expressing CHO cell assay

Described in the “Material and Methods” section of the chapter 1.

GLUTag cell assay

Described in the “Material and Methods” section of the chapter 1.

Animals

Male F344 and SD rats were purchased from CLEA Japan, Inc. and Charles river Laboratories Japan, Inc. (Yokohama, Japan), respectively. Male *Ffar1*$^{-/-}$, age-matched littermate (wild-type) mice **[65]** and female Wistar fatty rats **[66]** were obtained from RABICS, LTD. All rats and mice were housed in individual cages in a room with controlled temperature (23 °C), humidity (55 %) and lighting (lights on from 7:00 am to 7:00 pm) and were allowed free access to solid or powdered standard laboratory chow

diet (CE-2, CLEA) and tap water. The animals were fed a high fat diet (HFD; D12451, D12451M, Research diets, Inc.) if needed as described below. The care and use of the animals and experimental protocols used in this study were approved by the IACUC of Shonan Research Center, Takeda Pharmaceutical Company Limited. For animal studies, 0.5 % MC was used as vehicle. All blood samples used in the present study were obtained via the tail vein of animals.

***In vivo* hormone secretion by single dosing**

F344 rats were fed HFD from 5 weeks old in order to develop diet-induced obesity (defined as DIO-F344 rats). DIO-F344 rats (41 weeks old, baseline body weight 532 g) were randomized based on their body weight, and blood samples were collected at indicated time points after drug administration (N = 6). Plasma total GLP-1 and total GIP levels were measured.

Repeated dosing studies in DIO-F344 rats and *Ffar1*$^{-/-}$ mice

DIO-F344 rats (42 weeks old, baseline body weight 480 g) were randomized based on body fat mass measured, body weight and food intake, and were administered drugs once daily for 4 weeks (N = 6-7). After the repeated doses, blood samples were collected at the indicated time points after a single further dosing and plasma total GLP-1 levels were measured. *Ffar1*$^{-/-}$ and wild-type mice (23 weeks old, baseline body weight 45 g for wild and 44 g for *Ffar1*$^{-/-}$) fed HFD for 16 weeks were randomized based on their body weight and food intake, and were administered drugs once daily for 3 days (N = 5-6). Blood samples were collected before and 1 h after the 4th dosing and plasma parameters were determined.

c-Fos immunohistochemistry

SD rats (8 weeks old, baseline body weight 291 g) fed HFD for 2 weeks were randomized based on body weight, and were administered drugs (N = 4). Rats were dissected 3 h after the dosing to isolate the brain stem under sodium pentobarbital (50 mg/kg, *i.p.*, Kyoritsu, Tokyo, Japan) anesthesia. The obtained brain samples were fixed with 4 % paraformaldehyde for 24 h, followed by substitution with 30 % sucrose in phosphate-buffered saline (PBS) for 2 days. Immunostaining was performed by a free-floating method. Forty μm cryosections including NTS were incubated with anti-cFos rabbit polyclonal antibody (SC-52; Santa Cruz Biotechnology) diluted 1:4000 in PBS at pH 7.1 containing 1 % normal horse serum and 0.4 % Triton X-100 overnight. Immunoreactivity in the sections was visualized using diaminobenzidine after the treatment with an ABC Elite kit (PK-6101, Vector Laboratories, Inc., Burlingame, CA). After processing, the sections were mounted on a non-coated slide, dehydrated, and then cover-slipped. Photographs were captured with a 4 x objective lens using Nikon ECLIPSE 800 microscope equipped with Nikon ACT-1 version 2.12. c-Fos positive cells were counted using a computerized image analysis system (Image-Pro Plus version 4.5). c-Fos counts were performed on each of three sections from each animal. The mean of these three determinations was used for subsequent statistical analysis. In the same protocol as described above, *Ffar1*$^{-/-}$ and wild-type mice (46 weeks old, baseline body weight 50 g for wild and 55 g for *Ffar1*$^{-/-}$) fed HFD for 38 weeks were also used to confirm the GPR40 dependency (N = 5-6).

Surgical vagotomy

Overnight fasted SD rats (10 weeks old) pretreated with buprenorphine (0.01 mg/kg, *s.c.*, Otsuka, Tokyo, Japan) were operated for bilateral subdiaphragmatic vagotomy under sodium pentobarbital (50 mg/kg, *i.p.*) anesthesia as previously reported **[67]**. Eleven days after the operation, the rats began a HFD feeding regime until the end of the study (3 weeks of HFD feeding). Four weeks after the operation, the rats were randomized based on their food consumption (Pre) and body weight (561 g for sham and 484 g for vagotomy, N = 6). The drugs were orally administered, and food consumption for 24 h was calculated (% change from Pre). The success of surgical vagotomy was confirmed by means of the significant cancellation of the CCK-8 (3 μg/kg, *i.p.*)-induced anorectic effect for 30 min in the overnight fasted-refed experiment **[61, 68]**.

Drug-induced deafferentation

Wistar fatty rats (27 weeks old) pretreated with buprenorphine (0.01 mg/kg, *s.c.*) were administered a transient receptor potential vanilloid type 1 agonist, resiniferatoxin (RTX; 40 μg/kg, LC Laboratories, Woburn, MA) or vehicle (1 % ethanol, 1 % Tween80, 98 % saline) subcutaneously under isoflurane anesthesia (DS pharma, Osaka, Japan). A week after the injection of RTX or vehicle, rats were randomized based on their food consumption (Pre) and body weight (529 g for vehicle and 516 g for RTX, N = 6). The drugs were orally administered and food consumption for 24 h was calculated (% change from Pre). The following week, drugs were administered, and blood samples were collected before and 1 h after dosing for measurements of plasma incretin. The success of deafferentation by RTX was confirmed by the disappearance of capsaicin (0.1 mg/mL, FUJIFILM Wako)-induced eye-wipe reaction **[69]**.

Measurements

Plasma total GIP levels were determined using an ELISA kit (EZRMGIP-55K, Merck Millipore). *In vitro* secreted GLP-1 and plasma total GLP-1 were determined by an Active form assai kit (27784, IBL, Gunma, Japan) and using an ELISA established by Takeda Pharmaceutical Company Limited ("Material and Methods" section of the chapter 1.), respectively. Body composition was quantified by magnetic resonance imaging to directly measure total body fat mass and total body lean mass of rats that were not anaesthetized, at indicated ages (EchoMRI-900, Hitachi).

Statistics

Described in the "Material and Methods" section of the chapter 1. Two-way ANOVA was performed to evaluate combinational effects. When an interaction effect was significant ($P < 0.05$), the combinational effect was interpreted to be meaningful (the effects of combination treatment exceeds the sum of each effect) **[29]**.

Results

T-3601386 shows full agonistic activities for GPR40

Using the same *in vitro* assay system in CHO cells in the chapter 1, I evaluated the GPR40 agonistic activities of T-3601386 (Fig. 10A), AM-1638, fasiglifam and several free fatty acids on Ca^{2+} mobilization. While both T-3601386 and AM-1638 showed concentration-dependent increases in the intracellular Ca^{2+} mobilization in both clones (#104 and #2), fasiglifam increased intracellular Ca^{2+} only in high expressing clone #104. Free fatty acids also showed agonist activities in both clones only at high concentrations (Fig. 10B, C). More specifically, T-3601386 showed EC_{50} values of 1.2 nmol/L (clone #104) and 15 nmol/L (clone #2), and AM-1638 had EC_{50} values of 3.2 nmol/L (clone #104) and 138 nmol/L (clone #2). EC_{50} values in fasiglifam were 25 nmol/L (clone #104) and >1000 nmol/L (clone #2). In clone #2 CHO cells, the E_{max} value of T-3601386 (246 %, % γ-linolenic acid) was comparable to and higher than that of AM-1638 (229 %) and fasiglifam (15 %), respectively. To further characterize the full agonistic property of T-3601386, the effects of T-3601386 on GLP-1 secretion were examined in mouse enteroendocrine cells. T-3601386 elevated GLP-1 secretion in GLUTag cells at 10-fold lower concentration compared with AM-1638 (Fig. 10D). Next, I tested *in vivo* incretin secretion and clear increases in plasma levels of total GLP-1 and GIP were observed after oral dose of T-3601386 (10 and 30 mg/kg) in DIO-F344 rats (Fig. 11A, B). These results indicate that T-3601386 also elicits *in vivo* GPR40 full agonism. After the confirmation of these strong full agonistic *in vitro* and *in vivo* activities for GPR40, I performed subsequent experiments using T-3601386.

T-3601386 exerts anorectic and body weight-lowering effects with multiple dosing

To precisely evaluate weight-reducing effects of T-3601386, I selected DIO rats fed HFD for long term especially in multiple dosing study. Four weeks multiple treatment with T-3601386 (1, 3 and 10 mg/kg), but not fasiglifam (10 mg/kg), dose-dependently and significantly reduced food consumption and body weight in DIO-F344 rats (Fig. 11C, D), and the potency of T-3601386 at 10 mg/kg (-4.8 % vs. vehicle) was comparable to that of sibutramine (1 mg/kg) (-4.6 % vs. vehicle), a centrally-acting anti-obesity drug, as a positive control. In DIO-F344 rats given T-3601386 for 4 weeks, significant increases in plasma GLP-1 levels were observed following the single dose of T-3601386 (Fig. 11E), suggesting the continuous elevation of plasma GLP-1 levels during the repeated dosing study. These results are consistent with a previous report that a GPR40 full agonist exerts body weight-lowering effect with the augmentation of circulating GLP-1 **[39]**.

T-3601386 administration activates NTS

As mentioned above, many reports have demonstrated that GLP-1 and other gastro-intestinal hormones require the intact vagal nerve for their food intake suppression **[59-62]**. I hypothesized that elevated GLP-1 by a GPR40 full agonism can activate NTS, which receives peripheral projection via the afferent vagal nerve **[57, 58]**. A single dose of T-3601386 (30 mg/kg) in rats showed a mild but statistically significant increase in c-Fos immunoreactivity in the NTS (Fig. 12A, B, E) **[70]**, and the GLP-1 analogue liraglutide (100 μg/kg, *s.c.*) as a positive control also resulted in a marked increase in c-Fos immunoreactive cells in the NTS (Fig. 12C-E), suggesting that the afferent vagal pathway became activated after the administration of T-3601386.

Feeding suppression, incretinotropic effects, and NTS activation induced by T-3601386 are dependent on GPR40

To confirm the involvement of GPR40 in T-3601386-induced feeding suppression and weight loss, the effects of T-3601386 in *Ffar1*$^{-/-}$ and wild-type mice were evaluated. Three daily doses of T-3601386 (30 mg/kg) significantly reduced food intake and body weight in wild-type mice, but not in *Ffar1*$^{-/-}$ mice (Fig. 13A, B). Comparable decreases in food intake and body weight were observed both in wild-type and *Ffar1*$^{-/-}$ mice given sibutramine (10 mg/kg). After 3 days of administration, the significant elevation of plasma incretin after the dosing of T-3601386 in wild-type mice disappeared in *Ffar1*$^{-/-}$ mice (Fig. 13C, D). A GPR40 dependence of T-3601386-induced NTS activation was also examined by immunohistochemistry. As observed in rats, a single dosing of T-3601386 (30 mg/kg) significantly increased c-Fos immunoreactive cells in the NTS of wild-type mice (Fig. 13E, F, I), but this effect completely disappeared in *Ffar1*$^{-/-}$ mice (Fig. 13G-I).

Vagal nerve blockade attenuates T-3601386-induced feeding suppression and weight loss

To clarify the involvement of the vagal nerve in the feeding suppression of T-3601386, I evaluated the effects of T-3601386 in two models with surgical and pharmacological vagal nerve blockade, namely bilateral subdiaphragmatic vagotomy and RTX-induced deafferentation, respectively. Significant decreases in food intake (20 % inhibition vs. control) and body weight with T-3601386 (30 mg/kg) were observed in sham-operated rats, while T-3601386 administration in vagotomized rats showed relatively weak efficacy both in feeding suppression (11 % inhibition vs. control, P =

0.31) and weight loss (P = 0.13) (Fig. 14A, B). To demonstrate the success of the operation, I used CCK-8 which has been known to reduce food intake through intact vagal nerves as mentioned above. CCK-8-induced anorectic effect in sham-operated rats was significantly attenuated in vagotomized rats, indicating the successful surgical vagotomy in the present study (Fig. 14C). To demonstrate further universality and reproducibility of GPR40 full agonism-derived feeding suppression, I finally investigated the effects of T-3601386 in genetically-obese rats and a counteraction of pharmacological (RTX-treated) deafferentation on T-3601386-induced *in vivo* effects. Wistar fatty rats show a highly-obese phenotype without HFD feeding due to a mutation in the leptin receptor gene **[66]**. T-3601386 dose-dependently and significantly decreased food consumption in vehicle-treated Wistar fatty rats (% inhibition vs. control: 18, 40 and 43 % in 3, 10 and 30 mg/kg, respectively) (Fig. 14D). However, in rats treated with RTX, which induces afferent-specific denervation **[69]**, the effects of T-3601386 were markedly attenuated (% inhibition vs. control: 9, 16 and 18 % in 3, 10 and 30 mg/kg, respectively) (Fig. 14D). The significant body weight-lowering effect of T-3601386 in vehicle-treated rats also disappeared in RTX-treated rats (Fig. 14E). The results of a two-way ANOVA showed the significant interaction effects between T-3601386 and RTX (P < 0.01 both in food intake and body weight) (Fig. 14D, E), which precisely and strongly supported the impaired response of T-3601386 after the treatment of RTX. On the other hand, elevations of plasma GLP-1 and GIP after the dosing of T-3601386 were not attenuated in RTX-treated rats (Fig. 14F, G). Successful deafferentation by RTX treatment was confirmed by the disappearance of the capsaicin-induced eye-wipe reaction through the whole of this experiment.

Discussion

As a novel orally-available drug, a GPR40 full agonist is expected to exert not only anti-diabetic but also body weight-lowering effects through its incretin secretory activity in addition to a potent insulinotropic effect. In the present study, I showed that T-3601386 clearly reduces food intake and body weight accompanied by incretin secretion via GPR40. In addition, I also newly found that GPR40 full agonism induces an activation of afferent vagal nerves, which play an important role in full agonism-elicited feeding suppression and weight loss.

T-3601386 showed stronger *in vitro* activity than a partial agonist, fasiglifam, and a full agonist, AM-1638, in CHO cells and GLUTag cells. Next, *in vivo* studies demonstrated that T-3601386 has GPR40-dependent incretinotropic capacity and a beneficial effect on obesity. These results indicated that T-3601386 is a potent and orally available compound showing full agonistic activity for GPR40. Moreover, I have shown that T-3601386 reduces food intake and body weight not only in HFD-feeding obese rats and mice but also in genetically-obese rats, suggesting a clear universality of GPR40 full stimulation-derived feeding suppression. Recent report by Gorski JN. *et al.* indicated that GPR40 full agonist-induced food intake suppression requires GLP-1R activation **[55]**, and in the present study, a single dose of T-3601386 elevated plasma levels of total GLP-1, even after multiple treatments, with no desensitization. From these observations, I speculate that secreted GLP-1 lends major contributions to the durable weight reduction induced by T-3601386.

The elevation of c-Fos immunoreactivity after the dosing of T-3601386 in the present study is the first evidence for NTS activation following GPR40 agonism. NTS activation is known to result from the stimulation of afferent vagal nerve **[57, 58]**,

indicating that T-3601386 interacts with the afferent vagal nerve though unsolved, but GPR40-dependent, mechanisms. Regarding the direct action on vagal nerve, however, almost no expression of GPR40 was reported in vagal afferents compared with the well-known receptors for GLP-1, PYY and CCK **[71]**, indicating the low probability of direct stimulation of GPR40 on the vagal nerve. With respect to indirect effects via metabolic hormones, I have demonstrated in the chapter 1 that insulin, glucagon, GLP-1, GIP and PYY are secreted after *in vivo* GPR40 full agonist stimulation. Among them, similarly with GPR40, the expressions of glucagon and GIP receptors are not observed in vagal afferents **[71]**. Regarding insulin, although some studies reported the expression of insulin receptor in nodose ganglia **[72, 73]**, it remains unclear whether insulin-stimulated vagal afferents can transmit the signal to the NTS/brain and influence eventual physiological effects as GLP-1R lignads can. In my present study, I demonstrated clear *in vivo* NTS activation after dosing of T-3601386 in a GPR40-dependent manner, representing stark evidence for signal transmission from peripheral drug administration leading to activity changes in the brain stem. All of this being considered, it is reasonable to surmise that NTS neurons were activated mainly by secreted GLP-1 (and PYY unmeasured in this study) after GPR40 full stimulation. Of course, I cannot conclude that the elevation of GLP-1, rather than insulin, contributed to NTS activation until I conduct experiments using GLP-1R KO mice and/or GLP-1R antagonists as future studies.

The results in rats with surgical vagotomy and RTX-induced deafferentation clearly showed the involvement of the afferent vagal nerve in feeding suppression and weight loss with T-3601386. Considering at least no decreases in plasma GLP-1 and GIP levels with T-3601386, the peripheral incretin secretion normally occurred via GPR40 even after deafferentation, and subsequent transmission of the anorectic signal to the brain

through the vagal nerve was seemingly disrupted by vagal nerve blockade. As mentioned above, I speculate that the possible main factor for vagally-mediated feeding suppression by T-3601386 would be GLP-1. This hypothesis can be supported by our denervation experiments and the previous findings of high GLP-1R expression on vagal afferents **[71]**, GLP-1R-related appetite suppression through the vagal nerve **[59, 60, 74]** and the disappearance of GPR40-derived food reduction by GLP-1R depletion **[55]**. The incomplete cancellation of T-3601386-induced feeding suppression in models with vagal nerve blockade suggests the existence of other circulating factors delivered to the brain via non-neuronal pathways. Although more detailed mechanisms remain to be elucidated, the afferent vagal nerves, at least in part, contribute to GPR40 full agonism-induced feeding suppression and weight loss, which are probably mediated by secreted metabolic hormones.

In conclusion, a synthetic compound, T-3601386, exerts weight loss associated with its feeding suppression via GPR40. Moreover, I found for the first time that GPR40 full agonism evokes the activation of the vagal nerve-NTS pathway, which plays an important role for feeding suppression and weight loss. My present study provides a novel aspect of GPR40 pharmacology in feeding regulation and raises the possibility that a GPR40 full agonist may become a weight-reducing drug with less CNS adverse effects, since vagally-mediated weight reduction does not always require drug exposure to the CNS.

Figures

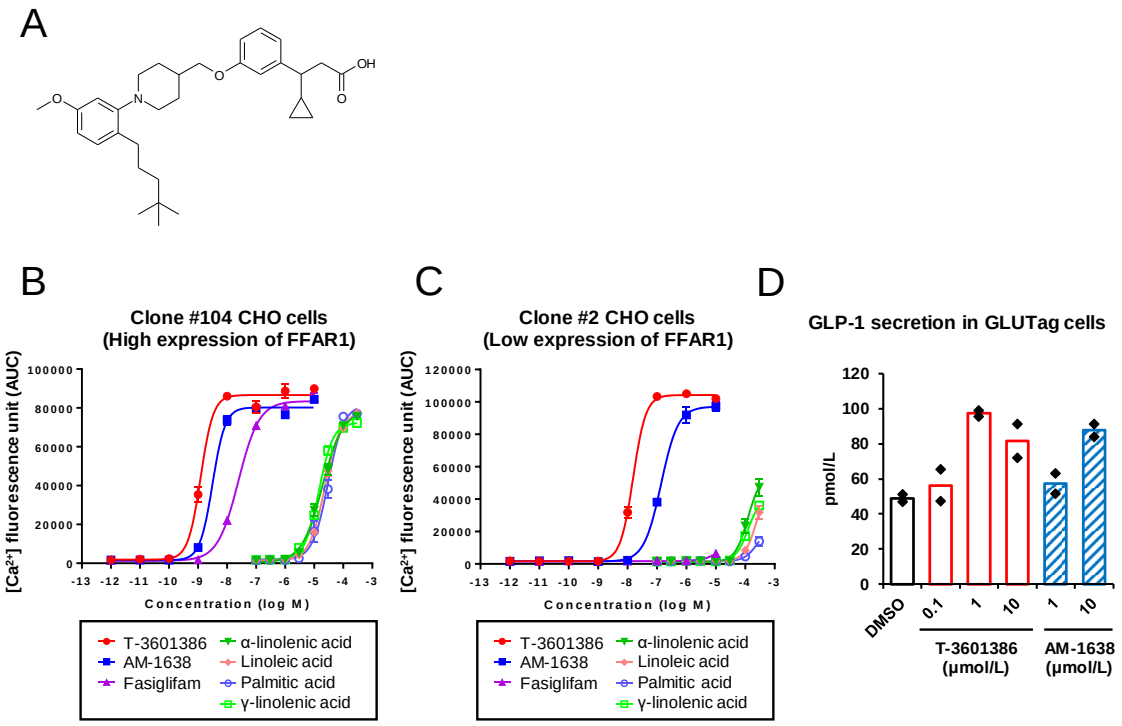


Figure 10. Chemical structure of T-3601386 and *in vitro* activities in CHO cells.

The chemical structure of T-3601386 (A). Intracellular Ca^{2+} concentrations were measured before and after adding samples using FLIPR in CHO cells with high (clone#104) (B) and low expression (clone#2) (C) of human *FFAR1*. Each data point represents the mean ± S.E.M. (quadruplicate). Comparable results were obtained by another independent experiment. Stimulation of GLP-1 secretion in GLUTag cells with 10 mmol/L glucose condition (duplicate) (D).

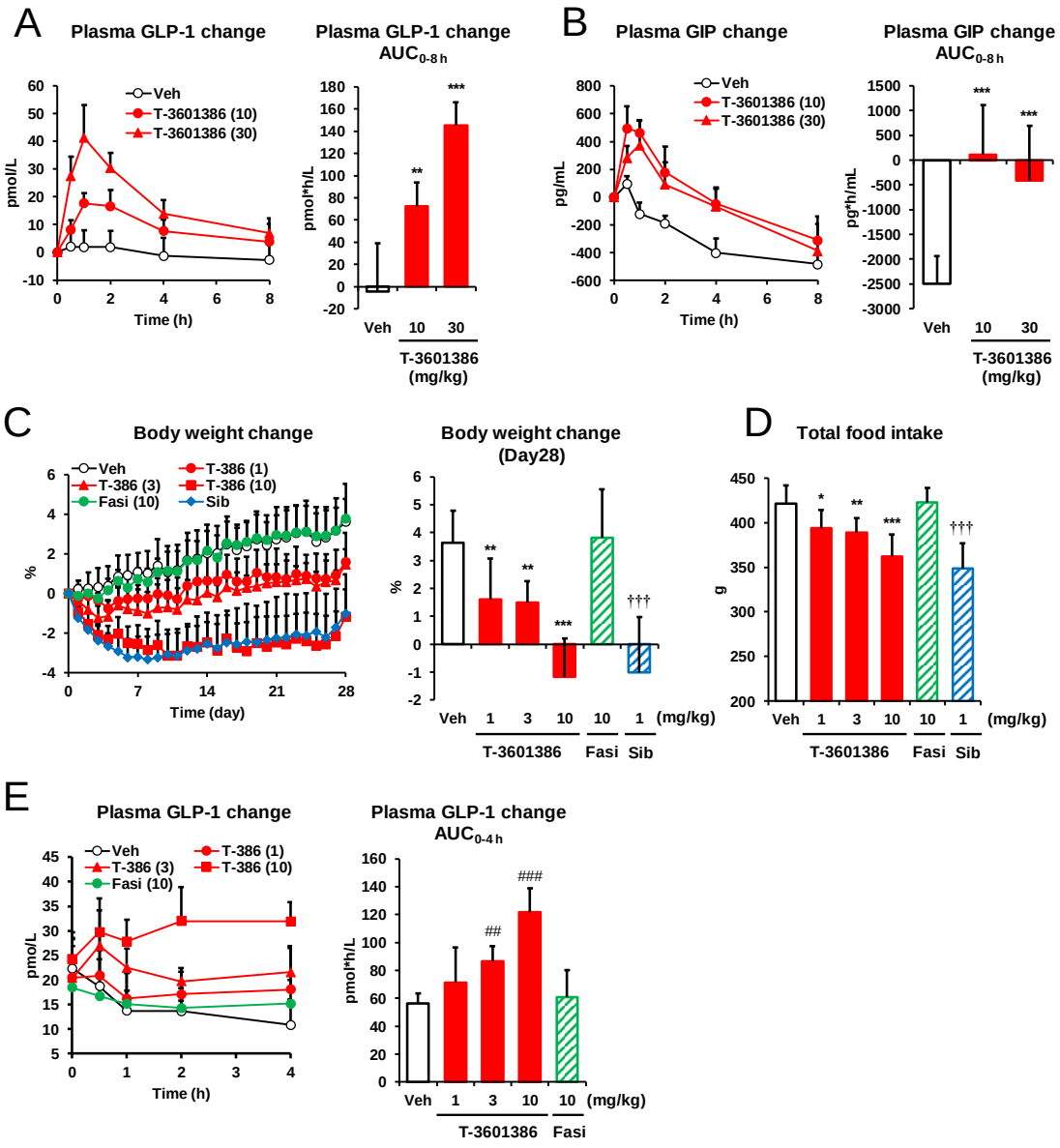


Figure 11. Effects of single or repeated administration of T-3601386 in DIO-F344 rats.

Vehicle or T-3601386 (10 or 30 mg/kg) were orally administered, and plasma level of total GLP-1 and GIP were monitored in DIO-F344 rats (A, B). Graphs showed the time-dependent changes and areas under the curve (0-8 h) of incremental plasma levels of total GLP-1 (A) and GIP (B) after drug administration. Four weeks multiple dosing of vehicle or T-3601386 (1-10 mg/kg), fasiglifam (10 mg/kg) and sibutramine (1 mg/kg) was

performed in DIO-F344 rats (C-E). Body weight change (C) and cumulative food consumption (D) were shown. Time-dependent changes and the area under the curve (0-4 h) of the plasma levels of total GLP-1 by the further single dose after the repeated doses were calculated (E). Veh, vehicle; T-386, T-3601386; Fasi, fasiglifam; Sib, sibutramine. Each data point represents the mean ± S.D. (N = 6 in A, B and N = 6-7 in C-E). * P<0.025, ** P<0.005, *** P<0.0005 and ## P<0.005, ### P<0.0005 vs. vehicle by one-tailed Williams' test and Shirley-Williams test, respectively. ††† P<0.001 vs. vehicle by Student's *t*-test.

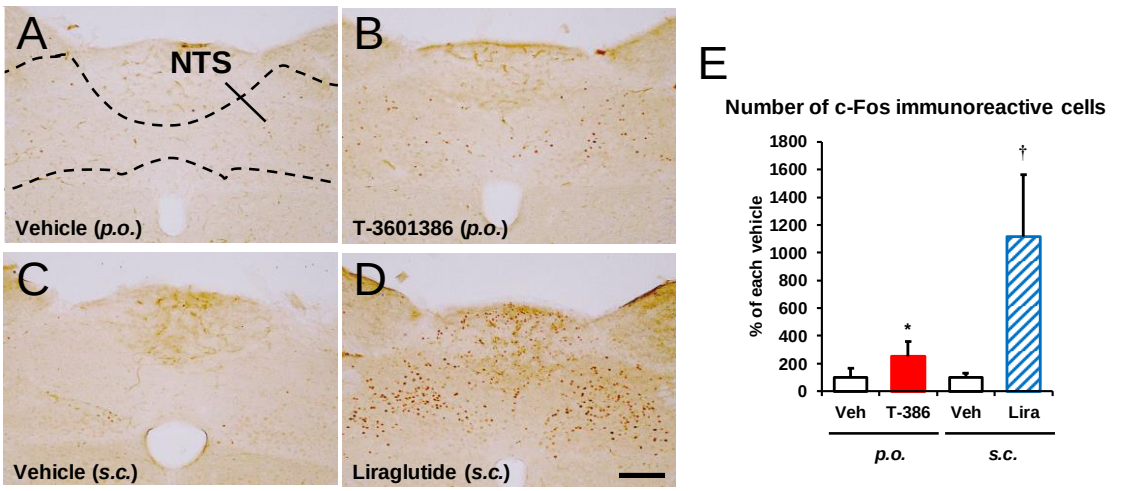


Figure 12. Effects of T-3601386 on c-Fos immunoreactivity in NTS.

SD Rats were dissected to obtain the brain stem regions 3 h after the oral or subcutaneous drug administration. The immunohistochemical images for c-Fos staining after the administration of vehicle (*p.o.*) (A), T-3601386 (*p.o.*) (B), vehicle (*s.c.*) (C) and liraglutide (*s.c.*) (D) were shown. The location of NTS in the brain section was shown in (A) according to the brain atlas **[70]**. c-Fos immunoreactive cells in NTS were automatically counted for their quantification as percentage of each vehicle (E). Bar: 200 μm. Veh, vehicle; T-386, 30 mg/kg of T-3601386; Lira, 100 μg/kg of liraglutide. Each data point represents the mean ± S.D. (N = 4). * $P<0.05$ and † $P<0.05$ vs. each vehicle by Student's *t*-test and Aspin-Welch test, respectively.

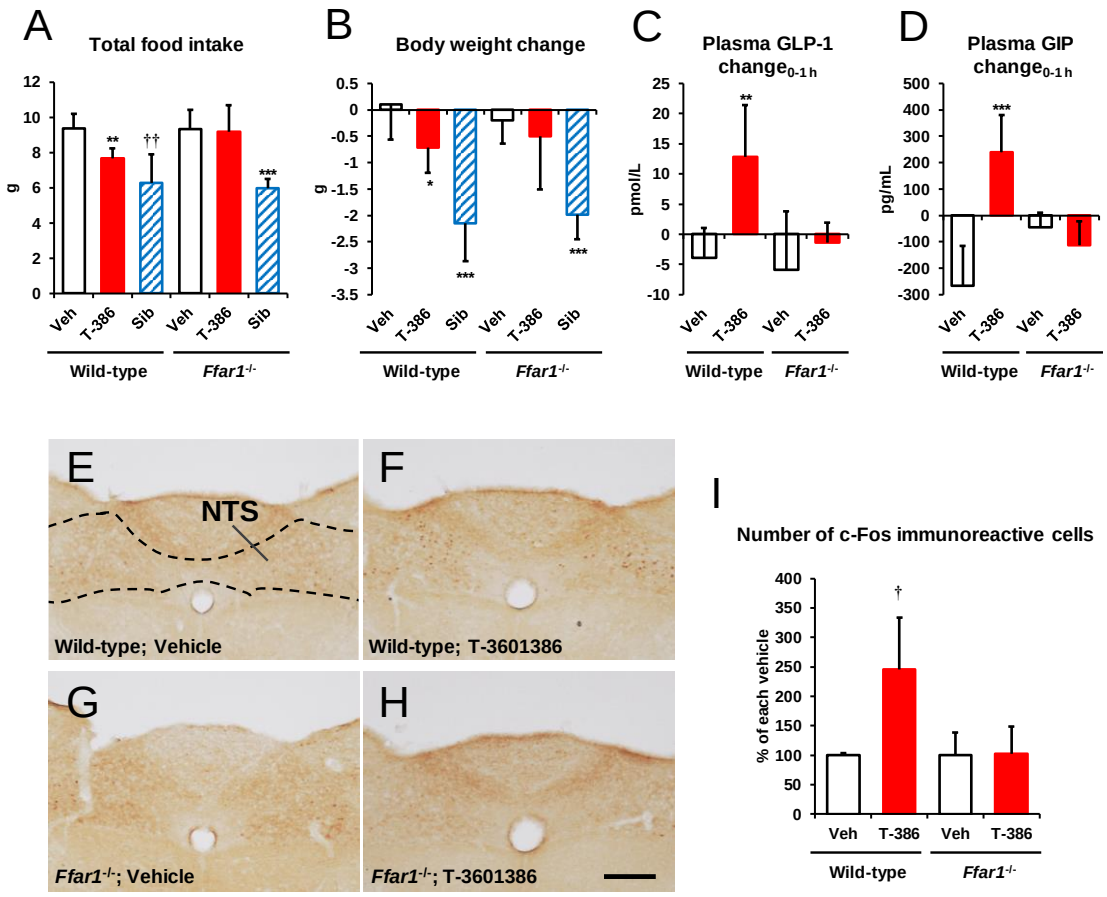


Figure 13. Effects of T-3601386 on food intake, body weight, plasma incretin levels and NTS activation in *Ffar1*$^{-/-}$ and wild-type mice fed HFD.

Vehicle, T-3601386 (30 mg/kg) or sibutramine (10 mg/kg) was orally administered for 3 days in *Ffar1*$^{-/-}$ and wild-type mice (A-D), and the cumulative food consumption (A) and body weight change at the end of experiment (B) were calculated. The plasma levels of total GLP-1 (C) and GIP (D) before and 1 h after the 4th drug administration were measured, and the incremental values of those hormones levels were calculated. *Ffar1*$^{-/-}$ and wild-type mice were dissected to obtain the brain stem regions 3 h after the oral drug administration (E-I). The immunohistochemical images for c-Fos staining after the administration of vehicle (E), T-3601386 (F) in wild-type mice, vehicle (G) and T-

3601386 (H) in *Ffar1*$^{-/-}$ mice were shown. The location of NTS in the brain section was shown in (E). c-Fos immunoreactive cells in NTS were automatically counted for their quantification as percentage of each vehicle (I). Each data point represents the mean ± S.D. (N = 5-6). Bar: 200 μm. Veh, vehicle; T-386, 30 mg/kg of T-3601386; Sib, 10 mg/kg of sibutramine. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and † $P<0.05$, †† $P<0.01$ vs. each vehicle by Student's *t*-test and Aspin-Welch test, respectively.

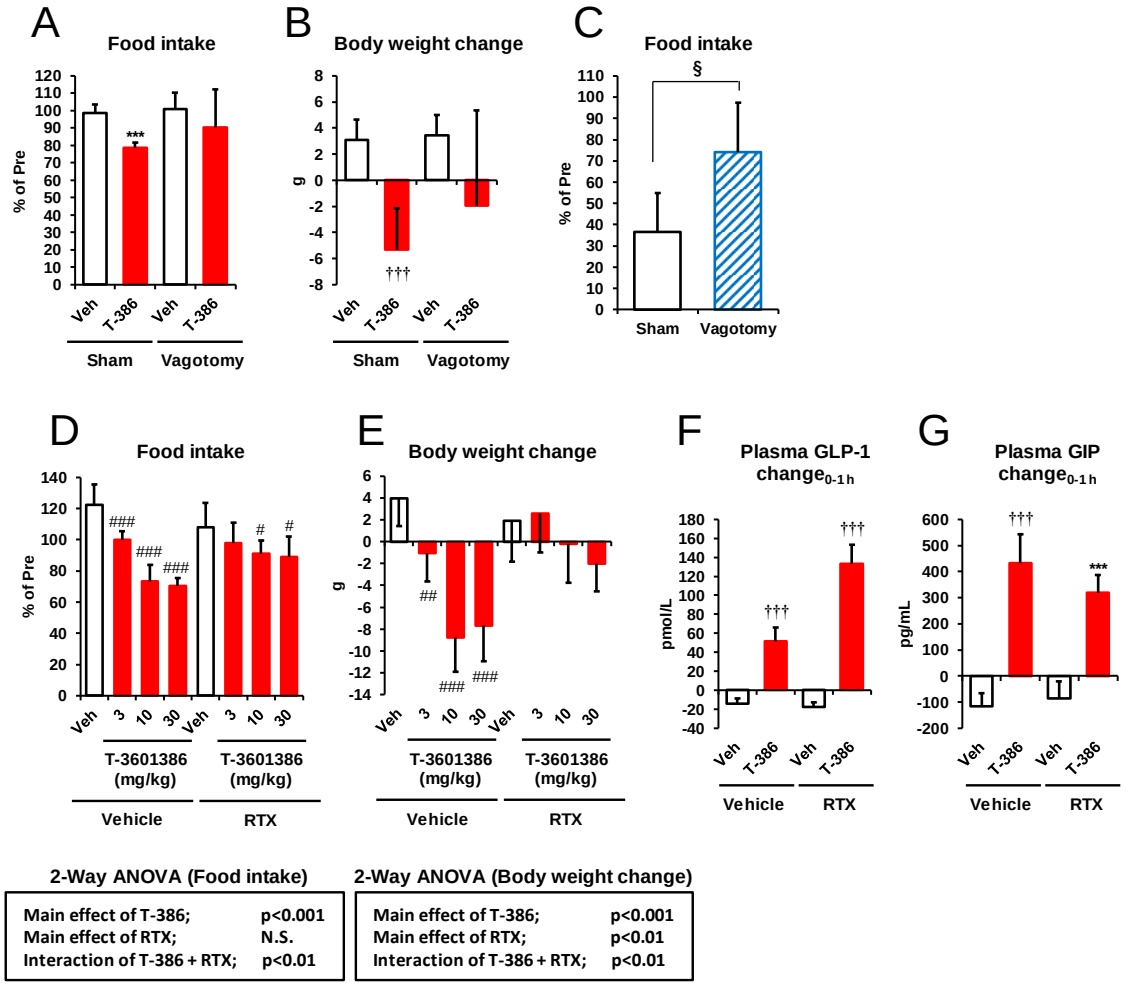


Figure 14. Effects of T-3601386 on food intake and body weight in rats with vagal nerve blockade.

Vehicle or T-3601386 (30 mg/kg) were orally administered in vagotomized or sham-operated rats, and the food consumption for 24 h (A) and body weight change (B) were calculated. As a positive control, CCK-8 (3 μg/kg, *i.p.*)-induced anorectic effects for 30 min in overnight fasted vagotomized or sham-operated rats were shown (C). Vehicle or T-3601386 (3-30 mg/kg) was administered orally to female Wistar fatty rats w/wo RTX treatment, and the food consumption for 24 h (D) and body weight change (E) were calculated. The plasma levels of total GLP-1 (F) and GIP (G) before and 1 hour after the

single administration of vehicle or T-3601386 (10 mg/kg) were measured, and the incremental values of those hormone levels were calculated. The results of two-way ANOVA in (D) and (E) were shown in each inset. Veh, vehicle; T-386, 30 (A, B) or 10 mg/kg (F, G) of T-3601386. Each data point represents the mean ± S.D. (N = 6). # $P<0.025$, ## $P<0.005$, ### $P<0.0005$ vs. vehicle by one-tailed Williams' test, *** $P<0.001$ and ††† $P<0.001$ vs. each vehicle by Student's *t*-test and Aspin-Welch test, respectively. § $P<0.05$ vs. Sham by Student's *t*-test.

General Discussion

Fasiglifam, a partial agonist of GPR40, has been shown to be effective in patients with type 2 diabetes **[30]**. As a novel orally-available drug, a GPR40 full agonist is expected to exert not only anti-diabetic but also body weight-lowering effects through its incretin secretory activity in addition to a potent insulinotropic effect. In the first chapter, I showed that SCO-267, a potent GPR40 full agonist, is highly effective in improving diabetes and obesity. In the second chapter, I newly found that GPR40 full agonism induces an activation of afferent vagal nerves, which play an important role in full agonism-elicited feeding suppression and weight loss.

In diabetes and obesity, sustained efficacy is important for a drug targeting GPCR to induce therapeutic benefits. When a GPCR is exposed to its agonist over a period of minutes to hours or days, the response is significantly reduced and is associated with decreased receptor expression at the plasma membrane. This process is referred to as downregulation **[75, 76]**. Some GPCRs undergo receptor desensitization after repeated stimulation, thus decreasing and, in some cases, abolishing the biological response to a drug **[75, 76]**. In the present study, repeated dosing of GPR40 full agonists resulted in sustained glucose lowering and weight reduction, and the efficacies were much better than those of fasiglifam. This suggests that the efficacies of SCO-267 and T-3601386 are strong and durable. Although the agonism of fasiglifam is partial, it has shown effectiveness in decreasing HbA1c levels in a 52-week study of patients with type 2 diabetes **[32]**. Therefore, GPR40 full agonists used in this study may induce similar durability and better efficacy in humans.

Detailed molecular mechanisms of GPR40 have been clarified in recent years. The crystal structure of human GPR40 bound to fasiglifam, a partial agonist, was revealed

with their unique binding mode, where fasiglifam entered to the non-canonical binding pocket, but not to the canonical orthosteric site as observed in other GPCR ligands **[77]**. In addition, recent studies on co-crystal structure of GPR40-lignad show that the receptor binding site of GPR40 full agonist is clearly distinct from that of a partial agonist like fasiglifam **[78]**, which can explain that a full and a partial agonist function as allosteric modulators on GPR40 each other. Regarding the downstream signaling after receptor-ligand binding, stimulation with GPR40 full agonists elevates not only intracellular Ca^{2+} levels but also cAMP levels, suggesting the activation both of G_q and G_s pathways, although partial agonists such as fasiglifam and AMG837 involve traditional G_q only **[79]**. GPR119 and TGR5 agonists are famous GPCR for their GLP-1 secretory capacity and they conjugate G_s. All things considered, SCO-267 and T-3601386 are speculated to bind to the full agonist-specific site and augment intracellular Ca^{2+} and cAMP levels followed by eventual GLP-1 secretion. Indeed, T-3601386 was demonstrated to bind to the full agonist site in the very recent paper **[80]**.

GLP-1, which is secreted through the specific machinery to full agonism, could contribute to the SCO-267-derived anti-diabetic effects via their insulinotropic actions in addition to the direct GPR40 stimulation on β cells as a main effect. In fact, AM-1638, a well-known GPR40 full agonist, was reported to show the attenuated insulinotropic and glucose-lowering effects in the presence of a GLP-1R antagonist **[37]**. Also regarding feeding suppression, future investigations using GLP-1R deficient mice and/or GLP-1R antagonists will reveal whether GLP-1R signaling plays an important role. On the other hand, GPR40 has been reported to co-localize not only with GLP-1 but also with other intestinal hormones in the gastro-intestinal tract **[81]**, and in fact, SCO-267 also secrete various kinds of hormones as shown in the chapter 1. Previous reports suggested that a

novel strategy through dual receptor agonism of GLP-1/GIP or GLP-1/PYY would lead to a greater weight loss with anti-diabetic effects than each mono-hormonal therapy **[82, 83]**. These results raise the possibility that GIP and/or PYY secreted by GPR40 full agonists potentiate food intake suppression by GLP-1, and that eventually SCO-267 and T-3601386 may behave as an indirect agonist for endogenous dual/triple (or more) receptors.

GPR40 full agonist also secretes glucagon and glucagon receptor signaling has a pivotal role in fat metabolism by promoting lipid oxidation and lowering lipid synthesis in the liver **[84]**. Therefore, the activation of liver glucagon signaling which decreases hepatic lipid would be beneficial in treating liver diseases such as non-alcoholic fatty liver disease (NAFLD) which is common among individuals with type 2 diabetes. In fact, SCO-267 improved NAFLD-related liver parameters independent of glycemic control and weigh loss **[85]**. Because the combination of glucagon receptor and GLP-1R agonisms was also known to improve hepatic steatosis when compared to GLP-1R agonism alone in rodents **[86]**, further studies are needed to clarify the main contributor to improved liver parameters by GPR40 full agonists. The mechanism of GPR40 full agonist is highly complicated due to its secretory capacity of multiple hormones as described so far, and remains to be elucidated with respect to the pharmacological and biological characteristics.

From the results of my present study using potent GPR40 full agonists, I can speculate the biological roles of GPR40, secreted GLP-1 and vagal afferents. In the second chapter, a GLP-1 analogue liraglutide (100 μg/kg) showed clearly stronger NTS activation compared with T-3601386, which probably means the difference in potency of GLP-1R agonism. The peak level of plasma GLP-1 was around 57 pmol/L after the

administration with T-3601386 in DIO rats while liraglutide is known to show efficacy at nmol/L order in rodents and human **[87, 88]**, suggesting that mild NTS activation itself is likely to reflect the endogenous factors-derived activation of afferent vagal nerve by the GPR40 full agonism. Regarding the involvement of vagal nerves with GLP-1 action, the liraglutide-induced anorectic effect was cancelled by subdiaphragmatic vagal deafferentation under the condition of a low dose (10 μg/kg) and short period (3 hours), but not under the condition of a high dose (50 μg/kg) and long period (24 hours) **[89]**, suggesting that the requirement of the vagal nerve in GLP-1R agonism depends on its concentration and/or period of time. All these considered, the degree of vagal nerve activation by endogenously-secreted hormones following GPR40 full stimulation is not so potent that such lower level of GLP-1 elicits an anorectic effect only via vagal afferents while the potent GLP-1R stimulation like exogenous injection of liraglutide also works on the central nervous system through circulation. It is possible that such physiological level of GLP-1 elevation by GPR40 full stimulation mimics a situation where food-derived free fatty acids act on enteroendocrine cells to release intestinal hormones. In fact, oral administration of corn oil including natural ligands for GPR40 induced GLP-1 secretion, which disappeared in *Ffar1*$^{-/-}$ mice **[90]**. In addition, the GLP-1 elevation only in the portal vein, but not in the peripheral blood, was reported to reduce food intake through vagal afferents **[74]**. Another study using rats with GLP-1R knockdown in vagal afferent neurons demonstrated increased meal size and postmeal glycemia **[91]**. These reports are important evidence of the interaction between physiological levels of GLP-1 and vagal afferents in the body. Therefore, GPR40-GLP-1 pathway is thought to play a biologically important role as one of the mechanisms of the vagally-mediated feeding regulation following nutrient sensing.

In conclusion, GPR40 full agonists have a potential to become a novel therapeutic agent for diabetes and obesity, and my research activity using the potent full agonists reveals new pharmacological and biological aspects of GPR40. While I expect GPR40 full agonists to show good efficacy/safety profiles in future clinical trials, additional biological studies will be required to clarify deeper mechanisms of GPR40.

The texts and figures used in this thesis were cited with some modifications from the original articles described below, based on Creative Commons License.

Reference [45]

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