Bioorganic & Medicinal Chemistry xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Orally active ghrelin receptor inverse agonists and their actions on a rat obesity model

Bitoku Takahashi *, Hideaki Funami, Takehiko Iwaki, Hiroshi Maruoka, Makoto Shibata, Makoto Koyama, Asako Nagahira, Yoshiyuki Kamiide, Satomi Kanki, Yoshiyuki Igawa, Tsuyoshi Muto

Asubio Pharma Co., Ltd, 6-4-3, Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan

ARTICLE INFO

Article history:
Received 20 February 2015
Revised 20 May 2015
Accepted 22 May 2015
Available online xxxx

Keywords:
GhrelinR
Inverse agonist
2-Aminoalkyl nicotinamide
Obesity

ABSTRACT

A series of 2-alkylamino nicotinamide analogs was prepared as orally active ghrelin receptor (ghrelinR) inverse agonists. Starting from compound **1**, oral bioavailability was improved by modifying metabolically unstable sites and reducing molecular weight. Brain-permeable compound **33** and compound **24** with low brain permeability were tested in rat models of obesity; 30 mg/kg of compound **33** suppressed weight gain. PK/PD analysis revealed that the anti-obesity effect of ghrelinR inverse agonists depends on their brain concentrations.

1. Introduction

Ghrelin is a gut-derived 28-amino-acid peptide that has an important role in food intake and energy homeostasis and is the endogenous ligand for growth hormone secretagogue receptor 1a (GHS-R 1a),[1] currently known as the ghrelin receptor (ghrelinR). It has been suggested that ghrelin acts as a feeding signal as the plasma ghrelin level increases before meals and declines after eating,[2] and ghrelin is the only peripheral orexigenic hormone identified to date.[3] Besides orexigenic action, ghrelin is known to regulate glucose homeostasis,[4] gastric motility[5] and addictive behavior.[6] Therefore, disruption of ghrelin signal is an attractive target for anti-obesity, anti-diabetes and anti-alcohol addiction therapeutics.[7]

In determining target profiles of small molecules that suppress ghrelinR signaling, we considered two things: antagonists or inverse agonists and peripheral or central. GhrelinR exhibits high constitutive activity, and the constitutive signal reaches almost half that of ghrelin-induced activity,[8] which implies that ghrelinR inverse agonists might suppress the signal more effectively than ghrelinR antagonists. Nevertheless, ghrelinR inverse agonists are relatively scarce and have been reported only recently by Merck,[14] AstraZeneca[15] and Pfizer.[16] Therefore, we decided to search for inverse agonists. It is said that orexigenic action of ghrelin is caused mainly via three routes: the vagal afferent nerves, the pituitary gland, and the central nervous system (CNS). Although how much each route works remains under dispute, some data showed that the vagal afferent nerves route is dominant[17,18] whereas other data showed that the CNS effect is dominant.[19–21] While almost all anorectic drugs act through CNS targets, from the viewpoint of drug discovery, a non-CNS anti-obesity drug is attractive because of the lower risk of CNS-related adverse effects. During the target validation, our in-house data reproduced the result that ghrelin-induced food intake was canceled in vagotomized rats,[17] which encouraged us to seek peripheral ghrelinR ligands.

Our first effort led to a non-CNS penetrable ghrelinR inverse agonist (**1**)[22] whose brain/plasma ratio (B/P ratio) was 0.005 (Fig. 1). This compound suppressed ghrelin-induced growth hormone (GH) release in rats, ghrelin-induced gastric mobility in mice, ghrelin-induced food intake in mice and cumulative food intake for two weeks in high-fat diet-induced obesity (HFDIO) mice, which further supports the idea that peripheral ghrelinR inhibition can be sufficient for reducing food intake. Since compound **1** is not orally available, we tried to optimize the structure to improve oral bioavailability.

2. Results and discussion

2.1. Chemistry

The 5-diazabicyclo pyridine analogs were prepared by the method depicted in Scheme 1. Optically pure (*S*)-2-aminomethyl-

* Corresponding author. Tel.: +81 78 306 5309; fax: +81 78 306 5047.
E-mail address: takahashi.bitoku.cz@asubio.co.jp (B. Takahashi).

http://dx.doi.org/10.1016/j.bmc.2015.05.047
0968-0896/

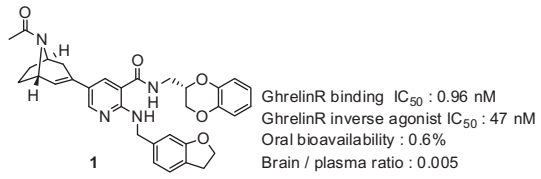
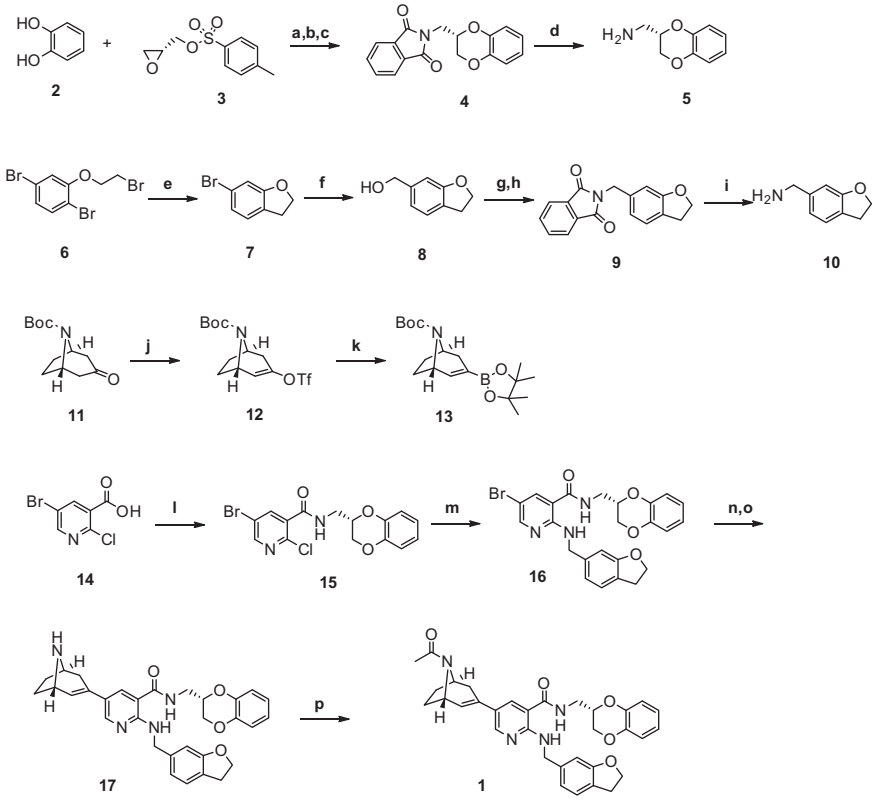


Figure 1. A previously reported ghrelinR inverse agonistic 2-aminoalkyl nicotinamide derivative.

1,4-benzodioxane (**5**) was prepared using a chiral glycidol derivative.[9] Catechol and *p*-toluenesulfonic acid (2*R*)-(−)-glycidyl ester were coupled to yield (*S*)-2-hydroxymethyl-1,4-benzodioxane and the hydroxyl group was converted to an amino group via a phthalimide intermediate (**4**). The dihydrobenzofuran ring of compound **7** was constructed by intramolecular C–C bond formation,[10] and the bromo group of compound **7** was converted to a hydroxymethyl group by lithiation, followed by paraformaldehyde treatment. The same procedure for compound **5** was adopted for the conversion of the hydroxymethyl group to the aminomethyl group to yield **10**. The diazabicyclo boronate intermediate (**13**) was prepared by the method described in the literature[11] with slight modification. Commercially available *N*-Boc-nortropine was converted to vinyl triflate **12** and the triflate was coupled with bis(pinacolate)diboron to yield the boronate. Finally, these intermediates (**5**, **10** and **13**) were coupled with the nicotinic acid scaffold (**14**). The amidation of compound **14** and compound **5** was performed using BOP reagent and the amination at the 2-position of the pyridine ring was accomplished by simply heating the mixture of reactants. Suzuki coupling of the aryl bromide (**16**) and the borate (**13**) proceeded smoothly, and following deprotection and acetylation yielded the final product **1**. The yield of each reaction was moderate to good, and the whole scheme was applicable to 10 g scale synthesis without difficulty.

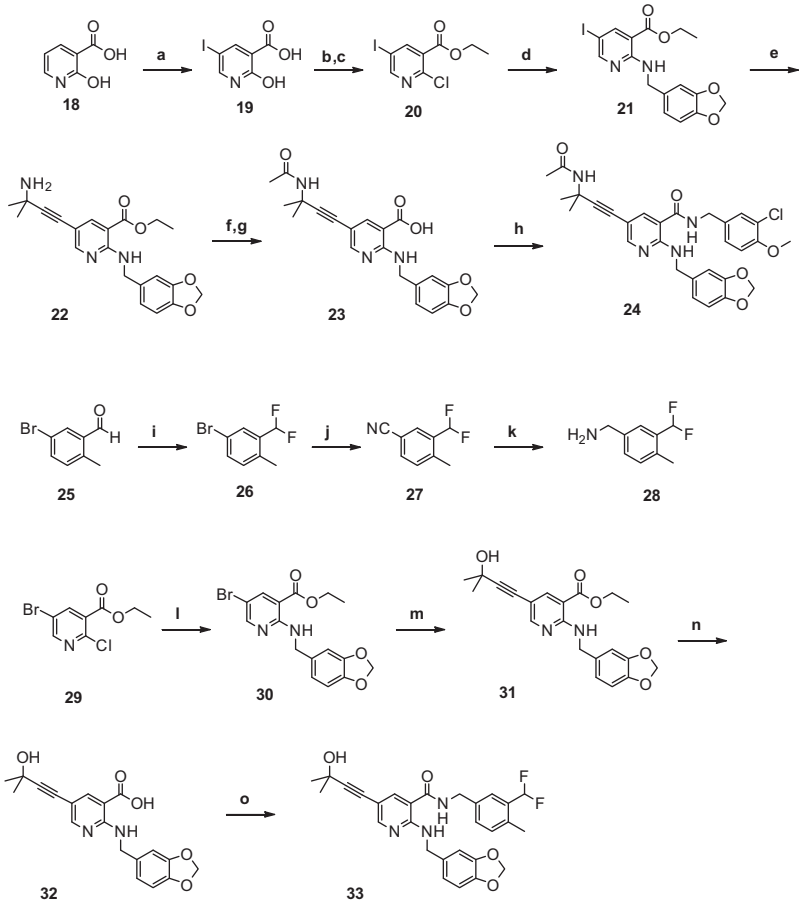
By analyzing the ^{1}H NMR spectrum of compound **1**, we found that protons at the pyridine ring and the diazabicyclo moiety were split into two peaks, and the integral ratio of the peaks was approximately 0.45:0.55. Because compound **1** is a mixture of diastereomers, each diastereomer was separated through a chiral column and analyzed by ^{1}H NMR spectroscopy. Interestingly, NMR spectra of both isomers were almost identical, and the splits of the peaks were also observed. We, therefore, concluded that the splits indicate *cis*- and *trans*-rotamers of the amide bond on the bridge-head nitrogen.

Preparation of the alkynyl pyridine analogs is described in Scheme 2. The key intermediate **20** was prepared from commercially available 2-hydroxy nicotinic acid **18** through 5-iodination using *N*-iodosuccinimide, chlorination using thionyl chloride, and esterification. Compound **20** was coupled with 3,4-(methylenedioxy)benzylamine by heating without solvent, and an alkynyl moiety was introduced by a Sonogashira reaction. No protection of 2-methyl-3-butyl-2-amine was necessary in the reaction, and compound **22** was obtained in good yield (90%). Acetylation, ester hydrolysis, and amide coupling with 3-chloro-4-methoxybenzylamine yielded compound **24**. The amide part of compound **33** was prepared from compound **25** through di-fluorination using *N*,*N*-diethylaminosulphur trifluoride (DAST), cuprate coupling to introduce a cyano group, and reduction of the cyano group. Starting from the 5-bromo intermediate **29**, compound **33** was prepared by applying the same procedure for compound **24**. All the



Scheme 1. Reagents and conditions: (a) K_2CO_3 DMF, 60 °C; (b) TsCl, pyridine, rt; (c) phthalimide potassium, DMF, 90 °C, 57% (3 steps); (d) hydrazine hydrate, MeOH, reflux, 88%; (e) *n*-BuLi, THF, paraformaldehyde, −74 °C, 74%; (f) *t*-BuLi, THF, paraformaldehyde, −74 °C, 74%; (g) $SOCl_2$ CH_2Cl_2, rt; (h) phthalimide potassium, DMF, 90 °C, 69% (2 steps); (i) hydrazine hydrate, MeOH, 77 °C, 80%; (j) LDA, THF, −78 °C, then, $PhNTf_2$ rt, 46%; (k) bis(pinacolato)diboron, $PdCl_2$(dppf), KOAc, dioxane, 80 °C, 67%; (l) **5**, BOP reagent, DIPEA, DMF, 0 °C–rt, 93%; (m) **10**, 160 °C, 74%; (n) **13** $Pd(pph_3)_4$, K_3PO_4, dioxane, H_2O, 100° C; (o) TFA, CH_2Cl_2, rt, 61% (2 steps); (p) AcCl, Et_3N, CH_2Cl_2, rt, 72%.

Please cite this article in press as: Takahashi, B.; et al. *Bioorg. Med. Chem.* (2015), http://dx.doi.org/10.1016/j.bmc.2015.05.047



Scheme 2. Reagents and conditions: (a) *N*-iodosuccinimide, DMF, 50 °C, 76%; (b) $SOCl_2$, DMF, 70 °C; (c) $SOCl_2$, DMF, EtOH, 80 °C, 95% (2 steps); (d) 3,4-(methylenedioxy)-benzylamine, 110 °C, then, CH_3CN, H_2O, 80 °C–rt, 96%; (e) 2-methyl-3-butyn-2-amine, $PdCl_2(PPh_3)_2$, CuI, Et_3N CH_3CN, 60 °C, 90%; (f) AcCl, Et_3N, CH_3Cl_3, 0 °C; (g) 1 N NaOH, MeOH, 50 °C, 95% (2 steps); (h) 3-chloro-4-methoxybenzylamine, BOP reagent, DIPEA, DMF, 0 °C, 85%; (i) DAST, CH_2Cl_2, 0 °C, 79%; (j) CuCN, pyridine, DMF, microwave (190 °C), 87%; (k) Pd–C, H_2, MeOH, rt, 97%; (l) 3,4-(methylenedioxy)-benzylamine, 110 °C, 45%; (m) 3-methyl-1-butyn-3-ol, $PdCl_2(PPh_3)_2$, CuI, Et_3N, 110 °C, 85%; (n) 1 N-NaOH, MeOH, 60 °C, 88%; (o) **28**, BOP reagent, DIPEA, DMF, 0 °C–rt, 89%.

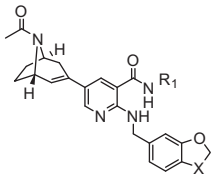
reactions proceeded in moderate to good yield and were no problem in 10 g scale synthesis.

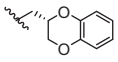
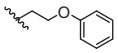
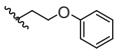
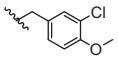
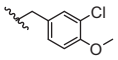
2.2. Structure–activity relationship

Because poor oral availability of compound **1** was considered to be derived from a high metabolic rate, structural modification was aimed at improving the metabolic stability while keeping the main activity. GhrelinR binding affinity was evaluated by a radiolabeled ghrelin substitution assay, and inverse agonist activity was evaluated by a reporter gene assay. Potencies of the compounds were depicted as IC_{50} values. As in a previous structure activity relationship (SAR) study, all compounds were pure inverse agonists, which almost completely suppressed the constitutive activity of ghrelinR at concentrations sufficiently higher than the IC_{50}. Metabolic sites were estimated by analyzing metabolites in an in vitro liver microsome assay, which showed that most metabolic reactions, mostly oxidations, occurred on the 3- and 2-position moieties of the pyridine ring. Therefore, we modified the structures of these parts (Table 1). Regarding the main activity, structural tolerance at the 2-position of the pyridine ring was very narrow, and we could only convert the carbon atom to an oxygen atom on the dihydrobenzodioxane ring. The 3-position of the pyridine ring was rather structurally tolerable, and the benzodioxane ring could be substituted for a simple phenoxy group. Although this modification seemed to be beneficial because the molecular weight was decreased and the chiral center was eliminated without loss of activity, the metabolic stability was not improved (**34a** and **34b**). Another substitution with potent activity was the 3,4-substituted benzyl group such as in **34c** and **34d**. Metabolic stability was not improved in combination with the dihydrobenzofuran moiety at the 2-position of the pyridine ring (**34c**). However, improvement of metabolic stability was observed in combination with 3,4-(methylenedioxy)benzylamine (**34d**). Although the metabolic rate was improved, the pharmacokinetic study revealed that oral bioavailability of compound **34d** was not improved (as discussed later). We then decided to put priority on seeking other substitutions at the 5-position of the pyridine ring rather than further analyzing the properties of diazabicyclo pyridine derivatives because of the chirality of the diazabicyclo moiety and the rotation barrier of amide bond discussed above, which can sometimes result in troublesome atropisomers.[12]

We aimed for a simpler structure with reduced molecular weight and found that the diazabicyclo ring can be replaced by propargyl amine (**24**) or propargyl alcohol type substitutions without loss of activity. These derivatives have lower molecular weights, no chirality, and, surprisingly, good oral bioavailability. The reason for the improvement of oral bioavailability remains to be elucidated. Further optimization of the substitution at the 3- and 5-position of the pyridine ring led to the highly potent compound **33** whose molecular weight is 507, which is not so high

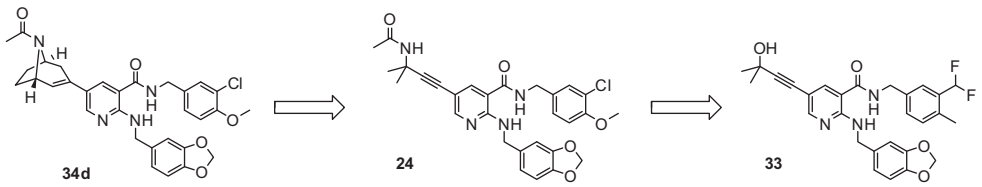
Table 1
GhrelinR binding affinity, inverse agonist IC_{50}, and metabolic rate for selected compounds varying R_1 and X



Compd	R_1	X	GhrelinR binding IC_{50} (nM)		Inverse agonist IC_{50}[a] (nM)	Metabolic rate[b] (pmol min^{-1} mg^{-1})
			Rat	Human		
1		CH_2	0.96	0.39	47	302
34a		CH_2	2.1	N.D.[c]	28	358
34b		O	9.1	N.D.[c]	80	202
34c		CH_2	5.9	0.42	9.4	211
34d		O	5.6	0.30	9.8	39

All values indicate mean of at least two independent measurements.
[a] All compounds showed the same efficacy (95% block).
[b] Metabolic rate in human liver microsomes.
[c] No data.

Table 2
GhrelinR binding affinity, inverse agonist IC_{50}, and metabolic rate, total body clearance, oral bioavailability, and brain/plasma ratio for selected compounds



Compd	Binding IC_{50} (nM)		Inverse agonist IC_{50}[a] (nM)	Agonist IC_{50}[b] (nM)		Metabolic rate[c] (pmol min^{-1} mg^{-1})	CLtot (L h^{-1} kg^{-1})	Oral BA (%)	B/P ratio[f]
	Rat	Human		Calcium	Pituitary GH				
34d	5.6	0.30	9.8	100	32.8	39	10.79[d]	7.2[d]	N.D.
24	6.6	3.0	26	34	36.2	81	1.67[e]	46.4[e]	0.07[e]
33	0.28	0.44	5.4	13	11.6	103	0.74[e]	23.8[e]	0.21[e]

All values indicate mean of at least two independent measurements.
[a] All compounds showed the same efficacy (>95% block).
[b] All compounds blocked >95% of ghrelin-induced activity.
[c] Metabolic rate in human liver microsomes.
[d] Measured in male ICR mice.
[e] Measured in male SD rats.
[f] Determined at 2 h after intravenous administration.

among ghrelinR ligands.[13] Antagonistic activity of the compounds was tested in a calcium efflux assay (FLIPR) and a GH release assay using primary rat pituitary cells. Because inverse agonist activity cannot be evaluated in these assays, we adopted them only for evaluating antagonist activity using ghrelin as an agonist. These compounds blocked ghrelin-induced calcium efflux and GH release almost completely at high concentrations, showing that they also have antagonist activity. Antagonist potency of **34d** was lower than inverse agonist potency, and antagonist potencies of **24** and **33** were similar to inverse agonist potencies.

2.3. In vivo evaluation

2.3.1. Pharmacokinetics

Pharmacokinetic data were obtained in normal male ICR mice (**34d**) or normal male SD rats (**24** and **33**) as depicted in Table 2. In spite of a lower metabolic rate in human liver microsomes (HLM), total body clearance (CLtot) of compound **34d** was very high, which resulted in low oral bioavailability (7%). CLtot of **24** and **33** were lower, and therefore, these compounds showed good oral bioavailability (46.4% and 23.8%,

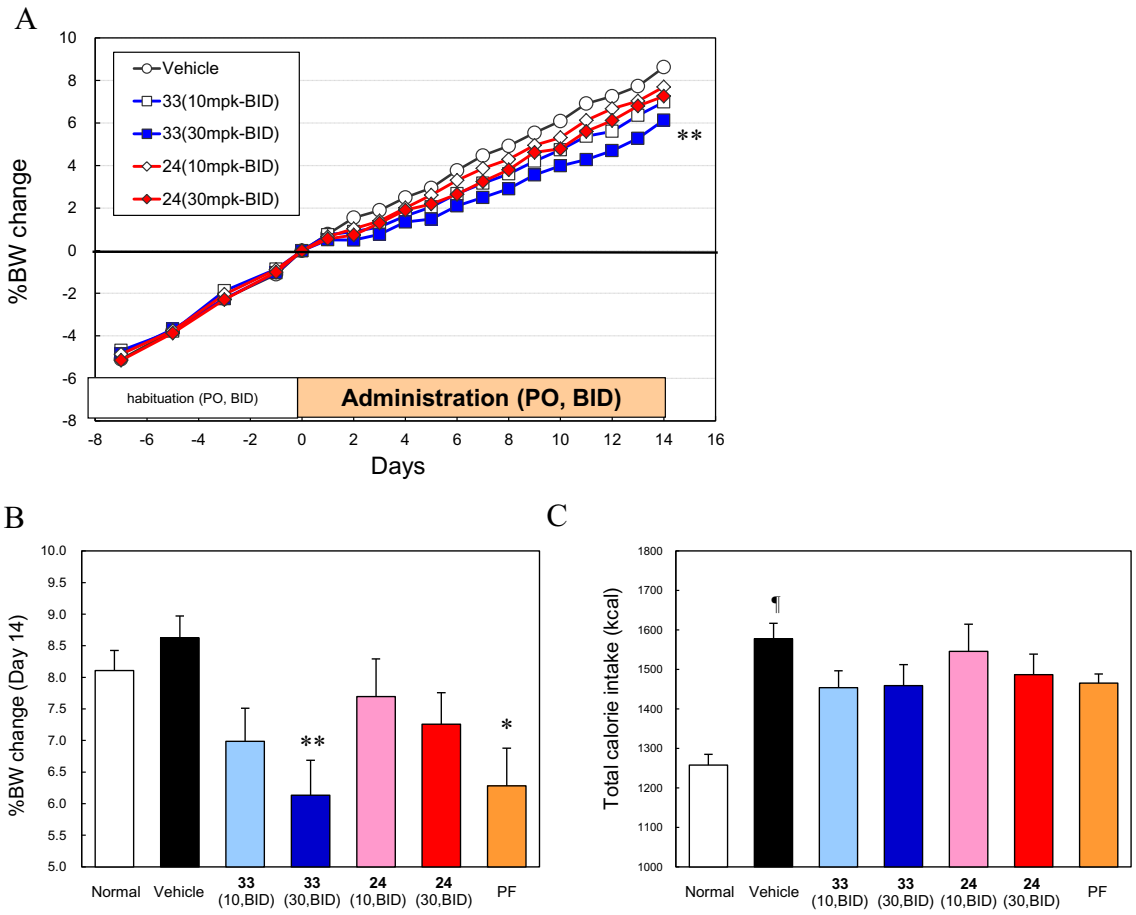


Figure 2. Effect of twice daily oral treatment of compounds **24** or **33** on body weight (A and B) and food intake (C) in HFDIO rats. (A) Daily body weight change before and after the treatment. (B) Body weight change on day 14 from day 0. (C) Total calorie intake between day 0 and day 14. PF indicates pair-fed group. *Denotes $p<0.05$ versus vehicle, **$p<0.01$ versus vehicle, and ¶denotes $p<0.001$ versus normal. (Dunnet's test) BW, body weight; BID, twice daily; mpk, mg/kg; PO, post oral; HFDIO, high-fat diet-induced obesity.

Table 3
Plasma and brain concentrations and their ratios against binding IC_{50} for PK/PD analysis

	Compound **33**		Compound **24**	
	10 mg/kg BID[d]	30 mg/kg BID[d]	10 mg/kg BID[d]	30 mg/kg BID[d]
Brain/plasma ratio	0.21		0.07	
Plasma protein binding (%)	99		99	
AUC_{0-24} (ng * h/mL)	1105	3314	24901	74704
Mean plasma concn[a] (nM)	91	272	1890	5670
Mean brain concn[b] (nM)	19	57	132	370
Mean plasma concn/binding IC_{50}	324	972	286	859
Mean brain concn/binding IC_{50}	68	203	19	58
Mean plasma free conc[c]/binding IC_{50}	3.2	97	2.9	86
Mean brain free conc[c]/binding IC_{50}	0.7	2.0	0.2	0.6

[a] Calculated by $AUC_{0-24}/24$ (h).
[b] Calculated by (mean plasma concn) * (B/P ratio).
[c] Free concentration was calculated by multiplying the free fraction with total concentration.
[d] Twice daily.

respectively). The B/P ratio of compound **24** was low (0.07) and that of **33** was moderate (0.21), both of which were higher than the lead compound **1**. However, we thought that a B/P ratio of 0.07 is acceptable for a peripheral drug if its pharmacological action depends on plasma concentration. Although compound **33** penetrated the blood-brain barrier to some extent, we evaluated this compound in comparison with compound **24** in an in vivo pharmacology experiment in order to prove our concept.

2.3.2. Pharmacology and PK/PD analysis

We chose HFDIO rats in this study because the roles of the vagal afferent nerve in ghrelin signaling have been well characterized in rats. Specifically, ghrelin-induced food intake and GH release were canceled in vagotomized rats,[17] and ghrelinR mRNA expression was increased in high-fat diet induced obesity-prone rats but not in low-fat diet rats or high-fat diet-induced obesity-resistant rats,[23] but these results were not reproduced in mice (in house data).

HFDIO rats were treated with 10 mg/kg or 30 mg/kg of compound **33** or **24** through twice daily oral administration. Body weight was increased constantly during the administration period in the vehicle group the same as during the habituation period (Fig. 2A). The daily weight increase was attenuated in treated groups and statistically significant attenuation was observed on day 14 only at 30 mg/kg of compound **33**. A slight decrease in total calorie intake for 14 days was observed in treated groups, but was not statistically significant. Although a statistically significant decrease in food intake was not observed, attenuation of body weight gain of rats treated with compound **33** was conceivably ascribed to the suppression of food intake because pair-fed animals showed similar body weight changes. Plasma concentration in an in vivo disease model was monitored at the first day of the administration and used for PK/PD analysis in combination with the B/P ratio in normal rats and in vitro plasma protein binding. The plasma and brain concentrations of the compounds and the ratio of each free concentration versus binding IC_{50} are described in Table 3. Considering the binding IC_{50} and B/P ratio, the difference between compounds **33** and **24** can be reduced to the difference in brain concentration. Mean plasma free concentration/binding IC_{50} were similar (3.2 vs 2.9 and 97 vs 86 in 10 mg/kg and 30 mg/kg, respectively), and mean brain free concentration/binding IC_{50} was three times higher for compound **33** than **24** (0.7 vs 0.2 and 2.0 vs 0.6 in 10 mg/kg and 30 mg/kg, respectively). Therefore, the anti-obesity activity of compound **33** seems to be derived from suppression of CNS ghrelinR activity.

3. Conclusions

Starting from compound **1**, we improved oral bioavailability by modifying the structures of metabolic sites and changing the substitution at the 5-position of the pyridine ring. The B/P ratio increased during the structural modification, and we chose two compounds with low and moderate brain penetration. Pharmacological evaluation in HFDIO rats revealed that the brain-permeable compound attenuated weight gain effectively. Therefore, we concluded that the anti-obesity activity of ghrelinR inverse agonists is attributed to the suppression of brain ghrelinR activity.

4. Experimental

4.1. Chemistry

Proton nuclear magnetic resonance spectra (^{1}H NMR) and carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded on Brucker ARX-400 or Brucker Avance III (400 MHz) spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per million relative to the internal standard tetramethylsilane. High-resolution mass spectra (HRMS) and fast atom bombardment (FAB) mass spectra were recorded on JEOL JMS-700 mass spectrometer. Electro-spray ionization (ESI) mass spectra were recorded on Agilent G1956A MSD spectrometer system. Purity was analyzed by Agilent 1100 HPLC system. HPLC conditions utilized are as follows; column: YMC-Pack Pro C18, 4.6 mm × 100 mm, gradient and mobile phase: 10% to 100% solvent B in solvent A for 8 min, solvent A = MeCN/H_2O/trifluoroacetic acid (5:95:0.1), solvent B = MeCN/trifluoroacetic acid (100:0.09), UV detector: 210 nm, flow rate: 1 mL/min. Retention times (t_R) are in minutes and purity is calculated as% total area. Other chemical reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, Kanto Kagaku or Nacalai tesque and used without purification. Flash column chromatography was performed using Merck Silica gel 60 (230–400 mesh) or Purif-Pack® SI 30 μm supplied by Shoko Scientific.

4.1.1. (*S*)-2-((2,3-Dihydrobenzo[*b*][1,4]dioxin-2-yl)methyl)isoindoline-1,3-dione (4)

To a solution of catechol (24.12 g, 219 mmol) and *p*-toluenesulfonic acid (2*R*)-(−)-glycidyl ester (50 g, 219 mmol) in DMF (500 mL) was added potassium carbonate (45.4 g, 329 mmol) and the mixture was stirred at 60 °C for 48 h. The mixture was concentrated in vacuo and quenched with water. After extraction with [EtOAc/hexane = 1:1] solution, the organic phase was washed with water and brine, then dried over Na_2SO_4, filtered and concentrated in vacuo to obtain (*S*)-(2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)methanol as a pale red solid (36.4 g). The solid was dissolved in pyridine (145 mL) and *p*-toluenesulfonyl chloride (50.1 g, 263 mmol) was added to the solution. The mixture was stirred at room temperature for 2 h, then 3 N HCl (1000 mL) was slowly added at 0 °C. After extraction with ether, the organic phase was washed with water and brine, then dried over Na_2SO_4, filtered and concentrated in vacuo to obtain (*R*)-(2,3-dihydrobenzo[*b*][1,4]-dioxin-2-yl)methyl 4-methylbenzenesulfonate as a pale yellow oil (60.45 g). The oil was dissolved in DMF (300 mL) and phthalimide potassium salt (45.4 g, 245 mmol) was added to the mixture. The mixture was stirred at 90 °C for 20 h and cooled to room temperature, then MeOH (1.5 L) was added with stirring. The resulting solid was filtered, washed with MeOH and dried in vacuo to give **4** as a white solid (36.9 g, 57% from catechol).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.88 (m, 2H), 7.75 (m, 2H), 6.86 (m, 4H), 4.51 (dq, *J* = 2.4, 6.4, 1H), 4.31 (dd, *J* = 2.4, 12.0 Hz, 1H), 4.10 (dd, *J* = 6.4, 14.4 Hz, 1H), 4.05 (dd, *J* = 6.4, 12.0 Hz, 1H), 3.90 (dd, *J* = 6.0, 14.4 Hz, 1H); MS (ESI+) 296.1 $(M+H)^+$.

4.1.2. (*S*)-(2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)methanamine (5)

To a solution of **4** (15 g, 50.8 mmol) in MeOH (300 mL) was added hydrazine hydrate (12.7 g, 254 mmol) and the mixture was stirred at reflux for 2 h. After cooling, chloroform (500 mL) was added to the mixture and insolubles were removed by filtration. The filtrate was concentrated in vacuo and treated with 0.5 N NaOH (200 mL), then extracted with ether. The organic phase was washed with 0.5 N NaOH, water and brine, then dried over Na_2SO_4, filtered and concentrated in vacuo to obtain **5** as a colorless oil (7.36 g, 88%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.86 (m, 4H), 4.27 (dd, *J* = 2.4, 11.4 Hz, 1H), 4.13 (m, 1H), 4.02 (dd, *J* = 7.8, 11.2 Hz, 1H), 2.99 (dd, *J* = 3.2, 5.6 Hz, 2H); MS (ESI+) 166.1 $(M+H)^+$.

4.1.3. 6-Bromo-2,3-dihydrobenzofuran (7)

To a solution of 1,4-dibromo-2-(2-bromoethoxy)benzene (22.3 g, 62.2 mmol) in THF (180 mL) was added 1.57 M *n*-BuLi in hexanes (40 mL, 62.8 mmol) under Ar at −76 °C. After stirring at −76 °C overnight, the mixture was quenched with sat. NH_4Cl aq and extracted with AcOEt. The organic phase was washed with brine and dried over Na_2SO_4. After filtration and evaporation, the residue was recrystallized from ethanol (28 mL) and water (3 mL) to obtain **7** as a white solid (9.4 g, 76%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.03 (d, *J* = 7.6 Hz, 1H), 6.97 (d, *J* = 1.6 Hz, 1H), 6.94 (dd, *J* = 1.6, 7.6 Hz, 1H), 4.59 (t, *J* = 8.8 Hz, 2H), 3.15 (t, *J* = 8.8 Hz, 1H); MS (ESI+) 200.1 $(M+H)^+$.

4.1.4. (2,3-Dihydrobenzofuran-6-yl)methanol (8)

To a solution of **7** (4.29 g, 21.6 mmol) in THF (43 mL) was added 1.76 M *t*-BuLi in pentane (25.7 mL, 45.2 mmol) under Ar at −74 °C. Paraformaldehyde (0.97 g, 32.3 mmol) was added to the mixture at −74 °C and the mixture was stirred at room temperature overnight. The mixture was quenched with satd NH_4Cl aq and extracted with AcOEt. The organic layer was washed with brine and dried over $MgSO_4$, then filtered. After removal of the solvent, the residue was recrystallized from CH_2Cl_2 (6.5 mL) and *n*-hexane (25.9 mL) to obtain **8** as a pale red solid (2.39 g, 74%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.16 (d, J = 7.2 Hz, 1H), 6.84 (d, J = 7.2 Hz, 1H), 6.81 (s, 1H), 4.63 (br s, 2H), 4.58 (t, J = 8.8 Hz, 2H), 3.20 (t, J = 8.8 Hz, 2H); MS (ESI+) 151.1 $(M+H)^+$.

4.1.5. 2-((2,3-Dihydrobenzofuran-6-yl)methyl)isoindoline-1,3-dione (9)

A solution of **8** (2.38 g, 15.8 mmol) in CH_2Cl_2 (15.8 mL) was cooled with ice bath and $SOCl_2$ (9.44 g, 79 mmol) was added dropwise. The mixture was stirred at room temperature for 1 h and concentrated in vacuo. The residue was stirred with phthalimide potassium salt (3.23 g, 17.4 mmol) and DMF (15.8 mL) at 90 °C for 2 h and cooled, then partitioned between AcOEt (80 mL) and water (60 mL). The aqueous phase was extracted with AcOEt and the combined organic phase was washed with brine and 1 N NaOH, then dried over $MgSO_4$ and filtered. After removal of the solvent in vacuo, the residue was recrystallized from MeOH (58 mL) and AcOEt (27 mL) to obtain **9** as a red solid (3.07 g, 69%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.84 (d, J = 8.4 Hz, 2H), 7.70 (d, J = 8.4 Hz, 2H), 7.11 (d, J = 8.0 Hz, 1H), 6.91 (dd, J = 1.6, 7.6 Hz, 1H), 6.84 (d, J = 1.6 Hz, 1H), 4.79 (s, 2H), 4.53 (t, J = 8.8 Hz, 2H), 3.15 (t, J = 8.8 Hz, 2H); MS (ESI+) 280.2 $(M+H)^+$.

4.1.6. (2,3-Dihydrobenzofuran-6-yl)methanamine (10)

A mixture of **9** (3.07 g, 11 mmol) and hydrazine hydrate (2.75 g, 55 mmol) in MeOH (45 mL) was stirred at 77 °C for 3 h. The mixture was concentrated in vacuo and partitioned between AcOEt (60 mL) and water (58 mL). The organic layer was washed with brine, dried over $MgSO_4$ and evaporated to afford **10** as a red solid (1.31 g, 80%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.14 (d, J = 8.8 Hz, 1H), 6.78 (d, J = 8.4 Hz, 1H), 6.75 (s, 1H), 4.56 (t, J = 8.8 Hz, 2H), 3.80 (s, 2H), 3.18 (t, J = 8.8 Hz, 2H); MS (ESI+) 150.1 $(M+H)^+$.

4.1.7. *tert*-Butyl 3-(((trifluoromethyl)sulfonyl)oxy)-8-azabicyclo[3.2.1]oct-3-ene-8-carboxylate (12)

A solution of diisopropylamine (0.34 mL, 2.74 mmol) in THF (10 mL) was treated with 1.6 M *n*-BuLi in hexanes at −78 °C and the mixture was stirred at −78 °C for 30 min. A solution of N-Boc-nortropinone **11** (411 mg, 1.83 mmol) in THF was added to the mixture and stirred at −78 °C for 3 h, then *N*,*N*-bis(trifluoromethanesulfonyl)aniline (717 mg, 2 mmol) in THF (5 mL) was added. The mixture was stirred at room temperature overnight, quenched with water and extracted with ether. The organic phase was washed with $NaHCO_3$ aq, water and brine, then dried over Na_2SO_4 and filtered. After removal of the solvent in vacuo, the residue was purified by silica gel column chromatography (Hexane/EtOAc; 67/33) to yield **12** as a white solid (297 mg, 46%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.10 (s, 1H), 4.46 (m, 2H), 3.08 (m, 1H), 2.24 (br s, 1H), 2.08 (m, 1H), 2.01 (m, 2H), 1.74 (br s, 1H), 1.45 (s, 9H); MS (ESI+) 258.0 (M−Boc+H) $^+$

4.1.8. *tert*-Butyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-8-azabicyclo[3.2.1]oct-3-ene-8-carboxylate (13)

A suspension of **12** (296 mg, 0.83 mmol), bis(pinacolato)diboron (252 mg, 0.99 mmol), $PdCl_2$(dppf) (60.6 mg, 0.083 mmol) and potassium acetate (244 mg, 2.49 mmol) in 1,4-dioxane (5 mL) was stirred at 120 °C under microwave irradiation for 20 min. After cooling, the mixture was partitioned between water and AcOEt. The organic phase was washed with water and brine, dried over Na_2SO_4 and filtered. The solvent was removed in vacuo and the residue was purified by silica gel column chromatography (Hexane/EtOAc; 80/20) to yield **13** as a colorless oil (186 mg, 67%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.76 (s, 1H), 4.30 (m, 2H), 3.08 (m, 1H), 2.78 (m, 1H), 2.11 (m, 1H), 1.92 (m, 3H), 1.44 (s, 9H), 1.25 (s, 12H); MS (ESI+) 236.0 (M−Boc+H) $^+$

4.1.9. (*S*)-5-Bromo-2-chloro-*N*-((2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)methyl)nicotinamide (15)

To a solution of 5-bromo-2-chloronicotinic acid (5 g, 21.2 mmol) and **5** (4.19 g, 25.4 mmol) in DMF (50 mL) were added DIPEA (11.08 mL, 63.4 mmol) and BOP reagent (10.29 g, 23.3 mmol) at 0 °C. The mixture was stirred at room temperature for 1 h, quenched with water (200 mL) and extracted with [EtOAc/hexane = 1: 1] solution. The organic phase was washed with brine, dried over Na_2SO_4 and filtered. After removal of the solvent in vacuo, the residue was recrystallized from AcOEt and *n*-hexane to yield **15** as a white solid (4.82 g, 93%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.53 (d, J = 2.4 Hz, 1H), 8.21 (d, J = 2.4 Hz, 1H), 6.96 (br s, 1H), 6.87 (m, 4H), 4.43 (m, 1H), 4.35 (dd, J = 2.2, 11.8 Hz, 1H), 4.05 (dd, J = 7.0, 11.8 Hz, 1H), 3.92 (ddd, J = 4.0, 6.4, 14.4 Hz, 1H), 3.71 (ddd, J = 1.2, 6.4, 14.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 163.6, 152.1, 145.6, 143.0, 142.5, 142.1, 131.7, 121.9, 119.6, 117.4, 117.3, 71.6, 65.6, 40.6; MS (ESI+) 385.0 $(M+H)^+$.

4.1.10. (*S*)-5-Bromo-*N*-((2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)-methyl)-2-(((2,3-dihydrobenzofuran-6-yl)methyl)amino)nicotinamide (16)

A mixture of **15** (192 mg, 0.5 mmol) and **10** (90 mg, 0.6 mmol) was stirred at 160 °C for 2 h. After cooling, the mixture was partitioned between AcOEt (6 mL) and satd $NaHCO_3$ aq (2 mL) and the organic phase was washed with brine and dried over Na_2SO_4. After filtration, the mixture was concentrated in vacuo and the residue was purified by silica gel column chromatography (Hexane/EtOAc; 60:40) to yield **16** as a yellow solid (184 mg, 74%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.32 (t, J = 7.4 Hz, 1H), 8.24 (d, J = 2.4 Hz, 1H), 7.64 (d, J = 2.4 Ha, 1H), 7.11 (d, J = 7.6 Hz, 1H), 6.85–6.95 (m, 4H), 6.82 (d, J = 7.6 Hz, 1H), 6.78 (s, 1H), 6.47 (t, J = 7.6 Hz, 1H), 4.60 (d, J = 5.6 Hz, 1H), 4.54 (t, J = 8.8, 2H), 4.36 (m, 1H), 4.30 (dd, J = 2.4, 11.4 Hz, 1H), 3.99 (dd, J = 6.8, 11.4 Hz, 1H), 3.80 (ddd, J = 4.0, 6.4, 14.4 Hz, 1H), 3.61 (ddd, J = 6.2, 6.6, 14.4 Hz, 1H), 3.17 (t, J = 8.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 167.5, 160.5, 156.3, 152.7, 143.0, 142.6, 139.6, 137.2, 125.8, 124.8, 121.8, 121.8, 119.6, 117.3, 117.3, 110.7, 108.6, 104.2, 71.8, 71.3, 65.7, 44.9, 40.2, 29.5; MS (ESI+) 498.0 $(M+H)^+$.

4.1.11. 5-(8-Azabicyclo[3.2.1]oct-3-en-3-yl)-*N*-(((*S*)-2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)methyl)-2-(((2,3-dihydrobenzofuran-6-yl)methyl)amino)nicotinamide (17)

A mixture of **16** (3.24 g, 6.53 mmol), **13** (2.63 g, 7.83 mmol), $Pd(PPh_3)_4$ (0.377 g, 0.326 mmol) and K_3PO_4 (2.77 g, 13.1 mmol) in 1,4-dioxane (40 mL) and water (20 mL) was stirred at 100 °C for 7 h. After cooling, the mixture was quenched with water and extracted with AcOEt. The organic phase was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo to yield a pale yellow solid. The solid was dissolved in CH_2Cl_2 (50 mL) and treated with TFA (10 mL). The mixture was stirred at room temperature for 1 h and concentrated in vacuo. The residue was purified by column chromatography on N-H silica gel ($MeOH/CH_2Cl_2$; 5:95) to yield **17** as a pale yellow solid (2.1 g, 61%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.55 (q, J = 5.6 Hz, 1H), 8.18 (d, J = 1.2 Hz, 1H), 7.67 (t, J = 2.4 Hz, 1H), 7.56 (br s, 1H), 7.07 (d, J = 7.6 Hz, 1H), 6.80–6.85 (m, 4H), 6.76 (s, 1H), 6.02 (t, J = 7.0 Hz, 1H), 4.63 (d, J = 5.2 Hz, 2H), 4.49 (t, J = 8.8, 2H), 4.23–4.30 (m, 1H), 4.20–4.23 (m, 1H), 4.05 (m, 1H), 3.93 (m, 1H), 3.90 (dd, J = 7.2, 11.4 Hz, 1H), 3.57 (m, 1H), 3.11 (t, J = 8.8 Hz, 2H), 3.03 (m, 1H), 2.20–2.32 (m, 2H), 2.07–2.15 (m, 2H), 1.71–1.79 (m, 1H); MS (ESI+) 525.2 $(M+H)^+$.

4.1.12. 5-(8-Acetyl-8-azabicyclo[3.2.1]oct-3-en-3-yl)-*N*-(((*S*)-2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)methyl)-2-(((2,3-dihydrobenzofuran-6-yl)methyl)amino)nicotinamide (1)

To a solution of **17** (2.2 g, 4.19 mmol) in CH_2Cl_2 were added triethylamine (0.877 mL, 6.29 mmol) and acetyl chloride (0.358 mL, 5.03 mmol), and the mixture was stirred at room temperature for 0.5 h. The mixture was concentrated in vacuo and the residue was purified by silica gel column chromatography (MeOH/AcOEt/*n*-hexane = 3:48.5:48.5) to yield **1** as a pale yellow solid (1.72 g, 72%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.33 (m, 1H), 8.25 (s, 0.54H), 8.18 (s, 0.46H), 7.61 (s, 0.46H), 7.53 (s, 0.54H), 7.11 (d, J = 7.6 Hz, 1H), 6.81–6.91 (m, 5H), 6.78 (s, 1H), 6.23 (d, J = 5.6 Hz, 1H), 4.94 (m, 0.46H), 4.87 (m, 0.54H), 4.64 (d, J = 5.2 Hz, 1H), 4.53 (t, J = 8.8 Hz, 2H), 4.34–4.41 (m, 2H), 4.32 (dd, J = 2.0, 12.0 Hz, 1H), 4.00 (dd, J = 7.6, 11.2 Hz, 1H), 3.75–3.84 (m, 1H), 3.60–3.68 (m, 1H), 3.15 (t, J = 8.8 Hz, 2H), 3.06–3.13 (m, 0.54H), 2.86–2.91 (m, 0.46H), 2.27–2.37 (m, 1H), 2.07–2.17 (m, 2H), 2.10 (s, 1.38H), 2.07 (s, 1.62H), 1.67–1.83 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 168.7, 167.1 [166.2], 160.4, 157.0 [157.1], 148.7 [148.2], 143.1, 142.8 [142.8], 139.9, 132.1 [130.0], 131.8 [132.2], 125.6, 124.7, 124.5 [126.0], 122.6 [122.3], 121.7, 121.6, 119.5, 117.3, 117.3, 109.0 [109.4], 108.5, 72, 71.3, 65.9 [65.9], 55.0 [53.6], 50.0 [50.9], 44.9, 40.3, 35.9 [37.3], 35.3 [33.9], 29.5, 28.7 [30.5], 21.6 [20.8], the minor counterparts of doubled signals due to rotamers are in the brackets; MS (ESI+) 567.2 $(M+H)^+$; HRMS (FAB) Calcd for $C_{33}H_{35}N_4O_5^+$: 567.2607; Found: 567.2574; HPLC purity 93.4% (4.95 min).

4.1.13. 2-Hydroxy-5-iodonicotinic acid (19)

A mixture of 2-hydroxynicotinic acid (24 g, 173 mmol), *N*-iodosuccinimide (50.5 g, 224 mmol) in DMF (300 mL) was stirred under shading at room temperature for 1 h and at 50 °C for 20 h. About 250 mL of DMF was removed in vacuo and water (400 mL) was added to the mixture. The mixture was stirred at room temperature for 1 h and at 0 °C for 1 h, and then the resulting solid was filtered. The solid was suspended in MeOH (200 mL) and stirred at room temperature for 2 h, then the solid was filtered, washed with MeOH (40 mL) and dried in vacuo to give **19** as a pale yellow solid (38.4 g, 76%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.41 (d, J = 2.4 Hz, 1H), 8.23 (d, J = 2.4 Hz, 1H); MS (ESI+) 265.9 $(M+H)^+$.

4.1.14. Ethyl 2-chloro-5-iodonicotinate (20)

The mixture of **19** (29.8 g, 107 mmol), DMF (1.5 mL) and $SOCl_2$ (78 mL, 10 eq.) was stirred at 70 °C for 4 h. The mixture was concentrated in vacuo and diluted with DMF (6 mL). Water (90 mL) was slowly added to the mixture and then satd $NaHCO_3$ aq (200 mL) was slowly added. The mixture was acidified to pH 4 by 1 N HCl (55 mL) and stirred at room temperature for 2 h and at 0 °C overnight. After filtration, the solid was washed with water and dried in vacuo to yield a crude mixture of 2-chloro-5-iodonicotinic acid as a pale yellow solid (27 g). The solid was suspended in $CHCl_3$ (135 mL) and $SOCl_2$ (13.9 mL) and the suspension was stirred at reflux for 0.5 h. DMF (0.5 mL) and $SOCl_2$ (27.8 mL) were added to make a clear solution and the mixture was stirred at reflux for 1.5 h, then cooled and concentrated in vacuo. EtOH (135 mL) was slowly added to the residue at 0 °C and the mixture was stirred at 0 °C for 15 min. and at room temperature for 30 min. The mixture was concentrated in vacuo and partitioned between AcOEt (270 mL) and satd $NaHCO_3$ aq (135 mL). The aqueous phase was extracted with AcOEt (135 mL) and the combined organic phase was washed with brine, dried over Na_2SO_4 and filtered. The mixture was concentrated in vacuo and the residue was purified by silica gel column chromatography (AcOEt/*n*-hexane; 0:100 to 20:80) to yield **20** as a white solid (27.25 g, 95%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.70 (d, J = 2.0 Hz, 1H), 8.41 (d, J = 2.0 Hz, 1H), 4.42 (q, J = 7.2 Hz, 2H), 1.42 (t, J = 7.2 Hz, 3H); MS (ESI+) 311.9 $(M+H)^+$.

4.1.15. Ethyl 2-((benzo[*d*][1,3]dioxol-5-ylmethyl)amino)-5-iodonicotinate (21)

A mixture of **20** (26.2 g, 84 mmol) and 3,4-(methylenedioxy)-benzylamine (25.4 g, 168 mmol) was stirred at 110 °C for 0.5 h and stood at 110 °C for 3 h. The mixture was cooled to 80 °C and treated with CH_3CN (260 mL) and water (260 mL), then stirred at 80 °C for 1 h and cooled to room temperature. The mixture was stirred at 0 °C for 1 h and the solid was filtered, washed with a 1:1 mixture of CH_3CN and water (30 mL) and dried in vacuo to yield **21** as a pale gray solid (32.42 g, 96%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.41 (d, J = 2.4 Hz, 1H), 8.33 (d, J = 2.4 Hz, 1H), 8.25 (br s, 1H), 6.84 (d, J = 1.2 Hz, 1H), 6.80 (dd, J = 1.2, 8.0 Hz, 1H), 6.75 (d, J = 8.0 Hz, 1H), 4.60 (d, J = 5.6 Hz, 2H), 4.31 (q, J = 7.2 Hz, 2H), 1.37 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 166.4, 158.9, 157.0, 147.8, 146.9, 146.7, 133.0, 120.8, 108.5, 108.3, 100.9, 61.2, 44.6, 14.2; MS (ESI+) 427.0 $(M+H)^+$.

4.1.16. Ethyl 5-(3-amino-3-methylbut-1-yn-1-yl)-2-((benzo[*d*][1,3]dioxol-5-ylmethyl)amino)nicotinate (22)

A mixture of **21** (25 g, 58.7 mmol), 2-methyl-3-butyn-2-amine (6.34 g, 76 mmol), $PdCl_2(PPh_3)_2$ (1.24 g, 1.76 mmol), copper(I) iodide (0.335 g, 1.76 mmol) and triethylamine (125 mL) in CH_3CN (125 mL) was stirred at 60 °C for 3 h. The mixture was diluted with AcOEt (500 mL) and water (500 mL) and filtered. The filtrate was diluted with AcOEt (500 mL), then the organic phase was separated and washed with 28% aqueous solution of NH_3, satd NH_4Cl aq and brine. The solution was dried over Na_2SO_4, filtered and concentrated, then the residue was recrystallized from CH_3CN (125 mL) and water (125 mL) to give **22** as a pale gray solid (20.2 g, 90%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.35 (br s, 1H), 8.31 (d, J = 2.0 Hz, 1H), 8.13 (d, J = 2.4 Hz, 1H), 6.85 (d, J = 1.2 Hz, 1H), 6.81 (dd, J = 1.2, 8.0 Hz, 1H), 6.75 (d, J = 8.0 Hz, 1H), 5.92 (s, 2H), 4.64 (d, J = 5.6 Hz, 2H), 4.31 (q, J = 7.2 Hz, 2H), 1.49 (s, 6H),1.38 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 167.0, 157.0, 156.1, 147.8, 146.7, 142.4, 133.1, 120.8, 108.3, 108.2, 107.4, 105.6, 100.9, 97.1, 77.0, 61.0, 45.8, 44.7, 32.0, 14.3; MS (ESI+) 382.1 $(M+H)^+$.

4.1.17. 5-(3-Acetamido-3-methylbut-1-yn-1-yl)-2-((benzo[*d*][1,3]dioxol-5-ylmethyl)amino)nicotinic acid (23)

A solution of acetyl chloride (4.9 mL, 68.7 mmol) in $CHCl_3$ (10 mL) was added dropwise to a solution of **22** in $CHCl_3$ (190 mL) and triethylamine (29.5 mL) at 0 °C. The mixture was stirred at 0 °C for 15 min. and at room temperature for 2 h, then concentrated in vacuo. The residue was partitioned between AcOEt (600 mL) and water (300 mL) and the organic phase was washed with satd $NaHCO_3$ aq, half-satd $NaHCO_3$ and half-sat NaCl aq. The organic solution was dried over Na_2SO_4, filtered and concentrated. The residue was treated with THF (100 mL) and concentrated in vacuo again to yield a red oil (23.84 g). The oil was dissolved in MeOH (106 mL) and THF (106 mL) and treated with 1 N NaOH (106 mL). The mixture was stirred at 50 °C for 1 h and cooled to 0 °C. The mixture was neutralized by 1 N HCl (106 mL) at 0 °C and concentrated in vacuo. AcOEt (660 mL) was added to the mixture and the organic phase was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was suspended in CH_3CN (210 mL), stirred at 80 °C for 1 h and cooled to room temperature. The suspension was further stirred at 0 °C for 1 h and the solid was filtered, washed with ice cooled CH_3CN and dried in vacuo to yield **23** as a pale yellow solid (19.87 g, 95%).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.52 (t, J = 5.6 Hz, 1H), 8.24 (d, J = 2.4 Hz, 1H), 7.98 (d, J = 2.4 Hz, 1H), 7.96 (s, 1H), 6.90 (d,

J = 1.2 Hz, 1H), 6.4 (d, J = 8.0 Hz, 1H), 6.80 (dd, J = 1.6, 8.0 Hz, 1H), 5.97 (s, 2H), 4.59 (d, J = 4.8 Hz, 2H), 1.80 (s, 3H), 1.56 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.5, 168.1, 156.7, 155.0, 147.3, 146.1, 142.3, 133.4, 120.5, 108.1, 108.0, 106.6, 106.0, 100.8, 94.3, 76.5, 46.7, 43.6, 29.0, 23.3; MS (ESI+) 396.1 $(M+H)^+$.

4.1.18. 5-(3-Acetamido-3-methylbut-1-yn-1-yl)-2-((benzo[*d*]-[1,3]dioxol-5-ylmethyl)amino)-N-(3-chloro-4-methoxybenzyl)-nicotinamide (24)

A solution of **23** (12.7 g, 32.1 mmol) and 3-chloro-4-methoxybenzylamine hydrochloride (8.02 g, 38.5 mmol) in DMF (127 mL) was cooled to 0 °C and treated with DIPEA (16.8 mL) and BOP reagent (15.63 g, 35.3 mmol). The mixture was stirred at 0 °C for 2 h and diluted with a solution of AcOEt (900 mL) and *n*-hexane (450 mL). The organic solution was washed with water (600 mL), half-satd $NaHCO_3$ aq (300 mL), water (600 mL) and brine (600 mL), then dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was suspended in CH_3CN (88 mL) with heating and water (88 mL) was added to the suspension. The suspension was stirred at 80 °C for 1 h and slowly cooled to 0 °C with stirring. The solid was filtered, washed with an ice-cooled mixture of [CH_3CN/water = 1:1] and dried under vacuum to yield **24** as a pale yellow solid (16 g, 91%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.60 (br s, 1H), 8.25 (d, J = 2.0 Hz, 1H), 7.62 (d, J = 2.0 Hz, 1H), 7.35 (d, J = 2.0 Hz, 1H), 7.19 (dd, J = 2.4, 8.4 Hz, 1H), 6.90 (d, J = 8.4 Hz, 1H), 6.85 (d, J = 1.6 Hz, 1H), 6.80 (dd, J = 1.6, 8.0 Hz, 1H), 6.74 (d, J = 8.0 Hz, 1H), 6.53 (br s, 1H), 5.92 (s, 2H), 5.65 (br s, 1H), 4.60 (d, J = 5.6 Hz, 2H), 4.45 (d, J = 5.6 Hz, 2H), 3.89 (s, 3H), 1.96 (s, 3H), 1.69 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.7, 167.6, 156.5, 154.8, 154.5, 147.7, 146.6, 137.8, 133.2, 131.3, 129.9, 127.4, 122.6, 120.8, 112.2, 109.1, 108.3, 108.2, 106.2, 100.9, 93.0, 78.2, 56.2, 48.2, 44.7, 42.8, 29.4, 24.2; MS (ESI+) 549.1 $(M+H)^+$; HRMS (FAB) Calcd for $C_{20}H_{30}ClN_4O_5^+$: 549.1905; Found: 549.1935; HPLC purity 97.2% (5.84 min).

4.1.19. 4-Bromo-2-(difluoromethyl)-1-methylbenzene (26)

DAST (6 mL, 45.4 mmol) was added dropwise to a solution of 5-bromo-2-methylbenzaldehyde (3.0 g, 15.1 mmol) in CH_2Cl_2 (60 mL) at 0 °C and the mixture was stirred at 0 °C for 1 h and at room temperature for 2 h. The mixture was diluted with CH_2Cl_2 (60 mL), cooled to 0 °C and quenched with water (30 mL). The organic phase was separated, washed with brine, dried over Na_2SO_4 and filtered. After removal of the solvent in vacuo, the residue was purified by silica gel column chromatography (AcOEt/*n*-hexane; 0:100 to 4:96) to yield **26** as a colorless oil (2.62 g, 79%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.64 (d, J = 0.8 Hz, 1H), 7.47 (dd, J = 0.8, 8.0 Hz, 1H), 7.10 (d, J = 8.0 Hz, 1H), 6.70 (t, J = 55.0 Hz, 1H), 2.37 (s, 3H); MS (ESI+): compound did not ionize under the condition.

4.1.20. 3-(Difluoromethyl)-4-methylbenzonitrile (27)

A mixture of **26** (2.62 g, 11.85 mmol) and CuCN (2.12 g, 23.7 mmol) in pyridine (1.3 mL) and DMF (1.3 mL) was stirred at 190 °C under microwave irradiation for 1.5 h. The mixture was cooled to 0 °C and insolubles were filtered off and washed with AcOEt (200 mL). The filtrate was diluted with *n*-hexane (65 mL), washed with water and brine, dried over Na_2SO_4 and filtered. The mixture was concentrated in vacuo and the residue was purified by silica gel column chromatography (AcOEt/n-hexane; 0:100 to 27:73) to give **27** as a white solid (1.73 g, 87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.81 (s, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.36 (d, J = 8.0 Hz, 1H), 6.76 (t, J = 55.0 Hz, 1H), 2.51 (s, 3H); MS (ESI+) 186.1 $(M+H_2O+H)^+$.

4.1.21. (3-(Difluoromethyl)-4-methylphenyl)methanamine (28)

A mixture of **27** (1.92 g, 11.52 mmol), Pd–C (5%, 386 mg) and 4 N 1,4-dioxane solution of HCl in (7.7 mL) in MeOH (77 mL) was stirred under H_2 atmosphere at room temperature for 6 h. The mixture was filtered and the filtrate was concentrated in vacuo. The residue was suspended in ether (26 mL) and stirred at room temperature overnight. The solid was filtered, washed with ether and dried in vacuo to yield **28** as a white solid (2.33 g, 97%).

^{1}H NMR (400 MHz, MeOD) δ = 7.63 (s, 1H), 7.48 (d, J = 8.0 Hz, 1H), 7.37 (d, J = 8.0 Hz, 1H), 6.95 (t, J = 55.0 Hz, 1H), 4.13 (s, 2H), 2.50 (s, 3H); MS (ESI+) 172.1 $(M+H)^+$.

4.1.22. Ethyl 2-((benzo[*d*][1,3]dioxol-5-ylmethyl)amino)-5-bromonicotinate (30)

A mixture of ethyl 5-bromo-2-chloronicotinate **29** (27 g, 102 mmol) and 3,4-(methylenedioxy)-benzylamine (30.9 g, 204 mmol) was stirred at 110 °C for 15 min. During the reaction a pale brown solution changed to a black solid. The black solid was pound with a mortar and suspended in AcOEt (500 mL). The suspension was stirred for 30 min. and the solid was filtered off. The filtrate was washed with water and brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$) to yield **30** as a white solid (17.34 g, 45%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.30 (d, J = 2.8 Hz, 1H), 8.24 (br s, 1H), 8.21 (d, J = 2.8 Hz, 1H), 6.84 (d, J = 1.2 Hz, 1H), 6.81 (dd, J = 1.6, 8.0 Hz, 1H), 6.75 (d, J = 8.0 Hz, 1H), 5.93 (s, 2H), 4.61 (d, J = 5.6 Hz, 2H), 4.32 (q, J = 7.2 Hz, 2H), 1.37 (t, J = 7.2 Hz, 3H); MS (ESI+) 381.0 $(M+H)^+$.

4.1.23. Ethyl 2-((benzo[*d*][1,3]dioxol-5-ylmethyl)amino)-5-(3-hydroxy-3-methylbut-1-yn-1-yl)nicotinate (31)

To a mixture of **30** (18.2 g, 48 mmol), 3-methyl-1-butyn-3-ol (12.11 g, 144 mmol) in triethylamine (182 mL) were added $PdCl_2(PPh_3)_2$ (1.68 g, 2.4 mmol) and cupper(I) iodide (0.914 g, 4.8 mmol) and the mixture was stirred at reflux for 2 h under Ar atmosphere. The mixture was cooled and concentrated in vacuo. The residue was suspended in AcOEt (364 mL) at 40 °C and filtered through celite. The filtrate was washed with satd NH_4Cl aq, water and brine, dried over Na_2SO_4 and filtered. The solution was concentrated in vacuo and the residue was recrystallized from CH_3CN (91 mL) and water (46 mL) to yield **31** as a white solid (15.56 g, 85%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.40 (t, J = 5.0 Hz, 1H), 8.38 (s, 1H), 8.33 (d, J = 2.0 Hz, 1H), 8.16 (d, J = 2.4 Hz, 1H), 6.85 (d, J = 1.6 Hz, 1H), 6.81 (dd, J = 1.6, 8.0 Hz, 1H), 6.75 (d, J = 8.0 Hz, 1H), 5.93 (s, 2H), 4.64 (d, J = 5.6 Hz, 2H), 4.31 (q, J = 7.2 Hz, 2H), 2.01 (s, 1H), 1.62 (s, 6H), 1.38 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 166.9, 157.2, 156.2, 147.8, 146.8, 142.6, 133.0, 120.8, 108.3, 108.3, 106.8, 105.7, 100.9, 94.1, 79.2, 65.7, 61.1, 44.7, 31.6, 14.2; MS (ESI+) 383.1 $(M+H)^+$.

4.1.24. 2-((Benzo[*d*][1,3]dioxol-5-ylmethyl)amino)-5-(3-hydroxy-3-methylbut-1-yn-1-yl)nicotinic acid (32)

A mixture of **31** (15.54 g, 40.6 mmol) and 1 N aqueous NaOH (81 mL, 81 mmol) in MeOH (22.4 mL) was stirred at 60 °C for 2 h. The organic solvent was removed in vacuo and the mixture was neutralized with 1 N HCl (81 mL) and extracted with AcOEt. The organic phase was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was recrystallized from CH_3CN to yield **32** as a pale yellow solid (12.7 g, 88%).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 13.4 (br s, 1H), 8.62 (br s, 1H), 8.26 (d, J = 2.4 Hz, 1H), 8.00 (d, J = 2.0 Hz, 1H), 6.90 (s, 1H), 6.84 (d, J = 8.0 Hz, 1H), 6.82 (d, J = 8.0 Hz, 1H), 5.97 (s, 2H), 4.58 (d, J = 5.2 Hz, 2H), 1.44 (s, 6H); MS (ESI+) 355.1 $(M+H)^+$.

4.1.25. 3-(Difluoromethyl)-4-methylbenzyl 2-((benzo[*d*][1,3]-dioxol-5-ylmethyl)amino)-5-(3-hydroxy-3-methylbut-1-yn-1-yl)nicotinate (33)

To a mixture of **32** (12.65 g, 35.7 mmol), **28** (8.9 g, 42.8 mmol) and DIPEA (18.7 mL, 107 mmol) in DMF (126 mL) was added BOP reagent (17.37 g, 39.3 mmol) over the period of 10 min at 0 °C. The mixture was stirred at 0 °C for 1 h and at room temperature for 1 h. The mixture was diluted with a solution of [AcOEt/*n*-hexane = 2:1] and washed with satd $NaHCO_3$ aq, water and brine. The solution was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was recrystallized from CH_3CN (65 mL) to yield **33** as a white solid (16.08 g, 89%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.60 (t, *J* = 5.4 Hz, 1H), 8.28 (d, *J* = 2.0 Hz, 1H), 7.59 (d, *J* = 2.4 Hz, 1H), 7.45 (s, 1H), 7.32 (dd, *J* = 3.2, 8.0 Hz, 1H), 7.23 (d, *J* = 8.0 Hz, 1H), 6.86 (d, *J* = 0.8 Hz, 1H), 6.81 (dd, *J* = 0.8, 8.0 Hz, 1H), 6.76 (t, *J* = 55.4 Hz, 1H), 6.74 (d, *J* = 4.0 Hz, 1H), 6.31 (t, *J* = 5.2 Hz, 1H), 5.93 (s, 2H), 4.61 (d, *J* = 5.6 Hz, 2H), 4.55 (d, *J* = 5.6 Hz, 2H), 2.43 (s, 3H), 1.59 (s, 3H), 1.56 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 167.4, 156.6, 154.8, 147.8, 146.7, 137.7, 135.9 (t, $^3J_{C-F}$ = 4.4 Hz), 135.7, 133.1, 132.8 (t, $^2J_{C-F}$ = 20.8 Hz), 131.7, 130.1, 125.3 (t, $^3J_{C-F}$ = 7.4 Hz), 120.9, 114.1(t, $^1J_{C-F}$ = 238 Hz), 108.9, 108.4, 108.2, 106.1, 100.9, 94.4, 79.2, 65.7, 44.8, 43.4, 31.5, 18.2; MS (ESI+) 508.1 $(M+H)^+$; HRMS (FAB) Calcd for $C_{28}H_{28}F_2N_3O_4^+$: 508.2048; Found: 508.2013; HPLC purity 99.6% (6.30 min).

4.1.26. 5-(8-Acetyl-8-azabicyclo[3.2.1]oct-3-en-3-yl)-2-(((2,3-dihydrobenzofuran-6-yl)methyl)amino)-*N*-(2-phenoxyethyl)nicotinamide (34a)

Starting form compound **14**, the title compound was prepared by the method described in the preparation of compound **1** with compound **5** replaced by 2-phenoxyethanamine.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.34 (m, 1H), 8.24 (d, *J* = 2.4 Hz, 0.55H), 8.17 (d, *J* = 2.4 Hz, 0.45H), 7.58 (d, *J* = 2.4 Hz, 0.45H), 7.51 (d, *J* = 2.4 Hz, 0.55H), 7.28–7.35 (m, 2H), 7.11 (d, *J* = 7.2 Hz, 1H), 6.95–7.00 (m, 1H), 6.92 (dd, *J* = 1.2, 8.8 Hz, 2H), 6.83 (d, *J* = 8.4 Hz, 1H), 6.79 (s, 1H), 6.74 (t, *J* = 6.0 Hz, 0.45H), 6.64 (t, *J* = 6.0 Hz, 0.55H), 6.22 (dd, *J* = 1.2, 5.6 Hz, 1H), 4.94 (t, *J* = 4.8 Hz, 0.45H), 4.87 (t, *J* = 4.8 Hz, 0.55H), 4.64 (d, *J* = 5.6 Hz, 2H), 4.54 (t, *J* = 8.8 Hz, 2H), 4.33–4.37 (m, 1H), 4.14 (t, *J* = 5.2 Hz, 2H), 3.81 (q, *J* = 5.2 Hz, 2H), 3.16 (t, *J* = 8.8 Hz, 2H), 3.11 (dd, *J* = 4.0, 17.2 Hz, 0.55H), 2.86 (dd, *J* = 4.0, 16.8 Hz, 0.45H), 2.25–2.37 (m, 1H), 2.11–2.19 (m, 2H), 2.10 (s, 1.35H), 2.07 (s, 1.65H), 1.89–1.97 (m, 1H), 1.70–1.85 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 168.4, 167.1 [166.2], 160.4, 158.5, 157.0 [157.1], 148.6 [148.3], 140, 132.1 [130.1], 131.5 [131.9], 129.6 [129.6], 125.6, 124.7, 124.6 [126.3], 122.6 [122.5], 121.3 [121.3], 119.6, 114.6, 109.4 [109.7], 108.6, 71.3, 66.6 [66.6], 55.0 [50.8], 49.9 [53.6], 44.9, 39.5, 36.0 [37.4], 35.3 [33.9], 29.5, 28.7 [30.6], 21.7 [20.8], the minor counterparts of doubled signals due to rotamers are in the brackets; MS (ESI+) 539.2 $(M+H)^+$; HPLC purity 92.2% (4.87 min).

4.1.27. 5-(8-Acetyl-8-azabicyclo[3.2.1]oct-3-en-3-yl)-2-((benzo-[*d*][1,3]dioxol-5-ylmethyl)amino)-*N*-(2-phenoxyethyl)nicotinamide (34b)

Starting form compound **14**, the title compound was prepared by the method described in the preparation of compound **1** with compound **5** replaced by 2-phenoxyethanamine and compound **10** replaced by 3,4-(methylenedioxy)benzylamine.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.33 (m, 1H), 8.24 (s, 0.55H), 8.17 (s, 0.45H), 7.61 (s, 0.45H), 7.53 (s, 0.55H), 7.26–7.32 (m, 2H), 6.95–7.00 (m, 1H), 6.91 (d, *J* = 8.0 Hz, 2H), 6.85 (s, 1H), 6.80 (d, *J* = 8.4 Hz, 1H), 6.72–6.82 (m, 1H), 6.73 (d, *J* = 8.0 Hz, 1H), 6.22 (br s, 1H), 5.91 (s, 2H), 4.92 (t, *J* = 4.8 Hz, 0.45H), 4.85 (t, *J* = 4.8 Hz, 0.55H), 4.60 (d, *J* = 6.0 Hz, 2H), 4.33–4.37 (m, 1H), 4.09–4.16 (m, 2H), 3.75–3.83 (m, 2H), 3.06 (d, *J* = 16.0 Hz, 0.55H), 2.88 (d, *J* = 16.0 Hz, 0.45H), 2.25–2.37 (m, 1H), 2.08–2.19 (m, 2H), 2.09 (s, 1.35H), 2.06 (s, 1.65H), 1.89–1.97 (m, 1H), 1.70–1.85 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.4, 167.1 [166.2], 158.5, 157.0 [156.9], 148.5 [148.2], 147.7, 146.6, 133.5, 131.7 [132.1], 129.6 [129.6], 129.6 [130.9], 124.6 [126.3], 122.7 [122.5], 121.3 [121.3], 120.7, 114.6, 109.4 [109.7], 108.3, 108.2, 100.9, 66.6 [66.6], 55.0 [50.8], 49.9 [53.6], 44.8, 39.5, 35.9 [37.4], 35.3 [33.8], 28.7 [30.5], 21.7 [20.8], the minor counterparts of doubled signals due to rotamers are in the brackets; MS (ESI+) 541.2 $(M+H)^+$; HPLC purity 99.1% (4.85 min).

4.1.28. 5-(8-Acetyl-8-azabicyclo[3.2.1]oct-3-en-3-yl)-*N*-(3-chloro-4-methoxybenzyl)-2-(((2,3-dihydrobenzofuran-6-yl)methyl)amino)nicotinamide (34c)

Starting form compound **14**, the title compound was prepared by the method described in the preparation of compound **1** with compound **5** replaced by 3-chloro-4-methoxybenzylamine.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.33 (q, *J* = 5.2 Hz, 1H), 8.22 (d, *J* = 2.0 Hz, 0.55H), 8.13 (d, *J* = 2.0 Hz, 0.45H), 7.59 (d, *J* = 2.0 Hz, 0.45H), 7.50 (d, *J* = 2.0 Hz, 0.55H), 7.34 (s, 1H), 7.20 (dt, *J* = 2.0, 8.8 Hz, 1H), 7.10 (d, *J* = 8.0 Hz, 1H), 6.90 (d, *J* = 8.0 Hz, 1H), 6.86 (t, *J* = 5.4 Hz, 0.45H), 6.82 (dd, *J* = 1.2, 8.0 Hz, 1H), 6.79 (s, 1H), 6.68 (t, *J* = 5.4 Hz, 0.55H), 6.20 (dd, *J* = 5.2, 12.0 Hz, 1H), 4.86 (t, *J* = 5.6 Hz, 0.45H), 4.80 (t, *J* = 5.2 Hz, 0.55H), 4.64 (t, *J* = 5.2 Hz, 2H), 4.53 (t, *J* = 8.8 Hz, 2H), 4.47 (d, *J* = 5.6 Hz, 2H), 4.25–4.35 (m, 1H), 3.89 (s, 3H), 3.16 (t, *J* = 8.8 Hz, 2H), 3.03 (d, *J* = 16.0 Hz, 0.55H), 2.85 (d, *J* = 16.0 Hz, 0.45H), 2.25–2.37 (m, 1H), 2.09–2.16 (m, 1H), 2.02–2.09 (m, 1H), 2.06 (s, 1.35H), 2.03 (s, 1.65H), 1.89–1.97 (m, 1H), 1.70–1.85 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 167.9, 167.1 [166.3], 160.4, 154.6, 139.9 (br), 132.1 (br), 131.8 [132.5], 131.2, 129.9 [129.9], 127.4 [127.4], 125.6, 124.7, 124.7 [126.5], 122.8, 122.6 [122.4], 119.6, 112.4, 108.6, 71.3, 56.2, 55.0 [50.8], 50.0 [53.6], 44.9, 42.9, 36.0 [37.4], 35.3 [33.8], 29.5, 28.7 [30.5], 21.6 [20.8], the minor counterparts of doubled signals due to rotamers are in the brackets (3 signals corresponding to pyridine ring were not observed due to line broadening most possibly caused by amide rotation), MS (ESI+) 573.1 $(M+H)^+$; HPLC purity 92.8% (5.03 min).

4.1.29. 5-((1*R*,5*S*)-8-Acetyl-8-azabicyclo[3.2.1]oct-3-en-3-yl)-2-((benzo[*d*][1,3]dioxol-5-ylmethyl)amino)-*N*-(3-chloro-4-methoxybenzyl)nicotinamide (34d)

Starting form compound **14**, the title compound was prepared by the method described in the preparation of compound **1** with compound **5** replaced by 3-chloro-4-methoxybenzylamine and compound **10** replaced by 3,4-(methylenedioxy)benzylamine.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.46 (q, *J* = 5.6 Hz, 1H), 8.22 (d, *J* = 2.4 Hz, 0.55H), 8.13 (d, *J* = 2.4 Hz, 0.45H), 7.65 (d, *J* = 2.4 Hz, 0.45H), 7.54 (d, *J* = 2.4 Hz, 0.55H), 7.35 (t, *J* = 2.0 Hz, 1H), 7.20 (dt, *J* = 2.0, 8.8 Hz, 1H), 7.15 (m, 0.45H), 6.95 (m, 0.55H), 6.88 (d, *J* = 8.0 Hz, 1H), 6.84 (d, *J* = 1.6 Hz, 1H), 6.80 (dd, *J* = 1.6, 8.0 Hz, 1H), 6.73 (d, *J* = 7.6 Hz, 1H), 6.21 (d, *J* = 5.2 Hz, 0.55H), 6.17 (d, *J* = 5.2 Hz, 0.45H), 5.91 (s, 2H), 4.82 (t, *J* = 5.2 Hz, 0.45H), 4.77 (t, *J* = 5.2 Hz, 0.55H), 4.60 (d, *J* = 5.6 Hz, 2H), 4.70 (d, *J* = 5.6 Hz, 2H), 4.29–4.36 (m, 1H), 3.88 (s, 3H), 2.99 (d, *J* = 16.8 Hz, 0.55H), 2.85 (d, *J* = 16.8 Hz, 0.45H), 2.07–2.35 (m, 2H), 2.02–2.07 (m, 1H), 2.04 (s, 1.35H), 2.02 (s, 1.65H), 1.65–1.92 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 168.0, 167.1 [166.3], 156.9 (br), 154.6, 148.5 (br), 147.8, 146.6, 133.4, 132.0 [130.1], 131.8 [132.6] (br), 131.2, 129.9 [129.9], 127.4 [127.4], 124.8 [126.5] (br), 122.7 [122.4], 122.7, 120.7 [120.8], 112.3, 109.3 (br), 108.3, 108.2, 100.8, 56.2, 55.0 [50.9], 50.0 [53.6], 44.8, 42.9, 36.0 [37.3], 35.3 [33.8], 28.7 [30.5], 21.6 [20.8], the minor counterparts of doubled signals due to rotamers are in the brackets; MS (ESI+) 575.2 $(M+H)^+$; HPLC purity 97.4% (4.97 min).

4.2. In vitro assays

4.2.1. Radioligand binding assay

Binding affinity of the compounds was determined based on the displacement of [^{125}I] human ghrelin from its receptors in the cell membrane fraction obtained from rat or human ghrelinR stably overexpressed in Chinese hamster ovary (CHO) cells. 3.34 μg protein of the membrane fraction, various concentrations of compounds and 0.04 nM [^{125}I] human ghrelin (PerkinElmer) were incubated at room temperature for 30 min in 50 mM Hepes (pH 7.5), 1 mM $CaCl_2$, 5 mM $MgCl_2$, 75 mM NaCl, and 0.5% w/v BSA in MultiScreen HTS, Durapore, HV, 96-well filter plates (Millipore) pretreated with 0.01% polyethyleneimine. Membranes were harvested and washed with 50 mM Hepes (pH 7.5), 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 75 mM NaCl using a vacuum manifold (Millipore). After addition of Microscint™-O, radioactivity was counted in the TopCount™ NXT HTS Microplate Scintillation and Luminescence Counter (Perkin Elmer).

4.2.2. Inverse agonist assay

HEK293 (human embryonic kidney) cells were seeded at 15,000 cells/well into a 96-well collagen-coated plate in RPMI1640 containing 10% FBS and cultured overnight at 37 °C. Growth medium was removed and prewarmed Opti-MEM (Life Technologies) was added to the plates. For transfection of cells in each well, 10 ng rat ghrelinR expression vector, 100 ng PathDetect pNFAT-Luc plasmid (Agilent Technologies) and 0.3 μL Lipofectamine 2000 (Life Technologies) were used. Plasmids and Lipofectamine were diluted separately and preincubated for 5 min with Opti-MEM before they were mixed together and incubated for 20 min. Then, they were added to the plates. After incubation for about 5 h at 37 °C, the plasmids-Lipofectamine solution was removed, compound solutions diluted to the desired final concentrations in RPMI1640 containing 0.1% dialyzed FBS were added, and then plates were incubated overnight at 37 °C. Luciferase activity was detected using the Steadylite HTS™ assay (PerkinElemer) and luminescence was measured with the EnVision multilabel reader (PerkinElmer). AlamarBlue (Life Technologies) was used to measure cell viability.

4.2.3. Intracellular Ca^{2+} mobilization assay

The FLIPR Calcium 3 Assay Kit (Molecular Devices) was used for the assay. CHO cells stably overexpressing rat ghrelinR were seeded at 20,000 cells/well into 96-well black-wall, clear-bottom plates in F-12 medium containing 10% FBS and were cultured overnight at 37 °C. Growth medium was removed, and plates were washed with calcium buffer (1 Hank's Balanced Salt solution with 20 mM Hepes, 2.5 mM probenecid and 1 mg/mL BSA). Calcium 3 reagent reconstituted with calcium buffer was added to the plates and cells were incubated for 2.5 h at room temperature. FLEXstation (Molecular Devices) was used for monitoring the fluorescence, adding compounds, and subsequently adding 1 nM human ghrelin. The values of the fluorescence increase were calculated as the peak fluorescence value minus the basal level.

4.2.4. Growth hormone secretion in rat primary pituitary cells

4.2.4.1. Rat pituitary cell culture[24]. Anterior pituitary glands were obtained from male Sprague-Dawley (SD) rats at 6–8 weeks old (Charles River Laboratories), and were minced into small fragments. The fragments were dispersed to single cells with two incubations of 3 mg/ml collagenase (Wako Pure Chemical Industries) at 37 °C for 30 min and Papain Dissociation System (Worthington Biochemical Corporation) at 37 °C for 30 min. The resultant cell suspension was isolated by gentle pipetting and passed through 40 μm filter. Cells were centrifuged at 300×*g* for 5 min, and the pelleted cells were subjected to a discontinuous density gradient to remove cell debris. Cells were suspended in the Dulbecco's Modified Eagle's Medium (DMEM), supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), HEPES (25 mM), 2.5% fetal bovine serum, and 10% horse serum. Cells were plated into 24-well plate at densities of 1.5×10^5 cells per well.

4.2.4.2. Growth hormone secretion assay. Pituitary cells were cultured for 2 days, and then were pre-incubated with the reaction buffer (Hanks's balanced salt solution, containing 1% bovine serum albumin, 0.25% glucose, and 25 mM HEPES) 1.5 h before tested compounds were applied. The reaction buffer was exchanged with a new one 15 min before the application of compounds. Cells were treated with compounds at 37 °C for 15 min, and then the supernatants were collected into the tubes containing 2% EDTA and 2000 U/ml Aprotinin. After centrifugation (2000*g* for 15 min at 4 °C), GH in the supernatants was measured with Growth hormone ELISA kit (LINCO Research). The percentage of inhibition caused by each compound was calculated from the formula $(C - E)/C \times 100$, where C stands for the GH concentration released with Ghrelin (1 nM) and E stand for that released with each compound.

4.2.5. Metabolic stability in liver microsomes

For the microsomal stability assay, 1 μM of each compound was incubated with human liver microsomes (0.5 mg protein/mL) in 50 mM phosphate buffer (pH 7.4) containing 1 mM NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) at 37 °C for 30 min. After the enzyme reaction was terminated by the addition of a 5-fold volume of acetonitrile, the reaction mixture was centrifuged at 4000 rpm for 5 min. The resultant supernatant was used as a test sample to measure the stability in human liver microsomes by quantitating the compound in the sample using LC/MS/MS (liquid chromatography tandem mass spectrometry). Metabolites were analyzed in the same sample.

4.2.6. Equilibrium dialysis to determine plasma protein binding

Compounds (10 μM) were mixed with human plasma. The mixtures then were subjected to equilibrium dialysis against Dulbecco's phosphate-buffered saline (pH 7.4) at 37 °C for 4 h using a Rapid Equilibrium Dialysis (Thermo Fisher Scientific K.K.). On completion of the dialysis period, both the plasma and buffer fractions were removed from the device. The plasma and buffer fractions were mixed with a 5-fold volume of acetonitrile and then the reaction mixture was centrifuged at 4000 rpm for 5 min. The resultant supernatant was used as a test sample to measure the protein binding in human plasma by quantitating the compound in the sample using LC/MS/MS.

4.3. In vivo assays

4.3.1. Pharmacokinetics

Non-fasted SD rats (n = 3 per treatment group) were administered compounds orally at a dose of 10 mg/kg or by the intravenous route (iv) at a dose of 1 mg/kg. Compounds were dissolved in a vehicle of 5% DMSO and 10% 2-hydroxypropyl-β-cyclodextrin (HPCD) in saline. Blood samples were collected with heparin as an anticoagulant at 0.08, 0.25,0.5 1, 2, 4, 6, 8, and 24 h following iv dosing and at 0.25, 0.50, 1, 2, 4, 6, 8, and 24 h following oral dosing. Samples were centrifuged, and the plasma was collected and stored at −80 °C before analysis. Samples were analyzed by LC/MS/MS. The pharmacokinetic parameters were calculated by noncompartmental analysis.

4.3.2. HFDIO rat model

Male SD rats were fed with a high-fat diet (containing 60% fat) for 10 weeks before the start of the study. All animals were habituated to oral administration with vehicle for three weeks and

animals that showed abnormal behavior during the habituation were excluded from the study. Animals with mean ± 2SD body weight were included in the study and were assigned randomly to vehicle, treatment, or pair-fed groups. Compound or vehicle was administered orally as a suspension in 0.5% hydroxypropyl cellulose (w/v) at 9:30 am and 6:00 pm. Body weight and daily food consumption were measured before the morning administration. In the pair-fed group, the amount of food was limited to that of the average food intake of compound-treated group the day before, and animals were treated with vehicle twice daily.

References and notes

1. Kojima, M.; Hosoda, H.; Date, Y.; Nakazato, M.; Matsuo, H.; Kangawa, K. *Nature* **1999**, *402*, 656.
2. Tschop, M.; Smiley, D. L.; Heiman, M. L. *Nature* **2000**, *407*, 908.
3. Al Massadi, O.; Lear, P. V.; Muller, T. D.; Lopez, M.; Dieguez, C.; Tschop, M.; Nogueiras, R. *Curr. Drug Metabol.* **2014**, *15*, 398.
4. Varhulst, P. J.; Depoortere, I. *World J. Gastroenterol.* **2012**, *18*, 3183.
5. Kojima, M.; Kangawa, K. *Physiol. Rev.* **2005**, *85*, 495.
6. Koopmann, A.; von der Goltz, C.; Grosshans, M.; Dinter, C.; Vitale, M.; Wiedemann, K.; Kiefer, F. *PsychoNeuroendocrinology* **2012**, *37*, 980.
7. Korbonits, M.; Goldstone, A. P.; Gueorguiev, M.; Grossman, A. B. *Front. Neuroendocrinol.* **2004**, *25*, 27.
8. Holst, B.; Cygankiewicz, A.; Jensen, T. H.; Ankeren, M.; Schwartz, T. W. *Mol. Endocrinol.* **2003**, *17*, 2201.
9. Delgado, A.; Lecerc, G.; Lobato, M.; Mauleona, D. *Tetrahedron Lett.* **1988**, *29*, 3671.
10. Mertinell, P. M.; Navarro, M. I.; Mormeneo, J. D. PCT Int. Appl. WO2009007399 A1.
11. Ghosh, S.; Kinny, W. A.; Gauthier, D. A.; Lawson, E. C.; Hudlicky, T.; Maryanoff, B. E. *Can. J. Chem.* **2006**, *84*, 555.
12. LaPlante, S. R.; Fader, L. D.; Fandrick, K. R.; Fandrick, D. R.; Hucke, O.; Kemper, R.; Miller, S. P. F.; Edwards, P. J. *J. Med. Chem.* **2011**, *54*, 70005.
13. Cameron, K. O.; Bhattacharya, S. K.; Loomis, A. K. *J. Med. Chem.* **2014**, *57*, 8671.
14. Pasternak, A.; Goble, S. D.; deJesus, R. K.; Hreniuk, D. L.; Chung, C. C.; Tota, M. R.; Mazur, P.; Feighner, S. D.; Howard, A. D.; Mills, S. G.; Yang, L. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6237.
15. McCoull, W.; Barton, P.; Broo, A.; Brown, A. J. H.; Clarke, D. S.; Coope, G.; Davies, R. D. M.; Dossetter, A. G.; Kelly, E. E.; Knerr, L.; MacFaul, P.; Holmes, J. L.; Martin, N.; Moore, J. E.; Morgan, D.; Newton, C.; Osterlund, K.; Robb, G. R.; Rosevere, E.; Selmi, N.; Stokes, S.; Svensson, T. S.; Ullah, V. B. K.; Williams, E. J. *Med. Chem. Commun.* **2013**, *4*, 456.
16. Bhattacharya, S. K.; Andrews, K.; Beveridge, R.; Cameron, K. O.; Chiliu, C.; Dunn, M.; Fernando, D.; Gao, H.; Hepworth, D.; Jackson, V. M.; Khot, V.; Kong, J.; Kosa, R. E.; Lapham, K.; Loria, P. M.; Londregan, A. T.; McClure, K. F.; Orr, S. T. M.; Patel, J.; Rose, C.; Saenz, J.; Stock, I. A.; Storer, G.; VanVolkenburg, M.; Vrieze, D.; Wand, G.; Xiao, J.; Zhang, Y. *ACS Med. Chem. Lett.* **2014**, *5*, 474.
17. Date, Y.; Murakami, N.; Toshinai, K.; Matsukura, S.; Niijima, A.; Matsuo, H.; Kangawa, K.; Nakazato, M. *Gastroenterology* **2002**, *123*, 1120.
18. le Roux, C. W.; Neary, N. M.; Halsey, T. J.; Small, C. J.; Martinez-Isla, A. M.; Ghatei, M. A.; Theodorou, N. A.; Bloom, S. R. *J. Clin. Endocrinol. Metabol.* **2005**, *90*, 4521.
19. Arnold, M.; Mura, A.; Langhans, W.; Geary, N. *J. Neurosci.* **2006**, *26*, 11052.
20. Mason, B. L.; Wang, Q.; Zigman, J. M. *Annu. Rev. Physiol.* **2014**, *76*, 519.
21. McClure, K. F.; Jackson, M.; Cameron, K. O.; Kung, D. W.; Perry, D. A.; Orr, S. T.; Zhang, Y.; Kohrt, J.; Tu, M.; Gao, H.; Fernando, D.; Jones, R.; Erasga, N.; Wang, G.; Polivkova, J.; Jiao, W.; Swartz, R.; Ueno, H.; Bhattacharya, S. K.; Stock, I. A.; Varma, S.; Bagdasarian, V.; Perez, S.; Kelly-Sullivan, D.; Wang, R.; Kong, J.; Cornelius, P.; Michael, L.; Lee, E.; Janssen, A.; Steyn, S. J.; Lapham, K.; Goosen, T. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 5410.
22. Takahashi, B.; Funami, H.; Iwaki, T.; Maruoka, H.; Nagahira, A.; Koyama, M.; Kamiide, Y.; Matsuo, T.; Muto, T.; Annoura, H. *Bioorg. Med. Chem. Lett.* **2015**. http://dx.doi.org/10.1016/j.bmcl.2015.04.040.
23. Paulino, G. *Am. J. Physiol. Endocrinol. Metab.* **2009**, *296*, E898.
24. (a) Cheng, K.; Chaung, L.-Y.; Chan, W. W.-S.; Butler, B.; Smith, R. G. *J. Endocrinol.* **1997**, *152*, 155; (b) Yamazaki, M.; Nakamura, K.; Kobayashi, H.; Matsubara, M.; Hayashi, Y.; Kangawa, K.; Sakai, T. *J. Neuroendocrinol.* **2002**, *14*, 156.