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Identification of piperazine-bisamide GHSR antagonists for the treatment of obesity

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ABSTRACT

Piperazine-bisamide analogs were discovered as partial agonists of human growth hormone secretagogue receptor (GHSR) in a high throughput screen. The partial agonists were optimized for potency and converted into antagonists through structure–activity relationship (SAR) studies. The efforts also led to the identification of potent antagonist with favorable PK profile suitable as a tool compound for in vivo studies.

Obesity affects life quality and endangers the general health of a large population, and it may replace tobacco as the number one health risk for developed societies.[1] It is estimated that about 127 million adults in the United States are overweight or obese,[2] and approximately 300,000 deaths per year are directly attributable to obesity, mainly due to associated heart disease, diabetes, or cancer.[3] Several other medical conditions can also be attributed to obesity, including asthma, sleep apnea, arthritis, reproductive complications, and psychological disturbances.[3]Over the years, many therapeutic interventions have been used for the treatment of obesity, but most of them are either complicated with safety issues[4] or limited in patient population.[5]

Ghrelin, a 28 amino acid peptide hormone bearing an octanoyl side chain at the third amino acid from its N-terminus (serine 3), is an endogenous ligand for the growth hormone secretagogue receptor (GHSR).[6] It is synthesized primarily in the stomach and found in the circulation of healthy humans. The GHSR was identified to be a G protein-coupled receptor (GPCR) located predominantly in the pituitary gland and the hypothalamus.[7] Ghrelin levels in plasma are influenced by nutritional status and regulate growth hormone (GH) secretion, appetite and fat deposition.[8] Administration of ghrelin to rats results in weight gain as a consequence of changes in energy intake and fuel utilization.[9] Moreover, systemic ghrelin administration in humans stimulates sensations of hunger and induces overeating.[10] Based on these findings, ghrelin is believed to play a crucial role in the regulation of appetite and body weight, and GHSR antagonists are expected to reduce appetite and food intake, and may provide treatment of eating disorders and obesity.[11] Thus, we and others[12] have been interested in identifying GHSR antagonists for the treatment of obesity. Herein, we report the discovery and optimization of piperazine-bisamide based GHSR antagonists.

High throughput screening of our chemical library was conducted with an aequorin flash luminescence (Aeq) assay using CHO cells stably expressing human GHSR.[13] This assay measured the inhibition of compounds on the intracellular calcium increases induced by ghrelin (1.0 μM) and identified the piperazine-bisamide **1a** (Fig. 1) as a potent antagonist (IC_{50} = 100 nM) for the GHSR receptor. However, further study of **1a** in a human inositol phosphate (IP) accumulation assay demonstrated an 18% increase in IP at 10 μM relative to ghrelin's maximal response.[14,15] Therefore, efforts to optimize the potency of compounds in the aequrin assay while reducing their partial agonist properties observed with the IP

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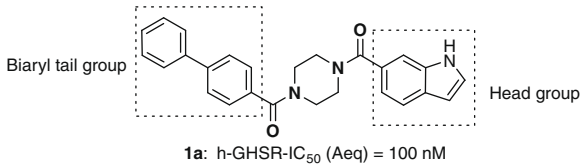


Figure 1. HTS hit from high throughput screening.

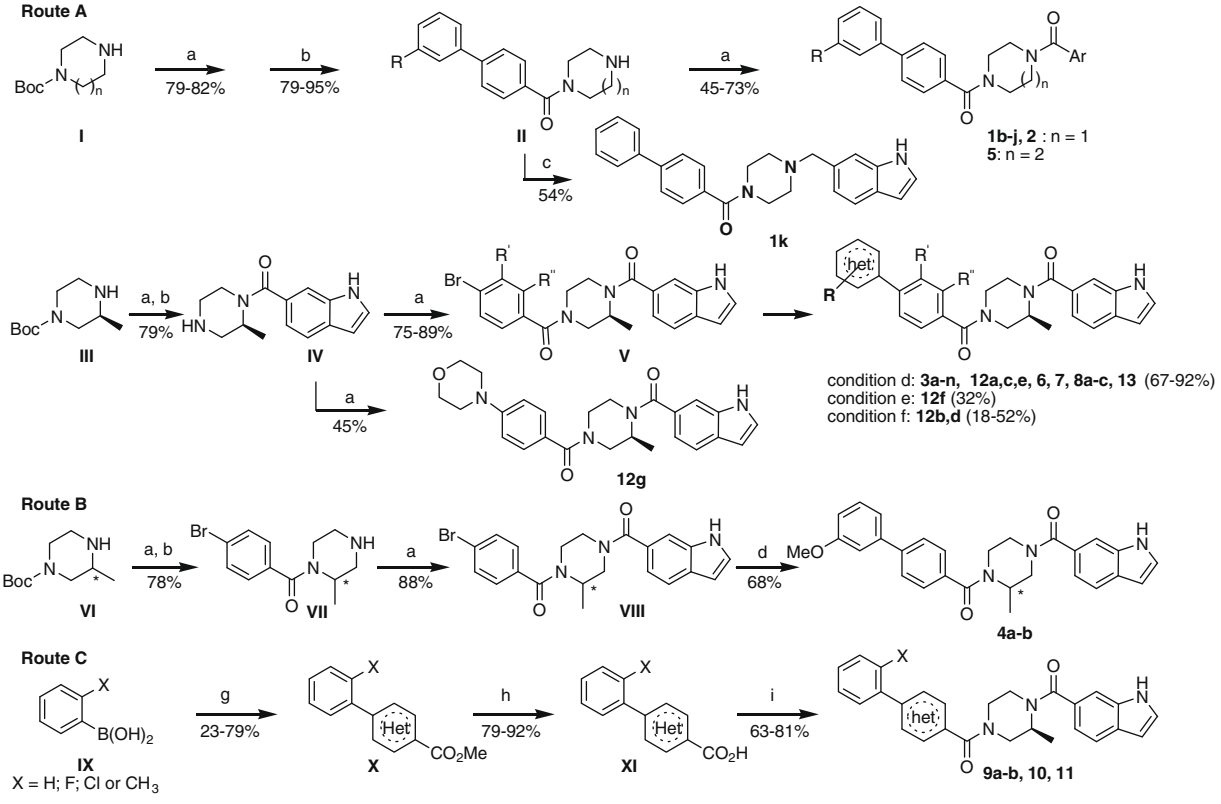
assay were pursued. All compounds tested in the human or rat IP assays were evaluated at 0.1 μM, 1.0 μM, and 10 μM. However, for purposes of comparing their maximal agonistic response in IP assays we chose to report the compounds activity at 10 μM.

The synthetic route to obtain these piperazine-bisamide compounds is outlined in Scheme 1. Following route **A**, treating commercially available **I** (tert-butyl piperazine-1-carboxylate:n = 1, or tert-butyl 1,4-diazepane-1-carboxylate:n = 2) with 4-biphenyl carboxylic acid or 3′-methoxy-biphenyl-4-carboxylic acid in the presence of HBTU and Hünig's base in DMF followed by boc-group deprotection afforded intermediate **II**, which upon second amide coupling with the corresponding aryl-carboxylic acid provided **1b–j**, **2**, and **5**, respectively. Reductive amination of the amine intermediate **II** with indole-6-carboxylaldehyde afforded **1k** in 54% yield. Starting from **III**, this synthetic approach provided access to bromo-substituted intermediate **V**, which after either Suzuki or Stille coupling reactions gave compounds **3a–n**, **12a**,**c**,**e**,**f**, **6**, **7**, **8a–c**, **13** or **12b**,**d**. The intermediate **IV** involved in this route also provided convenient access to compound **12g** after amide coupling with the corresponding commercial carboxylic acids. In contrast to this approach, while route **B** also shared the same starting material **VI** (either the *S* or *R* enantiomer), it switched the order of the amide bond formation and thus provided compounds **4a–b**. We also designed a synthesis for the middle B-ring replacements with heterocycles as shown on route **C**. Pre-installation of the heterocyclic B-ring onto the biaryl tail of the molecule through Suzuki coupling of the Br- or Cl-heteroaryl methyl carboxylates with the corresponding boronic acids **IX** followed by saponification provided intermediate carboxylic acid **XI**. Coupling of **XI** with **IV** offered the corresponding compounds **9a–b**, **10** and **11** in moderate to good yields.

Our initial lead optimization started with the modification of the indole head group as summarized in Figure 2. Extensive exploration indicated that there was limited tolerance of structural change in this region of the molecule. All of the modifications to the indole moiety, including *N* substitution (**1b**), linkage alteration (**1c–d**) or replacement of the indole with either closely related heterocycles (**1e–h**) or more diverse chemical motifs (**1i–k**) resulted in significant loss of potency (IC_{50} > 10 μM).

While no potency improvement was achieved from the indole head group modification, we quickly found that 3-methoxy biphenyl as the tail group improved the potency by four folds in compound **2** (Table 1). Using 3-methoxy biphenyl as the tail group, our optimization was then focused on the middle piperazine core. Compound **3a** with *s*-CH_3 group at R^3 position was twice as potent (IC_{50} = 12 nM) as compound **2**, but all the other methylated piperazines (**3b, 4a**, and **4b**) were detrimental toward potency. Enlarging the piperazine core as in compound **5** also failed to improve the activity. After this exploration, the methylated piperazine core in compound **3a** was selected as the template for subsequent modifications.

With the piperazine core optimized, we conducted a detailed study of modifications to the biphenyl tail group of the molecule.



Scheme 1. Reagents and conditions: (a) $ArCO_2H$, HBTU, $^iPr_2NEt$, DMF, rt, 0.5–3.5 h; (b) 20% TFA/CH_2Cl_2, rt to 50 °C 3.5–5.0 h or 4.0 N HCl/dioxane, rt, 1.5–2.0 h; (c) indole-6-carboxylaldehyde, $NaB(OAc)_3H$, dichloroethane, 55 °C, 45 min; (d) $ArB(OH)_2$, $Pd(PPh_3)_4$, DMF, Cs_2CO_3, 80–100 °C, 1.0–4.0 h; (e) 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole, $[Pd(PPh_3)_2Cl_2]_2$, DMF, Cs_2CO_3, 110 °C, 3.5 h, 32%; (f) $ArSnBu_3$, $[Pd(PPh_3)_2Cl_2]_2$, DME, K_3PO_4, 90–100 °C, 3.0–6.0 h, 18–52%; (g) Br-$ArCO_2Me$ or Cl-$ArCO_2Me$, $[Pd(PPh_3)_2Cl_2]_2$, Cs_2CO_3, DMF, 80–100 °C, 1.5–4.5 h; (h) LiOH, dioxane/H_2O (4:1), 1.5–3.0 h; (i) **IV**, HBTU, $^iPr_2NEt$, DMF, rt; 1.0 h.

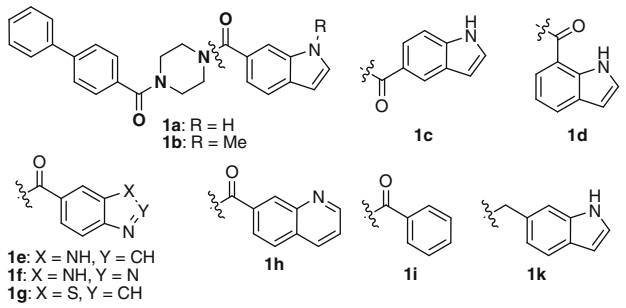
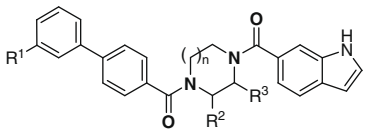


Figure 2. Early exploration of indole head group replacements.

Table 1
SAR of piperazine ring modification

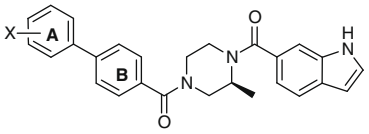


Compound	R^1	*n*	R^2	R^3	h-GHSR, Aeq IC_{50}[a,b] (nM)
1[*]	H	1	H	H	100
2	OMe	1	H	H	25
3a	OMe	1	H	-(*S*)-Me	12
3b	OMe	1	H	-(*R*)-Me	640
4a	OMe	1	-(*S*)-Me	H	280
4b	OMe	1	-(*R*)-Me	H	68
5	H	2	H	H	6500

[a] Values were the means of three determinations, standard derivation was ⩽30%.
[b] See Ref. 13 for assay protocol.

As illustrated in Table 2, with compound **3a** as a starting point, we investigated several other *meta* substituents for the terminal phenyl A-ring, but found that this *meta* position was much less tolerant of electron withdrawing groups, with 3–11 folds loss of potency on compounds **3c**–**e**. This was in contrast to the observation made at the *ortho* position, wherein almost all the substitutions (**3h**–**k**) studied improved the potency as compared to **3a**, except for the 2-methoxy group (**3g**). Substitutions on the *para* position had similar effects as those on the *meta* positions: the electron donating methoxy group (**3l**) was more potent than electron withdrawing ones (**3m**–**n**). It is noteworthy that the unsubstituted

Table 2
Substitution SAR of terminal phenyl ring



Compound	X	h-GHSR, Aeq IC_{50}[a,b] (nM)
3a	3-OMe	12.0
3c	3-OCF_3	46.5
3d	3-CF_3	77.0
3e	3-F	147.0
3f	H	6.0
3g	2-OMe	26.0
3h	2-F	9.1
3i	2-Cl	2.1
3j	2-CF_3	4.1
3k	2-Me	2.9
3l	4-OMe	7.0
3m	4-OCF_3	136.0
3n	4-F	64.0

[a] Values were the means of three determinations, standard derivation was ⩽30%.
[b] See Ref. 13 for assay protocol.

derivative **3f** with the optimized piperazine core was also a very potent compound.

With potency optimized, we decided to further evaluate the partial agonist activity. For this purpose, compound **3h** was selected as a tool compound partially based on its favorable PK properties.[16] Like our lead compound, **1a**, compound **3h** continued to behave as a partial agonist in the IP assay (Fig. 3 and Table 3). CHO cells expressing the human GHSR receptor treated with 10 μM, 1.0 μM or 0.1 μM concentration of compound **3h** afforded a 33%, 31%, and 21% increase in inositol phosphate relative to ghrelin's maximal increase.[14] Moreover, compound **3h** also acted as a partial agonist in rat IP assays[17] and in an ex vivo primary rat pituitary cell assay,[18] wherein it stimulated rat growth hormone (GH) secretion equivalent to 24% of the maximal ghrelin response at 1.0 μM. In addition, the partial agonist activity seemed rather prevalent on this lead series and all the potent compounds from Table 2 (**3a**, **3f**, and **3h–l**) demonstrated partial agonist activity in both the IP and rat ex vivo assays.

In order to decrease the agonist activity, we modified the phenyl B-ring of the biphenyl moiety (Table 4). Because of the good correlation of the agonist activity readouts across the three assays in Table 3, the rat IP assay was chosen to guide the lead optimization efforts. In short, several B-ring modifications decreased the agonist activity, such as in compounds **8a–11**. But many of these modifications also led to loss of potency. The fluorine substitution on the B-ring (**8a**) well maintained the potency.

The breakthrough of eliminating agonist activity came from further exploration on the A-ring of the biphenyl tail (Table 5).

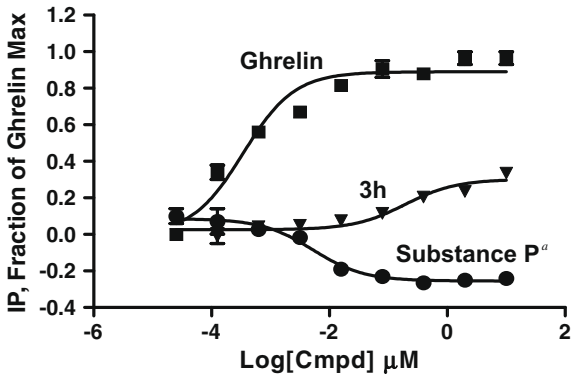


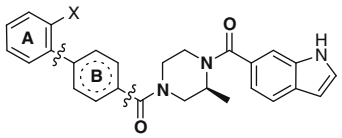
Figure 3. Partial agonist activity of compound **3h** in human IP assay. [a]The reference compound was chosen as substance P, a peptide inverse agonist of the GHSR; [b]values were the means of three determinations, p <0.005; [c]see Ref. 14 for assay protocol.

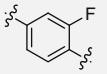
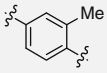
Table 3
Agonist activity as %fraction of maximal ghrelin response

Compound	In vitro IP[a,b,c]		Rat ex vivo pituitary[a,d,e]
	Human	Rat	
1a	18%	14%	—[f]
3h	33%	33%	24%
3a	45%	46%	40%
3f	29%	26%	—[f]
3i	35%	32%	24%
3j	24%	28%	21%
3k	32%	31%	18%
3l	33%	34%	—[f]

[a] Values were the means of three determinations, standard derivation was ⩽30%.
[b] %Fraction of maximal ghrelin response at 10 μM.
[c] See Ref. 14 for assay protocol.
[d] %Fraction of maximal ghrelin response at 1.0 μM.
[e] See Ref. 18 for assay protocol.
[f] Data not obtained.

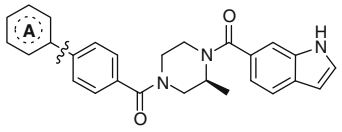
Table 4
SAR of middle B-ring to reduce agonism



Compound	X	B-ring	h-GHSR, Aeq IC_{50}[a,b] (nM)	Rat IP %agonism[a,c,d]
3h	F		9.1	33%
3k	CH_3		2.9	31%
6	H		17.7	43%
7	H		14.3	36%
8a	CH_3		9.0	16%
9a	H		20.0	19%
9b	Cl		13.0	14%
10	Cl		37.2	18%
11	F		33.7	14%

[a] Values were the means of three determinations, standard derivation was 30%.
[b] See Ref. 13 for assay protocol.
[c] %Fraction of maximal ghrelin response at 10 μM.
[d] See Ref. 14 for assay protocol.

Table 5
SAR of terminal phenyl A-ring to eliminate agonism



Compound	A-ring	h-GHSR, Aeq IC_{50}[a,b] (nM)	Rat IP %agonism[a,c,d]
3h		9.1	33%
12a		12.7	−2%
12b		42.2	12%
12c		143.0	21%
12d		65.0	5%
12e		244.6	5%
12f		123.0	17%
12g		338.7	—[c]

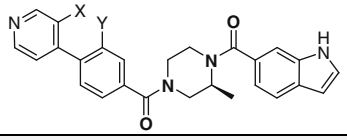
[a] Values were the means of three determinations, standard derivation was 30%.
[b] See Ref. 13 for assay protocol.
[c] %Fraction of maximal ghrelin response at 10 μM.
[d] See Ref. 14 for assay protocol.

Employing 4-pyridyl as A-ring provided compound **12a**, which maintained potency in the aequorin assay while showing no detectable agonist activity in the rat IP assay. Other heterocyclic replacements of phenyl A-ring all reduced the agonist activity, but not as completely or as potent as 4-pyridyl.

Combination of the optimal features discovered on biphenyl A-ring and B-ring, respectively provided a set of compounds (**8b–c**, **12a**, and **13**, Table 6) that were free of agonist activity on the IP assay. Compound **8b**[19] was the most potent in the rat and human aequorin assays, therefore, it was chosen for further evaluation. This compound displayed high in vitro metabolic stability across species. After 30 min incubation in liver microsomes, the percentage remaining of **8b** (initial concentration: 1.0 μM) was 78% and 87% for rat and human, respectively. The in vivo pharmacokinetic properties of **8b** were evaluated in mouse. After iv administration of **8b** at dosage of 0.5 mg/kg, the clearance (Cl) was 1.96 L/h/kg, the MRT was 0.70 h and the Vd_{ss} was 1.4 L/kg. This compound also had decent unbound free drug fraction in both mouse and rat plasma (f_{ub} = 4%, respectively). Although compound **8b** showed high affinity toward *p*-glycoprotein (*p*-gp) transporter (efflux ratio = 14) and low brain/plasma AUC ratio (0.16) in FVB mice, this compound was able to achieve excellent brain uptake in mdr1a knockout mice (brain/plasma AUC ratio = 2.3),[20] which ensured sufficient CNS exposure for in vivo proof-of-concept studies in this animal. In addition, compound **8b** also exhibited good selectivity over a screening against our internal receptor panel.[21]

The eradication of agonist activity in compound **8b** was also confirmed in the ex vivo growth hormone (GH) release experiment conducted in isolated primary rat pituitary cells as shown on Figure 4. Compound **8b** did not produce any noticeable GH

Table 6
Summary of top compounds



Compound	X	Y	Aeq IC_{50}[a,b] (nM)		Rat IP [a,c,d] %agonism
			Human	Rat	
12a	H	H	12.7	20.5	−2%
8b	H	F	12.2	10.3	−16%
8c	F	F	42.0	45.0	−1%
13	F	H	31.0	49.0	−2%

[a] Values were the means of three determinations, standard derivation was ⩽30%.
[b] See Ref. 13 for assay protocol.
[c] %Fraction of maximal ghrelin response at 10 μM.
[d] See Ref. 14 for assay protocol.

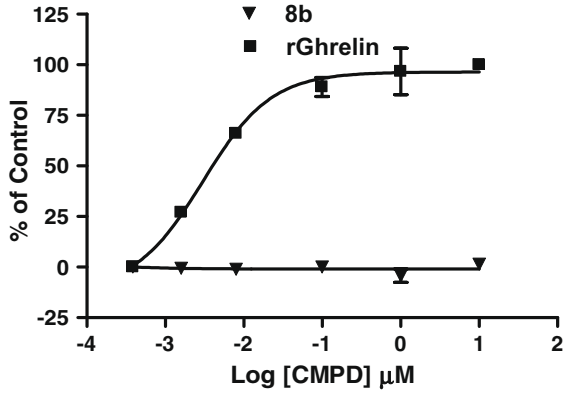


Figure 4. Agonist activity of ghrelin versus **8b** on growth hormone (GH) release in primary rat pituitary cells. Values were the means of three determinations, p <0.005; see Ref. 18 for assay protocol.

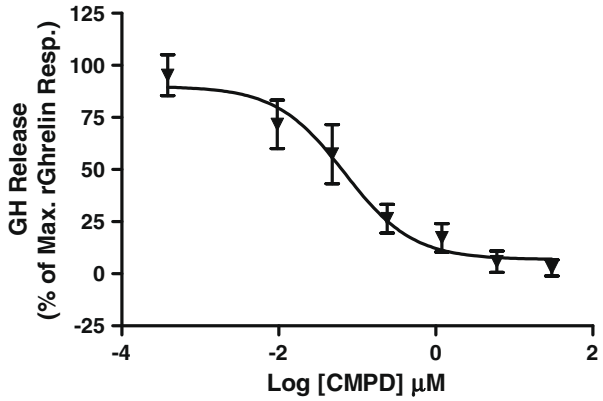


Figure 5. Antagonist effect of compound **8b** on rghrelin (0.2 μM) induced growth hormone (GH) release in primary rat pituitary cells. Values were the means of three determinations, p <0.005; see Ref. 18 for assay protocol.

secretion at up to 10 μM concentration. It could also antagonize the stimulating effect of ghrelin (0.2 μM) on GH release from rat pituitary cells (IC_{50} = 93 nM), as shown in Figure 5.

In summary, we optimized a series of piperazine-bisamide based GHSR inhibitors for potency and removed the partial agonist activity seen with the early lead compounds in the IP assays. The efforts led to the discovery of tool compound **8b**, which was featured with high potency, satisfactory PK profile and sufficient CNS exposure in mdr1a knockout mice. The compound was also confirmed to be an antagonist in the ex vivo study of GH release from isolated primary rat pituitary cells. Compound **8b** was proved to be a useful tool for evaluation in in vivo proof-of-concept studies in mouse, and the results will be published in due course.

Acknowledgments

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References and notes

1. Marshall, E. *Science* **2004**, *304*, 804.
2. (a) Ogden, C. L.; Carroll, M. D.; Curtin, L. R. *J. Am. Med. Assoc.* **2006**, *295*, 1549; (b) Melnikova, I.; Wages, D. *Nat. Rev. Drug Disc.* **2006**, *5*, 369; (c) Baskin, M. L.; Ard, J.; Franklin, F.; Allison, D. B. *Diabetes Rev.* **2005**, *6*, 5.
3. Gale, S. M.; Castracane, V. D.; Mantzoros, C. S. *J. Nutr.* **2004**, *134*, 295.
4. A representative example is the vascular heart disease associated with the combined use of fenfluramine and phentermine, see: (a) Connolly, H.; Cray, J. L.; Mcgoon, M. D.; Hensrud, D. D.; Edwards, B. S.; Edwards, W. D.; Schaff, H. V. *N. Eng. J. Med.* **1997**, *37*, 581–588; For other examples, see: (b) Padwal, R.; Majumdar, S. *The Lancet* **2007**, *369*, 71; (c) Nisoli, E.; Carruba, M. O. *Obes. Rev.* **2001**, *1*, 127; (d) Kolanowski, J. *Drug Safety* **1999**, *20*, 119.
5. Bray, G. A.; Greenway, F. L. *Endocr. Rev.* **1999**, *20*, 805.
6. Kojima, M.; Hosoda, H.; Date, Y.; Nakazato, M.; Matsuo, H.; Kangawa, K. *Nature* **1999**, *402*, 656.
7. (a) Jeffery, P. L.; Herington, A. C.; Chopin, L. K. *J. Endocrinol.* **2002**, *172*, R7; (b) Zigman, J. M.; Jones, F. E.; Lee, C. E.; Saper, C. B.; Elmquist, J. K. *J. Comp. Neurol.* **2006**, *294*, 528.
8. (a) Cummings, D. E.; Overduin, J.; Foster-Schubert, K. E. *Curr. Opin. Endocr. Diabetes* **2005**, *12*, 72; (b) Camina, J. P.; Carreira, M. C.; El Messari, S.; Llorens-Cortes, C.; Smith, R. G.; Casanueva, F. F. *Endocrinology* **2004**, *145*, 930–940; (c) Nakazato, M.; Murakami, N.; Date, Y.; Kojima, Y.; Matsuo, H.; Kangawa, K.; Matsukura, S. *Nature* **2001**, *409*, 194; (d) Wren, A. M.; Small, C. L.; Abbott, C. R.; Dhillo, W. S.; Seal, L. J.; Cohen, M. A.; Batterham, R. L.; Taheri, S. A.; Ghatei, M. A.; Bloom, S. R. *Diabetes.* **2001**, *50*, 2540; (e) Hataya, Y.; Akamizu, T.; Takaya, K.; Kanamoto, N.; Ariyasu, H.; Saijo, M.; Moriyama, K.; Shimatsu, A.; Kojima, M.; Kangawa, K.; Nakao, K. *J. Clin. Endocrinol. Metab.* **2001**, *86*, 4552; (f) Peino, R.; Baldelli, R.; Rodriguez-Garcia, J.; Rodriguez-Segade, S.; Kojima, M.; Kangawa, K.; Arvat, E.; Ghigo, E.; Dieguez, C.; Casanueva, F. F. *Eur. J. Endocrinol.* **2000**, *143*, R11; (g) TschoP, M.; Smiley, D. L.; Heiman, M. L. *Nature* **2000**, *407*, 908.
9. Davies, J. S.; Kotokorpi, P.; Eccles, S. R.; Barnes, S. K.; Tokarczuk, P. F.; Allen, S. K.; Whiteworth, H. S.; Guschina, I. A.; Evans, B. A. J.; Mode, A.; Zigman, J. M.; Welss, T. *Mol. Endocrinol.* **2009**, *23*, 914.
10. Wren, A. M.; Seal, L. J.; Cohen, M. A.; Brynes, A. E.; Frost, G. S.; Murphy, K. G.; Dhillo, W. S.; Ghatei, M. A.; Bloom, S. R. *J. Clin. Endocrinol. Metab.* **2001**, *86*, 5992.
11. For recent reviews, see (a) DeVriese, C.; Delporte, C. *Curr. Opin. Clin. Nutr.* **2007**, *10*, 615; (b) Cummings, D. E.; Foster-Schubert, K. E.; Overduin, J. *Curr. Drug Targets* **2005**, *6*, 153.
12. (a) Rosita, D.; DeWit, M. A.; Luyt, L. G. *J. Med. Chem.* **2009**, *52*, 2196; (b) Moulin, A.; Demange, L.; Ryan, J.; M'Kadmi, C.; Galleyrand, J.-C.; Martinez, J.; Fehrentz, J.-A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 164; (c) Moulin, A.; Demange, L.; Berge, G.; Gagne, D.; Ryan, J.; Mousseaux, D.; Heitz, A.; Perrissoud, D.; Locatelli, V.; Torsello, A.; Galleyrand, J.-C.; Fehrentz, J.-A.; Martinez, J. *J. Med. Chem.* **2007**, *50*, 5790; (d) Rudolph, J.; Esler, W. P.; O'connor, S.; Coish, P. D. G.; Wickens, P. L.; Brands, M.; Bierer, D. E.; Bloomquist, B. T.; Bondar, G.; Chen, L.; Chuang, C.; Claus, T. H.; Fathi, Z.; Fu, W.; Khire, U. R.; Kristie, J. A.; Liu, X.; Lowe, D. B.; McClure, A. C.; Michels, M.; Ortiz, A. A.; Ramsden, P. D.; Schoenleber, R. W.; Shelekhin, T. E.; Vakalopoulos, A.; Tang, W.; Wang, L.; Yi, L.; Gardell, S. J.; Livingston, J. N.; Sweet, L. J.; Bullock, W. H. *J. Med. Chem.* **2007**, *50*, 5202; (e) Esler, W. P.; Rudolph, J.; Claus, T. H.; Tang, W.; Barucci, N.; Brown, S.; Bullock, W.; Daly, M.; Decarr, L.; Li, Y.; Milardo, L.; Molstad, D.; Zhu, J.; Gardell, S. J.; Livingston, J. N.; Sweet, L. J. *Endocrinology* **2007**, *148*, 5175; (f) Serby, M. D.; Zhao, H.; Szczepankiewicz, B. G.; Kosogof, C.; Xin, Z.; Liu, B.; Liu, M.; Nelson, L. T. J.; Kaszubska, W.; Falls, H. D.; Schaefer, V.; Bush, E.; Shapiro, R.; Droz, B. A.; Knourek-Segel, V. E.; Fey, T. A.; Brune, M. E.; Beno, D. W. A.; Turner, T. M.; Collins, C. A.; Jacobson, P. B.; Sham, H. L.; Liu, G. *J. Med. Chem.* **2005**, *49*, 2568; (g) Xin, Z.; Zhao, H.; Serby, M. D.; Liu, B.; Schaefer, V. G.; Falls, D. H.; Kaszubska, W.; Colins, C. A.; Sham, H. L.; Liu, G. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1201; (h) Bernasconi, G.; Bromidge, S. M.; Carpenter, A. J.; D'Adamo, L.; Di Fabio, R.; Guery, S.; Pavone, F.; Pozzan, A.; Rinaldi, M.; Sabbatini, F. M.; St-Denis, Y. PCT Int. Appl. 2008, WO 2008148854 A1.; (i) Heightman, T. D. PCT Int. Appl. 2008, WO 2008148856 A1.
13. This aequorin flash luminescence (Aeq) assay used a CHO-K1 cell line expressing the human ghrelin receptor and the aequorin gene (purchased from Euroscreen, Belgium). The assay was performed as previously described by An et al. and Bandoh et al.: (a) An, S.; Bleu, T.; Zheng, Y.; Goetzl, E. J. *Mol. Pharm.* **1998**, *54*, 881; (b) Bandoh, K.; Aoki, J.; Hosono, H.; Kobayashi, S.; Kobayashi, T.; Murakami-Murofushi, K.; Tsujimoto, M.; Arai, H.; Inoue, K. *J. Biol. Chem.* **1999**, *274*, 27776.
14. These assays measured the relative magnitude of inositol phosphate (IP) accumulation that induced by subject compounds as percentage of the maximal ghrelin's response at concentrations of 10, 1.0, and 0.1 μM. The assay protocol was as the following: The CHO cells stably expressing human or rat GHSR placed in 96-well TC plates were incubated for 12 h with ^{3}H inositol (1 μCi/mL in DMEM solution). The cells were then incubated with ghrelin or subject compounds at 37 °C for 1.0 h followed by treatment with 20 mM formic acid at 4 °C for 4 h. The formic acid solution was extracted to Amersham SPA beads pre-placed in 96-well plates. After incubation in dark for 12 h, radioactivity was counted on Top Counting.
15. Compound **1a** also behaved as a partial agonist in the IP assay with CHO cells stably expressing rat GHSR. In this assay **1a** was evaluated at 10 μM, 1.0 μM, and 0.1 μM and afforded a 14%, 14%, and 24% in IP accumulation relative to ghrelin's maximal response, respectively.
16. Compound **3h** displayed reasonable rodent PK profile (rat iv @ 2.0 mg/kg: Cl = 2.1 L/h/kg, MRT = 1.9 h, Vd_{ss} = 3.7 L/kg; po @ 0.5 mg/kg: F = 17%), sufficient brain uptake (brain/plasma AUC ratio = 0.26 in FVB mice), and high potency across species (IC_{50} = 2.0 nM in rat aequorin assay). This compound had no hERG or CYP inhibition liability (IC_{50} > 10 μM).
17. Compound **3h** afforded a 33%, 42%, and 41% relative to ghrelin's maximal increase in the IP assay using CHO cells stably expressing rat GHSR at 10 μM, 1.0 μM, and 0.1 μM, respectively.
18. The assay protocol was as the following: pituitary cells freshly collected from 6–8 weeks old male SD rats were plated onto 96-well poly-D-lysine coated plates and incubated at 37 °C for 3 days. The cells were then washed with DMEM assay buffer containing 20 mM Hepes and 0.3% BSA, and incubated at 37 °C for 5 min. For agonist activity experiment, the cells were treated with rat ghrelin or subject compounds for 20 min. For antagonist activity experiment (**8b**), the cells were pretreated with the subject compounds for 10 min before co-incubation with rat ghrelin and the subject compounds for additional 20 min. The sample was taken for rat growth hormone secretion readout by RIA assay (LINCO Research).
19. *The analytic data for compound* **8b**: ^{1}H NMR (500 MHz, $CDCl_3$): δ ppm 1.24 (br s, 3H), 2.97–3.78 (m, 4H), 4.16–4.80 (m, 3H), 6.53 (br s, 1H), 7.08 (d, J = 8.1 Hz, 1H), 7.22–7.27 (m, 1H), 7.29 (dd, J = 7.7, 7.7, 2 Hz), 7.42–7.57 (m, 4H), 7.61 (d, J = 8.1 Hz, 1H), 8.69 (m, J = 5.0 Hz, 2H), 9.92 (br s, 1H); LC/MS: MS ESI (pos) m/e: 443.2 $(M+H)^+$.
20. The cell membrane permeability of this compound in the human MDR1–MDCK cell assay is: PaapB-A/PaapA-B = 1.7.
21. This lead series (e.g., **1a**, **3a**–**n**) is highly selective (K_i > 10 μM) over 5HT2α, 5HT2χ, SERT, Adrα2A, D2, D3, DAT, Opioid μ, Opioid κ, M3, NET, and H2.