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Identification of spirocyclic piperidine-azetidine inverse agonists of the ghrelin receptor

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ABSTRACT

The discovery of spirocyclic piperidine-azetidine inverse agonists of the ghrelin receptor is described. The characterization and redressing of the issues associated with these compounds is detailed. An efficient three-step synthesis and a binding assay were relied upon as the primary means of rapidly improving potency and ADMET properties for this class of inverse agonist compounds. Compound **10n** bearing distributed polarity in the form of an imidazo-thiazole acetamide and a phenyl triazole is a unit lower in log*P* and has significantly improved binding affinity compared to the hit molecule **10a**, providing support for further optimization of this series of compounds.

Introduction

The development of small molecules targeting the growth hormone secretagogue receptor 1a (GHS-R1a) goes back to the mid 1980s, predating the identification of the natural ligand, ghrelin.[1] While small molecule agonists of this peptide G-protein coupled receptor (GPCR) such as ibutamoren,[2] capromorelin,[3] and ulimorelin[4] have reached the clinic, none has reached the market. More recently interest has turned to the opposite functional profile. Although some aspects of the switch to functional antagonists are controversial,[5] the change has been driven by a steady stream of supporting evidence suggesting that ligand neutralization[6,7] or receptor deletion[8,9] can have beneficial effects on body weight and glucose homeostasis.[10–12]

Accompanying the move in functional profile to antagonists or inverse agonists is a decision to target a centrally acting agent capable of reaching receptors in the hypothalamus, or to target an agent that is more peripherally restricted. The agonist capromorelin with its peptidic character and multiple hydrogen bond donors is a peripherally restricted molecule that reaches its target in the pituitary, but does not reach other central nervous tissue.[13]

Based on the structures of the antagonists reported recently (see **1**,[14] **2**,[15] **3**,[16] **4**,[17] **5**,[18] **6**[19] in Fig. 1) the characteristics of the key N-terminal amino acid residues[20] of the endogenous peptide are less apparent than for the early agonist structures. Likely, this results from advances in the ability to rapidly screen this target and to identify novel chemical matter. Herein we report the identification and initial optimization of a series of spirocyclic piperidine-azetidines from an initial high throughput binding assay.

To identify hit compounds that possessed the potential to act as antagonists or inverse agonists at the ghrelin receptor, a two-stage screening sequence was established. Our primary screen was a radioligand binding assay based on ^{125}I-ghrelin and a scintillation proximity assay (SPA) format.[21] Although the ghrelin receptor is unusually prone to agonists[22] our plan to first assess ligand affinity was driven by our desire to start with the most efficient substrate, allowing maximum flexibility in the optimization process. This strategy contrasts with high throughput screening efforts elsewhere to identify lead matter using functional assays.[19] Our initial functional screening using a calcium mobilization FLIPR assay identified the same limitations described by Pasternak[18] and we were unable to properly discriminate an agonist from an inverse agonist. To fully characterize the functional profile of our binding hits we used a GTP-γ-S assay.[23] Interestingly, very few 'silent antagonists'[24] were identified using this screening paradigm. The vast majority of hits showed either partial agonist activity or

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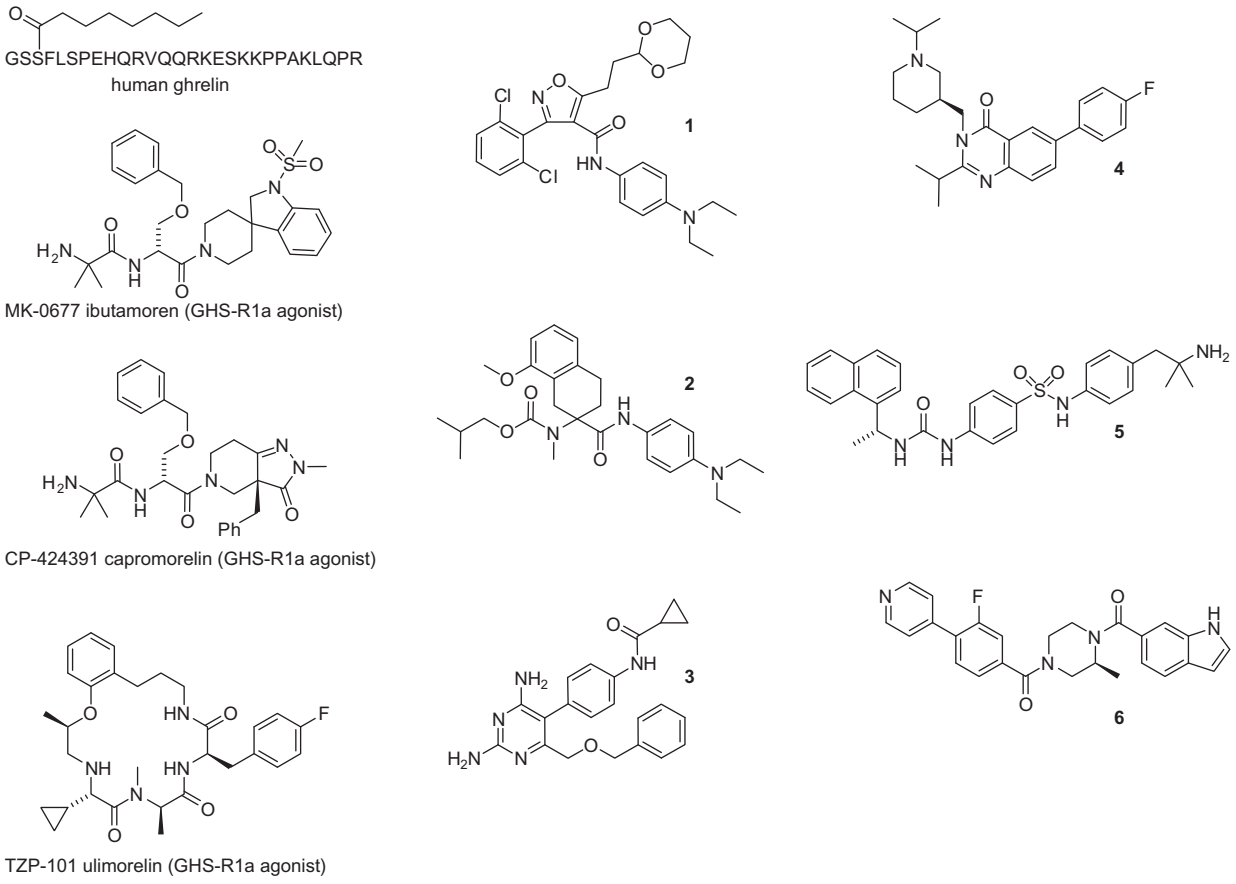


Figure 1. Ghrelin receptor agonists and antagonists.

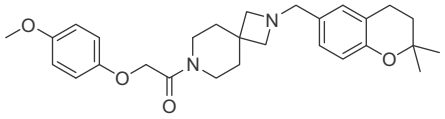
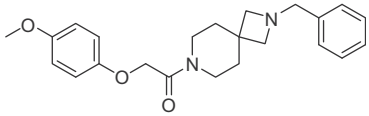
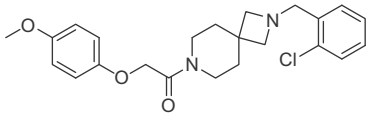
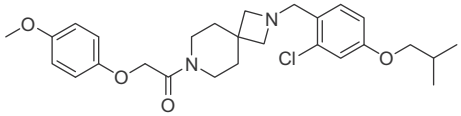
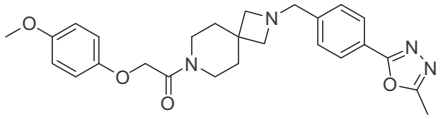
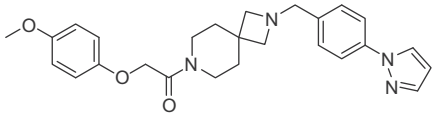
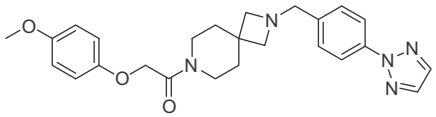
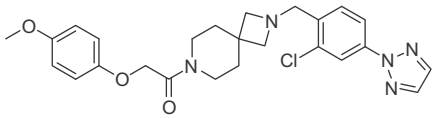
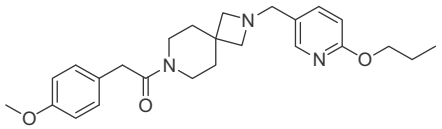
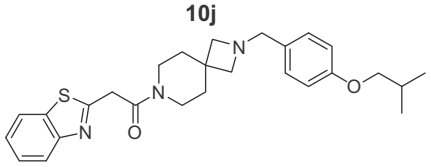
inverse agonist properties. Priority for lead follow-up was given to inverse agonists to ensure a non-agonist profile, and to compounds with a low hydrogen bond donor count and physicochemical properties consistent with achieving CNS penetration.

Compound **10a** (Table 1), identified from a screen of our corporate file, was an attractive hit because of its inverse agonist functional profile and moderate binding potency. Its physical properties (molecular weight 464, *c*log*P* 4.4) were modestly outside of our desired range for a hit that targeted CNS penetration, but our expectations were tempered by the known difficulties of finding low molecular weight ligands for peptidic GPCRs.[25] The structure of compound **10a**, a spiro-piperidine-azetidine with the two nitrogens functionalized respectively as an amide and a tertiary amine, provided an opportunity for efficient synthesis of analogues around the spiro-diamine core.[26] Starting from *tert*-butoxycarbonyl (Boc) protected diamine **7**,[27] we developed synthetic conditions that enabled the parallel synthesis of analogues as shown in Scheme 1. The identification of efficient synthetic conditions utilizing readily available reagent classes (carboxylic acids and aromatic aldehydes) that contain significant structural diversity enabled us to rapidly query structure-activity relationships with regard to potency, as well as with regard to absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties.

Our initial approach to the design of compounds was to move the physical properties of the hit compound toward lower lipophilicity and/or lower molecular weight chemical space, while also probing potency, functional profile, and ADMET properties (with a focus on stability in human liver microsomes (HLM) and on activity in a dofetilide binding assay as a proxy for hERG activity). Because one of our aims was to decrease molecular weight relative to the hit, we employed ligand efficiency[28] as a method complementary to absolute potency for assessing binding activity. To simplify design of compounds and analysis of specific structure-activity relationships, we focused primarily on making changes to one end of the molecule at a time, with the expectation of subsequent iterations of synthesis that would cross 'good' substituents from the initial analogues. Recognizing that most of the hydrophobicity in the compound lay in the dimethylchroman group of the lead compound **10a**, the majority of our initial compounds shown in Table 1 focused on changes to this region of the molecule, although the flexible synthetic route enabled us to probe concurrently changes to both ends of the molecule.

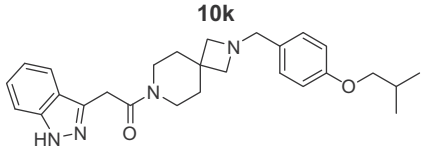
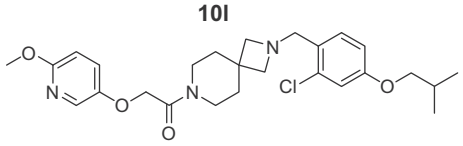
Several active compounds that replaced the dimethylchroman group in **10a** with lower molecular weight or lower lipophilicity groups were identified. The parent benzyl derivative **10b** was synthesized as a benchmark compound. General trends in SAR of the azetidine-benzyl substituent included the following (some data not shown). At the 2-position of the phenyl ring, small lipophilic groups such as a chlorine atom (**10c**), provided enhanced ligand efficiency relative to the hit compound; larger groups diminished both potency and ligand efficiency. Substitutions at the 4-position were preferred to those at the 3-position of the phenyl ring, based on both potency and ligand efficiency. At the 4-position, electron donating groups (e.g., isobutyoxy, **10d**) provided enhanced potency relative to electron withdrawing groups (e.g., oxadiazole, **10e**). A subset of compounds that spanned a range of structural types and physical properties (as well as a range of ghrelin receptor binding potencies) were tested in high throughput ADMET assays to establish a baseline understanding of structure-ADMET relationships. Within the initial set of compounds tested in ADMET assays, the oxadiazole **10e** was noted because it demonstrated relatively low HLM clearance and dofetilide binding.

Table 1
Lead compounds and initial SAR

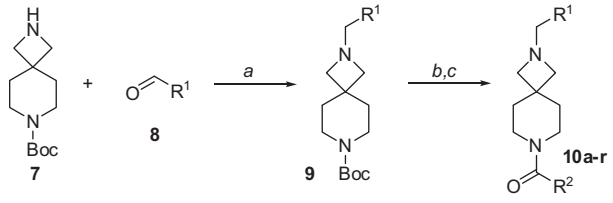
Compound	Human ghrelin receptor binding IC_{50}[a] (nM)	LE	Clog*P* (*E*log*D*[b])	HLM[c] (RLM)	Dofetilide[d]	Human ghrelin receptor K_i[a] (nM)	Funct. response[a,e]	Rat CL_p mL/min/kg (percent pulmonary extraction)
10a 	211	0.27	4.4	31 (71)	77	408	−22	
10b 	2980 *N*=1	0.27	2.9	21			−22 *N*=1	
10c 	231	0.31	3.7	47		445	−26	
10d 	86	0.29	4.3	88	106	20	−18	
10e 	1070	0.24	1.8 (1.5)	<8	46	52	−31	61 (22%)
10f 	442	0.26	3.0 (1.9)	20 (32)	94	152	−32	
10g 	102	0.29	3.0 (2.2)	22 (42)	76	14	−33	152
10h 	14	0.31	3.9 (4.1)	54 (189)	87	11	−40	83 (80%)
10i 	407	0.28	3.2 (2.6)	31 (123)	64	114	−19	447
10j 	86	0.29	4.2 (3.4)	42 (135)	102	25	−21	

(*continued on next page*)

Table 1 (*continued*)

Compound	Human ghrelin receptor binding IC_{50}[a] (nM)	LE	*C*log*P* (*E*log*D*[b])	HLM[c] (RLM)	Dofetilide[d]	Human ghrelin receptor K_i[a] (nM)	Funct. response[a,e]	Rat CL_p mL/min/kg (percent pulmonary extraction)
10k 	120	0.28	3.8 (3.3)	63 (105)		14	−27	
10l 	59	0.29	4.9 (4.4)	43 (107)	100	17	−17	238

[a] Unless otherwise indicated cumulative in vitro characterization is the result of 3 or more determinations run in duplicate or triplicate.
[b] Reference.[29]
[c] Human and rat liver microsome intrinsic apparent clearance, μL/min/mg.
[d] Dofetilide binding percent inhibition @ 10 μM.
[e] Reference[23], agonist mode maximum functional response (minimum asymptote) in GTP-γ-S functional assay. A negative value indicates an inverse agonist.



Scheme 1. Synthesis of piperidine-azetidine inverse agonists of the ghrelin receptor. Reagents and conditions: (a) $Na(OAc)_3BH$, 30–40%; (b) HCl, dioxane, 99%; (c) HBTU, DIEA, R_2CO_2H, DMF, 90%.

The initial SAR on the benzyl substituent suggested two distinct directions for follow-up; both were pursued simultaneously. One approach for compound design focused on the desirable ADMET properties of the phenyl-oxadiazole **10e**. The other approach built on the observed increases in ligand efficiency or potency: ortho substitution of the phenyl ring with a chlorine atom, and/or para substitution of the phenyl ring with an alkoxy group.

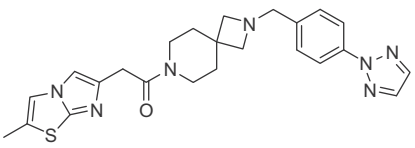
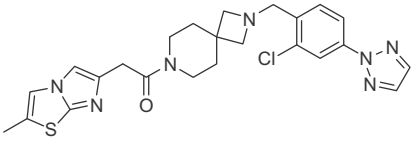
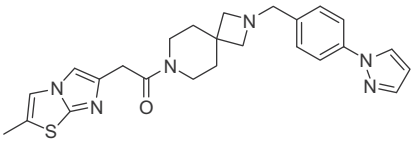
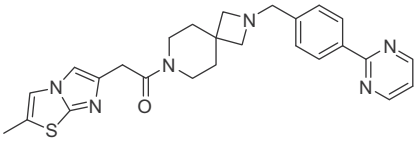
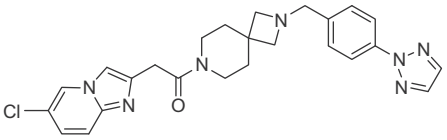
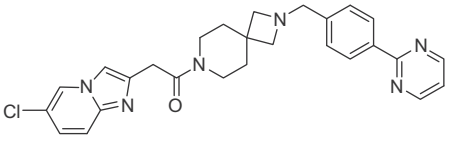
The relatively good ADMET properties of the phenyl-oxadiazole compound **10e** were hypothesized to be due to the lower lipophilicity (*c*log*P* 1.8; *e*log*D* 1.5) and/or the electron withdrawing nature of the oxadiazole substituent on the phenyl ring. A broad set of compounds that further pursued this hypothesis was synthesized. The first set of phenyl-heteroaryl derivatives focused on pyridine, imidazole, pyrazole, and triazole isomers in the terminal ring as representative compounds with varied overall lipophilicity as well as different spatial placement of heteroatom(s). The variation in ghrelin receptor binding potency across this set of compounds was interpreted to be evidence of specific interactions between this region of the molecule and the receptor. A subset of these five- and six-membered heteroaryl derivatives did demonstrate improved potency relative to the oxadiazole, and a partially overlapping subset also exhibited relatively good HLM stability. The balance between potency and clearance exhibited by this group of compounds, exemplified by the pyrazole and triazole derivatives (**10f** and **10g**), was appealing, although dofetilide binding remained a concern.

A subsequent iteration of synthesis to combine the previously identified potency-enhancing ortho-chloro substitution with a selection of the phenyl-heteroaryl derivatives provided compounds such as **10h**, which demonstrated the anticipated improvement in potency and ligand efficiency at the expected expense of increased lipophilicity and clearance. However, identifying specific structural changes (*para*-heteroaryl, *ortho*-chloro) that enabled us to predict relatively well the trade-offs between potency and clearance was a significant breakthrough as we continued to further optimize compounds in this series.

On the piperidine amide side of the molecule, concerns about possible metabolic pathways guided initial efforts focused on identifying structural alternatives to the masked hydroquinone functionality. A structurally diverse set of amides was synthesized, but only relatively small structural changes were found to retain significant ghrelin receptor binding activity. A one-atom deletion from phenoxyacetamide to phenylacetamide was tolerated (**10i**), as was replacement of the dialkoxyphenyl group by selected bicycloheteroaromatic rings (**10j** and **10k**). Simple substitution of pyridine for phenyl also provided adequate potency (**10l**; coupled with the potency-enhancing ortho-chloro benzyl substitution). These alternative heteroaryl acetamide derivatives in particular provided a structural direction in which we could further modulate overall lipophilicity of the compounds, as well as the electron-density in this aromatic ring. Importantly, from the initial iterations of synthesis to develop SAR around both the amide and amine substituents on the spiro-piperidine-azetidine core, all of the compounds tested in the functional assay displayed inverse agonist profiles.

With an initial understanding of the potency and ADMET SAR within this series, we then sought to benchmark selected compounds for in vivo pharmacokinetics. Our primary objective was to understand the overall properties of this series of amido-piperidine-azetidines, rather than to characterize a single lead compound. Thus, a subset of compounds representing the range of productive structural modifications was selected for rat pharmacokinetic studies properties using cassette dosing.[30,31] Up to 5 compounds were combined per cassette, ensuring at least 2.5 amu differences between the compounds in the cassette. Animals were dosed at 0.4 mg/kg for iv studies and 1 mg/kg for po studies. Disturbingly, all compounds tested in rat showed high clearance (in most cases, exceeding hepatic blood flow = 70 mL/min/kg; see Table 1). Several hypotheses were put forth to explain the high rate of clearance. Hypotheses that were subsequently disproved with experimental data included: instability of compounds in rat

Table 2
Compounds with improved overall profiles

Compound	Human ghrelin receptor binding IC_{50}[a] (nM)	LE	*C*log*P* (*E*log*D*[b])	HLM[c] (RLM)	Dofetilide[d]	Human ghrelin receptor K_i[a] (nM)	Funct. response[a,e]	Rat CL_p mL/min/kg (percent pulmonary extraction)
10m 	25	0.31	2.6 (1.9)	18 (19)	20	69	−22	48 (0%)
10n 	4.6	0.33	3.4 (3.6)	34 (159)	40	7.0	−35	41 (0%)
10o 	298	0.28	2.5 (1.5)	11 (<16)	56	206	−23	
10p 	90	0.30	2.1 (1.4)	14 (<16)	11	14	−23	
10q 	204	0.27	3.0 (2.4)	23	63	23	−27	17 (0%)
10r 	321	0.25	2.6 (1.7)	<8 (<14)	32	27	−28	

[a] Unless otherwise indicated cumulative in vitro characterization is the result of 3 or more determinations run in duplicate or triplicate.
[b] Reference.[29]
[c] Human and rat liver microsome intrinsic apparent clearance, μL/min/mg.
[d] Dofetilide binding percent inhibition @ 10 μM.
[e] Reference[23], Agonist mode maximum functional response (minimum asymptote) in GTP-γ-S functional assay. A negative value indicates an inverse agonist.

plasma, selective partitioning of compounds into blood from plasma, biliary excretion, and renal excretion.

Pulmonary extraction to explain the high in vivo clearance was the hypothesis best supported by our data. In our standard rat intravenous PK protocol, compounds were dosed via the jugular vein, with samples taken via the carotid artery. One hundred percent of the intravenous dose passes through the lungs before reaching systemic circulation. A modified protocol utilized intra-arterial dosing (and sampling) via the carotid artery, thus by-passing pulmonary first pass extraction. The comparison of AUCs for compound exposure between the intravenous and intra-arterial dose routes provided a measure of pulmonary extraction, as reported in Table 1.[32]

Though we did not experimentally test the possibility, we believed that if the observed pulmonary clearance were due to lung P450 enzymes,[33] we should emphasize structural changes that were likely to diminish CYP-mediated metabolism. We thus focused on electron rich phenyl rings and on relatively high overall lipophilicity, and crossed the general SAR findings from the two ends of the molecule – specifically, replacing alkoxyphenyl rings with heteroaryl derivatives, believing that the heteroaryl groups would enable tuning of both electronics and lipophilicity. Significant effort across multiple iterations of compound design and synthesis was dedicated to identifying heteroaryl derivatives that delivered the desired potency and ADMET properties. Ultimately, a set of lead compounds with desirable potency, HLM stability,

and dofetilide binding were identified. Further, selected compounds evaluated for rat in vivo clearance and pulmonary extraction provided support for the hypothesis that replacing the alkoxyphenyl rings with heteroaryl derivatives would improve in vivo PK properties. The most promising compounds from this series (Table 2) contained a fused bicyclic imidazo-acetamide group on the piperidine, and a heterobiaryl derivative on the azetidine. The rat in vivo PK properties of compound **10n** were further characterized; it demonstrated oral bioavailability of 43% (30 mg/kg dose in Sprague–Dawley rat), and it exhibited reasonable penetration into the brain ([free brain] = 324 nM, [free plasma] = 560 nM @ 30 min, 30 mg/kg, po).

In summary, we exploited an efficient three-step synthetic process that allowed variation at each end of a piperidine-azetidine core structure from readily accessible carboxylic acid and aromatic aldehyde starting materials. The ability to rapidly explore SAR hypotheses, around ghrelin receptor binding potency, in vitro ADMET, and in vivo rat PK, was critical to the identification of specific structural changes that enabled the improvement of potency and of ADMET properties. Decreased lipophilicity and replacement of alkoxyphenyl rings with heteroaryl derivatives were the key structural changes that drove this series toward more drug-like properties. A hypothesis of pulmonary extraction as a primary source of extrahepatic clearance among early compounds in this series was tested by the use of jugular vein and carotid artery cannulated rats.

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21. Ghrelin SPA binding assays were performed in 384-well plates in a final volume of 90 μL containing 250 ng human GHS-R1a (HEK293 tetracycline-inducible cell line expressing the human growth secretagogue receptor 1a; prepared as membranes) coupled to 0.5 mg SPA beads (wheat germ agglutinin coated, GE Healthcare, RPNQ0060) and 50 pM [^{125}I]-ghrelin (Perkin Elmer Life Sciences, NEX-388), plus varying concentrations of test compound or vehicle. Controls were included on each assay plate to define 0% effect (ZPE) where no compound was included in the binding reaction and 100% effect (HPE) where 2 μM unlabeled ghrelin was added to maximally displace the radioligand. All reagents were diluted in assay buffer (50 mM HEPES, 10 mM MgCl2, 0.2% BSA, EDTA-free protease inhibitors mix, pH 7.4) and reactions were incubated for 8 h at room temperature to allow binding to reach equilibrium. The amount of receptor–ligand complex was determined by liquid scintillation counting using a 1450 Microbeta Trilux (Wallac). Data analysis was performed using a proprietary software package. Briefly, the percent effect for each compound dose (Sample) was calculated from raw data as follows: % Effect = 100–100 × ((Sample-HPE)/(ZPE − HPE)) where HPE and ZPE values are averages of 16 wells each. The compound % effect values were then plotted versus concentration and the IC_{50} was determined using a standard 4-parameter fit algorithm.
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23. To measure the ability of test compounds to modulate the activity of human GHS-R1a (agonize, antagonize, partially agonize, inversely agonize), a DELFIA GTP-binding assay (Perkin Elmer, AD0260 and AD0261) was performed. The assay monitors the ligand-dependent exchange of GDP for GTP. GPCR activation results in an increase in fluorescence as receptor-bound GDP is replaced by Europium-labeled GTP. Antagonist binding prevents GDP-GTP exchange whereas binding of an inverse agonist pushes the receptor to the GDP bound (inactive) state, both resulting in decreased fluorescence. Ghrelin functional assays were performed in a final volume of 39.5 μL containing 720 ng human GHS-R1a (HEK293 tetracycline-inducible cell line expressing the human growth secretagogue receptor **1a**, prepared as membranes), 9 nM GTP-Europium and varying concentrations of test compound or vehicle. To test for receptor antagonism (antagonist mode), membranes were incubated in the presence of agonist ghrelin (Anaspec, 24158) at the EC_{80} concentration, plus test compound or vehicle. To test for receptor agonists (agonist mode), membranes were incubated in the absence of ghrelin. Briefly, test compounds were prepared in DMSO at room temperature in 384-well plates (Matrix, 4340) and then transferred to intermediate plates containing basal buffer (50 mM HEPES pH 7.4, 3.7 mM $MgCl_2$, 250 μM EGTA, 125 nM GDP) with and without EC_{80} concentration of ghrelin peptide. Controls were included on each assay plate to define 0% effect (ZPE) where no compound was included in the binding reaction and 100% effect (HPE) where either 10 μM ghrelin was added to determine maximal agonist activity or the EC_{80} concentration of ghrelin was omitted to determine maximal antagonist activity. Samples were then transferred to 384-well filter plates (Pall, 5071) containing hGHS-R1a membrane and 0.35 mg/mL saponin (Perkin Elmer, AD0261) in basal buffer. The mixture was incubated 24 min at room temperature with gentle shaking, followed by the addition of GTP-Europium in 50 mM HEPES pH 7.4. Samples were shielded from light and incubated for 90 minutes further at room temperature with gentle shaking. The reactions were suctioned dry with vacuum, washed three times with ice cold 1× GTP Wash Solution (Perkin Elmer, AD0261), and immediately read on the Envision 2101 Multilabel Reader (Perkin Elmer) using excitation filter 320 nm and emission filter 615 nm. Data analysis was performed using a proprietary software package. Briefly, the percent effect for each compound dose (Sample) was calculated as follows: %Effect = 100–100 × ((Sample-HPE)/(ZPE–HPE)) where HPE and ZPE values are averages of 16 wells each. The compound % effect values were then plotted vs. compound concentration and the K_i was determined for antagonists and inverse agonists as follows: $K_B = IC_{50}/(1 + ([\text{radioligand}]/K_d))$ where IC_{50} was determined from a standard 4-parameter fit algorithm, the [radioligand] = EC_{80} concentration ghrelin, and K_d is experimentally determined in each run by performing a ghrelin titration. Similarly, for compounds tested in agonist mode the % effect for each well is calculated based on the median values for the HPE and ZPE controls. The % effect values for each compound/dose are then plotted to generate a dose response curve for each compound/batch tested. The agonist compounds will have an increasing curve where % effect will increase with dose. Antagonist compounds will appear inactive. Inverse agonist compounds will have decreasing curves with the % effect values becoming more negative with increasing concentration of compound. The agonist activity of the compounds is quantitated by calculating an EC_{50} value.
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