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Novel isoxazole carboxamides as growth hormone secretagogue receptor (GHS-R) antagonists

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Abstract—Novel isoxazole carboxamides have been identified as growth hormone secretagogue receptor (GHS-R) antagonists. Substituent modification off the 5-position of the isoxazole ring led to analogues with potent binding affinity and functional antagonism of GHS-R. A potent analogue (**32**) with high aqueous solubility and good GPCR selectivity was also identified as a potential pharmacological tool for in vivo studies.

Ghrelin, a 28 amino acid peptide with an unique *n*-octanoyl modification on Ser 3, was recently identified as an endogenous ligand for growth hormone secretagogue receptor (GHS-R), a G-protein coupled receptor (GPCR).[1] Ghrelin is produced mainly in the upper intestinal tract as well as in the hypothalamus.[2] Besides being a potent growth hormone secretagogue and regulator of other endocrine functions, ghrelin is also implicated in the short- and long-term regulation of energy balance.[3] Its administration in near physiological doses in rodents[4] and humans[5] stimulates food intake. Chronic administration of ghrelin in freely feeding mice and rats results in increased body weight and decreased fat utilization.[6]

Consistent with ghrelin's involvement in long-term regulation of body weight, accumulating data suggest that ghrelin antagonism may lead to desired effects in an anti-obesity therapeutic regimen. It has been shown that single intracerebroventricular (icv) or intraperitoneal (ip) administration of anti-ghrelin IgG suppresses acute feeding in lean rats.[7,8] In addition, chronic twice-daily icv administration of anti-ghrelin IgG reduces body weight over a five-day period.[9] A recent study using a peptidic GHS-R antagonist, [D-Lys-3]-GHRP-6, showed a reduction of food intake and body weight gain in diet induced obese mice.[10] Based on the above observations in rodents and humans, administration of a selective small molecule GHS-R antagonist can potentially lead to reduced food intake, decreased adiposity, and body weight reduction in humans.

Only one small molecule GHS-R antagonist has been reported in the literature. Compound **1** is a 3-amino-2,3,4,5-tetrahydro-benzo[*b*]azepin-2-one derivative used in the preparation of a nonpeptidyl GHS.[11] We recently identified a novel isoxazole carboxamide derivative (**2**) from high throughput screening. It was confirmed as a pure, competitive GHS-R antagonist with an IC_{50} of 180 nM in a calcium flux cellular assay. Extensive medicinal chemistry efforts were initiated to explore the structure–activity relationship (SAR) of this series of compounds. In this paper we present the synthesis and biological profiles of analogues through substituent modification off the 5-position of the isoxazole core, which led to compounds with potent functional antagonism of GHS-R and good GPCR selectivity (Fig. 1).

The isoxazole carboxamide derivatives were prepared in a straightforward fashion as outlined in Scheme 1. Various β-ketoesters **3** were prepared through reacting the imidazolide of the corresponding carboxylic acids with magnesium monoethylmalonate followed by spontaneous decarboxylation.[12] The [2+3] cyclization between

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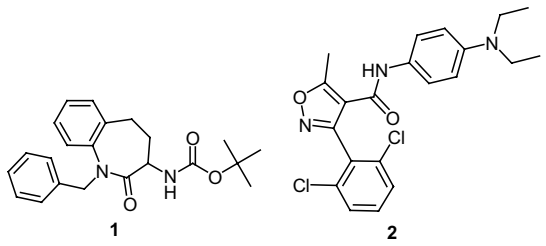
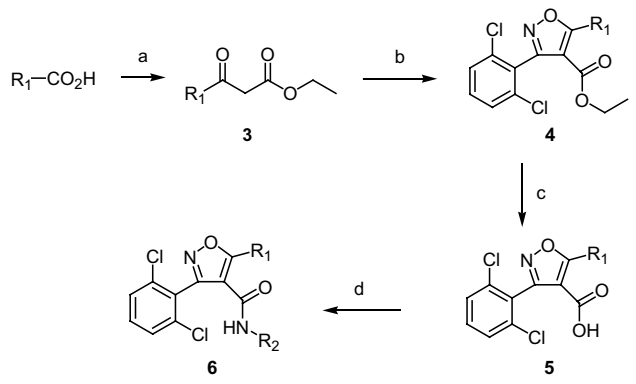


Figure 1. GHS-R antagonists from both literature and internal screening.



Scheme 1. Reagents and conditions: (a) CDI, THF, $MgCl_2$, EtO_2CCH_2COOK; (b) 2,6-dichloro-*N*-hydroxybenzenecarboximidoyl chloride, NaOMe, MeOH, rt; (c) NaOH, MeOH, H_2O; (d) R_2NH_2, TBTU, HOBT, Et_3N, DMF, rt.

ketoesters **3** and 2,6-dichloro-*N*-hydroxyarylcarboximidoyl chloride was accomplished with sodium methoxide in methanol to yield the isoxazole carboxylates **4**. Base hydrolysis of the esters **4** afforded the key isoxazole acids **5**, which were then coupled with amines to furnish isoxazole carboxamide derivatives **6**.[13]

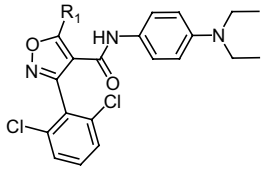
The amine **8** was prepared from *trans*-1,4-cyclohexyldiamine through monoprotection of the amine group to yield compound **7** (Scheme 2). Subsequent reductive amination and deprotection generated the amine **8** for coupling with the isoxazole acid core.

The primary binding assay for the compounds described in this study was a measurement of their ability to displace ^{125}I-radiolabeled ghrelin from its receptor using Chinese hamster ovarian (CHO)-K cell membranes permanently expressing human GHS-R. Ghrelin was found to have a K_d of 0.4 nM in this assay. A fluorescent calcium indicator assay (FLIPR) measuring the compounds' ability to inhibit a ghrelin-induced increase in intracellular $[Ca^{2+}]$ in CHO-K cells was utilized to determine functional antagonism.[14] Mouse and rat orthologues of GHS-Rs were also cloned and stable cell lines were generated for use in species selectivity assays on selected compounds.

The original screening lead **2** showed submicromolar activity in both binding and FLIPR assays. Initial extension of the 5-methyl group of the isoxazole ring revealed interesting SAR and prompted us to further explore this observation. Elongation of the methyl group of **2** to ethyl (**9**), propyl (**10**), and isopropyl (**11**) groups seemed to have little impact on the binding affinity of the resultant analogues, but such changes appeared to cause a decrease in FLIPR activity (Table 1). Compound **12** seemed to suggest that more hydrophilic groups, such as a hydroxyl group, would be tolerated off the extended alkyl groups. Additionally, methoxyethyl group-derived **13** appeared to be equipotent to **2** in terms of both binding affinity and FLIPR activity. Therefore, we decided to explore analogues featuring a variety of hydrogen bond acceptor-containing cyclic structures linked through an ethylene group to the isoxazole core.

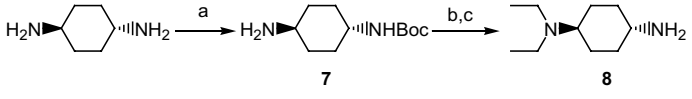
When 1,3-dioxane ethyl (**14**) was introduced into the 5-position of isoxazole ring, an over 20-fold increase in binding potency was observed; a similar degree of FLIPR activity improvement was also noted (Table 2). Corresponding 2-tetrahydropyran and 4-tetrahydropyran analogues (**15**–**16**) were only slightly better than **2** in binding affinity and were comparable in FLIPR activity. Five-membered dioxalane-derived analogue **17** was 3–4-fold weaker than the six-membered **14**, while tetrahydrofuran analogue **18** was almost equipotent to **14**. The largest potency improvement came from introduction of piperidinonylethyl to give antagonist **19** with low nanomolar IC_{50} values in both binding and FLIPR assays. In comparison, five-membered pyrrolidinone-

Table 1. SAR of 5-alkyl modification in isoxazole carboxamides



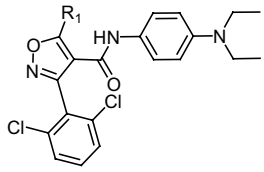
Compounds	R_1	Binding IC_{50} (nM)[a]	FLIPR IC_{50} (nM)[a]
2	CH_3	130	180
9	C_2H_5	147	319
10	*n*-C_3H_7	131	492
11	*i*-C_3H_7	150	861
12	*n*-C_3H_6OH	132	308
13	$C_2H_4OCH_3$	110	115

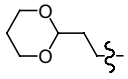
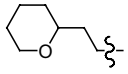
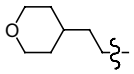
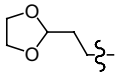
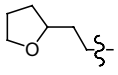
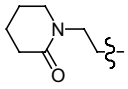
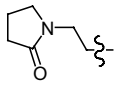
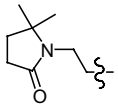
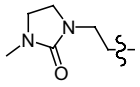
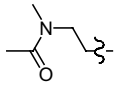
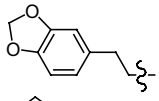
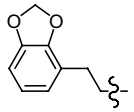
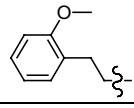
[a] Values are the means of at least two experiments against human GHS-R.



Scheme 2. Reagents and conditions: (a) Boc_2O (0.5 equiv), $CHCl_3$, rt; (b) CH_3CHO, $Na(OAc)_3BH$, AcOH, CH_2ClCH_2Cl, rt; (c) 4 N HCl in 1,4-dioxane, rt.

Table 2. SAR of 5-alkyl modification in isoxazole carboxamides



Compounds	R_1	Binding IC_{50} (nM)[a]	FLIPR IC_{50} (nM)[a]
14		6	19
15[b]		39	102
16		29	146
17		25	56
18[b]		57	57
19		9	8
20		18	18
21		39	15
22		21	10
23		34	19
24		122	98
25		47	93
26		162	564
27		389	714

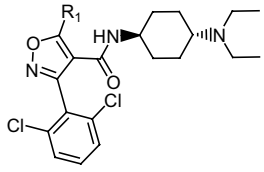
[a] Values are means of at least two experiments against human GHS-R.
[b] 1:1 mixture of two enantiomers.

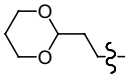
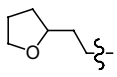
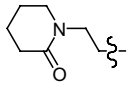
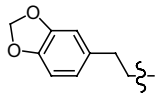
derived analogue **20** was about 2-fold less potent than the six-membered **19** in both binding and FLIPR. Further modification of the piperidinone only maintained the low double-digit nanomolar potency with 5,5-dimethyl pyrrolidinone (**21**), 3-*N*-methylimidazolinone (**22**), and acyclic acetamide (**23**) analogues. In contrast, pyrrolidinone linked via a methylene group to the isoxazole core (**24**) was almost 50-fold weaker than the corresponding ethylene analogue **20**.

A variety of methoxy-substituted phenyl groups were also examined as alternatives to H-bond acceptor-containing heterocycles. 3-Benzo[1,3]dioxolyl replacement yielded a slightly more potent analogue (**25**, Table 2) than **2**, while 2-benzo[1,3]dioxolyl substituent yielded a slightly less potent analogue **26**. In comparison, 2-methoxyphenylethyl substituent resulted in analogue **27** with a 4-fold loss of FLIPR activity.

For in vivo studies we would like to inject GHS-R antagonists directly into the brain of rodents, which requires small dosing volumes and highly aqueous soluble agents. In the course of SAR exploration of phenylenediamine amide off the isoxazole core of **2**, it was found that the phenylenediamine motif could be replaced with *trans*-cyclohexyldiamine with slight improvement of activity, as shown with compound **28** in Table 3. Analogue **28** was also found to possess much-improved aqueous solubility as determined by turbidity assay (>1.0 mM @ pH 7.4). Therefore, *trans*-cyclohexyldiamine was incorporated in selected isoxazole core modifications to explore SAR of more soluble analogues. In the context of *trans*-cyclohexyldiamine amides, 1,3-dioxane ethyl derivative (**29**) was found to be a fairly potent binder with strong ghrelin-like agonistic response.[15] Moderately potent pure antagonists were generated with tetrahydrofuranyl (**30**) and piperidinonyl (**31**)-based core modifications. Benzo[1,3]dioxyl-based core modification yielded potent, pure ghrelin antagonist **32** when

Table 3. SAR of the isoxazole *trans*-cyclohexyl diamine amides



Compounds	R_1	Binding IC_{50} (nM)[a]	FLIPR IC_{50} (nM)[a]
28	CH_3	53	57
29		87	3[b]
30[c]		194	103
31		139	190
32		32	54

[a] Values are means of at least two experiments.
[b] Exhibited a strong ghrelin-like agonistic response in the FLIPR assay.
[c] 1:1 mixture of two enantiomers.

combined with *trans*-cyclohexyldiamine amide. It had aqueous solubility of greater than 1 mg/mL, making it suitable for dosing icv through catheter injection.

Compound **32** was found to have FLIPR IC_{50} values of 12 and 7 nM, respectively, against rat and mouse GHS-Rs. Compound **32** was also tested against a panel of seven GPCRs, including adrenergic, histaminergic, muscarinic, and dopaminergic receptors. The FLIPR K_i values for **32** exceeded 4000 nM in these assays, suggesting good GPCR selectivity for this potent GHS-R antagonist.

In summary, a novel series of isoxazole carboxamides have been identified as potent GHS-R antagonists. Substituent modification off the 5-position of the isoxazole led to analogues with potent binding affinity and functional antagonism of GHS-R. The high aqueous solubility of **32** and its good selectivity profile suggest that this compound could be a useful pharmacological tool for investigating the effects of GHS-R antagonism in vivo.

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13. All target compounds were determined to be >95% pure by both ^{1}H NMR and LC/MS spectroscopy; the spectral data were consistent with the reported structure.
14. FLIPR assay: CHO-K cells expressing human GHS-R (Euroscreen) were cultured in Ultra-CHO medium from BioWhittaker supplemented with 1% dialyzed FCS, 1% penicillin/streptomycin/fungizone, and 400 μg/mL G418 (all from Life Technologies) at 37 °C in a humidified cell incubator containing 5% CO_2. Cells were plated in black 96-well plates with clear bottom (Costar) and cultured to confluency overnight. Prior to assay, cells were incubated in 100 μL of Dulbecco's phosphate-buffered saline (DPBS) containing 1000 mg/L D-glucose, 36 mg/L sodium pyruvate, without phenol red (Life Technologies) with 1.14 mM Fluo-4 AM (Molecular Probes) and 0.25 M probenecid (Sigma) for 1–3 h in the dark at room temperature. The dye solution was aspirated and the cells were washed twice with DPBS using the EL-450X cell washer (BioTech). After the last wash, 100 μL of DPBS was added to each well. Cell plates were then transferred to the FLIPR unit (Molecular Probes). Compound additions were 50 μL in duplicate or triplicate of 4× final concentration in DPBS containing 0.1% BSA and 4% DMSO. Fluorescence emissions from 96 wells were measured simultaneously at excitation and emission wavelength of 488 and 520 nm, respectively, for 3 min in 1-s intervals for the first minute and 5-s intervals thereafter. During this time agonist responses, if any, were recorded in the absence of ghrelin. Next, 50 μL in duplicate or triplicate of 4× final concentration of ghrelin in DPBS containing 0.1% BSA and 4% DMSO were delivered within 1 s by an integrated 96-well pipettor. Fluorescence emissions were measured for another 3 min as above. During this time the antagonist effects of compounds on ghrelin-stimulated calcium flux were recorded. Sigmoidal curves were fitted and IC_{50} and EC_{50} values were determined by GraphPad Prism software. Ghrelin shows an EC_{50} of 0.2 nM in this assay.
15. Compound **29** was found to give a strong ghrelin-like agonistic response in the FLIPR assay as described in Ref. 14 with a calculated EC_{50} value of 9 nM.