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New ligands of the ghrelin receptor based on the 1,2,4-triazole scaffold by introduction of a second chiral center

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ABSTRACT

Introducing a second chiral center on our previously described 1,2,4-triazole, allowed us to increase diversity and elongate the 'C-terminal part' of the molecule. Therefore, we were able to explore mimics of the substance P analogs described as inverse agonists. Some compounds presented affinities in the nanomolar range and potent biological activities, while one exhibited a partial inverse agonist behavior similar to a Substance P analog.

Ghrelin,[1] an orexigenic hormone essentially synthesized in the stomach, is the endogeneous ligand of the growth hormone secretagogue receptor (GHS-R1a).[2] Ghrelin is a peptide composed of 28 amino-acids, octanoylated on the seryl residue in position 3. This lipidation is essential for both binding to the receptor and biological activity.[3] Among its various biological functions, ghrelin stimulates the secretion of GH (Growth Hormone),[3] and food intake,[4] controls energy homeostasis[5] and gastrointestinal motility.[6] It has effects on cellular proliferation,[7] cardiovascular,[8] pancreatic, pulmonary and immune functions, memory and sleep.[9] More recently, it was established that ghrelin plays a role in addiction processes.[10] In our effort to find efficient ghrelin receptor ligands, we have developed a pseudo-peptide (JMV 1843),[11] which is a potent in vitro and in vivo agonist of the GHS-R1a. This compound contains a gem-diamino moiety (Fig. 1) and is orally active in man[12] as exemplified by an initial validation study.[13] Named macimorelin, it is evaluated in a test that detects adult growth hormone deficiency. A pivotal confirmatory study is currently recruiting participants.[14] We then focused on the search of GHS-R1a antagonists, which could lead to potential anti-obesity agents. We decided to explore non-peptide ligands of the GHS-R1a through heterocycles bearing the pharmacophore groups included in JMV 1843. We discovered that the 1,2,4-triazole scaffold presented an interesting approach to obtain ligands with high affinity toward the GHS-R1a (Fig. 1).[15,16]

The synthesis of the 1,2,4-triazole scaffold was efficiently performed starting from commercially available compounds. An easy access to tri-substituted 1,2,4-triazoles with four points of diversity was developed and optimized.[17] The first step of the synthesis included a *N*-protected α-amino acid whose configuration was conserved during the entire synthetic route. Several derivatives of these new active non-peptide compounds were synthesized. An intensive SAR study involving the four putative points of diversity was carried out. The following conclusions were made: (i) the preferred amino-acid starting material was D-tryptophan; (ii) on position 4 of the triazole, a 4-methoxy- or 2,4-dimethoxy-benzyl group preferentially led to receptor antagonists; (iii) a 2 carbon chain bearing a phenyl or an indole group was preferred in position 3; (iv) numerous acyl groups including α-aminoisobutyryl (Aib), pyridin-2-ylcarboxyl, and glycyl groups, could be introduced in R^3, modulating both the binding affinity and the biological activity.

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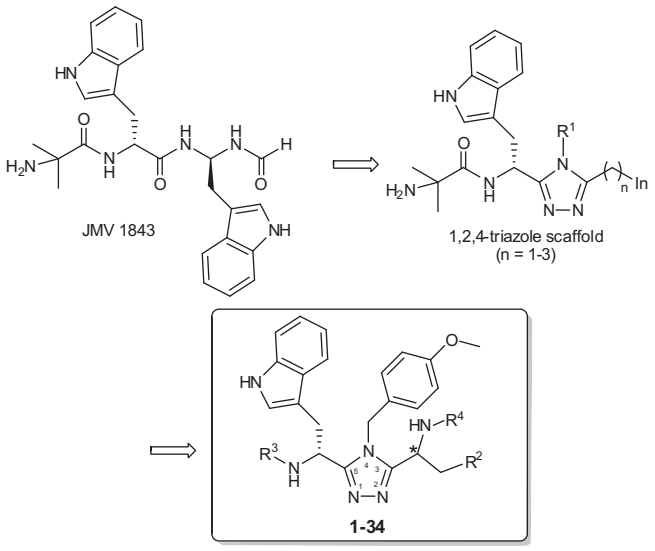


Figure 1. From pseudo-peptide to peptido-mimetic.

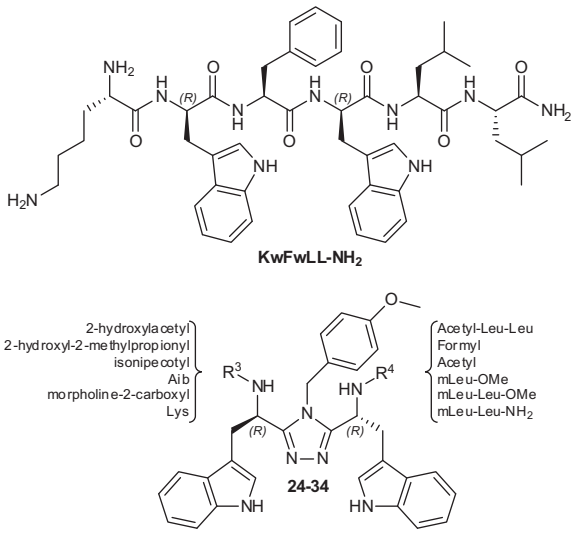


Figure 3. Similarity between KwFwLL-NH_2 and triazoles 24–34.

It is also interesting to notice that a simple atom change could shift an agonist compound into an antagonist (Fig. 2). Indeed, when the piperidine moiety of compound JMV 2951 [EC_{50} (Ca^{2+}) = 1.6 nM] was replaced by a tetrahydro-2*H*-pyran group (compound JMV 3168), the agonist character of the ligand was lost to the benefit of the antagonist character [IC_{50} (Ca^{2+}) = 60 nM].

As compound JMV 1843 includes in its C-terminal part a gem-diamino function, we then decided to introduce a chiral center in position 3 of our 1,2,4 triazole scaffold allowing the presence of an amine function at this position. This modification should lead to closer structures of compound JMV 1843 than the previous tri-substituted triazoles and could allow an additional point of diversity (Fig. 1). Moreover, a characteristic of this receptor is its high constitutive (ligands independent) activity, which reach about 50% of the maximal activity induced by ghrelin.[18] Although ghrelin receptor antagonists are able to reduce meal-associated food intake,[19] inverse agonists of the ghrelin receptor, by blocking the constitutive receptor activity, are expected to lower the set-point for hunger between meals.[20] The first GHS-R1a inverse agonist was reported by Holst et al. as a Substance P analog: [(D)Arg[1],(D)Phe[5],(D)Trp[7,9],Leu[11]]-substance P.[18] Later, extensive SAR studies identified new inverse agonist peptides with better specificity toward GHS-R1a than the Substance P analog.[21] In these papers, a core pentapeptide wFwLL-NH_2 was described as the minimal active sequence maintaining the inverse agonist activity. When a lysine residue was introduced at the N-terminal of this pentapeptide, a potent inverse agonist of the receptor was obtained.[21a] A striking structural similarity could be found between the hexapeptide KwFwLL-NH_2 described by Holst et al. and our JMV 1843 compound: the presence of two (D)tryptophan residues (Fig. 3). When comparing our triazole scaffold with this peptide, we can hypothesize that the benzyl group in position 4 of our scaffold can play the role of the phenylalanine residue. In that case, the new chiral center incorporating an amino function in position 3 of our triazole scaffold could allow the elongation with the Leu-Leu dipeptide sequence.

A new series of triazole derivatives was therefore designed and tested in order to find new efficient ligands, potentially inverse agonists, of the GHS-R1a.

In a first set of experiments (Table 1), the new chiral center was introduced by the reaction of the thioamide Boc-(D)Trp[S]-NH(pOMe)Benzyl with Cbz-(L) or (D)phenylalanine hydrazide or Cbz-(L) or (D)tryptophan hydrazide in the presence of silver benzoate to form the triazole scaffold bearing two chiral centers (Scheme 1). After removal of the Boc protecting group, diverse interesting R^3 acids were introduced to the amine function by conventional coupling. The Cbz protection was then removed by hydrogenolysis and the amino function was acylated or not with a formyl or acetyl group to lead to the final compounds. All final compounds were purified by reversed-phase preparative HPLC.[22] These compounds were tested for their affinity toward the GHS-R1a, their ability to induce intracellular calcium mobilization[15] and for confirmation of their agonist/antagonist character in a cyclic AMP response element CRE-luciferase reporter gene assay.[23]

Compounds **1** to **8** with the indole group as R^2 clearly showed that the R configuration of the new chiral center leads to ligands with a higher affinity than compounds with the S configuration of this carbon. The most potent compounds were obtained with Aib or picolinic acid at the N-terminus (compounds **5** and **8** with K_i values of 12 and 9 nM, respectively). As previously described, the picolinic acid was well tolerated at this position.[24] For compounds **9** to **18**, all containing the phenyl group as R^2, the best affinities were also obtained when the second chiral center was of R configuration. For this reason, compounds **19**–**23** were only synthesized in the R series. Acetylation of the amino function was preferred and only the N-terminal group was modulated. We introduced at this position acyl groups that gave good results in other series: 3-fluoro(pyridin-2-yl)carboxylic acid, 4,6-difluoro(pyridin-2-yl)carboxylic acid, 3,4-dihydro-2*H*-pyran-6-carboxylic acid, 2-hydroxylacetic acid, and (*S*) morpholine-2-carboxylic acid.[15] These modifications led to compounds with a high affinity toward the receptor, particularly compounds **22** and **23**, exhibiting

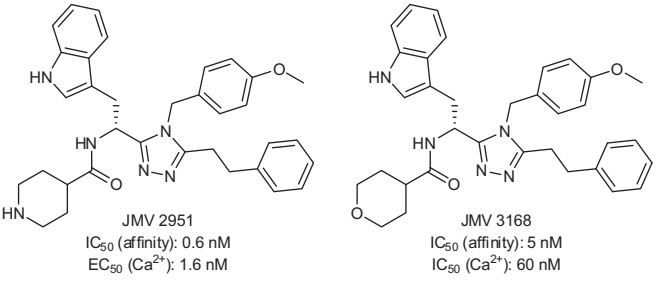
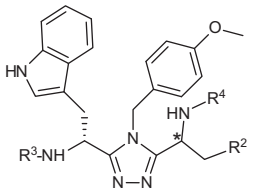


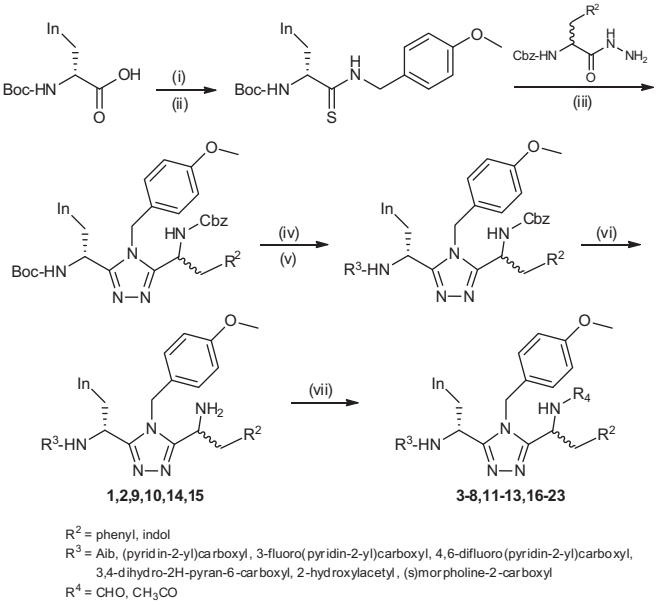
Figure 2. Shift from agonist (JMV2951) to antagonist (JMV3168).

Table 1
Binding affinity constants and biological activities of 1,2,4-triazole antagonists



Compounds	R^2	R^3	R^4	C* configuration	K_i (nM)[a]	IC_{50} $[Ca^{2+}]_i$ (nM)[b]	IC_{50} CRE/Luc (nM)[c]
1	Indole	Aib	H	S	>1000	>1000	>1000
2	Indole	Aib	H	R	131 ± 16	90	130
3	Indole	Aib	Formyl	R	46 ± 6	33	60
4	Indole	Aib	Acetyl	S	>1000	>1000	>1000
5	Indole	Aib	Acetyl	R	12 ± 3	19	22
6	Indole	(Pyridin-2-yl)carboxyl	Formyl	S	154 ± 20	1200	410
7	Indole	(Pyridin-2-yl)carboxyl	Acetyl	S	62 ± 9	190	100
8	Indole	(Pyridin-2-yl)carboxyl	Acetyl	R	9 ± 2	3	2
9	Phenyl	Aib	H	S	>1000	>1000	>1000
10	Phenyl	Aib	H	R	154 ± 18	135	115
11	Phenyl	Aib	Formyl	R	59 ± 7	43	113
12	Phenyl	Aib	Acetyl	S	>1000	>1000	>1000
13	Phenyl	Aib	Acetyl	R	46 ± 5	90	70
14	Phenyl	(Pyridin-2-yl)carboxyl	H	S	62 ± 8	150	320
15	Phenyl	(Pyridin-2-yl)carboxyl	H	R	18 ± 8	4	7
16	Phenyl	(Pyridin-2-yl)carboxyl	Acetyl	S	17 ± 4	70	360
17	Phenyl	(Pyridin-2-yl)carboxyl	Acetyl	R	10 ± 5	2	4
18	Phenyl	(Pyridin-2-yl)carboxyl	Formyl	R	8 ± 3	3	8
19	Phenyl	3-Fluoro(pyridin-2-yl)carboxyl	Acetyl	R	4 ± 1	2	2
20	Phenyl	4,6-Difluoro(pyridin-2-yl)carboxyl	Acetyl	R	11 ± 4	4	4
21	Phenyl	3,4-Dihydro-2*H*-pyran-6-carboxyl	Acetyl	R	9 ± 3	4	3
22	Phenyl	2-Hydroxylacetyl	Acetyl	R	0.8 ± 0.2	2	8
23	Phenyl	(*S*) morpholine-2-carboxyl	Acetyl	R	2 ± 0.4	3	9

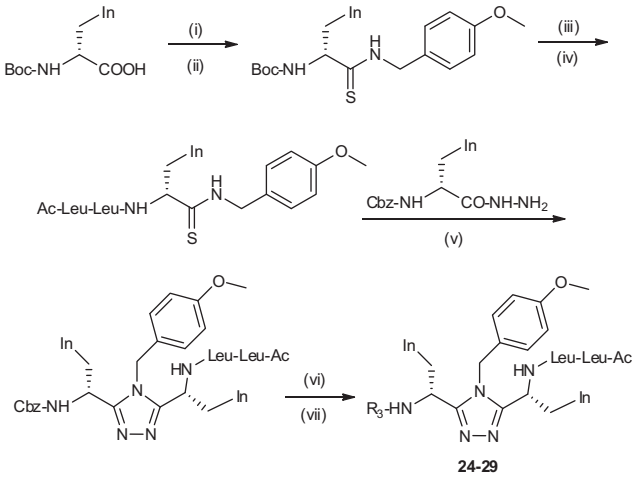
[a] Specific binding was determined by competition binding on membranes prepared from GHS-R1a transfected LLC PK-1 cells incubated with ^{125}I-His^9-ghrelin (0.3 nM) in the presence of increasing concentrations of compounds: K_i values were obtained from binding curves using GraphPad Prism Software.
[b] The ability of the antagonists to inhibit ghrelin signaling $[Ca^{2+}]_i$ through the GHS-R1a (measurement of fluorescence output), was assessed using Schild Plots, with increasing concentrations of ghrelin, alone or in the presence of a fixed antagonist concentration (10^{-8} M, 10^{-7} M or 10^{-6} M). Experiments were performed in quadruplicate.
[c] IC_{50} values were determined by competition using saturated dose of ghrelin and increasing amounts of tested compounds. Experiments were performed in quadruplicate.



Scheme 1. Reagents and conditions: (i) 4-methoxybenzyl amine, BOP, DIEA, DCM, rt; (ii) Lawesson's reagent, DME, 85 °C; (iii) AgBz, AcOH, DCM, rt; (iv) 4 N HCl/dioxanne, rt; (v) appropriate acid group, BOP, DIEA, DCM, rt; (vi) H_2, Pd/c, EtOH, rt; (vii) appropriate acylating agent, DCM, rt.

K_i values of 0.8 and 2 nM, respectively. All these compounds behaved as receptor antagonists, when tested in two functional assays. The best compounds of the series, presenting good affinities and potent biological activities were still of R configuration for the new chiral center. The most potent ligands with IC_{50}s in the nanomolar range were compounds **8**, **15** and **17**–**23**. Compounds **17**–**23** were latter evaluated in the IP3 turnover assay (data not shown) and they were all found partial agonists at 10^{-5} M, except compound **22** (containing hydroxyl-acetyl moiety at the N-terminus) which acted as a neutral antagonist, being unable to increase or decrease the constitutive IP3 activity of the ghrelin receptor.

In a second set of syntheses, the amino function of the second chiral center was elongated to mimic the Substance P analog and to potentially obtain inverse agonists. A first series of compounds, incorporating at the C-terminal part the acetyl-Leu-Leu sequence, was designed. This led to an inversed dipeptide sequence at the C-terminus compared to the peptidic sequence of substance P analog. For the synthesis, a first approach consisting in the condensation of N-acetyl-Leu-Leu-D-tryptophan hydrazide with the usual thioamide Boc-D-Trp[S]-NH(pOMe)Benzyl led to a poor yield of the desired triazole after a long time reaction (more than two months). As the 1,2,4-triazole scaffold includes a symmetry axis, we proposed a different synthetic pathway (Scheme 2). Boc-D-Trp-OH was coupled with the 4-methoxybenzylamine and then thionated as previously described. The obtained thioamide was then elongated step by step by conventional peptide synthesis to yield the corresponding tripeptide containing a thioamide bond: Ac-Leu-Leu-D-Trp[S]-NH(pOMe)Benzyl. This thioamide was reacted with the Cbz-D-Trp-NH-NH_2 to yield the desired triazole within two days. After hydrogenolysis of the Z protecting group, six different acylating moieties (R^3 in Table 2) were then introduced at the N-terminal part (compounds **24**–**29**). Keeping the



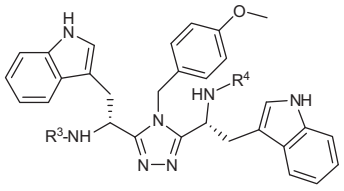
Scheme 2. Reagents and conditions: (i) 4-methoxybenzyl amine, BOP, DIEA, DCM, rt; (ii) Lawesson's reagent, DME, 85 °C; (iii) deprotection steps: 4 N HCl/dioxane, coupling steps: Boc-Leu-OH, BOP, DIEA, DCM; (iv) Ac_2O, DCM, rt; (v) AgBz, AcOH, DCM, rt; (vi) H_2, Pd/c, EtOH, rt; (vii) appropriate acid group, BOP, DIEA, DCM, rt.

lysine residue at the N-terminus, another series of compounds was designed to evaluate the influence of the C-terminal part. We first suppressed the two leucine residues and just formylated (compound **30**) or acetylated (compound **31**) the amino function. Then, to recover the peptide backbone similarity at the C-terminal part, we introduced a 2-isobutylmalonic methyl ester residue (as a racemic mixture) to get compound **32**. After saponification of the ester, elongation of the C-terminal part with a leucine methyl ester or leucine amide led to compounds **33** and **34**. All final compounds were purified by reversed-phase preparative HPLC and fully characterized.[25]

Pharmacological characterization of compounds **24**–**34** is reported in Table 2. To determine the binding affinities of the compounds, we used a fluorescence-based assay, called Tag-lite binding assay. This assay is based on a fluorescence resonance energy transfer (FRET) process between a terbium cryptate covalently attached to a SNAP-tag fused GHS-R1a (SNAP-GHS-R1a) and a high-affinity red fluorescent ghrelin ligand that was previously described.[26] As we were looking for compounds acting as inverse agonists, the constitutive activity was evaluated with the IP3 turnover assay as it could not be detected using measurement of intracellular calcium.[18] As reference, the hexapeptide KwFwLL-NH_2, described by Holst et al. was tested and included in Table 2. Concerning affinity, some compounds exhibited K_i in the nanomolar range (compounds **24**–**26**, **30** and **31**). In the first series of ligands with the Ac-Leu-Leu moiety, compounds **25**, **26**, **27** and **28** containing 2-hydroxyl-2-methylpropionyl, isonipecotyl, α-aminoisobutyryl and (*S*)morpholin-2-carboxyl groups respectively behaved as agonists in the IP3 assay, while compound **24** (containing the hydroxyacetyl moiety) presented a neutral antagonist character. Introduction of a lysine residue at the N-terminal part (compound **29**) induced a loss of binding affinity. However, this compound behaved as a partial inverse agonist, being able to partially inhibit the constitutive activity of the receptor (E_{max} = −37%). This confirmed the observation of Holst et al. on the significance of this residue for inverse agonist activity.[21b] With this interesting result, we decided to keep the lysine residue at the N-terminal part, and we then modulated the C-terminal part (compounds **30**–**34**). We first suppressed the two leucine residues and just formylated or acylated the amino-function. Both compounds (**30**, **31**) revealed good binding affinity in the nanomolar range but exhibited a partial agonist character in the IP3 assay, showing that the presence of the lysine residue at the N-terminus is not sufficient to get inverse agonists. To recover the peptide backbone similarity, we introduced 2-isobutylmalonic methyl ester residue (as a racemic mixture) to get compound **32**, which showed a slight decrease in affinity (K_i: 17 nM) and a partial agonist activity. Elongation of the C-terminal part with a leucine methyl ester or leucine amide (compounds **33**, **34**) gave a negative shift of one log in the affinity constants versus compound **32**. Both compounds were partial agonists. As a comparison, the K_i value of the hexapeptide KwFwLL-NH_2, reported by Holst et al.[21a] was inserted in Table 2. In our hands, this compound exhibited a K_i value of 255 nM and 55% inhibition of the basal response.

We have shown in this study that the introduction of a second chiral center in position 3 of our 1,2,4-triazole scaffold could lead

Table 2
Evaluation of the biological activity of the ligands after structural modulations on the second chiral center



Compounds	R^3	R^4	K_i (nM)[a]	E_{max} (%)[b]	EC_{50} (nM)[c]	Behavior
24	2-Hydroxylacetyl	Acetyl-Leu-Leu–	3 ± 0.2	0	–	Antagonist
25	2-Hydroxyl-2-methylpropionyl	Acetyl-Leu-Leu	3 ± 0.5	75	74	Agonist
26	Isonipecotyl	Acetyl-Leu-Leu	2 ± 0.3	108	4.4	Agonist
27	Aib	Acetyl-Leu-Leu	30 ± 8	80	0.8	Agonist
28	(*S*)morpholine-2-carboxyl	Acetyl-Leu-Leu	50 ± 7	75	60	Agonist
29	Lys	Acetyl-Leu-Leu	110 ± 12	−37	70	Inverse agonist
30	Lys	Formyl	8 ± 2	89	10	Agonist
31	Lys	Acetyl	2.5 ± 0.8	89	4	Agonist
32	Lys	mLeu-OMe	17 ± 6	81	20	Agonist
33	Lys	mLeu-Leu-OMe	220 ± 32	82	82	Agonist
34	Lys	mLeu-Leu-NH_2	210 ± 28	60	170	Agonist
KwFwLL-NH_2			255 ± 31	−55	100	Inverse agonist

[a] K_i values of compounds were determined from binding fluorescent assays as described in Ref. 26.
[b] The activity was determined by HTRF inositol phosphate assay: E_{max} values are expressed for agonists and antagonists as % of maximal stimulation promoted by ghrelin over basal, and for inverse agonists as % of maximal inhibition of basal (basal represents 50–60% of the maximal ghrelin stimulation).
[c] EC_{50} values were determined from analysis of dose response curves using GraphPad Prism Software.

to potent GHS-R1a ligands presenting good to high affinities toward the receptor. Some compounds (**8**, **15**, **17**–**23**) exhibited a nanomolar value of IC_{50} in two functional tests (calcium and CRE reporter assays). Furthermore, the presence of an amino function at the new chiral center allowed us to elongate the C-terminal part of the molecule and to introduce the Leu-Leu dipeptide sequence that is included in potent described inverse agonists. Unfortunately, most of the compounds incorporating this dipeptide behaved as agonists except compound **24** which is a neutral antagonist and compound **29** which exhibits a partial inverse agonist behavior similar to the hexapeptide KwFwLL-NH_2 described in the literature. Some discrepancies between the functional assays (calcium mobilization, CRE-luciferase and IP3 turnover) were observed, indicating the complex signaling of this receptor which is likely responsible for the diversity in biological effects elicited by ghrelin. We are currently investigating to try to understand how signaling efficacy and selectivity are regulated.[27] In conclusion, we demonstrated that the trisubstituted 1,2,4-triazole scaffold bearing a second chiral center can be an alternative to introduce more diversity and to obtain high affinity ligands.

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22. The purity assessed by analytical reversed phase C18 HPLC was found to be greater than 98% for most of the target compounds and greater than 95% for the rest, and the structures of the compounds were confirmed by MS (electrospray), ^1H and/or ^{13}C NMR. As an example: compound **8**. White powder, 51 mg (0.067 mmol, 32%). ^1H NMR (300 MHz, DMSO-d_6, 303 K): δ (ppm) 1.62 (s, 3H, $COCH_3$), 3.21–3.43 (m, 3H, CH_2 Trp (2H), CH_2 Trp (1H)), 3.47–3.62 (m, 4H, CH_2 Trp (1H), OCH_3), 4.95 (d, J = 16.8 Hz, 1H, $CH_2\Phi$ (1H)), 5.12–5.22 (m, 2H, $CH_2\Phi$ (1H), CH Trp), 5.36–5.45 (m, 1H, CH Trp), 6.50 (d, J = 8.7 Hz, 2H, CH aryl), 6.62 (d, J = 8.7 Hz, 2H, CH aryl), 6.76–6.90 (m, 2H, CH aryl), 6.91–7.07 (m, 4H, CH aryl), 7.16 (d, J = 7.9 Hz, 1H, CH aryl), 7.22 (d, J = 7.9 Hz, 1H, CH aryl), 7.24–7.31 (m, 2H, CH aryl), 7.51–7.58 (m, 1H, CH pyridin-2-yl), 7.82 (d, J = 7.7 Hz, 1H, CH pyridin-2-yl), 7.91 (dt, J = 7.7, J = 1.6 Hz, 1H, CH pyridin-2-yl), 8.53–8.61 (m, 2H, NH acetyl, CH pyridin-2-yl), 8.95 (d, J = 8.7 Hz, 1H, NHCO), 10.72–10.79 (m, 2H, NH Trp). ^{13}C NMR (75 MHz, DMSO-d_6, 303 K): δ (ppm) 22.1, 28.6, 28.7, 44.2, 44.8, 45.2, 54.9, 109.3, 109.7, 111.2, 111.3, 113.6 (2C), 118.0 (2C), 118.2, 118.3, 120.7, 120.9, 121.9, 123.9, 124.0, 126.6, 126.7 (2C), 126.9, 127.0, 127.1, 135.9, 136.0, 137.6, 148.2, 148.8, 154.9, 155.8, 158.3, 163.2, 168.8. Purity >98% (reverse phase HPLC). LC–MS (ES): m/z 533.2 $[M+H\text{-}4\text{-methoxybenzyl}]^+$, 653.4 $[M+H]^+$.
23. In the CRE/Luc reporter gene assay, mouse LTK-cells were stably transfected with a plasmid containing the CMV minimal promotor linked to three cAMP response elements (CRE) followed by a luciferase reporter gene. Based on this parental cell line, single cell clones stably overexpressing the human GHS-R1A were established and characterized. The cells were incubated for 6 h with 1 μM rolipram in the presence of different concentrations of the tested compounds and saturating concentration of ghrelin. Subsequently, cells were lysed and ATP bioluminescence was measured in the luminescence mode on FlexStation3 (Molecular Devices). All data were assessed in quadruplicate measurements and calculated in % inhibition according to cells treated with saturating concentrations of ghrelin (Polypeptide #sc1357) as negative (0% inhibition), and non-treated cells as positive control (100%). IC_{50} values were determined by using the GraphPad Prism analysis program (GraphPad Software).
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25. The purity assessed by analytical reversed phase C18 HPLC was found to be greater than 98% for most of the target compounds and greater than 95% for the rest, and the structures of the compounds were confirmed by MS (electrospray), ^1H and/or ^{13}C NMR. As an example: compound **29**. White powder, 38 mg (0.067 mmol, 27%). ^1H NMR (400 MHz, DMSO-d_6, 303 K): δ (ppm) 0.68 (d, J = 6.4 Hz, 3H, CH_3 Leu), 0.70 (d, J = 6.4 Hz, 3H, CH_3 Leu), 0.76 (d, J = 6.4 Hz, 3H, CH_3 Leu), 0.83 (d, J = 6.4 Hz, 3H, CH_3 Leu), 0.97 (m, 2H, CH_2 Lys), 1.22–1.33 (m, 3H, CH_2 Lys, $CH(CH_3)_2$), 1.36–1.40 (m, 4H, 2 CH_2 Leu), 1.52 (m, 1H, $CH(CH_3)_2$), 1.81 (s, 3H, $COCH_3$), 2.51 (m, 2H, CH_2 Lys), 3.09 (dd, J = 6.0 Hz, J = 14.0 Hz, 1H, CH_2 Trp (1H)), 3.18–3.29 (m, 2H, CH_2 Trp (1H), CH_2 Trp (1H), 3.38 (dd, J = 6.8 Hz, J = 14.0 Hz, 1H, CH_2 Trp (1H)), 3.56 (m, 1H, CH_2 Lys (1H)), 3.71 (m, 4H, OCH_3, CH_2 Lys (1H)), 4.15–4.27 (m, 3H, CH Lys, CH Leu, CH Leu), 4.92 (d, J = 16.4 Hz, 1H, $CH_2\Phi$ (1H)), 5.06 (d, J = 16.4 Hz, 1H, $CH_2\Phi$ (1H)), 5.17 (dd, J = 8.8 Hz, J = 14.8 Hz, 1H, CH Trp), 5.30 (dd, J = 8.0 Hz, J = 15.6 Hz, 1H, CH Trp), 6.77 (d, J = 8.8 Hz, 2H, CH aryl), 6.81–6.91 (m, 4H, CH aryl), 6.98–7.06 (m, 5H, CH aryl), 7.26–7.32 (m, 3H, CH aryl), 7.56 (d, J = 8.4 Hz, 1H, NH-CO), 7.71 (s, 3H, NH_3^+), 7.94 (s, 3H, NH_3^+), 7.98 (d, J = 8.4 Hz, 1H, NH-CO), 8.63 (d, J = 8.8 Hz, 1H, NH-CO), 9.17 (d, J = 8.8 Hz, 1H, NH-CO), 10.76 (d, J = 8.4 Hz, 2H, NH Trp). ^{13}C NMR (100 MHz, DMSO-d_6, 303 K): δ (ppm) 20.44, 21.31, 21.59, 22.37, 22.85, 22.89, 23.87, 24.06, 26.44, 28.78, 29.58, 30.07, 38.36, 40.21, 40.65, 43.95, 44.51, 45.00, 50.71, 50.95, 51.64, 54.99, 109.11, 109.67, 111.20, 111.24, 113.50, 114.00, 117.85, 118.00, 118.22, 118.26, 120.73, 120.93, 124.01, 124.20, 126.71, 126.96, 127.39, 127.49, 128.26, 135.88, 135.90, 154.63, 155.12, 158.73, 167.92, 169.28, 171.65, 171.72. Purity >96% (reverse phase HPLC). LC–MS (ES): m/z 451.89 $[M+2H]^{2+}$, 782.51 $[M+H\text{-}4\text{-methoxybenzyl}]^+$, 902.53 $[M+H]^+$.
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