Article

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Development of non-peptidic inverse agonists of the ghrelin receptor (GHSR) based on the 1,2,4-triazole scaffold

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ABSTRACT

GHSR controls, among others, growth hormone and insulin secretion, adiposity, feeding and glucose metabolism. Therefore, an inverse agonist ligand capable of selectively targeting GHSR and reducing its high constitutive activity appears to be a good candidate for the treatment of obesity-related metabolic diseases. In this context, we present a study that led to the development of several highly potent and selective inverse agonists of GHSR based on the 1,2,4-triazole scaffold. We demonstrate that, depending on the nature of the substituents on positions 3, 4 and 5,

this scaffold leads to ligands that exert an intrinsic inverse agonist activity on GHSR-catalyzed G protein activation through the stabilization of a specific inactive receptor conformation. Thanks to an in vivo evaluation, we also show that one of the most promising ligands not only exerts an effect on insulin secretion in rat pancreatic islets but also affects the orexigenic effects of ghrelin in mice.

INTRODUCTION

The ghrelin receptor, a G protein-coupled receptor identified as the growth hormone secretagogue receptor (GHSR),[1] is known for regulating numerous central and peripheral processes including, among others, growth hormone (GH) secretion, adiposity, feeding and glucose metabolism. Indeed, administration of its endogenous agonist, a stomach-derived 28-amino acid peptide named ghrelin,[2] induces blood glucose elevation and the reduction of circulating insulin in humans and rodents.[3] Moreover, ghrelin is the most potent orexigenic peptide hormone known and its administration to humans or rodents potently increases food intake.[4] Knowing that GHSR exhibits a high constitutive activity, an inverse agonist compound capable of selectively targeting the receptor and reducing its constitutive activity appears to be a good candidate for the treatment of obesity-related metabolic diseases. In an effort to find efficient GHSR ligands, we previously developed a heterocyclic series that presented high affinity and selectivity for this receptor. These new ligands, based on the 3,4,5-trisubstituted 1,2,4-triazole scaffold (Figure 1), provided four points of diversity ($R^{1'}$, $R^{2'}$, $R^{3'}$ and $R^{4'}$) and their synthesis, starting from an optically pure amino acid, comprised a few steps with moderate to good yields. We demonstrated that the configuration of the chiral center was retained during the synthetic process.[5-7] An intensive structure activity relationship study, carried out over several hundred triazole compounds, allowed us to evaluate the ability of these compounds to activate or inactivate GHSR signaling. Moreover, very interestingly, some of our antagonist compounds were able to

antagonize food intake elicited by an agonist such as hexarelin, while they were unable to antagonize GH secretion elicited by the same agonist. This was the case for JMV2959 (Figure 1, compound **1**), a GHSR antagonist which has since been broadly described in the literature[8] to hold potent anti-obesity but also anti-addictive properties.

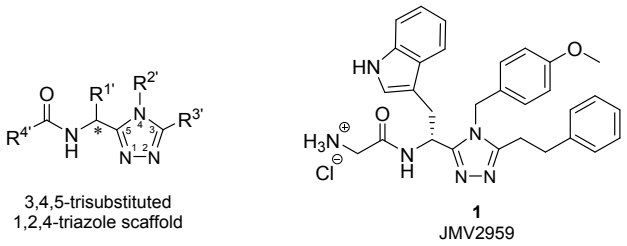


Figure 1. Structure of the 3,4,5-trisubstituted 1,2,4-triazole scaffold and compound **1** (JMV2959)

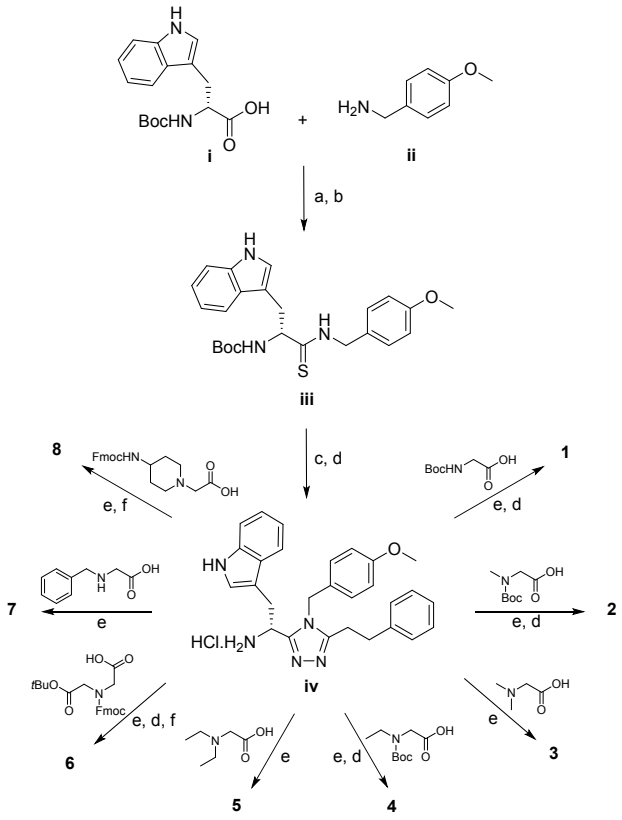
The first described inverse agonist of GHSR was an analog of substance P (SPA).[9] Even though [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]-substance P was able to selectively reduce the constitutive activity of the receptor, it also acted as a biased ligand toward neuropeptide and chemokine receptors.[10-11] However, after several truncations of the original sequence, Holst *et al.* identified the *C*-terminal pentapeptide wFwLL-NH_2 (in its carboxamide version) as the peptide core responsible for the inverse agonist activity.[12] Moreover, the presence of a lysine residue at the *N*-terminus of the peptide core K-(D-1-Nal)-FwLL-NH_2 led to a specific inverse agonist almost as potent as the substance P analog.[13] Most recently, our team found that LEAP2 (liver-expressed antimicrobial peptide 2), an endogenous ligand described as antagonist of GHSR,[14] behaved as inverse agonist of the receptor and as competitive antagonist of ghrelin-induced inositol phosphate production and calcium mobilization.[15] We also demonstrated that the entire sequence of LEAP2 was not necessary for its biological activity. Indeed, the *N*-terminal part alone, namely the LEAP2 (1-14-NH_2) analog, maintained receptor binding and biological activity, and was able to inhibit ghrelin-induced food intake in mice. In addition to these substance P and LEAP2 peptide analogs, only a

few non peptidic inverse agonists of GHSR has been reported in the literature so far (for a review see[16]). In order to overcome the lack of potent small molecules capable of selectively reducing the constitutive activity of GHSR, we decided to search for inverse agonists based on our 1,2,4-triazole scaffold. The first structural modulations with minor changes in the $R^{1'}$, $R^{2'}$, $R^{3'}$ and $R^{4'}$ groups did not lead to inverse agonists. However, the introduction of a secondary chiral center at position 3 of the triazole ring and the elongation with the Leu-Leu dipeptide sequence allowed us to obtain a partial inverse agonist with moderate affinity toward the receptor.[17] In light of these encouraging results, we decided to carry on our investigation by making structural changes on our initial antagonist, JMV2959. First, we chose to decorate its primary amine with alkyl or carbonyl groups. We were able to introduce various substituents including alkanes, alkenes, substituted aromatic rings and heterocycles. Then, we modulated the $R^{3'}$ substituent of the 1,2,4-triazole scaffold. Herein we report the design, the synthesis and the biological evaluation of this new series of ligands. Having characterized all the compounds by NMR, LC-MS and HRMS, an in vitro SAR study was performed in order to determine their pharmacological properties and their ability to reduce the basal activity of GHSR. In order to determine whether this specific 1,2,4-triazole scaffold was active on endogenously expressed GHSR, we tested one of the most effective ligands for its ability to modulate the insulinostatic effect of ghrelin in rat isolated pancreatic islets. At last, we investigated the ability of this lead compound to affect food intake under different experimental settings in mice.

RESULTS AND DISCUSSION

Design and Syntheses. We first designed a set of ligands (compounds **2-8**) on which the primary amine of JMV2959 (compound **1**) was substituted by various alkyl groups. Compounds **2-8** were synthesized following the same procedure previously described to provide compound **1**.[18] Briefly, the triazole derivative (**iv**) was obtained in four steps starting from Boc-(D)-Trp-OH as shown in Scheme 1. First, Boc-(D)-Trp-OH (**i**) was reacted with 4-methoxybenzylamine (**ii**) in the presence of BOP reagent and DIEA in DCM for 2 hours at room temperature. The resulted amide was transformed into the corresponding thioamide (**iii**) using the Lawesson's reagent in DME at 80 °C for 3 hours. The cyclization into the triazole heterocycle was performed by reacting the thioamide (**iii**) with 3-phenylpropanehydrazide in the presence of silver benzoate ($PhCO_2^-Ag^+$) and acetic acid (AcOH) in DCM at room temperature for 12 hours.[19] After removal of the Boc protecting group using a solution of 4N HCl in dioxane at 50 °C for 30 minutes, the structural diversity was introduced on the triazole derivative (**iv**) by coupling various *N*-substituted glycines in the presence of BOP reagent and DIEA in DCM for 2 hours at room temperature. Depending on the structure of the *N*-substituent on the glycine, a last deprotection step was required to afford the final compounds: a solution of 4N HCl in dioxane at 50 °C for the removal of the Boc and *t*Bu protecting groups and a solution of diethylamine (Et_2NH) in DMF at room temperature for the removal of the Fmoc protecting group.

Scheme 1. Synthesis of Compounds 1-8[a]

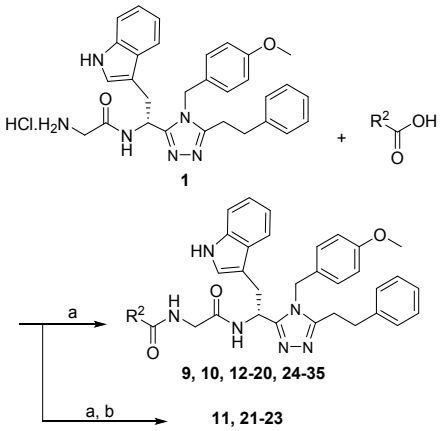


[a]Reagents and conditions: (a) BOP, DIEA, DCM, 2 h, 88%; (b) Lawesson's reagent, DME, 80 °C, 3h, 74%; (c) 3-phenylpropanehydrazide, $PhCO_2^-Ag^+$, AcOH, DCM, 12 h, 81%; (d) 4N HCl/dioxane, 50 °C, 30 min, 100%; (e) BOP, DIEA, DCM, 2 h, 14-86%; (f) Et_2NH, DMF, 2h, 100%.

The second set of ligands (compounds **9-35**) was also designed starting from compound **1** (Scheme 2). A classical peptide coupling reaction between the primary amine of **1** and a large panel of carboxylic acids allowed us to introduce the structural diversity on substituent R^2. The coupling reaction was conducted under basic conditions (DIEA) in the presence of HATU reagent in DCM for 2 hours at room temperature. In the case of ligands decorated with either an amino function (compounds **11** and **23**) or a hydroxyl group (compounds **21** and **22**), the protecting group,

required to carry on the coupling reaction, was removed in a final step using a solution of 4N HCl in dioxane at 50 °C for 30 min.

Scheme 2. Synthesis of Compounds 9-35[a]

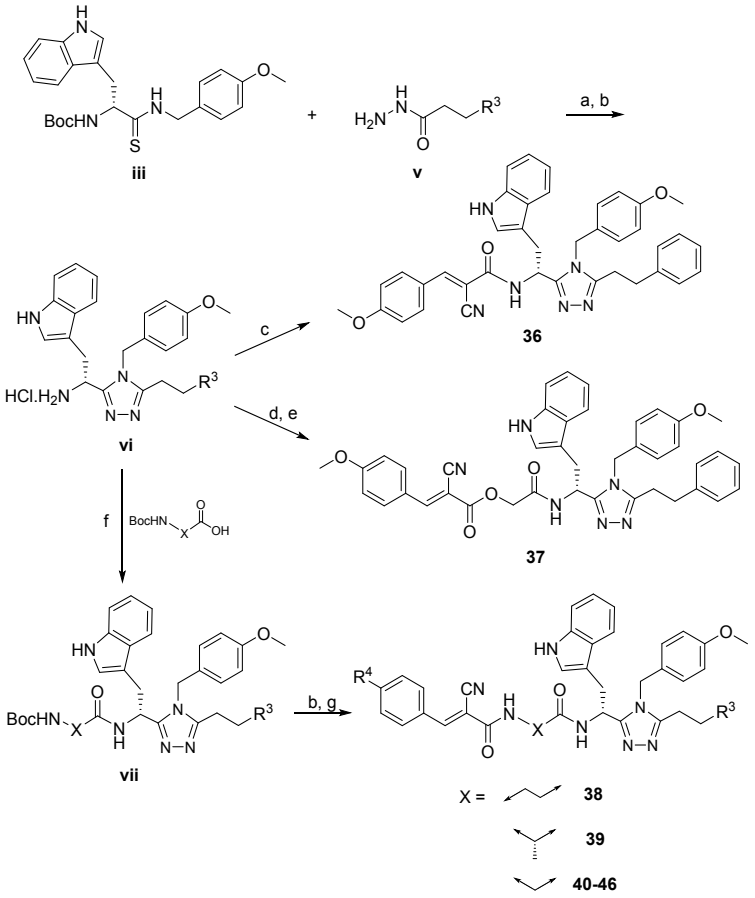


[a]Reagents and conditions: (a) HATU, DIEA, DCM, 2 h, 32-78%; (b) 4N HCl/dioxane, 50 °C, 30 min, 100%. For R^2, see Table 2.

The last set of ligands (compounds **36**-**46**) included several structural modulations but all were comprised of a α-cyano-β-phenylethylene moiety (potentially substituted in position para of the phenyl group) in place of substituent R^2 (Scheme 3). First, we varied the nature of substituent R^3. We decided to introduce either a phenyl group (compounds **36**-**39**), a morpholine group (compounds **40**-**42**), an indole group (compounds **43** and **44**), a piperidine group (compound **45**) or a diethylamine group (compound **46**). We used appropriate substituted hydrazides (intermediates **v**) that we reacted with thioamide (**iii**) in the presence of $PhCO_2^-Ag^+$ and AcOH in DCM at room temperature for 12 hours. Removal of the Boc protecting group led to triazole intermediates (**vi**). At this step of the syntheses, we had the possibility to modulate the spacer between these triazole intermediates and the 2-cyano-3-phenylacrylyl moiety bearing substituent

R^4. While we kept a glycine residue for most of the ligands, we modified the linker of compounds **36-39**. For instance, compound **36**, which had no spacer, was obtained by directly coupling alpha-cyano-4-methoxycinnamic acid with intermediate (**vi**) under the same conditions described above. The spacer of compound **37** was introduced by using 2-hydroxyacetic acid, which allowed us to make an ester bond with alpha-cyano-4-methoxycinnamic acid in contrast to the usual amide bond. For compounds **38** and **39**, we simply replaced the methylene bridge by an ethylene bridge (compound **38**) or an ethylidene bridge (compound **39**). At last, diversity of substituent R^4 resulted from the use of various para substituted alpha-cyanocinnamic acids that were reacted with deprotected beforehand intermediate (**vii**) via the same classical peptide coupling conditions previously described.

Scheme 3. Synthesis of Compounds 36-46[a]



[a]Reagents and conditions: (a) $PhCO_2^-Ag^+$, AcOH, DCM, 12 h, 71-88%; (b) 4N HCl/dioxane, 50 °C, 30 min, 100%; (c) alpha-cyano-4-methoxycinnamic acid, HATU, DIEA, DCM, 2 h, 36%; (d) 2-hydroxyacetic acid, HATU, DIEA, DCM, 2h, 58%; (e) alpha-cyano-4-methoxycinnamic acid, EDC, DIEA, DCM, 2 h, 39%; (f) HATU, DIEA, DCM, 2 h, 62%; (g) para substituted alpha-cyanocinnamic acids, HATU, DIEA, DCM, 2h, 32-72%. For R^3 and R^4, see Table 3.

All final compounds **2-46** (Figure 2) were purified by reversed-phase preparative HPLC. Depending on the intricacy of the purification, the purity assessed by analytical reversed phase C18 HPLC was at least 95% for most compounds and up to 99% for the rest, and the structures of the compounds were confirmed by HRMS, ^{1}H NMR, and ^{13}C NMR (see supporting information for more details).

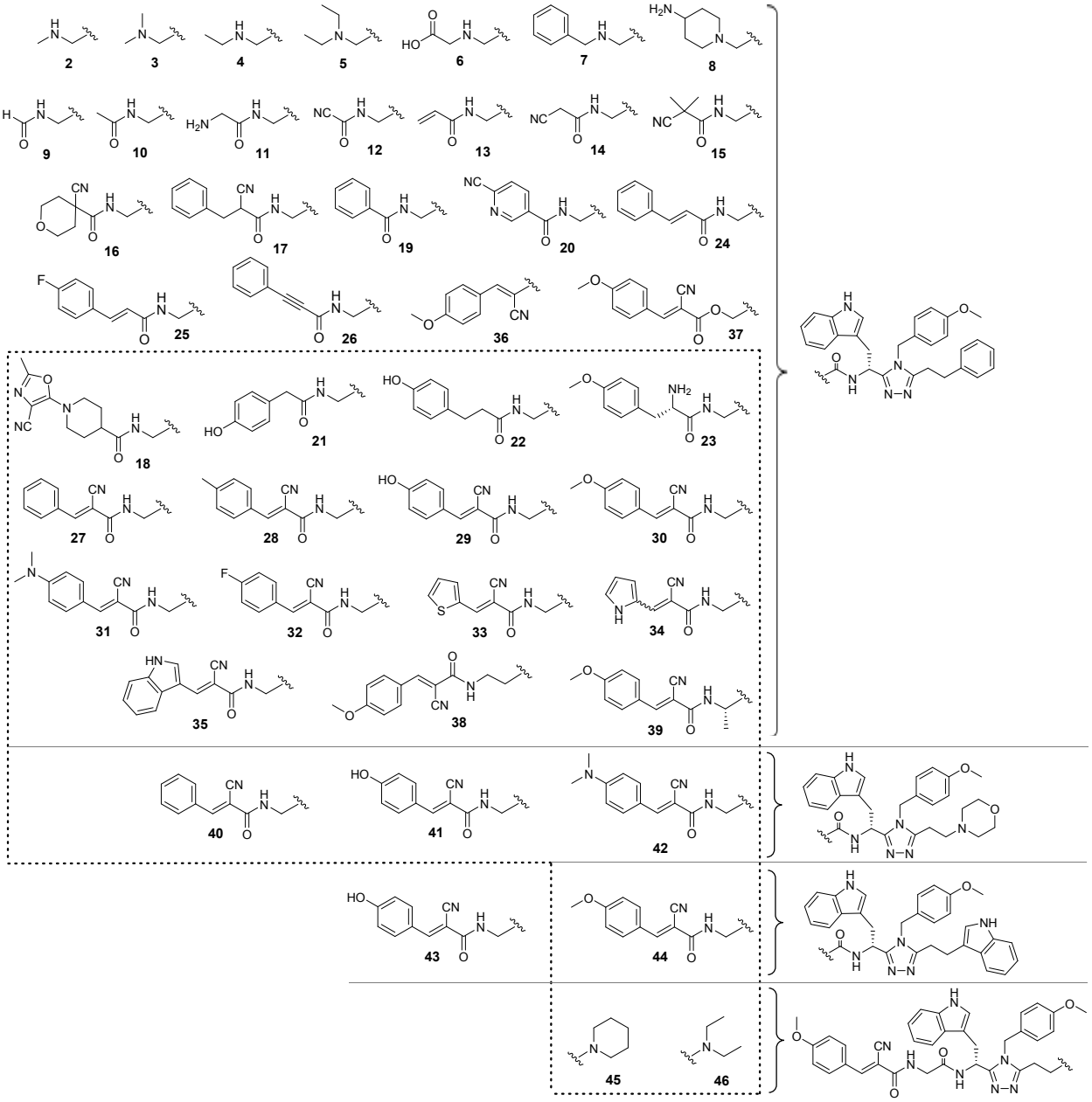


Figure 2. Structure of final ligands **2-46**. Inverse agonist ligands are framed by dotted lines.

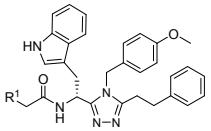
Evaluation of the Ligands in GHSR Expressing HEK293T Cells. *Binding and Pharmacological Activities of the Ligands*. We first analyzed in vitro whether the different compounds directly bound to GHSR stably expressed in HEK293T cells (Tables 1-3). *K*i values were determined from binding competition experiments with fluorescent ghrelin using the

homogenous time-resolved fluorescence (HTRF) assay as previously described.[20] We also measured the Inositol Phosphate 1 (IP1) response of each compound (10^{-5} M) and calculated it as a percentage of the maximal stimulation promoted by ghrelin (10^{-7} M). We compared this value with the percentage of the basal response of the receptor (value in square brackets) in order to determine the pharmacological activity of each compound toward the receptor. The high basal level of IP1 production in GHSR-expressing cells, representing in our in vitro experiments a range of 65−75% of the maximal IP1 production promoted by ghrelin, resulted from the high constitutive activity of GHSR as previously shown.[9, 21] A ligand was considered as agonist when it induced a minimum of 95% stimulation of the receptor compared to ghrelin, as inverse agonist when it reduced the basal activity of the receptor, and as antagonist when the IP1 response was identical to the basal level. We defined as "partial" any agonist/inverse agonist that elicited activity below the maximum level defined as that triggered by a reference compound, usually the natural ligand. Hence, in the case of agonists, the natural ligand ghrelin was considered as the reference full agonist that elicits the maximum signal at the interrogated pathway, here IP1 production. To take into account the experimental error, we considered as partial agonist any compound that induced a response comprised between the measured basal level and 94% of the maximal effect induced by ghrelin. The situation was more complex for the inverse agonist activity because of the lack of any clearly referenced compound. Therefore, in this case, we considered the natural GHSR inverse agonist LEAP2 as the reference compound that elicits the maximal activity. This peptide decreases the basal level of IP1 production by 50% (for more details see[15]). To be noted, this maximal effect is closely related to that also triggered by the reference synthetic inverse agonist compound K-(D-1-Nal)-FwLL-NH_2. In our assay, the basal activity of GHSR corresponded to 65-75% of the maximal IP1 production triggered by ghrelin. Hence, a 50% decrease would correspond to a final

level of IP1 production of ca. 35%. We thus considered as partial inverse agonist any compound with a response comprised between 35% and the basal level in the absence of ligand. Parent compound JMV2959 (ligand **1**) was taken as reference. First, we tested the compounds with alkylated amine (ligands **2**-**8**). As shown in Table 1, all ligands lost some affinity toward the receptor compared to JMV2959. Steric hindrance did not seem to play a role since ligands **3**, **4**, **5**, **7** and **8** presented comparable *K*i values while bearing more or less bulky substituents. However, the presence of a carboxyl group (ligand **6**) should be prohibited as it drastically reduced the affinity of the compound (*K*i = 617 nM). Concerning the activity of the ligands, all behaved as agonists or partial agonists of GHSR. In light of these results, alkylation of the amine function of compound JMV2959 did not appear as a valid approach to obtain inverse agonists of the receptor. Therefore, we decided to acylate the amine function with various carboxylic acids offering a wide range of substituents R^2 (Table 2). In terms of affinity, this approach seemed promising as it lowered the *K*i value for most of the ligands. Some ligands even showed *K*i values below the nanomolar range (ligands **14**, **30** and **32**). Only a cyano group directly linked to the amide bond prevented the compound to bind to the receptor (ligand **12**, *K*i = 1.6 μM). It is noteworthy that all the ligands bearing a α-cyanoethylene group substituted in position β by an unsaturated ring such as phenyl (ligands **27**-**32**), thiophene (ligand **33**), pyrrole (ligand **34**) or indole (ligand **35**), behaved as inverse agonists of the receptor. The substituent in position para of the phenyl group (ligands **28**-**32**) did not seem to affect the affinity nor the activity of the ligands. As shown in Table 3, modification of the spacer (Y) between the triazole core and the 2-cyano-3-phenylacrylyl moiety bearing substituent R^4 had an impact on the properties of the ligands. Indeed, shortening the spacer (ligand **36**) or replacing the amide bond by an ester bond (ligand **37**) cannot be envisaged since it leads to partial agonists. At last, replacement of a phenyl group by various R^3 substituents such as

morpholine, indole, piperidine and diethylamine, led to inverse agonists of the receptor (ligands **40-42**, **44-46**), except ligand **43**, which behaved as a partial agonist. For most ligands, these structural modifications maintained good affinities toward the receptor (*K*i = 6.3-14 nM). Nevertheless, the morpholine substituent seemed to be a little less well tolerated as it lowered affinities of ligands **40** and **41** (*K*i = 67 and 64 nM, respectively).

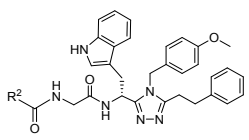
Table 1. Binding and Pharmacological Activity of Ligands 1-8[a]

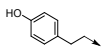
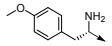
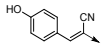
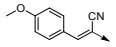
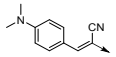
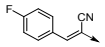
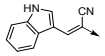


Ligand	R^1	*K*i[b] (nM)	IP-[basal][c] (%)	Pharmacological profile
1	$-NH_2.HCl$	43 ± 10	65-[65]	antagonist
2	$-NH-CH_3$	96 ± 22	100-[76]	agonist
3	$-N(CH_3)_2$	200 ± 28	96-[76]	agonist
4	$-NH-CH_2-CH_3$	300 ± 32	82-[61]	partial agonist
5	$-N(CH_2-CH_3)_2$	230 ± 31	88-[72]	partial agonist
6		617 ± 41	80-[72]	partial agonist
7		136 ± 19	88-[72]	partial agonist
8		220 ± 21	85-[72]	partial agonist

[a]Values reported in the table were obtained from experiments performed in HEK293T cells stably expressing GHSR. [b]*K*i values were determined from HTRF competition binding assays. Values are expressed as means ± SEM of two independent experiments, each experiment being performed in triplicate. [c]Activities were determined by HTRF inositol phosphate (IP) assay. IP response of each ligand (10^{-5} M) is given as percentage of the maximal stimulation promoted by ghrelin (10^{-7} M). The percentage in square brackets refers to the basal response of the receptor also given as percentage of the maximal ghrelin response.

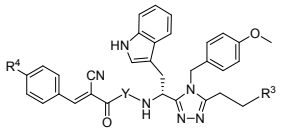
Table 2. Binding and Pharmacological Activity of Ligands 9-35[a]



Ligand	R^2	*Ki*[b] (nM)	IP-[basal][c] (%)	Pharmacological profile
9	-H	12 ± 2	79-[62]	partial agonist
10	$-CH_3$	10 ± 3	93-[62]	partial agonist
11	$-CH_2-NH_2$	130 ± 22	85-[64]	partial agonist
12	-CN	1600 ± 112	76-[67]	partial agonist
13		4 ± 1	90-[69]	partial agonist
14		0.45 ± 0.2	83-[79]	partial agonist
15		3.4 ± 1	70-[70]	antagonist
16		28 ± 8	77-[74]	partial agonist
17		166 ± 23	72-[70]	antagonist
18		6.6 ± 2	37-[74]	partial inverse agonist
19		115 ± 26	94-[66]	partial agonist
20		6.3 ± 2	81-[70]	partial agonist
21		23 ± 6	72-[77]	partial inverse agonist
22		35 ± 8	60-[77]	partial inverse agonist
23		17 ± 7	56-[67]	partial inverse agonist
24		370 ± 36	79-[71]	partial agonist
25		56 ± 15	74-[71]	antagonist
26		130 ± 26	95-[69]	agonist
27		9 ± 2	25-[77]	inverse agonist
28		1.0 ± 0.3	19-[74]	inverse agonist
29		3 ± 1	22-[77]	inverse agonist
30		0.5 ± 0.2	12-[77]	inverse agonist
31		2.4 ± 1	22-[70]	inverse agonist
32		0.6 ± 0.2	23-[74]	inverse agonist
33		9.7 ± 2	31-[70]	inverse agonist
34		8.5 ± 2	23-[70]	inverse agonist
35		6.3± 2	19-[74]	inverse agonist

[a]Values reported in the table were obtained from experiments performed in HEK293T cells stably expressing GHSR. [b]*K*i values were determined from HTRF competition binding assays. Values are expressed as means ± SEM of two independent experiments, each experiment being performed in triplicate. [c]Activities were determined by HTRF inositol phosphate (IP) assay. IP response of each ligand (10^{-5} M) is given as percentage of the maximal stimulation promoted by ghrelin (10^{-7} M). The percentage in square brackets refers to the basal response of the receptor also given as percentage of the maximal ghrelin response.

Table 3. Binding and Pharmacological Activity of Ligands 36-46[a]



Ligand	R^3	R^4	Y	*Ki*[b] (nM)	IP-[basal][c] (%)	Pharmacol. profile
36		-OCH_3		122 ± 23	95-[68]	partial agonist
37		-OCH_3		13 ± 3	80-[71]	partial agonist
38		-OCH_3		14 ± 6	15-[71]	inverse agonist
39		-OCH_3		3 ± 1	15-[71]	inverse agonist
40		-H		67 ± 12	44-[65]	partial inverse agonist
41		-OH		64 ± 11	33-[65]	inverse agonist
42		-$N(CH_3)_2$		6.3 ± 2	29-[65]	inverse agonist
43		-OH		30 ± 8	82-[65]	partial agonist
44		-OCH_3		1.4 ± 0.2	7-[71]	inverse agonist
45		-OCH_3		14 ± 2	25-[65]	inverse agonist
46		-OCH_3		10 ± 3	29-[65]	inverse agonist

[a]Values reported in the table were obtained from experiments performed in HEK293T cells stably expressing GHSR. [b]*Ki* values were determined from HTRF competition binding assays. Values are expressed as means ± SEM of two independent experiments, each experiment being performed in triplicate. [c]Activities were determined by HTRF inositol phosphate (IP) assay. IP response of each ligand (10^{-5} M) is given as percentage of the maximal stimulation promoted by ghrelin (10^{-7} M). The percentage in square brackets refers to the basal response of the receptor also given as percentage of the maximal ghrelin response.

Pharmacological Activities of the Inverse Agonist Ligands. We selected the ligands that behaved as inverse agonists and further tested their ability to affect the basal level of IP1 production in HEK293T cells stably expressing GHSR (Table 4, Figure 3). We determined E_{max} (maximal inhibition effect on the basal response promoted by the receptor) and EC_{50} values, and compared them to reference inverse agonists SPA and K-(D-1-Nal)-FwLL-NH_2. As shown in Table 4, most of our ligands were at least as potent as the reference compounds. Some were even more potent than SPA (ligands **28**-**30**, **44**). Moreover, ligands described as partial inverse agonists (ligands **21**-

23) showed less effect on the basal response (E_{max} values of between 18 and 43% inhibition of the basal response) than full inverse agonists which were able to reduce the basal response up to more than 80% (ligands **28**-**30**, **44**). As shown in Figure 3, ligands **28**, **29**, **30** and **44** presented better pharmacological profiles in term of E_{max} and/or EC_{50} values compared to SPA and K-(D-1-Nal)-FwLL-NH_2.

Table 4. Pharmacological Activity of Inverse Agonist Ligands 18, 21-23, 27-35, 38-42, 44-46[a]

Ligand	IP EC_{50} [b] (nM)	IP E_{max} [c] (%)	cLogP[d]	Ligand	IP EC_{50} [b] (nM)	IP E_{max} [c] (%)	cLogP[d]
SPA	66 ± 10	78	-	**33**	8.6 ± 4	65	5.41
K-(D-1-Nal)-FwLL-NH_2	12 ± 3	65	-	**34**	15.8 ± 6	73	4.49
18	8.2 ± 2	55	4.1	**35**	28 ± 8	79	5.59
21	155 ± 32	18	4.85	**38**	3.6 ± 1	75	5.57
22	19 ± 8	33	5.30	**39**	1.4 ± 0.6	78	5.91
23	62 ± 12	43	4.47	**40**	73 ± 12	49	3.13
27	45 ± 13	70	5.49	**41**	42 ± 9	59	2.82
28	21.5 ± 8	82	6.01	**42**	6 ± 2	64	3.23
29	27 ± 12	83	5.19	**44**	25 ± 9	86	5.44
30	50 ± 18	86	5.34	**45**	23 ± 7	76	4.04
31	89 ± 22	78	5.60	**46**	35 ± 10	69	3.90
32	14.2 ± 5	76	5.64				

[a]Values reported in the table were obtained from experiments performed in HEK293T cells stably expressing GHSR. [b]EC_{50} values were obtained from concentration-response curves of inositol phosphate (IP) production (HTRF assay) and are expressed as means ± SEM of three independent experiments, each experiment being performed in triplicate. [c]E_{max} values were obtained from these plots and are given as percentage inhibition of basal receptor response at a concentration of 10^{-5} M for each compound. [d]cLogP values were calculated using the MarvinSketch 20.7 software provided by ChemAxon.

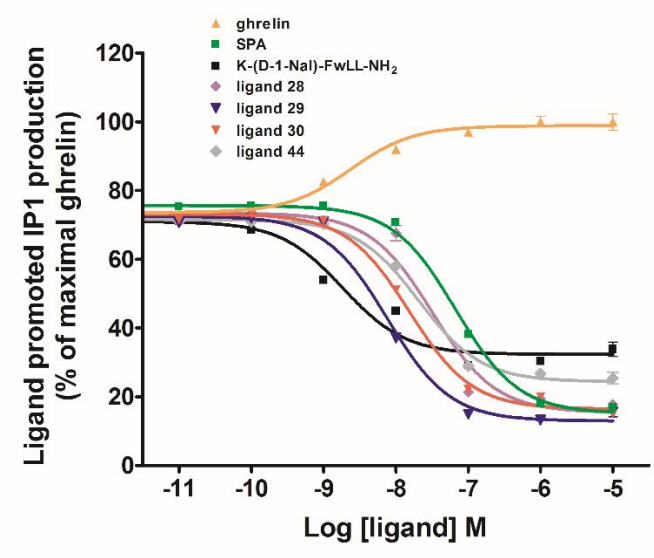


Figure 3. Concentration-response curves of inverse agonist ligands at promoting IP1 production. Data are expressed as the percentage of maximal effect of ghrelin on HEK293T cells expressing GHSR. In this experiment, basal IP1 production of GHSR-expressing cells represents 72.5 ± 1.5% of the maximal effect triggered by ghrelin. SPA and K-(D-1-Nal)-FwLL-NH_2 described in the literature as inverse agonists are given as references. Data are representative of two independent experiments, each performed in triplicate.

Activity of Inverse Agonists on the Purified GHSR. *GHSR-Mediated G Protein Activation.* We used the purified receptor assembled into lipid nanodiscs in order to evaluate whether the impact of selected ligands **27**, **29**, **30** and **44** on GHSR signaling observed in HEK293T cells resulted from a direct effect on the receptor independently of the cellular context. Note that ligand **28** was excluded due to solubility issues, and replaced by ligand **27** (same structure without the methyl substituent R^4 but with a lower cLogP value, see Table 4). The ability of the ligands to affect GHSR activation was assessed by monitoring GHSR-mediated G_q protein activation in a GTPγS binding assay (Figure 4). We fixed the maximal GHSR-catalyzed G protein activation with ghrelin, and we compared our ligands to reference inverse agonist SPA. As expected, the purified

apo GHSR assembled into lipid nanodiscs displayed a significant constitutive activity (Figure 4). Concerning ligands **27**, **29**, **30** and **44**, they were able to significantly reduce G_q protein activation by the receptor. Ligand **44** has proven to be even more potent than SPA as it almost fully inhibited the basal activity of the purified receptor. This indicated that our ligands exerted an inverse agonist activity on GHSR-dependent basal activation of G_q. Thus, these data suggest that the inverse agonist activity of our ligands observed in HEK293T cells is directly related to their interaction with GHSR independently of the cellular context.

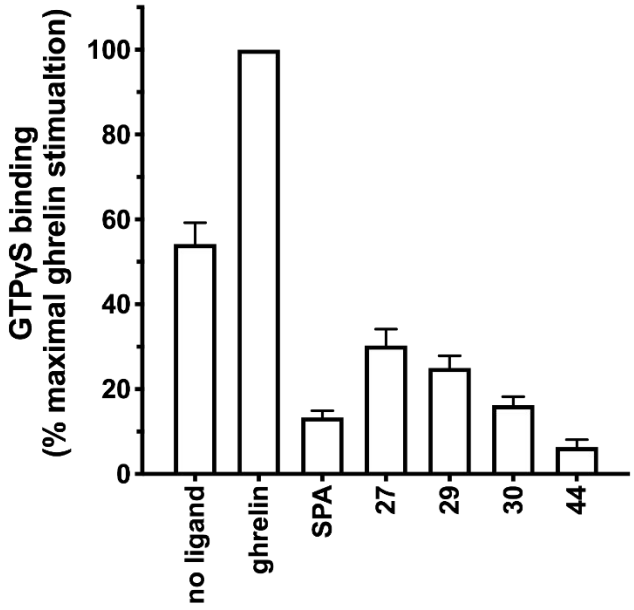


Figure 4. Effect of inverse agonist ligands on GHSR-catalyzed G_q protein activation. BODIPY-FL GTPγS binding to G_q protein induced by GHSR in lipid nanodiscs in the absence of ligand; in the presence of ghrelin and SPA (all ligands at 10^{-6} M); and in the presence of ligands **27**, **29**, **30** and **44** (all ligands at 10^{-5} M). Data are presented as the percentage of maximum BODIPY-FL fluorescence change measured in the presence of ghrelin, and represent the mean ± SEM from three independent experiments.

Impact of Inverse Agonists on GHSR Conformation. We previously reported that the pharmacological properties of GHSR ligands were intricately related to their ability to stabilize specific receptor conformations.[22] To assess whether the inverse agonist activity of ligands **27**, **29**, **30** and **44** was indeed associated with their ability to stabilize an inactive conformation similar to that observed with SPA, we analyzed the conformational features of GHSR using a fluorescence resonance energy transfer (FRET)-based approach (Figure 5).[22] To this end, the purified monomeric receptor stabilized into nanodiscs was labeled with a fluorescence acceptor (AF488) and donor (AF350) in the cytoplasmic ends of TM1 and TM6, respectively. As shown in Figure 5, addition of ghrelin or SPA at saturating concentrations induced a significant change in the FRET-monitored proximity ratio, indicative of a change in the distance between the two fluorescent probes. These variations were not due to changes in the mobility or orientation of the fluorophores, as the anisotropy of fluorophores attached to the receptor was not altered by treatment with ligands. Specifically, the full agonist ghrelin triggered a decrease in the proximity ratio, whereas the inverse agonist SPA had the opposite effect. As the FRET efficiency is directly related to the distance between the fluorophores, the increase in the proximity ratio indicates that SPA binding is associated to a change in GHSR conformation with a decrease in the distance between the cytoplasmic regions of TM1 and TM6. Such a decrease has been shown to be associated to the inactivation process of the ghrelin receptor.[23] All our ligands triggered an increase in the proximity ratio similar to that observed with SPA, suggesting that these compounds stabilize similar GHSR conformations. Correlated with GTPγS binding assay results, these data indicate that our ligands exert an intrinsic inverse agonist activity on GHSR-catalyzed G protein activation through the stabilization of a specific, inactive receptor conformation.

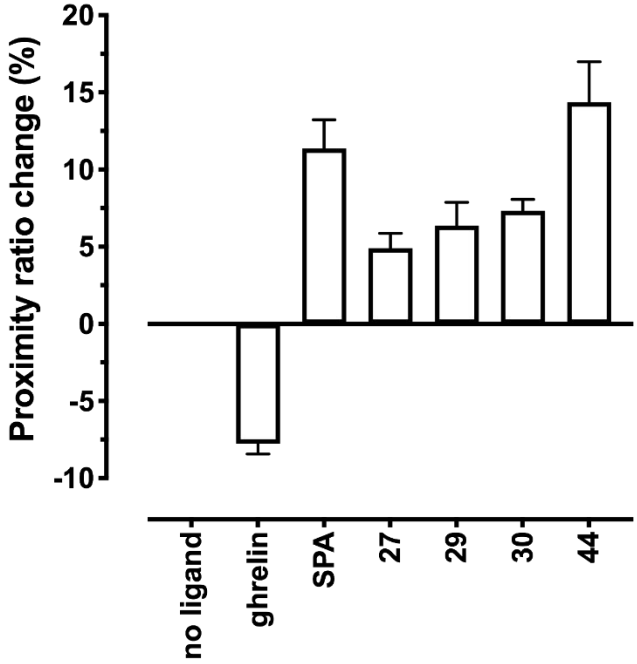


Figure 5. Effect of inverse agonist ligands on GHSR conformation. Proximity ratio changes induced by ghrelin, SPA, and inverse agonist ligands **27**, **29**, **30** and **44**. These changes were calculated from the FRET signal between the fluorophores in TM1 and TM6 of the purified ghrelin receptor assembled into lipid nanodiscs (see the Experimental section). The data are representative of two experiments performed in duplicate, and the error bars represent the SEM.

Evaluation of Ligand 29 on Endogenously Expressed GHSR. In order to perform in vivo experiments on endogenously expressed GHSR, we initially planned to select ligand **44**. Indeed, this ligand presented not only an excellent pharmacological profile (*K*i value of 1.4 ± 0.2 nM, E_{max} value of 86% inhibition of the basal level, and EC_{50} value of 25 ± 9 nM) but also the best results when tested on purified GHSR. Nevertheless, because of its low solubility in water (3.8 mg/L) we opted for ligand **29**. This ligand was selected because (i) it also appeared as one of the most

promising compounds in term of pharmacological profile (*K*i value of 3 ± 1 nM, E_{max} value of 83% inhibition of the basal level, and EC_{50} value of 27 ± 12 nM), and (ii) it presented an improved water solubility (6.5 times more soluble) compared to ligand **44**, probably due to the presence of a hydroxyl substituent on the aryl group (for more details on the water solubility of ligands **29** and **44**, see the Supporting Information).

Effect of Ligand 29 in Rat Isolated Pancreatic Islets. To determine whether the substituted 1,2,4-triazole scaffold was active on native GHSR, we tested the effect of ligand **29** on the modulation of insulin secretion in rat isolated pancreatic islets (Figure 6). Knowing that ghrelin-evoked GHSR activation inhibits glucose-stimulated insulin secretion in isolated islets of Langerhans,[24] we used this biological system to test ligand **29**. Isolated pancreatic islets were therefore incubated with a stimulating glucose concentration (8.3 mM) in the presence or absence of ghrelin (10 nM) and ligand **29** (1 μM) and then insulin release was quantified (Figure 6). As previously shown, ghrelin decreased glucose-stimulated insulin secretion. Importantly, simultaneous treatment with both ghrelin and ligand **29** blocked the inhibitory action of ghrelin on insulin secretion. These findings indicate that ligand **29** binds to pancreatic GHSR and prevents its ghrelin-evoked activation.

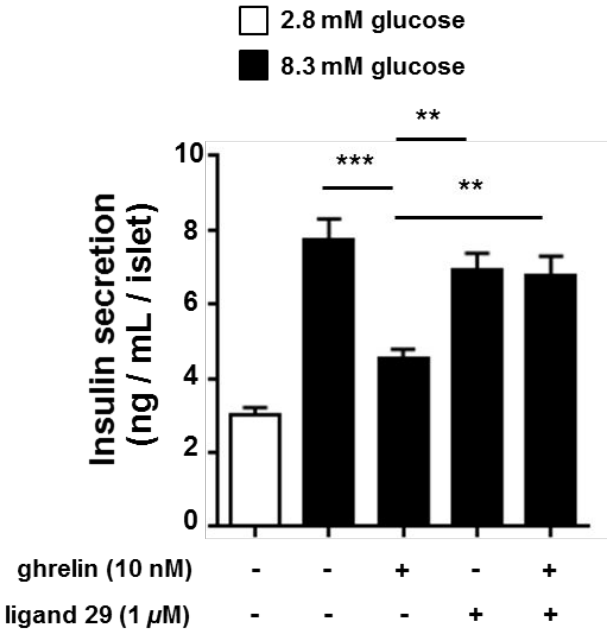


Figure 6. Effect of ligand **29** on ghrelin-induced inhibition of insulin secretion in rat pancreatic islets. Rat pancreatic islets were incubated under basal (2.8 mM glucose) or glucose-stimulated conditions (8.3 mM glucose) in the presence or absence of ghrelin (10 nM) and ligand **29** (1 μM). After 1h of incubation, the extracellular medium was collected and insulin concentration was quantified. The histogram depicts the mean ± SEM of insulin secretion expressed in ng/mL/islet. Two-way ANOVA performed with 8.3 mM glucose data points showed an interaction between ghrelin and ligand **29** [$p < 0.01$]. **$p < 0.01$; ***$p < 0.001$, Holm-Sidak multiple comparison test, n = 6.

Effect of Ligand 29 on Food Intake in Mice. First, we tested the effect of K-(D-1-Nal)-FwLL-NH_2 and ligand **29** on spontaneous food intake during the dark cycle, when mice consume the majority of their food. K-(D-1-Nal)-FwLL-NH_2 was used here as the reference inverse agonist because of its higher specificity compared to SPA. ICV administration of K-(D-1-Nal)-FwLL-NH_2 and ligand **29** significantly reduced ad libitum food intake in the early dark phase period, from

18:00 h to 23:00 h (Figure 7A) but did not affect overnight food intake, as compared to vehicle treatment (2623 ± 240, 2179 ± 333 and 2591 ± 197 mg, respectively). Importantly, K-(D-1-Nal)-FwLL-NH_2 and ligand **29** treatments did not affect body weight nor induce any sickness-like behavior such as a spiky coat, hunched posture, altered breathing rate, labored movements, reduced activity and/or subdued behavior. Then, we tested the ability of K-(D-1-Nal)-FwLL-NH_2 and ligand **29** to affect ghrelin-induced food intake (Figure 7B). Both GHSR ligands reduced ghrelin-induced food intake; however, K-(D-1-Nal)-FwLL-NH_2 treatment reduced ghrelin-induced food intake by 67.6 ± 5.6% while ligand **29** treatment reduced ghrelin-induced food intake by 30.3 ± 4.8%. Thus, these observations suggest that K-(D-1-Nal)-FwLL-NH_2 and ligand **29** behave as GHSR antagonists in vivo. Indeed, since the physiological impact of the constitutive GHSR activity is uncertain, demonstrating the ability of a given GHSR ligand to display inverse agonist activity in vivo has proved to be challenging. We have shown that the compensatory hyperphagia that follows a fasting event requires GHSR but occurs in a ghrelin-independent manner.[25] Thus, we investigated the ability of K-(D-1-Nal)-FwLL-NH_2 and ligand **29** to affect the compensatory hyperphagia in a fasting-refeeding protocol that follows a fasting event (Figure 7C-D). Notably, K-(D-1-Nal)-FwLL-NH_2 and ligand **29** treatments significantly decreased the cumulative food intake between days 1 and 5 of refeeding (Figure 7C). In particular, K-(D-1-Nal)-FwLL-NH_2 and ligand **29** reduced the compensatory hyperphagia by 20.1 ± 3.5 and 12.4 ± 3.9%, respectively, as compared to the vehicle treatment (Figure 7D). This in vivo evidence supports the notion that ligand **29** modulates ghrelin-independent aspects of GHSR activity.

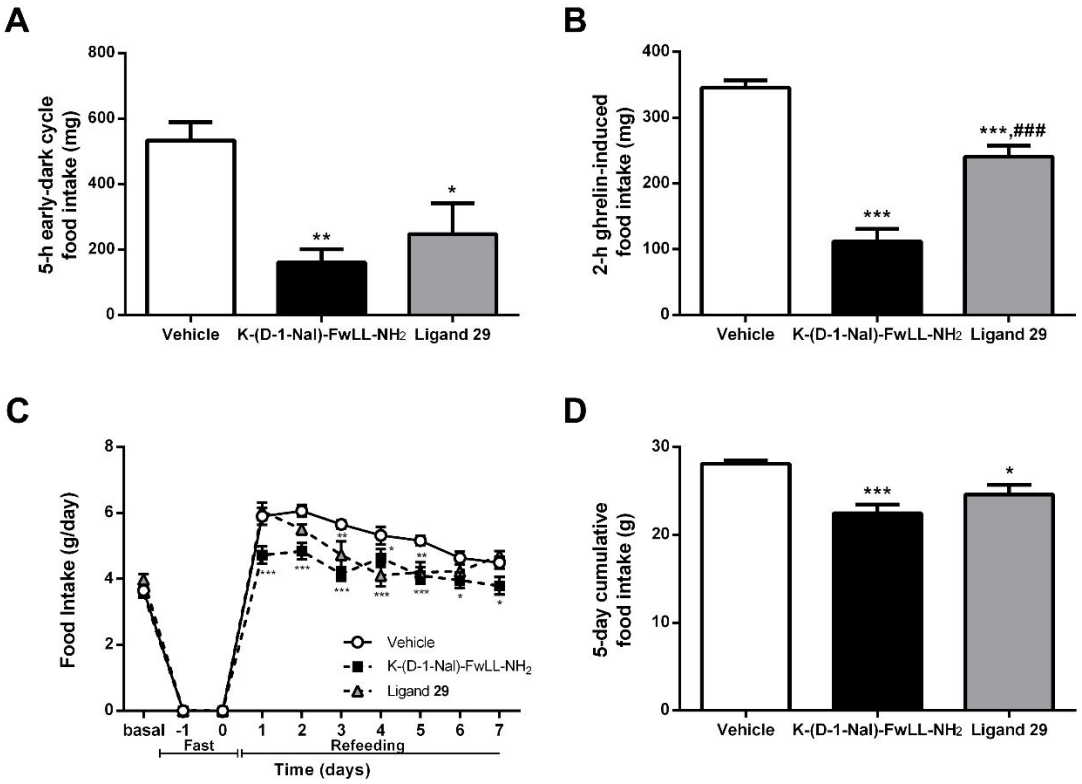


Figure 7. (A) 5-hour early dark cycle food intake in mice ICV-treated with vehicle, K-(D-1-Nal)-FwLL-NH_2 or ligand **29**. (B) 2-hour food intake in mice ICV-pretreated with vehicle, K-(D-1-Nal)-FwLL-NH_2 or ligand **29** and subsequently ICV-injected with ghrelin. (C) food intake and (D) 5-day cumulative food intake during refeeding of mice ICV-treated with vehicle, K-(D-1-Nal)-FwLL-NH_2 or ligand **29** during the fasting period. Data represent the mean ± SEM and were compared by one-way ANOVA. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. vehicle-treated mice and $^{###}p < 0.001$ vs. K-(D-1-Nal)-FwLL-NH_2-treated mice, n=33.

CONCLUSION

We synthesized a new series of 3,4,5-trisubstituted 1,2,4-triazole derivatives based on antagonist JMV2959 (compound **1**). We modulated the structure by introducing various substituents at

different positions on the scaffold and generated 45 new ligands. We carried out an in vitro SAR study in order to determine the pharmacological properties of the ligands and their ability to reduce the basal activity of GHSR. We showed that 21 ligands behaved as inverse agonists of GHSR. Among them, seventeen were at least as potent as the reference compounds K-(D-1-Nal)-FwLL-NH_2. More importantly, four ligands (ligands **28**-**30** and **44**) proved to be even more potent than SPA by inhibiting the basal response up to 86%. In order to show a direct interaction between the ligands and GHSR, we tested the activity of some selected inverse agonists on purified GHSR assembled into lipid nanodiscs. We proved that these ligands exerted an intrinsic inverse agonist activity on GHSR-catalyzed G protein activation through the stabilization of a specific inactive receptor conformation. At last, we investigated the effect of one of the most promising inverse agonist (ligand **29**) on both the modulation of insulin secretion in rat isolated pancreatic islets and food intake in mice. By directly binding to GHSR, ligand **29** revealed to block the inhibitory action of ghrelin on insulin secretion. Moreover, ICV administration of ligand **29** reduced ghrelin-induced food intake in mice, as well as the compensatory hyperphagia that follows a fasting event. In conclusion, this 3,4,5-trisubstituted 1,2,4-triazole ring has proven to be a promising scaffold for the development of efficient inverse agonists of GHSR in order to treat obesity-related metabolic diseases.

EXPERIMENTAL SECTION

General Chemistry Methods. All chemicals for synthesis were purchased from Sigma-Aldrich Chemical Co., Thermo Fisher GmbH, Fluka™ and Iris Biotech GmbH, and used without further purification. All solvents were purchased from Sigma Aldrich and Honeywell in gradient grade or reagent quality. Classical purifications were performed with flash column chromatography using

silica gel (VWR, 40-63 μm). HPLC purifications were run on a Waters 4000 preparative apparatus using a C18 Deltapak column (100 mm x 40 mm, 15μm, 100 Å), with UV detection at 214 nm and 254 nm, at a flow rate of 50 mL/min of a mixture of A, water with 0.1% TFA, and B, acetonitrile with 0.1% TFA, in gradient mode. Samples for LC-MS analyses were prepared in methanol (gradient grade). The LC-MS system consisted of a Waters Alliance 2695 HPLC coupled to a Water Micromass ZQ spectrometer (electrospray ionization mode, ESI+). All analyses were carried out using a Phenomenex Onyx reversed-phase column (25 x 4.6 mm). A flow rate of 3 mL/min and a gradient from 0 to 100% of B over 2.5 min were used. Eluent A: water/0.1% FA ; eluent B: acetonitrile/0.1% FA. UV detection was performed at 214 nm. The purity of the compounds was assessed by reversed-phase HPCL analyses (UV detection at 214 nm) performed on a Beckman apparatus (System Gold) using a Chromatolith High Resolution column (RP-18e, 50-4.6 mm) with a gradient mode of 100% of A (water with 0.1% TFA) to 100% of B (acetonitrile with 0.1% TFA) at a flow rate of 3 mL/min. All purities were found ≥95%. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker Advance spectrometers at 300, 400 or 500 MHz and 75, 100 or 125 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm) relative to the residual peak of the solvent (2.50 and 39.52 ppm for DMSO, 3.31 and 49 ppm for MeOD) according to the literature.[26] Data are reported as follows: chemical shift (δ in ppm), multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, br s=broad singlet, m=multiplet), coupling constants (J = Hz), integration and assignment. HRMS analyses were performed using a microTOF-Q spectrometer (electrospray ionization mode, ESI+). The lyophilizer from Cryonext (Saint Gely du Fesc, France) was used with a freezing trap at -90 °C to lyophilize compounds purified by reverse phase preparative chromatography.

General Procedure: Compound **1** (compound **4** in reference 18), triazoles (**iv**, **vi** and **vii**) and not commercially available hydrazides (**v**) were synthesized following the same procedures previously described.[18, 27]

General Procedure: Method A (for ligands 2-8). Triazole **iv** (100 mg, 0.2 mmol, 1eq) was coupled with carboxylic acids (1.1 equiv), in the presence of BOP (1.1 equiv) and DIEA (pH=9) for 2 h, in DCM. The mixture was then concentrated in vacuo, and the residue was dissolved in EtOAc. The organic layer was successively washed with aqueous solutions of 1M $KHSO_4$, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and condensed under reduced pressure to yield the desired compound. The compounds that did not require a final deprotection were purified by preparative HPLC on a C18 column using a water/acetonitrile/TFA 0.1% gradient.

General Procedure: Method B (for ligands 9-35). Compound **1** (100 mg, 0.18 mmol, 1eq) was coupled with carboxylic acids (1.1 equiv), in the presence of HATU (1.1 equiv) and DIEA (pH=9) for 2 h, in DCM. The mixture was condensed under reduced pressure, and the residue was dissolved in EtOAc. The organic layer was successively washed with aqueous solutions of 1M $KHSO_4$, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and condensed under reduced pressure to yield the desired compound. The compounds that did not require a final deprotection were purified by preparative HPLC on a C18 column using a water/acetonitrile/TFA 0.1% gradient.

General Procedure: Method C (for ligand 36). Appropriate triazole **vi** (100 mg, 1eq) was coupled with alpha-cyano-4-methoxycinnamic acid (1.1 equiv), in the presence of HATU (1.1 equiv) and DIEA (pH=9) for 2 h, in DCM. The mixture was condensed under reduced pressure,

and the residue was dissolved in EtOAc. The organic layer was successively washed with aqueous solutions of 1M $KHSO_4$, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and condensed under reduced pressure to yield the desired compound that was purified by preparative HPLC on a C18 column using a water/acetonitrile/TFA 0.1% gradient.

General Procedure: Method D (for ligand 37). Appropriate triazole **vi** (100 mg, 1eq) was coupled with 2-hydroxyacetic acid (1.1 equiv), in the presence of HATU (1.1 equiv) and DIEA (pH=9) for 2 h, in DCM. The mixture was condensed under reduced pressure and the residue was dissolved in EtOAc. The organic layer was successively washed with aqueous solutions of 1M $KHSO_4$, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and condensed under reduced pressure. The resulting compound was purified by flash chromatography on silica gel with a gradient of EtOAc to EtOAc/MeOH 9:1 as eluent and then reacted with alpha-cyano-4-methoxycinnamic acid (1.1 equiv), in the presence of EDC (1.1 equiv) and DIEA (pH=9) for 2 h, in DCM. After removal of solvents under reduced pressure, the residue was dissolved in EtOAc and the organic layer was successively washed with aqueous solutions of 1M $KHSO_4$, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and condensed under reduced pressure to yield the desired compound that was purified by preparative HPLC on a C18 column using a water/acetonitrile/TFA 0.1% gradient.

General Procedure: Method E (for ligands 38-46). Appropriate Boc-protected triazole **vii** (100 mg, 1eq) was treated with 4N HCl in dioxane (5 mL) for 30 min at 50 °C. When removal of the Boc-protecting group was confirmed by HPLC analysis, MeOH was added and the reaction mixture was condensed under reduced pressure. Additions of MeOH followed by evaporations were repeated several times in order to remove excess of HCl. The resulting deprotected amine was coupled with appropriate para-substituted alpha-cyano-cinnamic acid (1.1 equiv), in the

presence of HATU (1.1 equiv) and DIEA (pH=9) for 2 h, in DCM. The mixture was condensed under reduced pressure, and the residue was dissolved in EtOAc. The organic layer was successively washed with aqueous solutions of 1M $KHSO_4$, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and condensed under reduced pressure to yield the desired compound that was purified by preparative HPLC on a C18 column using a water/acetonitrile/TFA 0.1% gradient.

General Procedure: Method F (for ligands 2, 4, 6, 11, 21-23). Removal of the protecting group was performed at 50 °C with a solution of 4N HCl in dioxane (2 mL) for 30 min. The mixture was then condensed under reduced pressure, diluted with MeOH, and condensed again under reduced pressure. Additions of MeOH followed by evaporations were repeated several times in order to remove excess of HCl. The resulting deprotected compounds were then purified by preparative HPLC on a C18 column using a water/acetonitrile/TFA 0.1% gradient.

General Procedure: Method G (for ligands 6 and 8). Removal of the Fmoc protecting group was performed at room temperature with Et_2NH (10 eq, 1 mmol/ml) in DMF for 2 h. The mixture was then condensed under reduced pressure and the compounds were purified by preparative HPLC on a C18 column using a water/acetonitrile/TFA 0.1% gradient.

*(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-(methylamino)acetamide (**2**).* By methods A and F: 89 mg (0.17 mmol, 85%)**,** orange wax (purity HPLC = 99%). LC-MS (ES): t_R = 1.30 min, *m/z* 523.3 $[M+H]^+$. ^1H NMR (300 MHz, DMSO-d_6): δ 10.90 (d, J = 1.8 Hz, 1H, NH), 9.35 (d, J = 8.1 Hz, 1H, NH), 8.85-8.71 (m, 2H, NH_2 TFA salt), 7.35 (d, J = 8.1 Hz, 1H, CH_{aryl}), 7.32-7.11 (m, 6H, CH_{aryl}), 7.09-7.02 (m, 2H, CH_{aryl}), 6.91 (t, J = 7.3 Hz, 1H, CH_{aryl}), 6.71 (br s, 4H, CH_{aryl}), 5.34-5.24 (m, 1H, CH), 5.05 (d, J = 16.9 Hz, 1H,

CH_2), 4.99 (d, J = 16.9 Hz, 1H, CH_2), 3.69 (s, 3H, OCH_3), 3.66-3.50 (m, 2H, CH_2), 3.44 (d, J = 14.2 Hz, 7.3 Hz, 1H, CH_2), 3.32 (d, J = 14.2 Hz, 7.3 Hz, 1H, CH_2), 2.95-2.78 (m, 4H, 2 CH_2), 2.41 (br s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 164.8, 158.7, 154.4, 154.1, 140.3, 136.1, 128.3 (4C), 127.5 (2C), 127.0 (2C), 126.2, 124.3, 121.0, 118.5, 117.9, 114.1 (2C), 111.4, 109.2, 55.1, 48.7, 45.2, 44.9, 32.5, 32.2, 29.1, 26.0. HRMS (ESI): *m/z* calculated for $C_{31}H_{35}N_6O_2$ $[M+H]^+$: 523.2821. Found: 523.2819.

(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-(dimethylamino)acetamide ***(3)***. By method A: 71 mg (0.13 mmol, 66%), beige wax (purity HPLC > 99%). LC-MS (ES): t_R = 1.30 min, *m/z* 537.2 $[M+H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.89 (d, J = 1.6 Hz, 1H, NH), 9.79-9.63 (m, 1H, NH TFA salt), 9.43 (d, J = 8.3 Hz, 1H, NH), 7.38-7.30 (m, 2H, CH $_{aryl}$), 7.29-7.10 (m, 5H, CH $_{aryl}$), 7.08-7.01 (m, 2H, CH $_{aryl}$), 6.95-6.88 (m, 1H, CH $_{aryl}$), 6.73 (br s, 4H, CH $_{aryl}$), 5.37-5.27 (m, 1H, CH), 5.00 (s, 2H, CH_2), 3.78 (d, J = 13.4 Hz, 1H, CH_2), 3.72 (d, J = 13.4 Hz, 1H, CH_2), 3.70 (s, 3H, OCH_3), 3.44 (dd, J = 14.3 Hz, 6.9 Hz, 1H, CH_2), 3.33 (d, J = 14.3 Hz, 7.8 Hz, 1H, CH_2), 2.92-2.79 (m, 4H, 2 CH_2), 2.60 (br s, 6H, 2 CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.8, 158.7, 154.2, 154.1, 104.4, 136.1, 128.3 (4C), 127.4 (2C), 127.2, 127.0, 126.1, 124.2, 120.9, 118.4, 118.0, 114.1 (2C), 111.4, 109.3, 57.1, 55.1, 45.0, 44.8, 42.9 (2C), 32.2, 29.1, 26.1. HRMS (ESI): *m/z* calculated for $C_{32}H_{37}N_6O_2$ $[M+H]^+$: 537.2978. Found: 537.2979.

(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-(ethylamino)acetamide ***(4)***. By methods A and F: 62 mg (0.12 mmol, 58%), white amorphous powder (purity HPLC = 98%). LC-MS (ES): t_R = 1.33 min, *m/z* 537.3 $[M+H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.87 (d, J = 1.2 Hz, 1H, NH), 9.31 (d, J = 8.2 Hz, 1H, NH), 8.73-8.68 (m, 2H, NH_2 TFA salt), 7.34 (d, J = 8.1 Hz, 1H, CH $_{aryl}$), 7.31-7.11 (m, 6H, CH $_{aryl}$), 7.08-7.01 (m, 2H, CH $_{aryl}$), 6.90 (t, J = 7.4 Hz, 1H, CH $_{aryl}$), 6.71 (br s, 4H, CH $_{aryl}$), 5.33-5.22 (m, 1H, CH), 5.08-4.93

(m, 2H, CH_2), 3.69 (s, 3H, OCH_3), 3.65-3.48 (m, 2H, CH_2), 3.43 (dd, *J* = 14.2 Hz, 7.4 Hz, 1H, CH_2), 3.31 (dd, *J* = 14.2 Hz, 7.4 Hz, 1H, CH_2), 2.94-2.78 (m, 4H, 2 CH_2), 2.77-2.67(m, 2H, CH_2), 1.08 (t, *J* = 7.2 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 164.7, 158.7, 154.3, 154.0, 140.4, 136.0, 128.3 (4C), 127.3 (2C), 127.2, 126.9, 126.1, 124.2, 120.9, 118.4, 117.9, 114.1 (2C), 111.4, 109.3, 55.1, 46.8, 45.0, 44.9, 41.8, 32.2, 29.2, 26.0, 10.7. HRMS (ESI): *m/z* calculated for $C_{32}H_{37}N_6O_2$ $[M+H]^+$: 537.2978. Found: 537.2977.

*(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-(diethylamino)acetamide (**5**)*. By method A: 70 mg (0.13 mmol, 65%), orange wax (HPLC, purity > 96%). LC-MS (ES): t_R = 1.35 min, *m/z* 445.3 $[M+H - 4\text{-methoxybenzyl}]^+$, 565.3 $[M+H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.88 (s, 1H, NH), 9.40 (d, *J* = 8.5 Hz, 1H, NH), 9.32-9.21 (m, 1H, NH TFA salt), 7.37-7.29 (m, 2H, CH_{aryl}), 7.28-7.11 (m, 5H, CH_{aryl}), 7.09-7.01 (m, 2H, CH_{aryl}), 6.91 (t, *J* = 7.4 Hz, 1H, CH_{aryl}), 6.80-6.68 (m, 4H, CH_{aryl}), 5.41-5.30 (m, 1H, CH), 5.02 (s, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.64-3.55 (m, 2H, CH_2), 3.46-3.30 (m, 2H, CH_2), 3.29-2.70 (m, 8H, 4 CH_2), 1.11-0.90 (m, 6H, 2 CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 164.0, 158.7, 154.2 (2C), 140.4, 136.1, 128.3 (4C), 127.3 (2C), 127.0, 126.1, 124.3, 120.9, 118.4-118.0 (3C), 114.1 (2C), 111.4, 109.3, 55.1, 52.1, 48.2, 45.0, 44.8, 39.8, 32.2, 28.9, 26.0, 8.7, 8.4. HRMS (ESI): *m/z* calculated for $C_{34}H_{41}N_6O_2$ $[M+H]^+$: 565.3291. Found: 565.3290.

*(R)-2-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethylamino]acetic acid (**6**)*. By methods A, F and G: 16 mg (0.03 mmol, 14%), white amorphous powder (HPLC, purity > 95%). LC-MS (ES): t_R = 1.36 min, *m/z* 567.3 $[M+H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.84 (d, *J* = 1.6 Hz, 1H, NH), 9.32 (d, *J* = 8.1 Hz, 1H, NH), 7.34 (d, *J* = 7.6 Hz, 1H, CH_{aryl}), 7.28-7.12 (m, 7H, NH, CH_{aryl}), 7.08-7.00 (m, 2H, CH_{aryl}), 6.89 (t, *J* = 7.6 Hz, 1H, CH_{aryl}), 6.68 (s, 4H, CH_{aryl}), 5.28-5.18 (m, 1H, CH), 5.05-4.90 (m,

2H, CH_2), 3.78 (s, 4H, 2 CH_2), 3.69 (s, 3H, OCH_3), 3.42 (dd, J = 14.1 Hz, 7.6 Hz, 1H, CH_2), 3.29 (dd, J = 14.1 Hz, 7.1 Hz, 1H, CH_2), 2.89-2.73 (m, 4H, 2 CH_2). ^{13}C NMR (75 MHz, DMSO-d_6): δ 167.8, 164.5, 158.4, 154.2, 153.8, 140.5, 136.0, 128.3 (4C), 127.3 (2C), 126.9, 126.1, 124.4, 120.9, 118.4, 117.8, 114.0 (2C), 111.4, 109.3, 55.0, 47.0, 46.6, 45.3, 44.2, 40.5, 32.3, 29.3, 26.1. HRMS (ESI): *m/z* calculated for $C_{32}H_{35}N_6O_4$ $[M+H]^+$: 567.2720. Found: 567.2722.

*(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-(benzylamino)acetamide (**7**)*. By method A: 84 mg (0.14 mmol, 70%), orange amorphous powder (HPLC, purity > 98%). LC-MS (ES): t_R = 1.43 min, *m/z* 599.4 $[M+H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.86 (d, J = 0.9 Hz, 1H), 9.30 (d, J = 8.2 Hz, 1H), 9.26-9.15 (m, 1H, NH), 7.45-7.35 (m, 5H, CH_{aryl}), 7.34-7.10 (m, 7H, CH_{aryl}), 7.08-7.01 (m, 2H, CH_{aryl}), 6.90 (t, J = 7.4 Hz, 1H, CH_{aryl}), 6.71 (s, 4H, CH_{aryl}), 5.32-5.22 (m, 1H, CH), 5.02 (s, 2H, CH_2), 4.01-3.90 (s, 2H, CH_2), 3.68 (s, 3H, OCH_3), 3.63-3.47 (m, 2H, CH_2), 3.41 (dd, J = 14.2 Hz, 7.2 Hz, 1H, CH_2), 3.30 (dd, J = 14.2 Hz, 7.5 Hz, 1H, CH_2), 2.93-2.76 (m, 4H, 2 CH_2). ^{13}C NMR (75 MHz, DMSO-d_6): δ 164.6, 158.7, 154.3, 154.0, 140.3, 136.0, 131.3, 130.0 (2C), 129.1, 128.7 (2C), 128.3 (4C), 127.4 (2C), 127.1, 126.9, 126.2, 124.2, 121.0, 118.4, 117.9, 114.1 (2C), 111.4, 109.2, 55.0, 49.7, 46.5, 45.0, 44.9, 32.2, 29.2, 26.0. HRMS (ESI): *m/z* calculated for $C_{37}H_{39}N_6O_2$ $[M+H]^+$: 599.3134. Found: 599.3140.

*(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-(4-aminopiperidin-1-yl)acetamide (**8**)*. By methods A and G: 90 mg (0.15 mmol, 76%), orange wax (HPLC, purity > 95%). LC-MS (ES): t_R = 1.14 min, *m/z* 592.4 $[M+H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.90 (s, 1H, NH), 9.44 (d, J = 8.4 Hz, 1H, NH), 8.26 (br s, 3H, NH_3 TFA salt), 7.38-7.31 (m, 2H, CH_{aryl}), 7.29-7.10 (m, 5H, CH_{aryl}), 7.09-7.01 (m, 2H, CH_{aryl}), 6.92 (t, J = 7.4 Hz, 1H, CH_{aryl}), 6.72 (s, 4H, CH_{aryl}), 5.38-5.27 (m, 1H, CH), 5.00 (s, 2H, CH_2), 3.77-3.61 (m, 5H,

OCH_3 and CH_2), 3.43 (dd, J = 14.2 Hz, 7.0 Hz, 1H, CH_2), 3.34 (dd, J = 14.2 Hz, 7.9 Hz, 1H, CH_2), 3.28-3.14 (m, 2H, CH_2), 3.12-3.02 (m, 1H, CH), 3.01-2.76 (m, 6H, 3 CH_2), 2.05-1.93 (m, 2H, CH_2), 1.88-1.67 (m, 2H, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.5, 158.7, 154.1 (2C), 140.4, 136.1, 128.3 (4C), 127.3 (2C), 127.2, 127.0, 126.1, 124.3, 120.9, 118.5, 118.0, 114.1 (2C), 111.4, 109.3, 56.1, 55.1, 50.5-50.3 (3C), 45.0, 44.8, 32.3, 29.1, 26.7 (2C), 26.1. HRMS (ESI): *m/z* calculated for $C_{35}H_{42}N_7O_2$ $[M+H]^+$: 592.3400. Found: 592.3401.

*(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-formamidoacetamide (**9**)*. By method B: 56 mg (0.10 mmol, 58%), beige amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.44 min, *m/z* 537.2 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4): δ 8.12 (s, 1H, CHO), 7.35 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.32 (d, J = 7.5 Hz, 1H, CH_{aryl}), 7.22-7.15 (m, 3H, CH_{aryl}), 7.08 (t, J = 8.0 Hz, 1H, CH_{aryl}), 7.00-6.97 (m, 3H, CH_{aryl}), 6.93 (t, J = 8.0 Hz, 1H, CH_{aryl}), 6.63-6.58 (m, 3H, CH_{aryl}), 6.54 (d, J = 8.5 Hz, 1H, CH_{aryl}), 5.42-5.39 (dd, J = 9.5 Hz, 6.0 Hz, 1H, CH), 4.87 (d, J = 16.0 Hz, 1H, CH_2), 4.75 (d, J = 16.0 Hz, 1H, CH_2), 3.82 (d, J = 4.5 Hz, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.56-3.51 (dd, J = 14.0 Hz, 9.5 Hz, 1H, CH_2), 3.43-3.39 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.89-2.84 (m, 2H, CH_2), 2.78 (t, J = 7.0 Hz, 2H, CH_2). ^{13}C-NMR (125 MHz, methanol-d_4): δ 170.5, 164.1, 160.9, 157.0, 156.4, 140.8, 137.9, 129.6 (2C), 129.3 (2C), 128.7 (2C), 128.4, 127.6, 127.1, 125.2, 122.6, 120.1, 118.9, 115.3 (2C), 112.4, 110.2, 55.7, 47.3, 47.0, 41.7, 33.9, 30.3, 27.6. HRMS (ESI): *m/z* calculated for $C_{31}H_{33}N_6O_3$ $[M+H]^+$: 537.2614. Found: 537.2614.

*(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-acetamidoacetamide (**10**)*. By method B: 54 mg (0.10 mmol, 54%), orange amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.53 min, *m/z* 551.1 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4): 7.35 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.31 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.22-7.16 (m, 3H,

CH $_{aryl}$), 7.08 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 6.99-6.97 (m, 3H, CH $_{aryl}$), 6.93 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 6.60 (d, J = 8.5 Hz, 2H, CH $_{aryl}$), 6.54 (d, J = 8.5 Hz, 2H, CH $_{aryl}$), 5.42-5.39 (dd, J = 9.5 Hz, 6.0 Hz, 1H, CH), 4.86 (d, J = 16.5 Hz, 1H, CH_2), 4.75 (d, J = 16.5 Hz, 1H, CH_2), 3.77 (s, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.55-3.51 (dd, J = 14.0 Hz, 9.5 Hz, 1H, CH_2), 3.42-3.38 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.90-2.81 (m, 2H, CH_2), 2.79-2.76 (m, 2H, CH_2), 1.98 (s, 3H, CO-CH_3). ^{13}C-NMR (125 MHz, methanol-d_4): δ 173.8, 171.2, 160.8, 157.0, 156.4, 140.9, 137.9, 129.6 (2C), 129.3 (2C), 128.7 (2C), 128.4, 127.6, 127.3, 125.1, 122.6, 120.1, 118.9, 115.3 (2C), 112.4, 110.3, 55.6, 47.3, 46.9, 43.2, 33.9, 30.3, 27.6, 22.4. HRMS (ESI): *m/z* calculated for $C_{32}H_{35}N_6O_3$ $[M+H]^+$: 551.2771. Found: 551.2770.

*(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-(2-aminoacetamido)acetamide (**11**)*. By methods B and F: 65 mg (0.12 mmol, 64%), beige amorphous powder (HPLC, purity = 98%). LC-MS (ES): t_R = 1.34 min, *m/z* 446.2 [M+H – 4-methoxybenzyl]$^+$, 566.3 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4): 7.35 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.31 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.21-7.16 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 6.99-6.97 (m, 3H, CH $_{aryl}$), 6.92 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 6.60 (d, J = 8.5 Hz, 2H, CH $_{aryl}$), 6.53 (d, J = 8.5 Hz, 2H, CH $_{aryl}$), 5.41-5.38 (dd, J = 9.5 Hz, 6.0 Hz, 1H, CH), 4.76 (d, J = 16.5 Hz, 1H, CH_2), 4.66 (d, J = 16.5 Hz, 1H, CH_2), 3.91 (d, J = 16.5 Hz, 1H, CH_2), 3.85 (d, J = 16.5 Hz, 1H, CH_2), 3.73 (s, 2H, CH_2), 3.69 (s, 3H, OCH_3), 3.55-3.50 (dd, J = 14.0 Hz, 9.5 Hz, 1H, CH_2), 3.42-3.39 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.85-2.74 (m, 4H, 2 CH_2). ^{13}C-NMR (125 MHz, methanol-d_4): δ 1707, 167.9, 160.8, 156.8, 156.4, 141.0, 137.9, 129.6 (2C), 129.3 (2C), 128.6 (2C), 128.4, 127.6, 127.3, 125.2, 122.6, 120.1, 118.9, 115.3 (2C), 112.4, 110.3, 55.6, 47.1, 40.0, 47.9, 41.4, 34.0, 30.6, 27.6. HRMS (ESI): *m/z* calculated for $C_{32}H_{36}N_7O_3$ $[M+H]^+$: 566.2880. Found: 566.2881.

*(R)-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]carbamoyl cyanide (**12**)*. By method B: 45 mg (0.08 mmol, 45%), beige amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.62 min, *m/z* 561.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.35 (d, J = 8.4 Hz, 1H, CH $_{aryl}$), 7.30 (d, J = 8.0 Hz, 1H, CH aryl), 7.25-7.15 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 6.99-6.88 (m, 4H, CH $_{aryl}$), 6.61 (d, J = 8.8 Hz, 2H, CH $_{aryl}$), 6.55 (d, J = 8.8 Hz, 2H, CH $_{aryl}$), 5.41 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.95 (d, J = 16.4 Hz, 1H, CH_2), 4.83 (d, J = 16.4 Hz, 1H, CH_2), 3.83 (s, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.56-3.51 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.45-3.40 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.99-2.85 (m, 2H, CH_2), 2.78 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.4, 161.0, 157.3, 156.4, 155.1, 140.4, 137.9, 129.7 (2C), 129.3 (2C), 128.9 (2C), 127.8, 126.6, 125.2, 122.7, 120.2, 118.8, 115.4 (2C), 112.5, 109.9, 77.8, 76.6, 55.7, 47.8, 47.1, 43.2, 33.5, 30.1, 27.4. HRMS (ESI): *m/z* calculated for $C_{33}H_{33}N_6O_3$ $[M+H]^+$: 561.2614. Found, 561.2614.

*(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]acrylamide (**13**)*. By method B: 34 mg (0.06 mmol, 34%), beige amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.61 min, *m/z* 563.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.36 (d, J = 8.4 Hz, 1H, CH $_{aryl}$), 7.30 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.23-7.17 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 7.2 Hz, 1H, CH $_{aryl}$), 7.01-6.92 (m, 4H, CH $_{aryl}$), 6.63-6.55 (m, 4H, CH $_{aryl}$), 6.29-6.25 (m, 2H, CH $_{vinyl}$), 5.71-5.68 (dd, J = 9.2 Hz, 2.8 Hz, 1H, CH $_{vinyl}$), 5.42 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.93 (d, J = 16.8 Hz, 1H, CH_2), 4.80 (d, J = 16.8 Hz, 1H, CH_2), 3.87 (s, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.57-3.51 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.47-3.40 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.95-2.85 (m, 2H, CH_2), 2.78 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 171.1, 168.5, 161.0, 157.2, 156.5, 140.6, 137.9, 131.6, 129.7 (2C), 129.3 (2C), 128.8 (2C), 128.4, 127.7, 127.3, 126.9, 125.2, 122.7, 120.2, 118.9, 115.4 (2C),

112.5, 110.1, 55.7, 47.6, 47.1, 43.2, 33.7, 30.3, 27.5. HRMS (ESI): *m/z* calculated for $C_{33}H_{35}N_6O_3$ $[M+H]^+$: 563.2771. Found: 563.2772.

*(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-(2-cyanoacetamido)acetamide (**14**)*. By method B: 79 mg (0.14 mmol, 76%), white amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.61 min, *m/z* 56.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.36 (d, J = 8.4 Hz, 1H, CH $_{aryl}$), 7.31 (d, J = 8.0 Hz, 1H, CH aryl), 7.23-7.15 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 7.2 Hz, 1H, CH $_{aryl}$), 6.99-6.92 (m, 4H, CH $_{aryl}$), 6.64-6.57 (m, 4H, CH $_{aryl}$), 5.44 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.92 (d, J = 16.4 Hz, 1H, CH_2), 4.83 (d, J = 16.4 Hz, 1H, CH_2), 3.82 (s, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.60 (s, 2H, CH_2), 3.57-3.51 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.45-3.40 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.95-2.89 (td, J = 8.4 Hz, 7.6 Hz, 2H, CH_2), 2.78 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.1, 165.3, 161.0, 157.2, 156.5, 140.4, 137.9, 129.7 (2C), 129.3 (2C), 128.8 (2C), 128.4, 127.8, 126.7, 125.2, 122.7, 120.2, 118.9, 115.9, 115.4 (2C), 112.5, 110.0, 55.7, 47.8, 47.1, 43.5, 33.5, 30.1, 27.4, 25.8. HRMS (ESI): *m/z* calculated for $C_{33}H_{34}N_7O_3$ $[M+H]^+$: 576.2723. Found: 576.2724.

*(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-2-methylpropanamide (**15**)*. By method B: 74 mg (0.12 mmol, 68%), orange wax (HPLC, purity > 98%). LC-MS (ES): t_R = 1.70 min, *m/z* 604.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.36 (d, J = 8.4 Hz, 1H, CH $_{aryl}$), 7.30 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.23-7.16 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 7.2 Hz, 1H, CH $_{aryl}$), 7.00 (s, 1H, CH $_{aryl}$), 6.98-6.92 (m, 3H, CH $_{aryl}$), 6.64-6.57 (m, 4H, CH $_{aryl}$), 5.46-5.42 (dd, J = 9.2 Hz, 6.4 Hz, 1H, CH), 5.02 (d, J = 16.4 Hz, 1H, CH_2), 4.90 (d, J = 16.4 Hz, 1H, CH_2), 3.83 (s, 2H, CH_2), 3.71 (s, 3H, OCH_3), 3.56-3.50 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.47-3.42 (dd, J = 14.0 Hz, 6.4 Hz, 1H, CH_2), 3.04-2.90 (m, 2H, CH_2), 2.78 (t, J = 8.0 Hz, 2H, CH_2), 1.58 (s, 6H, $C(CH_3)_2$). ^{13}C-NMR (100 MHz,

methanol-d_4): δ 171.8, 170.7, 161.1, 157.4, 156.4, 140.1, 137.9, 129.8 (2C), 129.3 (2C), 128.9 (2C), 128.3, 127.9, 126.3, 125.2, 122.8, 122.7, 120.2, 118.8, 115. (2C), 112.5, 109.8, 55.7, 48.1, 47.1, 43.6, 40.6, 33.3, 30.1, 27.3, 25.2 (2C). HRMS (ESI): *m/z* calculated for $C_{35}H_{38}N_7O_3$ $[M+H]^+$: 604.3036. Found: 604.3038.

*(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-4-cyanotetrahydro-2H-pyran-4-carboxamide (**16**)*. By method B: 67 mg (0.10 mmol, 58%), orange wax (HPLC, purity > 98%). LC-MS (ES): t_R = 1.55 min, *m/z* 646.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.36 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.29 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.23-7.17 (m, 3H, CH_{aryl}), 7.09 (t, J = 7.2 Hz, 1H, CH_{aryl}), 7.00 (s, 1H, CH_{aryl}), 6.96-6.92 (m, 3H, CH_{aryl}), 6.63-6.57 (m, 4H, CH_{aryl}), 5.47-5.43 (dd, J = 9.6 H, 6.4 Hz, 1H, CH), 5.01 (d, J = 16.4 Hz, 1H, CH_2), 4.89 (d, J = 16.4 Hz, 1H, CH_2), 3.95-3.92 (m, 2H, CH_2), 3.86 (s, 2H, CH_2), 3.70-3.62 (m, 5H, CH_2, OCH_3), 3.56-3.50 (dd, J = 14.0 Hz, 9.6 Hz, 1H, CH_2), 3.47-3.42 (dd, J = 14.0 Hz, 6.4 Hz, 1H, CH_2), 3.01-2.90 (m, 2H, CH_2), 2.77 (t, J = 7.4 Hz, 2H, CH_2), 2.08 (m, 2H, CH_2), 1.95 (d, J = 14.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.7, 170.0, 161.1, 157.4, 156.4, 140.0, 137.9, 129.8 (2C), 129.3 (2C), 129.0 (2C), 128.2, 127.9, 126.1, 125.3, 122.7, 120.5, 120.2, 118.8, 115. (2C), 112.6, 109.7, 65.1 (2C), 55.7, 48.2, 47.1, 44.7, 43.7, 33.6, 33.5, 33.1, 30.0, 27.2. HRMS (ESI): *m/z* calculated for $C_{37}H_{40}N_7O_4$ $[M+H]^+$: 646.3136. Found: 646.3145.

*N-[2-((R)-2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-phenylpropanamide (**17**)*. By method B: 74 mg (0.11 mmol, 62%), orange amorphous powder (HPLC, purity > 98%). LC-MS (ES): t_R = 1.84 min, *m/z* 666.3 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.36 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.33-7.16 (m, 9H, CH_{aryl}), 7.10 (t, J = 7.6 Hz, 1H, CH_{aryl}), 7.00 (s, 1H, CH_{aryl}), 6.98-6.93 (m, 3H, CH_{aryl}), 6.64-

6.59 (m, 4H, CH $_{aryl}$), 5.48-5.42 (m, 1H, CH), 5.01-5.95 (dd, J = 16.4 Hz, 6.0 Hz, 1H, CH_2), 4.92-4.87 (dd, J = 16.4 Hz, 4.4 Hz, 1H, CH_2), 3.94-3.81 (m, 1H, CH_2), 3.80 (d, J = 8.0 Hz, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.57-3.51 (m, 1H, CH_2), 3.47-3.41 (m, 1H, CH_2), 3.26-2.21 (m, 1H, CH_2), 3.15-2.08 (m, 1H, CH_2), 3.03-2.89 (m, 2H, CH_2), 2.78 (t, J = 8.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.6, 167.9, 161.1, 157.4, 156.5, 140.1, 137.9, 137.6, 130.1 (2C), 129.8 (2C), 129.7 (2C), 129.3 (2C), 129.0, 128.9, 128.5, 128.3, 127.9, 126.3, 125.3, 122.7, 120.2, 118.8, 118.5, 115.5 (2C), 112.5, 109.8, 55.7, 48.1, 47.1, 43.5, 41.4, 37.3, 33.3, 30.0, 27.3. HRMS (ESI): *m/z* calculated for $C_{40}H_{40}N_7O_3$ $[M+H]^+$: 666.3187. Found: 666.3196 .

(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-1-(4-cyano-2-methyloxazol-5-yl)piperidine-4-carboxamide ***(18)***. By method B: 47 mg (0.06 mmol, 36%), red amorphous powder (HPLC, purity > 95%). LC-MS (ES): t_R = 1.63 min, *m/z* 726.4 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4, rotamers): δ 7.36 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.30 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.23-7.16 (m, 3H, CH $_{aryl}$), 7.10 (t, J = 7.2 Hz, 1H, CH $_{aryl}$), 7.00-6.92 (m, 4H, CH $_{aryl}$), 6.65-6.60 (m, 4H, CH $_{aryl}$), 5.47-5.43 (dd, J = 9.2 Hz, 6.4 Hz, 1H, CH), 5.01 (d, J = 16.4 Hz, 1H, CH_2), 4.92 (d, J = 16.4 Hz, 1H, CH_2), 3.99 and 3.96 (2s, 2H, CH_2), 3.79 (s, 2H, CH_2), 3.71 (s, 3H, OCH_3), 3.55-3.49 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.46-3.41 (dd, J = 14.0 Hz, 6.4 Hz, 1H, CH_2), 3.19-2.12 (m, 2H, CH_2), 3.02-2.91 (m, 2H, CH_2), 2.79 (t, J = 8.0 Hz, 2H, CH_2), 2.55-2.48 (m, 1H, CH), 2.29 and 2.04 (2s, 3H, CH_3), 1.80-1.56 (m, 4H, 2 CH_2). ^{13}C-NMR (100 MHz, methanol-d_4, rotamers): δ 177.3, 171.3, 162.1, 161.1, 157.5, 156.5, 153.2, 140.1, 137.9, 129.8 (2C), 129.3 (2C), 129.0 (2C), 128.3, 127.9, 126.3, 125.2, 122.7, 120.2, 118.8, 116.9, 115.5 (2C), 112.5, 109.8, 85.8, 55.7, 48.1, 47.4 (2C), 47.1, 43.2, 42.6, 33.2, 30.0, 28.7, 28.6, 27.3, 13.1. HRMS (ESI): *m/z* calculated for $C_{41}H_{44}N_9O_4$ $[M+H]^+$: 726.3511. Found: 726.3512.

*(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino]-2-oxoethyl)benzamide (**19**)*. By method B: 86 mg (0.14 mmol, 78%), white amorphous powder (HPLC, purity > 99%). LC-MS (ES): t_R = 1.71 min, *m/z* 613.3 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4): δ 7.85 (d, J = 8.0 Hz, 2H, CH $_{aryl}$), 7.55 (t, J = 7.0 Hz, 1H, CH $_{aryl}$), 7.46 (t, J = 8.0 Hz, 2H, CH $_{aryl}$), 7.35 (d, J = 8.0 Hz, 1H, CH aryl), 7.30 (d, J = 8.0 Hz, 1H, CH aryl), 7.22-7.15 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 7.0 Hz, 1H, CH $_{aryl}$), 7.00-6.91 (m, 4H, CH $_{aryl}$), 6.60-6.55 (m, 4H, CH $_{aryl}$), 5.47-5.44 (dd, J = 9.5 Hz, 6.0 Hz, 1H, CH), 4.98 (d, J = 16.5 Hz, 1H, CH_2), 4.86 (d, J = 16.5 Hz, 1H, CH_2), 4.04-3.96 (m, 2H, CH_2), 3.67 (s, 3H, OCH_3), 3.57-3.53 (dd, J = 14.0 Hz, 9.5 Hz, 1H, CH_2), 3.47-3.43 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.95-2.85 (m, 2H, CH_2), 2.76 (t, J = 8.0 Hz, 2H, CH_2). ^{13}C-NMR (125 MHz, methanol-d_4): δ 171.5, 170.5, 161.0, 157.3, 156.4, 140.4, 137.9, 134.9, 133.0, 129.7 (2C), 129.6 (2C), 129.3 (2C), 128.9 (2C), 128.5 (2C), 128.3, 127.8, 126.7, 125.2, 122.7, 120.2, 118.9, 115.4 (2C), 112.5, 110.0, 55.6, 47.8, 47.1, 43.7, 33.5, 30.1, 27.5. HRMS (ESI): *m/z* calculated for $C_{37}H_{37}N_6O_3$ $[M+H]^+$: 613.2922. Found: 613.2917.

*(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-6-cyanonicotinamide (**20**)*. By method B: 59 mg (0.09 mmol, 51%), red amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.70 min, *m/z* 639.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 9.09 (s, 1H, CH $_{aryl}$), 8.35 (dd, J = 8.4 Hz, 2.4 Hz, 1H, CH $_{aryl}$), 7.96 (dd, J = 8.4 Hz, 1.2 Hz, 1H, CH $_{aryl}$), 7.35 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.29 (d, J = 8.0 Hz, 1H, CH aryl), 7.24-7.16 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 7.4 Hz, 1H, CH $_{aryl}$), 7.01 (s, 1H, CH $_{aryl}$), 6.97-6.92 (m, 3H, CH $_{aryl}$), 6.62 (s, 4H, CH $_{aryl}$), 5.50-5.46 (dd, J = 9.2 Hz, 6.4 Hz, 1H, CH), 5.02 (d, J = 16.4 Hz, 1H, CH_2), 4.93 (d, J = 16.4 Hz, 1H, CH_2), 4.03 (s, 2H, CH_2), 3.69 (s, 3H, OCH_3), 3.58-3.52 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.49-3.44 (dd, J = 14.0 Hz, 6.4 Hz, 1H, CH_2), 3.02-

2.90 (m, 2H, CH_2), 2.78 (t, J = 8.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 171.1, 166.8, 161.1, 157.5, 156.5, 151.0, 140.0, 137.9, 137.8, 136.7, 133.7, 129.8 (2C), 129.6, 129.3 (2C), 129.0 (2C), 128.3, 127.9, 126.2, 125.3, 122.7, 120.2, 118.8, 117.8, 115.5 (2C), 112.5, 109.7, 55.7, 48.2, 47.2, 43.7, 33.2, 30.0, 27.3. HRMS (ESI): *m/z* calculated for $C_{37}H_{35}N_8O_3$ $[M+H]^+$: 639.2827. Found: 639.2831.

(R)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-(2-(4-hydroxyphenyl)acetamido)acetamide ***(21)***. By methods B and F: 62 mg (0.10 mmol, 54%), white amorphous powder (HPLC, purity > 99%). LC-MS (ES): t_R = 1.64min, *m/z* 643.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.35 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.29 (d, J = 8.0 Hz, 1H, CH aryl), 7.23-7.15 (m, 3H, CH $_{aryl}$), 7.11-7.07 (m, 3H, CH $_{aryl}$), 6.98-6.92 (m, 4H, CH $_{aryl}$), 6.73 (d, J = 8.8 Hz, 2H, CH aryl), 6.62-6.55 (m, 4H, CH $_{aryl}$), 5.42 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.94 (d, J = 16.8 Hz, 1H, CH_2), 4.82 (d, J = 16.8 Hz, 1H, CH_2), 3.76 (s, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.54-3.48 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.45 (s, 2H, CH_2), 3.42-3.37 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.97-2.86 (m, 2H, CH_2), 2.77 (t, J = 8.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 175.2, 171.3, 161.1, 157.6, 157.3, 156.5, 140.2, 137.9, 131.3 (2C), 129.7 (2C), 129.3 (2C), 128.9 (2C), 128.3, 127.8, 127.2, 126.4, 125.2, 122.7, 120.2, 118.8, 116.4 (2C), 115.4 (2C), 112.5, 109.9, 55.7, 48.0, 47.1, 43.4, 42.7, 33.3, 30.3, 27.4. HRMS (ESI): *m/z* calculated for $C_{38}H_{39}N_6O_4$ $[M+H]^+$: 643.3027. Found: 643.3037.

(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-3-(4-hydroxyphenyl)propanamide ***(22)***. By methods B and F: 66 mg (0.10 mmol, 56%), white amorphous powder (HPLC, purity > 99%). LC-MS (ES): t_R = 1.66 min, *m/z* 657.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.36 (d, J = 8.4 Hz, 1H, CH $_{aryl}$), 7.30 (d, J = 8.0 Hz, 1H, CH aryl), 7.23-7.15 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 7.6 Hz, 1H, CH $_{aryl}$), 7.02-6.92

(m, 6H, CH $_{aryl}$), 6.69 (d, J = 8.4 Hz, 2H, CH aryl), 6.64-6.58 (m, 4H, CH $_{aryl}$), 5.43 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.97 (d, J = 16.4 Hz, 1H, CH_2), 4.87 (d, J = 16.4 Hz, 1H, CH_2), 3.76 (s, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.55-3.50 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.45-3.39 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 3.01-2.87 (m, 2H, CH_2), 2.83-2.76 (m, 4H, CH_2), 2.48 (t, J = 8.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 176.0, 171.4, 161.1, 157.4, 156.8, 156.5, 140.2, 137.9, 132.9, 130.2 (2C), 129.7 (2C), 129.3 (2C), 128.9 (2C), 128.3, 127.8, 126.4, 125.3, 122.7, 120.2, 118.8, 116.2 (2C), 115.5 (2C), 112.5, 109.8, 55.7, 48.0, 47.1, 43.2, 38.9, 33.3, 31.8, 30.0, 27.4. HRMS (ESI): *m/z* calculated for $C_{39}H_{41}N_6O_4$ $[M+H]^+$: 657.3184. Found: 657.3199.

*(S)-N-[2-((R)-2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-amino-3-(4-methoxyphenyl)propanamide (**23**)*. By methods B and F: 52 mg (0.08 mmol, 42%), orange amorphous powder (HPLC, purity > 97%). LC-MS (ES): t_R = 1.48 min, *m/z* 686.3 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.36 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.30 (d, J = 8.0 Hz, 1H, CH aryl), 7.20-7.15 (m, 5H, CH $_{aryl}$), 7.09 (t, J = 7.6 Hz, 1H, CH $_{aryl}$), 7.00-6.91 (m, 4H, CH $_{aryl}$), 6.88 (d, J = 8.4 Hz, 2H, CH $_{aryl}$), 6.62-6.56 (m, 4H, CH $_{aryl}$), 5.41 (dd, J = 9.6 Hz, 6.0 Hz, 1H, CH), 4.80 (d, J = 16.8 Hz, 1H, CH_2), 4.73 (d, J = 16.8 Hz, 1H, CH_2), 4.06 (t, J = 7.6 Hz, 1H, CH_2), 3.95 (d, J = 16.4 Hz, 1H, CH_2), 3.75 (s, 3H, OCH_3), 3.75-3.70 (m, 1H, CH_2), 3.69 (s, 3H, OCH_3), 3.52 (dd, J = 14.0 Hz, 9.6 Hz, 1H, CH_2), 3.42 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 3.16 (dd, J = 14.0 Hz, 6.4 Hz, 1H, CH_2), 2.99 (dd, J = 14.0 Hz, 8.0 Hz, 1H, CH_2), 2.91-2.84 (m, 2H, CH_2), 2.77 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.5, 170.3, 160.9, 160.8, 157.0, 156.4, 140.6, 137.9, 131.6 (2C), 129.7 (2C), 129.3 (2C), 128.8 (2C), 128.4, 127.7, 127.3, 126.9, 125.2, 122.7, 120.1, 118.8, 115.5 (2C), 115.4 (2C), 112.5, 110.1, 55.9, 55.7 (2C), 47.4, 47.1, 42.9, 37.6, 33.7, 30.5, 27.5. HRMS (ESI): *m/z* calculated for $C_{40}H_{44}N_7O_4$ $[M+H]^+$: 686.3449. Found: 686.3477.

*(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]cinnamamide (**24**)*. By methods B: 72 mg (0.11 mmol, 63%), white amorphous powder (HPLC, purity = 96%). LC-MS (ES): t_R = 1.82 min, *m/z* 639.3 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.58-7.54 (m, 3H, CH $_{aryl}$), 7.40-7.30 (m, 6H, CH $_{aryl}$, CH $_{vinyl}$), 7.22-7.16 (m, 3H, CH $_{aryl}$), 7.08 (t, J = 7.2 Hz, 1H, CH $_{aryl}$), 7.00-6.92 (m, 4H, CH $_{aryl}$), 6.64-6.57 (m, 4H, CH $_{aryl}$, CH $_{vinyl}$), 5.45 (dd, J = 9.2 Hz, 6.4 Hz, 1H, CH), 4.95 (d, J = 16.4 Hz, 1H, CH_2), 4.85 (d, J = 16.4 Hz, 1H, CH_2), 3.93 (s, 2H, CH_2), 3.69 (s, 3H, OCH_3), 3.57-3.52 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.47-3.42 (dd, J = 14.0 Hz, 6.4 Hz, 1H, CH_2), 2.94-2.88 (m, 2H, CH_2), 2.78 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 171.3, 169.0, 161.0, 157.3, 156.5, 152.1, 146.3, 142.4, 140.4, 137.9, 136.2, 131.0, 130.0 (2C), 129.7 (2C), 129.3 (2C), 128.9 (2C), 128.4, 127.8, 126.7, 125.2, 122.7, 121.3, 120.2, 118.9, 115.4 (2C), 112.5, 110.0, 55.7, 47.8, 47.1, 43.5, 33.6, 30.2, 27.5. HRMS (ESI): *m/z* calculated for $C_{39}H_{39}N_6O_3$ $[M+H]^+$: 639.3078. Found: 639.3086.

*(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-3-(4-fluorophenyl)acrylamide (**25**)*. By methods B: 68 mg (0.10 mmol, 58%), white amorphous powder (HPLC, purity > 96%). LC-MS (ES): t_R = 1.85 min, *m/z* 657.3 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.62-7.51 (m, 3H, CH $_{aryl}$), 7.35 (d, J = 8.4 Hz, 1H, CH $_{aryl}$), 7.33 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.22-7.07 (m, 6H, CH $_{aryl}$, CH $_{vinyl}$), 7.00-6.91 (m, 4H, CH $_{aryl}$), 6.63-6.57 (m, 5H, CH $_{aryl}$, CH $_{vinyl}$), 5.46-5.43 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.95 (d, J = 16.8 Hz, 1H, CH_2), 4.84 (d, J = 16.8 Hz, 1H, CH_2), 3.92 (s, 2H, CH_2), 3.69 (s, 3H, OCH_3), 3.57-3.51 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.47-3.42 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.96-2.84 (m, 2H, CH_2), 2.78 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 171.3, 168.9, 166.3, 163.8, 161.0, 157.3, 156.5, 141.0, 140.5, 137.9, 132.6, 131.0, 130.9, 129.7 (2C),

129.3 (2C), 128.9 (2C), 127.8, 126.7, 125.2, 122.7, 121.2, 120.2, 118.9, 117.0, 116.7, 115.4 (2C), 112.5, 110.0, 55.7, 47.8, 47.1, 43.5, 33.6, 30.2, 27.5. ^{19}F-NMR (376 MHz, methanol-d_4): δ -112.89. HRMS (ESI): *m/z* calculated for $C_{39}H_{38}FN_6O_3$ $[M+H]^+$: 657.2984. Found: 657.2992.

*(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-3-phenylpropiolamide (**26**)*. By methods B: 50 mg (0.08 mmol, 43%), white amorphous powder (HPLC, purity > 95%). LC-MS (ES): t_R = 1.84 min, *m/z* 637.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.56 (d, J = 6.8 Hz, 2H, CH $_{aryl}$), 7.49-7.31 (m, 5H, CH $_{aryl}$), 7.23-7.17 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 7.6 Hz, 1H, CH $_{aryl}$), 7.00-6.92 (m, 4H, CH $_{aryl}$), 6.63-6.55 (m, 4H, CH $_{aryl}$), 5.46-5.42 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.95 (d, J = 16.4 Hz, 1H, CH_2), 4.83 (d, J = 16.4 Hz, 1H, CH_2), 3.89 (s, 2H, CH_2), 3.70 (s, 3H, OCH_3), 3.58-3.52 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.47-3.42 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.98-2.84 (m, 2H, CH_2), 2.78 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.5, 161.0, 157.2, 156.5, 156.1, 140.4, 137.9, 133.5 (2C), 131.5, 129.8 (2C), 129.7 (2C), 129.3 (2C), 128.8 (2C), 128.4, 127.8, 126.7, 125.2, 122.7, 121.3, 120.2, 118.9, 115.4 (2C), 112.5, 110.0, 86.7, 83.4, 55.7, 47.8, 47.1, 43.4, 33.6, 30.2, 27.5. HRMS (ESI): *m/z* calculated for $C_{39}H_{37}N_6O_3$ $[M+H]^+$: 637.2922. Found: 637.2924.

*(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-phenylacrylamide (**27**)*. By methods B: 93 mg (0.14 mmol, 78%), white amorphous powder (HPLC, purity > 97%). LC-MS (ES): t_R = 1.90 min, *m/z* 664.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 8.21 (s, 1H, CH $_{vinyl}$), 7.96 (d, J = 6.8 Hz, 2H, CH $_{aryl}$), 7.59-7.51 (m, 3H, CH $_{aryl}$), 7.35 (d, J = 8.4 Hz, 1H, CH aryl), 7.31 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.23-7.17 (m, 3H, CH $_{aryl}$), 7.08 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.00-6.92 (m, 4H, CH $_{aryl}$), 6.63-6.56 (m, 4H, CH $_{aryl}$), 5.45 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.97 (d, J = 16.4 Hz, 1H, CH_2), 4.86 (d, J = 16.4 Hz, 1H, CH_2), 3.96 (s, 2H, CH_2), 3.69 (s, 3H, OCH_3), 3.58-3.52 (dd, J = 14.0 Hz, 9.2

Hz, 1H, CH_2), 3.48-3.43 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.95-2.89 (m, 2H, CH_2), 2.78 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.8, 163.7, 161.0, 157.3, 156.5, 153.2, 140.5, 137.9, 133.8, 133.3, 131.6 (2C), 130.3 (2C), 129.7 (2C), 129.3 (2C), 128.8 (2C), 128.4, 127.7, 126.7, 125.2, 122.7, 120.2, 118.9, 117.0, 115.4 (2C), 112.5, 110.0, 106.2, 55.7, 47.8, 47.2, 44.0, 33.6, 30.2, 27.5. HRMS (ESI): *m/z* calculated for $C_{40}H_{38}N_7O_3$ $[M+H]^+$: 664.3031. Found: 664.3035.

*(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-p-tolylacrylamide (**28**)*. By methods B: 45 mg (0.07 mmol, 36%), orange amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.85 min, *m/z* 678.4 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 8.16 (s, 1H, CH_{vinyl}), 7.86 (d, J = 8.0 Hz, 2H, CH aryl), 7.36-7.30 (m, 4H, CH_{aryl}), 7.23-7.15 (m, 3H, CH_{aryl}), 7.08 (t, J = 8.0 Hz, 1H, CH_{aryl}), 7.00-6.92 (m, 4H, CH_{aryl}), 6.63-6.56 (m, 4H, CH_{aryl}), 5.47-5.43 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.98 (d, J = 16.4 Hz, 1H, CH_2), 4.86 (d, J = 16.4 Hz, 1H, CH_2), 3.95 (s, 2H, CH_2), 3.68 (s, 3H, OCH_3), 3.58-3.52 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.48-3.43 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.95-2.89 (m, 2H, CH_2), 2.78 (t, J = 7.6 Hz, 2H, CH_2), 2.42 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.9, 164.0, 161.0, 157.3, 156.5, 153.2, 145.4, 140.3, 137.9, 131.8 (2C), 131.0 (2C), 130.6, 129.7 (2C), 129.3 (2C), 128.9 (2C), 128.3, 127.8, 126.6, 125.2, 122.7, 120.2, 118.8, 117.3, 115.4 (2C), 112.5, 109.9, 104.7, 55.7, 47.9, 47.2, 44.0, 33.5, 30.1, 27.4, 21.7. HRMS (ESI): *m/z* calculated for $C_{41}H_{40}N_7O_3$ $[M+H]^+$: 678.3187. Found: 678.3182.

*(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(4-hydroxyphenyl)acrylamide (**29**)*. By methods B: 39 mg (0.06 mmol, 32%), white amorphous powder (HPLC, purity = 97%). LC-MS (ES): t_R = 1.74 min, *m/z* 679.4 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4, isomers E/Z): δ 8.09 and 7.43 (2s, 1H, CH

$_{vinyl}$), 7.89 and 7.54 (2d, J = 8.8 Hz, 2H, CH $_{aryl}$), 7.35 (d, J = 8.0 Hz, 1H, CH aryl), 7.30 (d, J = 7.6 Hz, 1H, CH $_{aryl}$), 7.23-7.15 (m, 3H, CH $_{aryl}$), 7.09 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.01-6.94 (m, 4H, CH $_{aryl}$), 6.90 and 6.74 (2d, J = 8.8 Hz, 2H, CH $_{aryl}$), 6.63-6.57 (m, 4H, CH $_{aryl}$), 5.47-5.43 (m, 1H, CH), 5.05-4.99 (m, 1H, CH_2), 4.94-4.89 (m, 1H, CH_2), 3.94 and 3.90 (2s, 2H, CH_2), 3.69 (s, 3H, OCH_3), 3.58-3.52 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.48-3.43 (dd, J = 14.0 Hz, 6.4 Hz, 1H, CH_2), 3.01-2.89 (m, 2H, CH_2), 2.78 (t, J = 8.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4, isomers E/Z): δ 171.1, 164.6, 163.8, 161.2, 157.5, 156.5, 153.2, 150.9, 140.2, 137.9, 134.5 (2C), 134.2, 129.8 (2C), 129.3 (2C), 128.9, 128.3, 127.8, 125.3, 124.7, 120.3, 118.8, 117.9, 117.2 (2C), 116.8, 115.5 (2C), 112.5, 109.9, 101.0, 55.7, 48.2, 47.2, 44.0, 33.4, 30.0, 27.3. HRMS (ESI): *m/z* calculated for $C_{40}H_{38}N_7O_4$ $[M+H]^+$: 680.2980. Found: 680.2996.

*(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(4-methoxyphenyl)acrylamide (**30**)*. By methods B: 70 mg (0.10 mmol, 56%), orange amorphous powder (HPLC, purity > 95%). LC-MS (ES): t_R = 3.66 min, *m/z* 694.2 $[M+H]^+$. ^{1}H-NMR (300 MHz, methanol-d_4): δ 8.13 (s, 1H, CH $_{vinyl}$), 7.97 (d, J = 8.4 Hz, 2H, CH $_{aryl}$), 7.36 (d, J = 8.4 Hz, 1H, CH aryl), 7.31 (d, J = 7.8 Hz, 1H, CH $_{aryl}$), 7.25-7.17 (m, 3H, CH $_{aryl}$), 7.11-7.05 (m, 3H, CH $_{aryl}$), 7.01 (s, 1H, CH $_{aryl}$), 6.98-6.92 (m, 3H, CH $_{aryl}$), 6.63-6.57 (m, 4H, CH $_{aryl}$), 5.45 (t, J = 7.5 Hz, 1H, CH), 5.02 (d, J = 16.8 Hz, 1H, CH_2), 4.90 (m, 1H, CH_2), 3.96 (s, 2H, CH_2), 3.88 (s, 3H, OCH_3), 3.67 (s, 3H, OCH_3), 3.60-3.42 (m, 2H, CH_2), 2.98-2.90 (m, 2H, CH_2), 2.77 (t, J = 7.5 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 171.0, 165.0, 164.3, 161.1, 157.4, 156.5, 152.9, 140.2, 137.9, 134.1 (2C), 129.8 (2C), 129.3 (2C), 128.9 (2C), 128.3, 127.8, 126.4, 125.9, 125.3, 122.7, 120.2, 118.8, 117.7, 115.8 (2C), 115.5 (2C), 112.5, 109.8, 102.2, 56.2, 55.7, 48.1, 47.2, 44.0, 33.3, 30.3, 27.4. HRMS (ESI): *m/z* calculated for $C_{41}H_{40}N_7O_4$ $[M+H]^+$: 694.3136. Found: 694.3144.

*(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(4-(dimethylamino)phenyl)acrylamide (**31**)*. By methods B: 80 mg (0.11 mmol, 63%), orange amorphous powder (HPLC, purity > 98%). LC-MS (ES): t_R = 1.91 min, *m/z* 707.3 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 8.00 (s, 1H, CH $_{vinyl}$), 7.87 (d, J = 9.2 Hz, 2H, CH $_{aryl}$), 7.35 (d, J = 8.0 Hz, 1H, CH aryl), 7.31 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.23-7.14 (m, 3H, CH $_{aryl}$), 7.08 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 6.99-6.91 (m, 4H, CH $_{aryl}$), 6.76 (d, J = 9.2 Hz, 2H, CH $_{aryl}$), 6.62-6.55 (m, 4H, CH $_{aryl}$), 5.44 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.98 (d, J = 16.4 Hz, 1H, CH_2), 4.87 (d, J = 16.4 Hz, 1H, CH_2), 3.93 (s, 2H, CH_2), 3.68 (s, 3H, OCH_3), 3.57-3.51 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.47-3.42 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 3.08 (s, 6H, $N(CH_3)_2$), 2.95-2.84 (m, 2H, CH_2), 2.77 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 171.2, 165.4, 161.0, 157.3, 156.5, 155.1, 153.3, 140.4, 137.9, 134.6 (2C), 129.7 (2C), 129.3 (2C), 128.9 (2C), 128.4, 127.8, 126.7, 125.2, 122.7, 120.5, 120.2, 119.0, 118.9, 115.4 (2C), 112.6 (2C), 112.5, 110.0, 96.2, 55.7, 47.9, 47.2, 44.0, 40.0 (2C), 33.5, 30.1, 27.5. HRMS (ESI): *m/z* calculated for $C_{42}H_{43}N_8O_3$ $[M+H]^+$: 707.3453. Found: 707.3458.

*(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(4-fluorophenyl)acrylamide (**32**)*. By methods B: 69 mg (0.10 mmol, 61%), beige amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.80 min, *m/z* 682.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 8.18 (s, 1H, CH $_{vinyl}$), 8.03 (d, J = 9.2 Hz, 1H, CH $_{aryl}$), 8.02 (d, J = 8.8 Hz, 1H, CH $_{aryl}$), 7.35 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.31 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.27 (t, J = 8.8 Hz, 2H, CH $_{aryl}$), 7.21-7.17 (m, 3H, CH $_{aryl}$), 7.08 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.00-6.91 (m, 4H, CH $_{aryl}$), 6.63-6.56 (m, 4H, CH $_{aryl}$), 5.44 (dd, J = 9.2 H, 6.4 Hz, 1H, CH), 4.97 (d, J = 16.4 Hz, 1H, CH_2), 4.85 (d, J = 16.4 Hz, 1H, CH_2), 3.96 (s, 2H, CH_2), 3.68 (s, 3H, OCH_3), 3.58-3.52 (dd, J = 14.4 Hz, 9.2 Hz, 1H, CH_2), 3.47-3.42 (dd, J = 14.4 Hz, 6.4 Hz,

1H, CH_2), 2.94-2.89 (m, 2H, CH_2), 2.78 (t, J = 7.6 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.8, 167.7, 165.2, 163.6, 161.0, 157.3, 156.5, 151.8, 140.4, 137.9, 134.3, 134.2, 129.7 (2C), 129.3 (2C), 128.9 (2C), 128.4, 127.8, 126.7, 125.2, 122.7, 120.2, 118.9, 117.5, 117.3, 117.0, 115.4 (2C), 112.5, 110.0, 105.9, 55.7, 47.8, 47.2, 44.0, 33.5, 30.2, 27.5. HRMS (ESI): *m/z* calculated for $C_{40}H_{37}FN_7O_3$ $[M+H]^+$: 682.2936. Found: 682.2957.

(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(thiophen-2-yl)acrylamide ***(33)***. By methods B: 69 mg (0.10 mmol, 57%), beige amorphous powder (HPLC, purity = 96%). LC-MS (ES): t_R = 1.84 min, *m/z* 670.3 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 8.35 (s, 1H, CH_{vinyl}), 7.93 (d, J = 5.2 Hz, 1H, CH_{aryl}), 7.80 (d, J = 3.6 Hz, 1H, CH_{aryl}), 7.35 (d, J = 8.0 Hz, 1H, CH aryl), 7.30 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.26-7.15 (m, 4H, CH_{aryl}), 7.08 (t, J = 8.0 Hz, 1H, CH_{aryl}), 6.99-6.91 (m, 4H, CH_{aryl}), 6.62-6.55 (m, 4H, CH_{aryl}), 5.44 (dd, J = 9.6 Hz, 6.0 Hz, 1H, CH), 4.96 (d, J = 16.4 Hz, 1H, CH_2), 4.85 (d, J = 16.4 Hz, 1H, CH_2), 3.94 (s, 2H, CH_2), 3.68 (s, 3H, OCH_3), 3.57-3.51 (dd, J = 14.0 Hz, 9.6 Hz, 1H, CH_2), 3.47-3.42 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.94-2.88 (m, 2H, CH_2), 2.77 (t, J = 8.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 170.9, 163.8, 161.0, 157.3, 156.4, 145.6, 140.4, 138.6, 137.9, 137.5, 135.8, 129.7 (2C), 129.5, 129.3 (2C), 128.9 (2C), 128.4, 127.8, 126.6, 125.2, 122.7, 120.2, 118.8, 117.2, 115.4 (2C), 112.5, 109.9, 55.7, 49.8, 47.9, 47.2, 44.0, 33.5, 30.1, 27.4. HRMS (ESI): *m/z* calculated for $C_{38}H_{36}N_7O_3S$ $[M+H]^+$: 670.2595. Found: 670.2598.

(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(1H-pyrrol-2-yl)acrylamide ***(34)***. By methods B: 74 mg (0.11 mmol, 63%), orange amorphous powder (HPLC, purity > 99%). LC-MS (ES): t_R = 1.60 and 1.70 min, *m/z* 653.5 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4, rotamers): δ 8.04 (s, 1H, CH_{vinyl}),

7.39-7.33 (m, 2H, CH $_{aryl}$), 7.30 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.23-7.16 (m, 4H, CH $_{aryl}$), 7.08 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.00 (d, J = 3.6 Hz, 1H, CH aryl), 6.97-6.83 (m, 3H, CH $_{aryl}$), 6.64-6.55 (m, 4H, CH $_{aryl}$), 6.43 and 6.34 (2m, 1H, CH $_{pyrrole}$), 5.43 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 5.01-4.95 (2d, J = 16.4 Hz, 1H, CH_2), 4.89-4.84 (m, 1H, CH_2), 3.93 (s, 2H, CH_2), 3.68 and 3.67 (2s, 3H, OCH_3), 3.57-3.52 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.47-3.42 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.95-2.87 (m, 2H, CH_2), 2.77 (t, J = 8.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4, rotamers): δ 171.1, 165.0, 161.0, 157.4, 156.5, 142.4, 141.9, 140.3, 137.9, 129.7 (2C), 129.3 (2C), 128.9 (2C), 128.3, 128.0, 127.8, 126.5, 125.9, 125.2, 122.7, 120.2, 118.9, 118.8, 115.5 (2C), 113.7, 113.0, 112.5, 109.9, 55.7, 48.0, 47.2, 43.9, 33.4, 30.1, 27.4. HRMS (ESI): *m/z* calculated for $C_{38}H_{37}N_7O_3S$ $[M+H]^+$: 653.2983. Found: 653.3003.

*(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(1H-indol-3-yl)acrylamide (**35**)*. By methods B: 71 mg (0.10 mmol, 58%), orange amorphous powder (HPLC, purity > 95%). LC-MS (ES): t_R = 1.84 min, *m/z* 703.3 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 8.55 (s, 1H, CH $_{vinyl}$), 8.48 (s, 1H, CH $_{vinyl(tryp)}$), 7.80 (d, J = 7.6 Hz, 1H, CH $_{aryl}$), 7.48 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.36-7.14 (m, 7H, CH $_{aryl}$), 7.08 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.00 (s, 1H, CH $_{aryl}$), 6.97-6.91 (m, 3H, CH $_{aryl}$), 6.62-6.56 (m, 4H, CH $_{aryl}$), 5.47-5.44 (dd, J = 9.2 Hz, 6.0 Hz, 1H, CH), 4.98 (d, J = 16.4 Hz, 1H, CH_2), 4.87 (d, J = 16.4 Hz, 1H, CH_2), 3.97 (s, 2H, CH_2), 3.66 (s, 3H, OCH_3), 3.58-3.52 (dd, J = 14.0 Hz, 9.2 Hz, 1H, CH_2), 3.49-3.43 (dd, J = 14.0 Hz, 6.0 Hz, 1H, CH_2), 2.93-2.87 (m, 2H, CH_2), 2.77 (t, J = 7.2 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 171.3, 165.5, 161.0, 157.4, 156.5, 145.3, 140.3, 137.9, 137.7, 131.8, 129.7 (2C), 129.3 (2C), 128.9 (3C), 128.8, 128.4, 127.8, 126.6, 125.2, 124.7, 123.1, 122.7, 120.2, 119.5, 119.1, 118.9, 115.5 (2C), 113.5, 112.5, 111.7, 109.9, 96.4, 55.7,

47.9, 47.2, 44.0, 33.5, 30.1, 27.4. HRMS (ESI): *m/z* calculated for $C_{42}H_{39}N_8O_3$ $[M+H]^+$: 703.3140. Found: 703.3134.

*(R,E)-N-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethyl]-2-cyano-3-(4-methoxyphenyl)acrylamide (**36**)*. By method C: 42 mg (0.07 mmol, 33%), white amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.82 min, *m/z* 637.3 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4): δ 7.88 (d, J = 9.0 Hz, 2H, CH_{aryl}), 7.81 (s, 1H, CH_{vinyl}), 7.40 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.35 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.22-7.17 (m, 3H, CH_{aryl}), 7.11-6.95 (m, 7H, CH_{aryl}), 6.64 (br s, 4H, CH_{aryl}), 5.56 (t, J = 7.5 Hz, 1H, CH), 5.00 (d, J = 17.0 Hz, 1H, CH_2), 4.93 (d, J = 17.0 Hz, 1H, CH_2), 3.87 (s, 3H, OCH_3), 3.67-3.62 (m, 1H, CH_2), 3.58-3.54 (m, 4H, OCH_3, CH_2), 3.00-2.97 (m, 2H, CH_2), 2.86-2.84 (m, 2H, CH_2). ^{13}C-NMR (125 MHz, methanol-d_4): δ 165.1, 164.0, 160.9, 157.0, 156.8, 152.7, 140.3, 137.9, 134.2 (2C), 129.7 (2C), 129.4 (2C), 128.6 (2C), 128.4, 127.8, 126.5, 125.7, 125.2, 122.7, 120.2, 118.9, 117.3, 115.7 (2C), 115.5 (2C), 112.5, 110.0, 102.0, 56.2, 55.5, 47.8 (2C), 33.4, 29.5, 27.4. HRMS (ESI): *m/z* calculated for $C_{39}H_{37}N_6O_3$ $[M+H]^+$: 637.2922. Found: 637.2934.

*(R,E)-2-[2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino]-2-oxoethyl 2-cyano-3-(4-methoxyphenyl)acrylate (**37**)*. By method D: 54 mg (0.08 mmol, 39%), beige amorphous powder (HPLC, purity > 99%). LC-MS (ES): t_R = 1.73 min, *m/z* 695.2 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4): δ 8.28 (s, 1H, CH_{vinyl}), 8.06 (d, J = 12.0 Hz, 2H, CH_{aryl}), 7.35 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.33 (d, J = 8.0 Hz, 1H, CH_{aryl}), 7.21-7.18 (m, 3H, CH_{aryl}), 7.10-7.08 (m, 3H, CH_{aryl}), 7.02 (s, 1H, CH_{aryl}), 6.99-6.92 (m, 3H, CH_{aryl}), 6.63 (d, J = 8.5 Hz, 2H, CH_{aryl}), 6.57 (d, J = 8.5 Hz, 2H, CH_{aryl}), 5.47-5.44 (dd, J = 9.0 Hz, 6.5 Hz, 1H, CH), 4.95 (d, J = 16.5 Hz, 1H, CH_2), 4.84 (d, J = 16.5 Hz, 1H, CH_2), 4.69-4.62 (m, 2H, CH_2), 3.91 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 3.58-3.54 (dd, J = 14.0 Hz, 9.0 Hz, 1H, CH_2), 3.48-3.44 (dd, J =

14.0 Hz, 6.0 Hz, 1H, CH_2), 3.00-2.88 (m, 2H, CH_2), 2.81 (t, J = 8.0 Hz, 2H, CH_2). ^{13}C-NMR (125 MHz, methanol-d_4): δ 169.1, 165.8, 163.9, 161.0, 157.0, 156.6, 156.5, 140.5, 137.9, 135.0 (2C), 129.7 (2C), 129.4 (2C), 128.7 (2C), 128.4, 127.8, 126.8, 125.6, 125.2, 122.7, 120.2, 118.9, 117.0, 115.9 (2C), 115.4 (2C), 112.5, 110.0, 99.3, 64.3, 56.3, 55.7, 47.7, 46.9, 33.7, 30.0, 27.5. HRMS (ESI): *m/z* calculated for $C_{41}H_{39}N_6O_5$ $[M+H]^+$: 695.2976. Found: 695.2981.

*(R)-N-[3-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-3-oxopropyl]-2-cyano-3-(4-methoxyphenyl)acrylamide (**38**)*. By method E: 82 mg (0.12 mmol, 65%), white amorphous powder (HPLC, purity = 96%). LC-MS (ES): t_R = 1.80 and 1.96 min, *m/z* 708.2 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4, isomers E/Z): δ 8.07 and 7.44 (2s, 1H, CH_{vinyl}), 7.96 and 7.50 (2d, J = 8.4 Hz, 2H, CH_{aryl}), 7.34 (d, J = 8.4 Hz, 2H, CH_{aryl}), 7.21-7.14 (m, 3H, CH_{aryl}), 7.09-7.02 (m, 3H, CH_{aryl}), 6.99 (s, 1H, CH_{aryl}), 6.96-6.91 (m, 3H, CH_{aryl}), 6.62-6.52 (m, 4H, CH_{aryl}), 5.48-5.44 (dd, J = 9.2 Hz, 6.4 Hz, 1H, CH), 4.96 (d, J = 16.8 Hz, 1H, CH_2), 4.85 (d, J = 16.8 Hz, 1H, CH_2), 3.88 and 3.77 (2s, 3H, OCH_3), 3.68 (s, 3H, OCH_3), 3.57-3.43 (m, 4H, 2 CH_2), 2.93-2.88 (m, 2H, CH_2), 2.78-2.73 (m, 2H, CH_2), 2.44-2.41 (td, J = 6.0 Hz, 2H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4, isomers E/Z): δ 173.4 and 172.8 (1C), 164.9, 163.7, 160.9, 152.5, 149.4, 140.6, 137.9, 134.0 (2C), 133.3, 129.7 (2C), 129.3 (2C), 128.8 (2C), 128.4, 127.7, 126.9, 125.9, 125.1, 122.7, 120.2, 118.9, 117.8, 115.8 (2C), 115.4 (2C), 112.5, 110.3, 102.6, 56.1 and 56.0 (1C), 55.7, 47.7, 46.9, 37.6 and 37.3 (1C), 36.0 and 35.6 (1C), 33.6, 30.2, 27.5. HRMS (ESI): *m/z* calculated for $C_{42}H_{42}N_7O_4$ $[M+H]^+$: 708.3293. Found: 708.3295.

*(E)-N-[(S)-1-((R)-2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-phenethyl-4H-1,2,4-triazol-3-yl)ethylamino)-1-oxopropan-2-yl]-2-cyano-3-(4-methoxyphenyl)acrylamide (**39**)*. By method E: 58 mg (0.08 mmol, 46%), beige amorphous powder (HPLC, purity > 95%). LC-MS (ES): t_R = 1.94 min, *m/z* 708.2 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4): δ 8.04 (s, 1H, CH_{vinyl}), 7.95 (d, J =

9.0 Hz, 2H, CH $_{aryl}$), 7.36 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.34 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.22-7.16 (m, 3H, CH $_{aryl}$), 7.10-7.03 (m, 4H, CH $_{aryl}$), 7.00-6.94 (m, 3H, CH $_{aryl}$), 6.65 (m, 4H, CH $_{aryl}$), 5.53 (t, J = 8.0 Hz, 1H, CH), 5.08-5.01 (m, 2H, CH_2), 4.30-4.26 (q, J = 7.0 Hz, 1H, CH), 3.88 (s, 3H, OCH_3), 3.65 (s, 3H, OCH_3), 3.56-3.52 (dd, J = 14.5 Hz, 8.0 Hz, 1H, CH_2), 3.49-3.45 (dd, J = 14.5 Hz, 8.0 Hz, 1H, CH_2), 2.98-2.95 (m, 2H, CH_2), 2.85-2.81 (m, 2H, CH_2), 1.19 (d, J = 7.0 Hz, 3H, CH_3). ^{13}C-NMR (125 MHz, methanol-d_4): δ 174.2, 165.0, 163.4, 160.9, 157.2, 156.7, 152.4, 140.4, 137.9, 134.1 (2C), 129.7 (2C), 129.3 (2C), 128.7 (2C), 128.4, 127.8, 126.8, 125.9, 125.1, 122.6, 120.1, 119.0, 117.7, 115.8 (2C), 115.4 (2C), 112.4, 110.1, 102.5, 56.2, 55.6, 51.0, 47.9, 46.4, 33.5, 29.7, 27.5, 18.2. HRMS (ESI): *m/z* calculated for $C_{42}H_{42}N_7O_4$ $[M+H]^+$: 708.3293. Found: 708.3299.

*(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-(2-morpholinoethyl)-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-phenylacrylamide (**40**)*. By method E: 62 mg (0.09 mmol, 51%), white amorphous powder (HPLC, purity > 95%). LC-MS (ES): t_R = 1.46 min, *m/z* 553.3 $[M+H - 4\text{-methoxybenzyl}]^+$, *m/z* 673.3 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4): δ 8.20 (s, 1H, CH $_{vinyl}$), 7.97 (d, J = 7.0 Hz, 2H, CH $_{aryl}$), 7.60-7.53 (m, 3H, CH $_{aryl}$), 7.33 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.30 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.07 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.03 (s, 1H, CH $_{aryl}$), 6.92 (t, J = 7.0 Hz, 1H, CH $_{aryl}$), 6.65-6.61 (m, 4H, CH $_{aryl}$), 5.46-5.43 (dd, J = 9.0 Hz, 6.0 Hz, 1H, CH), 5.03 (d, J = 16.5 Hz, 1H, CH_2), 4.89 (d, J = 16.5 Hz, 1H, CH_2), 3.94 (s, 2H, CH_2), 3.86 (br s, 4H, 2 CH_2), 3.69 (s, 3H, OCH_3), 3.59-3.54 (dd, J = 14.0 Hz, 9.0 Hz, 1H, CH_2), 3.51-3.41 (m, 3H, 2 CH_2), 3.26 (br s, 4H, 2 CH_2), 3.07-3.01 (m, 1H, CH_2), 2.92-2.86 (m, 1H, CH_2). ^{13}C-NMR (125 MHz, methanol-d_4): δ 170.7, 163.7, 160.8, 157.6, 153.1, 152.6, 137.9, 133.8, 133.3, 131.6 (2C), 130.3 (2C), 128.7 (2C), 128.4, 127.5, 125.2, 122.5, 120.0, 119.0, 117.0, 115.3 (2C), 112.4,

110.5, 106.3, 65.0 (2C), 55.6, 54.6, 53.3 (2C), 47.0 (2C), 43.9, 30.4, 20.8. HRMS (ESI): *m/z* calculated for $C_{38}H_{41}N_8O_4$ $[M+H]^+$: 673.3245. Found: 673.3246.

(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-(2-morpholinoethyl)-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(4-hydroxyphenyl)acrylamide ***(41)***. By method E: 88 mg (0.13 mmol, 71%), white amorphous powder (HPLC, purity = 99%). LC-MS (ES): t_R = 1.30 and 1.34 min, *m/z* 569.2 $[M+H–4\text{-methoxybenzyl}]^+$, *m/z* 689.3 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4, isomers E/Z): δ 8.09 and 7.43 (2s, 1H, CH $_{vinyl}$), 7.90 and 7.53 (2d, J = 8.5 Hz, 2H, CH $_{aryl}$), 7.33 (d, J = 8.5 Hz, 1H, CH $_{aryl}$), 7.29 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.08 (t, J = 7.0 Hz, 1H, CH $_{aryl}$), 7.03 (s, 1H, CH $_{aryl}$), 6.93-6.90 and 6.74 (m, 3H, CH $_{aryl}$), 6.65-6.60 (m, 4H, CH $_{aryl}$), 5.46-5.42 (dd, J = 9.0 Hz, 6.0 Hz, 1H, CH), 5.05-5.02 (m, 1H, CH_2), 4.91-4.87 (m, 1H, CH_2), 3.92 (s, 2H, CH_2), 3.87 (br s, 4H, 2 CH_2), 3.67 (s, 3H, OCH_3), 3.59-3.49 (m, 1H, CH_2), 3.46-3.42 (m, 3H, 2 CH_2), 3.26 (br s, 4H, 2 CH_2), 3.07-3.00 (m, 1H, CH_2), 2.92-2.86 (m, 1H, CH_2). ^{13}C-NMR (125 MHz, methanol-d_4, isomers E/Z): δ 170.8 and 170.1 (1C), 164.5 and 163.8 (1C), 160.8, 157.6, 153.1, 152.6, 137.9, 134.5 and 134.2 (2C), 128.8, 128.7 and 128.4 (2C), 127.4, 125.1, 124.7, 122.5, 120.0, 119.0, 117.9, 117.2 (2C), 116.7, 115.4 (2C), 112.4, 110.5, 101.0, 64.9 (2C), 55.6, 54.6, 53.3 (2C), 47.1, 47.0, 43.9 and 43.7 (1C), 30.4, 20.8. HRMS (ESI): *m/z* calculated for $C_{38}H_{41}N_8O_5$ $[M+H]^+$: 689.3194. Found: 689.3188.

(R,E)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-(2-morpholinoethyl)-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(4-(dimethylamino)phenyl)acrylamide ***(42)***. By method E: 41 mg (0.06 mmol, 32%), red wax (HPLC, purity = 95%). LC-MS (ES): t_R = 1.50 min, *m/z* 596.3 $[M+H–4\text{-methoxybenzyl}]^+$, *m/z* 716.4 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 7.99 (s, 1H, CH $_{vinyl}$), 7.88 (d, J = 8.8 Hz, 2H, CH $_{aryl}$), 7.32 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.29 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.06 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.02 (s, 1H, CH $_{aryl}$), 6.92 (t, J = 8.0 Hz,

1H, CH $_{aryl}$), 6.78 (d, J = 8.8 Hz, 2H, CH $_{aryl}$), 6.65-6.61 (m, 4H, CH $_{aryl}$), 5.46-5.42 (dd, J = 8.4 Hz, 6.4 Hz, 1H, CH), 5.03 (d, J = 16.8 Hz, 1H, CH_2), 4.89 (d, J = 16.8 Hz, 1H, CH_2), 3.91 (s, 2H, CH_2), 3.86 (br s, 4H, 2 CH_2), 3.68 (s, 3H, OCH_3), 3.58-3.30 (m, 4H, 2 CH_2), 3.25-3.21 (m, 4H, 2 CH_2), 3.09 (s, 6H, $N(CH_3)_2$), 3.06-3.00 (m, 1H, CH_2), 2.94-2.86 (m, 1H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 171.0, 165.4, 160.9, 157.6, 155.1, 153.2, 152.7, 137.9, 134.5 (2C), 128.7 (2C), 128.4, 127.4, 125.2, 122.5, 120.5, 120.0, 119.0, 115.4 (2C), 112.8, 112.6 (2C), 112.4, 110.5, 96.3, 64.9 (2C), 55.7, 54.6, 53.3 (2C), 47.1, 47.0, 44.0, 40.1 (2C), 30.4, 20.8. HRMS (ESI): *m/z* calculated for $C_{40}H_{46}N_9O_4$ $[M+H]^+$: 716.3667. Found: 716.3682.

*(R,E)-N-[2-(1-(5-(2-(1H-indol-3-yl)ethyl)-4-(4-methoxybenzyl)-4H-1,2,4-triazol-3-yl)-2-(1H-indol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(4-hydroxyphenyl)acrylamide (**43**)*. By method E: 39 mg (0.05 mmol, 32%), orange powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.72 min, *m/z* 719.3 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 8.09 (s, 1H, CH $_{vinyl}$), 7.88 (d, J = 8.8 Hz, 2H, CH $_{aryl}$), 7.33 (d, J = 8.0 Hz, 2H, CH $_{aryl}$), 7.27 (d, J = 7.2 Hz, 2H, CH $_{aryl}$), 7.10-7.06 (m, 2H, CH $_{aryl}$), 6.98-6.89 (m, 5H, CH $_{aryl}$), 6.81 (1s, 1H, CH $_{aryl}$), 6.53 (d, J = 8.8 Hz, 2H, CH $_{aryl}$), 6.44 (d, J = 8.4 Hz, 2H, CH $_{aryl}$), 5.40-5.37 (dd, J = 8.8 Hz, 6.8 Hz, 1H, CH), 4.90-4.87 (m, 1H, CH_2), 4.73-4.69 (d, J = 16.4 Hz, 1H, CH_2), 3.93 (s, 2H, CH_2), 3.65 (s, 3H, OCH_3), 3.52-3.48 (dd, J = 14.4 Hz, 8.8 Hz, 1H, CH_2), 3.44-3.39 (dd, J = 14.4 Hz, 6.8 Hz, 1H, CH_2), 3.03-2.99 (m, 4H, 2 CH_2). ^{13}C-NMR (125 MHz, methanol-d_4): δ 171.0, 164.6, 163.9, 161.0, 157.4, 157.1, 153.2, 138.1, 137.9, 134.6 (2C), 134.3, 128.7 (2C), 128.3, 128.0, 126.4, 125.2, 124.7, 123.6, 122.7 (2C), 120.2, 120.0, 118.8, 117.9, 117.2 (2C), 116.7, 115.3 (2C), 113.0, 112.5, 110.9, 100.9, 55.7, 47.9, 47.1, 43.9, 30.0, 26.8, 23.8. HRMS (ESI): *m/z* calculated for $C_{42}H_{39}N_8O_4$ $[M+H]^+$: 719.3089. Found: 719.3108.

*(R,E)-N-(2-(1-(5-(2-(1H-indol-3-yl)ethyl)-4-(4-methoxybenzyl)-4H-1,2,4-triazol-3-yl)-2-(1H-indol-3-yl)ethylamino)-2-oxoethyl)-2-cyano-3-(4-methoxyphenyl)acrylamide (**44**)*. By method E: 74 mg (0.10 mmol, 59%), yellow amorphous powder (HPLC, purity > 96%). LC-MS (ES): t_R = 1.86 min, *m/z* 733.3 $[M+H]^+$. ^{1}H-NMR (400 MHz, methanol-d_4): δ 8.11 (s, 1H, CH $_{vinyl}$), 7.94 (d, J = 8.8 Hz, 2H, CH $_{aryl}$), 7.33 (d, J = 8.0 Hz, 2H, CH $_{aryl}$), 7.28-7.26 (dd, J = 7.6 Hz, 2.0 Hz, 2H, CH $_{aryl}$), 7.10-7.02 (m, 4H, CH $_{aryl}$), 6.97-6.90 (m, 3H, CH $_{aryl}$), 6.81 (s, 1H, CH $_{aryl}$), 6.53 (d, J = 8.8 Hz, 2H, CH $_{aryl}$), 6.44 (d, J = 8.8 Hz, 2H, CH $_{aryl}$), 5.41-5.37 (dd, J = 8.8 Hz, 6.4 Hz, 1H, CH), 4.90-4.86 (d, J = 16.4 Hz, 1H, CH_2), 4.72-4.68 (d, J = 16.4 Hz, 1H, CH_2), 3.93 (s, 2H, CH_2), 3.86 (br s, 3H, OCH_3), 3.65 (s, 3H, OCH_3), 3.53-3.47 (dd, J = 14.0 Hz, 8.8 Hz, 1H, CH_2), 3.44-3.39 (dd, J = 14.0 Hz, 6.4 Hz, 1H, CH_2), 3.04-2.97 (m, 4H, 2 CH_2), 3.06-3.00 (m, 1H, CH_2), 2.94-2.86 (m, 1H, CH_2). ^{13}C-NMR (100 MHz, methanol-d_4): δ 171.0, 164.9, 164.3, 161.0, 157.4, 156.9, 152.8, 138.0, 137.8, 134.1 (2C), 128.8 (2C), 128.2, 127.9, 125.8, 125.7, 125.3, 123.6, 122.7, 120.2, 120.0, 118.8, 118.7, 117.8, 115.7 (2C), 115.4 (2C), 112.7, 112.5 (2C), 109.7, 102.0, 56.1 (2C), 55.6 (2C), 47.1, 43.9, 29.8, 26.6, 23.4. HRMS (ESI): *m/z* calculated for $C_{43}H_{41}N_8O_4$ $[M+H]^+$: 733.3245. Found: 733.3256.

*(R)-N-[2-(2-(1H-indol-3-yl)-1-(4-(4-methoxybenzyl)-5-(2-(piperidin-1-yl)ethyl)-4H-1,2,4-triazol-3-yl)ethylamino)-2-oxoethyl]-2-cyano-3-(4-methoxyphenyl)acrylamide (**45**)*. By method E: 91 mg (0.13 mmol, 72%), white amorphous powder (HPLC, purity > 95%). LC-MS (ES): t_R = 1.43 and 1.49 min, *m/z* 581.3 $[M+H–4\text{-methoxybenzyl}]^+$, *m/z* 701.4 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4, isomers E/Z): δ 8.12 and 7.47 (2s, 1H, CH $_{vinyl}$), 7.98 and 7.61 (2d, J = 9.0 Hz, 2H, CH $_{aryl}$), 7.36 and 7.29 (2d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.33 (d, J = 8.0 Hz, 1H, CH $_{aryl}$), 7.07 and 6.84 (d, J = 9.0 Hz, 3H, CH $_{aryl}$), 7.04-7.03 (2s, 1H, CH $_{aryl}$), 6.95-6.89 (m, 1H, CH $_{aryl}$), 6.64-6.60 (m, 4H, CH $_{aryl}$), 5.51-5.48 and 5.46-5.43 (dd, J = 9.0 Hz, 6.0 Hz, 1H, CH), 5.04-5.99 (2d, J = 16.5

Hz, 1H, CH_2), 4.90-4.80 (2d, J = 16.5 Hz, 1H, CH_2), 3.94-3.86 (m, 4H, OCH_3, CH_2), 3.78-3.68 (m, 4H, OCH_3, CH_2), 3.58-3.54 (dd, J = 14.0 Hz, 9.5 Hz, 1H, CH_2), 3.46-2.35 (m, 5H, 3 CH_2), 3.04-2.98 (m, 1H, CH_2), 2.89-2.83 (m, 3H, 2 CH_2), 1.99-1.38 (m, 6H, 3 CH_3). ^{13}C-NMR (125 MHz, methanol-d_4, isomers E/Z): δ 170.8 and 170.1 (1C), 165.2 and 165.0 (1C), 164.3 and 163.8 (1C), 160.8, 157.6 and 157.4 (1C), 152.8, 152.7 and 150.3 (1C), 137.9, 134.1 (2C), 133.9, 128.8, 128.7 (2C), 128.4, 127.5, 126.2 and 125.9 (1C), 122.5, 120.0, 119.0, 117.7, 115.8 (2C), 115.3 (2C), 112.4, 110.6 and 110.5 (1C), 102.3, 56.2 and 55.9 (1C), 55.6, 54.4 (2C), 54.3, 47.0, 43.9 and 43.7 (1C), 30.4, 24.2 (2C), 22.5, 21.1. HRMS (ESI): *m/z* calculated for $C_{40}H_{45}N_8O_4$ $[M+H]^+$: 701.3558. Found: 701.3585.

*(R)-2-cyano-N-[2-(1-(5-(2-(diethylamino)ethyl)-4-(4-methoxybenzyl)-4H-1,2,4-triazol-3-yl)-2-(1H-indol-3-yl)ethylamino)-2-oxoethyl]-3-(4-methoxyphenyl)acrylamide (**46**)*. By method E: 71 mg (0.10 mmol, 56%), white amorphous powder (HPLC, purity = 95%). LC-MS (ES): t_R = 1.43 and 1.47 min, *m/z* 569.3 $[M+H–4\text{-methoxybenzyl}]^+$, *m/z* 689.4 $[M+H]^+$. ^{1}H-NMR (500 MHz, methanol-d_4, isomers E/Z): δ 8.13 and 7.48 (2s, 1H, CH $_{vinyl}$), 7.99 and 7.62 (2d, J = 9.0 Hz, 2H, CH $_{aryl}$), 7.38 and 7.29 (m, 2H, CH $_{aryl}$), 7.09-7.04 and 6.85 (m, 4H, CH $_{aryl}$), 6.92 (t, J = 8.0 Hz, 1H, CH $_{aryl}$), 6.65-6.61 (m, 4H, CH $_{aryl}$), 5.52-5.44 (m, 1H, CH), 5.02 (d, J = 17.0 Hz, 1H, CH_2), 4.90 (d, J = 17.0 Hz, 1H, CH_2), 3.93-3.86 (m, 4H, OCH_3, CH_2), 3.79-3.69 (m, 4H, OCH_3, CH_2), 3.59-3.54 (dd, J = 14.0 Hz, 9.5 Hz, 1H, CH_2), 3.47-2.37 (m, 3H, 2 CH_2), 3.19-3.15 (q, J = 7.5 Hz, 4H, 2 CH_2), 3.02-2.96 (m, 1H, CH_2), 2.87-2.81 (m, 1H, CH_2), 1.24 (t, J = 7.5 Hz, 6H, 2 CH_3). ^{13}C-NMR (125 MHz, methanol-d_4, isomers E/Z): δ 170.8 and 170.1 (1C), 165.3 and 165.1 (1C), 164.3 and 163.9 (1C), 160.9, 157.7 and 157.5 (1C), 152.9 and 152.7 (1C), 150.3, 137.9, 134.2 and 133.9 (2C), 128.9, 128.8 and 128.4 (2C), 127.4, 125.9, 125.2, 122.6, 120.0, 119.0, 117.7, 115.8 (2C), 115.4 (2C), 115.3, 112.4, 110.4, 102.3, 56.2 and 55.9 (1C), 55.7, 49.4, 48.7, 47.1, 47.0, 43.9 and

43.7 (1C), 30.4, 21.1, 9.0 (2C). HRMS (ESI): *m/z* calculated for $C_{39}H_{45}N_8O_4$ $[M+H]^+$: 689.3558. Found: 689.3574.

Materials for Biology. Ghrelin (16/28) was purchased from PolyPeptide Laboratories. [D-Arg^1-D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P (SPA) was from Bachem, and K-(D-1-Nal)-FwLL-NH_2 was synthesized at the Institut des Biomolecules Max Mousseron. The IP-One HTRF kit, benzyl guanine-Tb^{3+}-cryptate, and the insulin ultrasensitive kit were provided by Cisbio. HEK293T stable cell line expressing a SNAP-Tag-GHSR (SNAP-GHSR) was a generous gift from Eric Trinquet (Cisbio Bioassays, Codolet, France). BODIPY-FL GTPγS was from Invitrogen. Chemical reagents used for buffer preparation, namely, collagenase V, bovine serum albumin (BSA), and phosphate-buffered saline (PBS) with $MgCl_2$ and $CaCl_2$, were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture. HEK293T cells (stably expressing SNAP-human GHSR) were maintained in DMEM Glutamax (Invitrogen) supplemented with 1 mg/mL Geneticin (Gibco), 50 μg/mL penicillin, 50 μg/mL streptomycin, 2 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1% nonessential amino acids, and 10% heat-inactivated fetal calf serum.

Ligand Binding Assay. *K*i values were determined from binding competition experiments performed on the intact HEK293T cell line expressing the SNAP-GHSR using homogenous time-resolved fluorescence (HTRF) assay in a 96-well plate format at a density of 50 000 cells/well, as previously described.[20] The HTRF signal was collected using a PHERAstar microplate reader (BMG LABTECH). *K*i values were obtained from binding curves using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego).

Inositol Phosphate Assay. The inositol phosphate accumulation assay was carried out on the adherent HEK293T cell line expressing the SNAP-GHSR in a 96-well plate format at a density of

50 000 cells/well. IP1 production was measured using the IP-One HTRF kit (Cisbio Bioassays ref 621PAPEC) as previously described.[20] Briefly, cells were stimulated for 30 min at 37 °C with the ligand to be tested in 70 μL of the IP1 stimulation buffer. An anti-IP1 antibody labeled with Lumi4-Tb (15 μL) and an IP1-d2 derivative (15 μL) were added to the cells. The medium was incubated for 1 h at room temperature. Signals at 665 and 620 nm were detected using a PHERAstar (BMG LABTECH) fluorescence reader. EC_{50} values from dose-response curves were obtained using GraphPad Prism 5.0 software.

Receptor Preparation and Labeling. For GTPγS binding assays, the monomeric wild-type human GHSR in lipid nanodiscs was prepared as previously described.[28] For FRET measurements, the ghrelin receptor with a TAG amber codon at the position encoding F71 and a single reactive cysteine at position 255 was produced in lipid nanodiscs and subsequently labeled with Click-IT Alexa Fluor 488 DIBO Alkyne (LifeTechnologies) and Alexa Fluor 350 maleimide, as previously described.[23]

G Protein Preparation. His_6-tagged Gα_q was expressed in Sf9 cells and purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography.[29] Gβ_1 was expressed with His_6-tagged Gγ in Sf9 cells and the G$\beta\gamma$ dimer purified using Ni-NTA chromatography combined with ion exchange chromatography.[29]

GTPγS Binding. GTPγS binding experiments were carried out using the fluorescent BODIPY-FL GTPγS analog.[30] Association of BODIPY-FL GTPγS to the G protein was monitored using a fluorescence spectrophotometer (Cary Eclipse, Varian) with the excitation wavelength set at 500 nm and the emission wavelength at 511 nm. Reaction conditions were 100 nM Gα_q, 500 nM G$\beta_1\gamma_2$, 100 nM BODIPY-FL GTPγS, and 20 nM receptor in lipid nanodisks. The receptor, the ligands (10

μM final concentration), and the G protein were first incubated for 30 min at room temperature, and fluorescence was measured after addition of BODIPY-FL GTPγS and 10 min incubation at 15 °C.

FRET Measurements. For FRET measurements, the receptor, the ligand, and the $G\alpha_q\beta_1\gamma_2$ protein were incubated for 30 min at room temperature. GHSR and ligand concentrations were in the 0.5 and 10 μM range, respectively, with a 1:5 receptor-to-G protein molar ratio. Fluorescence emission spectra were recorded between 360 and 700 nm on a Cary Eclipse spectrofluorimeter (Varian) with an excitation at 346 nm. Buffer contributions were systematically subtracted. The proximity ratio was calculated from the emission spectra as previously described.[31]

Insulin Secretion Experiments in Isolated Rat Pancreatic Islets. All animal care and experiments complied with the directive 2010/63/EU and were approved by the local animal care and use committee Languedoc-Roussillon under reference CEEA-LR-19002. Male Wistar rats (Charles River, France) were housed in a temperature and humidity-controlled room under a 12 h light/dark cycle and had food and water ad libitum. Rats weighed 280-320 g at the time of the experiments. Rats were anesthetized with isoflurane and killed by decapitation. The common bile duct was cannulated, and the pancreas was filled by injection of the collagenase V solution (10 mL, 1 mg/mL), excised, and digested at 37 °C for 10 min. After three successive sedimentations using PBS and 5 mM glucose, islets of Langerhans were hand-picked under a microscope. Then, islets were stabilized for 1 h (5% CO_2, 37 °C) in buffer A (120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 24 mM $NaHCO_3$, and 0.1% (w/v) BSA, pH 7.4) containing 2.8 mM glucose. Batches of five islets were incubated for 1 h at 37 °C, in 1 mL of buffer A with 0.1% (w/v) BSA containing 2.8 or 8.3 mM glucose in the absence or presence of the tested ligands. At the end of the incubation period, supernatants were collected and stored at -20

°C until the insulin assay. Experiments were performed in quintuplicate. Insulin was quantified using the Cisbio insulin ultrasensitive kit according to the manufacturer's instructions.

Food Intake Experiments in Mice. Experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Research Council (2011, Guide for the care and use of laboratory animals) and all efforts were made to minimize suffering. All protocols received approval from the Institutional Animal Care and Use Committee of the IMBICE (ID 10-0112). Studies were performed using 3- to 5-month-old C57BL6/J wild type male mice generated in the animal facility of the IMBICE. Mice were housed under controlled room temperature (22 ± 1°C) and photoperiod (12 h light/dark cycle from 6:00 h to 18:00 h) with regular chow and water available ad libitum, except when indicated. Thirty-three mice were implanted with intracerebroventricular (ICV) cannulas and then used to test the effect of K-(D-1-Nal)-FwLL-NH_2 or ligand **29** on food intake under three experimental conditions: spontaneous ad libitum food intake, ghrelin-induced food intake and fast-refeeding. Briefly, mice were first stereotaxically implanted with an ICV single indwelling guide cannula in the lateral brain ventricle (placement coordinates: antero-posterior: -0.34 mm, medio-lateral: +1.0 mm and dorso-ventral: -2.3 mm) as previously described.[25] After surgery, mice were individually housed and allowed to recover for at least 5 days. In these days, mice were made accustomed to handling by removal of the dummy cannula and connection to an empty cannula connector. For the experiments, mice were ICV-injected with 2 μl of vehicle alone (artificial cerebrospinal fluid) or containing 1 nmol/mouse of K-(D-1-Nal)-FwLL-NH_2 or ligand **29**. The dose of K-(D-1-Nal)-FwLL-NH_2 was chosen based on previous studies showing that it reduces both ad libitum food intake in the early dark phase period and ghrelin-induced food intake in wild-type mice while it does not affect food intake in GHSR-deficient mice.[13, 25] All ICV injections were made over 2 min

through a 30-gauge needle that extend 0.5 mm below the guide cannula, which was connected by polyethylene tubing to a Hamilton syringe. The needle was left in place for 2 min following the injection, to prevent back flow of the injected solution. To test the effect of the GHSR ligands on spontaneous food intake, mice were ICV-injected with vehicle (n=11), K-(D-1-Nal)-FwLL-NH_2 (n=11) or ligand **29** (n=11) right before lights turn off (at 18:00 h), and food intake was assessed 5 and 12 h later. After 3 days of recovery, mice were randomly used to test the effect of the GHSR ligands on ghrelin-induced food intake. Specifically, mice were ICV-injected with vehicle (n=11), K-(D-1-Nal)-FwLL-NH_2 (n=11) or ligand **29** (n=11) around 9:00 h, and then ICV-injected with 0.02 nmol/mouse of ghrelin 15 min later. Food intake was assessed 2 h after ghrelin treatment. After 3 days of recovery, mice were randomly used to test the effect of the GHSR ligands on a fast-refeeding condition. In particular, food was removed from the home cages at 10:00 h, and mice ICV-treated with vehicle (n=13), K-(d-1-Nal)-FwLL-NH_2 (n=13) or ligand **29** (n=7) every 8 h, starting around 16:00 h of the first day of fasting and finishing around 08:00 h of the second day of fasting. Thus, each mouse received 6 ICV injections during the 48 h fasting period. Mice were refed at 10:00 h, and food intake was monitored every 1 h, until 14:00 h of the first day of refeeding. Then, body weight and food intake were daily assessed at 10:00 h during 7 days after refeeding. In all the experiments, food intake was assessed by subtracting the remaining weight of the pellets, at any given time, to the initial weight. At the end of the experiment, mice were euthanized and perfused. Brains were extracted, frozen, coronally cut and visualized in a microscope to verify the correct location of ICV cannulas.

Statistical Analysis. Data were expressed as mean ± standard error of the mean (SEM). Depending on the design of the experiment, data were analyzed with one-way or two-way ANOVA followed by the Tukey's post hoc test to assign significance to the comparisons between groups.

Equality of variance was analyzed using Bartlett's test. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software). Differences were considered statistically significant when $p < 0.05$.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Copies of ^{1}H and ^{13}C NMR spectra of all final ligands **1**-**46**, LC-MS chromatograms and determination of water solubility of key target ligands **27**, **29**, **30** and **44** (PDF)

SMILES and Biological data for all final ligands **1**-**46** (CSV)

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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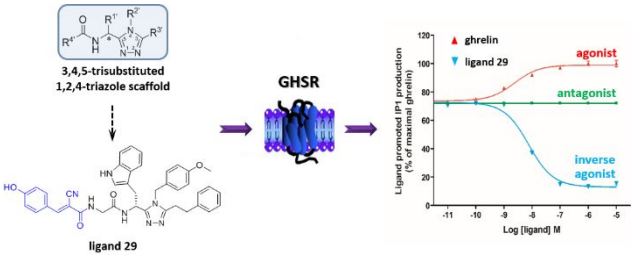
ABBREVIATIONS

AcOH, acetic acid; BOC, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DME, 1,2-dimethoxyethane, DMF, *N*,*N*-dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Et_2NH, diethyl amine; EtOAc, ethyl acetate; Fmoc, fluorenylmethyloxycarbonyl; FA, formic acid; FRET, fluorescence resonance energy transfer; GHSR, growth hormone secretagogue receptor; HATU, (1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide; HCl, hydrochloride

acid; HTRF, homogenous time-resolved fluorescence; ICV, intracerebroventricular; IP, inositol phosphate; MeOH, methanol; SPA, substance P analog; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid.

TOC



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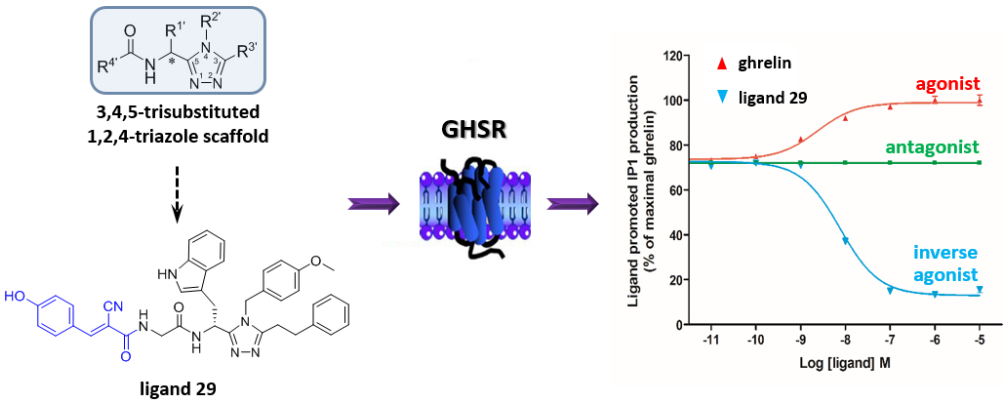

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