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N-Terminal Liver-Expressed Antimicrobial Peptide 2 (LEAP2) Region Exhibits Inverse Agonist Activity toward the Ghrelin Receptor

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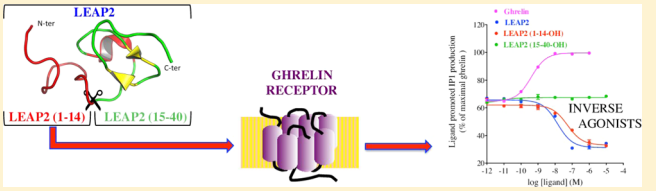
Supporting Information

ABSTRACT: The ghrelin receptor or growth hormone secretagogue receptor (GHSR) is a G-protein-coupled receptor that controls growth hormone and insulin secretion, food intake, and reward-seeking behaviors. Liver-expressed antimicrobial peptide 2 (LEAP2) was recently described as an endogenous antagonist of GHSR. Here, we present a study aimed at delineating the structural determinants required for LEAP2 activity toward GHSR. We demonstrate that the entire sequence of LEAP2 is not necessary for its actions. Indeed, the N-terminal part alone confers receptor binding and activity to LEAP2. We found that both LEAP2 and its N-terminal part behave as inverse agonists of GHSR and as competitive antagonists of ghrelin-induced inositol phosphate production and calcium mobilization. Accordingly, the N-terminal region of LEAP2 is able to inhibit ghrelin-induced food intake in mice. These data demonstrate an unexpected pharmacological activity for LEAP2 that is likely to have an important role in the control of ghrelin response under normal and pathological conditions.



INTRODUCTION

Ghrelin[1] is a stomach-derived 28-amino acid peptide that plays a major role in the regulation of body energy homeostasis. It controls, among others, growth hormone secretion, blood glucose homeostasis, gastrointestinal tract motility, and reward-related behaviors.[2] Notably, ghrelin is the only known orexigenic peptide hormone and its administration to humans or rodents potently increases food intake.[2] The biological effects of ghrelin are due to its interaction with a prototypical class A G-protein-coupled receptor (GPCR), named the ghrelin receptor or growth hormone secretagogue receptor (GHSR).[3] GHSR is a peculiar receptor as it displays a strikingly high constitutive activity.[4,5] Observations in animal models as well as in a family with a naturally occurring mutation of GHSR that selectively abolishes constitutive activity without altering ghrelin-evoked activity suggest that constitutive GHSR activity plays a role in vivo, independent of ghrelin action.[6−8]

Liver-expressed antimicrobial peptide 2 (LEAP2)[9] is a bicyclic, cationic peptide predominantly expressed in the liver and small intestine. This peptide is initially produced as a 77-residue precursor and subsequently processed, through different steps, into a mature 40-residue peptide. NMR-based structural analysis indicates that the mature peptide is composed of a disordered, hydrophobic N-terminal region and a compact central part with two disulfide bridges connected in a I−III, II−IV pattern. This peptide was originally reported to display antimicrobial activities with inhibitory concentrations in the hundreds of μM range.[10] However, such effective antimicrobial concentrations are much higher than the physiological levels of LEAP2.[10] It has also been suggested that LEAP2 modulates fibroblast growth factor signals.[11] Very recently, LEAP2 was reported to also have noncompetitive allosteric antagonistic activity toward GHSR.[12] This antagonist activity was directly related to the inhibition of ghrelin effects in vivo. Notably, at physiological levels, LEAP2 is able to bind to GHSR and impair the action of ghrelin. Thus, it is now recognized that endogenous LEAP2 plays a major role as an antagonist of ghrelin action.

To illuminate the mechanisms responsible for the biological actions of LEAP2 on ghrelin signaling, we carried out a detailed analysis of the pharmacological properties of this peptide and of its different structural domains, namely, the unstructured N-terminal part and the compact central core. For this, we evaluated different peptide fragments in a panel of model systems ranging from purified GHSR in lipid nanodiscs to GHSR-expressing HEK293T cells, ex vivo and in in vivo rodent models. By doing so, we found that LEAP2 is a competitive antagonist of ghrelin at GHSR and an inverse agonist toward GHSR constitutive activity. Moreover, we

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demonstrated that the activities of LEAP2 are localized to its N-terminal region.

RESULTS AND DISCUSSION

In all synthesized peptides (Figure 1), the N-terminal methionine residue was replaced by its norleucine isostere to avoid oxidation of the methionine residue. In a first set of experiments, due to the presence of a glycyl residue at position 14, we decided to synthesize three peptides: (1) full-length LEAP2, (2) an N-terminal LEAP2 fragment (1−14-OH), hereafter named LEAP2 (1−14-OH), and (3) a C-terminal LEAP2 structured fragment (15−40-OH) with the two disulfide bonds, hereafter named LEAP2 (15−40-OH). In a second set of experiments, we downsized the sequence of the N-terminal part of LEAP2. Peptides were assembled on a solid support using Fmoc chemistry and starting from 2-chlorotrityl resin or an Agilent Amphisphere 40 RAM resin for acid peptides or amide peptides, respectively. All peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) and characterized by liquid chromatography−mass spectrometry (LC−MS) and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS)/MS with a purity >95% (see the Supporting Information).

LEAP2: NleTPFWRGVSLRPIGASCRDDSECTIRLCRKRRCSLSVAQE-OH

LEAP2 (1-14-OH): NleTPFWRGVSLRPIG-OH

LEAP2 (15-40-OH): ASCRDDSECTIRLCRKRRCSLSVAQE-OH

LEAP2 (1-14-NH_2): NleTPFWRGVSLRPIG-NH_2

LEAP2 (1-12-NH_2): NleTPFWRGVSLRP-NH_2

LEAP2 (1-10-NH_2): NleTPFWRGVSL-NH_2

LEAP2 (1-8-NH_2): NleTPFWRGV-NH_2

LEAP2 (1-6-NH_2): NleTPFWR-NH_2

Figure 1. Sequences of the synthesized peptides.

Binding and Activity of LEAP2-Related Peptides. *Binding.* We first analyzed whether the different LEAP2 variants directly bind to the ghrelin receptor (GHSR) expressed in HEK293T cells. K_i values were thus determined from binding competition experiments using the homogenous time-resolved fluorescence (HTRF) assay previously described.[13] As a reference, we included the peptide K-(D-1-Nal)-FwLL-NH_2, which is described as an inverse agonist.[14] As shown in Figure 2 and Table 1, LEAP2 displaced labeled ghrelin from its binding site with a K_i value in the nanomolar range (1.26 ± 0.05 nM). Interestingly, LEAP2 (1−14-OH) also displayed high affinity for GHSR (K_i 3.66 ± 0.64 nM), whereas LEAP2 (15−40-OH) was unable to compete with ghrelin up to a 10^{-5} M concentration.

Intrinsic Activity. As LEAP2 and some of its structural domains appeared to compete with ghrelin for binding to GHSR, we subsequently investigated their possible impact on ghrelin-independent GHSR signaling. To this end, we first tested the ability of LEAP2 variants to affect the basal level of inositol phosphate 1 (IP1) production in HEK293T cells expressing GHSR (Figure 3 and Table 1).[5,15] As seen in Figure 3, the high basal level of IP1 production in GHSR-expressing cells, representing 60−70% of the maximal IP1 production promoted by ghrelin, resulted from the constitutive activity of

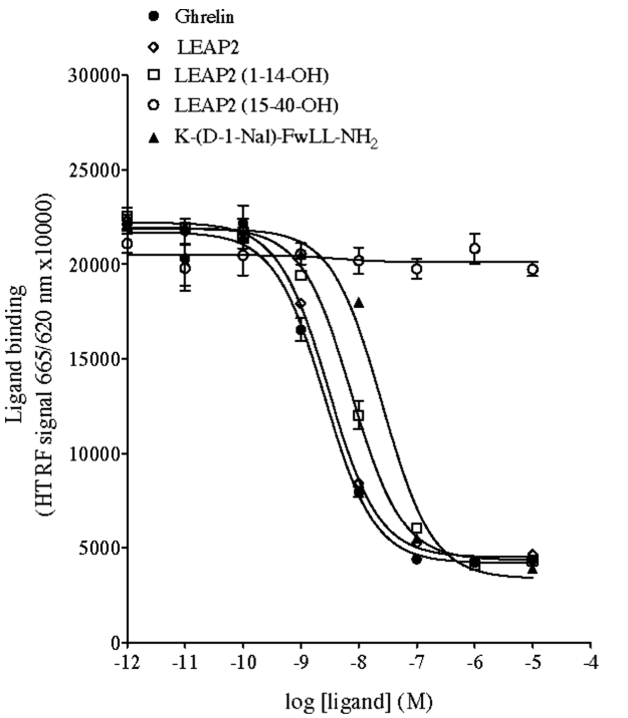

Figure 2. Competition binding curves obtained with LEAP2-related peptides, ghrelin, and the K-(D-1-Nal)-FwFwLL-NH_2 inverse agonist. Data are representative of two to three independent experiments, each performed in triplicate. Error bars correspond to standard deviation from the mean of triplicates. K_i values are collected in Table 1.

GHSR as previously shown.[4,15] LEAP2 decreased the basal level of IP1 production by 50% (E_{max}: 52.3 ± 6.6%) promoted by GHSR with an EC_{50} in the 20 nM range (22.8 ± 7.8 nM). This indicates that LEAP2 displays an inverse agonist activity toward basal IP1 production promoted by GHSR. Interestingly, the maximal inverse agonist effect of LEAP2 was very similar to that of the reference inverse agonist compound K-(D-1-Nal)-FwLL-NH_2[14] (E_{max}: 48.7 ± 1.2%). Similarly, LEAP2 (1−14-OH) reduced GHSR-promoted basal IP1 production with an EC_{50} of 76.4 ± 6.3 nM and a maximal effect of 50.8 ± 6.7%. In contrast, LEAP2 (15−40-OH) did not modify the basal level of IP1 production, indicating that this fragment had no activity of its own, in agreement with the absence of binding of this peptide to GHSR. These data indicate that LEAP2 behaves as an inverse agonist toward GHSR signaling at least on the GHSR-promoted IP1 pathway and that its linear N-terminal part is both necessary and sufficient for its binding and its activity.

To our knowledge, current data are the first indication that LEAP2 acts as an inverse agonist of GHSR. Strikingly, the study by Ge and colleagues[12] found that LEAP2 did not affect GHSR-mediated β-arrestin recruitment and concluded that LEAP2 had no inverse agonist activity. Unfortunately, the referred study did not report the use of known inverse agonists of GHSR as positive controls to confirm that the sensitivity of the β-arrestin recruitment assay was sufficient to unmask the inverse agonist activity of LEAP2.[12] Such controls are important because β-arrestin recruitment at GHSR is low under basal conditions.[5,15] In contrast, our conclusions are based on the data derived from the IP1 assay, which is recognized as a very sensitive assay for measuring the

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Table 1. Binding and Activity of LEAP2-Related Peptides[a]

ligand	binding K_i (nM)	inositol phosphate 1 EC_{50} (nM)	inositol phosphate 1 E_{max} (% basal inhibition)
LEAP2 (1−14-OH)	3.66 ± 0.64	76.4 ± 6.3	50.8 ± 6.7
LEAP2 (15−40-OH)	not detected	not detected	0
LEAP2	1.26 ± 0.05	22.8 ± 7.8	52.3 ± 6.6
ghrelin	1.28 ± 0.36	1.51 ± 0.85	
K-(D-1-Nal)-FwLL-NH_2	3.7 ± 0.05	5.5 ± 2.6	48.7 ± 1.2

[a]Values reported in the table were obtained from experiments performed in HEK293T cells stably expressing GHSR. K_i values were determined from HTRF competition binding assays. EC_{50} and E_{max} values were obtained from dose−response curves of inositol phosphate (IP1) production in HEK293T cells expressing GHSR. Values are mean ± S.E. of two and three independent experiments for binding and activity, respectively, each experiment being performed in triplicate.

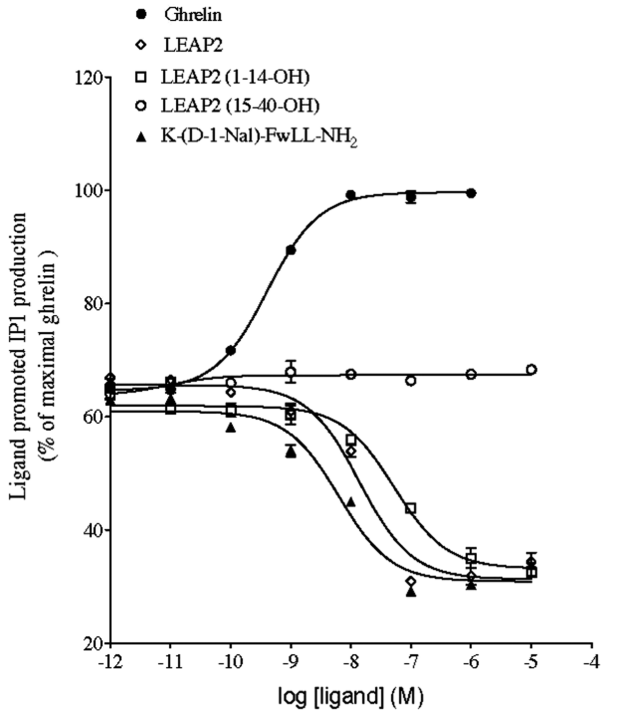

Figure 3. Dose−response curves of ligands at promoting IP1 production. Data are expressed as the percentage of maximal effect of ghrelin on HEK293T cells expressing GHSR. In this experiment, basal IP1 production of GHSR-expressing cells represents 64.5 ± 1.5% of the maximal effect triggered by ghrelin. K-(D-1-Nal)-FwLL-NH_2 described in the literature as an inverse agonist is given as reference. Data are representative of two experiments, each performed in triplicate.

constitutive activity of GPCRs and studying the inverse agonist activity of compounds.[10,16]

Antagonist Effect of LEAP2 Peptides on Ghrelin-Induced Activation of GHSR. *IP1 Production.* We first tested the antagonist effect of LEAP2-related peptides on ghrelin-induced IP1 production. To evaluate whether the interaction between LEAP2 and ghrelin is competitive or noncompetitive, we constructed dose−response curves of ghrelin in the absence or presence of increasing concentrations of LEAP2 or LEAP2 (1−14-OH). As shown in Figure 4, increasing the concentration of LEAP2 increased the EC_{50} value of ghrelin from 0.36 ± 0.01 to 16.6 ± 5.5 nM in the absence or the presence of 1 μM LEAP2, respectively (Figure 4A). Similarly, increasing the concentration of LEAP2 (1−14-OH) up to 1 μM increased the EC_{50} value of ghrelin from 0.40 ± 0.05 to 2.5 ± 0.3 nM (Figure 4B). pA2 values of 8.83 ± 0.22 and 7.46 ± 0.21 were obtained for LEAP2 and LEAP2 (1−14-

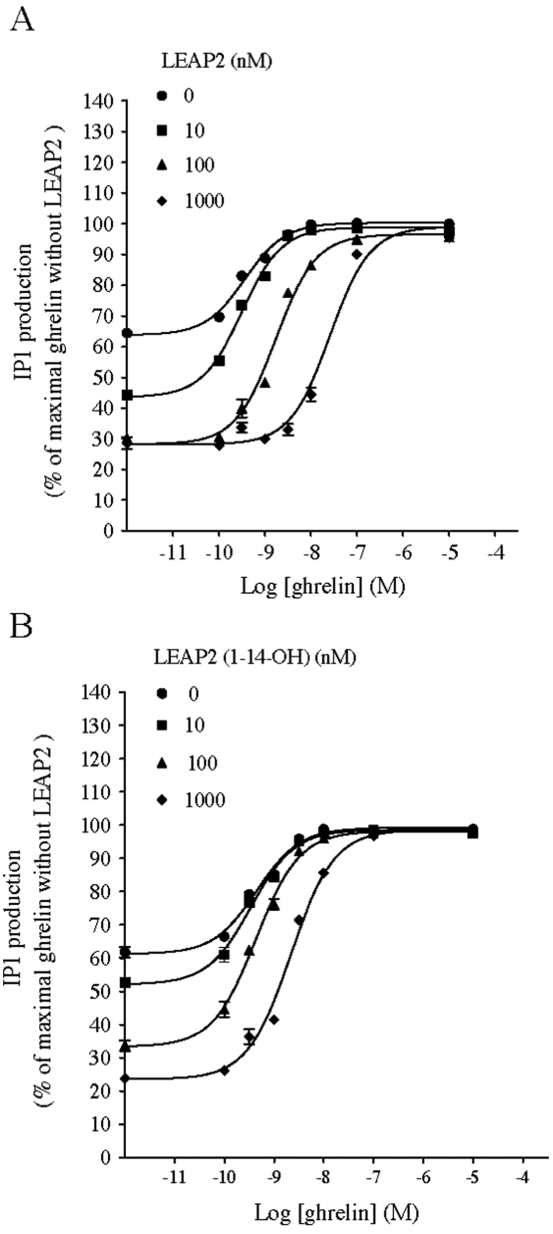

Figure 4. Antagonist effect of LEAP2 peptides on ghrelin-induced IP1 production in HEK293T cells expressing GHSR. Dose−response curves of ghrelin-induced IP1 production in the presence of increasing concentrations of LEAP2 (A) and LEAP2 (1−14-OH) (B). Data are expressed as the percentage of maximal ghrelin response in HEK293T cells stably expressing GHSR, where zero represents basal IP1 production of mock-transfected HEK293T cells. Data are representative of two experiments, each performed in triplicate.

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OH), respectively. In contrast, neither LEAP2 nor LEAP2 (1−14-OH) changed the maximal effect elicited by ghrelin (Figure 4). These data strongly suggest that LEAP2 and its N-terminal (1−14) domain are competitive antagonists for ghrelin with regard to IP1 production.

Calcium Release. We then tested whether the antagonist competitive character of LEAP2-related peptides existed also on calcium release following activation of GHSR by ghrelin. As for the IP1 assay, we constructed dose−response curves of ghrelin-induced calcium release in the presence of increasing concentrations of LEAP2-related peptides in the same GHSR-expressing cell system. As shown in Figure 5A, the EC_{50} value of ghrelin increased in parallel with the increase of LEAP2 concentration from 0.41 ± 0.13 to 27.7 ± 13 nM. Similarly, increasing concentrations of LEAP2 (1−14-OH) up to 1 μM increased the EC_{50} value of ghrelin from 0.34 ± 0.12 to 18 ± 4 nM (Figure 5B). pA2 values of 7.99 ± 0.15 and 7.05 ± 0.05 were calculated for LEAP2 and LEAP2 (1−14-OH), respectively. Moreover, as it was for IP1 production, LEAP2 and LEAP2 (1−14-OH) did not affect the maximal effect elicited by ghrelin (Figure 5). It has to be mentioned that, in contrast to IP1 production, no reduction of the basal signal was observed for calcium levels. This is not inconsistent with the fact that LEAP2-related peptides are inverse agonists. Indeed, it is well established that the inverse agonist activity of compounds cannot be detected with this calcium assay because of both the inability of this assay to produce an elevated basal signal and the nonequilibrium experimental conditions of the assay.[17]

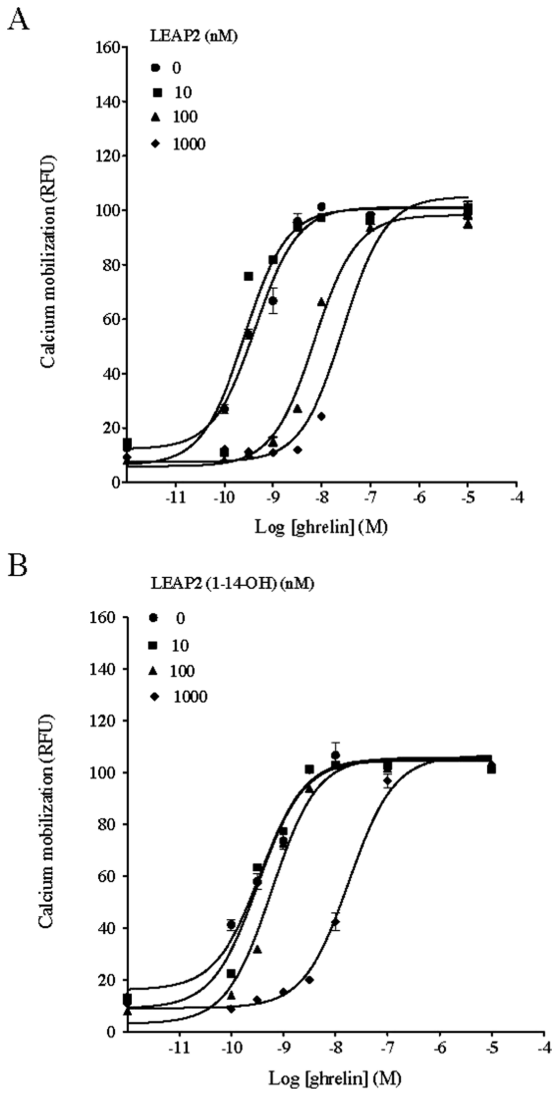

Figure 5. Antagonist effect of LEAP2-related peptides on ghrelin-induced calcium release in HEK293T cells expressing GHSR. Dose−response curves of ghrelin-induced GHSR activation at promoting intracellular calcium release in the presence of increasing concentrations of LEAP2 (A) and LEAP2 (1−14-OH) (B). Data are expressed as the percentage of maximal ghrelin response in HEK293T cells expressing GHSR, where zero represents the basal IP1 production of mock-transfected HEK293T cells. Data are representative of two experiments, each performed in triplicate.

Overall, these data indicate that both LEAP2 and LEAP2 (1−14-OH) are competitive antagonists of ghrelin at GHSR, as increasing concentrations of LEAP2 and its N-terminal domain resulted in an increase of EC_{50} values of ghrelin with no change in its maximal effect for both IP1 production and calcium mobilization. The reason for the difference between our data and those previously reported on the competitive behavior of LEAP[12] is not clear so far, and the exact nature of this behavior is still an open question at the present stage of the analysis.

Binding and Biological Activity of N-Terminal Truncated Fragments. Our data above indicate that LEAP2 (1−14-OH) contains the structural determinants for recognition and activation of GHSR. To assess the minimal active sequence within LEAP2, we prepared different N-terminal truncated LEAP2-related variants. These were synthesized as their terminal amide analogues to mask the carboxylic charge that is not present in LEAP2. We then evaluated their binding properties and their ability to modulate the GHSR activity on IP1 production (Table 2). LEAP2 (1−14-OH) and LEAP2 (1−14-NH_2) exhibited almost the same affinity, and their inverse agonist potency and efficacy were similar. All LEAP2 N-terminal fragments displayed K_i values in the same range. Only LEAP2 (1−6-NH_2) displayed a 20-fold increased K_i value compared to that of LEAP2 (1−14-NH_2). In terms of IP1 production, truncation of LEAP2 did not affect the ability of the peptides to act as inverse agonists of GHSR; however, their potency decreased with the reduction in their size. Indeed, a significant increase of EC_{50} was observed for LEAP2 (1−10-NH_2) and LEAP2 (1−8-NH_2), whereas their efficacy remained unchanged, as compared to those for both LEAP2 (1−14-OH) and LEAP2 (1−14-NH_2) as well as to those for the reference inverse agonists substance P analogue (SPA)[4] and K-(D-1-Nal)-FwLL-NH_2. Downsizing of only the N-terminal fragment of LEAP2 to six residues dramatically decreased both its potency and efficacy (EC_{50}: 6344 ± 2155 nM, E_{max}: 26 ± 7%), whereas its binding affinity was much less affected. These data suggest that the binding determinants of LEAP2 are contained within the (1−8) N-terminal part of LEAP2, whereas a longer N-terminal sequence encompassing at least the 1−12 region is required for maximal potency and efficacy.

LEAP2 Activity on the Purified Ghrelin Receptor. We then used the purified ghrelin receptor assembled into lipid nanodiscs to evaluate whether the impact of LEAP2 on GHSR signaling observed in HEK293T cells was associated with a direct effect of this peptide on the receptor. To this end, the ability of LEAP2 and its N-terminal fragments to affect GHSR activation was assessed by the ability of the purified receptor to

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Table 2. Binding Affinities and Inverse Agonist Activities of Truncated N-Terminal Peptides of LEAP2[a]

ligand	binding K_i (nM)	inositol phosphate 1 EC_{50} (nM)	inositol phosphate 1 E_{max} (% basal inhibition)
LEAP2	1.26 ± 0.05	22.8 ± 7.8	52.3 ± 6.6
LEAP2 (1−14-OH)	3.66 ± 0.64	76.4 ± 6.3	50.8 ± 6.7
LEAP2 (1−14-NH_2)	3.05 ± 0.15	48.0 ± 5.4	50.3 ± 1.5
LEAP2 (1−12-NH_2)	2.7 ± 0.1	46 ± 18	49.3 ± 1.5
LEAP2 (1−10-NH_2)	3.4 ± 0.01	134 ± 48	50.3 ± 1.5
LEAP2 (1−8-NH_2)	6.45 ± 0.25	368 ± 63	45.3 ± 1.5
LEAP2 (1−6-NH_2)	55 ± 4	6633 ± 2212	26 ± 7.2
K-(D-1-Nal)-FwLL-NH_2	3.7 ± 0.05	5.5 ± 2.6	48.7 ± 1.2
rPKPfQwFwLL-NH_2 (SPA)	76.7 ± 12.6	98.7 ± 20.0	51 ± 13
ghrelin	1.28 ± 0.36	1.51 ± 0.85	

[a]K_i values were determined by the HTRF competition binding assay on HEK293T cells expressing GHSR. For activity, EC_{50} and E_{max} values were obtained from dose−response curves of inositol phosphate (IP1) production in HEK293T cells expressing GHSR. Values are mean ± S.E. of two and three independent experiments for binding and activity, respectively, each performed in triplicate.

activate purified G proteins in a GTPγS binding assay. The purified apo GHSR in lipid nanodiscs displayed a significant constitutive activity at both G_q and G_{13} (Figure 6). Ghrelin

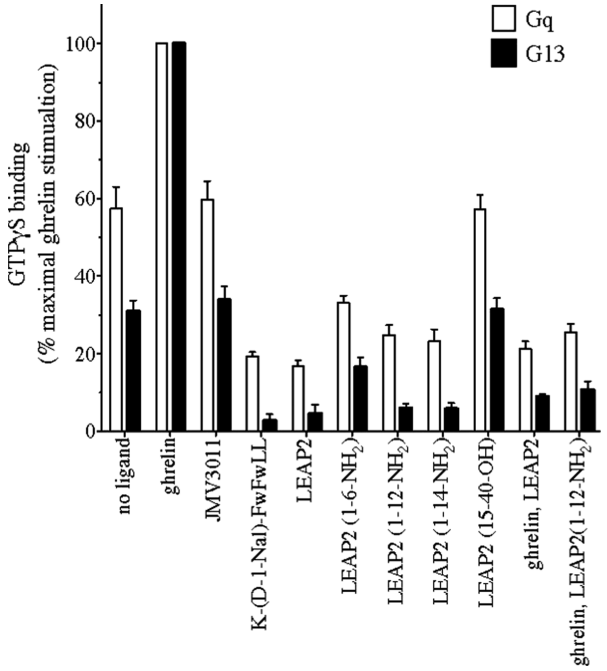

Figure 6. Effect of LEAP2 peptides on GHSR-catalyzed G protein activation. BODIPY-FL GTPγS binding to G_q and G_{13} proteins induced by GHSR in lipid disks in the absence of ligand; in the presence of ghrelin, JMV 3011, and K-(D-1-Nal)-FwLL-NH_2 (all ligands at 10^{-6} M); and in the presence of LEAP2, its N-terminal amide peptides, and C-terminal LEAP2 (15−40-OH) (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 ± S.E. from three independent experiments.

further enhanced these G-protein-activation properties, whereas the K-(D-1-Nal)-FwLL-NH_2 inverse agonist decreased GTPγS binding for G_q and G_{13}.[20] Finally, the neutral antagonist JMV 3011[15] did not change basal GTPγS binding for both G proteins. As shown in Figure 6, the full-length LEAP2 decreased the constitutive activity of GHSR for both G_q and G_{13} in the absence and in the presence of ghrelin. The degree of decrease in the basal activity with LEAP2 was similar to the one detected with K-(D-1-Nal)-FwLL-NH_2. This indicates that LEAP2 by itself exerts an inverse agonist activity on GHSR-dependent basal activation of G_q and G_{13}. Importantly, this inverse agonist activity was preserved with all of the N-terminal truncated fragments of LEAP2, although it was slightly diminished with LEAP2 (1−6-NH_2) (Figure 6). Thus, these data suggest that the inverse agonist activity of LEAP2 observed in HEK293T cells is directly related to its interaction with the ghrelin receptor independent of the cellular context.

Impact of LEAP2 on GHSR Conformation. We previously reported that the pharmacological properties of GHSR ligands are intricately related to their ability to stabilize specific receptor conformations.[18] To assess whether the inverse agonist activity of LEAP2 is indeed associated with its ability to stabilize an inactive conformation similar to that observed in the presence of K-(D-1-Nal)-FwLL-NH_2, we analyzed the conformational features of GHSR using a fluorescence resonance energy transfer (FRET)-based approach (Figure 7).[19] To this end, the purified monomeric receptor in nanodiscs was labeled with a fluorescence donor (AF350) and an acceptor (AF488) in the cytoplasmic ends of TM1 and TM6. As shown in Figure 7, addition of ghrelin or K-(D-1-Nal)-FwLL-NH_2 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 neutral antagonist JMV 3011 did not change this ratio and the inverse agonists triggered an increase in this ratio. As shown in Figure 7, all LEAP2-related peptides also triggered an increase in the proximity ratio very similar to that observed with K-(D-1-Nal)-FwLL-NH_2, suggesting that these compounds stabilize similar GHSR conformations. As in the case of the GTPγS binding assays, the proximity ratio was essentially unaffected by the deletion of the central domain of LEAP2, indicating that the interaction with the N-terminal region of LEAP2 is sufficient to stabilize the conformation of GHSR associated with its reduced basal activity. Altogether, these data indicate that the N-terminal domain of LEAP2 exerts an intrinsic inverse agonist activity on GHSR-catalyzed G protein activation through the stabilization of a specific, inactive receptor conformation.

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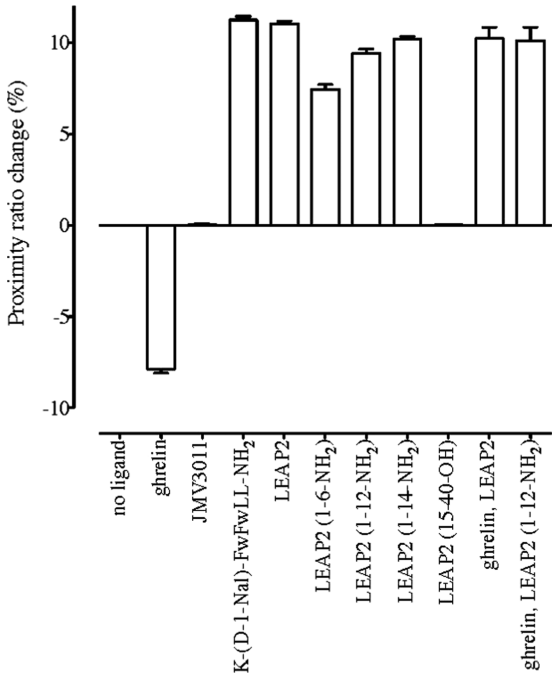

Figure 7. Effect of LEAP2 on GHSR conformation. Proximity ratio changes induced by ghrelin, JMV 3011, K-(D-1-Nal)-FwLL-NH_2, LEAP2, its N-terminal amide peptides, and C-terminal LEAP2 (15−40-OH). These changes were calculated from the FRET signal between the fluorophores in TM1 and TM6 of the purified ghrelin receptor in lipid disks (see the Materials and Methods section). The data are representative of two experiments performed in duplicate, and the error bar represents the S.E.

Effect of LEAP2 (1−12-NH_2) on "Ex Vivo" Endogenously Expressed GHSR. Next, we aimed at determining whether the N-terminal domain of LEAP2 was also active on endogenously expressed GHSR. To do so, we tested rat pancreatic islets that express GHSR.[20,21] Indeed, ghrelin is known to inhibit glucose-induced insulin secretion in pancreatic islets.[22] Therefore, insulin release was quantified after incubating isolated rat pancreatic islets with a stimulating (8.3 mM) glucose concentration in the presence or absence of ghrelin and LEAP2 (1−12-NH_2). LEAP2 (1−12-NH_2) was chosen because its inverse agonist potency and efficacy were equivalent to those of LEAP2 (1−14-NH_2) (Figure S1 in the Supporting Information). As expected, ghrelin attenuated glucose-stimulated insulin secretion (Figure S2 in the Supporting Information). Importantly, co-treatment of pancreatic islets with ghrelin and LEAP2 (1−12-NH_2) abolished the insulinostatic action of ghrelin. These data indicated that LEAP2 (1−12-NH_2) binds to endogenous GHSR and blocks its activation by ghrelin.

In Vivo Evaluation of LEAP2-Related Peptides. Finally, we tested the ability of LEAP2, LEAP2 (1−12-NH_2), and LEAP2 (15−40-OH) to affect the orexigenic effects of ghrelin in mice. We performed this experiment using similar experimental conditions as those used by Ge and colleagues[12] to compare the outcomes of the studies. Thus, we tested a ghrelin to LEAP2-related peptides ratio of 1:10 and assessed cumulative food intake 2 h after treatment, as the entire orexigenic response to the hormone occurs within this time window.[23] In particular, mice were initially subcutaneously (s.c.) injected with the vehicle alone or containing the different LEAP2 variants (0.6 nmol/g BW) and injected with ghrelin (0.06 nmol/g BW, Figure 8) 10 min later. Food intake was assessed 2 h after treatment. As compared to that in vehicle

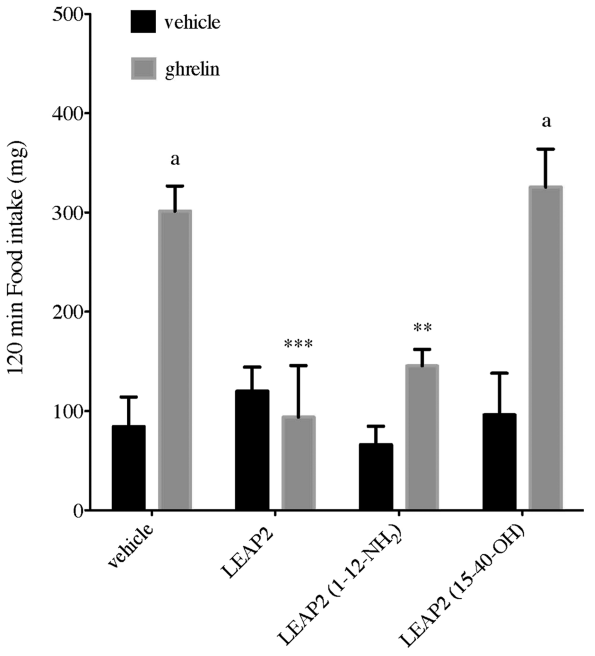

Figure 8. Food intake monitored at 2 h after acute subcutaneous administration of LEAP2-related peptides in the presence or absence of ghrelin. ***$p < 0.001$; **$p < 0.01$ versus vehicle + ghrelin group. [a]$p < 0.05$ versus the same initial treatment (either vehicle, LEAP2, LEAP2 (1−12-NH_2), or LEAP2 (15−40-OH)) + vehicle. n = 5−9 per group.

treatment, ghrelin increased food intake, whereas LEAP2, LEAP2 (1−12-NH_2), and LEAP2 (15−40-OH) alone did not affect food intake. LEAP2 fully inhibited ghrelin-induced food intake as previously described. More interestingly, LEAP2 (1−12-NH_2) also inhibited ghrelin-induced food intake in a similar fashion as seen for LEAP2, whereas LEAP2 (15−40-OH) did not affect ghrelin-induced food intake, as expected based on our in vitro data.

These in vivo data confirmed that the N-terminal part of LEAP2 preserves the biological effects of the whole peptide on food intake.

CONCLUSIONS

We delineated here the active part of LEAP2 and showed that the activity of this peptide toward the ghrelin receptor is restricted to its N-terminal region. Moreover, our data provide evidence that both LEAP2 and its N-terminal part act as inverse agonists of GHSR by stabilizing an inactive conformation of the receptor. LEAP2 and its 1−14 N-terminal region also appeared to be competitive antagonists of ghrelin at promoting IP1 production and calcium mobilization in HEK293T cells expressing GHSR. Accordingly, LEAP2 (1−12-NH_2) blocks ghrelin-induced insulin level reduction in rat pancreatic islets, indicating that LEAP2 antagonizes ghrelin action ex vivo at endogenous GHSR. Moreover, s.c. administration of LEAP2 and LEAP2 (1−12-NH_2) in mice was associated with a decrease of ghrelin-induced food intake. One can hypothesize from our in vitro pharmacological data that LEAP2 and/or its N-terminal part may behave as endogenous inverse agonists of GHSR. Indeed, GHSR displays

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very high constitutive activity and it may be possible that LEAP2 regulates physiologically the tonic activity of GHSR, a hypothesis that certainly deserves to be tested in future studies. Interestingly, besides our LEAP2 inverse agonist of the GHSR, another endogenous inverse agonist, the agouti-related protein, has been reported for the melanocortin receptor.[24] In this line, LEAP2-related peptides developed in the present study should be useful tools to further investigate the physiological role of these potential endogenous inverse agonists of GHSR and to design new potential antiobesity drugs.

Altogether, our observations shed light on the control of ghrelin-mediated regulation of food intake and certainly pave the way for future studies.

EXPERIMENTAL SECTION

Materials and Methods. Ghrelin (1−28) 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 as described previously. The IP-One HTRF kit, benzyl guanine-Tb^{3+}-cryptate, and the insulin ultrasensitive kit were provided by Cisbio. Cell line: HEK293T stable cell line expressing a SNAP-Tag-GHSR (SNAP-GHSR) was a generous gift of 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).

Peptide Synthesis and Characterization. Peptides were synthesized by solid-phase peptide synthesis using Fmoc chemistry, a 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxide hexafluorophosphate/diisopropylethylamine system for coupling, and piperidine/DMF for deprotection. A double coupling with 5 equivalents of reagents was used for each step, and the final deprotection was performed with a trifluoroacetic acid/triisopropylsilane/H_2O (95:2.5:2.5) mixture for 2−3 h. After purification by preparative RP-HPLC, the identity of all peptides was evaluated by LC−MS, MALDI-MS, and MS−MS analyses and UV purity was assessed to be >95% (see in the Supporting Information).

Cell Culture. HEK293T cells 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 as previously described.[13] 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 described previously.[13] 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.

Intracellular Ca^{2+} measurement. HEKTSA cells stably expressing GHSR were seeded with fresh media (100 μL/well) with 10^5 cells into a 96-well black-wall/clear-bottom plate (Corning 3603) precoated with poly-D-lysine. After 24 h, cells were washed and incubated with the assay buffer (100 μL/well) containing Hanks' balanced salt solution, 0.5% BSA, 20 mM HEPES, 1 mM $MgSO_4$, 3.3 mM Na_2CO_3, 1.3 mM $CaCl_2$, and 2.5 mM probenecid (pH 7.4) supplemented with 1 μM fluo 4-AM (Interchim). Cells were incubated at 37 °C and 5% CO_2 with fluo 4-AM for 60 min followed by 15 min incubation with LEAP2 peptides or the control buffer. Then, cells were washed twice with 100 μL of the assay buffer containing LEAP2 peptides and the assay buffer (50 μL/well) was added. The plate containing the cells was placed into the FlexStation (a benchtop scanning fluorometer, Molecular Devices, Sunnyvale, CA), and the reading started by automatic addition of 50 μL/well of the assay buffer containing ligands. The fluorescence output (Exc 495 nm/Em 520 nm) was measured for 1 min. Data were expressed as relative fluorescence units corresponding to the signal obtained by subtracting the baseline from the max calcium signal. EC_{50} values were obtained from dose−response curves using GraphPad Prism (version 5.0).

Receptor Preparation and Labeling. For GTPγS binding assays, the monomeric wild-type GHSR in lipid nanodiscs was prepared as described.[5] For FRET measurements, the ghrelin receptor with a TAG amber codon at the position encoding F^{71} 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 described.[19]

G Protein Preparation. $G\alpha_{13}$ was purchased from Kerafast. His_6-tagged $G\alpha_q$ was expressed in Sf9 cells and purified using nickel−nitrilotriacetic acid (Ni−NTA) affinity chromatography.[25] $G\beta_1$ 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.[25]

GTPγS Binding. GTPγS binding experiments were carried out using the fluorescent BODIPY-FL GTPγS analog.[26] 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 $G\alpha_q$ or $G\alpha_{13}$, 500 nM $G\beta_1\gamma_2$, 100 nM BODIPY-FL GTPγS, and 20 nM receptor in lipid disks. 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 described.[27]

Animals. In vivo testing of LEAP2-related peptides was performed using 3−4 month old C57BL/6J male mice, which were single-housed under a 12 h light/dark cycle with regular chow and water available ad libitum. Studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council and received approval from the IACUC committee of the IMBICE (ID:10-0112).

Insulin Secretion Experiments in Isolated Rat Pancreatic Islets. All animal care and experiments adhered to the Directive 2010/63/EU of the European Parliament and of the Council. 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

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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 in Mice. The ability of LEAP2-related peptides to affect ghrelin-induced food intake was tested. Mice were subcutaneously injected with saline alone or containing 600 pmol/g BW of LEAP2, LEAP2 (1−12-NH_2), or LEAP2 (15−40-OH) and 10 min later subcutaneously injected with saline alone or containing 60 pmol/g BW of ghrelin (n = 5−9 per group). The dose of ghrelin and LEAP2-related peptides was chosen on the basis of previous studies showing that this ghrelin dose reliably increases food intake and that a ghrelin to LEAP2-related peptides ratio of 1:10 would be enough to unmask the effect of LEAP2.[3,27] In all cases, food intake was assessed at 10:00 a.m. in ad libitum-fed mice, which were subjected to the different treatments and exposed to a single preweighed chow pellet in the floor of the home cages. Mice remained undisturbed for 2 h, and then, chow pellets were collected and weighed. Weighing was performed in calibrated scales that had a precision of 1 mg. Two hour food intake was calculated by subtracting the remaining weight of the pellet from the initial weight and expressed in milligram.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01644.

Purification conditions and mass spectrometry characterization; Figure S1, inverse agonist and antagonist activity of LEAP2 peptides on ligand-independent or ghrelin-dependent IP1 production in HEK293T cells expressing GHSR; Figure S2, effect of LEAP2 (1−12-NH_2) on ghrelin-induced inhibition of insulin secretion in rat pancreatic islets (PDF)

Molecular formula strings (CSV) (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

GHSR, growth hormone secretagogue receptor; DIPEA, *N*,*N*-diisopropylethylamine; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HTRF, homogeneous time-resolved fluorescence

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