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Design and characterization of a triazole-based growth hormone secretagogue receptor modulator inhibiting the glucoregulatory and feeding actions of ghrelin

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ABSTRACT

The growth hormone secretagogue receptor (GHSR) is a G protein-coupled receptor that regulates essential physiological functions. In particular, activation of GHSR in response to its endogenous agonist ghrelin promotes food intake and blood glucose increase. Therefore, compounds aimed at blocking GHSR signaling constitute potential options against obesity-related metabolic disorders. We have previously developed potent ligands of GHSR based on a triazole scaffold. Here, we report a new 3,4,5-trisubstituted 1,2,4-triazole compound, named JMV 6616, that potently blocks GHSR activity *in vitro* and *in vivo*. Specifically, in HEK293T cells JMV 6616 behaves as an inverse agonist since it binds to GHSR and inhibits its ghrelin-independent signaling. Accordingly, using purified labeled GHSR assembled into lipid nanodiscs we found that JMV 6616 decreases GHSR-catalyzed G protein activation and stabilizes an inactive receptor conformation. Importantly, JMV 6616 also acts on native GHSR since it blocks the insulinostatic effect of ghrelin in pancreatic islets. In mice, JMV 6616 inhibits blood glucose-raising effects of ghrelin treatment and the orexigenic actions of acute ghrelin administration. Together, our data suggest that this triazole-derived modulator of GHSR holds promise to mitigate several pathological features associated with eating and metabolic disorders.

1. Introduction

Ghrelin is an acylated peptide mainly produced and secreted from the stomach that was originally identified for its capacity to stimulate growth hormone secretion [1]. At present, ghrelin is mostly known for its potent orexigenic action [2,3]. Besides feeding, ghrelin regulates a variety of other centrally- and peripherally-controlled physiological functions related to energy homeostasis [4,5]. For instance, administration of ghrelin promotes weight gain and adiposity. It also raises blood glucose in rodents and human [6–8]. Hence, ghrelin is a multifaceted

regulator of energy metabolism and targeting the ghrelinergic system is being considered a therapeutic option to treat eating- and obesity-related disorders [4,9,10].

Ghrelin is the endogenous agonist of the G protein-coupled receptor (GPCR) named growth hormone secretagogue receptor (GHSR) and, as such, the physiological effects of ghrelin result from its interaction with GHSR. In line with the orexigenic activity of ghrelin, GHSR is enriched in hypothalamic neurons that control food intake and in different nuclei of the reward circuitry [11,12]. GHSR is also expressed in peripheral tissues and, in particular, the blood glucose-raising action of ghrelin has

GHSR, Growth Hormone Secretagogue Receptor; GPCR, G protein-coupled receptor; LEAP2, Liver-Expressed Antimicrobial Peptide-2; HTRF, Homogenous Time-Resolved Fluorescence; FRET, Fluorescence Resonance Energy Transfer; ICV, Intra-Cerebro-Ventricular

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been attributed, at least in part, to its capacity to inhibit glucose-stimulated insulin secretion from pancreatic islets [7,13–15].

GHSR is a versatile receptor coupled to various G proteins of which Gq is the primary. In addition, GHSR signals in response to, but also in the absence of ghrelin [16,17]. This ligand-independent activity of GHSR, including allosteric modulation of other GPCRs and constitutive activity, are likely to also contribute to GHSR-mediated physiological processes in rodents [18–20] and human [21–23]. The constitutive activity of GHSR can be altered by the co-expression of other membrane proteins [24,25] and, importantly, it can be modulated by pharmacological agents. In particular, since inverse agonists not only exert antagonism by blocking agonist-evoked receptor activation but also inactivate constitutive receptor signaling, such compounds have been developed and administered to animals in order to antagonize and/or reverse the actions of ghrelin [18,26–30]. The potential of such pharmacological strategy has been recently strengthened by the description of an endogenous ligand of GHSR named liver-expressed antimicrobial peptide-2 (LEAP2) [31]. Indeed, LEAP2 behaves as both an antagonist and an inverse agonist and interestingly its circulating plasma concentration is sensitive to energy status and is globally inversely correlated to that of ghrelin [9,32–34]. The finding that GHSR signaling is dynamically modulated by two circulating ligands exerting opposite effects on the receptor activity makes synthetic inverse agonists of GHSR attractive candidates for regulating food intake and glucose metabolism [9,11,35].

With the aim of modulating the ghrelinergic system, we and other have conceived non-peptide molecules that act as potent GHSR ligands [27,28,36,37]. In particular, we have shown that the 1,2,4-triazole moiety constitutes a suited scaffold to build up compounds exhibiting either agonist or antagonist activities toward GHSR depending on the substituents that occur on the heterocycle [38–40]. Recently, a structure–activity relationship study led us to develop triazole-based compounds exhibiting GHSR inverse agonist activity [41]. Based on these results, we designed and synthesized a new 3,4,5-trisubstituted 1,2,4-triazole compound named JMV 6616, and we studied its pharmacological properties. We show that JMV 6616 potently inhibits constitutive GHSR signaling in different biological systems. In addition, we report that JMV 6616 is active *in vivo* and shows promise to attenuate different ghrelin-mediated effects that relate to energy balance.

2. Materials and methods

2.1. Synthesis of JMV 6616

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). Final HPLC purification was run on a RP-HPLC Waters 4000 preparative apparatus using a C18 Deltapak column (100 mm × 40 mm, 15 μm, 100 Å), with UV detection at 214 nm and 254 nm, at a flow rate of 50 mL/min with a gradient of eluent A (water/0.1% TFA) and eluent B (ACN/0.1% TFA). After lyophilization (Cryonext, France), the purity of the final compound was assessed by RP-UPLC-MS analyses (UV detection at 214 nm). The LC/MS system consisted of a UPLC Acquity H-Class (Waters) coupled to a SQD2 (Waters) equipped with an ESI source. Analyses were carried out using a Phenomenex Kinetex column (C18, 100 Å, 100x2.1 mm, 2.6 μm). A flow rate of 0.5 mL/min and a gradient of 0–100% B were used: eluent A, water/0.1% HCO₂H; eluent B, ACN/0.1% HCO₂H. Positive-ion electrospray (ESI +) mass spectra were acquired from 100 to 1500 *m/z* with a scan time of 0.2 s. Nitrogen was used for both the nebulizing and drying gas. Retention time (*t_R*) is given in minutes. ¹H NMR and ¹³C NMR spectra were recorded on Bruker Advance spectrometers

at 500 MHz and 125 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm) relative to the residual peak of the solvent (3.31 and 49 ppm). 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.

Compound JMV 6616 was prepared starting from Boc-(D)Trp-OH according to a synthetic pathway previously described [41]. Briefly, Boc-(D)Trp-OH was coupled with 4-methoxybenzyl amine using classical peptide coupling reagents. After treatments and purification, the amide was treated with Lawesson's reagent and converted into the corresponding thioamide which was then reacted with 3-morpholinopropanehydrazide in the presence of silver benzoate and acetic acid. After cyclisation, the Boc protecting group was removed under acidic conditions and the resulting unprotected triazole was reacted with Boc-Gly-OH in the presence of HATU and DIEA. After treatments and purification, the intermediate compound was treated with 4 N HCl in dioxane and the resulting free amine was coupled with alpha-cyano-4-methoxycinnamic acid using the same peptide coupling reagents. After treatments, the final compound was purified by preparative HPLC on a C18 column using a water/acetonitrile/TFA 0.1% gradient, yielding 105 mg of JMV 6616 as a white powder (0.15 mmol). UPLC, purity = 97%. ¹H NMR (500 MHz, methanol-*d*₄): δ 8.11 (s, 1H, CH vinyl), 7.98 (d, *J* = 9.0 Hz, 2H, CH aryl), 7.33 (d, *J* = 8.0 Hz, 1H, CH aryl), 7.29 (d, *J* = 8.0 Hz, 1H, CH aryl), 7.06–7.03 (m, 4H, CH aryl), 6.91 (t, *J* = 8.0 Hz, 1H, CH aryl), 6.64–6.60 (m, 4H, CH aryl), 5.46–5.43 (dd, *J* = 9.5 Hz, 6.5 Hz, 1H, CH), 5.03 (d, *J* = 16.5 Hz, 1H, CH₂), 4.98 (dd, *J* = 16.5 Hz, 1H, CH₂), 3.93 (s, 2H, CH₂), 3.88 (s, 3H, OCH₃), 3.87–3.82 (m, 4H, 2 CH₂), 3.68 (s, 3H, OCH₃), 3.58–3.53 (dd, *J* = 14.0 Hz, 9.0 Hz, 1H, CH₂), 3.51–3.41 (m, 3H, 2 CH₂), 3.25 (br s, 4H, 2 CH₂), 3.07–3.00 (m, 1H, CH₂), 2.92–2.86 (m, 1H, CH₂). ¹³C NMR (125 MHz, methanol-*d*₄): δ 170.8, 165.0, 164.3, 160.9, 157.6, 152.7 (2C), 152.6, 137.9, 134.2 (2C), 128.7 (2C), 128.4, 127.4, 125.9, 125.2, 122.5, 120.0, 119.0, 117.7, 115.8 (2C), 115.3 (2C), 112.4, 110.5, 102.3, 64.9 (2C), 56.2, 55.6, 54.6, 53.3 (2C), 47.0, 43.9, 30.4, 20.8. UPLC-MS (ESI +): *t_R* = 1.34 min, *m/z* 703.3 [M + H]⁺.

2.2. Materials

Because ligand binding, inositol phosphate, and GTP turnover assays as well as FRET measurements were performed using GHSR from human origin, human ghrelin was used for these experiments and was purchased from Santa Cruz Biotechnology (#sc-364689). Rat/mouse ghrelin for insulin secretion experiments and blood glucose monitoring was from Tocris (#1465, Bristol, UK). Rat/mouse ghrelin for food intake experiments was from Global Peptides (# PI-G-03, Fort Collins, USA). We confirmed that rat/mouse ghrelin (60 nmol/kg) from these two providers displayed equivalent orexigenic activity as assessed two hours after subcutaneous administration in mice. The synthesis and characterization of the fluorescent ghrelin variant (GSD(octanoylamide)FLSPEHQVRVQQRKESC-(DY-647P)) (hereafter named ghrelin (1–18)DY) were previously reported [42]. The reference inverse agonist of GHSR developed by Els et al K-(D-1-Nal)-FwLL-NH₂ [29] was also synthesized at the IBMM, Montpellier France. Except otherwise stated, chemical reagents used for buffer preparation were obtained from Sigma-Aldrich (Saint-Louis, USA). The IP-One Gq kit (#62IPAPEC), SNAP-Lumi4-Tb labeling reagent (#SSNPTBD), and the insulin ultrasensitive kit (#62IN2PEH) were purchased from Cisbio (Codolet, France). Chemical reagents used for pancreatic islets isolation and treatments were obtained from Sigma-Aldrich (Saint-Louis, USA).

2.3. Animals

Animals were group-housed in enriched cages in a temperature and humidity-controlled animal facilities under a 12 h light/dark cycle (lights on at 07:00 am) with food and water available *ad libitum*.

At Montpellier, for insulin secretion assays, 8- to 9-week old male Wistar rats (Charles River, France) weighing 280–300 g at the time of the experiments were used to isolate pancreatic islets. For blood glucose monitoring, 3-month old C57BL6/J wild type male mice (Charles River, France) weighing 25–30 g at the time of the experiments were used.

At La Plata, C57BL6/J wild type male mice were generated in the animal facility of the IMBICE and maintained as mentioned above. Chow was provided by Gepsa (Grupo Pilar) and provided 2.5 kcal/g. For food intake experiments, 3- to 5-month old mice were used.

All experiments involving animals 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).

2.4. Cell culture

HEK293T cells which stably express SNAP-human GHSR (SNAP-GHSR) were a generous gift from Eric Trinquet (Cisbio Bioassays, Codolet, France). Cells were maintained in a humidified atmosphere (5% CO₂, 37 °C) in DMEM Glutamax (Invitrogen) supplemented with 1 mg/mL geneticin (Gibco) and containing 50 µg/mL penicillin, 50 µg/mL streptomycin, 2 mM HEPES, 1% non-essential amino acids and 10% heat-inactivated fetal calf serum. Passage numbers were from P8 to P22. No mycoplasma contamination was detected.

2.5. Ligand binding assay

Competition binding assays were performed essentially as previously described [43]. Briefly, SNAP-GHSR HEK293T cells were covalently labeled with Lumi4-Tb following the provider's instruction and then frozen at –80 °C until used. Binding experiments were carried out using a final concentration of 20 nM ghrelin(1–18)DY (K_d = 20 nM) in the presence of increasing concentrations of unlabeled ligands. Assays were initiated by the addition of 50,000 cells per well. After 1 h of incubation at 37 °C, Homogenous Time-Resolved Fluorescence (HTRF) signal was collected at 665 and 620 nm (excitation at 337 nm) using with a PHERAstar reader (BMG LABTECH). Each independent experiment was performed in triplicate.

2.6. Inositol phosphate assay

Inositol phosphate 1 (IP1) accumulation was assayed using HEK293T cell line that stably expresses the SNAP-GHSR. 50,000 cells/well were plated in a 96-well plate. IP1 production was measured using the IP-One HTRF kit as recommended by the provider. Briefly, cells were stimulated for 30 min at 37 °C with the appropriate ligand in 70 µL of IP1 stimulation buffer. An anti-IP1 antibody labeled with Lumi4-Tb (15 µL) and an IP1-d2 derivative (15 µL) were added to each well. After 1 h of incubation at room temperature, signals at 665 and 620 nm (excitation at 337 nm) were detected using a PHERAstar (BMG LABTECH) fluorescence reader. Each point was performed in triplicate. IP1 production was calculated as previously reported and data were expressed as the percentage of maximal effect of ghrelin [41,44].

2.7. Receptor preparation and labeling

The monomeric human ghrelin receptor was expressed in *E. coli* and assembled into POPC/POPG (3/2 M ratio) using the MSP1E3D1 scaffolding protein as previously described [45]. For Fluorescence Resonance Energy Transfer (FRET) measurements, a GHSR mutant with an

azido-Phe residue at position 71^{1,60} (superscript numbers follow Ballesteros-Weinstein numbering; [46]) and a single reactive cysteine at position 255^{6,27} was produced and subsequently labeled with Click-IT Alexa Fluor 488 DIBO Alkyne and Alexa Fluor 350 maleimide, as described [47].

2.8. G protein preparation and GTP turnover assay

A Gαqβ1γ2 heterotrimer composed of the wild type rat Gαq, bovine Gβ1 subunits and bovine Gγ2 subunit tagged with an hexahistidine was expressed in *sf9* cells and purified as described [48]. GTP turnover was assessed using a GTPase-Glo assay [49]. Briefly, the receptor was first incubated at 15 °C with the isolated G protein and, when applicable, the ligand (10 µM) for 60 min in 25 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, pH 7.5. GTP turnover was then started by adding GTP (5 µM) and GDP (10 µM), and the amount of remaining GTP was assessed by measuring luminescence after 40 min incubation at 15 °C using the GTPase-Glo assay. The luminescence signal was normalized to that measured with receptor-free nanodiscs (100%).

2.9. FRET measurements

For FRET measurements, the receptor, the ligand, and the Gαqβ1γ2 heterotrimer were incubated for 60 min at 15 °C. 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 then recorded between 360 and 700 nm on a Cary Eclipse spectrofluorimeter (Varian) equipped with a Peltier-controlled temperature device with an excitation at 346 nm. Buffer contributions were systematically subtracted. The proximity ratio was calculated from the emission spectra as described [50].

2.10. Insulin secretion experiments

Rats were anaesthetized with isoflurane and killed by decapitation. Isolation and digestion of the pancreas using collagenase V as well as treatment of rat pancreatic islets and insulin assays were performed as previously described [34]. Briefly, isolated islets were incubated in a buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 24 mM NaHCO₃, 0.1% (w/v) BSA, pH 7.4) containing 2.8 mM or 8.3 mM glucose in the absence or presence of ghrelin, K-(D-1-Nal)-FwLL-NH₂ or JMV 6616 as depicted in the figure. When islets were incubated with two GHSR ligands, they were added simultaneously. After 1 h of incubation at 37 °C, supernatants were collected and stored at –20 °C until insulin assay. Basal insulin secretion was measured at 2.8 mM glucose concentration. Insulin quantification was performed using the insulin ultrasensitive kit from Cisbio according to the manufacturer's instructions. Experiments were performed in quintuplicate. Data were expressed as the percentage of response obtained under basal condition.

This protocol was approved by the Institutional Animal Care and Use Committee Languedoc-Roussillon (ID #19002).

2.11. Blood glucose monitoring

Mice were habituated to handling several days before the beginning of the experiments. At 09:00 am *ad libitum* fed mice were intraperitoneally (i.p.) administered with vehicle (1.5% DMSO in saline), ghrelin (at a concentration of 7.5 nmol/mL to achieve a final dose of 60 nmol/kg body weight), JMV 6616 (at a concentration of 150 nmol/mL to achieve a final dose of 1.2 µmol/kg body weight) or concomitantly with both ghrelin and JMV 6616 (60 nmol/kg and 1.2 µmol/kg, respectively). A drop of blood was collected from tail snips at 0, 15, 30, 60 and 120 min following i.p. administration to determine glycemia us-

ing a NovaStatstrip Xpress glucometer (DSI). Animals had no access to food throughout blood glucose monitoring.

This protocol was approved by the Institutional Animal Care and Use Committee Languedoc-Roussillon (ID #32440).

2.12. Food intake

Here, mice were single-housed and stereotaxically implanted with an intra-cerebro-ventricular (ICV) single indwelling sterile guide cannula (Plastics One, Roanoke, USA) into the lateral ventricle (coordinates: AP -0.3 mm, ML + 1.0 mm and DV -2.3 mm). During and after surgery, mice were injected with flunixin (2.5 mg/kg, Tecnofarm, Villa Lynch, Argentina) to relief surgery-associated pain and allowed to recover for at least 5 days. Mice were made accustomed to handling by removal of the dummy cannula and connection to an empty cannula connector. To test the effect of JMV 6616 on spontaneous food intake, a set of mice was ICV injected with 2 μ L of vehicle alone or containing 2 nmol/mouse of JMV 6616 at 15 min before lights turn off. Food intake was measured 5 and 12 h after treatment, by weighing the remaining pellet and subtracting it to the initial weight. To test the effect of JMV 6616 on ghrelin-induced food intake, mice were first ICV-injected with 2 μ L of either vehicle (artificial cerebrospinal fluid, aCSF) or with 2 nmol/mouse of JMV 6616 at 10:00 am. Fifteen minutes later, mice were ICV-administered with vehicle or with 0.02 nmol/mouse of ghrelin. Immediately after the second administration, mice were exposed to a pre-weighed chow pellet and food intake was assessed 2 h after, by weighing the remaining pellet and subtracting it to the initial weight.

This protocol was approved by the Institutional Animal Care and Use Committee of the IMBICE (ID #10-0112).

2.13. Data and statistical analyses

Data are expressed as mean \pm Standard Deviation (SD). Curve fitting, statistical analyses, calculations of pKi, pEC₅₀ and areas under the

curve (AUCs) were performed using GraphPad Prism v6 software. The AUCs were determined by subtracting the baseline area (by subtracting the basal blood glucose value of each mouse from the value at each time point) as described [51].

Except otherwise stated, data were analyzed with one-way or two-way ANOVAs after assessing the equality of variances using the Brown-Forsythe's test. When ANOVAs showed $P < 0.05$, Holm-Sidak's multiple comparisons test was used to assign significance between groups. For the data presented in the Fig. 5D, statistical tests are referred in the figure legend. Groups were considered to differ significantly when multiple comparisons tests showed $p < 0.05$ (depicted in the figures as *, # or \$). Group sizes "n" represent the number of experimental independent repeats.

3. Results

3.1. Binding and functional activities of JMV 6616 in GHSR-Expressing HEK293T cells

We recently reported the synthesis of triazole-based inverse agonists of GHSR that derivate from the well-defined GHSR antagonist named JMV 2959 [41]. In light of these results, we carried on the chemical modulation of the structure of JMV 2959 by both acylating its amine function with an α -cyano- β -4-methoxyphenylethylene group and by replacing the phenylethyl substituent in position 3 of the triazole ring with a morpholinoethyl substituent (Fig. 1). These modifications led to the 3,4,5-trisubstituted 1,2,4-triazole derivative that we named JMV 6616 (Fig. 1). JMV 6616 has a cLogP equal to 2.97 as calculated using the MarvinSketch 20.7 software provided by ChemAxon.

First, we assessed the pharmacological features of JMV 6616 using HEK293T cells stably expressing human SNAP-GHSR. We evaluated the affinity of JMV 6616 for GHSR through the determination of its pKi by competition binding experiments on intact cells using a typical HTRF assay [34,41]. We found that JMV 6616 displaced fluorescent ghrelin

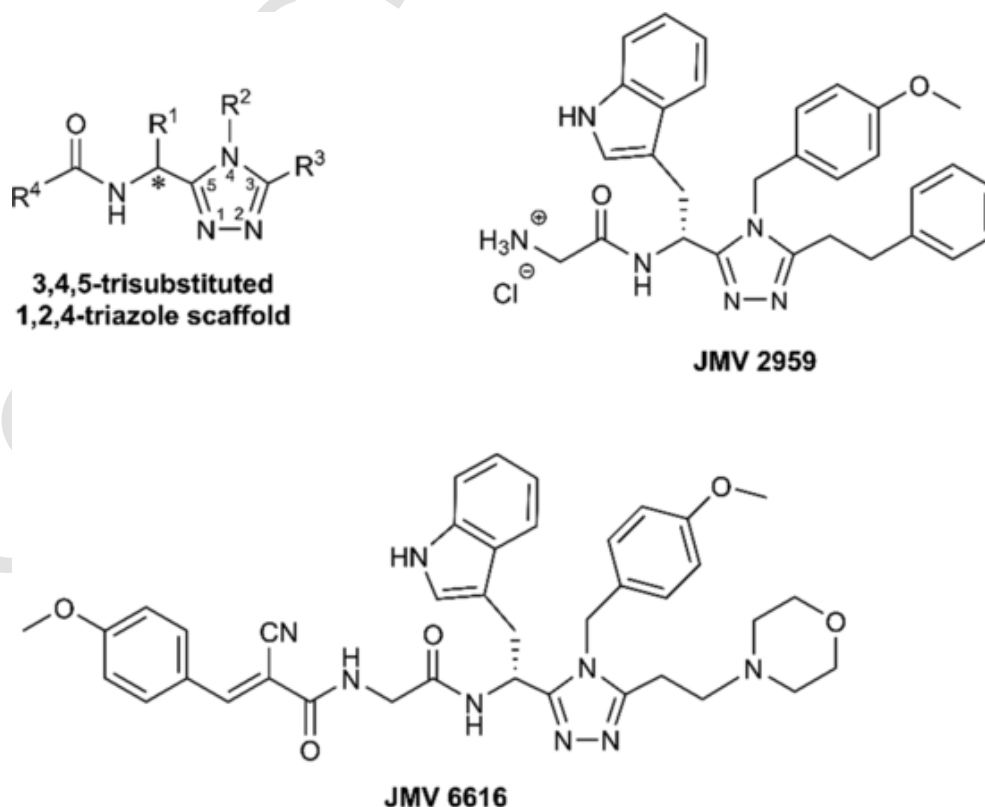


Fig. 1. Structures of the 3,4,5-trisubstituted 1,2,4-triazole scaffold, JMV 2959 and JMV 6616.

(1–18)DY from GHSR with a $pK_i = 8.4 \pm 0.1$, similar to that of ghrelin and K-(D-1-Nal)-FwLL-NH₂, a reference inverse agonist of GHSR [29] (Fig. 2A).

Next, we evaluated the effect of JMV 6616 on inositol phosphate 1 (IP1) production in HEK293T cells expressing SNAP-GHSR, in order to characterize its activity on Gq-associated signaling [52]. As expected, IP1 production was elevated under basal conditions, which is indicative of the prominent constitutive signaling exerted by GHSR [17,34,44], and ghrelin promoted IP1 production (Fig. 2B). JMV 6616 reduced the basal level of IP1 production by more than 50 % ($E_{max} = 58 \pm 1\%$) with a $pEC_{50} = 7.8 \pm 0.1$. Compared to K-(D-1-Nal)-FwLL-NH₂, JMV 6616 was less potent to reduce the basal IP1 production generated by GHSR (pEC_{50} comparison, $[F_{(1,42)} = 85.05, P < 0.0001]$). These data showed that JMV 6616 binds to GHSR and displays an inverse agonist activity toward GHSR-mediated IP1 production.

3.2. Activity of JMV 6616 on isolated GHSR

To further test the inverse agonist behavior of JMV 6616 and assess whether the impact of this compound on IP1 production in HEK293T cells was directly related to its interaction with GHSR, we used the purified GHSR assembled into lipid nanodiscs. This isolated receptor recapitulates the behavior of native GHSR with regard to ligand binding and G protein activation [45]. To this end, we first analyzed receptor-catalyzed Gq activation using a GTP turnover assay [49]. In this assay, GTPase activity is inversely correlated to the amount of free GTP remaining after GTP hydrolysis. As shown in Fig. 3A, the purified apo GHSR triggered a significant GTP turnover for Gq, consistent with its

constitutive activity. As expected, ghrelin increased GTP turnover. In contrast, K-(D-1-Nal)-FwLL-NH₂ and JMV 6616 both triggered a similar decrease in basal GTP binding to Gq, indicating that both compounds have a similar pharmacological profile with regard to the constitutive activity of the ligand-free isolated GHSR.

To assess whether the inverse agonist properties of JMV 6616 were associated with its ability to shift the equilibrium toward the receptor inactive state, we analyzed the conformational features of GHSR using a FRET-based approach [47]. To this end, the purified receptor in nanodiscs was labeled with a fluorescence acceptor (AF488) and donor (AF350) in the cytoplasmic end of the transmembrane helices TM1 and TM6, respectively. As previously shown, the FRET signal between these two probes reports on the distance between the corresponding regions of the receptor, and as such is a hallmark of the conformational state of GHSR [47]. As shown in Fig. 3B, addition of ghrelin or K-(D-1-Nal)-FwLL-NH₂ at saturating concentrations induced a decrease or an increase in the proximity ratio, respectively. JMV 6616 triggered an increase in the proximity ratio closely related to that observed with the reference inverse agonist K-(D-1-Nal)-FwLL-NH₂, suggesting that both compounds have a similar impact on the conformational equilibrium of GHSR.

3.3. Activity of JMV 6616 on pancreatic GHSR

Next, we asked whether JMV 6616 was active on native GHSR. Since ghrelin-mediated pancreatic GHSR activation exerts an insulinos-tatic action [53,54], we performed a concentration-effect relationship of JMV 6616 (1; 10 and 100 nM) on glucose-stimulated insulin secre-

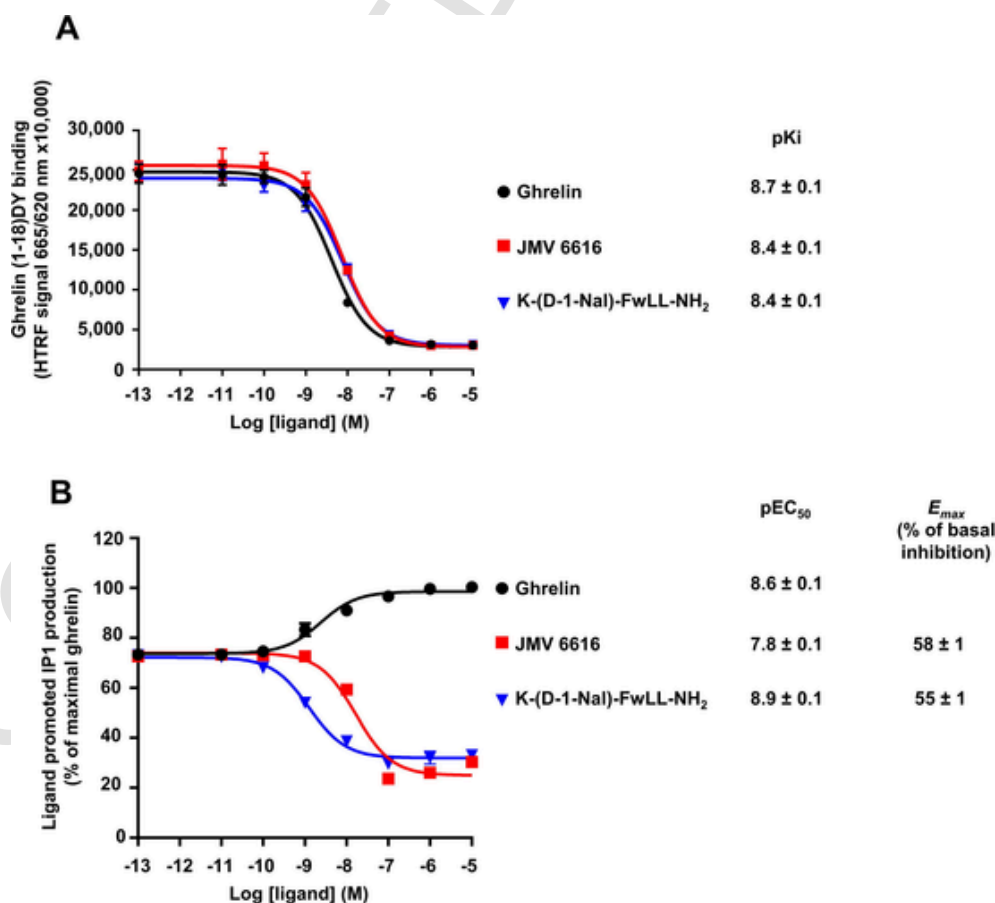


Fig. 2. Pharmacological characterization of JMV 6616 in HEK293T cells stably expressing GHSR. (A) Competition binding curves obtained with ghrelin, JMV 6616 and K-(D-1-Nal)-FwLL-NH₂. Fluorescent ghrelin(1–18)DY was used as a tracer, $n = 3$. (B) Concentration-response curves of ligands at promoting IP1 production, $n = 3$. E_{max} values are given as percentage inhibition of basal receptor response at a concentration of 10^{-5} M for each ligand. Data (mean \pm SD) are expressed as the HTRF signal ratio in (A) and the percentage of maximal effect of ghrelin in (B).

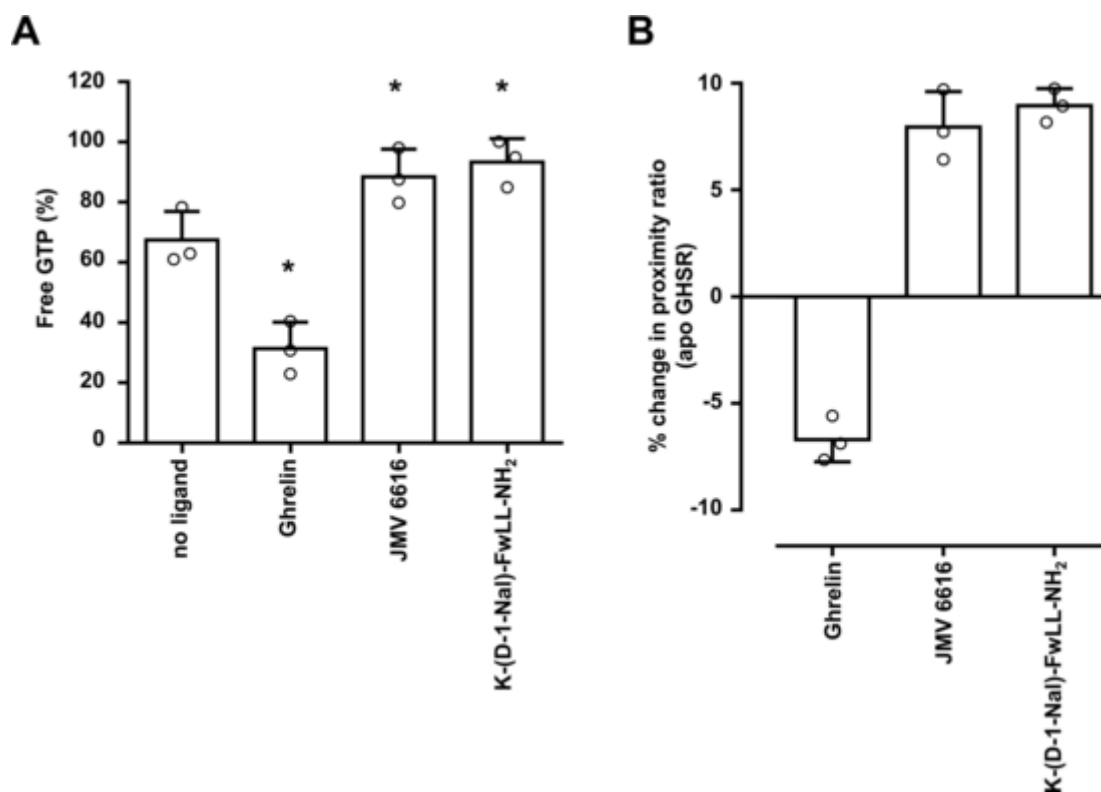


Fig. 3. Effects of JMV 6616 on GHSR-catalyzed Gq protein activation and on GHSR conformation. (A) GTP turnover catalyzed by GHSR in nanodiscs in the absence of ligand or in the presence of 10 μ M ghrelin, JMV 6616 or K-(D-1-Nal)-FwLL-NH₂. The signal (mean \pm SD) was normalized to that obtained for the Gq protein in the presence of empty nanodiscs, $n = 3$. (B) Proximity ratio changes induced by 10 μ M ghrelin, JMV 6616 or K-(D-1-Nal)-FwLL-NH₂. Results (mean \pm SD) are presented as the percentage change in proximity ratio after ligand binding, as calculated from the FRET signal between the fluorophores in TM1 and TM6 of the purified GHSR assembled into lipid nanodiscs, $n = 3$. In (A), one-way ANOVA shows an effect of the ligands, * $p < 0.05$ vs no ligand (Holm-Sidak post hoc test).

tion using rat pancreatic islets. Here, a glucose concentration of 8.3 mM was used because under this experimental condition, the level of insulin secretion is intermediate which is appropriate to disclose a potential regulatory effect of a compound [55,56]. As shown in Fig. 4A, JMV 6616 was unable to affect insulin secretion regardless of its concentration. Then, we tested whether JMV 6616 could antagonize the insulinostatic effect of ghrelin. To do so, pancreatic islets were incubated with 8.3 mM glucose in the presence or absence of ghrelin and JMV 6616 and then insulin release was assayed (Fig. 4B). As expected, we found that ghrelin inhibited glucose-stimulated insulin secretion whereas JMV 6616 prevented the inhibitory action of ghrelin on glucose-stimulated insulin secretion. Interestingly, JMV 6616 and K-(D-1-Nal)-FwLL-NH₂ similarly affected insulin secretion (Fig. 4C). Together, these results suggest that JMV 6616 targets native GHSR and exerts an antagonist activity on ghrelin-evoked actions of pancreatic GHSR.

3.4. Activity of JMV 6616 in mice

Next, we tested the activity of JMV 6616 *in vivo*. We first evaluated the ability of JMV 6616 to affect the blood glucose-raising action of ghrelin in mice [7,8]. Because the anatomical targets mediating the glucose-regulatory effect of ghrelin are diverse and likely differ depending on the metabolic status [6,7], GHSR ligands were systemically delivered in order to reach all the putative organs involved in ghrelin actions. *Ad libitum* fed animals were intraperitoneally (i.p.) administered with ghrelin and/or JMV 6616 and then blood glucose was monitored for 2 h (Fig. 5A and 5B). Ghrelin was administered at 60 nmol/kg because this dose robustly promotes feeding [34], and JMV 6616 was tested in 1:20 molar ratio, as previously done by Ge et al to unmask the capacity of LEAP2 to block the action of ghrelin in mice [31], in order to circum-

vent a potential low bioavailability of the triazole-derived compound following systemic administration. As expected, ghrelin increased glycemia with blood glucose peaking to 238 ± 26 mg/dL at 30 min after treatment (Fig. 5A). Besides, JMV 6616 alone did not affect glycemia but inhibited the blood glucose-raising effect of ghrelin reached 30 min following its administration. The corresponding AUCs further corroborated the blocking action of JMV 6616 (Fig. 5B).

Finally, we examined the ability of JMV 6616 to affect food intake (Fig. 5C and 5D). Since ghrelin-induced food intake implicates brain actions [12], ghrelin and JMV 6616 were centrally delivered in these studies. As compared to vehicle treatment, JMV 6616 ICV-injected 15 min before the onset of the dark phase in *ad libitum* fed mice did not affect spontaneous food intake in the early dark phase period (Fig. 5C) neither overnight food intake (2.623 ± 0.240 g, and 2.591 ± 0.197 g, respectively). Importantly, ICV treatment with JMV 6616 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. For ghrelin-evoked food intake, ghrelin produced a ~ 4 -fold increase in 2 h food intake of mice, compared to vehicle-treated animals, whereas JMV 6616 alone did not affect food intake (Fig. 5D). Interestingly, ghrelin induced a ~ 2 -fold increase of food intake, as compared to vehicle-treated mice, in animals pre-treated with JMV 6616, indicating that JMV 6616 partially blocks ghrelin-induced food intake.

4. Discussion

In the present study, we report the synthesis and the pharmacological properties of JMV 6616, a 3,4,5-trisubstituted 1,2,4-triazole. We showed that JMV 6616 behaved as an inverse agonist of nonnative

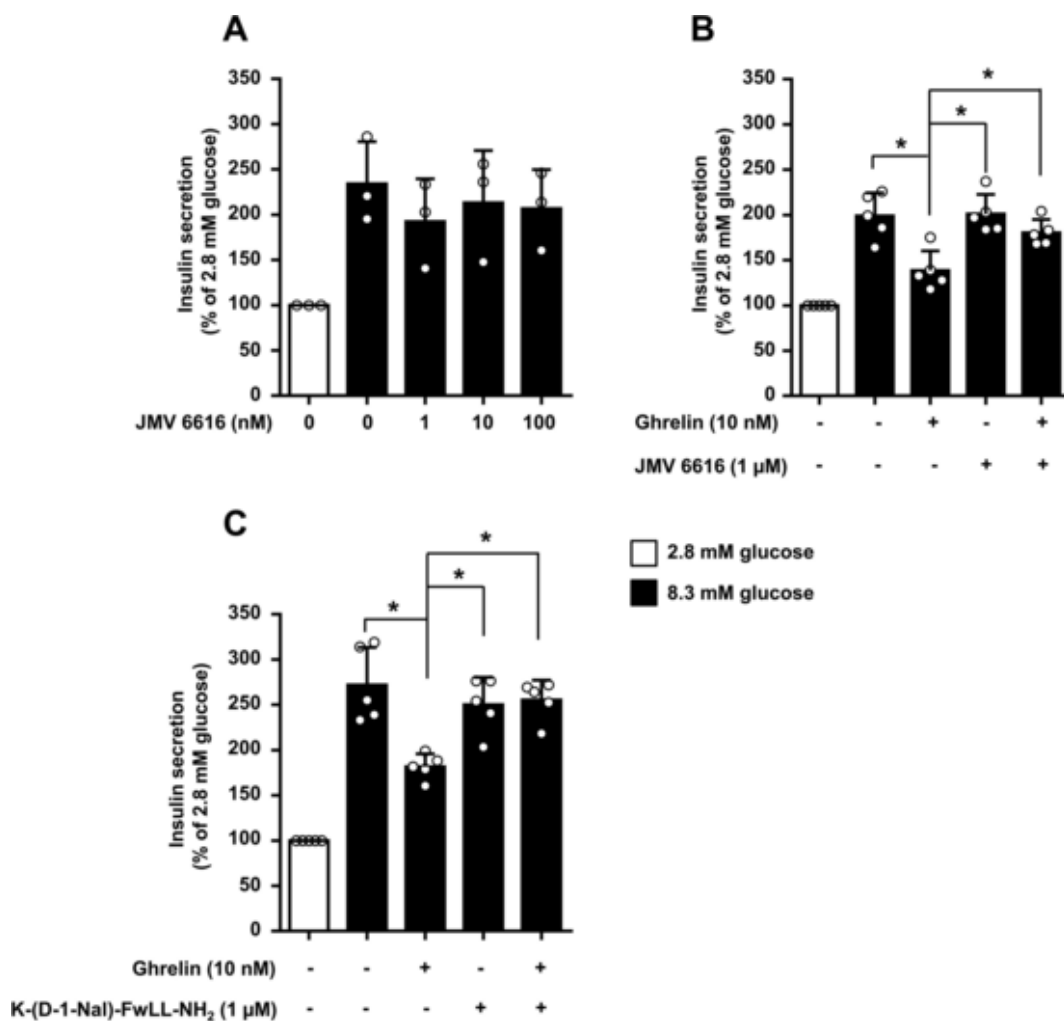


Fig. 4. Effects of JMV 6616 on insulin secretion in rat isolated pancreatic islets. Rat pancreatic islets were incubated under 2.8 mM glucose (basal) or 8.3 mM glucose-stimulated conditions in the presence of (A) different concentrations of JMV 6616; in the presence or absence of ghrelin (10 nM) and (B) JMV 6616 (1 μM) or (C) K-(D-1-Nal)-FwLL-NH₂ (1 μM). After 1 h of incubation, the extracellular medium was collected and insulin concentration was quantified. The histogram depicts insulin secretion (mean ± SD) expressed as the percentage of response obtained under basal condition. In (A), one-way ANOVA performed with 8.3 mM glucose data shows no effect of JMV 6616, $n = 3$. In (B), two-way ANOVA performed with 8.3 mM glucose data shows an effect of ghrelin and JMV 6616, $n = 5$. In (C), two-way ANOVA performed with 8.3 mM glucose data shows an interaction between ghrelin and K-(D-1-Nal)-FwLL-NH₂, $n = 5$. * $p < 0.05$ vs ghrelin (Holm-Sidak post hoc test).

GHSR since it inhibited its constitutive signaling in different assays *in vitro*. We also observed that JMV 6616 inhibited the glucoregulatory and orexigenic actions of ghrelin in mice suggesting that this synthetic ligand may serve in conditions linked to overweight or obesity.

JMV 6616 originates from a rational medicinal chemistry approach that has been harnessing the 1,2,4-triazole moiety to produce ligands with a wide range of activities toward GHSR [39]. One of the pioneer ligands, JMV 2959, illustrates the pharmacological value of triazole-based compounds to explore the biology of the ghrelinergic system since it is one of the most widely used GHSR antagonists. The 1,2,4-triazole scaffold is advantageous as it can be readily decorated with a wide variety of substituents through an optimized chemistry with good yields [38,41]. Indeed, this template presents at least three potential points of diversity on positions 3, 4, and 5 of its heterocycle. Our previous study suggests that the presence of a 2-cyano-3-(4-methoxyphenyl) acrylamidomethyl group in place of the R⁴ substituent (Fig. 1) could confer to JMV 6616 its inverse agonist activity [41]. This possibility requires further evaluations which would likely benefit from the recent structures of GHSR in complex with the inverse agonist PF-5190457 [57]. Nevertheless, using purified GHSR we showed that JMV 6616 stabilized a receptor conformation in the same way as the reference in-

verse agonist K-(D-1-Nal)-FwLL-NH₂. This conformation is distinct from the one obtained with ghrelin and is associated with a reduction in GHSR-catalyzed Gq activation. Therefore, our data indicate that the inverse agonist activity of JMV 6616 is independent of the cellular context and rather results from its binding to GHSR which in turn stabilizes an inactive receptor conformation [58].

Given that inverse agonists have the capacity to block agonist effects and to inhibit constitutive receptor signaling, they have emerged as pertinent pharmacological tools to leverage GHSR constitutive activity and thus to curb GHSR-mediated processes [9,59]. Accordingly, a number of GHSR inverse agonists have been tested in preclinical models with promising results on eating behavior [60]. For instance, K-(D-1-Nal)-FwLL-NH₂ [29], the small non peptidergic molecules GHSR-IA1 and GHSR-IA2 [26] were found to decrease spontaneous food intake whereas other compounds, such as JMV 6616, did not block overnight food intake but blocked feeding elicited with GHSR agonists [28,34]. Importantly, several GHSR antagonists, including the ones conceived by our group (e.g. JMV 2844 or JMV 2959), also altered food intake [36,61,62]. For instance, i.p. administration of JMV 2959 fully inhibits ghrelin-evoked food intake in *ad libitum* fed mice so does the antagonist/inverse agonist LEAP2 [61]. In most cases, it is therefore specula-

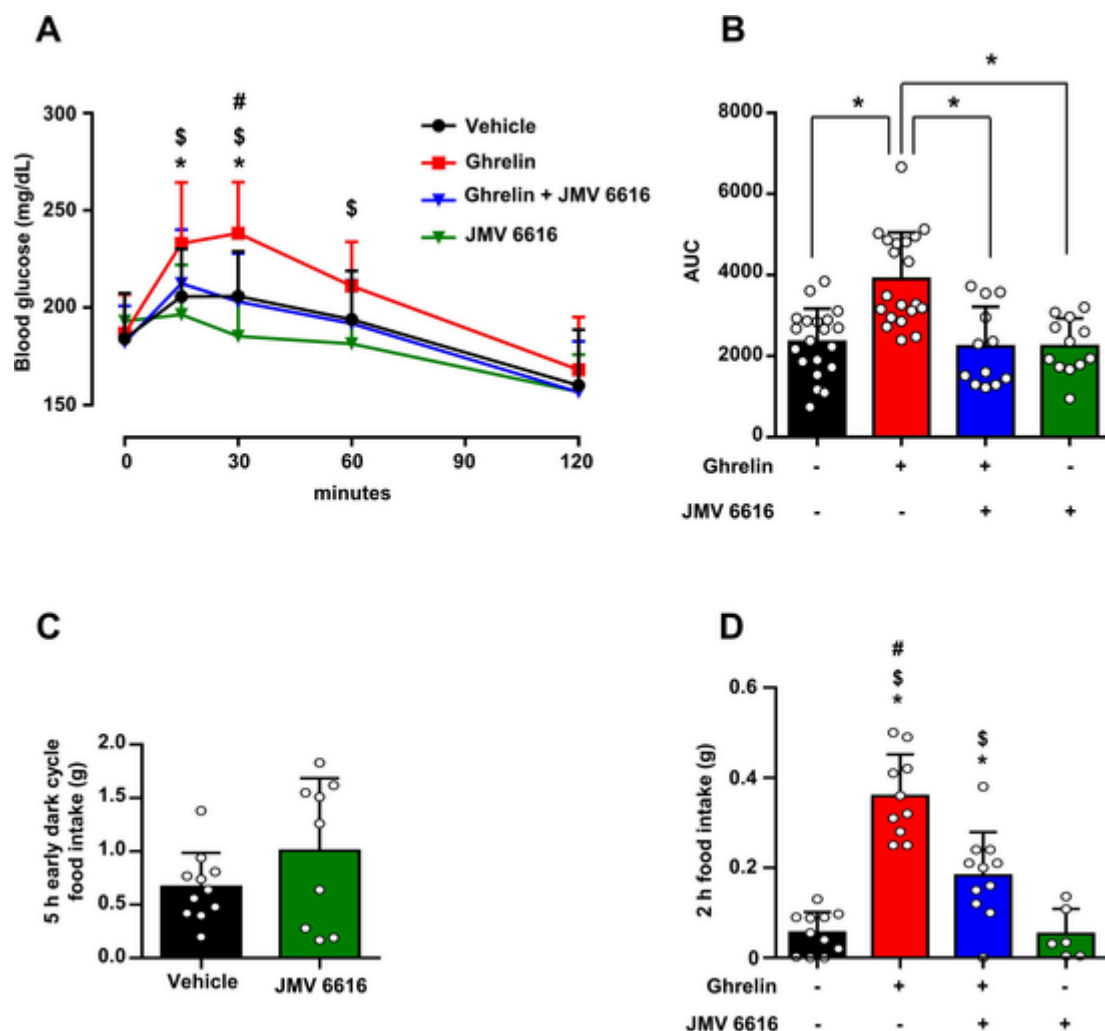


Fig. 5. JMV 6616 blocks ghrelin actions on blood glucose and food intake in *ad libitum* fed mice. (A) shows blood glucose (mean \pm SD) in mice administered (i.p.) with vehicle, 60 nmol/kg ghrelin, 1.2 μ mol/kg JMV 6616 or simultaneously with both ghrelin and JMV 6616. (B) shows the AUCs (mean \pm SD) of blood glucose from panel (A). (C) shows 5-h early dark cycle food intake (mean \pm SD) in mice ICV-injected with vehicle (n = 11) or 2 nmol/mouse JMV 6616 (n = 9) at 15 min before the onset of the dark period. (D) shows cumulative 2 h food intake (mean \pm SD) in mice ICV-treated with vehicle or with 2 nmol/mouse JMV 6616 and, 15 min later, ICV-administered with vehicle or with 0.02 nmol/mouse of ghrelin. (A) Two-way ANOVA shows an interaction between time and treatments [$F_{(12, 240)} = 4.93, P < 0.0001$]; * $p < 0.05$ ghrelin-treated mice vs vehicle-treated mice, \$ $p < 0.05$ ghrelin-treated mice vs JMV 6616-treated mice and # $p < 0.05$ ghrelin-treated mice vs ghrelin + JMV 6616-treated mice (Holm-Sidak post hoc test). (B) Two-way ANOVA shows an interaction between ghrelin and JMV 6616 [$F_{(1, 60)} = 4.93, P < 0.01$], * $p < 0.05$ vs ghrelin-treated mice (Holm-Sidak post hoc test). Vehicle group, n = 20; ghrelin group, n = 20; ghrelin + JMV 6616 group, n = 12; JMV 6616 group, n = 12. (D) Brown-Forsythe and Welch ANOVA test [$F_{(3,16,17)} = 30.81, P < 0.0001$] followed by Dunnett's T3 multiple comparisons test: * $p < 0.05$ vs vehicle + vehicle-treated mice; \$ $p < 0.05$ vs JMV 6616 + vehicle-treated mice; # $p < 0.05$ vs JMV 6616 + ghrelin-treated mice. Vehicle + vehicle group, n = 10; vehicle + ghrelin group, n = 10; JMV 6616 + ghrelin group, n = 10; JMV 6616 + vehicle group, n = 6.

tive to attribute the *in vivo* action of inverse agonists to their capacity to inactivate constitutive GHSR signaling. In other words, *in vivo* effects of JMV 6616 could be simply the consequence of its inherent and confounding antagonism [63] instead of its capacity to disable GHSR constitutive activity. Disentangling the contribution of either inverse agonism or antagonism to the observed modification in eating behavior is challenging and would require careful pharmacological and genetic manipulations of the ghrelinergic system [18].

Even though our data indicate that JMV 6616 displays inverse agonist properties toward nonnative GHSR, we did not observe any intrinsic effect of this ligand on native GHSR. Therefore, it remains unknown whether the effects of JMV 6616 in pancreatic islets and *in vivo* are due to its inverse agonism. Firstly, a wide range of JMV 6616 concentrations was ineffective on glucose-stimulated insulin secretion from rat pancreatic islets. We gathered similar results with K-(D-1-Nal)-FwLL-NH₂ and other peptidergic and non-peptidergic compounds displaying an inverse agonist activity toward GHSR-mediated IP1 production [34,

41,55]. In contrast, a gut-derived LEAP2 fragment corresponding to the (1–10) N-terminal part of LEAP2, which also acts as an inverse agonist of GHSR with low potency, increased glucose-stimulated insulin secretion from human pancreatic islets [64]. This observation suggested that GHSR constitutive activity or intra-islet ghrelin-evoked pancreatic GHSR activation exerts a tonic inhibition on insulin release from human β -cells. A study by Gray et al reported that pancreatic islets from ghrelin (*Ghrl*^{-/-}) and GHSR (*Ghsr*^{-/-}) deficient mice secreted insulin similarly to pancreatic islets from wild type mice in response to glucose and other secretagogues [65] suggesting that GHSR constitutive activity at the mouse pancreas plays a minor, if any, role in controlling insulin secretion in lean, chow-fed adult mice. This could be explained by the finding that the insulinostatic action driven by GHSR in mouse pancreatic islets requires the co-expression of the Melanocortin Receptor Accessory Protein 2, a membrane protein which represses its constitutive activity [24,66]. Secondly, we have not detected any intrinsic effect of JMV 6616 *in vivo*. Unlike insulin secretion experiments, we only tested

a single dose of JMV 6616 and therefore we cannot rule out the possibility that an effective dose was missed. Furthermore, the physiological role of GHSR constitutive activity is likely to be more sophisticated than anticipated *in vivo*. This is well exemplified by the fact that food intake is not decreased in mice expressing a mutant form of GHSR devoid of any constitutive activity [19]. It is also important to underline that only *ad libitum* fed mice were used throughout our study, a condition where GHSR signaling is probably endogenously downregulated owing to an elevated circulating LEAP2/ghrelin ratio [11,31]. Conversely, under food restriction, GHSR signaling is upregulated owing to a low circulating LEAP2/ghrelin ratio [11] which may reshape the pharmacological behavior of JMV 6616 *in vivo*. As the ghrelinergic system is particularly engaged in the regulation of metabolism under extreme energy deficit [3,13], the pharmacological exploration of JMV 6616 merits further investigations under fasting or more stressful metabolic conditions.

Although we recently presented other triazole-based molecules exhibiting inverse agonist activity on nonnative GHSR [41], JMV 6616 is the first of these compounds shown to be active *in vivo* following systemic administration and to have the potential to curb different physiological effects mediated by ghrelin. In addition, JMV 6616 may have favorable physicochemical properties for further preclinical development owing to a cLogP of 2.97 [67]. These features make JMV 6616 a promising compound for more advanced preclinical studies since lowering ghrelin-evoked GHSR activity is presumably beneficial in the setting of obesity-related metabolic disorders [9]. Specifically, JMV 6616 blocked the blood glucose-raising and the orexigenic actions of ghrelin. At this point, we do not know if JMV 6616 is brain penetrant. However, it has been postulated that GHSR ligands modulate glucose homeostasis mainly by acting in the periphery [4,68] in particular when mice are satiated [7]. As a result, it is probable that *i.p.* administration of JMV 6616 blocks the insulinostatic effect of ghrelin and thus its glucoregulatory action by preventing its binding to pancreatic GHSR. Even though we did not monitor insulinemia, our secretion experiments performed in isolated pancreatic islets support this hypothesis. Of note, we cannot rule out that systemically-injected JMV 6616 also impaired the hyperglycemic effects of ghrelin by reducing its stimulatory actions on the secretion of growth hormone or glucocorticoid [1,69], which also favor increments of the glycemia. In any case, it is interesting to note that JMV 6616 fully blocked the glucose-raising effects of ghrelin but only partially reduced the orexigenic effects of ghrelin, despite that a higher JMV 6616:ghrelin molar ratio was used in the food intake studies, suggesting that the new developed GHSR ligand may be used to mainly abrogate the effects of ghrelin on the glucose homeostasis rather than on appetitive behavior.

In conclusion, using a combination of pharmacological and biochemical experiments, we showed that JMV 6616 behaves as an inverse agonist of nonnative GHSR. The parallel observation that JMV 6616 blocks ghrelin actions *in vivo* agrees with the notion that targeting native GHSR with this compound affects physiology although further studies are required to assign the observed effects to the inverse agonism or any other modulatory role of JMV 6616 on GHSR. Nevertheless, JMV 6616 blocks ghrelin effect on feeding and glycemic control which are two processes involving distinct circuitries and mechanisms. This duality paves the way for testing JMV 6616 in animal models to evaluate its potential to tackle the pleiotropic pathological manifestations associated with obesity-related metabolic disorders. Our study also further documents a new chemical class of GHSR ligands based on the triazole scaffold. The usefulness of these GHSR modulators and others could go beyond the management of energy imbalance as reflected by PF-5190457 which is currently under clinical trial for the treatment of heavy alcohol drinking [59].

CRediT authorship contribution statement

Sylvie Péraldi-Roux: Conceptualization, Methodology, Validation, Investigation, Data curation. **Morgane Bayle:** Conceptualization, Methodology, Validation, Investigation, Data curation. **Céline M'Kadmi:** Conceptualization, Methodology, Validation, Investigation, Data curation. **Marjorie Damian:** Conceptualization, Methodology, Validation, Investigation, Data curation. **Justine Vaillé:** Investigation. **Gimena Fernandez:** Conceptualization, Methodology, Validation, Investigation, Data curation. **Maria Paula Cornejo:** Conceptualization, Methodology, Validation, Investigation, Data curation. **Jacky Marie:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Supervision. **Jean-Louis Banères:** Conceptualization, Methodology, Validation, Data curation, Writing – original draft, Supervision, Funding acquisition. **Khoubaib Ben Haj Salah:** Conceptualization, Methodology, Validation, Investigation, Data curation. **Jean-Alain Fehrentz:** Conceptualization, Methodology, Validation, Data curation, Supervision, Funding acquisition. **Sonia Cantel:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Supervision. **Mario Perello:** Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – review & editing, Supervision, Funding acquisition. **Séverine Denoyelle:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – review & editing, Supervision. **Catherine Oiry:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – review & editing, Supervision. **Jérémy Neasta:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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