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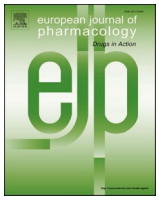

In vitro pharmacological characterization of growth hormone secretagogue receptor ligands using the dynamic mass redistribution and calcium mobilization assays

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ABSTRACT

Ghrelin modulates several biological functions via selective activation of the growth hormone secretagogue receptor (GHSR). GHSR agonists may be useful for the treatment of anorexia and cachexia, while antagonists and inverse agonists may represent new drugs for the treatment of metabolic and substance use disorders. Thus, the identification and pharmacodynamic characterization of new GHSR ligands is of high interest. In the present work the label-free dynamic mass redistribution (DMR) assay has been used to evaluate the pharmacological activity of a panel of GHSR ligands. This includes the endogenous peptides ghrelin, desacyl-ghrelin and LEAP2 (1–14). Among synthetic compounds, the agonists anamorelin and HM01, the antagonists HM04 and YIL-781, and the inverse agonist PF-05190457 have been tested, together with HM03, R011, and H1498 from patent literature. The DMR results have been compared to those obtained in parallel experiments with the calcium mobilization assay. Ghrelin, anamorelin, HM01, and HM03 behaved as potent full GHSR agonists. YIL-781 behaved as a partial GHSR agonist and R011 as antagonist in both the assays. LEAP2(1–14) resulted a GHSR inverse agonist in DMR but not in calcium mobilization assay. PF-05190457, HM04, and H1498 behaved as GHSR inverse agonists in DMR experiments, while they acted as antagonists in calcium mobilization studies. In conclusion, this study provided a systematic pharmacodynamic characterization of several GHSR ligands in two different pharmacological assays. It demonstrated that the DMR assay can be successfully used particularly to discriminate between antagonists and inverse agonists. This study may be useful for the selection of the most appropriate compounds to be used in future studies.

1. Introduction

The growth hormone secretagogue receptor (GHSR) is the receptor for the endogenous peptide ghrelin (Howard et al., 1996; Kojima et al., 1999). GHSR is widely expressed in the brain and in the periphery (Guan et al., 1997; Kamegai et al., 1999; Zigman et al., 2006). GHSR mainly signals through $G_{q/11}$ proteins (Damian et al., 2012), although the additional coupling to $G_{i/0}$, $G_{12/13}$, and to β-arrestin has been reported (Holst et al., 2005; Sivertsen et al., 2011). Ghrelin is released in response to caloric restriction or stress from gastric enteroendocrine cells. Ghrelin is acylated at Ser^3 with an octanoyl fatty acid, that is essential to bind and activate GHSR (Kojima et al., 1999; Bednarek et al., 2000; Gutierrez et al., 2008; Yang et al., 2008; Abizaid and Hougland, 2020). Via GHSR activation, ghrelin modulates several biological functions, including feeding behaviors, energy homeostasis, reword (Panagopoulos and Ralevski, 2014; Koopmann et al., 2018), cognition (Beck and Pourié,

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2013; Hsu et al., 2016), and emotion (Lutter et al., 2008; Meyer et al., 2014). Interestingly enough, GHSR exhibits strong constitutive activity (Holst et al., 2003), that seems important for growth hormone secretion (Pantel et al., 2006), feeding, and energy storage (Pantel et al., 2006; Petersen et al., 2009; Abegg et al., 2017). Liver-Expressed Antimicrobial Peptide 2 (LEAP2) is an endogenous peptide, mainly produced in the liver and small intestine, that behaved as GHSR inverse agonist (Ge et al., 2018; M'Kadmi et al., 2019).

Several therapeutic indications have been identified for GHSR ligands (Colldén et al., 2017; Giorgioni et al., 2022). GHSR agonists may be useful for the treatment of anorexia and cachexia (Müller et al., 2010; Northrup et al., 2013; Pietra et al., 2014; Borner et al., 2016; Villars et al., 2017; Hamauchi et al., 2019; Miyake et al., 2020). Additionally, GHSR agonists may be of useful for the treatment of gastric motility diseases and constipation (Masuda et al., 2000; Camilleri and Atieh, 2021), for the control of inflammatory and neuropathic pain (Sibilia et al., 2006; N Mohammadi et al., 2020) and for the management of some neurological diseases (i.e. epilepsy (Coppens et al., 2016; Buckinx et al., 2019, 2021b), Alzheimer's (Jeon et al., 2019) and Parkinson's (Jiang et al., 2008; Karasawa et al., 2014; Minalyan et al., 2019) diseases). GHSR antagonists and inverse agonists may be of interest for the treatment of metabolic and substance use disorders (Jerlhag and Engel, 2011; Jerabek et al., 2017; Havlickova et al., 2018; Dunn et al., 2019; Sustkova-Fiserova et al., 2020; Charalambous et al., 2021; Edvardsson et al., 2021).

As nicely review by Giorgioni et al. (2022), numerous GHSR ligands, with different chemical structures, have been generated in the past years and some of them reached clinical trials. For instance, the GHSR agonist anamorelin entered clinical trials for the treatment of cancer cachexia (Temel et al., 2016; Currow et al., 2017; Blum et al., 2019; Hamauchi et al., 2019; Miyake et al., 2020; Wakabayashi et al., 2021; Yennurajalingam et al., 2022; Rezaei et al., 2023), and the GHSR inverse agonist PF-05190457 is under development for the treatment of alcohol abuse (Denney et al., 2017; Farokhnia et al., 2018, 2020; Lee et al., 2020a, 2020b; Cobbina et al., 2021).

To date, GHSR ligands have been analyzed in vitro primarily through classical assays, i.e. calcium mobilization and inositol phosphate (IP) assays. These methods may be reductionist, potentially resulting in incomplete pharmacological profiles. Label-free assays provide the ability to non-invasively observe a comprehensive range of cellular responses following receptor activation. The dynamic mass redistribution (DMR) assay is a label-free technique utilizing optical biosensor technology (Schröder et al., 2010; Grundmann and Kostenis, 2015). Using a resonant waveguide grating, DMR detects alterations in the refractive index of the basal portion of the cell layer. Various intracellular events can produce a DMR signal, i.e. protein recruitment, receptor internalization and recycling, second messenger modulation, cytoskeletal remodeling, and alterations in cell adhesion. In the present study the pharmacological profile of the GHSR receptor has been investigated using DMR and calcium mobilization assays in HEK293 cells stably expressing the human GHSR receptor ($HEK293_{GHSR}$). The panel of compounds investigated includes ghrelin and its unacetylated form (desacyl-ghrelin), the GHSR agonists anamorelin (Pietra et al., 2014), and HM01 (Karasawa et al., 2014), the GHSR antagonists YIL-781 (Esler et al., 2007) and HM04 (Garcia Rubio et al., 2019; Richardson et al., 2023), as well as the inverse agonists PF-05190457 (Bhattacharya et al., 2014) and LEAP2(1–14), a LEAP2 fragment that maintains the same pharmacological activity of the parental compound (M'Kadmi et al., 2019). We also selected from patent literature R011 (Brandt et al., 2005), while HM03 and H1498 are two new compounds from Helsinn Healthcare SA, (Lugano, Switzerland) (Garcia Rubio et al., 2012; Giuliano et al., 2017).

2. Material and methods

2.1. Drugs and reagents

Ghrelin (Cat. No. 1463/1), desacyl-ghrelin (Cat. No. 2260), YIL-781 hydrochloride (Cat. No. 3959/5) and PF-05190457 (Cat. No. 6350/5) were purchased from Tocris Bioscience (Bristol, UK). HM01, HM03, HM04, H1498, R011, and anamorelin were kindly provided by Helsinn Healthcare SA, (Lugano, Switzerland) with a purity of ≥98%. LEAP2 (1–14) (Nle-TPFWRGVSLRPIG) was synthesized by automated Fmoc/tBu solid-phase peptide synthesis using the automated synthesizer Syro I. The Fmoc-Gly-Wang resin (200 mg, 0.58 mmol/g) was used as polymeric support; N,N-diisopropylcarbodiimide and 1-hydroxybenzotriazole (DIC/HOBt) were used as coupling reagents. The protected peptide-resin was treated with TFA/H_2O/Et_3SiH (95:2.5:2.5) for 4 h at room temperature. The crude peptide was purified by preparative reverse-phase HPLC, using a Waters Prep 600 HPLC System equipped with a Jupiter C18 column (250 × 30 mm, 300 Å, 15 μ m particle size), to yield a white powder after freeze drying. The molecular weight of the compound was determined using an electrospray mass spectrometer (ESI MICROMASS ZQ). All the compounds were dissolved in DMSO (1 mM stock solutions for ghrelin and PF-05190457, 10 mM stock solutions for HM01, HM03, HM04, H1498, R011, and YIL-781). Carbachol (Cat. No. 212385-M) was purchased from Merck (Darmstadt, Germany) and was dissolved in distilled water at 1 mM concentration. Stock solutions were kept at – 20 °C until use, for a maximum period of 8 weeks. Serial dilutions were made in assay buffer (Hanks' Balanced Salt solution (HBSS)/HEPES 20 mM, containing 0.01 % BSA and 0.1% DMSO). Brilliant black (Cat. No. 11220) and bovine serum albumin (BSA, Cat. No. A3059-50G) were from Merck KGaA, (Darmstadt, Germany). All cells culture media and supplements were from Euroclone (Pero, Italy).

2.2. Cells

To produce cells stably expressing GHSR, 90,000 HEK293T cells were transduced at multiplicity of infection of five (MOI5) with a lentiviral vector encoding the GHSR sequence driven by the CMV promoter and the tdTomato gene controlled by an IRES sequence. HEK293T cells were then cultured in DMEM supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin-Streptomycin for three weeks. After this period, cells that express tdTomato are considered as cells bearing the viral vector integrated in the genome. To isolate the population of tdTomato positive cells, cells were sorted on a BD FACSAria Fusion (BD Biosciences), equipped with 4 lasers (blue (488 nm), yellow-green (561 nm), red (640 nm) and violet (405 nm)), while gating was performed with BD FACS Diva software. Cells were sorted with a 100 μm nozzle tip. Sheat fluid pressure was set at 20 psi. A highly pure sorting modality (4-Way Purity) was chosen. Cells were sorted directly into 5 ml tubes containing PBS, then plated in complete culture medium and expanded for up to 4 weeks, then used for further experiments.

2.3. Dynamic mass redistribution assay

Confluent cells were sub-cultured using trypsin/EDTA and used for experiments. Cells were seeded at a density of 20,000 cells/well in 30 μl into fibronectin-coated EnspireTM-LC 384-wells plates and cultured 20 h to form a confluent monolayer. The day of the experiment cells were manually washed twice and maintained with 30 μl assay buffer (HBSS with 20 mM HEPES, 0.01% BSA) for 90 min before DMR experiments. DMR was monitored in real time with a temporal resolution of 44 s throughout the assay. Experiments were performed at 37 °C, using an EnSight Multimode Plate Reader (PerkinElmer). Agonism protocol: before adding ligand, DMR signal is measured for 5 min and the average of the signal recorded during this period is set as baseline (pm = 0). Compounds are added manually in a volume of 10 μl and the compound triggered DMR signal is recorded for 60 min. Antagonism protocol:

antagonists were added manually 55 min before reading the 5 min baseline. After baseline establishment (pm = 0), ghrelin or PF-05190457 were injected and DMR signal was recorded for 60 min. Antagonist properties of ligands were measured by assessing the concentration-response curve to the agonist in the absence and in presence of a fixed concentration of compound. Responses were described as pm shifts over time (seconds) following subtraction of values from vehicle-treated wells. Maximum picometers (pm) modification (peak) were used to determine agonist response.

2.4. Calcium mobilization assay

Cells were seeded at a density of 50,000 cells/well in 100 μl into 96-well black, clear-bottom plates. The following day, cells were incubated with medium supplemented with 2.5 mM probenecid, 3 μM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C. After that time the loading solution was aspirated and 100 μl of HBSS supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 μM Brilliant Black were added. Serial dilutions of compounds were carried out in HBSS/HEPES (20 mM) buffer containing 0.01% BSA. Cell culture and drug plates were placed into a fluorimetric imaging plate reader (FlexStation II, Molecular Devices, Sunnyvale, CA) and fluorescence changes were measured. On-line additions were carried out in a volume of 50 μl/well. Antagonist properties of ligands were measured by assessing the concentration-response curve to ghrelin in the absence and in presence of a fixed concentration of compound. Antagonists were injected into the wells 24 min before adding ghrelin. To facilitate drug diffusion into the wells the present studies were performed at 37 °C and three cycles of mixing (25 μl from each well moved up and down 3 times) were performed immediately after antagonist injection to the wells. Agonist effects were expressed as maximum change in percent over the baseline fluorescence. Baseline fluorescence was measured in wells treated with vehicle.

2.5. Data analysis and terminology

The terminology adopted in this paper to indicate ghrelin and its receptor follow the guidelines reported by Perelló et al. (2023). All data were analyzed using Graph Pad Prism 9.4 (La Jolla, CA, USA). Concentration-response curves were fitted using the four parameters log logistic equation. Data are expressed as mean ± sem of n experiments performed in duplicate. Agonist potency was expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. In antagonism experiments R011 was tested, at different concentrations (0.1, 1, and 10 μM), for its ability to shift to the right the concentration-response curve to ghrelin (classical Schild protocol). Antagonist potency was expressed as pA_2 derived from the Schild analysis. When antagonists potencies were assayed at single concentrations against the concentration-response curve to the agonist, pA_2 was derived assuming a competitive type of antagonism, using the following equation: $pA_2 = \log(CR\text{-}1)\text{-}\log[A]$, where CR is the ratio between agonist potency (EC_{50}) in the presence and in absence of antagonist and [A] is the molar concentration of antagonist (Kenakin, 2018). After testing normal distribution using the Shapiro-Wilk test, data were analyzed using Student *t*-test or one-way ANOVA followed by Dunnett's post hoc test, as specified in table and figure legends.

3. Results

Agonism experiments - the natural peptide ghrelin was able to induce a concentration-dependent positive DMR signal in $HEK293_{GHSR}$ cells; from these experiments a pEC_{50} of 9.02 and E_{max} of 432 pm have been derived. Under the same experimental conditions, the standard GHSR inverse agonist PF-05190457 produced a concentration-dependent negative DMR signal, with pEC_{50} and E_{max} of 8.80 and −405 pm, respectively (Fig. 1). In calcium mobilization experiments ghrelin evoked an increase of intracellular calcium levels with pEC_{50} and E_{max} of 9.21 and 155 % over the basal values, respectively. PF-05190457 did not modulate intracellular calcium levels in $HEK293_{GHSR}$ cells under these experimental conditions (Fig. 1).

Both in DMR and in calcium experiments desacyl-ghrelin was inactive or only poorly active (Fig. 2). Anamorelin, HM01, and HM03 produced a positive response in the DMR assay (Fig. 2), similar to that elicited by ghrelin, with potency and efficacy values reported in Table 1. Similar results were obtained in parallel experiments performed with the calcium mobilization assay. The maximal effects elicited by ghrelin and by the three GHSR ligands were not statistically different in both the assays. Of note, HM01 and HM03 resulted ~ 3-fold more potent than ghrelin in DMR assay but displayed similar potency in the calcium mobilization test. Supplementary information S1 shows the average DMR response elicited by increasing concentrations of anamorelin, HM01, and HM03 over a 60 min measurement period. No major differences have been detected between the DMR response evoked over time by ghrelin and by these compounds.

When the activity *per se* of HM04, R011, YIL-781, H1498, and LEAP2 (1–14) has been tested, both R011 and YIL-781 produced a concentration-dependent positive DMR response (pEC_{50} of 6.23 and E_{max} of 100 pm for R011; pEC_{50} of 8.27 and E_{max} of 120 pm for YIL-781). On the contrary, HM04, H1498, and LEAP2(1–14) evoked a concentration-dependent negative DMR response with pEC_{50} and E_{max} reported in Table 1 (Fig. 3). Different results were obtained in calcium mobilization experiments where R011 and HM04 resulted inactive, while YIL-781 produced a concentration-dependent increase in intracellular calcium levels with pEC_{50} of 7.10 and E_{max} of 29 % over the baseline (Fig. 3). Supplementary information S2 shows the average DMR response elicited by increasing concentrations of HM04, YIL-781, R011, H1498, and LEAP2(1–14) over a 60 min measurement period. To test the involvement of the GHSR receptor in ligands action, all the compounds producing a DMR response in $HEK293_{GHSR}$ cells have been evaluated at the highest concentration in wild-type HEK293 cells (Table 2) where they failed to induce statistically significant DMR responses. Carbachol, tested in parallel as positive control, produced a concentration-dependent positive DMR response in HEK293 cells (pEC_{50} = 5.47 (4.87–6.07), E_{max} = 429 ± 47 pm). In $HEK293_{GHSR}$ cells carbachol evoked a positive DMR response only at higher concentrations tested (at 100 μM 131 ± 29 pm, supplementary information S3).

Antagonism experiments – Based on its very low efficacy, R011 has been selected as antagonist and further investigated in both the assays using the Schild protocol. R011 concentration-dependently shifted the concentration response curves to ghrelin to the right without significantly affecting E_{max} (Fig. 4) in both the assays. pA_2 from Schild plot extrapolation was 6.26 and 6.82 in DMR and calcium experiments, respectively (Table 1).

Additionally, R011 3 μM has been tested in DMR experiments for its ability to shift to the right the concentration-response curve to the inverse agonists PF-05190457. In parallel experiments R011 shifted to the right both the concentration-response curve to ghrelin and to PF-05190457, without changing their maximal effects. pA_2 values of 6.80 (6.35–7.24) and 7.01 (6.50–7.52) have been derived for R011 vs ghrelin and vs PF-05190457, respectively (Fig. 5).

When tested as antagonists in DMR and calcium mobilization experiments, HM04, YIL-781, PF-05190457, and H1498 shifted to the right the concentration-response curve to ghrelin with different potency values displayed in Table 1. Of note, YIL-781 induced a statistically significant reduction of ghrelin E_{max} in both the assay, while PF-05190457 reduced ghrelin E_{max} only in calcium mobilization experiments. H1498 increased ghrelin E_{max} in calcium mobilization assay (Fig. 6). Desacyl-ghrelin 1 μM did not modify the concentration-response curve to ghrelin (supplementary information S4).

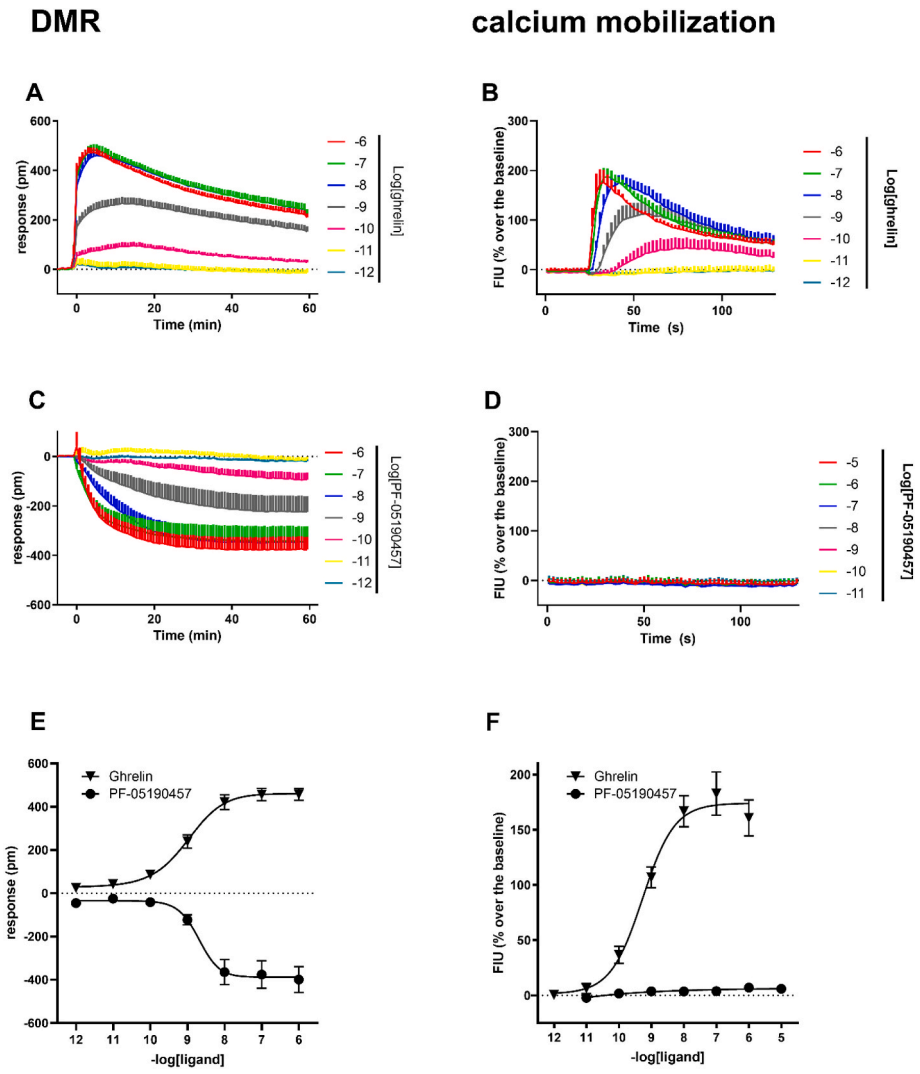

Fig. 1. Effects of ghrelin and PF-05290457 in DMR and calcium mobilization experiments in HEK293$_{GHSR}$ cells. Panels A and C show the time course of the DMR signal evoked by different concentrations of ghrelin (A) and of PF-05190457 (C). Panels B and D show the time course of the calcium mobilization signal evoked by different concentrations of ghrelin (C) and of PF-05190457 (D). In panels E and F the same data are shown as concentration-response curves. Data are the mean ± sem of N ≥ 5 experiments performed in duplicate.

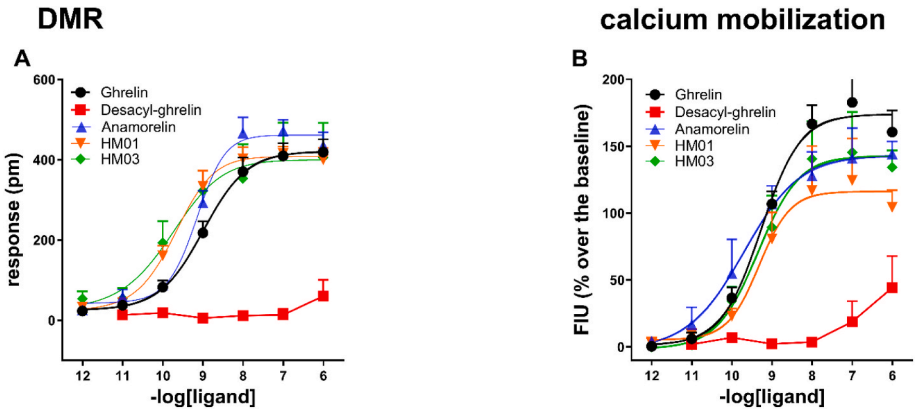

Fig. 2. Concentration-response curves to GHSR agonists in DMR and calcium mobilization experiments in HEK293$_{GHSR}$ cells. Concentration-response curves to ghrelin, desacyl-ghrelin, anamorelin, HM01, and HM03 in the DMR (panel A) and calcium mobilization (panel B) assays. Data are shown as the mean ± sem of N ≥ 5 experiments performed in duplicate.

Table 1
Pharmacological activities of GHSR ligands.

	DMR assay			calcium mobilization assay		
	Agonism		Antagonism	Agonism		Antagonism
	pEC_{50}	E_{max}	pA_2	pEC_{50}	E_{max}	pA_2
Ghrelin	9.04 (8.78–9.31)	423 ± 34	ND	9.21 (8.48–9.94)	155 ± 9	ND
PF-05190457	8.80 (8.66–8.94)	−405 ± 65	6.79 (6.38–7.20)	inactive		7.24 (6.81–7.67)
Desacyl-ghrelin	inactive		<6	crc incomplete, 1 μM: 44 ± 24		<6
Anamorelin	9.16 (8.96–9.36)	435 ± 33	ND	9.31 (8.65–9.97)	144 ± 9	ND
HM01	9.62 (9.29–9.94)	399 ± 29	ND	9.30 (8.88–9.72)	104 ± 12	ND
HM03	9.86 (9.37–10.35)	405 ± 86	ND	8.79 (7.69–9.88)	134 ± 12	ND
HM04	8.43 (7.62–9.25)	−212 ± 41	6.95 (6.65–7.25)	inactive		7.61 (7.25–7.96)
YIL-781	8.27 (7.99–8.56)	120 ± 27	6.88 (6.52–7.24)	7.10 (6.39–7.81)	29 ± 4	7.75 (7.38–8.12)
R011	6.23 (5.56–6.89)	100 ± 26	6.26 (5.84–6.67)	inactive		6.82 (5.80–7.84)
H1498	7.96 (7.49–8.42)	−379 ± 61	6.97 (6.45–7.49)	inactive		7.55 (6.94–8.16)
LEAP2(1–14)	7.39 (6.92–7.85)	−395 ± 33	ND	inactive		ND

pEC_{50} and pA_2 values are expressed as mean (CL95%) of N ≥ 5 experiments performed in duplicate; E_{max} values are expressed as mean ± sem of N ≥ 5 experiments performed in duplicate.

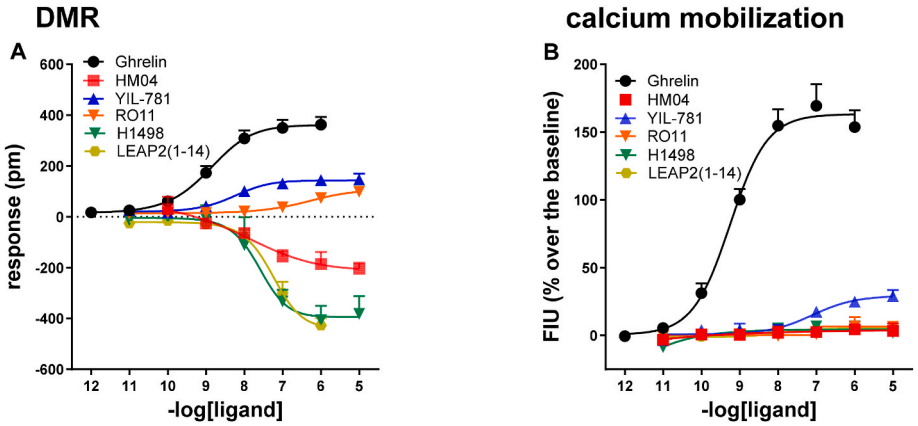

Fig. 3. Concentration-response curves to HM04, YIL-781, R011, and LEAP2(1–14) in DMR and calcium mobilization experiments in $HEK293_{GHSR}$ cells. Concentration-response curves to ghrelin, HM04, YIL-781, R011, and LEAP2(1–14) in the DMR (panel A) and calcium mobilization (panel B) assays. Data are shown as the mean ± sem of N ≥ 5 experiments performed in duplicate.

Table 2
DMR response (pm) evoked by a single concentration of tested compounds in HEK293 cells.

	$HEK293_{GHSR}$	HEK293
Vehicle	−45 ± 25	−33 ± 15
Ghrelin	431 ± 40 [a]	27 ± 11
Anamorelin	485 ± 38 [a]	24 ± 12
PF-05190457	−396 ± 25 [a]	−69 ± 28
HM01	396 ± 31 [a]	18 ± 9
HM03	459 ± 26 [a]	14 ± 7
HM04	113 ± 21 [a]	7 ± 4
YIL-781	193 ± 24 [a]	11 ± 3
R011	103 ± 20 [a]	12 ± 6
H1498	−251 ± 28 [a]	−26 ± 17
LEAP2(1–14)	−317 ± 75 [a]	−57 ± 30
Carbachol	131 ± 29 [a]	429 ± 42 [a]

Ghrelin, anamorelin, HM01, HM03, PF-05190457, LEAP2(1–14): 1 μM; HM04, YIL-781, R011, H1498: 10 μM; carbachol: 100 μM. Data are the mean ± sem of 5 experiments performed in duplicate. One-way ANOVA followed by Dunnet's post hoc test revealed an effect of treatment in $HEK293_{GHSR}$ ($F(11,47) = 90$) and HEK293 ($F(11,47) = 48$).

[a] $p < 0.05$ vs vehicle.

4. Discussion

In the present study the pharmacological profile of the GHSR receptor has been investigated using the DMR label free assay and the calcium mobilization assay, and a panel of ligands encompassing full, partial and inverse agonist pharmacological activities.

In a first series of experiments the compounds have been tested in agonism protocols. All the DMR responses to the compounds were recorded in $HEK293_{GHSR}$ but no in wild-type cells, demonstrating that the pharmacological actions discussed below, are exclusively due to GHSR receptor modulation. The results obtained in agonism studies clearly demonstrated that DMR and calcium mobilization assays have the same utility for the characterization of full and partial agonists while only the DMR assay can be used for the identification of inverse agonists. In fact, PF-05190457, HM04, H1498, and LEAP2(1–14) produced a concentration-dependent negative DMR signal, being completely inactive in calcium mobilization experiments. The fact that calcium mobilization assay precludes the detection of inverse agonism activity is not surprising, considering previous studies performed with GHSR (Bdioui et al., 2018; Ramirez et al., 2019) and other GPCRs (Altenbach et al., 2007; Lee et al., 2014; Ruzza et al., 2023). In particular, Bdioui et al. demonstrated that the GHSR inverse agonist activity of [D-Arg^1,D-Phe^5, D-$Trp^{7,9}$,Leu^{11}]-substance P is detectable with the inositol phosphate (IP) but not calcium mobilization assay. Of note, the amount of the negative DMR response to PF-05190457, H1498, and LEAP2(1–14) is similar to the positive response to ghrelin; this results is in line with the evidence that GHSR displays high levels of constitutive activity (Holst et al., 2003).

The efficacy and potency values obtained for anamorelin, HM01, and YIL-781 are similar to those previously published (Esler et al., 2007; Perdonà et al., 2011; Karasawa et al., 2014; Pietra et al., 2014; Mende

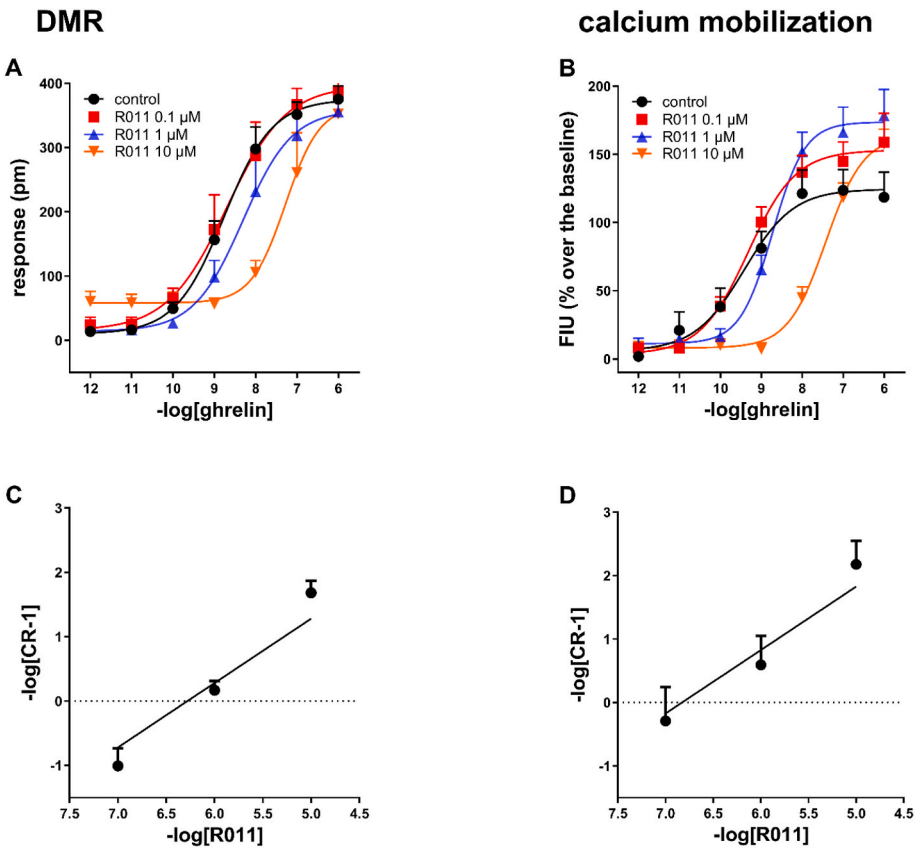

Fig. 4. Concentration-response curves to ghrelin in the absence and in the presence of different R011 concentrations in DMR and calcium mobilization experiments in HEK293$_{GHSR}$ cells. Concentration-response curves to ghrelin in the absence and in the presence of R011 0.1–10 μM in DMR assay (panel A) and calcium mobilization assay (panel B). The corresponding Schild plots are shown in panels C and D. Data are the mean ± sem of N ≥ 5 experiments performed in duplicate.

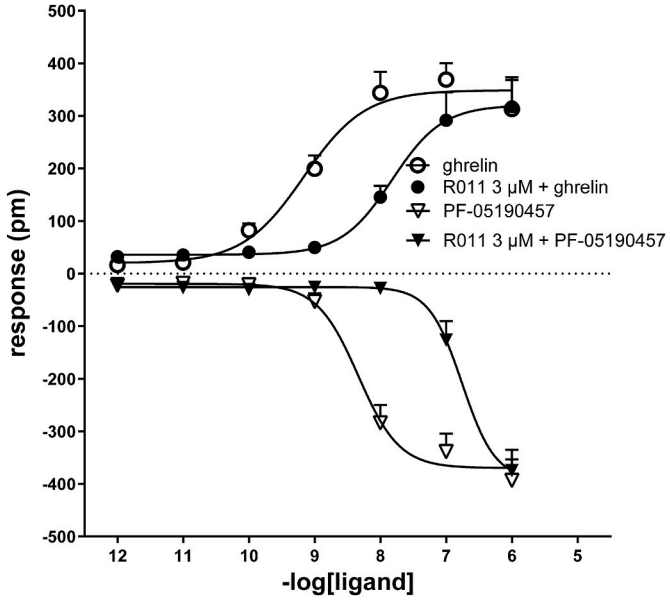

Fig. 5. Concentration-response curves to ghrelin and PF-05190457 in the absence and in the presence of 3 μM of R011 in DMR experiments in HEK293$_{GHSR}$ cells. Concentration-response curves to ghrelin and to PF-05190457 in the absence and in the presence of R011 3 μM in DMR assay. Data are the mean ± sem of 6 experiments performed in duplicate.

et al., 2018), and the same can be said for PF-05190457 (Bhattacharya et al., 2014; Kong et al., 2016) and LEAP2(1–14) (M'Kadmi et al., 2019). Desacyl-ghrelin resulted inactive both as agonist and antagonist in line with the well consolidated finding that ghrelin octanoylation is essential to bind and activate GHSR (Kojima et al., 1999; Hosoda et al., 2000; Zhao et al., 2010; Shiimura et al., 2020; Qin et al., 2022). Anyway, desacyl-ghrelin is now not considered simply the inactive form of ghrelin, but a peptide able to modulates several biological functions, although its mechanism of action is still unknown (Iwakura et al., 2023).

In antagonisms experiments R011 behaved as a competitive GHSR antagonist showing similar values of potency in both the assays. Moreover, R011 blocked PF-05190457 and ghrelin effects with similar potency demonstrating, together with wild-type cells findings mentioned above, that PF-05190457 produced its negative DMR response through the GHSR receptor. PF-05190457, HM04, YIL-781, and H1498 at 1 μM concentration shifted the ghrelin curve, thus behaving as GHSR antagonists. The rank order of potency is similar in DMR and calcium mobilization assays. In general, a tendency of DMR to estimate lower antagonist potency values compared to calcium mobilization assay can be noted. Similar results have been previously reported for different receptors (i.e. NPSR (Ruzza et al., 2018), LPA_1 and LPA_2 receptors (Ruzza et al., 2023), UT receptor (Lee et al., 2014)), but the reason for this slight difference among the two assays is still not know. Comparing our data with the literature, for YIL-781 the potency value obtained in calcium mobilization assay is similar to those previously reported (Esler et al., 2007; Perdonà et al., 2011), while for PF-05190457 it is approximately 30-fold lower. Moreover, PF-05190457 has been reported as a competitive GHSR antagonist (Kong et al., 2016) and recent structural data demonstrated the same binding pocket for ghrelin and PF-05190457 (Qin et al., 2022). In apparent contrast with these

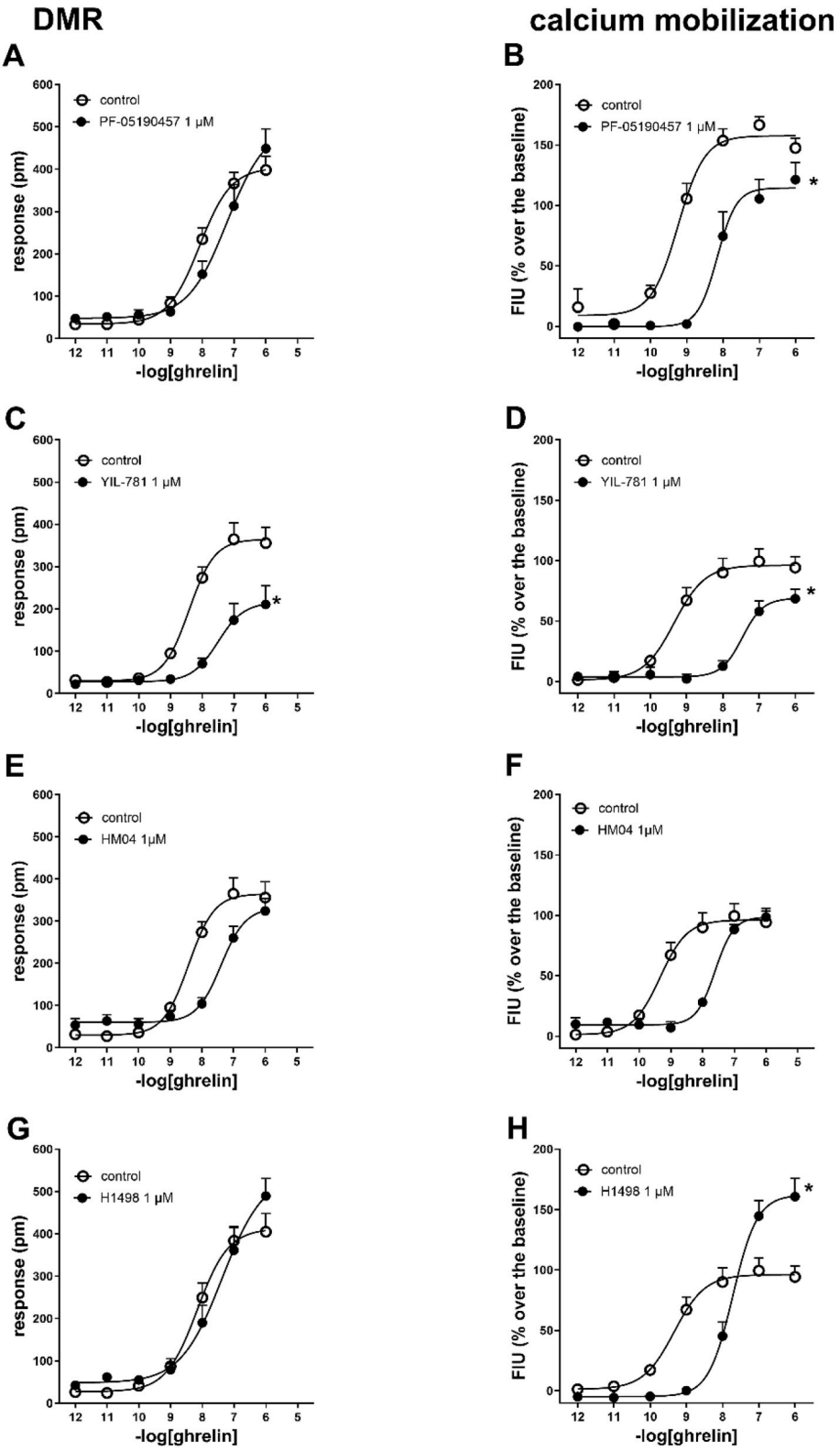

Fig. 6. Concentration-response curves to ghrelin, in the absence and in the presence of 1 μM of PF-05190457, YIL-781, HM04, and H1498 in DMR and calcium mobilization experiments in HEK293$_{GHSR}$ cells. Concentration-response curves to ghrelin in the absence and in the presence of PF-05190457 1 μM (panels A and B); concentration-response curves to ghrelin in the absence and in the presence of YIL-781 1 μM (panels C and D); concentration-response curves to ghrelin in the absence and in the presence of HM04 1 μM (panels E and F); concentration-response curves to ghrelin in the absence and in the presence of H1498 1 μM (panels G and H). Data are the mean ± sem of 5 experiments performed in duplicate. E_{max} has been analyzed using unpaired, two-tailed, Student *t*-test. *$p < 0.05$ vs control; panel B: $t = 4.40$, $df = 8$; panel C: $t = 4.20$, $df = 8$; panel D: $t = 3.46$, $df = 8$; panel H: $t = 6.41$, $df = 8$.

findings, in the present study PF-05190457 induced a significant decrease of ghrelin E_{max} in calcium mobilization experiments. It is worth of mention at this regards that the binding kinetic of PF-05190457 is very slow (Kong et al., 2016); thus the different incubation time used in this study (50 and 24 min, in DMR and calcium mobilization, respectively) may explain the different antagonist potency detected in our study. Additionally, because of the rapid and transient nature of calcium peaks, the calcium mobilization assay is characterized by hemi-equilibrium conditions and this may lead to the appearance of unsurmountable behavior of competitive antagonists, especially when the antagonist slowly dissociates from the receptor (Charlton and Vauquelin, 2010). These considerations may explain the reduction of ghrelin E_{max} induced by PF-05190457 in the calcium mobilization assay.

Similar potency values are expected for partial and inverse agonists when tested as agonists (pEC_{50}) and as antagonists (pA_2) (Kenakin, 2018). Of note, comparing the pEC_{50} and the pA_2 values obtained in DMR experiments some discrepancies emerge. All compounds resulted 10- to 100-fold more potent as partial/inverse agonists than as antagonists and the extent of this difference is not related to their potency or efficacy. This large difference is not expected, considering for instance previous IP accumulation studies performed with PF-05190457 (Kong et al., 2016) or with [D-Arg^1,D-Phe^5,D-$Trp^{7,9}$,Leu^{11}]-substance P (Bdioui et al., 2018). A recent DMR study on LPA_1 receptor shows a similar trend of inverse agonists to display higher potency when tested as agonists than as antagonists (Ruzza et al., 2023). Thus, despite the fact we do not have any hypothesis to explain these findings, it can be speculated that it derives from the assay features rather than from the compound pharmacological properties.

Another point that deserves attention is the ability of the pretreatment with partial and inverse agonists to induce a change in ghrelin E_{max}. In fact, the full inverse agonists PF-05190457 and H1498 show a clear tendency to increase ghrelin E_{max} in DMR and, for H1498, also in calcium mobilization assay; on the contrary YIL-781 decreased ghrelin E_{max} in both the assays. We propose that these effects are due to the changes in the baseline elicited by partial (baseline increase) and inverse agonists (baseline depression), that modify the net amount of signal evocable by a full agonist. Of note, while certain assays allow to appreciate the baseline modifications induced by partial and inverse agonists (i.e. IP accumulation and GTPγS binding), this is not possible in DMR and calcium assays. Anyway, the change induced in agonist E_{max} may be considered as a direct reflex of the partial or inverse agonist action exerted by these compounds.

Interestingly, cells expressing GHSR resulted less sensitive to the stimulant effects of carbachol. This might be due the presence of constitutive active GHSRs that may cause a reduction of the responses mediated by other GPCRs sharing the same coupling mechanism (IP – calcium). A similar phenomenon has been already reported in the literature: the expression in *Xenopus laevis* oocytes of a constitutive active form of the TRH receptor induced a reduction of acetylcholine effects (Grimberg et al., 1999). Similar findings were obtained with the constitutively active Kaposi's Sarcoma-associated Herpesvirus-GPCR co-expressed with THR, M_1, and GRP receptors (Matus;Leibovitch et al., 1995; Lupu-Meiri et al., 2001). Thus, we hypothesized that a similar heterologous desensitization may be evoked by GHSRs. Considering the high degree of constitutive activity displayed by this receptor and its wide distribution in the body, this phenomenon deserves to be further investigated for its possible implications in physiology and pathology.

Collectively, the present study characterized and compared the pharmacodynamic profile of several GHSR ligands of possible utility for several pathological conditions. The therapeutic potential of full GHSR agonists for the treatment of anorexia and cachexia is well documented, as well as the utility of antagonists/inverse agonists for the management of metabolic and substance use disorders (Giorgioni et al., 2022). However, it remains uncertain whether GHSR inverse agonists exhibit greater effectiveness compared to antagonists. GHSR receptor low efficacy partial agonists, antagonists, and inverse agonists were all effective in term of reduction of food (Esler et al., 2007; Moulin et al., 2007; McCoull et al., 2014; Takahashi et al., 2015; Daina et al., 2018; Mende et al., 2018; Haj Salah et al., 2020) and alcohol intake (Gomez and Ryabinin, 2014; Richardson et al., 2023), thus suggesting that the blockage of the ghrelin agonist action is sufficient to obtain these effects. Interestingly enough, a different in vivo effectiveness between GHSR inverse agonists and antagonists has been reported in the field of epilepsy, where the former but not the latter compounds produced anti-seizure effects (Portelli et al., 2012; Buckinx et al., 2021a; Beheshti et al., 2023). However, studies carried out in different laboratories and under different experimental conditions do not allow a precise comparison of the in vivo effectiveness of different receptor ligands. To investigate the hypothetical higher effectiveness of GHSR inverse agonists vs antagonists for the management of metabolic and substance use disorders studies should be performed by testing the different compounds in parallel under the same experimental conditions.

In conclusion, we demonstrated that the DMR assay can be successfully used to pharmacologically characterize GHSR ligands and particularly to discriminate between antagonists and inverse agonists. Additionally, this study provided a systematic pharmacodynamic characterization of novel and standard GHSR ligands. To the best of our knowledge, this is the first time that a rather large panel of GHSR ligands including full and partial agonists as well as antagonists and inverse agonists have been studied in parallel and systematically compared in different pharmacological assays. Such a detailed analysis may be useful for the selection of the most appropriate compounds to be used in preclinical and, eventually, in clinical studies aimed to investigate the therapeutic potential of GHSR ligands.

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CRediT authorship contribution statement

Chiara Sturaro: Writing – review & editing, Visualization, Investigation, Formal analysis. **Chiara Ruzza:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Conceptualization. **Federica Ferrari:** Visualization, Investigation, Formal analysis. **Pietro Pola:** Investigation. **Michela Argentieri:** Investigation. **Alessia Frezza:** Investigation. **Erika Marzola:** Methodology. **Barbara Bettegazzi:** Methodology. **Stefano Cattaneo:** Methodology. **Claudio Pietra:** Resources, Conceptualization. **Davide Malfacini:** Writing – review & editing, Conceptualization. **Girolamo Calò:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial or non-financial interests which may be considered as potential conflicts of interest: During the study Claudio Pietra was an employee of Helsinn Healthcare SA. All the other authors declare no conflict of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2024.176880.

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