Research article

Drug Testing and Analysis

Received: 3 August 2015 Revised: 5 November 2015 Accepted: 10 December 2015 Published online in Wiley Online Library

(www.drugtestinganalysis.com) DOI 10.1002/dta.1947

Structure-activity relationship for peptídic growth hormone secretagogues

P. Ferro,[a] G. Krotov,[b] I. Zvereva,[b] G. Rodchenkov[b] and J. Segura[a,c]*

Growth hormone releasing peptides (GHRPs) could be widely used by cheating athletes because they produce growth hormone (GH) secretion, so may generate an ergogenic effect in the body. Knowledge of the essential amino acids needed in GHRP structure for interaction with the target biological receptor GHSR1a, the absorption through different administration routes, and the maintenance of pharmacological activity of potential biotransformation products may help in the fight against their abuse in sport. Several GHRPs and truncated analogues with the common core Ala-Trp-(D-Phe)-Lys have been studied with a radio-competitive assay for the GHSR1a receptor against the radioactive natural ligand ghrelin. Relevant chemical modifications influencing the activity for positions 1, 2, 3, and 7 based on the structure aa-aa-aa-Ala-Trp-(D-Phe)-Lys have been obtained. To test *in vivo* the applicability of the activities observed, the receptor assay activity in samples from excretion studies performed after nasal administration of GHRP-1, GHRP-2, GHRP-6, Hexarelin, and Ipamorelin was confirmed. Overall results obtained allow to infer structure-activity information for those GHRPs and to detect GHSR1a binding (intact GHRPs plus active metabolites) in excreted urines. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: growth hormone secretagogues; growth hormone releasing peptides; radio receptor assay; doping substances; nasal administration

Introduction

Growth hormone secretagogues (GHSs), able to interact with the GHSR1a receptor, have been regarded as an alternative to support diagnostics and to treat diseases related to GH deficiency due to their ability to release this hormone in the body.[1,2] A widespread variety of compounds, including growth hormone releasing peptides (GHRPs)[1–6] and non-peptidic compounds[5,7,8] have been developed by the pharmaceutical industry.[9] However, so far only the peptide GHRP-2 (also known as Pralmorelin) has been clinically tested and approved in Japan for diagnostic purposes, while a few other GHSs are still in different stages of development.

In the world of sport, these compounds are being searched out by some athletes because of their ergogenic ability, so they are becoming abused substances either because the potential effect of the direct GH released or to their ability to mask the illicit usage of recombinant GH. The latter is based on the fact that they simultaneously increase the production of the different endogenous GH isoforms,[10] leaving the ratio between them unchanged, and avoiding the isoforms approach to detect GH abuse.[10,11] In fact, several of these GHRPs can be easily purchased on the black market via the Internet, specially GHRP-2, GHRP-6, Hexarelin, and Ipamorelin.[12,13] Therefore, since 2013. the World Anti-Doping Agency (WADA) has incorporated GHS and GHRPs in its Prohibited List of doping substances in Section S2.[14]

Different analytical methods have been proposed to effectively detect these compounds in biological samples. These methods are based on mass spectrometry (LC-MS),[15–19] especially for those GHS known to be abused and for which the structure and their metabolism are known.

Interestingly, further studies are being developed by pharmaceutical companies with new, and often yet unknown, GHS in preclinical and clinical trials. A common aspect for these compounds would be their interaction with the GHSR1a receptor, for which the endogenous natural ligand is ghrelin, a 3-octanoylated 28 amino acid peptide. Accordingly, a useful tool for research and development in this area would be competition assays between the potential GHS and a labelled, usually ghrelin itself, ligand.[20–22] These competitive labelled approaches do not need previous knowledge about any specific structure as far as the tested compounds (parent drug and/or metabolites) maintain GHSR1a receptor binding activity.

Studies on the structure of ghrelin itself and truncated ghrelin analogues that maintain the activity at receptor level have been carried out.[5,23,24] Also, pharmaceutical companies and other researchers have investigated a homologous series of non-peptidic[8,25–29] or peptidic[4,24,30–32] compounds. However, as some specific GHRPs such as GHRP-1, GHRP-2, GHRP-4, GHRP-5, GHRP-6, Hexarelin, or Alexamorelin are among the most available to cheating athletes, and bear a common core chemical structure, some further specific insight into their particular structure-activity relationship seems appropriate. In this work a study to determine the implication of core-flanking amino acids and chemical groups in those GHRPs regarding their interaction with the receptor GHSR1a is presented. The group of GHRPs, together with potential

* *Correspondence to: Jordi Segura, IMIM (Hospital del Mar Medical Research Institute), PRBB, carrer Dr. Aiguader 88, 08003 Barcelona, Spain. E-mail: jsegura@imim.es*

a *Bioanalysis Research Group, Neuroscience Research Program, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain*

b *Antidoping Centre, Moscow, Russia*

c *Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona, Spain*

metabolites or variants, was studied and their affinity for the receptor was evaluated by using a radio-competitive binding assay.[21] It is common in pharmacology to find an inverse relationship between *in vitro* molecular activity and *in vivo* dosage. Usually, less active *in vitro* compounds will need a higher *in vivo* dose to have a comparable activity to more active compounds. In a series of homologous compounds, assuming relatively similar disposition and metabolism, it is also expected that those compounds administered with higher doses will have a higher absolute amount of compound excreted in the urine in dosed subject. Activity of excreted parent compound plus active metabolites could be ascertained by using the radio-competitive binding assay applied to the urines. To confirm such potentiality, already verified when GHRP-2 was administered intravenously,[33] the radio-competitive binding assay was also used as a proof of principle to analyze urine samples from excretion studies with GHRP-1, GHRP-2, GHRP-6, Hexarelin and Ipamorelin administered by nasal route in healthy males.[20]

Methods and materials

Chemicals

GHRP-1, GHRP-2, GHRP-4, GHRP-5, GHRP-6, Hexarelin, Alexamorelin, Ipamorelin and their metabolites were obtained as described in Semenistaya *et al.*[20] The amino acid sequences of peptides are shown in Table 1. Radio labelled ghrelin ([^{125}I-His$_9$]ghrelin) was purchased from Perkin Elmer (Waltham, MA, USA). All other chemicals were of the highest grade commercially available.

Cell culture, membranes preparations, and competition binding assay

Cell culture, membrane preparations and competition binding assay parameters have previously been published by Pinyot *et al.* and Ferro *et al.*[20,34] Briefly, HEK293 cells stably expressing GHSR1a was obtained from Dr R. Smith (Baylor College of Medicine, Houston, TX, USA) through Dr F. Casanueva (University of Santiago de Compostela, Spain). Cells were cultured in 100-mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 U/mL), 2 mM glutamine (Invitrogen, Paisley, UK) and G418 (Invitrogen, 800 μg/mL). Cells were grown under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C up to 70-80% confluence. Cultured cells were trypsinized and pelleted by centrifugation at 1000g for 5 min and frozen at −80 °C in DMEM with 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) at a concentration of 5×10^6 cells/mL.

Membranes were prepared as follows: one aliquot of 5×10^6 cells was thawed and centrifuged at 4 °C for 10 min at 4000 g. The cell pellet was re-suspended in 1 mL of homogenization buffer (50 mM Tris–HCl and 10% sucrose, pH 7.4) and sonicated for 5 min. Disrupted cells were centrifuged again at 4 °C for 10 min at 4000g. Membrane pellet was re-suspended in 5 ml of binding buffer (25 mM Hepes, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM ethylenediaminetetraacetic acid [EDTA] and 0.4% bovine serum albumin [BSA], pH 7.4).

For competition binding assays, membranes obtained from 50000 cells were incubated in triplicate with [^{125}I-His$_9$]ghrelin (at a concentration of 15 pM; specific radioactivity 2200 Ci/mMol) and different GHS or extracts of urine samples (processing see below) in a final volume of 300 μL of fresh binding buffer. Prior addition to the membranes, [^{125}I-His$_9$]ghrelin aliquots were counted in a 1470 Wizard gamma scintillation counter (Perkin Elmer) to obtain the value of total radioactivity initially present in each experiment. The samples were incubated for 40 min at 25 °C under continuous shaking. The reaction was stopped by rapid centrifugation at 16000g for 5 min at 4 °C. The cell pellet was rinsed once with ice-cold 50 mM Tris-HCl pH 7.4 and centrifuged again at 16000g for 3 min at 4 °C to prevent the pellet from re-suspending. Finally, the supernatant was removed and the pellet was measured in the gamma counter and the results were analyzed with GraphPad Prism 5 software (San Diego, CA, USA) and graphs were produced with the same software.

Table 1. Elemental composition of GHRPs and their metabolites.

		POSITION AT GENERAL SEQUENCE							
COMPOUND CODE	**TRIVIAL NAME**	**1** aa-	**2** aa-	**3** aa-	**4** Ala-	**5** Trp-	**6** (D-Phe)-	**7** Lys-	**C-terminal form** NH_2/OH
1	**GHRP-1**	Ala-	His-	(D-β-Nal)-	Ala-	Trp-	(D-Phe)-	Lys-	NH_2
2	**GHRP-1 (2-7)**	-	His-	(D-β-Nal)-	Ala-	Trp-	(D-Phe)-	Lys-	NH_2
3	**GHRP-1 (2-7) FA**	-	His-	(D-β-Nal)-	Ala-	Trp-	(D-Phe)-	Lys-	OH
4	**GHRP-1 (3-7)**	-	-	(D-β-Nal)-	Ala-	Trp-	(D-Phe)-	Lys-	NH_2
5	**GHRP-1 (3-7) FA**	-	-	(D-β-Nal)-	Ala-	Trp-	(D-Phe)-	Lys-	OH
6	**GHRP-1 (3-6) FA**	-	-	(D-β-Nal)-	Ala-	Trp-	(D-Phe)-	-	OH
7	**GHRP-2**	-	D-Ala-	(D-β-Nal)-	Ala-	Trp-	(D-Phe)-	Lys-	NH_2
8	**GHRP-4**	-	-	(D-Trp)-	Ala-	Trp-	(D-Phe)-	-	NH_2
9	**GHRP-4 FA**	-	-	(D-Trp)-	Ala-	Trp-	(D-Phe)-	-	OH
10	**GHRP-5**	-	Tyr-	(D-Trp)-	Ala-	Trp-	(D-Phe)-	-	NH_2
11	**GHRP-6**	-	His-	(D-Trp)-	Ala-	Trp-	(D-Phe)-	Lys-	NH_2
12	**HEXARELIN**	-	His-	(D-Mrp)-	Ala-	Trp-	(D-Phe)-	Lys-	NH_2
13	**HEXARELIN FA**	-	His-	(D-Mrp)-	Ala-	Trp-	(D-Phe)-	Lys-	OH
14	**HEXARELIN (2-6)**	-	-	(D-Mrp)-	Ala-	Trp-	(D-Phe)-	Lys-	NH_2
15	**ALEXAMORELIN**	Ala-	His-	(D-Mrp)-	Ala-	Trp-	(D-Phe)-	Lys-	NH_2
16	**IPAMORELIN**	Aib-	His-	(D-2-Nal)-	-	-	(D-Phe)-	Lys-	NH_2
17	**IPAMORELIN FA**	Aib-	His-	(D-2-Nal)-	-	-	(D-Phe)-	Lys-	OH
18	**IPAMORELIN (1-4) FA**	Aib-	His-	(D-2-Nal)-	-	-	(D-Phe)-	-	OH

Reference samples

Two reference samples in binding buffer were included in each competition binding experiment, one as blank or negative control (containing cells and all reagents but not spiked with GHS) as the maximum possible binding (100% of relative specific binding, RSB) and a second sample or positive control, spiked with 7.5 μM GHRP-2, as the minimal possible specific binding (0% RSB), respectively. All sample binding values were calculated relative to these limits.

Excretion study

Urine samples from GHS excretion studies with GHRP-1, GHRP-2, GHRP-6, Hexarelin and Ipamorelin (Thermo Fisher Scientific, Ulm, Germany) were collected by the Antidoping Centre in Russia. This study was performed in five male volunteers (one for each compound). Each volunteer received a single nasal dose of one of the peptides GHRP-1, GHRP-2, GHRP-6, Hexarelin or Ipamorelin (5 μg/kg dose, solution in 200 μL water for administration) and spontaneous urine samples were collected prior to and during 48 h after administration as described by Semenistaya *et al.*[20] The urine samples were stored at −20°C until analyses.

For analyses, urine samples were processed following the protocol established by Pinyot *et al.*[37] Briefly, urine samples were thawed and centrifuged for 15 min at 3500 g. After desalting, in which 2.5 mL of urine were loaded in a 3-mL Oasis HLB solid phase extraction (SPE) cartridge (Waters, Mildford, MA, USA), the elute obtained with methanol was dried in a N2-evaporator TurboVap LV (Caliper, Hopkinton, MA, USA). They were reconstituted in 1 mL freshly prepared binding buffer, sonicated for 5 min and secretagogues were purified by receptor affinity. Eventually, samples were reconstituted in 150 μL of binding buffer and analyzed by competition binding assay.

Results

In this study we applied the radio-competitive binding assay methodology already developed and validated,[34] to study the affinity for the GHSR1a receptor of different GHRPs and some truncated forms (potential metabolites) in order to ascertain those core flanking amino acids and chemical groups essential for the interaction with the receptor and consequently important to produce GH release. Samples from excretion studies after a single nasal dose with five different GHS were also tested to evaluate the detectability of these compounds (and/or their metabolites) using this radio-competitive method.

Based on a common basic structure for GHS of 6-7 aminoacids, the scheme displayed in Table 1 was used to enumerate the amino acids evaluated in this study from position 1 (N-terminal) to position 7 (C-terminal). All compounds, exception of Ipamorelin related compounds, had a common core structure Ala-Trp-(D-Phe) at positions 4, 5, and 6. The study to determine the amino acids and chemical groups essential for the interaction was based in the comparison of competition curves from pairs of nearly identical GHS which had only one change in an amino acid or chemical group. As presented in Table 1, some studied compounds lacked aminoacids at positions 1, 2, 3, 4, 5, or 7. The latter (C-terminal) could also be either in the form of amide (-NH_2) or free acid (-OH).

Influence of Ala at position 1 to the receptor interaction

To test the influence of Ala at position 1, two pairs of GHS and metabolites were tested: GHRP-1 (with alanine at position 1) and GHRP-1 (2-7) (a metabolite from GHRP-1 without amino acid at this position), and Alexamorelin (with alanine at position 1) and Hexarelin (which has not any amino acid at this position). After competition binding curves, Ki values were calculated for each compound and these were compared between each pair. Results, displayed in Figure 1, showed an increase of the affinity for the receptor (lower Ki) in those compounds with no amino acid at position 1, so Ala at this position seems to produce a reduction of the interaction with the receptor.

Presence of D-alanine, histidine or tyrosine (at position 2 in our scheme) as N-terminal amino acids

D-alanine, histidine and tyrosine as N-terminal aminoacids were tested in GHS with these amino acids at position 2 (N-terminal) in front of metabolites without amino acids at this position. Presence or absence of alanine was studied through GHRP-2 and GHRP-1 (3-7), of histidine through metabolites GHRP-1 (2-7) and GHRP-1 (3-7), and through Hexarelin and Hexarelin (2-8); and tyrosine through GHRP-5 and GHRP-4. Results showed a high decrease in the affinity for the receptor in those GHRPs lacking N-terminal amino acid at position 2, as clearly seen in Ki values obtained in Figure 2; so D-alanine, histidine or tyrosine appear to be relevant for the interaction at this position, most probably interacting with Glu124 in the 3rd transmembrane domain of GHSR1a structure.[35,36] Other basic aminoacids, not so common among families of GHRPs, were not tested.

Relevance of the amino acid at position 3 for interaction with the GHSR1a receptor

GHRP-1 (2-7), Hexarelin and GHRP-6 have the same amino acid sequence, but with a difference at position 3. GHRP-1 (2-7) has D-β-Nal (β-(*2*-naphthyl)-D-alanine), Hexarelin has D-Mrp (D-methyl-tryptophane) and GHRP-6 has D-Trp (D-tryptophane) at this position.

After competition binding assays, compounds with β-D-Nal and D-Trp showed similar Ki values and lower than Ki values obtained from compounds with D-Mrp, so GHS with β-D-Nal and D-Trp seem to offer better affinity for the GHSR1a receptor, as shown in Figure 3.

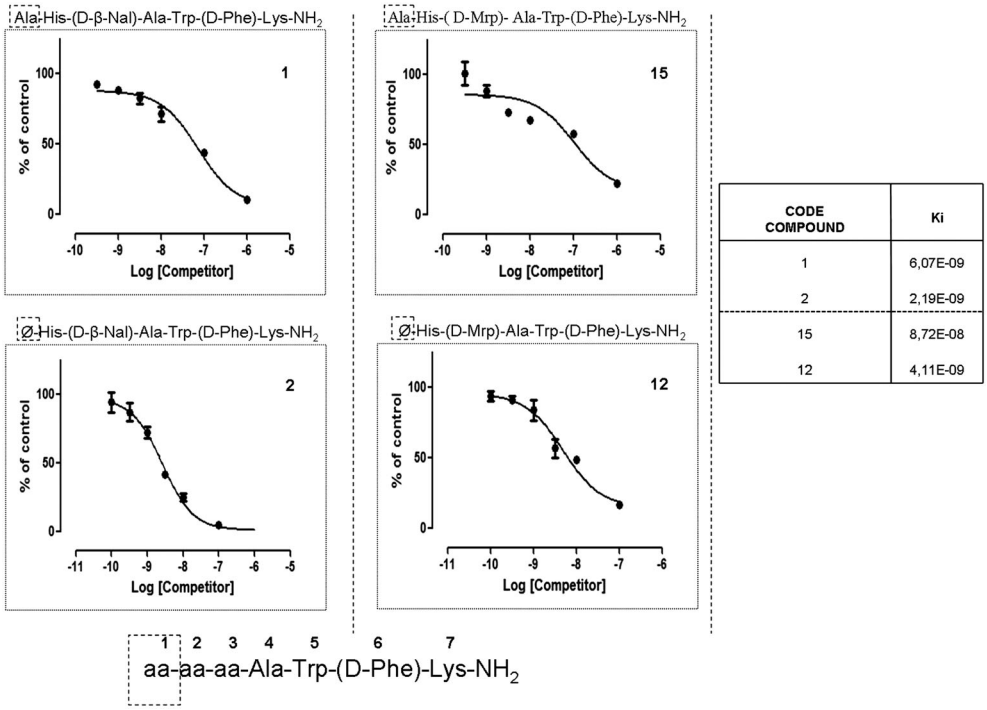
Evaluation of the presence of lysine at position 7

Two groups of GHS and metabolites were evaluated to determine the relevance of a lysine amino acid at position 7 (C-terminal) for the interaction with the GHSR1a receptor: a group of C-terminal amidated GHRPs (GHRP-6 and GHRP-5) and a group of C-terminal free acid GHRPs (Ipamorelin FA, Ipamorelin (1-4) FA, GHRP-1 (3-7) FA and GHRP-1 (3-6) FA). These compounds were compared with its corresponding counterpart who had the same sequence but lacked lysine at C-terminal final position.

Competition binding assays showed very high Ki values for compounds without lysine at this position, as displayed in Figure 4, so this amino acid is essential for the interaction with the receptor. In fact, some of the metabolites without lysine lost all capacity for the interaction with the receptor and it was not possible to establish a Ki value for these compounds.

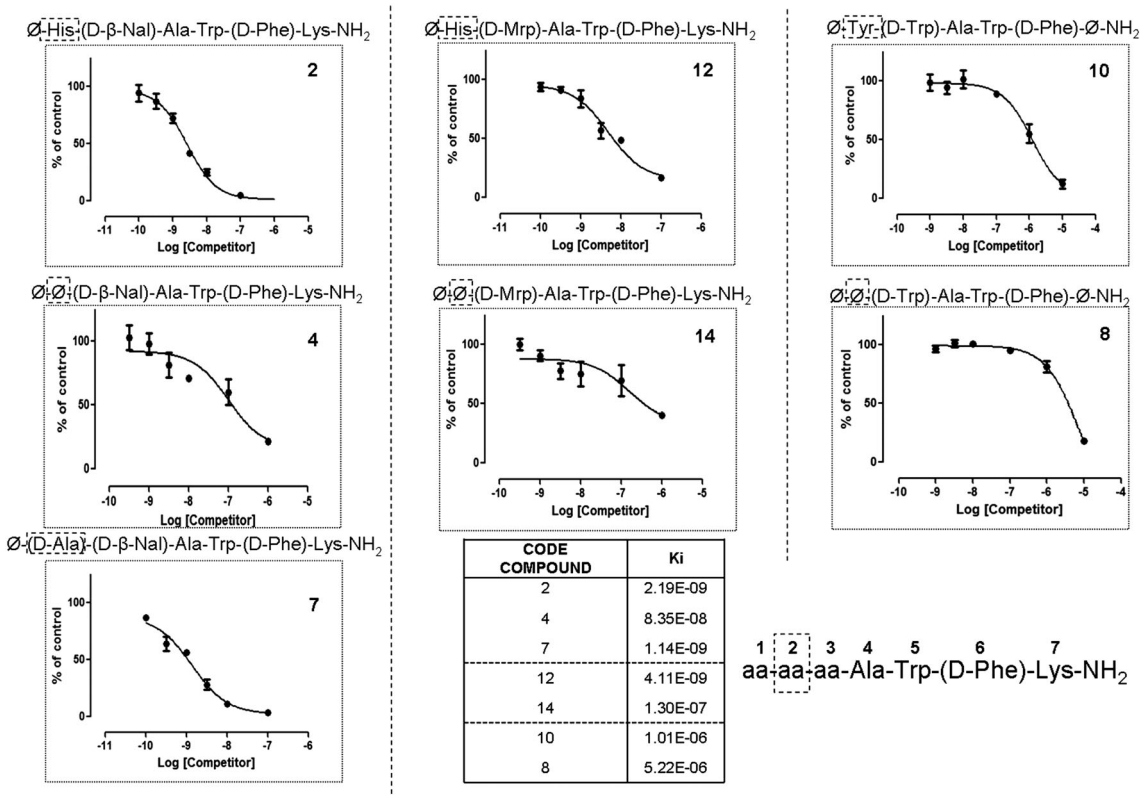
Substitution of the amide group with a free acid at the C-terminal end position and its relevance for the interaction

Several GHS and metabolites were evaluated to establish the significance of the exchange of an amide group by a free acid at C-terminal in the interaction with the receptor. Therefore,



CODE COMPOUND	Ki
1	6,07E-09
2	2,19E-09
15	8,72E-08
12	4,11E-09

Figure 1. Evaluation of the importance of an amino acid at position 1 by competition binding curves to GHSR1a receptor. GHRP- 1 (**1**) and Alexamorelin (**15**) have an alanine and, GHRP-1 (2-7) (**2**) and Hexarelin (**12**) do not have an amino acid at this position.



CODE COMPOUND	Ki
2	2.19E-09
4	8.35E-08
7	1.14E-09
12	4.11E-09
14	1.30E-07
10	1.01E-06
8	5.22E-06

Figure 2. Competition binding curves to test the affinity to the GHSR1a receptor in GHS with D-alanine, histidine or tyrosine at position 2 (N-terminal, no amino acid at position 1). GHRP- 2 (**7**) has an alanine, GHRP-1 (2-7) (**2**) and Hexarelin (**12**) have a histidine, GHRP-5 (**10**) has a tyrosine, and GHRP-1 (3-7) (**4**), Hexarelin (2-6) (**14**) and GHRP-4 (**8**) do not have any amino acids at this position.

compounds which exhibited the same amino acid sequence were tested, but the only difference between these compounds was the presence of an amide group (GHRP-1 (2-7), GHRP-1 (3-7), GHRP-4, Hexarelin and Ipamorelin) or a free acid (GHRP-1 (2-7) FA, GHRP-1 (3-7) FA, GHRP-4 FA , Hexarelin FA and Ipamorelin FA) at the C-terminal position.

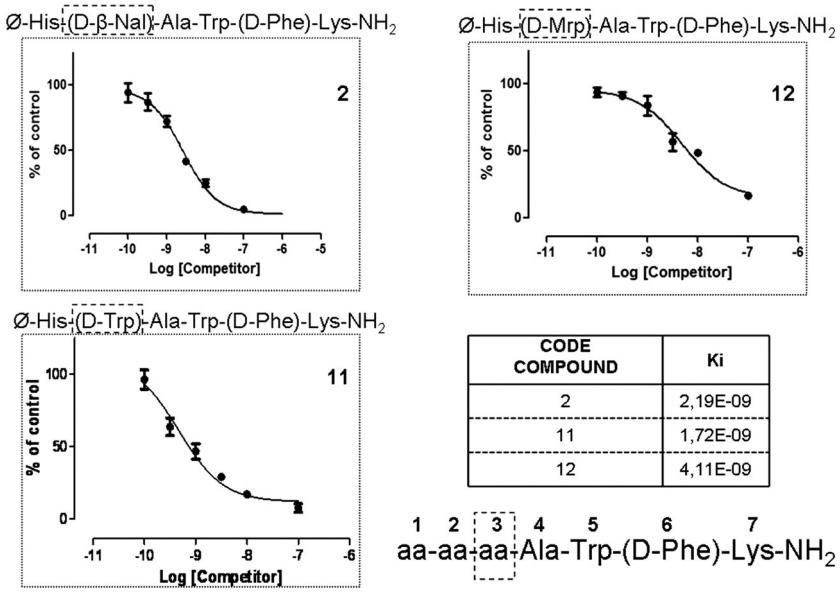


Figure 3. Competition binding curves to test the affinity to the GHSR1a receptor in GHS with D-β-Nal, D-Mrp or D-Trp at position 3. GHRP-1 (2-7) (**2**) has D-β-Nal, Hexarelin (**12**) has D-Mrp and GHRP-6 (**11**) has D-Trp.

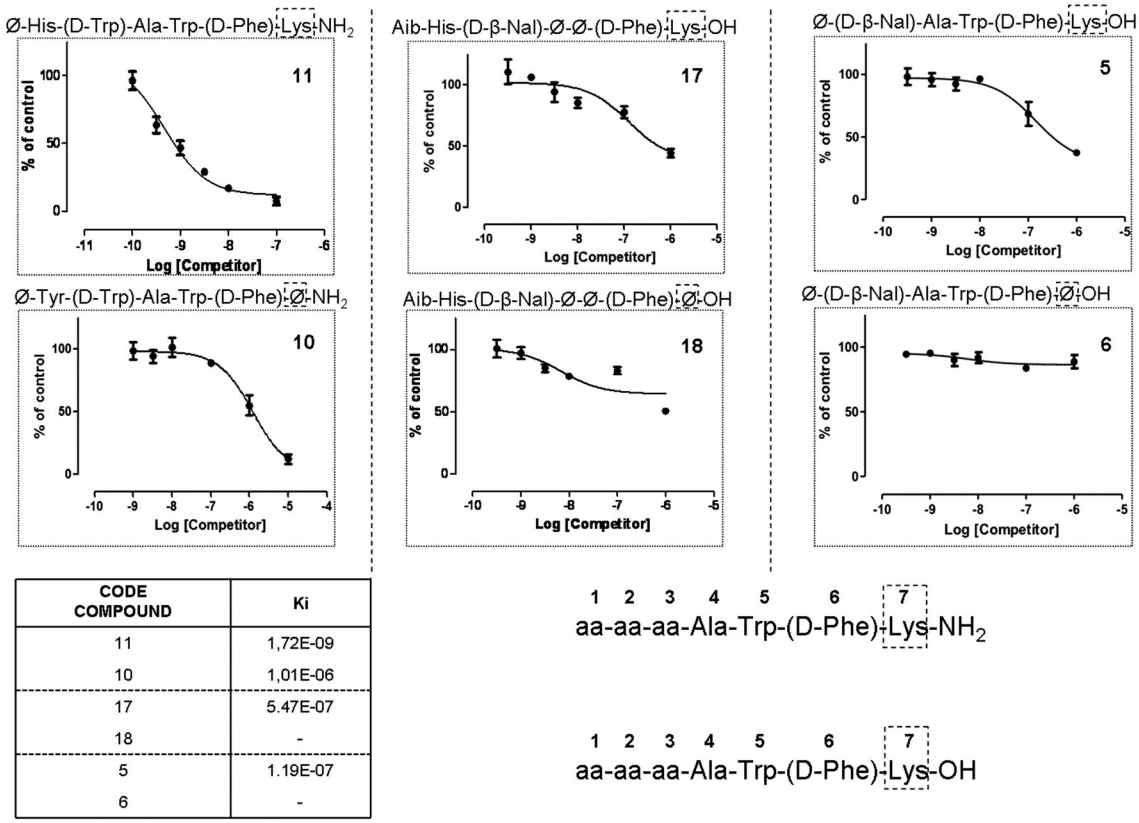
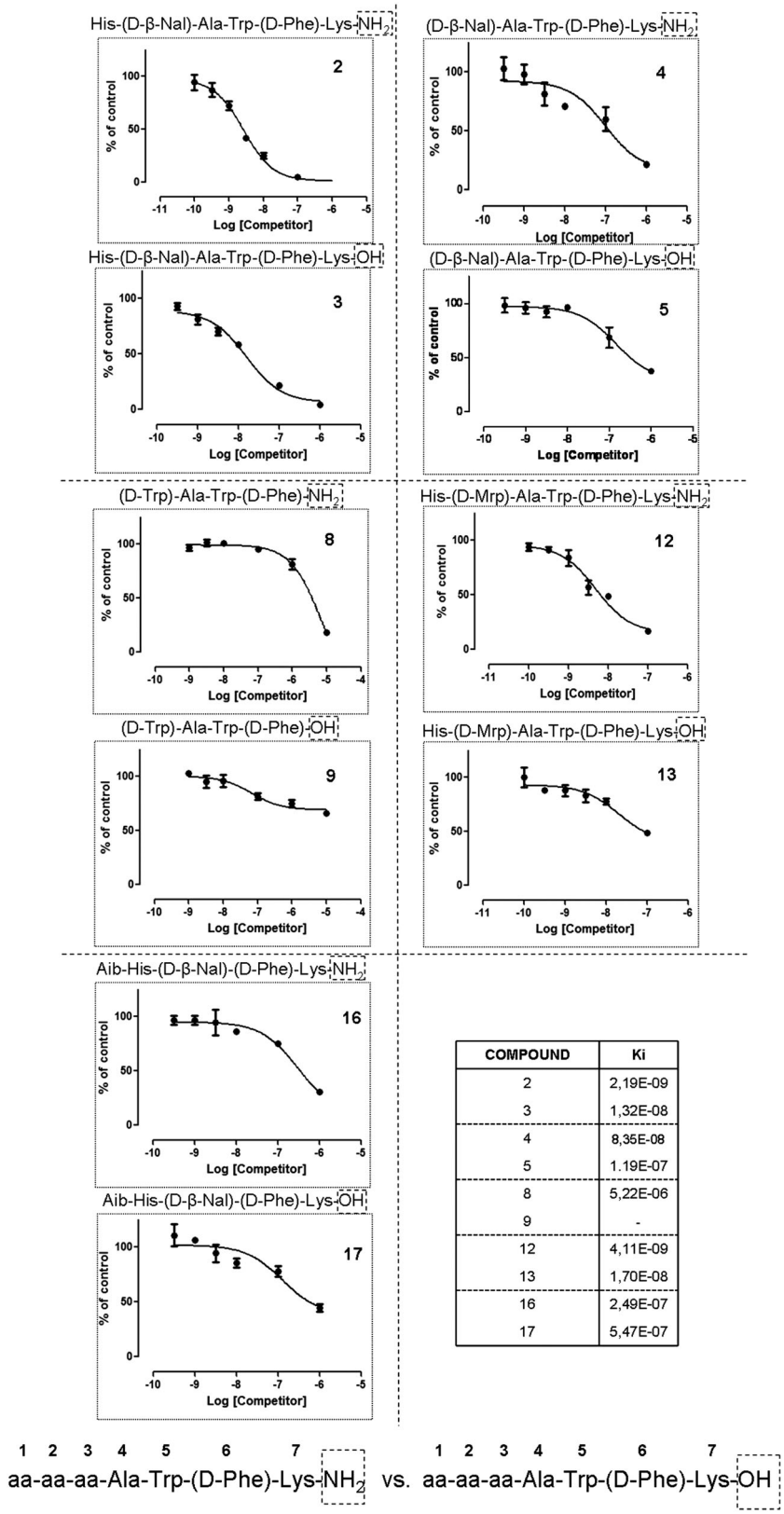


Figure 4. Evaluation of the importance of lysine at final position by competition binding curves to GHSR1a receptor. GHRP-6 (**11**), Ipamorelin FA (**17**) and GHRP-1 (3-7) FA (**5**) have lysine at this position and, GHRP-5 (**10**), Ipamorelin (1-4) FA (**18**) and GHRP-1 (3-6) FA (**6**) do not have amino acids at this position.

Competition binding curves were performed for each compound and Ki values were calculated (Figure 5). Results showed a low Ki value for all compounds with an amide group at final position and a substantially high Ki value in those compounds with a free acid at this position. Even, in the case of compound GHRP-4 FA, it was not possible to calculate the Ki value because the affinity for the receptor was very low. Hence, the amidated carboxyl group appears relevant for a strong interaction.



COMPOUND	Ki
2	2,19E-09
3	1,32E-08
4	8,35E-08
5	1.19E-07
8	5,22E-06
9	-
12	4,11E-09
13	1,70E-08
16	2,49E-07
17	5,47E-07

Figure 5. Relevance in the interaction with the GHSR1a receptor of the exchange of the amide group at C-terminal end position by a free acid. GHRP-1 (2-7) (**2**), GHRP-1 (3-7) (**4**), GHRP-4 (**8**), Hexarelin (**12**) and Ipamorelin (**16**) have an amide group at end position and, GHRP-1 (2-7) FA (**3**), GHRP-1 (3-7) FA (**5**), GHRP-4 FA (**9**), Hexarelin FA (**13**) and Ipamorelin FA (**17**) have a free acid at this position.

Insight into the GHSR1a activity in urine after nasal administration of 500 μg of GHRP-1, GHRP-2, GHRP-6, Hexarelin, and Ipamorelin

Urine samples from male subjects after nasal administration of a dose of 500 μg of five different GHRPs (GHRP-1, GHRP-2, GHRP-6, Hexarelin, and Ipamorelin) were tested by competition binding assay. Competitive binding activity was detected in urine samples for all compounds (Figure 6), The shape of the activity profile resembled the pharmacokinetic profiles obtained by HPLC-MS by Semenistaya *et al.*[20] with the same samples, with activities different from pre-administration even up to 18 hours thereafter for some compounds (GHRP-2 and GHRP-1). Strictly speaking, all compounds presented samples with positive activity although Ipamorelin showed only a sample at time 3 h (74.70% ± 8.53 RSB) which was at the limit of the threshold established by Pinyot *et al.*[37] (76.3% RSB).

Discussion

Due to the variety of GHRPs developed currently by the pharmaceutical industry and the number keeps growing, it is important to study the chemical groups or amino acids essentially implicated in the interaction with the receptor and GH release. This knowledge could help in the future for the development of compounds with improved therapeutic activity. On the other hand, the knowledge about different routes of administration regarding the increase in the time of excretion of these compounds from the body can be also very interesting.

Some previous studies have been made on the structure of ghrelin to identify the active core required for efficient binding and activation of the receptor.[24,38,39] These studies showed, after examine partially digested ghrelin and synthetic ghrelin derivatives, that the activity is located in the N-terminal penta- or tetra- peptide in which is the n-octanoyl group in serine at position 3.[24,39] The minimal amino acid sequence determined for ghrelin as active core may be regarded as similar to the sequence of most GHRPs and metabolites tested in this study. In fact, according to the results here obtained, the GHRP with common core Ala-Trp-(D-Phe) with best affinity for the receptor seem to be a peptide with the following properties: no more than 6 amino acids (no alanine at position 1 in our scheme); D-alanine, histidine or tyrosine at position 2 (other basic aminoacids not studied); D-β-Nal or D-Trp at position 3 and lysine amidated in C-terminal position. Referring to the last property, previous results obtained in ghrelin derivatives showed that positive charges in C-terminal structures are contributing to the activity possibly in the process of GHSR1a recognition,[34] so this aspect may explain the worse results obtained with the GHRPs which present free acid in C-terminal and therefore neutralization of the Lys positive net charge at this position. Other important small changes which could affect to the affinity for the receptor can be found at the position 3 where a methyl group in the D-tryptophan produces a decrease in this affinity with Ki value higher than those GHRPs without this methyl chemical group.

GHRPs and several of the peptides studied in this work to establish the essential amino acids in the interaction with the receptor have been detected as parent compounds or metabolites in urine samples from excretion studies conducted by Semenistaya *et al.*[20] Some of these metabolites have showed affinity for the receptor in our radio-competitive binding assay and therefore is not unexpected that they show receptor activity in excretion urines. A cautionary note on the interpretation of the radio-competitive binding assay here presented is that binding to a specific receptor does not imply always agonist activity. Specifically for GHSR1a, antagonist and inverse agonists are well known, including synthetic and natural other natural substances such as cortistatin and ghrelin variants or other synthetic compounds.[5,36,40]

The routes of administration tested to date for GHRP-2 have been oral and intravenous route. Studies on oral administration have been addressed to determining the levels of GH release, but no GHRP-2 itself. Only in intravenous route it was determined both GH and GHRP-2 levels, establishing the detection window for this secretagogue in about 4.5 h.[10,37] The results shown by Semenistaya *et al.* in which they evaluated different kinds of GHRPs and a new

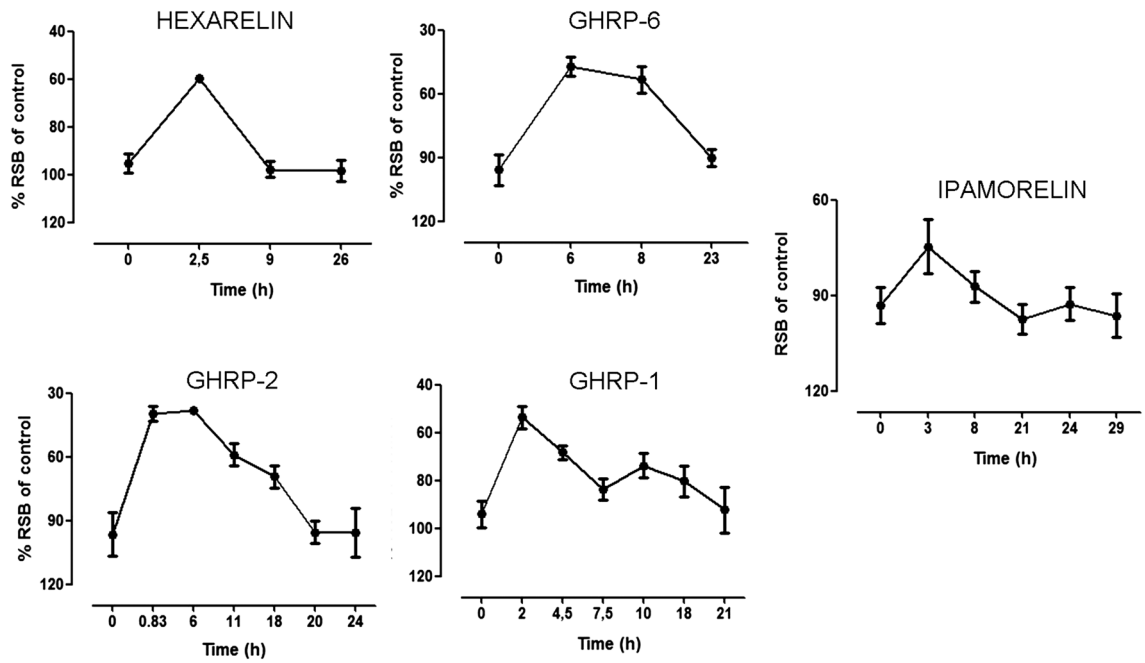


Figure 6. RSB profile in human urine after 500 μg nasal dose of Hexarelin, GHRP-1, GHRP-2, GHRP-6 and Ipamorelin by competition binding assay.

route of administration (nasal), shows the metabolism of other GHRPs and determine that this new route of administration present a detection window which may be larger than others routes, although excretion studies with more participants are needed.[20] Samples from the same excretion studies were evaluated by the radio-competitive binding assay and similar profiles of excretion were obtained with this method when these results were compared with the results obtained by Semenistaya *et al*.[20] from GHRPs and their metabolites by HPLC-MS. In spite of only one subject tested for each compound, it appears that the radio-competitive assay is probably detecting both unchanged GHRPs and those metabolites that retain receptor affinity. A larger detection window was established when the results obtained for GHRP-2 from this nasal administration study were compared with an intravenous study performed by Okano *et al*.[10] with 100 μg of GHRP-2 in 9 subjects, which had been also evaluated by HPLC-MS and by the same radio-competitive method.[37] These data support the idea about an alternative route of administration which could be as effective or even more than other routes previously studied. This nasal route would be minimally invasive in the body and would not need qualified staff to be administered in patients, so would be a great alternative in the use GHRPs for therapeutic or diagnostic purposes. In summary, a study which determines essential amino acids of GHRPs with the common core Ala-Trp-(D-Phe)-Lys in the interaction with the GHSR1a receptor and an understanding of the structure of GHRPs for such activity is presented in this work. These results could help to consider future GHRPs for therapeutic purposes based on inferences from activity obtained from *in vitro* radio-competitive binding results. Furthermore the study on urine samples from an excretion study after nasal administration endorses a new route of administration which appears to ensure greater viability of the compounds in the body and longer detection windows.

Acknowledgements

The authors thank F. Casanueva and R. Smith for HEK293 cells expressing GHS-R1a. This work was carried out with the financial support of the Ministerio de Ciencia e Innovación (DEP2009-09717) and the Catalan Government (DIUE 2009SGR492). The authors express also their gratitude to United State Anti-Doping Agency (USADA) and to the World Anti-Doping Agency (WADA) for the initial previous support to the radio-competitive approach.

References

[1] C. Y. Bowers, F. A. Momany, G. A. Reynolds, A. Hong. On the *in vitro* and *in vivo* activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology*. **1984**, *114*, 1537.

[2] C. Y. Bowers, D. K. Alster, J. M. Frentz. The growth hormone-releasing activity of a synthetic hexapeptide in normal men and short statured children after oral administration. *J. Clin. Endocrinol. Metab*. **1992**, *74*, 292.

[3] G. Muccioli, C. Ghè, M. C. Ghigo, M. Papotti, E. Arvat, M. F. Boghen, M. H. Nilsson, R. Deghenghi, H. Ong, E. Ghigo. Specific receptors for synthetic GH secretagogues in the human brain and pituitary gland. *J. Endocrinol*. **1998**, *157*, 99.

[4] V. Guerlavais, D. Boeglin, D. Mousseaux, C. Oiry, A. Heitz, R. Deghenghi, V. Locatelli, A. Torsello, C. Ghé, F. Catapano, G. Muccioli, J. C. Galleyrand, J. A. Fehrentz, J. Martinez. New active series of growth hormone secretagogues. *Med. Chem*. **2003**, *46*, 1191.

[5] C. Chollet, K. Meyer, A. G. Beck-Sickinger. Ghrelin--a novel generation of anti-obesity drug: design, pharmacomodulation and biological activity of ghrelin analogues. *J. Pept. Sci*. **2009**, *15*, 711.

[6] L. Maletínská, M. Pýchová, M. Holubová, M. Blechová, Z. Demianová, T. Elbert, B. Železná. Characterization of new stable ghrelin analogs with prolonged orexigenic potency. *J. Pharmacol. Exp. Ther*. **2012**, *340*, 781.

[7] J. Nagamine, R. Nagata, H. Seki, N. Nomura-Akimura, Y. Ueki, K. Kumagai, M. Taiji, H. Noguchi. Pharmacological profile of a new orally active growth hormone secretagogue, SM-130686. *J. Endocrinol*. **2001**, *171*, 481.

[8] A. L. Blayo, M. Maingot, B. Aicher, C. M'Kadmi, P. Schmidt, G. Müller, M. Teifel, E. Günther, D. Gagne, S. Denoyelle, J. Martinez, J. A. Fehrentz. New trisubstituted 1,2,4-triazoles as ghrelin receptor antagonists. *Bioorg. Med. Chem. Lett*. **2015**, *25*, 20.

[9] A. Moulin, J. Ryan, J. Martinez, J. A. Fehrentz. Recent developments in ghrelin receptor ligands. *Chem Med Chem*. **2007**, *2*, 1242.

[10] M. Okano, Y. Nishitani, M. Sato, A. Ikekita, S. Kageyama. Influence of intravenous administration of growth hormone releasing peptide-2 (GHRP-2) on detection of growth hormone doping: growth hormone isoform profiles in Japanese male subjects. *Drug Test. Anal*. **2010**, *2*, 548.

[11] WADA. **2015**. *Mailing List*. Available at: https://wada-main-prod.s3.amazonaws.com/resources/files/wada-tdssa-v2.2-en.pdf [Accessed 24 December 2015]

[12] M. Kohler, A. Thomas, H. Geyer, M. Petrou, W. Schänzer, M. Thevis. Confiscated black market products and nutritional supplements with non-approved ingredients analyzed in the Cologne Doping Control Laboratory 2009. *Drug Test. Anal*. **2010**, *2*, 533.

[13] A. Thomas, M. Kohler, J. Mester, H. Geyer, W. Schänzer, M. Petrou, M. Thevis. Identification of the growth-hormone-releasing peptide-2 (GHRP-2) in a nutritional supplement. *Drug Test. Anal*. **2010**, *2*, 144.

[14] WADA. The 2015 Prohibited List, Available at: https://www.wada-ama.org/en/resources/science-medicine/prohibited-list [Accessed 24 December 2015]

[15] M. L. Constanzer, C. M. Chavez-Eng, B. K. Matuszewski. Determination of a novel growth hormone secretagogue (MK-677) in human plasma at picogram levels by liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry. *J. Chromatogr. B Biomed. Sci. Appl*. **1997**, *693*, 131.

[16] M. Okano, M. Sato, A. Ikekita, S. Kageyama. Determination of growth hormone secretagogue pralmorelin (GHRP-2) and its metabolite in human urine by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom*. **2010**, *24*, 2046.

[17] A. Thomas, S. Höppner, H. Geyer, W. Schänzer, M. Petrou, D. Kwiatkowska, A. Pokrywka, M. Thevis. Determination of growth hormone releasing peptides (GHRP) and their major metabolites in human urine for doping controls by means of liquid chromatography mass spectrometry. *Anal. Bioanal. Chem*. **2011**, *401*, 507.

[18] M. Thevis, A. Thomas, W. Schänzer. Doping control analysis of selected peptide hormones using LC–MS(/MS). *Forensic Sci. Int*. **2011**, *213*, 35.

[19] M. Thevis, A. Thomas, V. Pop, W. Schänzer. Ultrahigh pressure liquid chromatography–(tandem) mass spectrometry in human sports drug testing: Possibilities and limitations. *J. Chromatogr. A*. **2013**, *1292*, 38.

[20] E. Semenistaya, I. Zvereva, A. Thomas, M. Thevis, G. Krotov, G. Rodchenkov. Determination of growth hormone releasing peptides metabolites in human urine after nasal administration of GHRP-1, GHRP-2, GHRP-6, Hexarelin, and Ipamorelin. *Drug Test. Anal*. **2015**. DOI:10.1002/dta.1787.

[21] A. Pinyot, Z. Nikolovski, J. Bosch, J. Segura, R. Gutiérrez-Gallego. On the use of cells or membranes for receptor binding: growth hormone secretagogues. *Anal. Biochem*. **2010**, *399*, 174.

[22] J. P. Leyris, T. Roux, E. Trinquet, P. Verdié, J. A. Fehrentz, N. Oueslati, S. Douzon, E. Bourrier, L. Lamarque, D. Gagne, J. C. Galleyrand, C. M'kadmi, J. Martinez, S. Mary, J. L. Banères, J. Marie. Homogeneous time-resolved fluorescence-based assay to screen for ligands targeting the growth hormone secretagogue receptor type 1a. *Anal. Biochem*. **2011**, *408*, 253.

[23] Y. Liu, X. X. Shao, L. Zhang, G. Song, Y. L. Liu, Z. G. Xu, Z. Y. Guo. Novel bioluminescent receptor-binding assays for peptide hormones: using ghrelin as a model. *Amino Acids*. **2015** [Epub ahead of print]. DOI:10.1007/s00726-015-2009-y.

[24] P. Cassoni, M. Papotti, C. Ghè, F. Catapano, A. Sapino, A. Graziani, R. Deghenghi, T. Reissmann, E. Ghigo, G. Muccioli. Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. *J. Clin. Endocrinol. Metab*. **2001**, *86*, 1738.

[25] M. A. Bednarek, S. D. Feighner, S. S. Pong, K. K. McKee, D. L. Hreniuk, M. V. Silva, V. A. Warren, A. D. Howard, L. H. Van Der Ploeg, J. V. Heck. Structure-function studies on the new growth hormone-releasing

peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *J. Med. Chem.* **2000**, *43*, 4370.

[26] J. J. Li, H. Wang, F. Qu, C. Musial, J. A. Tino, J. A. Robl, D. Slusarchyk, R. Golla, R. Seethala, K. Dickinson, L. Giupponi, G. Grover, P. Sleph, N. Flynn, B. J. Murphy, D. Gordon, M. Kung, R. Stoffel. Tetrahydroisoquinoline 1-carboxamides as growth hormone secretagogues. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1799.

[27] Z. Lu, J. R. Tata, K. Cheng, L. Wei, W. W. Chan, B. Butler, K. D. Schleim, T. M. Jacks, G. Hickey, A. A. Patchett. Highly potent growth hormone secretagogues. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3657.

[28] A. S. Hernández, P. T. Cheng, C. M. Musial, S. G. Swartz, R. J. George, G. Grover, D. Slusarchyk, R. K. Seethala, M. Smith, K. Dickinson, L. Giupponi, D. A. Longhi, N. Flynn, B. J. Murphy, D. A. Gordon, S. A. Biller, J. A. Robl, J. A. Tino. Discovery, synthesis, and structure-activity studies of tetrazole based growth hormone secretagogues. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5928.

[29] J. Li, S. Y. Chen, S. Tao, H. Wang, J. J. Li, S. Swartz, C. Musial, A. A. Hernandez, N. Flynn, B. J. Murphy, B. Beehler, K. E. Dickinson, L. Giupponi, G. Grover, R. Seethala, P. Sleph, D. Slusarchyk, M. Yan, W. G. Humphreys, H. Zhang, W. R. Ewing, J. A. Robl, D. Gordon, J. A. Tino. Design and synthesis of tetrazole-based growth hormone secretagogue: the SAR studies of the O-benzyl serine side chain. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1825.

[30] W. McCoull, P. Barton, A. Broo, A. J. H. Brown, D. S. Clarke, G. Coope, R. D. M. Davies, A. G. Dossetter, E. E. Kelly, L. Knerr, P. MacFaul, J. L. Holmes, N. Martin, J. E. Moore, D. Morgan, C. Newton, K. Österlund, G. R. Robb, E. Rosevere, N. Selmi, S. Stokes, T. S. Svensson, V. B. K. Ullah, E. J. Williams. Identification of pyrazolo-pyrimidinones as GHS-R1a antagonists and inverse agonists for the treatment of obesity. *Med Chem Comm.* **2013**, *4*, 456.

[31] F. A. Momany, C. Y. Bowers, G. A. Reynolds, D. Chang, A. Hong, K. Newlander. Design, synthesis, and biological activity of peptides which release growth hormone in vitro. *Endocrinology*. **1981**, *108*, 31.

[32] N. Boutard, A. G. Jamieson, H. Ong, W. D. Lubell. Structure-activity analysis of the growth hormone secretagogue GHRP-6 by alpha- and beta-amino gamma-lactam positional scanning. *Chem. Biol. Drug Des.* **2010**, *75*, 40.

[33] L. Maletínská, R. Matyšková, J. Maixnerová, D. Sýkora, M. Pýchová, A. Spolcová, M. Blechová, J. Drápalová, Z. Lacinová, M. Haluzík, B. Zelezná. The Peptidic GHS-R antagonist [D-Lys(3)]GHRP-6 markedly improves adiposity and related metabolic abnormalities in a mouse model of postmenopausal obesity. *Mol. Cell. Endocrinol.* **2011**, *343*, 55.

[34] P. Ferro, R. Gutiérrez-Gallego, J. Bosch, M. Farré, J. Segura. Fit-for-purpose radioreceptor assay for the determination of Growth Hormone Secretagogues in urine. J. Biomol. Screen. 2015, doi: 1087057115594590

[35] S. D. Feighner, A. D. Howard, K. Prendergast, O. C. Palyha, D. L. Hreniuk, R. Nargund, D. Underwood, J. R. Tata, D. C. Dean, C. P. Tan, K. K. McKee, J. W. Woods, A. A. Patchett, R. G. Smith, L. H. Van der Ploeg. Structural requirements for the activation of the human growth hormone secretagogue receptor by peptide and nonpeptide secretagogues. *Mol. Endocrinol.* **1998**, *12*, 137.

[36] B. Callaghan, J. B. Furness. Novel and conventional receptors for ghrelin, desacyl-ghrelin, and pharmacologically related compounds. *Pharmacol. Rev.* **2014**, *66*, 984.

[37] A. Pinyot, Z. Nikolovski, J. Bosch, G. Such-Sanmartín, S. Kageyama, J. Segura, R. Gutiérrez-Gallego. Growth hormone secretagogues: out of competition. *Anal. Bioanal. Chem.* **2012**, *402*, 1101.

[38] M. Matsumoto, Y. Kitajima, T. Iwanami, Y. Hayashi, S. Tanaka, Y. Minamitake, H. Hosoda, M. Kojima, H. Matsuo, K. Kangawa. Structural similarity of ghrelin derivatives to peptidyl growth hormone secretagogues. *Biochem. Biophys. Res. Commun.* **2001**, *284*, 655.

[39] M. Matsumoto, H. Hosoda, Y. Kitajima, N. Morozumi, Y. Minamitake, S. Tanaka, H. Matsuo, M. Kojima, Y. Hayashi, K. Kangawa. Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. *Biochem. Biophys. Res. Commun.* **2001**, *287*, 142.

[40] M. Volante, R. Rosas, E. Allia, R. Granata, A. Baragli, G. Muccioli, M. Papotti. Somatostatin, cortistatin and their receptors in tumours. *Mol. Cell. Endocrinol.* **2008**, *286*, 219.