Review

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Ghrelin – a novel generation of anti-obesity drug: design, pharmacomodulation and biological activity of ghrelin analogues

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Ghrelin is a unique bioactive peptide with respect to both the structure and its biological function. This 28-amino acid peptide is modified with an *n*-octanoyl group at serine-3, and accordingly is the only lipidated biologically active peptide hormone known so far. Ghrelin binds to the so-called ghrelin or GHS receptor, a member of the class A of G-protein coupled receptors, which leads to Ca^{2+} release intracellularly due to the activation of the Gq-system. Interestingly, the ghrelin receptor shows a significant constitutive activity which means that in addition to agonists and antagonists, inverse agonists play an important role in receptor modulation. In this review, the major activities of ghrelin are summarized with a strong focus on the regulation of food intake. So far reported agonists, antagonists and inverse agonists are shown and structure activitiy relationships are discussed. Furthermore, the application of ghrelin ligands as novel anti-obesity drugs is outlined and the state of the art in this field is summarized. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: ghrelin; GHS receptor; structure-activity studies; obesity; inverse agonist; peptide drug

Introduction

Obesity

Obesity is the fastest growing health problem in the western world. In the United States, obesity will soon overtake tobacco (435 000 deaths) as cause of preventable mortality (365 000 deaths in the year 2000) and already has overtaken alcohol consumption (85 000 deaths), infectious diseases (75 000) and motor vehicle crashes (43 000) [1]. In Europe, an increasing part of the adult population is overweight [body mass index (BMI) $>25\ kg/m^2$] and obesity affects up to one-third of the adult population (BMI $>30\ kg/m^2$) [2]. The average BMI in Europe is approximately $26.5\ kg/m^2$. If prevalence increases at the current rate, about 150 million adults in Europe are likely to be obese by 2010. In addition, childhood obesity is an acute health crisis. In Europe, about 20% of the children are overweight, and one-third of them are obese.

Once considered a problem only in high-income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries, particularly in urban settings. World Health Organization's (WHO) latest projections indicate that globally, in 2005, around 1.6 billion adults (age 15+) were overweight, at least 400 million adults were obese and at least 20 million children under the age of 5 years were overweight. Moreover, WHO further projects that by 2015, approximately 2.3 billion adults will be overweight and more than 700 million will be obese.

This would create a major economic burden through loss of productivity and income, and would consume 2–8% of overall health care budgets. Indeed, obesity has become one of the greatest public health challenges of the 21st century.

So far, therapy approaches are available in terms of bariatric surgery and a few pharmaceuticals with low efficacy. Actually, there are only few drugs available for obesity treatment and they are characterised by (i) low efficacy (approximately 5% weight reduction) [3], (ii) poor tolerance and/or (iii) side effects. The only efficient treatment for obesity so far is the bariatric surgery, which is also very effective against type 2 diabetes. GI bypass operations permit a mean weight reduction of 35–40%, and benefits are observed for up to 10 years [4]. However, the risks of surgery are considerable and the surgery benefits are not permanent as a cumulative reoperation rate of 32% is observed within 7 years after the first surgery. Indeed, there is an urgent need of alternative therapies [5].

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__Abbreviations used:__ AgRP, Agouti-related peptide; BBB, blood-brain barrier; BW, body weight; CNS, central nervous system; CRE, cAMP response element; DHFR, dihydrofolate reductase; DIO, diet-induced obesity; DMPK, drug metabolism and pharmacokinetics; GI, gastrointestinal; GH, growth hormone; ghrl, ghrelin; GHS, growth hormone secretagogues; GHS-R, growth hormone secretagogues receptor (ghrelin receptor); GHRH, growth hormone releasing hormone; GLP1, glucagon-like peptide 1; GPCR, G-protein coupled receptor; HTS, high throughput screening; icv, intracerebroventricular; IGF, Insulin-like growth factor; ip, intraperitoneal; IPGTT, IP glucose tolerance test; IP_3, inositol triphosphate; IUPHAR, International Union of Basic and Clinical Pharmacology; iv, intravenous; KO, knock-out; FI, food intake; FLIPR, Fluorometric Imaging Plate Reader (Calcium accumulation assay); HFD, high fat diet; MSP, modified substance P; NPY, neuropeptide Y; PLC, phospholipase C; POMC, Pro-opiomelanocortine; PK, pharmacokinetic; PP, pancreatic polypeptide; SAR, structure-activity relationship; TM, transmembrane.

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Biography

Constance Chollet was born in Paris in 1979. She graduated from Paris Descartes University, School of Pharmacy in February 2005. Then, she pursued her Ph.D. in Medicinal Chemistry in the School of Pharmacy of the University Paris South, and was graduated in December 2006. From 2007 to 2008, she got a post-doctoral position at University of Dundee as a medicinal chemist. She joined Annette Beck-Sickinger's group at Leipzig University in 2009 and was awarded with a Humboldt research fellowship.

Karolin Meyer (born in 1984) studied biochemistry at Leipzig University (Germany). She did her diploma thesis with Annette Beck-Sickinger in the field of ghrelin research. Currently, she performs her Ph.D. at the University of Zurich (Switzerland) with Michael O. Hottiger on PARP-1. She is a member of the Life Science Zurich Graduate School and participates in the Cancer Biology PhD Program.

Annette G. Beck-Sickinger was born in 1960. She studied Chemistry (diploma) and Biology (diploma) at the University of Tübingen and graduated with Günther Jung. After her habilitation (Biochemistry) and fellowships with Richard Houghten (Scripps Institute and Research Foundation, La Jolla), Ernest Carafoli (ETH Zürich) and Thue Schwartz (University of Copenhagen), she was assistant professor of Pharmaceutical Biochemistry at the ETH Zürich. Since 1999, she is professor of Biochemistry and Bioorganic Chemistry at Leipzig University.

Regulation of Food Intake

Multiple forms of endocrine sensory cells, located in the gut and the pancreas, release mainly peptide hormones. Those peptides are involved in the signalling pathways between the different parts of the GI tract and between the GI tract and the CNS. They mediate a short- and long-term regulation of hunger and satiety, FI, energy homeostasis and BW. Leptin and insulin are long-term adiposity signals, which indicate the size of adipose tissue to the brain and regulate glucose homeostasis [6,7]. Other peptides are direct satiety signals with a short-term effect on appetite regulation. The most important peptides, PYY_{3-36}, oxyntomodulin, GLP1 and PP [8,9] are all anorexigenic signals. A counterpart mediating orexigenic signalling has been unknown for a long time until the identification of ghrelin.

Discovery of Ghrelin

The discovery of ghrelin has been the achievement of a remarkable example of a reverse pharmacology approach [10]. In the eighties, some synthetic opioid-like peptides were found to be able to release GH from isolated pituitary cells, without activating the GHRH receptor or involving the release of other pituitary hormones [11]. Thus, research groups and companies, interested in the discovery of new GH stimulating agents, widely developed peptidic and non-peptidic GHS molecules [12]. It led, in 1996, to the identification of the putative receptor of these GHS peptides, the GHS-R, by reverse pharmacology [13]. Finally in 1999, following an orphan receptor strategy, Kojima *et al*. isolated the GI peptide ghrelin from rat stomach and identified it as the endogenous ligand of the GHS-R [14].

Ghrelin rapidly appeared to have numerous other functions than GH release stimulation and mainly to be involved in appetite regulation and energy homeostasis. It is currently the only endogenous orexigenic signal known from the GI tract, and thus, a remarkable lead for the development of new anti-obesity agents.

With this review, we want to provide an overview on ghrelin pharmacology, regulation and its role in energy homeostasis and appetite. A particular highlight will be made on the different families of ghrelin analogues, such as agonists, antagonists and inverse agonists of ghrelin, as well as their SAR studies.

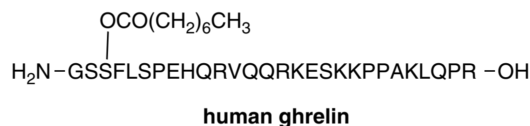

Figure 1. Sequence und lipidisation of ghrelin.

Structure, Distribution and Expression of Human Ghrelin

Ghrelin is a 28-amino acids peptide, *n*-octanoylated on serine-3, isolated in 1999 from rat stomach (Figure 1) [14].

Ghrelin is mainly produced by the X/A-cells in the oxyntic mucosa of the stomach [15], but small amounts are also found in the hypothalamus, the pituitary, the thyroid, the pancreas, the kidney, the liver, the heart, the lung and the testes [16].

The human ghrelin gene is located on chromosome 3p25-26 and comprises 5 exons. The first exon is only 20-bp long and encodes the 5′-untranslated region [17,18]. Then, the messenger RNA (mRNA) (transcript A) is transcripted from exons 2–5 and encodes a 117-amino acid precursor called preproghrelin. Preproghrelin is cleaved into proghrelin (1–94), and further into desacyl-ghrelin (1–28) [19]. The enzyme responsible for the proteolytic cleavage of preproghrelin remains unknown; thus, recent studies suggest that proghrelin is cleaved by the endoprotease prohormone convertase 1/3 in mouse stomach [20,21].

Post-translationally, the *n*-octanoylation of serine-3 is achieved by ghrelin-*O*-acyltransferase (GOAT) [22,23]. GOAT was recently shown to be restricted in its effect to the stomach and the pancreas, but not in its function concerning the type of fatty acids [23]. The post-translational *n*-octanoylation of ghrelin had never been observed on any other known endogenous peptides. It is essential for binding GHS-R as des-acyl ghrelin is a weak ligand of GHS-R [24], and it is also important for ghrelin circulation in blood [25].

Ghrelin has also been identified in many species belonging to the mammals, birds, fish and amphibians, and its sequence is well conserved throughout the evolution [26].

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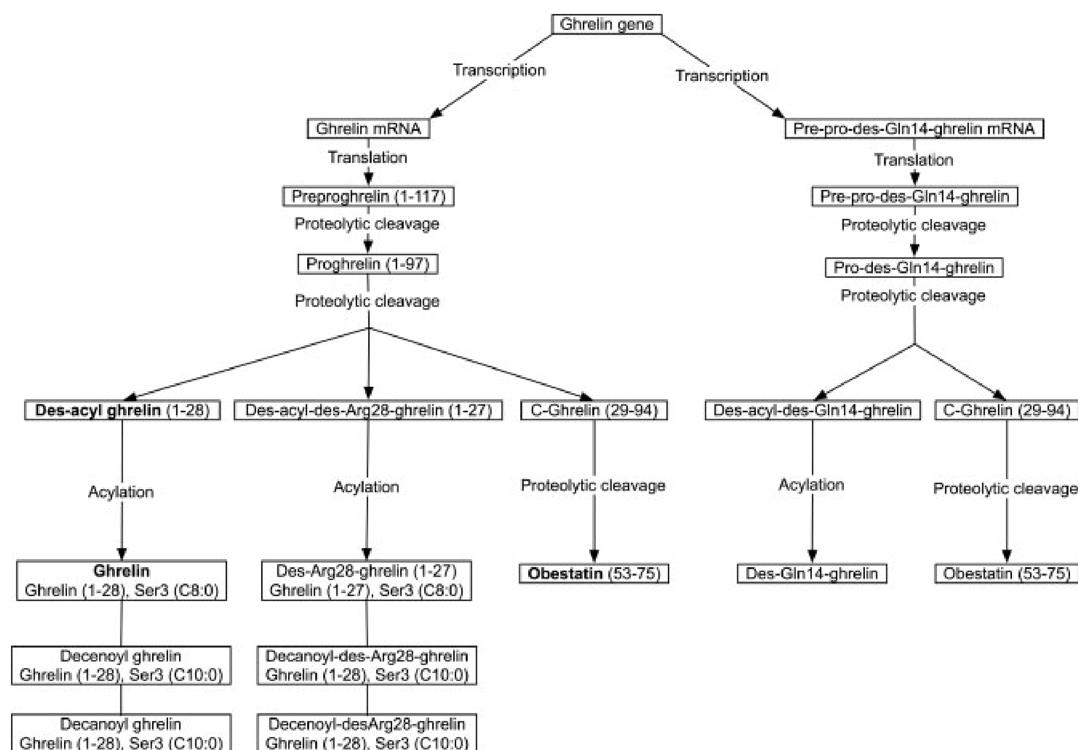

Figure 2. Ghrelin gene-derived peptides in humans.

Ghrelin Gene-derived Peptides

The ghrelin gene can generate, besides ghrelin, various bioactive molecules such as des-acyl ghrelin and obestatin obtained from (i) two transcription initiation sites, (ii) alternative splicing events and (iii) various post-translational modifications [19,27–30]. Their biological activities are still unclear and highly discussed [31].

In some mammals (rat, mouse, pig), a ghrelin peptide lacking glutamine-14, des-Gln14-ghrelin, has been isolated; it is found in negligible amount even in human stomach [27]. In rat, ghrelin(1–27) lacking the *C*-terminal arginine-28 has also been identified, but is present at very low level in rat stomach [28]. Interestingly, another peptide, obestatin, has been extracted from rat stomach [30]. It is derived from the *C*-terminal 66 amino acids portion of proghrelin (C-Ghrelin(29–94)) that is alternatively spliced into obestatin. Its sequence corresponds to 23 amino acids fragment (53–75) of preproghrelin.

Although in humans the post-translational acylation of serine-3 occurs mainly with an *n*-octanoyl group (C8 : 0) and leads to ghrelin, even the fattyacyl groups decanoyl (C10 : 0) or decenoyl (C10 : 1) are introduced [28] (Figure 2).

In summary, ghrelin analogues identified in humans are octanoyl-ghrelin(1–28), octanoyl-ghrelin(1–27), decanoyl-ghrelin(1–28), decanoyl-ghrelin(1–27), decenoyl-ghrelin(1–28), des-acyl-ghrelin(1–28) and des-acyl-ghrelin(1–27). All forms are found in stomach and human plasma, but the major active forms seem to be octanoyl-ghrelin (1–28) and des-acyl-ghrelin (1–28), C-ghrelin (29–94) and obestatin (53–75).

GHS-R – Ghrelin Receptor

The GHS-R was identified in 1996, before the discovery of its endogenous ligand ghrelin [13]. It is well conserved among vertebrate species [32] and belongs to a family of receptors including motilin, neurotensin 1 and 2, neuromedin 1 and 2 and the orphan GPR39 receptors. They all have functions in GI processes [26].

Two different variants of the receptor exist due to alternative splicing. The mRNA consists of two exons. The fully functional variant GHS-R1a is made up from two exons. The shorter variant GHS-R1b is composed of one exon, and lacks the two last transmembrane domains [13]. It does not bind ghrelin or other GHS and its function and ligand are still unknown [16].

The GHSR1a is mainly expressed in the brain, the hypothalamus and the pituitary gland. It is also found in other parts of the brain and peripheral tissues such as spleen, pancreas, stomach and some cells of the immune system. Moreover, its distribution almost always matches with ghrelin distribution [16,33,34].

The GHS-R is a classical GPCR presenting seven TM helices with an extracellular *N*-terminus and intracellular *C*-terminus. It shows typical conserved sites such as cysteine residues in the first two extracellular loops [26]. The GHS-R, when involved in appetite stimulation, is coupled to the G-protein type of $G_{\alpha q/11}$. Its stimulation results in the activation of PLC and the activation of the CRE. PLC cleaves phophatidylinositol biphosphate into IP_3 and diacylglycerol. Those factors lead to different signalling cascades such as releasing the second messenger Ca^{2+}. The subsequent IP_3 degradation after activation of the receptor and the calcium turnover can be exploited in functional assays. It is an excellent method to display activity and affinity of receptors to their ligands.

A remarkable characteristic of the receptor is its high constitutive and ligand-independent activity, which is around 50% of the activity introduced by its endogenous ligand ghrelin. Therefore, it follows the same functional output as ghrelin, but with a lower intensity. Constitutive activity was demonstrated using the CRE reporter assay and the IP_3 turnover assay, whereas it could not be detected using measurement of intracellular calcium [35]. Other

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receptors of the same family such as the neurotensin receptor 2 display constitutive activity as well [36].

Functions of Ghrelin

Ghrelin was initially identified as an endogenous GHS. Its impact on GH secretion is still of great interest for drug discovery, although the relevance of this physiological role is still investigated [37].

Ghrelin was described to have effects on several body processes such as glucose homeostasis, hormone secretion, GI motility, cell proliferation, cardiovascular, pancreatic, pulmonary and immune functions, memory, reproduction and sleep [20,37,38].

Indeed, from the numerous studies achieved since its discovery, it has clearly emerged that the main function of ghrelin is its central role in the regulation of food intake, energy expenditure and energy homeostasis [39]. It is the only known circulating orexigenic hormone and thus, a target of choice for the development of new obesity treatments.

Ghrelin and Energy Homeostasis

Mode of action: central pathways

The generation of ghrelin (ghrelin$^{-/-}$) and GHS-R (GHS-R$^{-/-}$) ko mice demonstrated that the orexigenic and GH-stimulatory effect of ghrelin were mediated through GHSR1a [40,41]. Produced in the oxyntic mucosa of the stomach, ghrelin binds its receptor GHSR1a mainly located on the membrane of NPY/AgRP neurons in the hypothalamic arcuate nucleus [16]. The subsequent release of NPY and AgRP is a known orexigenic brain signal [42]. NPY activates the orexigenic neurons in the lateral hypothalamic area (LHA) through its receptors Y_1 and Y_5 [43,44]. AgRP is an inverse agonist of the melanocortin receptors MC_4 and MC_3 which are – when activated – responsible for the anorexigenic signalling in the CNS [45,46]. Thus, the POMC neurons are indirectly inhibited by ghrelin [47].

The diffusion of ghrelin into CNS is not yet elucidated but it may occur via either or both hormonal and neural pathways. In a hormonal pathway, circulating ghrelin may directly cross the BBB as BBB is not fully impermeable at the arcuate nucleus [48]. It may also reach its receptor in the area postrema of the hindbrain where there is no BBB. Moreover, small quantities of ghrelin directly produced in the hypothalamus could regulate neurons involved in energy homeostasis [42,47,49]. In the neural pathway, ghrelin's orexigenic signal may be communicated to the hindbrain and the hypothalamus via the vagus nerve [50].

In all cases, it has been shown that the ghrelin receptor is expressed very selectively in brain centres known to be involved in energy homeostasis [34,51]. Ghrelin is involved in the short-term regulation of food intake as well as in the long-term regulation of energy homeostasis. Thus, it can be defined as both a hunger signal and an adiposity signal [39].

Hunger signal

As a hunger signal, ghrelin contributes to preprandial hunger and meal initiation [52]. Pre-meal ghrelin cue is independent from nutrient signal [53], thereby preprandial ghrelin secretion may be stimulated by the sympathetic nervous system, and be a cephalic response [54]. The level of circulating ghrelin strongly correlates to the feeding states in humans and rodents [44,55–58]. Preprandial ghrelin level always increases before meal, either planed or freely taken, and reaches concentrations known to stimulate appetite and food intake [53,59–62]. The duration of prandrial ghrelin suppression is correlated to the amount of ingested calories [63,64]. Interestingly, carbohydrates and proteins suppress ghrelin level more efficiently than lipids [65,66]. Intestinal osmolarity and enteric neural signalling may also contribute to ghrelin suppression.

Adiposity signal

Ghrelin is also involved in the long-term regulation of energy homeostasis. It is the orexigenic counter part of leptin and insulin, and these three peptides are the only known adiposity signals of the organism [39,67]. Adiposity signals communicate the statue of energy stores in the body to the brain and fulfil criteria defined by Schwartz *et al*. [68].

1. Adiposity signal levels in circulation are proportional to body fat content and fluctuate in the same way than these stores. Indeed, ghrelin levels inversely correlate to adiposity [55,69–73], increase with weight loss resulting form low calorie diet, lifestyle modification and various pathophysiological conditions [38,74,75], and decrease with weight gain, forced by over-feeding, HFD, glucocorticoids [76–78]. Finally, GHS-R expression in hypothalamus increases with fasting and chronic food restriction [79].
2. Adiposity signals alter food intake and/or energy expenditure if they are administrated exogenously, and they alter body weight if they are chronically administrated. On the contrary, the opposite effects should be observed if they are blocked. Concerning ghrelin, peripheral or central administration increases short-term food intake, and chronic or repeated infusions increase body weight [44,56,59,60,80]. It affects all pathways related to energy homeostasis such as promotion of adipogenesis, decrease of energy expenditure, fat catabolism [56,81,82]. Interestingly, the opposite is much more controversial and has not been demonstrated yet (it will be discussed later in Section on Ghrelin and Obesity). In fact, it is not clear whether suppression of ghrelin decreases appetite and/or body weight. Studies on ghrelin and GHS-R ko mice (ghrl$^{-/-}$ and GHS-R$^{-/-}$) described neither major phenotypic differences, nor alteration of spontaneous food intake comparing to wild-type mice [41,83,84]. However, ghrl$^{-/-}$ and GHS-R$^{-/-}$-mice exposed to a HFD, were protected from weight gain and presented a decreased adiposity and increased energy expenditure compared to wild mice [85,86]. In addition, both null-mice strains overused fat as fuel source toward other metabolic substrate and tended to have a higher lean body mass than wild mice [84,86]. But those observations were recently questioned by a study on a different strain of ghrl^{-}/$^{-}$ and GHS-R^{-}/$^{-}$-mice [87]. It appeared that protection against DIO in null-mice might be more related to strain or age than to ghrelin deficiency. Moreover, in the same study, null- and wild-type mice under caloric restriction presented no differences in weight loss nor in protection to weight regain after weight loss. It has been hypothesised that compensatory physiologic pathways may be created during development; however, further studies need to be performed to determine the real impact of a ghrelin deficiency. This is particularly important in regard to the development of ghrelin inhibitor against obesity.
3. Adiposity signals enter the CNS proportionally to their plasma level, and they target brain centres known to regulate body weight. Accordingly, as previously described, ghrelin main

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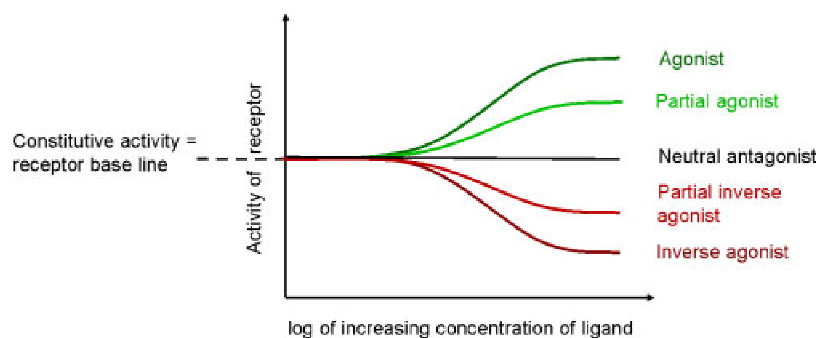

Figure 3. Diagram of theoretical concentration-response curves of different ligands in a constitutively active system. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

targets are NPY/AgRP neurons in the hypothalamic arcuate nucleus (see Section on Mode of Action).

Ghrelin and Obesity

Although ghrelin levels are low in obese subjects [73], weight loss is correlated to an increase of ghrelin. Several studies showed that weight loss resulting from low-calories diet (i) rises ghrelin levels [88]; (ii) increases expression of GHS-R in hypothalamus as well as hypothalamic response to GHS-R agonist [89,90] and (iii) enhances the sensitivity to the orexigenic action of ghrelin [91]. In addition, genetic studies in human showed a correlation between polymorphisms in the ghrelin gene and the risk or protection for developing obesity [92,93], and also a correlation between GHS-R mutations, and a short stature and obesity [94,95].

Moreover, ghrelin level is decreased or totally suppressed after gastric bypass surgery [88,96–103]. It has been hypothesised that ghrelin reduction contributes to the durable appetite reduction following the surgery, and thus to its success, although it is controversial because ghrelin suppression after bypass is not universally observed, and may be related to the technique used [104–106].

Another important fact is the high level of ghrelin found in subjects suffering from the Prader-Willi syndrome. The patients show an extreme hyperphagia which leads to severe obesity [70,107].

Ghrelin – A Lead for Developing New Anti-obesity Agents

Ghrelin and obesity are correlated, but ghrelin as a drug-target for developing anti-obesity agent is still an open question [108]. As reported above, studies on ko mice and GHS-R ko mice did not show any significant change in the phenotype, meaning that ghrelin suppression may not be essential or could be compensated by other signalling pathways. Only an impairment of glucose homeostasis was observed, which might be a hint to the insulin sensitivity affected by ghrelin [87,109]. However, many studies clearly demonstrated that ghrelin blockade decreases spontaneous food intake and body weight in the short- and long-term. Ghrelin inhibition was achieved by generation of transgenic rat expressing an antisense GHS-R mRNA [40], administration of antighrelin antibodies [80], immunoconjugates [110], RNA spiegelmer oligonucleotides [111] or GHS-R antagonists [112–117].

At least, the physiological relevance of the constitutive activity of GHS-R remains unclear. Interestingly, an 8-fold up-regulation of the hypothalamic receptor is observed during fasting [79], and may suggest an effect on food intake. In addition, a natural but very rare mutation in the aromatic cluster from phenylalanine in TM-VI to leucine shows an appearance of short stature and obesity [118]. It was hypothesised that a constant activation of the receptor itself causes a permanent perhaps subliminal appetite stimulation. It could be a reason for appetite and food intake between meals [36]. This highlights the major interest in development of drugable ghrelin inhibitors.

Inversely, the central role of ghrelin in appetite regulation and energy homeostasis has revitalised the interest in developing GHSR1a agonists. Agonists, previously called GHS and initially developed to stimulate GH-release, are reconsidered for treatment of cachexia, and disorder in GI motility [59,119,120].

Ghrelin Analogues and Their Biological Activity

Agonism, Antagonism and Inverse Agonism

GPCR can be stimulated by a broad set of ligands. Based on the variety of these ligands, different actions on the receptor can be observed. Ligands can show partial or full agonistic, neutral antagonistic or inverse agonistic function. The latter can occur if the receptor shows ligand-independent signalling. Figure 3 shows a diagram as an example of concentration-response curves displaying the different functions. The point of inflexion is calculated and displays the EC_{50} value, which is the half-maximal effective concentration. It gives information about the affinity of a ligand for a receptor.

According to the IUPHAR receptor nomenclature and drug classification [121], an agonist binds its target receptor and generates a biological response. Conventionally, an agonist increases receptor activity. A full agonist induces a maximal response, as it turns all receptors into the active conformation. An inverse agonist reduces the fraction of receptor in the active conformation, and then reduces the biological response of the receptor. It can occur that some of the receptors are in their active form without induction of agonists. A partial agonist cannot produce the maximum effect.

An antagonist reduces the action of an agonist. A competitive antagonist binds the same binding site or an overlapping region as the agonist, whereas a non-competitive antagonist reduces or prevents the action of an agonist while they can simultaneously bind the receptor. Antagonism may also result from combination with the substance being antagonised (chemical antagonist).

Neutral antagonists are another class of ligands for a constitutively active receptor. The neutral antagonist has the same affinity

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for both active and inactive states of the receptor. Thereby, it binds the receptor without changing its conformation in any direction, and the activity stays at the baseline. To prove the neutral antagonistic function, binding assays have to be applied.

The idea of constitutive activity and, consequently, inverse agonism emerged from experimental studies where drugs showed a reducing effect on the system. Evidence was found first on ionotropic receptors, such as $GABA_A$ receptors [122], but a broad range of GPCRs also underlie these effects [123,124]. Constitutive activity is based on spontaneous change of the receptor between an active and inactive state without the support of any ligand. The active/inactive ratio depends on the receptor itself, but normally the inactive state is overbalanced. The inverse agonist tends to push the receptor further into the inactive state.

Concerning ghrelin, GHSR1a presents one of the strongest constitutive activities ever observed. Indeed, the development of inverse agonists acting on the ghrelin receptor can be of great interest.

Ghrelin Agonists Refer also to GHS

Ghrelin agonists were developed before the discovery of ghrelin and its receptor, in the aim to generate molecules able to activate GH release, independently from the GHRH pathway. Thus, the so-called GHS were widely produced in the 80 s, as peptidic or non-peptidic derivatives [12,125,126].

First, the hexapeptide GHRP-6 **1** was developed by Bowers *et al*. from C-amidated Met- and Leu-enkephalins (Figure 4) [11]. It was a potent amplifier of GH release [127], but it presented a poor biopharmaceutical profile, i.e. specifically a poor oral bioavailability (0.3%) and short *in vivo* half-life (20 min) in humans [125,128,129]. Consequently, new non-peptidic motifs with improved bioavailability were sought, based on SAR derived from GHRP-6 **1** and analogues. The critical requirements for stimulation of GH release appeared to be (i) a basic amine at position 1; (ii) a preference for aromatic amino acid at positions 2, 4 and 5 and (iii) a D-Trp at position 2 [130]. Using those parameters, the screening of Merck chemical sample collection spotted a racemic benzolactam **2** as a potential lead for GH release stimulation (EC_{50} = 3 μM). Further pharmacomodulation of **2** converged in the synthesis of L-692,429 **3**, highly active *in vitro* (EC_{50} = 30 nM) and *in vivo* [131–133]. It succeeded preclinical safety and toxicology studies and was active in human [134,135]. At last, it possessed superior oral bioavailability than GHRP-6 **1**, although its oral bioavailability in dog was less than 5% [136].

In parallel, the spiropiperidine **4** was found to be another privileged structure for stimulating GH release (EC_{50} = 50 nM) [137]. Gain of oral bioavailability was achieved with replacement of the ureiquinuclidine by an aminoisobutyric moiety (compound **5**, EC_{50} = 14 nM). Potency and bioavailability were ultimately optimised in the design of MK-0677 **6** where *O*-benzyl-D-serine replaced D-Trp and containing a methylsulfonyl amido group. MK-0677 was the best GHSR1a agonist so far. It showed very high potency *in vitro* (EC_{50} = 3 nM), high oral bioavailability and long plasma half-life ($T_{1/2}$ in dogs = 4.7 h) [137]. It also efficiently stimulated GH release *in vivo* tested in dogs. In clinical assays, acute oral administration of MK-0677 by humans resulted in a dose-dependent GH secretion [138]. Moreover, MKF-0677 was highly selective. Importantly, the synthesis of the radioligand ^{35}S-MK-0677 led to the identification of the GHS-R and it is now the agonist of reference for binding assays [139].

Other efficient GHS were synthesised in the 90s and went into clinical trials. The peptidomimetic NN703 **8** derived from Ipamorelin **7** was orally bioavailable and dose-dependently increased GH release and the gain of weight in dogs but was less efficient than MK-0677 in humans (Scheme 1) [140,141]. Further pharmacomodulation of NN703 **8** did not succeed in improving *in vivo* efficacy [142,143]. Capromorelin (or CP-429391) **9** was developed with other pyrazolinone–piperidine analogues [50,144]. *In vivo*, it increased GH release and weight gain in dogs in a dose-dependent manner and was selected for clinical studies because of its good pharmacological profile and its oral bioavailability [145]. In recent studies, it was reported that oral administration of capromorelin dose-dependently increased insulin-like growth factor I (IGF-I) concentration in older adults [146]. The oxindole derivative SM130686 **10** was also identified as a potent GH release enhancer and presented a good pharmaceutical profile. Oral administration of **10** dose-dependently increased body weight in rats [147,148]. Moreover, a recent analogue **11**, showed a 7-fold higher binding affinity than ghrelin for GHSR1a (EC_{50} = 0.02 nM *vs* 0.14). It exhibited a partial agonist activity in a Ca^{2+} accumulation assay (functional assay), with 91% of ghrelin maximal response and an EC_{50} = 17 nM. *In vivo*, oral administration of **11** increased body weight in rats more efficiently than **10** at the corresponding doses [149].

More recently, new potent ghrelin agonist structures were identified by HTS. SAR optimisation of an indoline amide series, originally developed as $5\text{-}HT_{1B}$ receptor antagonists, led to an *in vitro* potent GHSR-1a agonist **12** (Scheme 2) [150]. As its high lipophilicity and poor solubility resulted in a low oral exposure a modified analogue **13** was synthesised that was equally potent *in vitro* but presented a good selectivity profile, a low blood clearance (24 ml/min/kg) and an excellent bioavailability (F = 75%) in rats [151]. Moreover, feeding experiments in rats showed an increase in food intake after oral administration of **13**. A close analogue **14** developed lately also gave promising *in vitro* activity and presented an encouraging profile following i.v. dosing, although its poor oral bioavailability required further optimisation [152].

Ghrelin Antagonists

Few ghrelin antagonists were first generated simultaneously to GHS development. [D-Lys-3]-GHRP-6 **15** and [D-Arg-1, D-Phe-5, D-Trp-7, 9, Leu-11]Substance P **16** were the only two peptidic GHSR1a antagonists identified in the nineties (Scheme 3) [153–155], and were used to demonstrate the inhibition of food intake through ghrelin antagonism [112]. In addition, one small non-peptidic GHSR1a antagonist **17** was reported in the literature [154]. As the focus at that time was to develop GH release activators, investigation and biological studies of those antagonists were not pursued. Interest in development of ghrelin antagonist re-emerged with the discovery of the orexigenic role of ghrelin, and thus, the pharmaceutical attraction of synthesising antagonists that may interfere with body weight regulation.

Isoxazole carboxamide and tetralin carboxamide

The isoxazole carboxamide derivative **18** was the first pure competitive ghrelin antagonist reported in the literature, since the discovery of ghrelin. It was isolated from a HTS in Abbott laboratories, aiming to identify ghrelin antagonist motifs [156].

Pharmacomodulation of the lead was initially undertaken at the 5-position of the isoxazole core to improve its efficiency (Scheme 4) [156]. SAR studies showed that elongation (R_1 = Ethyl,

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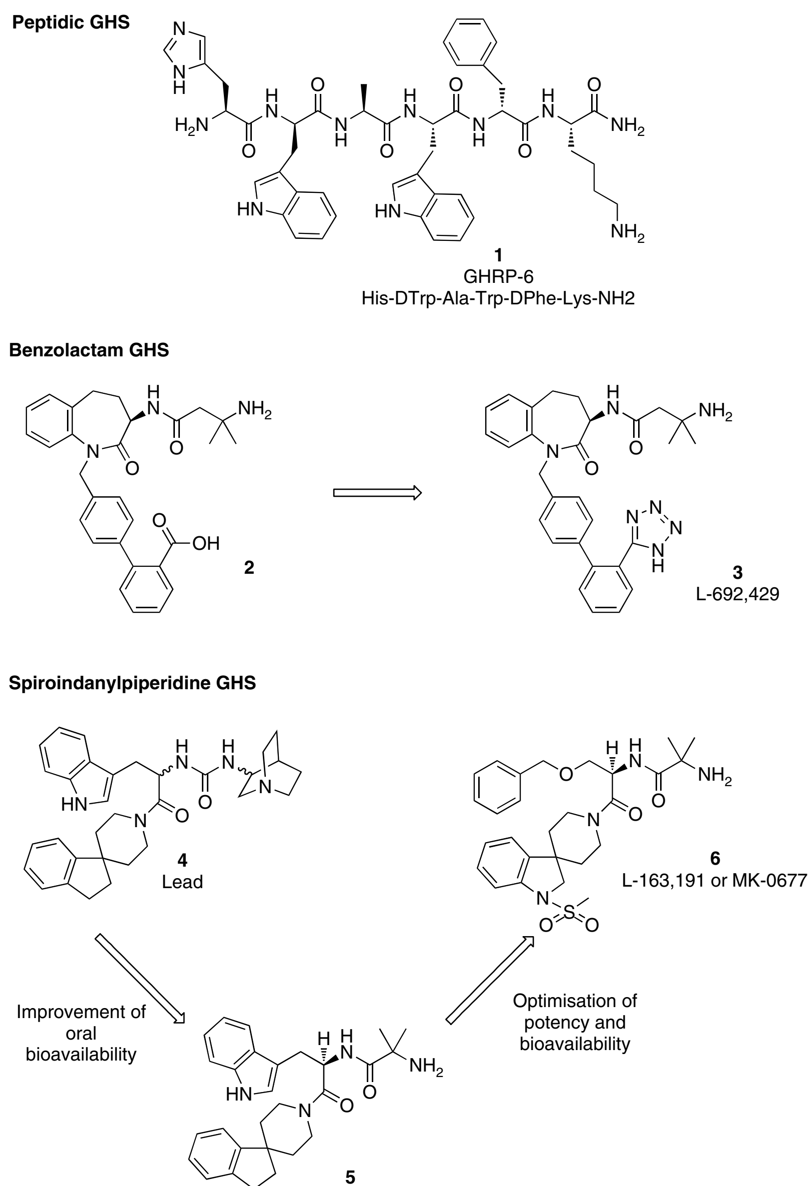
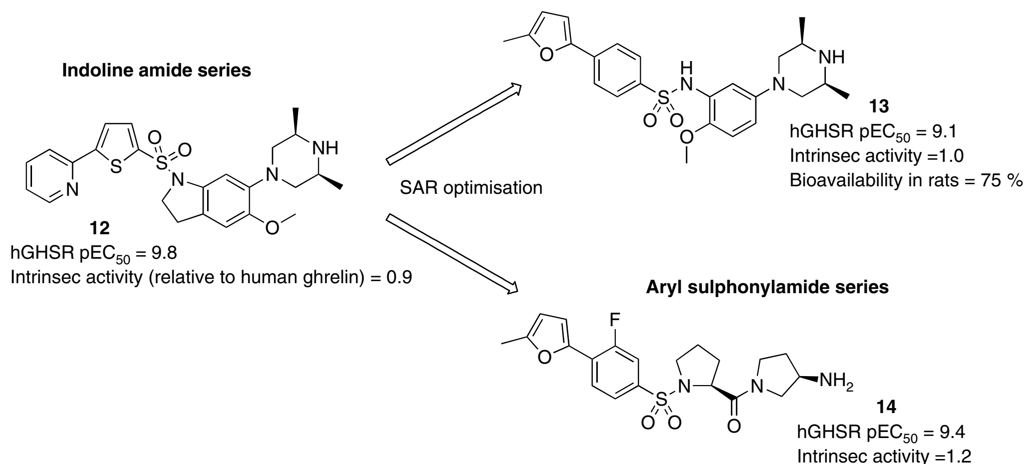


Figure 4. GHS or GHSR1a agonists.



Scheme 1. The most recent GHSR1a agonist motifs.

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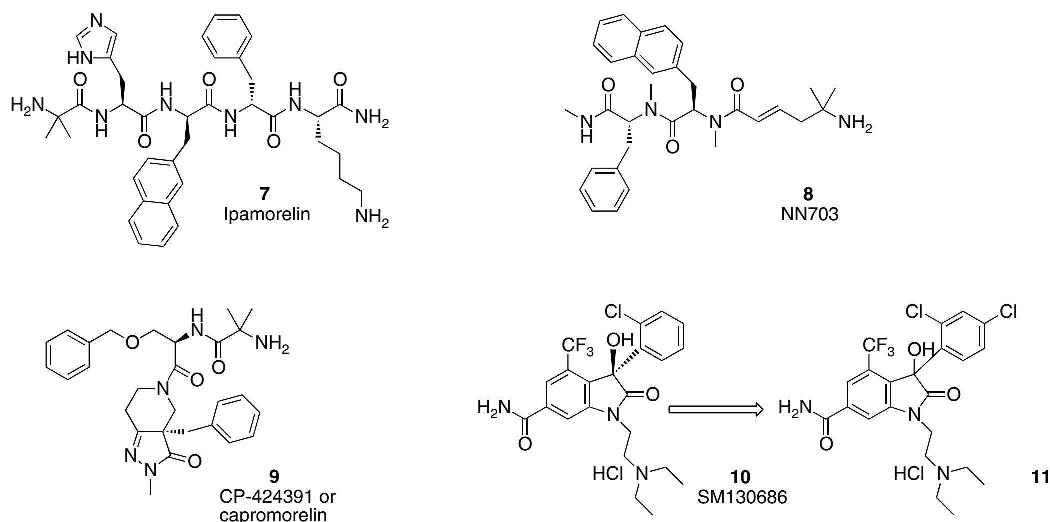
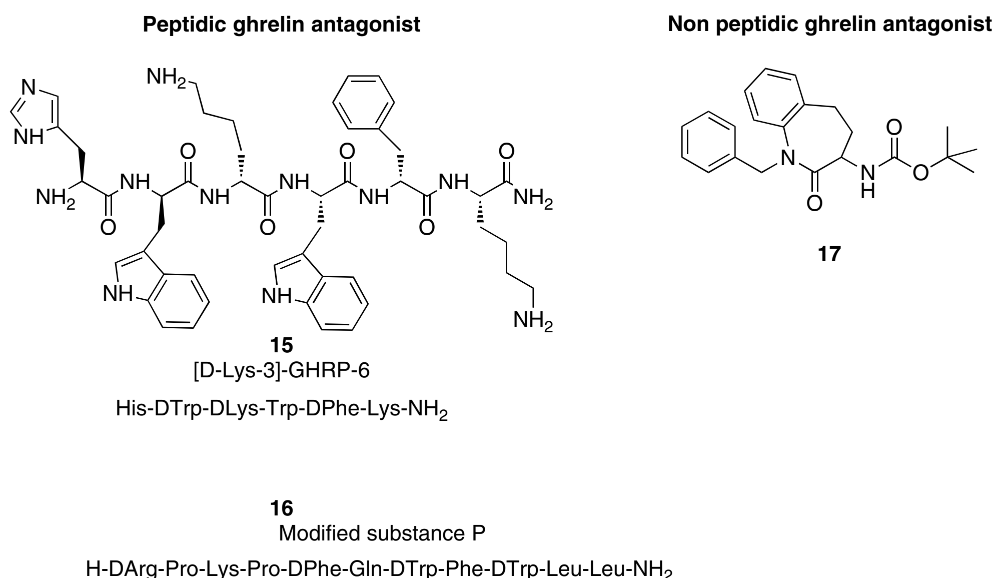


Scheme 2. New GHS.



Scheme 3. First ghrelin antagonists described in the literature.

propyl, isopropyl) and introduction of a hydrophilic moiety (propanol) were tolerated at this position. Introduction of cyclic structures containing H-bond acceptor, led to compound **19** presenting a high binding affinity and antagonist potency (IC_{50}, respectively, of 9 and 8 nM). With the aim to increase aqueous solubility, the phenylenediamine motif was replaced with *trans*-cyclohexyldiamine without any high impact on the potency or affinity of the compounds. The derivative **20** was the most active in this series, with an improved aqueous solubility greater than 1 mg/ml, making it suitable for intracerebroventricular (i.c.v.) injection.

Variation of substituents on dichlorophenyl ring was achieved without any improvement of the activity [157]. Finally, SAR study was pursued on the *N*,*N*-diethylaniline ring. Introduction of an electron-withdrawing group (CN, CF_3) at R_2 was unfavourable, whereas a methyl or ethyl group gave access to high antagonistic potency (Compound **21**, IC_{50} = 39 nM).

Despite an improvement of their potency, the isoxazole carboxamide derivatives remained poorly orally available. Incubation of the lead **18** with rat liver microsomes permitted to identify its major inactive metabolite **22**, produced by des-ethylation of the *N*,*N*-diethylaniline motif. Thus, replacement of the *N*,*N*-diethylaniline was carried out, although it was found to be very sensitive to modification. A broad screening of amide derivatives only identified **23** as a submicromolar active antagonist (Scheme 5).

To overcome the poor stability of isoxazole carboxamides, modification of the isoxazole ring itself was then investigated [158]. Indeed, 5–6 membered rings were found to be tolerated, leading to the conclusion that the main role of isoxazole was to hold A and C rings in the right orientation toward each other. The search for new scaffold, while maintaining the same distance between the A ring and the amide group, identified the tetraline carboxamide **24** as an appropriate hit. Efforts to quartenarise the amide α-carbon to gain stability toward hydrolysis led to the carbamate **25** with improved binding affinity, antagonist potency

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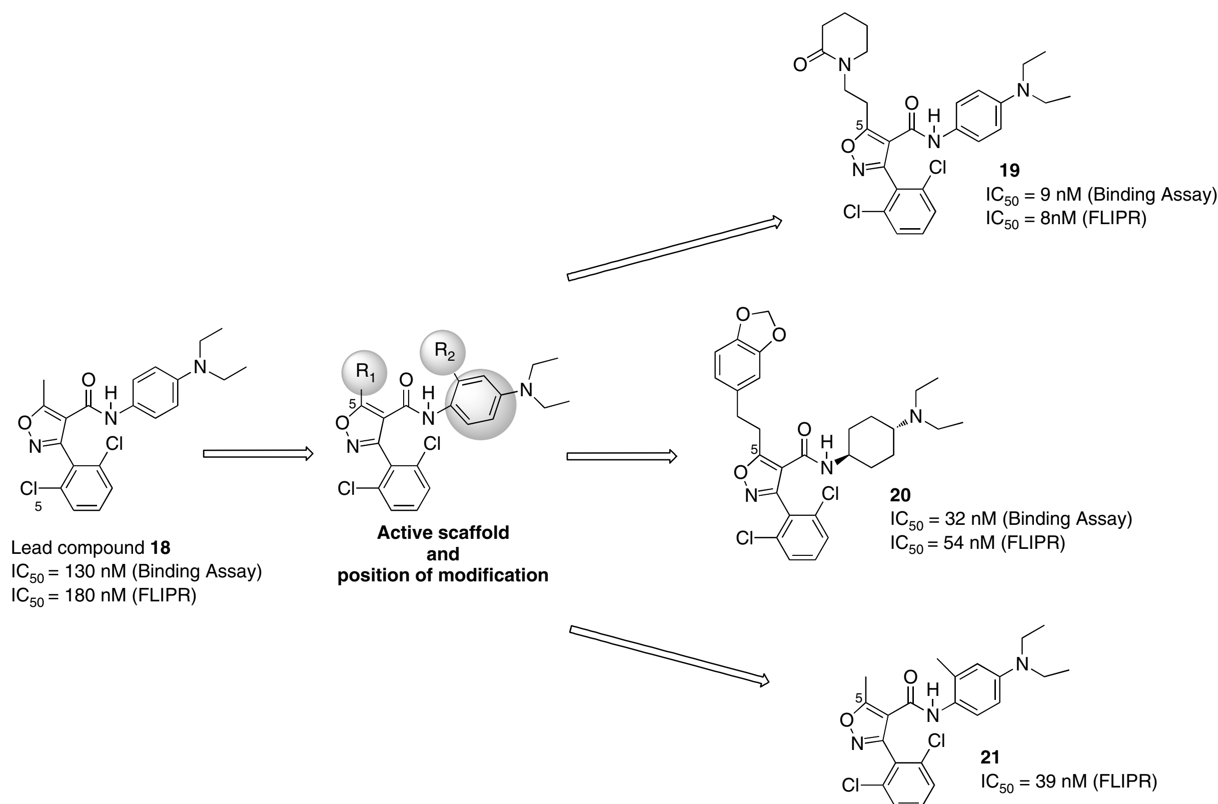
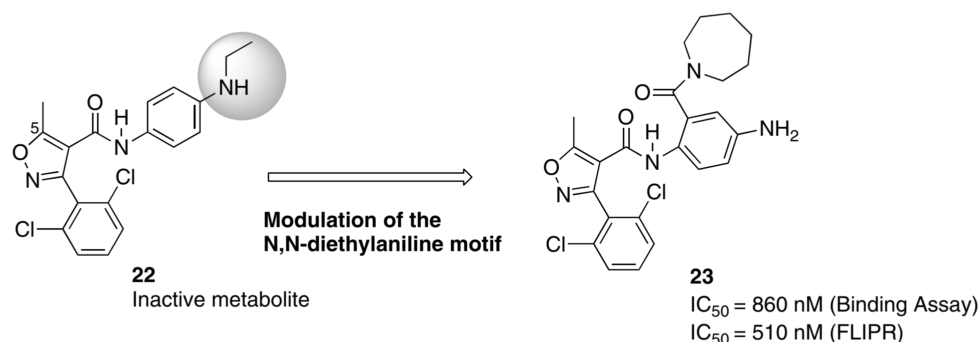


Scheme 4. Isoxazole carboxamide derviatives, pharmacomodulation of the lead.



Scheme 5. Isoxazole carboxamide derivatives, modulation of the *N*,*N*-diethylaniline motif.

(Scheme 6, IC_{50}, respectively, of 16 and 29 nM) and reasonable rat bioavailability (19%). In this series, as for isoxazole carboxamides, further modification or replacement of *N*,*N*-diethylaniline motif did not show any improvement in potency [159].

2,4-Diaminopyrimidine

The 2,4-diaminopyridine lead **26** was identified from a second HTS of Abbott laboratories compound collection (Scheme 7) [160]. An extensive SAR study was then performed with different substitutions on the central phenyl ring (R_2 and R_3). The most active substitution at R_3 appeared to be a benzylamine or benzylether moiety with small hydrophilic group in para position (Scheme 8, compounds **26, 27, 28**). Inversion of the nitrogen and the benzylic carbon atom of the benzylamine (compound **29**) or methylation of the nitrogen (compound **28**) did not significantly modify the antagonistic potency. Thus, this sequence was hypothesised not to be critical for the receptor binding and to act possibly as spacer holding the diaminopyrimidine and the phenyl ring in the right orientation. Halogenation at R_2 was also tolerated.

PK and *in vivo* studies were performed on the more potent compounds. Most of the 2,4-diaminopyridine derivatives presented a good PK profile ($t_{1/2}$ = 5.2 h for **26**, 3.9 h for **27**). Moreover, **26** and **27** presented high brain distribution ($[\mathbf{26}]_{Brain}$ = 2.74 µg/g after 1 h, $[\mathbf{27}]_{Brain}$ = 1.07 µg/g), indicating their ability to cross the BBB. Finally, *in vivo* studies were performed with **27**. i.p. administration of the compound caused a significant decrease of cumulative food intake and body weight in free fed or fasted rats.

The main concern about 2,4-diaminopyridine derivatives was their known toxicity through DHFR inhibition. For this purpose, a comparative *in vivo* study was carried out between **27** and a structurally close analogue **30**, 160-fold less potent for binding GHSR1a but equally potent toward DHFR inhibition and possessing a high brain distribution. As no decrease in food intake was

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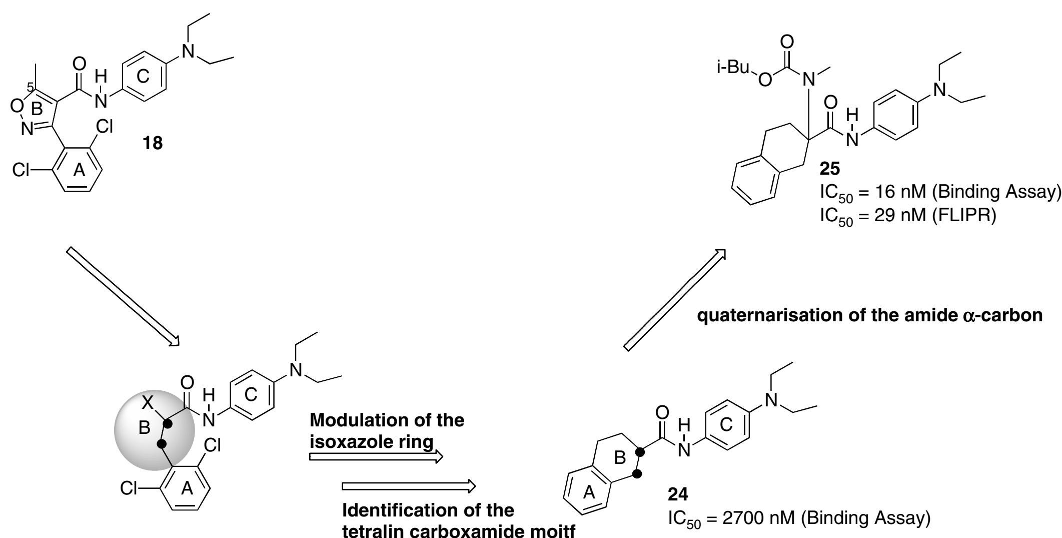
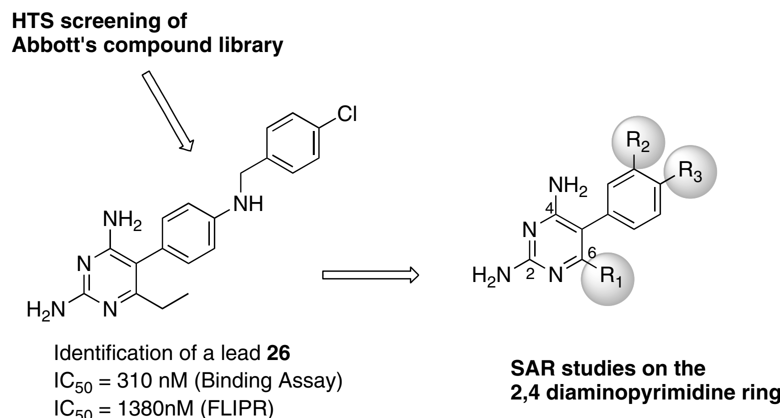


Scheme 6. Tetralin carboxamide derivatives.



Scheme 7. Identification of the 2,4-diaminipyridine motif for ghrelin antagonism.

observed after administration of **30**, it was concluded that **27** decreased food intake and body weight gain through GHSR1a blockade *versus* DHFR inhibition.

To overcome the DHFR selectivity in the 2,4-diaminopyrimidine series, pharmacomodulation of the 2,4-diaminopyrimidine ring itself was then undertaken [161]. Replacement of the pyrimidine totally eliminated GHSR1a activity and substitutions on 2- and 4-nitrogen were unfavourable. The modulation of position 6 was solely tolerated. Replacement of the ethyl group of the initial lead **26**, with different lipophilic structures gave access to potent antagonists. Initially, benzyloxymethyl group was found to maintain or increase the affinity and antagonist potency (for example, **27** *vs* **26**). It was speculated that the initial 6-ethyl group of the first derivatives might occupy the same binding site than octanoyl group of ghrelin. Indeed, this position is essential for binding to the receptor, as demonstrated by the lack of activity of the phenethyl analogue **30**. Further derivation of the position 6 with straight or branched aliphatic chain substituents led to analogues without high improvement of the antagonism potency against GHSR1a neither decrease of DHFR selectivity. Improvement of the selectivity toward DHFR was only observed with bulky substituents such as norborene (**31**) or tetrahydrofuran (**32**). Nevertheless, abolishment of the DHFR inhibition by this series of compounds needs to be pursued.

Quinazolinone

HTS of Bayer's compound library identified a novel GHSR1a ligand, the quinazoline derivative **33** (Scheme 9) [116]. It possessed a nanomolar affinity toward the receptor ($K_i = 36$ nM) but presented agonistic activity in functional activity testing (GTPγS binding assay). *N*-alkylation of the piperidine nitrogen was found to reverse the functional activity profile while maintaining a high affinity toward the receptor and led to the full antagonist **34** ($K_i = 20$ nM). Despite its good potency **34** presented a very low plasma exposure ($C_{max} < 20$ nM at 10 mg/kg, p.p., rat) probably caused by a fast metabolisation of the *N*-methyl-*N*-butyl moiety.

SAR study on different position of the quinazolinone core was achieved to reach favourable DMPK properties. The preferred sequence on R_1 for antagonistic activity was *N*-ethyl substituted methylpiperidine. The potency increased with enantiopure (*S*)-isomers (compound **37** *vs* **35**). Interestingly when the alkyl group was larger, inverse agonistic activity was observed (compound **36**). At R_2, an ortho-substituted phenyl group appeared to be the

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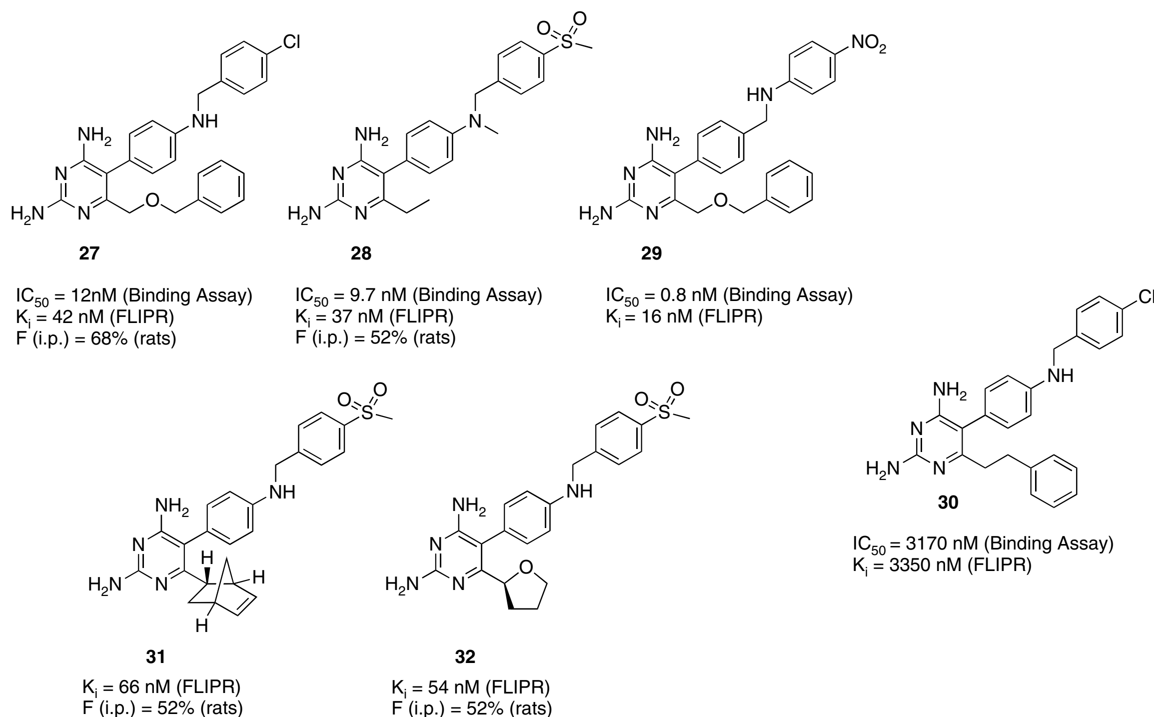

Scheme 8. Pharmacomodulation of the 2,4 diaminopyrimidine ring.

best substituent. Decreasing the size of R_2 moderately decreased the activity of the resulting compounds, while it enhanced their solubility and selectivity toward GHSR1a receptor (Scheme 9 and Table 1, compounds **38** and **39**). At R_3, a bulky substituent was necessary, and the more rigid analogues bore less potential for partial agonism (compound **35**).

Finally, compounds **38** and **39** were selected for *in vivo* studies on rats, as they presented good antagonistic potencies ($K_b = 14$ and 11 nM, respectively), high bioavailability, moderate clearances and high volumes of distribution (see Scheme 9). Both compounds were orally administrated to fasted-re-fed and DIO mice and they significantly decreased food intake and body weight, with **27** being more efficient than **39**. One hypothesis might be the difference in brain exposure of the two compounds with **38** presenting higher concentration in brain than **39**.

Correlation between GHSR1a inhibition and insulin secretion was also studied with quinazolinone derivatives. Indeed, previous studies demonstrated that insulin secretion was inhibited by ghrelin through GHSR1a. Thus, ghrelin antagonists should reverse insulin blockage and have a beneficial effect on glucose homeostasis and treatment of type 2 diabetes. Promotion of glucose-dependent insulin secretion in pancreatic islets was demonstrated *in vitro* for quinazolinone derivatives [113], and was confirmed *in vivo* in an IPGTT in rats. In fact, oral administration of compounds **38** and **39** to rats, subsequent to glucose administration, significantly decreased glucose excursion. Moreover, no hypoglycemia was observed when a high dose of the same compounds was orally administrated to fasted mice. Noticeably, the analogue **40** (Table 1) with a poor mouse brain exposure (1%) had a modest effect on feeding but was fully active in the rat IPGTT model. This is consistent with the hypothesis that ghrelin and ghrelin antagonists influenced the glucose homeostasis peripherally through pancreatic islet.

Nevertheless, quinazolinone derivatives were the first reported ghrelin antagonists being orally available and active *in vivo* on food intake, weight loss and glucose tolerance animal models.

Trisubsituted 1,2,4-triazoles

Trisubsituted 1,2,4-triazoles were developed recently by the teams of J.-A. Fehrentz and J. Martinez. The chemical modulation of a highly potent peptidic GHS **41**, orally active in human, led to the identification of a new active non-peptidic-scaffold containing a 1,2,4-triazole motif (Scheme 10) [115,162,163]. Many derivatives of the scaffold were synthesised and an intensive SAR study toward R_1, R_2 and R_3 modifications was carried out. As shown in Scheme 10, the best active templates were (i) on R_2, a 2 carbon chain bearing a benzyl (**42, 43**), or an indol group (compounds **44,45,46**); (ii) on R_3, a benzyl group, a 4-methoxybenzyl group (**42, 44**) or a 2,4-methoxybenzyl group (compounds **45,46,47**) and (iii) on R_3, an amino acid moiety. Introduction of α-aminoisobutyryl (Aib) moiety led to very potent compounds (**42,44**), but other substitutions such as pyridin-2-ylcarboxyl (**43**), pyrazine-2-carboxyl (**46**), were also tolerated.

Interestingly, the derivatives developed appeared to be agonist, antagonist or partial agonist of GHSR1a, without any clear understanding of the SAR study.

The most potent compounds were tested *in vivo* on rats to measure their influence on food intake. Graded doses of compounds were administrated subcutaneously (s.c.), alone or followed by an administration of hexarelin (N-alkyl derivative of GHRP6 **1**, Figure 4) to stimulate food intake. Some compounds clearly inhibit food intake. Surprisingly, there was no clear correlation between *in vitro* and *in vivo* results. For example, **44** was defined *in vitro* as a partial agonist, but was one of the most active inhibitors of food intake *in vivo*. Compounds **43** and **46** were also able to inhibit hexarelin-stimulated food intake at

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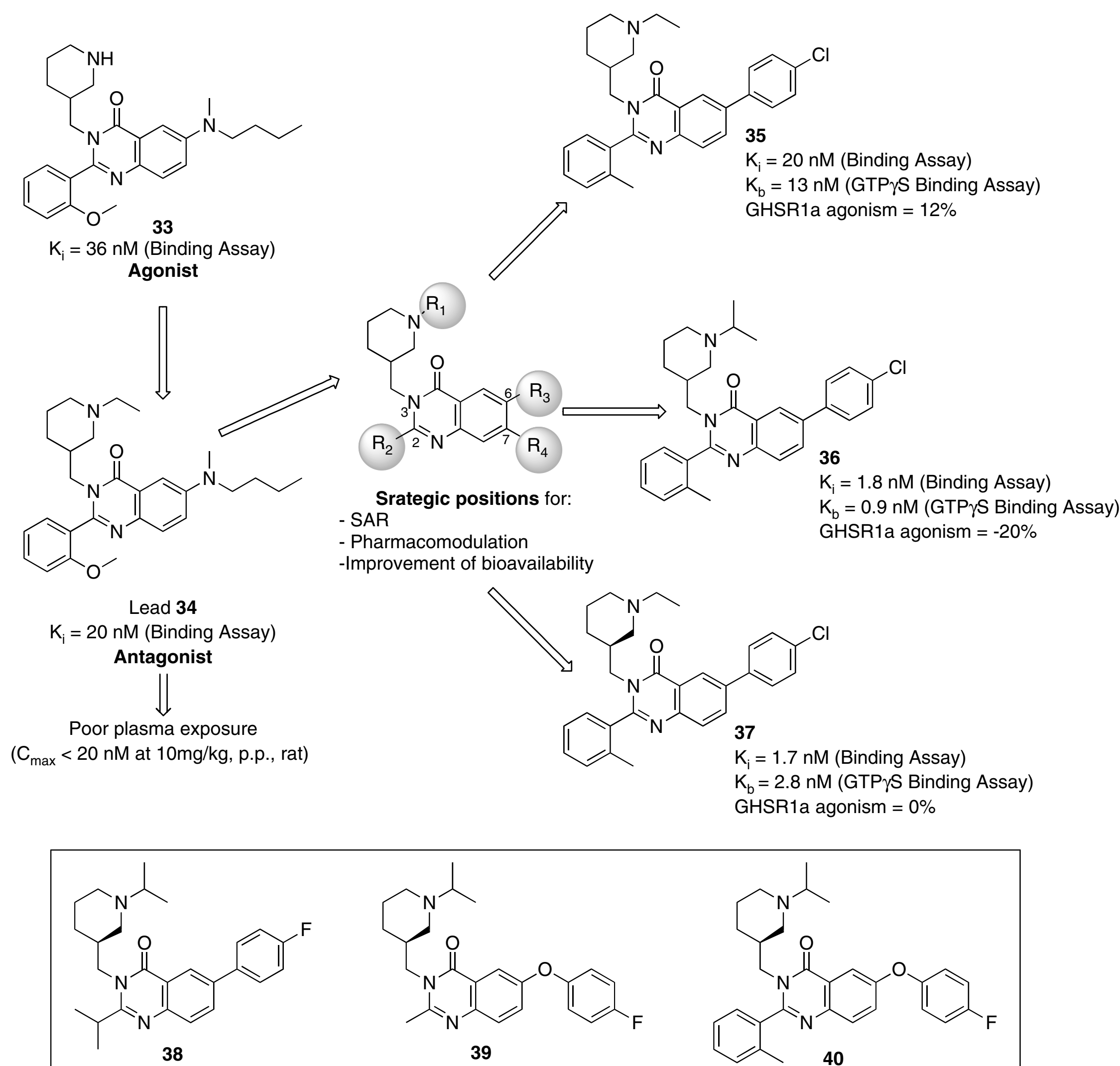

Scheme 9. Quinazolinone series: Identification of the lead 1, pharmacomodulation and SAR studies.

Table 1. PK properties and *in vivo* studies on quinazolinone derivatives

	GHS-R1a binding *Ki* (nM)	GTPγS binding assay (antagonism) *Kb* (nM)	GHSR1a agonism *E*/*E*max (%)	Aqueous solubility (pH 7.2, µM)	Rat PK C_{max} (µM)	Brain level (% of plasma concentration)	Change in food intake (30 mg/kg) (%)	Change in blood glucose (10 mg/kg) (%)
38	27	26	15	280	0.15	76	−17	−20
39	17	11	11	500	13	19	−12	−23
40	0.8	0.2	22	250	0.04	1	−7	−19

really low concentration (47 and 44%, respectively, inhibition of hexarelin-stimulated food intake with 20 µg/kg).

Ghrelin Inverse Agonists: Modified Substance P and Analogues

The GHS-R possesses a naturally high ligand-independent signalling representing 50% of its maximal activity. The physiological importance of this constitutive activity was partially elucidated with the discovery of a naturally occurring mutation that selectively eliminated the constitutive activity without affecting the affinity, potency or efficacy of the endogenous ghrelin. This mutation correlates with the development of short nature and obesity [94,164]. Consequently, synthesis of GHS-R inverse agonists has emerged as an interesting strategy in the development of anti-obesity agents. Indeed, considering the high ligand-independent signalling of GHS-R, a neutral antagonist could have limited benefice compared with an antagonist possessing inverse agonist activity. Hypothetically, a specific inverse agonist able to reduce appetite between meals could be in fact an efficient pharmaceutical for weight reduction. In addition, the blockade of the receptor should increase the sensitivity for inhibitory peptides such as leptin and insulin [165].

Since the discovery of GHS-R, the modified substance P **16** (MSP) was one of the first peptidic motifs identified as a ghrelin

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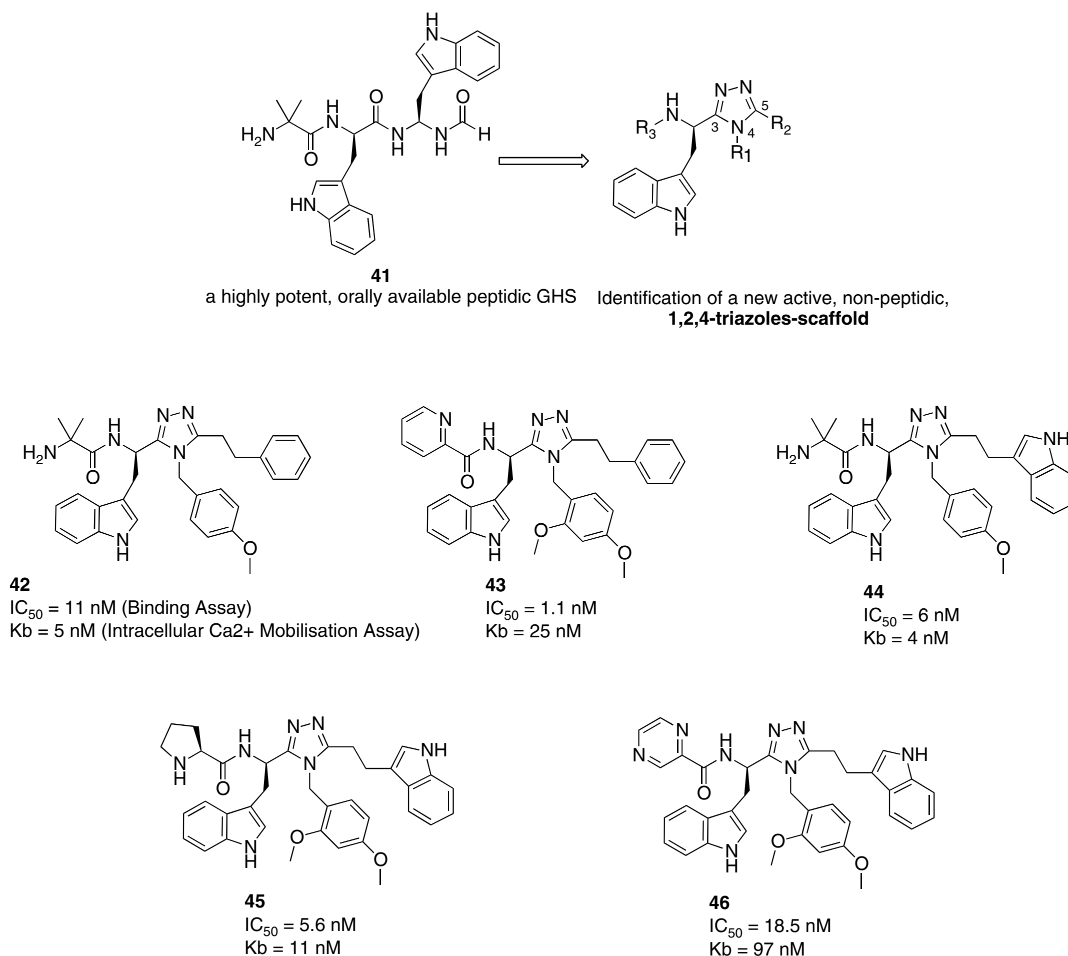

Scheme 10. 1,2,4-Triazole derivatives.

antagonist (Figure 5) [155]. Although it presented low antagonism efficiency, it appeared to be a 100-fold more potent inverse agonist [35]. Although the pharmaceutical interest of MSP **16** was limited considering its low specificity, an extensive SAR study was conducted to identify new peptidic inverse agonists, with high specificity toward GHS-R [118,166].

First, a systematic truncation of *N*-terminal residues identified the *C*-terminal heptapeptide fQwFwLL **47** to be the minimal active sequence maintaining the inverse agonist activity of MSP **16**. Then systematic alanine substitutions showed that Gln^6 and Leu^{11} were of minor importance for function and binding, whereas D-Trp^7, D-Trp^9 and Leu^{10} were necessary for the inverse agonist activity and replacement of Phe^8 decreased the activity. D-Phe^5 appeared to be a critical amino acid for inverse agonist properties. Whereas deletion of the *N*-terminal pentapeptide (i.e. D-Phe^5) resulted in a 55-fold decrease in potency, the sole deletion of D-Phe^5 from the MSP **16** or substitution with an L-Phe^5 had minimal effect toward the inverse agonist activity. Moreover, alanine substitution of D-Phe^5 resulted in a 19-fold reduction in inverse agonist activity. Thus, D-Phe^5 appeared not to be in itself an essential residue and could be replaced with L-Ala, L-Phe or a tetrapeptide D-Arg-Pro-Lys-Pro without any dramatic loss of potency.

According to these observations, it was hypothesised that D-Phe^5 may contribute to the inverse agonist potency of the core pentapeptide wFwLL **48**, by introducing a positive charge at a certain distance from the pentapeptide core. Indeed, this hypothesis was confirmed by the synthesis of the hexapeptide KwFwLL **49** presenting the highest inverse agonist activity (IC_{50} = 36 nM in functional assay). Replacement of Lys^1 with Arg^1 or His^1 had no effect on the potency whereas introduction of diaminopropionic acid resulted in a 6-fold decrease in potency and a 66-fold decrease in binding affinity (compound **50**, Figure 5). Interestingly, when Lys^1 was replaced with Ala^1, the activity swap to high agonist activity (compound **51**, IC_{50} = 15 nM). Those results were consistent with the activity of the core pentapeptide wFwLL **48** that presented a dual mode of action. At low concentration around 10 nm, it functioned as a partial agonist, whereas it behaved as a partial inverse agonist at higher concentration around 100 nM.

Keys toward Structure-Activity Relationships

Since the discovery of GHSR1a and ghrelin, SAR studies were attempted to identify the active core of peptides for binding GHSR1a, to determine agonist binding location(s) and to elucidate GHSR1a mode of activation.

The active core of ghrelin for binding GHSR1a was first identified [24]. As desacyl-ghrelin poorly activated GHSR1a, even at micromolar concentration (IC_{50} > 10 μM for binding and functional assay), the importance of the *n*-octanoylation at Ser^3 was investigated. A large hydrophobic group on Ser^3 appeared to

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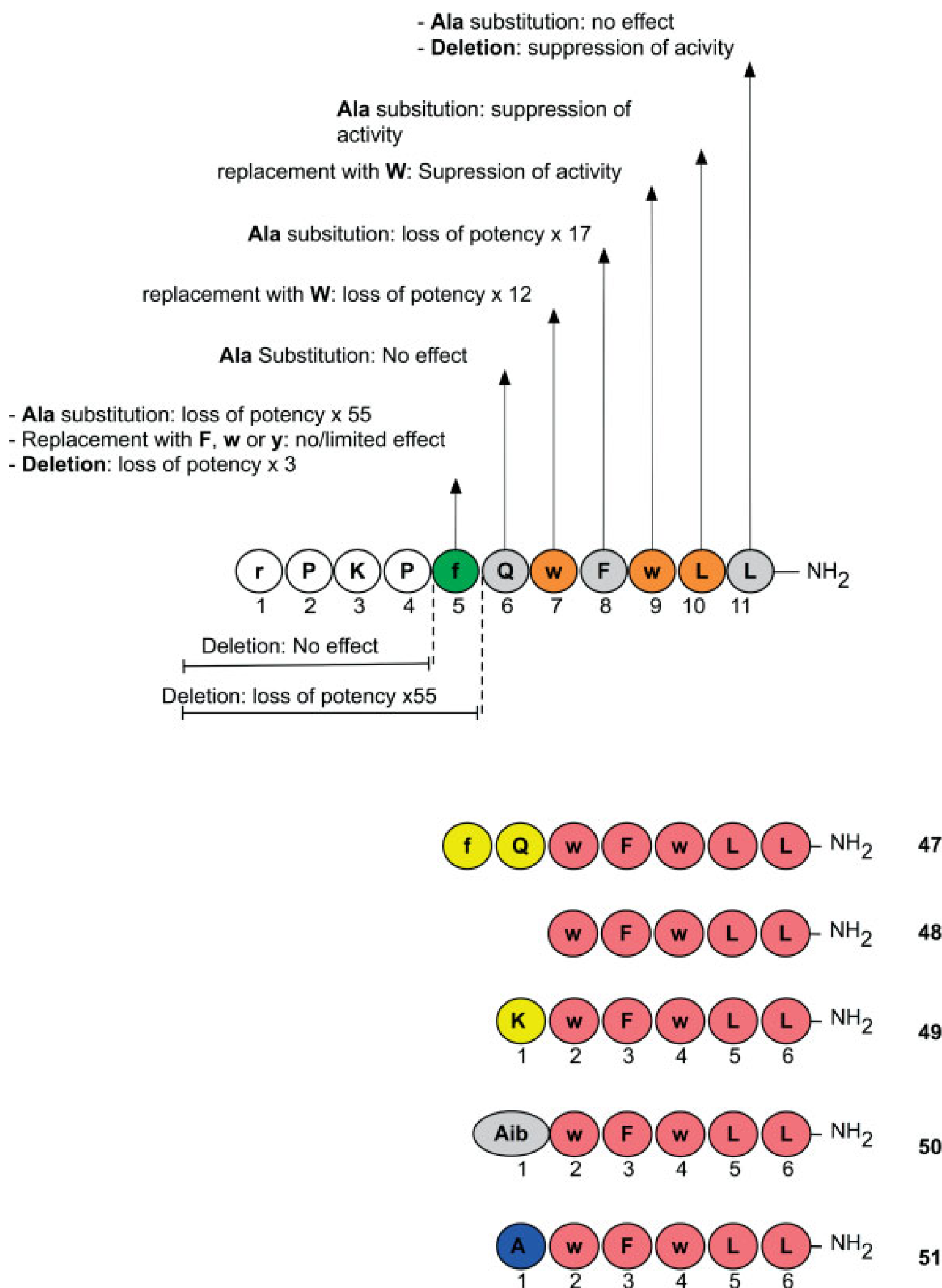

Figure 5. Design of peptidic inverse agonists.

be mandatory to maintain both binding and activity. Longer fatty acid or rigid hydrophobic substituents such as adamantane were tolerated on Ser^3, whereas shorter or polar aliphatic chains decreased or suppressed the agonist activity. Replacement of the ester bond with amide, ether or thioether linkage did not affect the activity. The position of the *n*-octanoyl group on the ghrelin sequence was also important. Ghrelin analogues *n*-octanoylated on Ser^6 or Ser^{18} had poor activity, whereas *n*-octanoyl Ser^2 analogue was equipotent to ghrelin, revealing that GHSR1a did not recognise the exact location of the *n*-octanoyl group in the *N*-terminal segment of ghrelin. Then, systematic *C*-terminal truncation of ghrelin identified the *N*-terminal pentapeptide GSS(*n*-octanoyl)FL to be the minimal active sequence with equipotent activity of ghrelin (Figure 6). In addition, amidation of the *C*-terminus enhances the agonist activity of ghrelin fragments [24,167]. Other reports demonstrated that acetylation at the N^α position decreased drastically the activity suggesting the N^α-terminal positive charge being essential for the activity [168,169]. Interestingly, the activity was maintained when the Gly^1-Ser^2 segment of the truncated ghrelin(1–7)-amide was replaced with 5-aminopentanoic acid, maintaining the same distance between the N^α-amino group and the octanoyl group.

From these reports, requirements for GHSR1a recognition emerged as a positive charge at N^α-amino group and a

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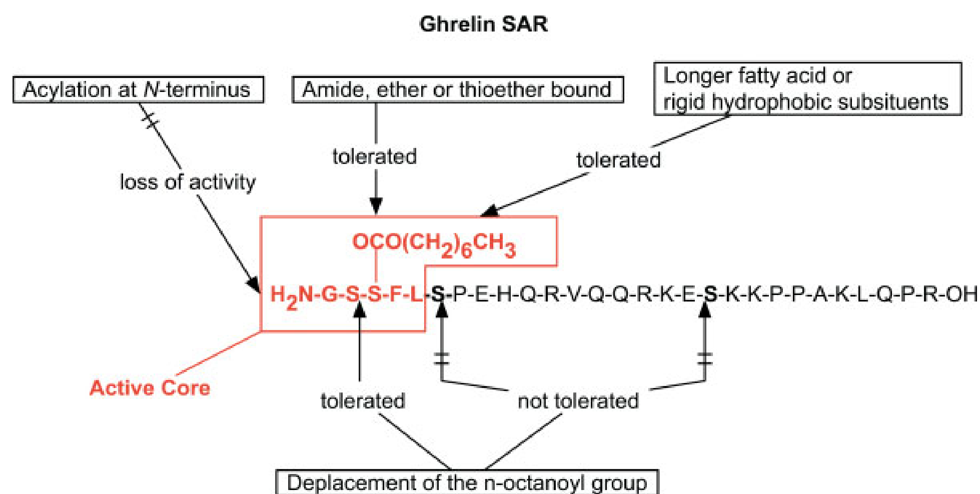

Figure 6. Schematic summary of the known structure–activity relationships of ghrelin.

hydrophobic group placed at a privileged distance from each other (9 atoms). In this context, the Gly^1-Ser^2 segment could only work as a spacer that maintained the favourable distance between the N^{α}-amino group and the octanoyl group.

In addition, intensive SAR studies with agonists and inverse agonists, and mutational mapping of GHSR1a, partially lightened the molecular activation of the receptor [118,166,170,171]. The key interaction sites of ghrelin and ghrelin agonists may consist in an aromatic cluster located in TM-VI and TM-VII and TM-III. When activated with agonists, the TM-VI and TM-VII follow an inward movement towards TM-III. Moreover, these aromatic residues were believed to be a sort of covalent tethered ligand, maintaining the extracellular segments in the active conformation, and thus mediating the high constitutive activity of the receptor. Conversely, inverse agonists bind in a larger pocket, extended from TM-II, across TM-III/VI/VI to TM-V and -IV. They prevent the spontaneous activation of the receptor by a deep insertion across the main ligand binding pocket, thus sterically blocking the inward movement of TM-VI and TM-VII towards TM-III. This mechanism is in accordance with the global toggle switch model for 7TM receptor activation proposed by Schwartz *et al*. [172].

Conclusion

Taken together, ghrelin is a peptide with some very unique properties: its octanoylation as well as its constitutively active receptor. It plays an important role in the regulation of food intake and compounds that act as antagonists or inverse agonists are promising molecules for the treatment of obesity, whereas agonists might address cachexia.

Acknowledgement

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