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Targeting the Ghrelin Receptor

Orally Active GHS and Cortistatin Analogs

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Ghrelin has been discovered as a natural ligand of the receptor specific for synthetic GH secretagogues (GHS). Ghrelin as well as synthetic GHS not only possess a remarkable GH-releasing activity but are also endowed with other endocrine and nonendocrine activities including orexigenic action, influence on gastro-entero-pancreatic functions, and cardiovascular and anti-proliferative effects. Based on these data, particular effort has been focused on the isolation of new putative natural ligands of the GHS-receptors (GHS-R) and on the identification of synthetic compounds endowed with agonistic or antagonistic activity. For instance, ghrelin analogs acting as agonists or antagonists would be able to enhance or reduce appetite and food intake; these molecules would receive obvious interest for treatment of eating disorders and obesity, respectively. Ghrelin and its orally active, agonistic analogs could have prespectives for diagnosis and treatment of GH insufficiency. In this context, EP1572, a selective, orally active, peptidomimetic GHS as well as cortistatin, another putative, natural ligand of the GHS-R, and its analogs, are currently under investigation.

Key Words: Ghrelin; GH secretagogues; cortistatin; EP1572.

Introduction

Synthetic GHS are a family of peptidyl and non-peptidyl molecules *(1–3)*. The first molecules were non-natural peptides (GH-releasing peptides, GHRP), which were developed in the late 1970s as met-enkephalin. derivatives devoid of any opioid activity *(1,2)*.

GHRP-6 was the first hexapeptide that was active in releasing GH in vivo even after oral administration, though with low bioavailability and short-lasting effect *(1–3)*. With the aim to select orally molecules with better bioavailability and longer half lives, further research led to the synthesis of orally active non-peptidyl molecules, the most representative of which was the spiroindoline MK-0677 that showed impressive bioavailability and was able to enhance 24-h GH secretion after a single oral administration *(1–6)*.

Noteworthy, MK-0677 allowed the discovery and cloning of the specific GHS receptor, the existence of which had been indicated by binding studies *(1,2,7)*. GHS receptor is expressed by a single gene found at chromosomal location 3q26.2 that encodes for two types of receptors: the GHS receptor type 1a, which consists of 366 amino acids with seven-transmembrane regions and a molecular mass of approx 41 kDa, and the GHS receptor type 1b, which consists of 289 amino acids with only a five-transmembrane regions *(1,7,8)*. Unlike the GHS receptor type 1a, the GHS receptor type 1b fails to bind GHS and its functional role remains unknown *(9)*.

The GHS receptor type 1a is expressed in the hypothalamus and anterior pituitary gland and also in other areas of the central nervous system and in multiple peripheral endocrine and nonendocrine organs *(8,10,11)*. Based on this knowledge, in 1999, about 20 yr after the synthesis of the first non-natural GHS, ghrelin was discovered as the natural ligand of the GHS-receptor type 1a *(12)*.

Ghrelin: The Discovery of the First Endogenous Ligand for the Orphan GHS Receptors

Ghrelin is a 28-amino-acid octanoylated peptide predominantly produced by the stomach but also at other levels such as bowel, pancreas, kidneys, lung, placenta, thyroid, testis, pituitary, and hypothalamus *(11–21)*. The acylation of the peptide is essential for binding the GHS receptor type 1a and for its GH-releasing and other endocrine actions *(12, 22–24)*. In fact, non-acylated ghrelin, which circulates in a higher amount than the acylated form *(25)*, does not displace radiolabeled ghrelin from its hypothalamic or pituitary binding sites *(24)* and has no endocrine activities *(12,26)*.

Another endogenous ligand for the GHS-R1a has been isolated from the stomach and named Des-Gln14-ghrelin being homologous to ghrelin except for missing one glutamine and sharing the same activity of ghrelin *(25)*. Interestingly, GHS receptor is also bound and activated by adenosine *(27,28)*. Thus, it has to be considered that ghrelin

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might be not the only natural ligand of the GHS-R, while, on the other hand, the GHS receptor type 1a might be only one out of a group of receptors for such ligands.

In fact, there is evidence pointing toward the existence of additional receptor subtypes *(29–31)*. The existence of a ghrelin receptor different from GHS-R1a is indicated by evidence that there are ghrelin receptors that are also recognized by the non-acylated form, which usually does not bind the GHS-R1a *(31–33)*; note that these receptors likely mediate some biological, nonendocrine actions of unacylated ghrelin *(32–34)*. Specific binding sites for peptidyl GHS with an higher or at least overlapping density than in the pituitary have been found in rat and human heart *(35–37)*, as well as in a wide range of other nonendocrine peripheral human tissues such as lung, arteries, skeletal muscle, kidney, and liver *(10)*. These binding sites are not ghrelin receptors, as they show very low binding affinity for ghrelin and non-peptidyl GHS MK-0677 *(10)*.

In agreement with the existence of different binding sites specific for ghrelin and/or synthetic GHS, these latter molecules do not always share the same neuroendocrine and extra-neuroendocrine activities; for instance, the existence of molecules belonging to the GHS family that lack GH-releasing activity but maintain orexigenic action or cardiotropic activity have been described *(29,30,38,39)*. Thus, the possibility exists that molecules displaying selective action among those exerted by ghrelin are generated and act as agonists or antagonists *(40,41)*.

Ghrelin and Synthetic GHS: Do They Have Potential Perspectives for Clinical Applications in Endocrine and Nonendocrine Diseases?

As anticipated, both natural (i.e., ghrelin) and synthetic GHS possess various endocrine and nonendocrine biologic activities that are generally unrelated to their GH-releasing property. In fact, besides potent GH-releasing action, ghrelin as well as synthetic GHS have other remarkable activities including: (i) stimulation of lactotroph and corticotroph secretion as well as inhibition of gonadotropin secretion; (ii) orexigenic effect coupled with control of energy expenditure; (iii) control of gastric motility and acid secretion as well as influence on the exocrine pancreatic function; (iv) influence on the endocrine pancreatic function, and glucose and lipid metabolism; (v) influence on gonadal function; (vi) cardiovascular actions; (vii) modulation of cell proliferation and apoptosis; (viii) influence on behavior; (ix) influence on sleep *(31,42,43)*.

GH Releasing Activity

Both ghrelin and synthetic GHS possess strong and dose-related GH-releasing activity mainly acting at the hypothalamic level via mechanisms, at least partially, distinct from those of GHRH, although a preserved GHRH activity is needed for a full GH-releasing effect of GHS and ghrelin *(3,44)*. Both ghrelin and synthetic GHS have been reported ineffective in modifying hypothalamic somatostatin (SS) release, but there are data indicating that these molecules act as functional somatostatin antagonists at either the pituitary or the hypothalamic level *(3,44–47)*. In agreement with this action, in humans the GH response to both natural and synthetic GHS is not modified by substances acting via SS inhibition and is partially refractory to the inhibitory effect of substances acting via stimulation of hypothalamic SS and even of exogenous SS *(31,48,49)*.

The GH-releasing effect of ghrelin and synthetic GHS undergoes marked age-related variations decreasing in aging *(3,50–52)*. The most important mechanism accounting for reduced GH-releasing activity of GHS in aging is probably represented by age-related variations in the neural control of somatotroph function including GHRH hypoactivity and somatostatinergic hyperactivity but a reduction in hypothalamic GHS receptors in human aged brain has also been reported *(3,50)*.

The theoretical diagnostic and therapeutic usefulness of ghrelin and synthetic GHS has been investigated especially taking into account their strong and reproducible GH-releasing effect even after oral administration. Particularly when combined with GHRH, GHS represent one of the most potent and reliable tests to evaluate the pituitary GH releasable pool for the diagnosis of GH deficiency *(6,53,54)*. Testing with GHS is as sensitive and specific as an insulin tolerance test and GHRH + arginine, the two "gold standard" tests for the diagnosis of GH deficiency, provided that appropriate cut-off limits are assumed *(6,54)*.

Regarding the therapeutical perspectives, the potential usefulness of GHS as growth-promoting factor in children with GH deficiency has been limited by the results of some trials showing that their efficacy is not comparable with that of recombinant human GH (rhGH) *(55)*. On the other hand, GHS has also been suggested for anabolic treatment in frail elderly subjects with somatopause *(50)*. In fact, it has already been reported that chronic treatment with MK-0677 (i) in elderly subjects restores IGF-I levels to the normal young range *(4)*; (ii) counteracts the alterations in sleep pattern in aging *(56,57)*; (iii) exerts anabolic effect in young volunteers *(58)* and increases fat-free mass and energy expenditure in obese patients *(59)*; (iv) alone or in combination with alendronate, a biphosphonate, attenuates the indirect suppressive effect of alendronate on bone formation and increases bone mineral density at the femoral neck *(60)*.

Orexant Activity and Regulation of Energy Metabolism

As anticipated, in addition to the hypothalamus–pituitary unit, GHS-R are also distributed in other central and peripheral tissues, explaining other biological activities and suggesting other potential clinical perspectives *(3,19,31)*.

Among these activities, a modulatory role of ghrelin in the regulation of energy balance and glucose metabolism has been reported both in humans and in animals *(43,61)*. In humans, ghrelin induces a significant increase in plasma glucose levels that is surprisingly followed by a reduction in insulin secretion *(62,63)*. Coupled with the observation that acute, as well as chronic treatment with GHS, particularly non-peptidyl derivatives, induced hyperglycemia and insulin resistance in a considerable number of elderly subjects and obese patients, these data strongly suggest that ghrelin exerts a significant role in the fine-tuning of insulin secretion, affects glucose metabolism and plays a major role in managing the neuroendocrine and metabolic response to variations in the energy balance *(42,43,61,64)*.

This peripheral metabolic action is coupled with the clear stimulatory effect of ghrelin and synthetic GHS, at least some of them, on appetite and food intake *(31,42,64,65)*. Exogenous ghrelin induces weight gain in rodents by increasing food intake and reduces fat utilization *(31,42,66)*. Stimulation of appetite and food intake has also been demonstrated in humans *(31,42,43)*. The orexigenic action of ghrelin and GHS is independent of their GH-releasing action and likely to be mediated by a specific central network of neurons, mostly within the arcuate nucleus of the hypothalamus, that is also modulated by leptin *(31,43,47,67–69)*. Ghrelin and leptin might really be complementary players of one regulatory system that has developed to inform the central nervous system about the status of energy balance *(31,42,43,65,69,70)*. Ghrelin-containing cells are also present in the mediobasal hypothalamus, where the neuroendocrine network regulating energy balance is located *(31,67)*. It is important to note, however, that in humans circulating ghrelin levels are decreased in chronic (obesity) and acute (feeding) states of positive energy balance, while are increased by fasting and in patients with anorexia nervosa *(31,71–76)*. Premeal rise of circulating ghrelin levels suggests its role as a hunger signal triggering meal initiation, and this action would be mediated by GHS-R subtypes as suggested by evidence that GHS analogs devoid of any GH-releasing effect stimulate food intake *(31,72)*.

The orexigenic action of ghrelin has increased interest by suggesting the possibility that synthetic GHS analogs acting as agonists or antagonists on the appetite would have perspectives as drug intervention in eating disorders and obesity.

New GHS-R Agonists and Antagonist as Potential Clinical Tools

Particular interest has been given to identification of new natural or synthetic ligands of the GHS-R endowed with agonistic or antagonistic activity eventually active even after oral administration. Natural and synthetic ligands with selective affinity for a GHS-R subtype and selective action among those displayed by ghrelin could have potential clinical applications.

Cortistatin: A New Putative Natural GHS-R Ligand

Cortistatin (CST) is a recently described peptide expressed in cerebral cortex and hippocampus but also in peripheral tissues such as fetal heart and lung, prostate, colon, and the immune system *(77–81)*. Pre-pro-CST shows high structural homology with pre-pro-somatostatin (pre-pro-SS), particularly in the carboxyl terminus from which SS-14 and SS-28 are enzymatically processed *(79)*. Interestingly, rat pre-pro-CST may also be cleaved to pro-CST from which the two mature products CST-14 and CST-29 can be generated in rat *(78,80)* and CST-17 and CST-29 in human *(78–80)*. CST-14 shares 11 of the 14 amino acid residues with SS-14, although these peptides are encoded by distinct genes *(80,82)*.

SS exerts its biological effects via membrane-bound receptors, the so-called SS receptors (sst-r), of which five subtypes (sst-r 1–5) have been cloned *(83,84)*. The sst-r are expressed in the brain and periphery *(83,84)* and mediate multiple SS activities including neurotransmission, neuromodulation, regulation of endocrine and exocrine secretions, and the inhibition of tumor growth *(83,84)*.

CST binds to all sst-r subtypes with an affinity (1–2 n*M*) quite close to that of SS and therefore is expected to have similar biological activities. However, the existence of specific receptors that selectively bind SS or CST has been hypothesized *(85)* and, in fact, CST possesses central activities that are not shared by SS *(80,85–87)*. For instance, CST, unlike SS, reduces locomotor activity and induces slow-wave sleep *(80,82)*. Moreover, CST and SS are often coexpressed in the same neurons but are regulated by different stimuli *(77)*.

Interestingly, although native somatostatin does not bind GHS-R, it has been surprisingly observed that some synthetic somatostatin (SS) octapeptide agonists (mainly lanreotide, octreotide, and vapreotide) displaced ^{125}I-Tyr-Ala-hexarelin from pituitary binding sites *(24,40)*. This evidence suggested the working hypothesis that an endogenous factor related to SS might exist and interact with the GHS-R, thus representing another natural ligand of these receptors. To clarify this hypothesis, the ability of different SRIH-like peptides such as various SS fragments (SRIH 3-14, SRIH 7-14, SRIH 7-10, SRIH 2-9) and of CST-14 and -17 to compete with ^{125}-Tyr-Ala-hexarelin binding sites of human pituitary gland in comparison with hexarelin and ghrelin has been investigated and recently described in rat and human brains *(40,41,88)*.

The different SRIH fragments were chosen as potential metabolites *(41)*, whereas CST, although it shares high structural homology with SS *(79,80)* and binds all five sst-r subtypes *(41,82,85)*, possesses several effects that do not parallel those of SS and might therefore related to the activation of another SS-unrelated receptor *(80)*.

Results showing that CST, like hexarelin and ghrelin, but not various fragments of native SS, displaces ^{125}I-Tyr-Ala-

hexarelin from its pituitary receptors, suggested that this factor may play a role in modulating the activity of GHS-R. The evidence that CST, like hexarelin and ghrelin, binds to GHS-R suggests that this peptide could represent another endogenous GHS ligand; this hypothesis could, in turn, imply that GHS-R is another specific receptor for CST.

Following the results of binding studies, in vivo animal studies have been performed in order to verify if CST may exert some endocrine effect and if the specific binding to the GHS-R might reveal some peculiar activities not related to the activation of the sst-r. Preliminary studies showed that CST-14 is as active as SS in vivo to induce dose-related inhibition of GH secretion in normal male anaesthetized rats *(88)*.

The effects of CST-14 on basal GH, insulin, glucose, and ghrelin secretion as well as on the somatotroph responsiveness to GHRH or ghrelin in normal young volunteers have been also studied. The effects of CST were compared with those of the same dose of SS-14. The effects of ghrelin both alone and during infusion of either CST or SS on PRL, ACTH, cortisol, insulin, and glucose levels were also evaluated *(89,90)*.

CST, like SS, remarkably inhibited GH, insulin, and spontaneous ghrelin secretion to the same extent in humans. The inhibitory effect (of approx 55%) of CST and SS on circulating ghrelin levels followed that on GH and insulin secretion. At the end of CST or SS infusion GH and insulin secretion almost immediately recovered despite persistent inhibition of ghrelin levels *(89,90)*. CST also inhibited GHRH- and ghrelin-stimulated GH secretion to the same extent as SS. The GH response to ghrelin was however partially refractory to the inhibitory effect of both CST and SS *(90)*.

Similar inhibitory activity of CST and SS on both GH and insulin secretion does not rule out peculiar GHS-R mediated actions of cortistatin; in fact, this action would be simply masked by the overriding actions triggered by the activation of sst-r *(90)*.

CST analogs able to selectively bind only the GHS-R might probably clarify the result, if any, of the peculiar CST binding to the GHS-R. The search for analogs with selective activity on GHS-R led to the octapeptide Pro-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Lys-NH2 (named CST-8), which does not displace SS from its receptors but has high affinity on the ghrelin receptor *(91)*. This compound is currently under investigation.

EP1572: A New Peptidomimetic Synthetic GHS-R Ligand with Selective GH-Releasing Activity

EP1572 (JMV 1843, [Aib-DTrp-DgTrp-CHO]) is a new peptidomimetic GH secretagogue (GHS) derived from a chemical–pharmacological research program on down-sized analogs of peptidyl GHS *(92)*.

Following previous orally active GHS, it was generated taking into account the potential clinical perspectives of ghrelin analogs: (i) diagnostic potential; (ii) therapeutic potential in GH-related disorders for treatment of short stature and as an anabolic intervention in somatopause and catabolic states; (iii) theoretical therapeutic potential in non-GH-related disorders taking into account the wide spectrum of ghrelin biological activities *(3,31)*.

Binding studies showed that EP1572 displaces ^{125}I-labeled ghrelin in a dose-dependent manner in human pituitary and hypothalamus with binding potency similar to that of ghrelin and hexarelin *(93,94)*. Studies in rats in vivo demonstrated that the subcutaneous administration of EP1572 induces a potent increase of GH levels that is similar to that induced by hexarelin, a potent synthetic peptidyl GHS *(93)*.

Based on these results, the endocrine effects of EP1572 were tested in humans. In agreement with the results in animal studies, in a preliminary study in humans, we showed that the acute intravenous administration of EP1572 induced a prompt and specific increase of GH to very high levels at variance with ghrelin that also stimulates PRL, ACTH, and cortisol secretion *(3,31)*. This selectivity could be related to the possibility that this synthetic GHS could bind different pockets of the GHS-R1a or activate GHS-R subtypes selectively devoted to the GH-releasing activity *(1,31)*. A potent and reproducible GH-releasing effect of EP1572 was observed even after oral administration at doses ranging from 0.06 to 0.5 mg/kg *(93,94)*.

In all, EP1572 is a new peptidomimetic GHS with potent and selective GH-releasing activity. It is very active even after oral administration of very low doses and likely undergoes reproducible adsorption. Based on these preliminary results EP1572 has been selected for further clinical investigation.

Conclusions

Looking for new natural and synthetic ligands of the GHS acting as ghrelin agonists or antagonists is, at present, one of the most interesting issues in the ghrelin field. These molecules eventually showing specific action among those displayed by ghrelin acting with specificity would have perspectives in term of clinical applications in the context of growth disorders, somatopause, catabolism, eating disorders, and obesity. Studies in the future will clarify these hopes but, even before, will progressively allow the clarification of many physiological and pathophysiological aspects.

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