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Lipidated analogues of luteinizing hormone-releasing hormone (LHRH) reduce serum levels of follicle-stimulating hormone (FSH) after oral administration

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ABSTRACT

A diverse range of diseases involving the reproductive system are treated with luteinizing hormone-releasing hormone (LHRH) agonists which must be administered daily. Currently, an efficient oral delivery system is not available. Here, we show the facile inclusion of lipoamino acids into the peptide sequence of LHRH, rendering it more stable towards enzymatic degradation, as well as enhancing permeability across Caco-2 cell monolayers. Selected LHRH derivatives were tested *in vivo* by daily oral administration to rats. The size and weight of the sex organs remained unchanged and the levels of LH were stable over the course of the experiment. However, some of the lipidic peptides (3, 8 and 9) were able to reduce serum levels of follicle-stimulating hormone (FSH), an important finding towards the development of orally available LHRH agonists.

1. Introduction

The development of the reproductive system and maintenance of its correct function are controlled by the neurohormone luteinizing hormone-releasing hormone (LHRH). This decapeptide is produced in the hypothalamus, secreted into the blood stream and transported to the pituitary where it binds to its receptor. Activation of the LHRH receptor stimulates the release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn elicit the secretion of gonadal steroids, androgens in males and estrogens in females (Jeong and Kaiser, 2006).

Over-stimulation of the LHRH receptors results in receptor down-regulation and desensitisation, subsequently leading to gonadal suppression (Handelsman and Swerdloff, 1986). This approach has a variety of clinical applications in the treatment of reproductive disorders like endometriosis and precocious puberty, as well as hormone-sensitive cancers like prostate and breast cancer (Engel and Schally, 2007). Desensitisation of the LHRH receptor requires prolonged agonist action which cannot be provided by native LHRH with its very short half-life of 4–8 min (Barron et al., 1982; Pimstone et al., 1977; Redding et al., 1973). Therefore, following the elucidation of the LHRH amino acid sequence (**1**, Fig. 1) by Schally's research group in 1971 (Matsuo et al., 1971), thousands of LHRH analogues were synthesized with the aim of producing agonists with higher activity and increased enzymatic stability (Karten and Rivier, 1986).

The main cleavage sites of LHRH are the Tyr^5-Gly^6 and Pro^9-Gly^{10} bonds, and, to a lesser extent, the $pGlu^1$-His^2 and Gly^6-Leu^7 bonds (Griffiths and McDermott, 1983; Karten and Rivier, 1986; Molineaux et al., 1988). The most common modifications of the LHRH peptide target the stabilisation of these bonds. The overwhelming majority of LHRH agonists, including all clinically approved agonists, contain a D-amino in place of glycine at position 6 (Beyer et al., 2011; Limonta et al., 2003; Moreau et al., 2006). Not only does this increase metabolic stability (Karten and Rivier, 1986), it also enhances receptor binding as the D-amino acid in this position stabilizes the type II β-turn conformation important for interaction with the receptor (Laimou et al., 2010; Nikiforovich and Marshall, 1993; Sealfon et al., 1997). The other widespread modification involves the C-terminal glycine amide, which in some compounds is replaced with either an ethyl group or azaglycine amide (Moreau et al., 2006). There are also a few examples of substitutions in position 7 resulting in active agonists (Ling and Vale, 1975). It has been noted that, while none of the amino acids in the LHRH sequence appear to be essential for receptor activation, modifications of all other positions have not resulted in potent agonists (Karten and Rivier, 1986).

None of the currently used LHRH analogues are orally available (Handelsman and Swerdloff, 1986), all of them are administered

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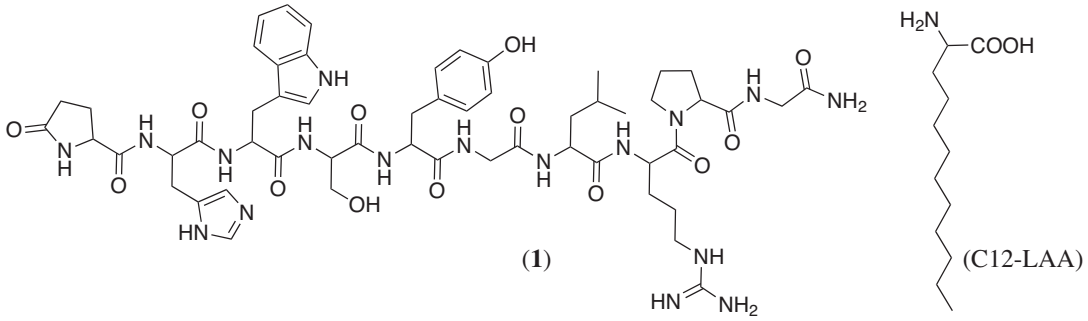

Fig. 1. Structure of native mammalian LHRH (**1**) and structure of C12-lipoamino acid (C12-LAA) used to modify this peptide.

either intranasally, by injection, or using depot formulations (Beyer et al., 2011; Moreau et al., 2006). Although oral delivery of peptide drugs is tremendously challenging, it is also a very attractive route of administration due to high patient acceptance and compliance (Hamman et al., 2005; Morishita and Peppas, 2006), especially for drugs like LHRH agonists that require daily administration. It has been suggested that by increasing the lipophilicity of LHRH analogues, oral bioavailability could be improved (Handelsman and Swerdloff, 1986). Consequently, several derivatives were synthesized with the aim of achieving increased hydrophobicity and enhanced enzymatic stability (Flinn et al., 1996a, 1996b; Haviv et al., 1992, 1993; Hillery et al., 1996). It was shown that oral uptake can be increased markedly by conjugation of LHRH to polymer nanoparticles (Hillery et al., 1996) or lipoamino acids (Flinn et al., 1996a, 1996b), with analogues detectable in blood and several organs for at least 12 h after administration. Furthermore, it has been suggested that a lipoamino acid-LHRH conjugate can act as a prodrug through the slow release of LHRH (Blanchfield et al., 2005). Taken together, these results show the potential of combining lipoamino acids with LHRH for the oral delivery of this peptide.

Here, we present a small library of lipidic derivatives of LHRH designed according to the essential features of agonists, discussed above. The peptides were initially analysed *in vitro*, to assess stability against enzymatic degradation in Caco-2 cell homogenates and permeability across a Caco-2 cell monolayer to estimate potential drug absorption. Based on this data, we selected four candidates for *in vivo* testing. Changes in serum levels of LH and FSH were monitored during a three week period of daily oral administration of agonist candidates to male rats, and the sex organs were subsequently examined for histological changes.

2. Materials and methods

2.1. General

Dimethylformamide (DMF), trifluoroacetic acid (TFA), and piperidine of peptide synthesis grade were purchased from Merck Biosciences (Kilsyth, VIC, Australia). HPLC-grade acetonitrile was purchased from RCI Labscan Ltd. (Bangkok, Thailand).

Fmoc-protected amino acids and Rink amide MBHA resin (100–200 mesh, 0.4–0.8 mmol/g loading) were obtained from Novabiochem (Melbourne, VIC, Australia) or Mimotopes (Clayton, VIC, Australia). All media and supplements for cell culture work were purchased from Life Technologies.

Preparative HPLC was carried out on a Shimadzu system equipped with a CBM-20A controller, LC-20AT pump, SIL-10A autosampler, SPD-20A UV/Vis detector set to a wavelength of 230 nm and a FRC-10A fraction collector. The analytical HPLC was a Shimadzu instrument with an LC-20AB pump, a SIL-20AHT autosampler and an SPD-M10A detector set to a wavelength of 214 nm. Electrospray ionisation mass spectrometry was carried out on a PE Sciex API3000 triple quadrupole instrument connected to a Shimadzu HPLC system with two LC-20AD pumps and a SIL-20AHT autosampler. UV/Vis spectrometry was carried out using a Varian Cary 50 Bio. Plates were read on a Molecular Devices SpectraMax 250.

2.2. Peptide synthesis

2.2.1. Solid-phase peptide synthesis

Peptides were assembled on Rink amide MBHA resin using the *in situ* neutralisation protocol for Fmoc solid phase peptide synthesis (Alewood et al., 1997). Nα-Fmoc-protected amino acids (4.2 eq.) were activated with HBTU (4 eq.) and DIPEA (5 eq.) and coupled twice for at least 45 min. The following side chain protecting groups were used: Arg(Pbf), Tyr(tBu), Ser(tBu), Trp(Boc), His(Trt) and Gln(tBu). The Fmoc protecting group was removed by treatment with 20% piperidine in DMF for 10 min and 20 min. The C12-lipoamino acid was synthesized according to a previously published procedure (Gibbons et al., 1990) and Nα-protected with 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl (Dde) (Ross et al., 2008) which was removed by treatment with 2% hydrazine hydrate in DMF (3 × 5 min).

Once the peptide sequence was complete, the resin was washed with DMF, dichloromethane and methanol, and dried under vacuum overnight. The peptide was cleaved by stirring the resin in a mixture of TFA (95%), water (2.5%) and triisopropyl silane (2.5%) for 3 h. Addition of cold diethyl ether precipitated the peptide, the solvent was discarded, and the peptide dissolved in a mixture of acetonitrile and water (1:1) containing 0.1% TFA and lyophilized.

2.2.2. Purification

The crude peptides were purified by preparative RP-HPLC using a Vydac C18 column (22 mm × 250 mm) with HPLC gradient P1 (Table 1, peptides **1** and **6**), or a Vydac C8 column (22 mm × 250 mm) with HPLC gradient P2 (Table 2, peptides **2**–**5** and **7**–**9**) at a flow rate of 10 ml/min. The collected fractions were analysed by analytical HPLC using a Vydac C18 (4.6 mm × 250 mm, 5 μm), a Vydac C8 column (4.6 mm × 250 mm, 5 μm) and a Vydac C4 column (4.6 mm × 250 mm, 5 μm) with a gradient of 100% A1 (water, 0.1% TFA) to 100% B1 (90% acetonitrile in water, 0.1% TFA) over 30 min at a flow rate of 1 ml/min. Fractions containing pure peptide were combined and lyophilized.

Table 1
HPLC gradient P1.

Time [min]	Solvent A1 [%]	Solvent B1 [%]
0	100	0
5	100	0
10	90	10
80	40	60