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Calorimetry and Langmuir–Blodgett studies on the interaction of a lipophilic prodrug of LHRH with biomembrane models

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ABSTRACT

The interaction between an amphiphilic luteinizing hormone-releasing hormone (LHRH) prodrug that incorporated a lipoamino acid moiety (C12-LAA) with biological membrane models that consisted of multilamellar liposomes (MLVs) and phospholipid monolayers, was studied using Differential Scanning Calorimetry (DSC) and Langmuir–Blodgett film techniques. The effect of the prodrug C12[Q^1]LHRH on the lipid layers was compared with the results obtained with the pure precursors, LHRH and C12-LAA.

Conjugation of LHRH with a LAA promoiety showed to improve the peptide interaction with biomembrane models. Basing on the calorimetric findings, the LAA moiety aided the transfer of the prodrug from an aqueous solution to the biomembrane model.

1. Introduction

The decapeptide luteinizing hormone-releasing hormone (LHRH) plays a pivotal role in the development and normal functioning of the reproductive system [1]. Several analogues of this peptide have been approved for therapeutic use in the treatment of diseases such as endometriosis, precocious puberty and hormone-sensitive cancers. However, all of these require administration by injection or as depot formulations [2,3]. Oral administration is the preferred method of drug delivery due to ease of administration and patient compliance. Our research efforts have been directed toward improving the oral bioavailability of LHRH analogues.

It was recently shown that rats administered continuous oral doses of an LHRH conjugated with a C12-lipoamino acid at the C-terminus (C12[Q^1]LHRH, Fig. 1) lowered the levels of circulating reproductive hormones [4], the secretion of which is directly controlled by LHRH.

Differential scanning calorimetry (DSC) and Langmuir–Blodgett (LB) techniques were used to study the interaction between C12[Q^1]LHRH (compared to the parent peptide) with biomembrane models. Both techniques are very important tools for studying the nature and depth of interaction between bioactive compounds and biomembrane models [5,6], providing a way to understand correlations between the chemical structure/variations and interaction, and to predict their biological and/or toxicological profile [5,7].

Biological membranes are too complex to effectively examine drug–membrane interactions, thus simplified systems that mimic natural lipid membranes are commonly used as an alternative [8,9]. In this study, DSC experiments were performed using multilamellar liposomes (MLVs) made of dimyristoylphosphatidylcholine (DMPC), an established 3-D model for cell membranes [10,11], while the Langmuir–Blodgett technique was used to determine the disposition and organization of the prodrug in a phospholipid monolayer that consisted of pure DMPC [12].

2. Experimental

2.1. Chemicals and materials

1,2-Dimiristoyl-*sn*-glicero-3-phosphatidylcholine (DMPC) was purchased from Genzyme Pharmaceuticals (Liestal, Switzerland;

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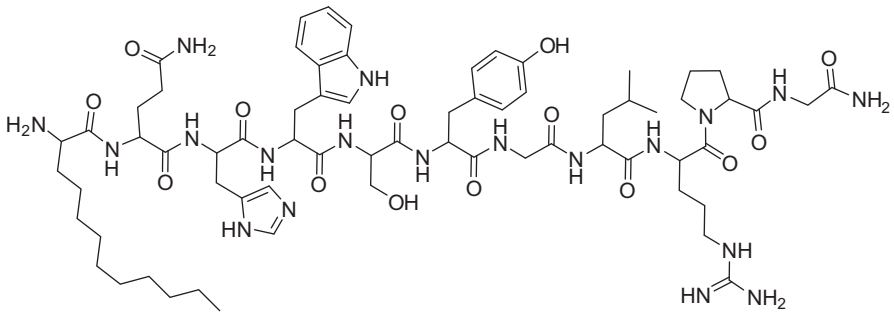

Fig. 1. Structure of C12[Q^1]LHRH.

a purity of 99.9% by HPTLC method was reported by the supplier). Lipid concentration in the liposomes was measured by phosphorous analysis. A 50 mM solution of tris(hydroxymethyl)aminomethane (TRIS) (Sigma–Aldrich Chimica srl, Milan, Italy) was used for MLVs preparation; the pH was adjusted to 7.4 with HCl. The subphase used for Langmuir–Blodgett film studies consisted of 5 mM TRIS buffer, adjusted to pH 7.4 with HCl. It was prepared in ultrapure water with a resistivity of 18.2 MΩ cm and a surface tension of 72.2 mN/m (Simplicity 185 system, Millipore, Vimodrone, Italy).

2.2. Chemistry

All peptides were assembled on Rink amide MBHA resin following the *in situ* neutralization protocol for Fmoc solid phase peptide synthesis [13]. Fmoc-protected amino acids (4.2 eq.) with the following side chain protecting groups: Arg(Pbf), Tyr(tBu), Ser(tBu), Trp(Boc), His(Trt) and Gln(tBu) were activated with 4 eq. HBTU and 5 eq. DIPEA, and double-coupled for at least 30 min. After each coupling step, the Fmoc protecting group was removed by treatment with 20% piperidine in DMF for 10 min and 20 min. The C12-lipoamino acid (C12-LAA) was synthesized according to a previously published procedure [14]. 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, v/v) containing 0.1% TFA, then lyophilized.

The crude peptides were purified by preparative RP-HPLC using a Vydac C18 column (22 × 250 mm) with HPLC gradient P1 (Table 1, LHRH), or a Vydac C8 column (22 × 250 mm) with HPLC gradient P2 (Table 2, C12[Q^1]LHRH) at a flow rate of 10 ml/min on a Shimadzu system. Fractions were analyzed by mass spectrometry on an AB Sciex API3000 instrument and by analytical HPLC using a Vydac C18 (4.6 × 250 mm, 5 μm) or Vydac C8 column (4.6 × 250 mm, 5 μm) with a gradient of 100% A (water, 0.1% TFA) to 100% B (90% acetonitrile in water, 0.1% TFA) over 30 min at a flow rate of 1 ml/min on a Shimadzu system. Fractions containing pure peptide were combined and lyophilized.LHRH: yield 69.8 mg (59%); HPLC retention time: C18 Vydac column (14.0 min); exact mass [M+H]$^+$ calculated: 1182.58, found: 1182.58.

Table 1
HPLC gradient P1 for LHRH.

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
5	100	0
10	90	10
80	40	60

Table 2
HPLC gradient P2 for prodrug C12[Q^1]LHRH.

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
5	100	0
10	80	20
80	30	70

C12[Q^1]LHRH: yield 95.0 mg (68%); HPLC retention time: C8 Vydac column (17.7/18.2 min); exact mass [M+H]$^+$ calculated: 1396.78, found: 1396.78.

2.3. Preparation of liposomes

MLVs made of pure DMPC were used as a reference, while liposomes that contained C12[Q^1]LHRH, LHRH or C12-LAA were prepared at different molar fractions of the host compounds (0.015, 0.03, 0.045, 0.06 or 0.09). Liposomes were produced by a classical thin lipid film method [15]: appropriate amounts of DMPC and of the test compounds were separately solubilized in a chloroform/methanol mixture (1:1, v/v). Aliquots of known concentration of DMPC (7 mg, 1.0325 × 10^5 moles) and of each of the compounds were mixed in glass tubes to get an exact molar fraction of the compound with respect to the lipid. Solvents were removed under a nitrogen stream and the resulting phospholipid film was dried under high vacuum for 8 h.

The lipid films were then hydrated with TRIS buffer (pH 7.4) placed at 37 °C for 1 min, and then vortex-mixed for one additional minute; the entire cycle was repeated three times. Samples were subsequently left at 37 °C for 1 h to allow homogenizing and annealing of the vesicles. This procedure created liposomes where the test compound was homogeneously dispersed in all the bilayers.

2.4. DSC analysis

2.4.1. DSC analysis of samples produced in an organic phase

A 120 μl aliquot of the aqueous liposomal dispersion, prepared in the presence or absence of the test compounds, was placed in 160 μl aluminum pans. The pans were hermetically sealed and submitted to three cycles of heating scan from 5 to 37 °C, at a heating rate of 2 °C/min, and cooling from 37 to 5 °C at 4 °C/min. The T_m and ΔH values obtained for pure DMPC vesicles were taken as reference parameters to evaluate the effect of a single molar fraction of each compound on the liposomes.

2.4.2. Contact kinetic experiments

Contact kinetic measurements were made to ascertain the ability of the compounds to cross the aqueous medium, interact with,