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In vitro evaluation of leptin fragments activity on the ob receptor

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Abstract: In an attempt to identify regions in the leptin molecule responsible for its bioactivity, we tested six related-leptin peptide fragments denoted: Ac-hLEP$_{23-47}$-NH$_2$ (**I**), Ac-hLEP$_{48-71}$-NH$_2$ (**II**), Ac-hLEP$_{72-88}$-NH$_2$ (**III**), Ac-hLEP$_{92-115}$-NH$_2$ (**IV**), Ac-[Ser^{117}]-hLEP$_{116-140}$-NH$_2$ (**V**), Ac-hLEP$_{141-164}$-NH$_2$ (**VI**) and their correspondent disulfide bridged dimer forms. The activity of the fragments was evaluated in comparision to leptin, by their ability to interact with leptin receptor using a cytosensor microphysiometer. Our results indicated that the fragments **IV** and **V** and [D-Leu^4]-OB$_3$ and its human sequence analog were recognized by leptin receptor present in HP-75 cells, in agreement with the results obtained by other workers, validating that this region of the molecule contain the functional epitope of the leptin molecule. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: leptin; synthetic peptides; cytosensor; circular dichroism; obesity; related-leptin peptide fragments

INTRODUCTION

Leptin, the product of the *ob gene* [1,2] is a 167-amino acid plasma protein that is produced predominantly by white adipocytes [3,4], although leptin synthesis has also been demonstrated in the gastric epithelium and placental trophoblast [5]. Leptin plays an important role in the regulation of a variety of physiological functions, including food intake [6], energy expenditure, reproductive [7] and respiratory [8] functions, body-temperature and body-weight maintenance [9,10]. Total absence or resistance to leptin causes morbid obesity, diabetes [11] and hypogonadism [12]. The energy balance in mammals ought to be controlled by a feedback loop in which the amount of stored energy is sensed by the hypothalamus, that in turn adjusts food intake [13] and energy expenditure to maintain a constant body weight [14].

The weight-reducing effects of leptin are likely mediated through interaction with a receptor for leptin (OB-R), the product of the *db gene* [15,16], which is expressed predominantly in the hypothalamus, a region of the brain associated with regulation of body weight.

The tertiary structure of the leptin molecule reveals the existence of a four-helix bundle that is characteristic of the short-helix cytokines [17,18]. In fact, leptin receptors belong to the class I cytokine receptor family; they are found in many hypothalamic regions including arcuate, dorsomedial and ventromedial nuclei [19].

In the past years, different groups reported their attempts to identify bioactive leptin fragments. Samson and coworkers, based on the predicted biological cleavage sites present in the leptin molecule, designed and tested three fragments. The *N*-terminal leptin fragment, OBGRP 22–56, inhibited food intake after central administration in adult male rats; however, OBGRP 57–92 and OBGRP 116–167 presented very poor activities [20]. In 1997, Grasso and coworkers, by testing six overlapping leptin-related peptide amides encompassing the residues 106–167, found three fragments 106–120, 116–130 and 126–140 that were able to reduced food intake and body weight of C57BL/6J *ob/ob* mouse, when administered by ip injections [21]. In 1999, the same group of researchers, designed and tested 14-overlapping peptide amides encompassing the complete leptin molecule sequence. They confirmed that the domain encompassing the residues 106–140 contain the functional epitope of the leptin molecule [22] and that the most potent fragment, LEP-(116–130)-NH$_2$, does not require peptide interaction with the OB-R$_b$ to reduce body weight, food intake in *ob/ob* and *db/db* mice [23]. They also found that an even smaller leptin fragment, LEP-(116–122), denominated OB$_3$ and its analog [D-Leu^4]-OB$_3$ were also able to reduce food and water consumption in C57BL/6J *ob/ob* mice [24].

Recently, we also performed a detailed study, which tested all regions of the leptin molecule, synthesizing six peptides based on the protein three-dimensional structure [17,18]. In this study, the synthetic peptides were injected intravenously in rat and their ability to induce Fos-ir was compared with that of the recombinant human leptin. The results

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showed that Ac-[Ser^{117}]-Lep$_{116-140}$-NH$_2$ (**V**) induced Fos-ir in hypothalamic nuclei that expresses leptin receptor long form [25]. Interestingly, this fragment encompasses the same region found to be active by Grasso and coworkers [21], however, different from LEP-(116–130)-NH$_2$, the fragment **V** was able to interact to OB-R$_b$. No similar ability was observed for the other five fragments. These results suggest that this fragment acts in the same group of neurons that mediates leptin response.

On the basis of these results, we decided to study the six leptin-related peptides: Ac-Lep$_{23-47}$-NH$_2$ (**I**), Ac-Lep$_{48-71}$-NH$_2$ (**II**), Ac-Lep$_{72-88}$-NH$_2$ (**III**), Ac-Lep$_{92-115}$-NH$_2$ (**IV**), Ac-[Ser^{117}]-Lep$_{116-140}$-NH$_2$ (**V**) and Ac-Lep$_{141-164}$-NH$_2$ (**VI**) and their corresponding dimer forms (except fragment **II**) which were generated by a disulfide-bridge formation. Herein, we report the synthesis, chemical characterization and biological responses found for the fragments in comparison to that expected for the native leptin and for [D-Leu^4]-OB$_3$ [26], and its human sequence analog. The activity of the fragments was evaluated by their ability to interact with the leptin receptor present in HP-75 cells and alter the extracellular acidification rate, using cytosensor microphysiometer.

MATERIALS AND METHODS

Peptide Synthesis, Purification and Chemical Characterization

All leptin-related peptide monomer (Table 1) and dimer fragments (Table 2) were synthesized using common protocols for manual solid-phase methodology and *t*-Boc strategy [27,28]. MBHA-resins, with substitutions varying from 0.4 to 0.8 mequiv/g, were employed [29]. The N^{α}-terminal temporary protecting group was removed with 50% TFA in DCM in the presence of 2% anisole for 20 min. Couplings were carried out using 2.5 excess of DIC in DCM-DMF (1 : 1, v/v) and were monitored by Kaiser ninhydrin test [30]. Coupling times were 1–2 h. Recouplings of 1 h were done, when needed, using 2.5 excess TBTU in the presence of excess DIEA in DCM-NMP (1 : 1, v/v). Acetylations were performed with 50% acetic anhydride in DMF for 15 min when required. The dry protected peptidyl-resins were exposed to anhydrous HF in the presence of 10% anisole at 0 °C for 1.5 h. Excess HF and scavenger were eliminated under high vacuum. The crude peptides were precipitated with anhydrous diethyl ether, separated by filtration and extracted from the resin with 50% acetic acid in H_2O and lyophilized. To obtain the dimer-leptin fragments, disulfide-bridge formation was achieved by dissolving the lyophilized crude peptides (100 mg/l). The pH was adjusted to 6.8–7.0 by addition of ammonium hydroxide. The solution was vigorously stirred with air bubbling at 5 °C. Cyclization reaction was monitored by LC/ESI-MS. After 72 h, the reaction medium was acidified with acetic acid to pH range 4.5–5.0 and lyophilized. The crude lyophilized peptides were purified in two steps [Triethylammonium phosphate (TEAP) pH = 2.25 and 0.1% TFA] by preparative RP-HPLC on a Waters Associates system (Model Prep 4000), using linear gradients (slope 0.33% B/min). Briefly, they were loaded on a Vydac C_{18} preparative RP-HPLC column (22 × 250 mm, 15 μm particle size, 300 Å pore size) at a flow rate of 10.0 ml/min and eluted with TEAP (pH = 2.25)/CH_3CN, and detected at 220 nm. Selected fractions were collected and converted to the TFA salt by loading on a preparative column as mentioned above and eluted using a linear gradient (slope 0.33 %B/min) containing a mixture of solvents A (0.1% TFA in H_2O) and B [0.1% TFA in CH_3CN/H_2O (75 : 25)] at a flow rate of 10.0 ml/min. Selected fractions

Table 1 Monomer leptin fragments characterization

No.	Fragments	Sequence[a]	Purity (%)		PM $[M+H]^+$	
			HPLC[b]	CE[c]	Obtained[d]	Calcd
	[D-Leu^4]-mOB$_3$	Ac-SCS**l**PQT-NH$_2$[e]	99	98	776	776
	[D-Leu^4]-hOB$_3$	Ac-SCH**l**PWA-NH$_2$[f]	99	99	853	854
I	Ac-hLEP$_{23-47}$-NH$_2$	Ac-PIQKVQDDTKTLIKTIVTRINDISH-NH$_2$	98	97	2918	2918
II	Ac-hLEP$_{48-71}$-NH$_2$	Ac-TQSVSSKQKVTGLDFIPGLHPILT-NH$_2$	98	98	2609	2608
III	Ac-hLEP$_{72-88}$-NH$_2$	Ac-LSKMDQTLAVYQQILTS-NH$_2$	98	99	1980	1980
IV	Ac-hLEP$_{92-115}$-NH$_2$	Ac-RNVIQISNDLENLRDLLHVLAFSK-NH$_2$	98	97	2851	2849
V	Ac-[Ser^{117}]-hLEP$_{116-140}$–NH$_2$	Ac-SSHLPWASGLETLDSLGGVLEASGY-NH$_2$	99	98	2589	2588
VI	Ac-hLEP$_{141-164}$-NH$_2$	Ac-STEVVALSRLQGSLQDMLWQLDLS-NH$_2$	97	98	2732	2731

[a] Bold and lower case letters correspond to D amino acid and Z stands for a norleucine.
[b] Column: Waters Nova-Pak C_{18} {(2, 1 × 150 mm), 60 Å, 4 μm}; Solvents: A: 0.1% TFA in H_2O and B: 0.1% TFA in CH_3CN/H_2O (75 : 25); Flow: 0.4 ml/min; λ = 190–300 nm; injection volume: 30 μl and sample concentration: 1.0 mg/ml.
[c] CE = capillary electrophoresis. Sodium phosphate buffer pH = 2, 5; λ = 214 nm; capillary: silica (75 μm × 60 cm); voltage: 20 KV; hydrostatic injection time: 25 s and sensibility: 0.005.
[d] LC/ESI-MS conditions: mass range = 500–3930 Dalton, injection volume: 20 μl, sample concentration = 1.0 mg/ml and deconvolution of 4000–8000 Da.
[e] Mice Sequence.
[f] Human sequence.

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Table 2 Dimer leptin fragments characterization

No.	Sequence[a]	Purity (%)		PM $[M+H]^+$	
		HPLC[b]	CE[c]	Obtained[d]	Calculated
I-I	Ac-VPIQKVQDDTKTLIKTIVTRINDISHC-NH_2 | Ac-VPIQKVQDDTKTLIKTIVTRINDISHC-NH_2	99	99	6240	6239
III-III	Ac-LSKZDQTLAVYQQILTSC-NH_2 | Ac-LSKZDQTLAVYQQILTSC-NH_2	98	98	4130	4129
IV-IV	Ac-CZPSRNVIQISNDLENLRDLLHVLAFSK-NH_2 | Ac-CZPSRNVIQISNDLENLRDLLHVLAFSK-NH_2	99	98	6494	6494
V-V	Ac-SSHLPWASGLETLDSLGGVLEASGYC-NH_2 | Ac-SSHLPWASGLETLDSLGGVLEASGYC-NH_2	98	97	5380	5379
VI-VI	Ac-STEVVALSRLQGSLQDZLWQLDLSC-NH_2 | Ac-STEVVALSRLQGSLQDZLWQLDLSC-NH_2	99	98	5630	5630

[a] Z stands for a norleucine.
[b] Column: Waters Nova-Pak C_{18} {(2, 1 × 150 mm), 60 Å, 4 μm}; Solvents: A: 0.1% TFA/H_2O and B: 0.1% TFA in CH_3CN/H_2O (75 : 25); Flow: 0.4 ml/min; λ = 190–300 nm; injection volume: 30 μl and sample concentration: 1.0 mg/ml.
[c] Sodium phosphate buffer pH = 2, 5; λ = 214 nm; capillary: silica (75 μm × 60 cm); voltage: 20 KV; hydrostatic injection time: 25 s and sensibility: 0.005.
[d] LC/ESI-MS conditions: mass range = 500–3930 Dalton, injection volume: 20 μl, sample concentration = 1.0 mg/ml and deconvolution of 4000–8000 Daltons.

containing the purified peptide were pooled and lyophilized. The purified peptides were characterized, as shown in Tables 1 and 2, by Reversed-phase liquid chromatography (RP-HPLC) and by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS). RP-HPLC was performed on a Waters Associated Alliance system using a linear gradient of 5–95% B for 30 min of CH_3CN in two aqueous buffers: TEAP pH = 2.25 and 0.1% TFA, at 1.5 ml/min, a Vydac C_{18} column (4.6 × 150 mm, 5 μm particle size, 300 Å pore size) at 215 nm. The LC/ESI-MS data were obtained on a Micromass instrument, model ZMD coupled on a Waters Alliance model 2690 system using a Waters Nova-Pak C_{18} column (2.1 × 150 mm, 4 μm particle size, 60 Å pore size): solvent A (0.1% TFA/H_2O); solvent B [0.1% TFA in CH_3CN/H_2O (75:25)], gradient 5–95%, 30 min, λ range 190–300 nm mass range: 500–3930 m/z.

Cell Culture and Cell Transfection

The pituitary adenoma human cells (HP-75 cells) obtained from American Type Culture Collection, ATCC (Manassas, VA), were maintained in Dulbecco's modified Eagle's medium, DMEM (GIBCO-BRL) supplemented with 15% fetal bovine serum and 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen) and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Extra Cellular Acidification Rate Measurements

The cytosensor microphysiometer is a powerful technique to evaluate the responsiveness of different cell lines to a variety of bioactive ligands measuring small changes in the concentration of H^+ ions in the sensor chamber [31]. This method has been used extensively to measure the effects of signal transduction in cell surface receptor ligand agonist and analogs for many families of G protein-coupled receptors [32,33]. The HP-75 cells expressing the leptin receptor ($OB-R_b$) were plated onto membrane polycarbonate capsule cups at a density of 5×10^5 cells/cup in a DMEM medium, approximately 12–16 h prior to the experiment. The capsule cup/insert assembly was placed into the sensor chamber of the cytosensor system (Molecular Devices Corporation) which contained the pH-sensitive system [31–33]. The DMEM medium not supplemented with fetal bovine serum was pumped through the chambers, bathing the cells, at a rate of 100 μl/min. This running medium was devoid of sodium bicarbonate and had a buffering capacity of 1 mM supplied by phosphate. Physiological osmolarity was obtained by the addition of NaCl. The assembled cup was then transferred to sensor chambers containing 1 ml of low-buffered (1 mM sodium phosphate) DMEM with 0.1% BSA and without bicarbonate but containing 2 mM glutamine and additional 44.4 mM NaCl to replace bicarbonate and adjust osmolarity.

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The sensor chambers were placed on the cytosensor and allowed to equilibrate for more than 30–40 min before the beginning of the experiment. The medium was run through the chambers at a rate of 100 µl/min and pump cycle was 90 s on and 20 s off at 37 °C. To assess shifts in the extracellular acidification rate, cells were stimulated over a period of 30 s with the drugs at concentrations of 10^{-7} M. All experiments were performed in triplicate.

Circular Dichroism Studies

CD spectra were acquired on a Jasco J-810 spectropolarimeter (Tokyo, Japan) thermostated at 20 °C and continually flushed with ultra-pure nitrogen. CD spectra were recorded using a 1-mm path length rectangular quartz cell, with four accumulations at 50-nm/min scan speed, 8 s response time, 0.5 nm spectral bandwidth, and a wavelength range of 190–260 nm. The results are expressed in terms of molar ellipticity [θ] in units of degrees cm^2/dmol. The spectra of the fragments were obtained in water, and in the presence of SDS (3 and 10 mM) and TFE (40 and 80%). Samples were prepared by diluting the peptide stock solution (1 mM solution in water, pH = 6,0) to obtain a final concentration of 0.1 mM.

RESULTS

Peptide Synthesis, Purification and Chemical Characterization

Peptide synthesis, accomplished by manual solid-phase methodology using the *t*-Boc strategy, presented difficulties in some amino acid residues couplings. Improvements on the amino acid couplings, and consequently on the purities of the crude peptides formed were obtained when the synthesis was performed on a MBHA resin with low degree of substitution (0.4 mmol/g).

The two-step purification using different solvent systems (TFA and TEAP) was very effective. The overall purity of the peptides was over 97 and 98% as judged by analytical RP-HPLC and capillary electrophoresis (CE), respectively, and their molecular masses determined by LC/ESI-MS were in agreement with those theoretically expected (Tables 1 and 2).

Extracellular Acidification Rate Measurements

Bioassays using the microphysiometer cytosensor measured the acidification rate of the medium in contact with the HP-75 cells (pituitary adenoma human). We have compared the effect caused by the leptin fragments with those obtained with the human recombinant leptin and with the human and rat [D-Leu^4]-OB$_3$ (Figures 1 and 2). The results revealed that fragments **IV** and **V**, in both the monomer and dimer forms were able to activate the OB-receptor, similarly to leptin and to human and rat [D-Leu^4]-OB$_3$. Conversely, the fragments **I, II, III** and **VI** could not activate the receptor, and consequently, alter the extracellular acidification rate.

Circular Dichroism (CD)

CD spectra of the leptin monomer fragments (**I** to **VI**) in water, pH = 6.0 are shown in Figure 3(a). In an attempt to verify possible conformational changes of the fragment **IV**, we performed CD experiments in different pHs (Figure 3(b)). CD spectra of the fragments (**I** to **VI**), obtained in 10 mM SDS and in 80% TFE are shown in Figure 3(c) and (d), respectively. Circular dichroism spectra of dimer-leptin fragments were obtained in

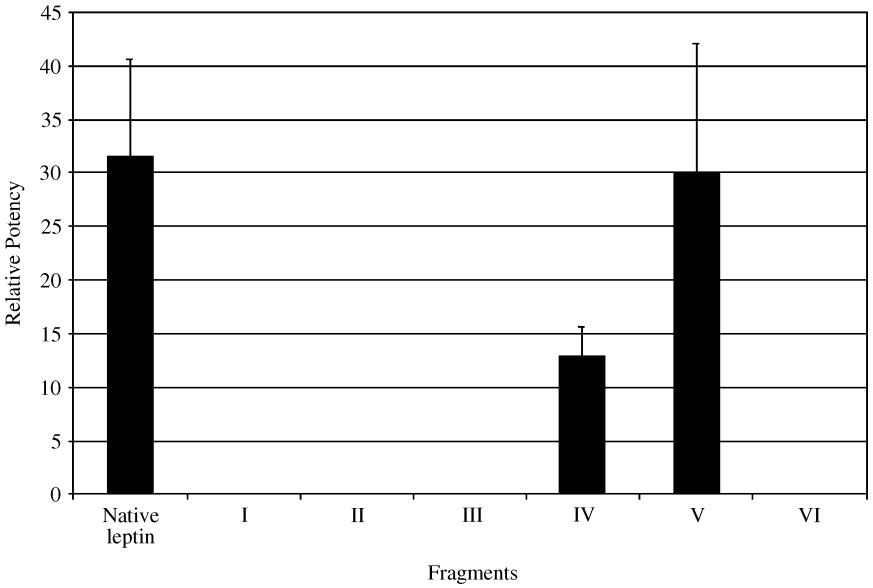

Figure 1 Effect of recombinant leptin and leptin monomer fragments (10^{-7} M) on extracellular acidification rate assays in HP-75. The activation is expressed as a percentage of the maximum values obtained for the recombinant leptin (mean ± SE, $n = 4–18$). Fragments **I, II, III** and **VI** were not active.

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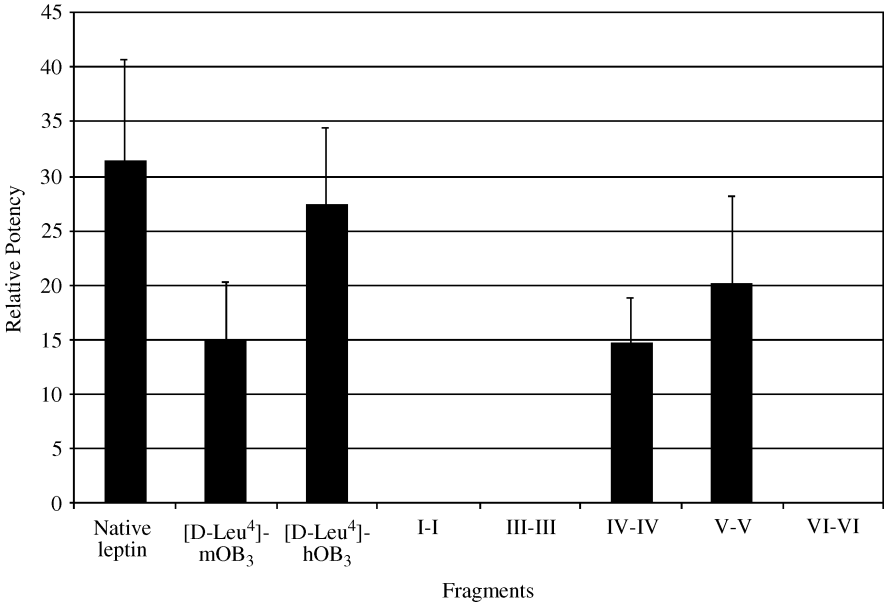

Figure 2 Effect of recombinant leptin, [D-Leu^4]-OB$_3$ (mice and human sequence) and leptin dimer fragments (10^{-7} M) on extracellular acidification rate assays in HP-75. The activation is expressed as a percentage of the maximum values obtained for the recombinant leptin (mean ± S.E., $n = 4–18$). Fragments **I-I, III-III** and **VI-VI** were not active.

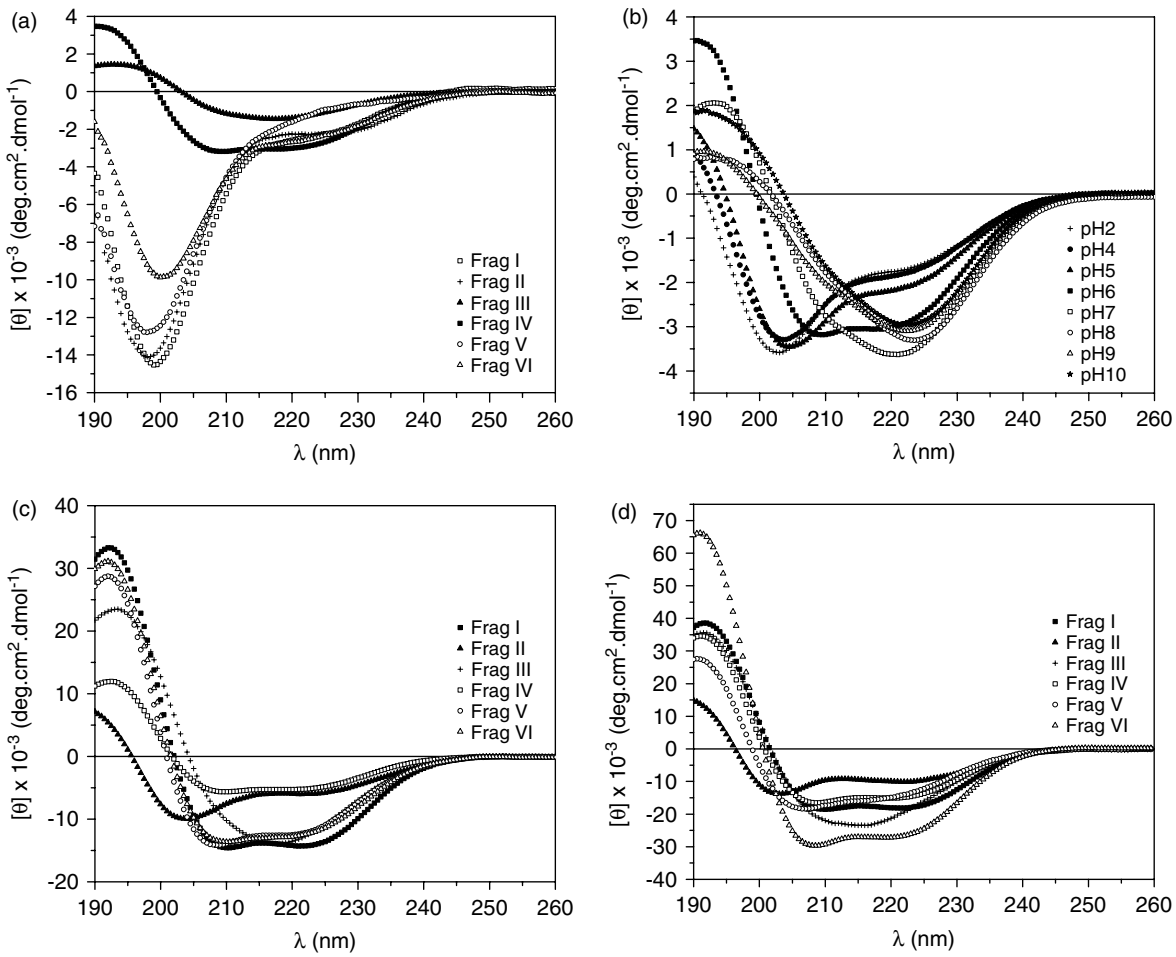

Figure 3 CD spectra: (a) Monomer leptin fragments in water pH = 6.0; (b) Monomer fragment **IV** at different pH; (c) and (d) Monomer leptin fragments in 10 mM SDS and 80% TFE, respectively.

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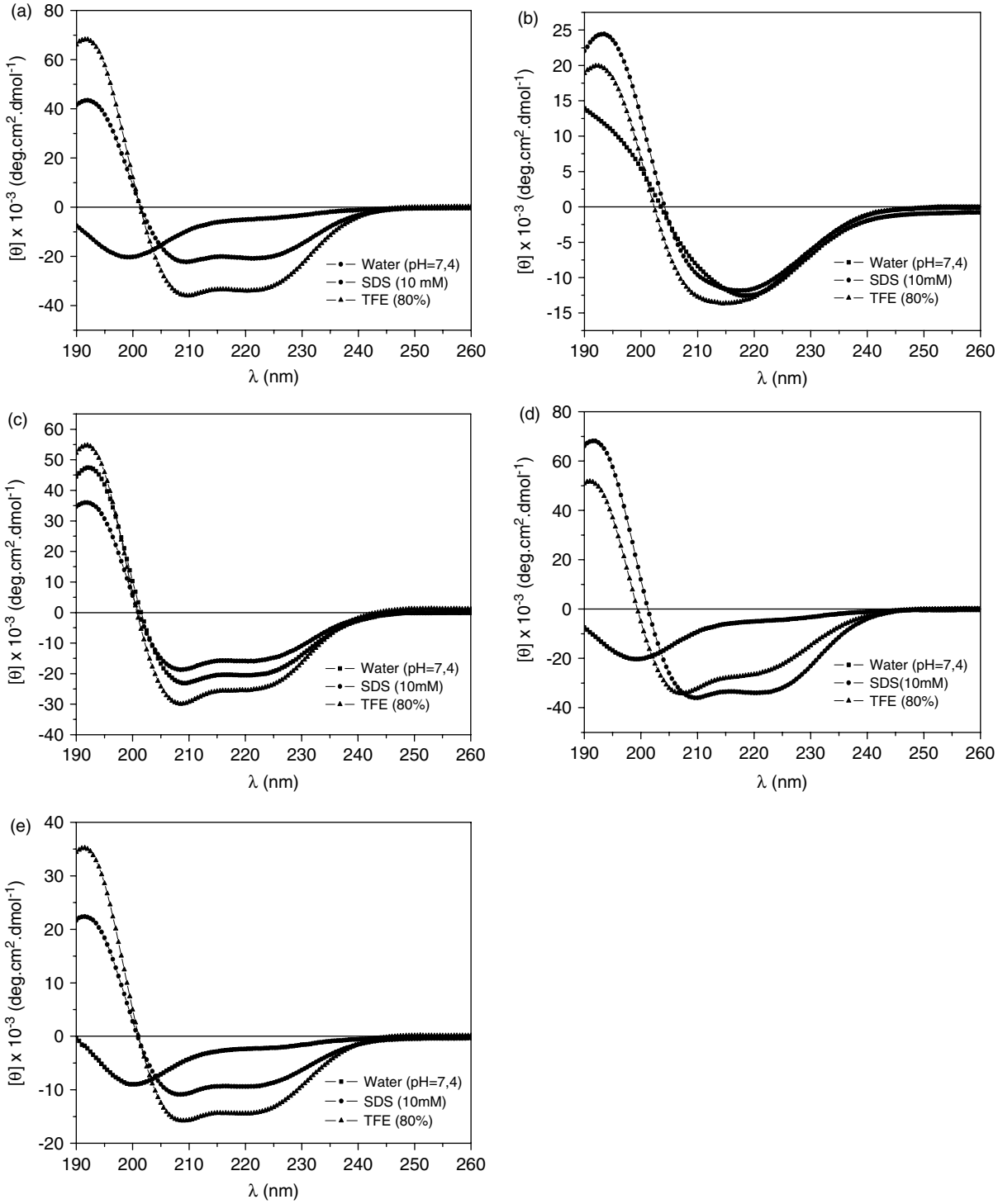

Figure 4 CD spectra in water (pH = 6.0), 10 mM SDS and 80% TFE of the dimer-leptin Fragments: (a) **I-I**; (b) **III-III**; (c) **IV-IV**; (d) **V-V** and (e) **VI-VI**.

water, 10 mM SDS and 80% TFE and are presented in the Figure 4(a)–(e).

DISCUSSION

In the past few years, synthetic peptide fragments of leptin have been employed in an attempt to identify leptin-like agonists [25]. This kind of approach takes into consideration that the activation of the long form of the leptin receptor may not be required for certain biological activities, such as food intake and body weight gain [23] and that short peptides could bypass more easily the blood-brain barrier. Grasso *et al.* [21] have shown that the *C*-terminal region

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of molecule, corresponding to amino acid residues 116–130, reduces body-weight gain and food intake in ob/ob mice. The same peptide was tested in male rats and showed to be able to stimulate prolactin and luteinizing hormone secretion, when administrated (i.c.v.) [34]. Two even shorter fragments (Ser^{116}-Cys-Ser-Leu-Pro^{120}-Gln-Thr, denoted OB_3 and its analog D-Leu^4-OB_3) were also described. They showed to be able to induce weight reduction when administered to female C57BL/6J ob/ob mice [24]. On the other hand, Samson *et al.* [20] hypothesized the satiety effects of leptin reside in the *N*-terminal region of the protein sequence. They described a significant inhibition of food intake in rats following central administration of the 35 amino acid fragment, named OBGRP 22–56.

The effects of human-leptin fragment (126–140) on spontaneous secretion of pituitary hormones and endogenous leptin, as well as on growth hormone releasing hormone (GHRH)-induced GH secretion were examined in eight healthy, non-obese men [35]. The author observed no alteration on the endogenous leptin levels following the administration of the leptin fragment. Nevertheless, the fragment had an inhibitory role in GH secretion when administered exogenously, with significantly dose-response suppression in GHRH-induced GH secretion.

Presently, we report the design, synthesis and chemical characterization of the leptin fragments described in this work and evaluated the neuronal responses of the fragments when compared to that expected for the whole protein. This comparison was made by using expression of Fos-immunoreactivity, a marker for cellular activation following intravenous administration of leptin [19]. The results showed that only the fragment Ac-[Ser^{117}]-$Lep_{116-140}$-NH_2 (**V**) induced Fos expression in hypothalamic nuclei albeit in lesser extension and intensity compared to the leptin molecule [25]. Interestingly, this peptide encompasses the region of the leptin molecule capable of reducing body-weight gain, described by Grasso *et al.* [21].

To investigate whether Fos-ir was induced in the same neuronal group activated by leptin, we proceeded with a dual label immunohistochemistry for cocaine- and amphetamine-regulated transcript (CART), a neuropeptide related to leptin action in rat hypothalamus. We found that Ac-[Ser^{117}]-$Lep_{116-140}$-NH_2 (**V**) activate CART neurons differently through the rostrocaudal extension of the arcuate nucleus. Such effect may be due to a direct interaction with leptin receptor long form, contrary to the results observed with *db/db* mice [23]. Thus, we decided to study the interaction of the fragments with the OB-receptor *in vitro*.

This study was achieved using peptide fragments (**I** to **VI** – Table 1) selected on the basis of three-dimensional structure of leptin. Their corresponding dimer forms (except fragment **II**), generated by a disulfide bridge formation, were also included (Table 2). The leptin secondary structure proposed by crystallography [18] and NMR [17] analyses present four antiparallel α–helical motifs that correspond to fragments Pro^{23}-His^{47}; Leu^{72}-Ser^{88}; Arg^{92}-Lys^{115} and Ser^{141}-Ser^{164}, and also two loops encompassing the residues Thr^{48}-Thr^{71} and Ser^{116}-Tyr^{140}.

The cellular activity of the leptin fragments were evaluated in the microphysiometer cytosensor and were compared to the activity of the recombinant human leptin. The activity was determined using a culture of HP-75 cells (human pituitary cell), which has been described as a cell line expressing the active leptin receptor OB-R_b [36,37]. Biological assays using the microphysiometer has been reported as an effective assay in the evaluation of the cellular activity of proteins [38,39] and peptides [40,41]. This assay was employed for all the leptin fragments (monomers and dimers) and for the peptides [D-Leu^4]-OB_3 (mice and human sequence).

The analysis of the biological activity of the monomer-leptin fragments **I** to **VI** showed that only fragments **IV** and **V** presented activity (Figure 1). In fact, fragment **V** showed to be as active as the native leptin. These results are in agreement with the ones obtained by Rozhavskaya-Arena [24] and by Oliveira *et al.* [25] by using expression of Fos-immunoreactivity. In fact, Grasso *et al.* had suggested that this region of the molecule could contain the functional epitope that presented the effects observed for the leptin. In their structure–activity relationship (SAR) study performed with this region of the molecule, they showed that modification in the stereochemical configuration of the amino acid residues, induced either drastic alteration in the biological activity or completed inactivation [24]. The results obtained with the dimer-leptin fragments confirmed that the region is important for the leptin fragments activity, since only the dimer-leptin fragments **IV-IV** and **V-V** were active (Figure 2) and the dimer fragment **IV-IV** was as active as leptin.

Also in agreement with the results obtained by Grasso *et al.* [24,26], both fragments [D-Leu^4]-mOB_3 and [D-Leu^4]-hOB_3 activated the cells. The mice sequence was 2-fold less potent and the human equipotent in comparison with activation caused by the native leptin.

In water, the CD spectra of the monomer fragments, except for the fragments **III** and **IV**, showed a negative band at 198 nm and a shoulder at 215–220 nm, indicative of unordered structure (Figure 3(a)). CD spectrum of the fragment **III** presented a positive band at 195 nm and a negative one at 216 nm, typical of a β-sheet structure. CD spectra of the fragment **IV** showed a more drastic pH-dependent conformational changes, varying from coexisting conformations (negative band at 204 nm and a shoulder at 220 nm) to a β-turn structure (positive band at 195 nm, a shoulder at 210 nm and a negative band at 225 nm)(Figure 3(a) and (b)). The

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CD spectra of the dimer fragments obtained in water revealed that only the dimer fragment **IV-IV** presented a great tendency to adopt an α-helix conformation (positive band at 190 nm, and two negative bands at 208 and 222 nm) while the dimer fragments **I-I, V-V** and **VI-VI** did not have a well-defined conformation and the CD spectra of the dimer fragment **III-III** presented a positive band at 195 nm and a negative one at 216 nm, characteristic of β-sheet conformation (Figure 4(a)–(e)).

In 10 mM SDS, the CD spectra of the fragments **I, V** and **VI** were typical of an α-helical conformation with a positive band at 90 nm and two negative bands at 208 and 222 nm (Figure 3(c)). The CD spectra of the dimer fragment **III-III** presented a positive band at 195 nm and a negative one at 216 nm, characteristic of β-sheet conformation in 10 mM SDS (Figure 3(c)); while the conformation of the dimer fragment **IV-IV** was not well defined.

In 80% TFE, the CD spectra revealed a tendency of the fragments **I, IV**, and **V** to adopt an α-helix conformation; undefined conformation for the fragment **II**, a typical α-helix conformation for the fragments **VI,** and a characteristic β-sheet conformation for the fragment **III**.

The CD spectra in both 10 mM SDS and 80% TFE, revealed a typical α-helix conformation for the dimer fragments **I-I, IV-IV, V-V** and **VI-VI**, and a β-sheet structure for the dimer fragment **III-III** (Figure 4(a)–(e))

Many similar studies of conformational behavior of protein fragments are found in the literature. Callihan *et al.* [42] investigated the structure of seven protein FK506 fragments (Tacormilus), using CD and RMN while Luo *et al.* [43], by using circular dichroism, studied the Vpr protein (Protein accessory of HIV-1) using peptide fragments, which corresponded to α-helix structures of the molecule. In both the works, conformational changes were verified in the protein regions that presented well-defined secondary structures. These fragments presented totally random coil or in some cases, maintained part of the original structure, probably due to absence of the interactions among the protein chains. The use of different concentrations of TFE and SDS in these works stabilized the secondary structures of the fragments studied, which adopt α-helix conformation or beta structures.

In conclusion, our results suggested that the fragments **IV** and **V** and the peptide [D-Leu^4]-OB$_3$ and its human-sequence analog present the region of the molecule containing the functional epitope of the leptin molecule, in agreement with the resuts obtained by Grasso *et al.* [21] and by Oliveira *et al.* [25]. However, they were shown to be recognized by leptin receptor present in HP-75 cells, not in agreement with the results obtained by Grasso *et al.* [22]. No clear correlation was found between bioactivity and CD results.

The peptide fragments design is a kind of approach which offers the basis for the development of leptin-related compounds with potential application in human obesity.

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