

Drug Development-targeted Screening of Leptin Agonist Glycopeptides

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Abstract A glycosylated dodecapeptide fragment corresponding to the hypothalamus-active cytokine leptin exhibits agonistic properties to the leptin receptor (ObR) in vitro and penetrates into the brain in vivo. In order to characterize the drug development potential of the lead peptide and to optimize it for pharmacological applicability, a series of biochemical screening assays were custom-tailored to the leptin/ObR system. To identify peptides that bind the extracellular domain of ObR, we characterized the optimal conditions for an ELISA-type assay where the leptin fragments were immobilized to the plates. With this technology we could identify low-dose binder peptidomimetics which, according to a comparison of the conventional cell proliferation assay and a measure of metabolically active cells, revealed that agonists identified by these cellular assays may not necessarily induce the expected growth characteristics in ObR expressing cells. The original glycopeptide lead displayed a 2 h half life in 25% diluted mouse serum but poor stability in mouse brain extract. Fifteen percent of the glycopeptide crossed a dual

endothelial/astrocyte cell layer (representing an in vitro model of blood-brain-barrier) in 30 min, and the coexistence of the two cell types appeared necessary to quantify the level of brain accessibility. Finally, in an in vivo mouse model, a Cy5.5 labeled glycopeptide was more evenly distributed all over the body, including the brain, than a similarly labeled full-sized leptin protein.

Keywords Binding · Biodistribution · Blood-brain-barrier penetration · Glycoamino acid · Leptin deficiency · Leptin receptor · Obesity · Signal transduction

Abbreviations

Ab	Antibody
ATCC	American type culture collection
BBB	Blood-brain barrier
BSA	Bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
ERK 1/2	Extracellular signal-regulated kinases 1 and 2
Fmoc	9-Fluorenyl-methoxy-carbonyl
GSK	Glycogen synthase kinase
Ip	Intraperitoneally
JAK	Janus kinase
MALDI-MS	Matrix-assisted laser ionization/desorption mass spectroscopy
ObR	Leptin receptor
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.5% Tween 20
PI-3K	Phosphoinositide 3 kinase
PLC	Phospholipase C
PKC	Protein kinase C

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RP-HPLC	Reversed-phase high performance liquid chromatography
Sc	Subcutaneously
SFM	Serum-free medium
STAT	Signal transducer and activator of transcription
TBST	Tris-buffered saline containing 0.1% Tween 20
TFA	Trifluoroacetic acid
VEGF	Vascular endothelial growth factor
MALDI	Matrix-assisted laser desorption/ionization

Introduction

Leptin, a hormone produced by adipose tissue, regulates food intake and energy balance in the hypothalamus (Wauters et al. 2000). In addition to its role as a neuro-hormone, in the periphery leptin can modulate immune response, fertility, and hematopoiesis, acting as a mitogen, metabolic regulator, or pro-angiogenic factor (Garofalo and Surmacz 2006). The existence of disorders related to leptin deficit and leptin overabundance calls for the development of drugs activating or inhibiting the leptin receptor (ObR) (Baicy et al. 2007). Leptin, in replacement doses, normalizes neuroendocrine, metabolic and immune functions in patients with states of relative leptin deficiency including lipoatrophy and some forms of hypothalamic amenorrhea but further clinical studies are required to determine the long-term efficacy and safety of the full recombinant protein (Brennan and Mantzoros 2006). As most forms of obesity are associated with diminished responsiveness to the appetite-suppressing effects of leptin (Muntzberg and Myers 2005), recently leptin agonists emerged as sought after intervention measures against a newly coined term ‘central leptin insufficiency syndrome’ (Kalra 2007).

Leptin activates the extracellular domain of its transmembrane receptor ObR and induces multiple signaling pathways including the Janus kinase and signal transducer and activator of transcription (JAK–STAT) pathway, the mitogen-activated protein kinase (MAPK) cascade, and the phosphatidylinositol 3-kinase (PI3-K) and adenosine-monophosphate-activated kinase (AMPK) pathways (Hegyí et al. 2004). Due to the necessity for leptin to cross the blood-brain-barrier (BBB), ObR signaling is traditionally targeted through deactivation of negative-feedback inhibitors in the central nervous system (CNS) rather than inhibition of the receptor itself (Peelman et al. 2006). However, we recently identified a glycopeptide derivative, H-Tyr(I₂)-Ser(Glc)-Thr-Glu-Val-Val-Ala-Leu-Ser-Arg-Leu-Dap(Ac)-NH₂ (termed *EIFree*), corresponding after

sequence modifications to amino acids 119–130 of human leptin. *EIFree* is a full agonist of ObR, crosses an epithelial/astrocyte cell layer in a BBB penetration model, and is distributed into the brain of Balb/c mice after intraperitoneal administration (Otvos et al. 2008). Thus, the burden of identification of leptin agonists can now return to screening of direct peptide (or peptidomimetic)–ObR interactions ensuing pharmacological evaluation of the lead peptides. As many of the assays in the past gave only qualitative suggestions for the therapeutic efficacy of leptin agonists and/or were time consuming, we concentrated our efforts on developing and applying measures compatible with industrial drug development programs.

Materials and Methods

Peptide Synthesis

Amino acids protected with 9-fluorenyl-methoxy-carbonyl group (Fields and Noble 1990) were used for the synthesis of the peptides. The peptide chain assembly was carried out on either a Rainin PS3 or a CEM Liberty automated synthesizer. After trifluoroacetic acid (TFA) cleavage, peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) in a water/acetonitrile/TFA elution system, until matrix-assisted laser-desorption/ionization (MALDI) mass spectra revealed only single species. The carbohydrate protecting acetyl groups were removed with a 10-min treatment with 0.01 M NaOH, the reaction mixtures were immediately neutralized with an equal amount of 0.01 M HCl and the free glycopeptides were repurified by RP-HPLC.

Enzyme-linked Immunosorbent Assay (ELISA) for Measuring Peptide Binding to ObR

The leptin fragments were dissolved in electroblot transfer buffer (25 mM Tris and 192 mM glycine buffer containing 20% methanol) and 1–50 μg of the peptides were dried down to ELISA plates at 37°C. The wells were blocked with 5% bovine serum albumin (BSA) in a phosphate-buffered saline—0.5% Tween 20 buffer (PBST) overnight at room temperature and the plates were washed 3 times with PBST. Then the plates were incubated with 50 μl of 1–100 nM solutions of human IgG Fc-conjugated ObR extracellular domain (R&D Systems) dissolved in Tris-buffered saline—0.1% Tween 20 buffer (TBST) containing 1% BSA for 2 h at 37°C. The plates were washed 3 times with PBST. Fifty μl of a goat monoclonal antibody (Ab), recognizing the N-terminus of human ObR (Santa Cruz Biotechnology) was added as a primary antibody at 1:100 dilution overnight at

4°C and the plates were washed 3 times with PBST. Then the wells were incubated with 50 µl of horseradish peroxidase- (HRP) conjugated anti-goat Fc mouse antibody in 1:100 dilution for 2 h at room temperature and washed 5 times with PBST. The ELISA was developed with 100 µl/well of an HRP ELISA kit featuring tetramethylbenzidine as substrate and sulfuric acid as stop solution. Absorbance values were determined at 405 and 595 nm (respectively before and after adding the stop solution) on a BioTek Instruments EL311 microplate reader. The binding assays were done in triplicate and the experiments repeated at least 4 times except in cases when very high peptide concentrations were used.

Cell Proliferation

Human leptin was purchased from R&D Systems and used at the concentration of 100 ng/ml (approximately 6 nM). MCF-7 cells naturally expressing ObR were grown in a standard medium DMEM:F12 plus 5% fetal bovine serum. Seventy percent confluent cultures were synchronized in serum-free medium (SFM) (DMEM plus 10 µM FeSO₄, plus 0.5% bovine serum albumin, BSA) for 24 h and then treated with leptin and/or peptides (different doses) for 3 or 5 days. Cell numbers before and after treatment were determined by counting the cells with trypan blue exclusion. All assays were done in triplicate and repeated 3–6 times. The changes in cell number vs control SFM were determined as percentage decrease/increase. Statistically significant ($P < 0.05$) stimulation or inhibition over 10% vs. SFM were considered as evidence of agonistic or antagonistic activity, respectively.

XTT Assay

Alternatively, the biological activity of the peptides was tested using the XTT Cell Proliferation Kit (Roche, Mannheim, Germany), following the manufacturer's instructions. Briefly, MCF-7 cells were plated in 96-well culture plates at 4×10^3 cells/well in normal culture medium. After 24 h, the medium was replaced with SFM for 24 h. Then, the cells were exposed to 50 µl of peptide solutions for 24 h. Next, the cells were incubated with XTT labeling mixture (final XTT concentration 0.3 mg/ml) for 2 h at 37°C. The formation of orange formazan dye product by metabolically active cells was quantified on a BioTek Instruments EL311 microplate reader; the absorbance was read at 450 nm using a reference wavelength at 650 nm. Cells stimulated with 100 ng/ml leptin for 24 h were used as controls. In some cases the incubation period was extended to 2–3 days. Each peptide dose was tested in triplicate and the experiments were repeated at least 3 times. Statistically significant ($P < 0.05$) stimulation or

inhibition over 10% vs. SFM were considered as evidence of agonistic or antagonistic activity, respectively. Partial agonists/antagonists are defined as analogs that change the cell viability profile depending upon the presence or absence of full agonists.

Serum Stability

For serum stability studies, 250 µl of an aqueous peptide stock solution containing about 0.8 mg/ml peptide was added to 2.5 ml 25% aqueous pooled mouse serum (Powell et al. 1993). The peptide-serum mixture was thermostated at 37°C. After 0 min, 45 min or 60 min, 90 min, 2 h, 4 h, and 8 h, three 210 µl samples of each peptide were taken, and precipitated by the addition of 40 µl 15% aqueous trichloroacetic acid (TCA). The samples were stored at 4°C for 20 min and centrifuged. The supernatants were immediately frozen on dry-ice and 220 µl of each was analyzed on RP-HPLC and/or MALDI-MS.

Peptide Stability in Brain Extract

The brains of 2 female Balb/c mice (killed by CO₂ inhalation) were placed in 2 ml of sterile PBS, homogenated in a blender and sonicated. The peptides were added to the brain homogenates (either before or after sonication) at 6 µg/ml final concentration. At each incubation timepoint (0, 5, 15, 30, 60 and 120 min), a 30-µl aliquot was removed and treated with 8 µl 15% TCA and incubated for 20 min at 4°C, to allow precipitation of the peptide. After centrifugation at 13,000 rpm for 5 min, the supernatant was removed and the solution samples were submitted to MALDI-MS in order to identify the presence of the original peptides or their metabolites.

In vitro Model of BBB Penetration

A confluent monolayer of human astrocytes (designation C8D30, ATCC) or brain capillary endothelial cells (designation bEND.1, ATCC) or a dual layer of both cell types (first astrocytes followed by endothelial cells) were grown at 37°C on polycarbonate filters (Costar, Transwell, 0.4 µm) pretreated with collagen. Under these conditions, the endothelial cells retain the characteristics of BBB which include complex tight junctions, low rate of pinocytosis and enzyme levels (Lundquist et al. 2002). A solution of 0.1 mM peptides was added to the upper compartment of the Transwell plates and samples from the bottom compartments were taken at 0, 5, 10, 30, and 120 min intervals. The presence and amount of peptides penetrated through the cell layers were analyzed by RP-HPLC and MALDI-MS.

In vivo Biodistribution

Peptide *EIFree* and leptin protein were coupled to an N-hydroxy-succinimide activated derivative of the near-infrared absorbing fluorescent dye Cy5.5. Forty μg of the labeled polyamides were injected subcutaneously (sc) into shaved and isoflurane anesthetized female Balb/c mice. The animals were placed into the fluorescence microscope chamber under continuous isoflurane exposure. Fluorescence exposure pictures were taken with an IVIS microscope set to 695 nm emission wavelength at every minute in the first 10 min after peptide addition and every 5 min afterwards until 65 min.

Results and Discussion

Assay Development and Validation

Conventional drug development proceeds through the molecular—cellular—animal screening pathway of lead molecules. Pharmaceutically preferred molecular binding assays are conducted in solution where the free unbound ligand is not separated from the bound fraction (Otvos et al. 1998). However, in our hands, the low solubility of the commercially available extracellular domain of ObR prevented even qualitative measurements of leptin peptide binding to ObR in fluorescence polarization assays (Otvos et al. 2008). Therefore, the binding constant of the *EI* peptides (*EIFree* or its carbohydrate-acetylated version *EIAc*) was estimated based on the amount of peptide needed to stimulate typical leptin-dependent signal transduction events in a cellular environment. The peptide/ObR binding was then confirmed in vitro by a dot-blot assay. In the cellular measures, both *EI* peptides induced ERK1/2 activation in ObR-positive MCF-7 cells between 100 and 200 nM concentrations.

For future identification of potential ObR agonists from peptide libraries or a larger set of individual peptides, we developed a medium-throughput solid-phase screening assay. The assay followed the usual direct ELISA format in which peptide antigens are dried down to the wells overnight (Otvos and Szendrei 1996). After blocking the peptide-free surfaces of the wells, the extracellular domain of ObR was added and the incubation was extended to 2–12 h at room temperature. The peptide/ObR interaction was monitored by using an anti-ObR monoclonal Ab in 1:100 dilution. In general, at 2 h incubation time, detection of *EIAc* was more efficient than that of *EIFree* (Fig. 1). The lower detection limits of our assay were 10 μg leptin peptide, 10 nM solution of ObR and 2 h incubation time (Fig. 1) for *EIAc* and 50 μg peptide or 100 nM ObR for *EIFree*. The negative control antibacterial peptide

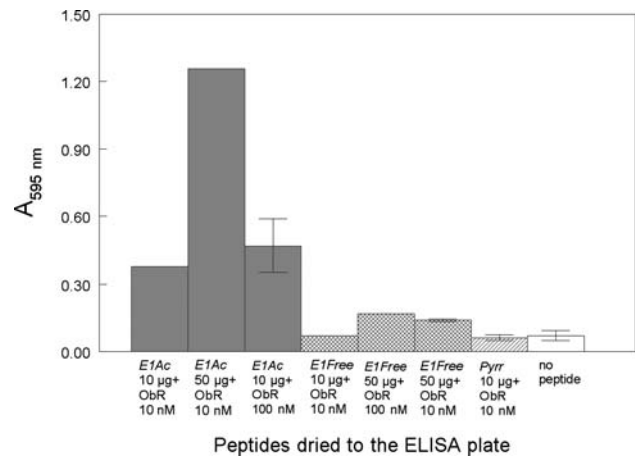


Fig. 1 Binding of leptin agonist peptides and controls to the extracellular domain of the leptin receptor. The peptide amounts and ObR concentrations in the wells are printed under each bar. The error bars indicate the standard deviation. Other details of the screening assay are outlined in the Materials and Methods and Results and Discussion sections. Pyrr stands for the negative control antibacterial peptide pyrrocorticin

pyrrocorticin at 10–50 μg had ELISA readings identical to peptide-free wells. In several repeat experiments the highest 100 nM ObR dose was inhibitory for binding to peptide *EIAc* and *EIFree* (Fig. 1 and data not shown) possibly due to receptor aggregation around this concentration, as we observed in the fluorescence polarization assay (Otvos et al. 2008). It is unlikely that the generally higher ELISA readings of the acetylated peptide compared to the deacetylated analog resulted from better recognition by the receptor. After all, the sugar moiety was artificially introduced only to promote penetration through the BBB and was not expected to interfere with ObR binding. Rather, the increasingly hydrophobic acetylated analog was more efficiently absorbed by the polypropylene plate surface. In our experience different peptides directly bound to hydrophobic ELISA plates or more hydrophilic solid surfaces show greatly variable binding efficacies and their binding conformations influence how proteins recognize them (Lang et al. 1994). In fact, our solid-phase binding assays of different site III leptin analogs suggested increased receptor recognition for peptides with long C-terminal hydrophobic tails, although these alkyl chains were located well outside the presumably binding pharmacophore sequence (data not shown).

While we managed to quantitate the *EI* peptide–ObR interactions to some degree, the 10–50 μg purified peptide needed for the assay described above exceeds the usual drug amounts produced by massively parallel synthetic methods. Thus, the assay is suitable only for medium-throughput screening of purified leptin agonists and antagonists. To validate the technology, we assayed the binding of 4 leptin site III analogs, 2 designer agonist and 2

Table 1 Binding of 10 µg leptin site III analogs to 100 nM of the extracellular domain of ObR

Peptide sequence	Absorbance at 595 nm
H-Tyr(I ₂)-Ser(GlcAc ₄)-Thr-Glu-Val-Val-Ala-Leu-Ser-Arg-Leu-Dap(Ac)-NH ₂ (<i>EIAc</i>)	0.589
H-Tyr(I ₂)-Ser(Glc)-Thr-Glu-Val-Val-Ala-Leu-Ser-Arg-Leu-Dap(Ac)-NH ₂ (<i>EIFree</i>)	0.106
H-Tyr(I ₂)-Ser-Thr(GlcAc ₄)-Glu-Val-Val-Ala-Leu-Ser-MeArg-Nva-NH ₂ (<i>Analog 1</i>)	0.144
H-Tyr(I ₂)-Ser(Bzl)-Thr-Glu-Val-Val-Ala-Leu-Ser-MeArg-Nva-NH ₂ (<i>Analog 4</i>)	0.150
Ac-Glu-Val-Val-Ala-Leu-Ser-Arg-Leu-Dap(Ac)-NH ₂	0.053
Ac-Thr-Glu-Val-Val-Ala-Leu-Ser-Arg-Leu-Dap(Ac)-NH ₂	0.068
Pyrrhocoricin	0.059
No peptide	0.052

antagonist candidates to ObR. Table 1 shows the peptide sequences and the ELISA reading values. In these assays, 10 µg peptides were plated and incubated with 100 nM ObR extracellular domain. The data indicated that the two shorter peptides did not bind to ObR. In contrast, the two longer peptides bound ObR, just like the positive control *EI* peptides did. Apparently the location of the carbohydrate moiety (Ser- or Thr-linked) or the identity of the serine side chain (carbohydrate or benzyl) did not influence the ability of the peptides to bind ObR. Because of the low sensitivity of the assay, it would be premature to draw further quantitative structure-activity relationship conclusions. Nevertheless, these assays confirmed that the receptor binding site is located somewhere in the middle of the sequence of the *EI* drug development candidates and the presence of the N-terminal Tyr-Ser dipeptide enhances receptor binding. As membranes are less hydrophobic than ELISA plates, the sensitivity of the assay may increase if using dot-blot (Otvos et al. 2008), but the binding can no longer be quantified.

Cellular Assays

The biological effects of the two new peptides that bound to ObR in the molecular assay were tested in ObR expressing MCF-7 breast cancer cells. Traditionally, the cellular activity of ObR agonists and antagonists are accessed with the cell proliferation assay. During these

experiments, the cells are incubated with the peptides for up to 5 days and the numbers of viable cells are counted after trypan blue exclusion (Otvos et al. 2008). Once again, while this is the most appropriate assay to evaluate peptide activity on cell growth, it is not compatible with high- or even medium throughput screening programs. A significantly faster alternative appeared to be an XTT assay, which quantifies metabolic activity of viable cells as a surrogate marker of cell number, and could be read after 24–36 h of treatment (Buttke et al. 1993). This assay is based on the reduction of the tetrazolium salt XTT by viable cells in the presence of an electron coupling reagent resulting in a soluble formazan salt. In this measure, in the absence of exogenous leptin, the Thr-linked glycopeptide (*Analog 1*) was a full agonist in the entire 10 nM–1 µM examination range, and the benzylated serine analog (*Analog 4*) was a full agonist at the lowest dose and a partial agonist at higher doses (Table 2). When 6 nM exogenous leptin was added to the cells, both peptides showed partial agonist/antagonist properties. However, when the same peptides were submitted to the conventional cell proliferation assay, neither of them produced any significant growth effects (Table 2). This suggested that metabolic response to ObR agonists or antagonists cannot be directly translated into cell number and used as a measure of cell growth effects. At this point we returned to the *EIFree* peptide that is a full agonist in the proliferation assay regardless whether leptin is present or not (Otvos

Table 2 Comparison of the effect of ObR agonist peptides on the cell proliferation and XTT cell viability assays

Assay type	Peptide <i>EIFree</i>	Peptide <i>Analog 1</i>	Peptide <i>Analog 4</i>
Proliferation no leptin added	Agonist 10 nM–1 µM	No effects	No effects
XTT no leptin added	Agonist 10–100 nM	Agonist 10 nM–1 µM	Agonist 10 nM
	Partial agonist 1 µM		Partial agonist 100 nM–1 µM
XTT 6 nM leptin added	Agonist 10–100 nM	Partial agonist 10–100 nM	Variable results, generally partial agonist/antagonist
	Antagonist 1 µM	Agonist 1 µM	

Cell proliferation and XTT assays were done and agonistic/antagonistic activities determined as described in Materials and Methods. The peptide sequences are found in Table 1

et al. 2008). Interestingly, in the XTT assay, the *EI Free* peptide is an agonist at 10 nM and 100 nM in all circumstances, but at 1 μ M is a partial agonist without leptin and an antagonist in the presence of 6 nM endogenous leptin protein. In summary, the comparison of the cell proliferation and XTT assays suggest that the faster measure is unable to fully replace the longer assay when it gets to accurately characterizing the effects of drugs on the growth of ObR expressing cells. Regarding the two new agonist analogs, animal models of obesity or other leptin-deficiency diseases will be needed to verify or disprove the positive effects produced by the XTT cell viability assay on ObR expressing cells.

Stability of the *EI Free* Peptide in Biological Environment

As the *EI Free* peptide shows agonistic properties to MCF-7 cells, penetrates the BBB model and is distributed into the brain of Balb/c mice (Otvos et al. 2008), we continued the detailed pharmaceutical evaluation of this peptidomimetic. First we submitted the peptide to quantitative stability assays in 25% diluted mouse serum. Dilution of serum linearly decreases the decomposition rate (makes it manageable for chromatographic analysis) without changing the cleavage sites or overall kinetics (Powell et al. 1992). The *EI Free* glycopeptide was remarkably stable in 25% mouse serum exhibiting a half-life of approximately 2 h (Fig. 2). Calculating with undiluted serum, this means that the peptide's estimated half life in vivo is 30 min, a period well exceeding the time needed to cross the BBB and each the hypothalamus. Indeed mass spectrometry identified *EI Free* in the bottom compartment of the BBB cellular model after as little as 10 min, and in the in vivo assay, the glycopeptide was biodistributed into the head in less than 30 min (Otvos et al. 2008).

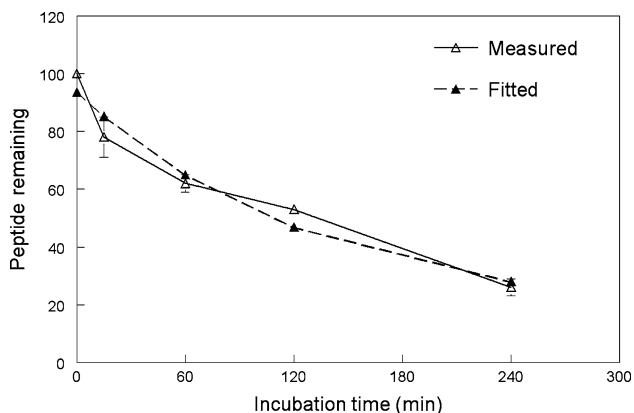


Fig. 2 Stability of peptide *EI Free* in 25% diluted mouse serum. The peptide exhibits an approximate half-life of 2 h

In our current study we tried to confirm the published BBB penetration cellular model mass spectrometry data (Otvos et al. 2008) by HPLC. We grew brain capillary endothelial cells or human astrocytes, or a confluent layer of the two cell types in Transwell filters and measured the quantity of peptide *EI Free* in the bottom compartment. While the peptide was detectable in the flow-through of either astrocytes or endothelial cells after 10 min, the combined layer allowed peptide penetration only at the next time point assessed, i.e. 30 min (Fig. 3). At 30 min, approximately 15% of the glycopeptide placed in to the top Transwell filter compartment was identified in the bottom compartment by HPLC. Since 5–8% of the peptide applied crossed the individual cell types already after 10 min, but none passed through the dual layer, we concluded that the synergism between the two cell types was needed to truly model the BBB characteristics. In our previous report, mass spectrometry (MS) identified some *EI Free* peptide in the bottom compartment already after 10 min (Otvos et al. 2008), which can be explained by the increased sensitivity of MS compared to HPLC. The peptide amount penetrated through the dual cell layer and detectable by MS is sufficient to induce leptin-dependent signal transduction in MCF-7 cells (Otvos et al. 2008). However, the peptide load in the in vitro BBB penetration assay is significantly higher (over 100-fold) than in the assay that is used for measuring cell growth. With an expected efficacious in vivo dose of 1 mg/kg or less, we designed our earlier biodistribution assay at 2 mg/kg. Then, we detected about 10–20% peptide in the head 30 min after intraperitoneal (ip) peptide administration, suggesting that the *EI Free* concentration in the brain should exceed doses needed to stimulate

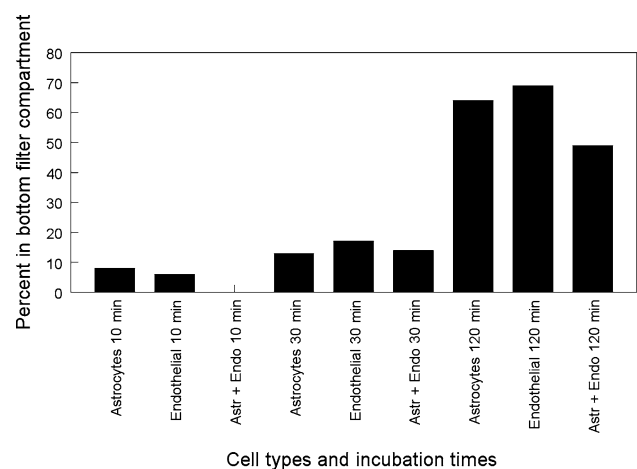


Fig. 3 Penetration of glycopeptide *EI Free* across cell layers grown in Transwell filters. The peptide was allowed to penetrate through either human brain astrocytes, capillary endothelial cells, or a confluent layer of the two cell types by gravitation. The high amount of peptide passed after 2 h might suggest the limited biological activity of intracellular barriers over extended time periods

ObR + cells. Indeed, this peptide dose is very similar to the amount contained in the 10–20% peptide fraction that penetrated the BBB, as we detected by HPLC in the current study.

As important *in vitro* serum stability studies in serum for peptide drug development are, some peptides undergo significantly more intensive degradation *ex vivo* or *in vivo* (Noto et al. 2008). To study the stability of the *EI* peptides in the brain tissue environment, the physiological site of peptide action, we incubated the peptides with a mouse brain extract preparation for various times and looked for the presence of unmodified peptides by MS. To distinguish peptide stability in the presence of intact brain cells and in cell extracts, some of the brain material was sonicated before and some after peptide addition. In brain cell fragments (cell sonication and supernatant removal before peptide addition), *EI*Free could be detected only after 5 min, but even then in very low quantities (approximately 1%, Fig. 4, left panel). No *EI*Free was visible in the presence of intact brain cells (cell sonication and supernatant removal after peptide addition) at any time point (data not shown). We hypothesized that the glycopeptide remained attached to carbohydrate receptors in brain cells and thus escaped our surveillance measure. To verify this, the acetylated peptide *EIAc* was submitted to identical assay conditions. As the acetyl groups and not the sugar moiety or the peptide backbone are the main cleavage points in acetylated *EI* peptides in serum (Otvos et al. 2008), the presence of uncleaved *EIAc* in the brain preparations would indicate loss of *EI*Free signal during the assay procedure rather than glycopeptide decomposition. Indeed, in both intact and fractionated cells about 15% of the *EIAc* peptide could be detected after 5 min incubation time (Fig. 4, right panel), and even 5% peptide was visible after 15 min (acknowledging that MS peak heights are not

linearly dependent upon the amounts of analytes present in the samples). Although we do believe that the increased amount of acetylated peptide compared to the deacetylated analog reflected less stable binding to brain carbohydrate receptors, it needs to be added that increasing hydrophobicity does promote BBB penetration (Egleton et al. 2000). In control experiments, leptin protein could be detected in the brain extract in times identical to peptide *EI*Free (data not shown). It is a recurrent problem in peptide and protein drug development how to assess stability in brain tissue. After destroying the tissues brain proteases are quickly released and these can cleave peptide and protein drugs regardless of the target brain compartments of the drugs or the local activity of the enzymes in functional brain.

Biodistribution

The pharmaceutical promise of peptide *EI*Free over leptin protein is that the glycopeptide reaches the hypothalamus more efficiently than the protein. Because the BBB cellular model and the brain stability studies could not fully support this claim, once again we returned to the biodistribution assay. We labeled both leptin protein and the glycopeptide with the near infrared dye Cy5.5 and injected the peptidomimetic and the protein into Balb/c mice subcutaneously under the shoulder blade. As Fig. 5 indicates, 60 min after inoculation most samples remained at the site of drug administration. Leptin protein was fairly evenly distributed (light blue-green color on the left mouse picture) except the head where the protein was less detectable than at other body parts (medium blue image). Lesser amount of the glycopeptide was distributed throughout the body in general, but whatever traveled through the body reached all organs equally, including the head (medium blue image on the right mouse picture). These assays verified that the

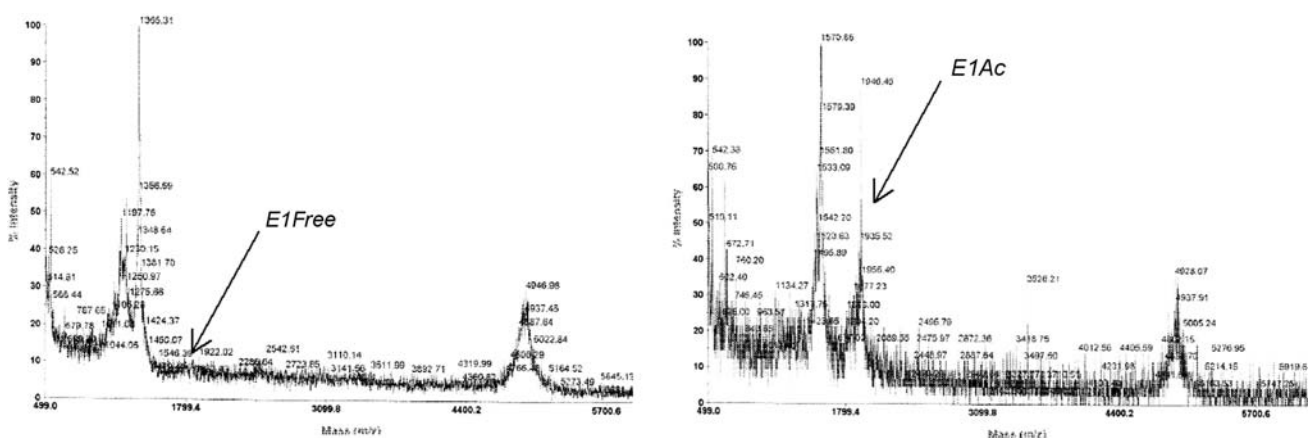


Fig. 4 Mass spectral detection of peptides *EI*Free (left panel) or *EIAc* (right panel) in mouse brain extracts after a 5 min incubation period. The arrows point to the expected M/z value of unmodified

samples. As the deacetylated peptide is significantly more stable in serum than the acetylated analog, the loss of signal likely indicates binding to the brain cells during the assay procedure

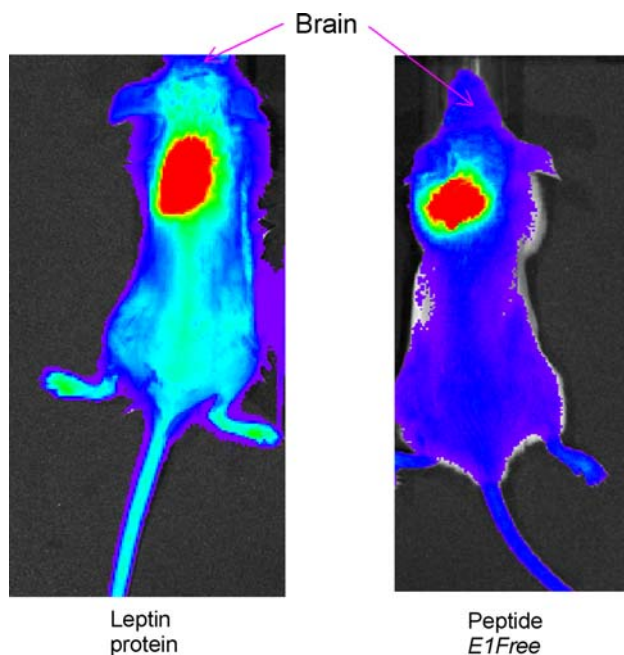


Fig. 5 Biodistribution of Cy5.5 labeled leptin protein (left panel) and peptide *EIFree* in Balb/c mice. While the peptide was evenly distributed all over the body including the head, the protein reached other organs more preferably

glycopeptide may reach the hypothalamus more efficiently than the full leptin protein that demonstrated poor BBB penetration in clinical trials. A comparison of images obtained after subcutaneous (sc) peptide administration of this study, and ip *EIFree* inoculation reported earlier (Otvos et al. 2008) indicates that the peptide is more readily absorbed when injected into the abdominal cavity. In support of this possibility, in our antibacterial peptide development program significantly better protection against bacterial challenge is achieved when the peptides are administered ip than if they are added sc. Thus, future *EIFree* therapy should use ip peptide treatment.

Conclusions

The leptin agonist glycopeptide *EIFree* exhibits very attractive biochemical and pharmacological properties. The strenuous work of lead optimization would require fast screening assays but these are currently not available for the leptin/ObR system. Although we managed to develop a low sensitivity medium-throughput binding assay, the 5-day cell proliferation assay cannot be fully replaced with faster measures assessing metabolic cell rate. Nevertheless, the serum stability and biodistribution profile of the *EIFree* derivative warrants detailed efficacy and toxicity studies in appropriate animal models of obesity, lipodystrophy, and other central leptin deficiency-related diseases.

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