Amino Acids (2014) 46:873–882
DOI 10.1007/s00726-013-1650-6

ORIGINAL ARTICLE

The designer leptin antagonist peptide Allo-aca compensates for short serum half-life with very tight binding to the receptor

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Received: 25 November 2013 / Accepted: 12 December 2013 / Published online: 24 December 2013
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Abstract The leptin receptor antagonist peptide Allo-aca exhibits picomolar activities in various cellular systems and sub-mg/kg subcutaneous efficacies in animal models making it a prime drug candidate and target validation tool. Here we identified the biochemical basis for its remarkable in vivo activity. Allo-aca decomposed within 30 min in pooled human serum and was undetectable beyond the same time period from mouse plasma during pharmacokinetic measurements. The C_{max} of 8.9 μg/mL at 5 min corresponds to approximately 22 % injected peptide present in the circulation. The half-life was extended to over 2 h in bovine vitreous fluid and 10 h in human tears suggesting potential efficacy in ophthalmic diseases. The peptide retained picomolar anti-proliferation activity against a chronic myeloid leukemia cell line; addition of a C-terminal biotin label increased the IC_{50} value by approximately 200-fold. In surface plasmon resonance assays with the biotin-labeled peptide immobilized to a NeutrAvidin-coated chip, Allo-aca exhibited exceptionally tight binding to the binding domain of the human leptin receptor with $k_a = 5 \times 10^5\ M^{-1}\ s^{-1}$ and $k_{diss} = 1.5 \times 10^{-4}\ s^{-1}$ values. Peptides excel in terms of high activity and selectivity to their targets, and may activate or inactivate receptor functions considerably longer than molecular turnovers that take place in experimental animals.

Keywords Anti-proliferation · Dissociation constant · Metabolic stability · Peptide therapeutics · Receptor activation

Abbreviations

Aβ	Amyloid β peptide
CML	Chronic myeloid leukemia
Dde	Dimethyl-dioxo-cyclohexylidene
EDTA	Ethylene diamine tetraacetate
Fmoc	Fluorenyl-methoxycarbonyl

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G-CSF	Granulocyte-colony stimulating factor
IL-6	Interleukin-6
ip	Intraperitoneally
LBD	Leptin binding domain
LOQ	Limit of quantification
MALDI TOF–MS	Matrix-assisted laser ionization/desorption time-of-flight mass spectrometry
ObR	Leptin receptor
PK	Pharmacokinetics
RP-HPLC	Reversed-phase high performance liquid chromatography
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TNBC	Triple negative breast cancer
sc	Subcutaneously
SPR	Surface plasmon resonance

Introduction

Leptin is a neurohormone that acts in the hypothalamus and regulates energy balance and food intake (Wauters et al. 2000), and thus the leptin/leptin receptor (ObR) system is considered a prime pharmaceutical target (Inui and Meguid 2003). Central leptin deficiency or inefficient activity of endogenous leptin leads to obesity and hyperinsulinemia, and has been linked to infertility, impaired cognitive function, and osteoporosis (Kalra 2008). On the other hand, leptin signaling in specific peripheral tissues promotes cancer development (Gonzalez et al. 2006). Similar to other hormone receptors involved in metabolic functions and appetite control such as adiponectin (Otvos et al. submitted) and ghrelin (Moulin et al. 2013) ObR antagonists are being developed either as mutants of the full protein or peptide fragments representing single receptor binding sites. Superposition of leptin's sequence with other cytokines, such as human IL-6 (interleukin-6), bovine G-CSF (granulocyte-colony stimulating factor) or human oncostatin M reveals three potential bivalent ObR-binding sites (sites I–III) (Peelman et al. 2004). While site I protein mutants do exhibit orexigenic properties (Shpilman et al. 2011), a site II-derived peptide inhibits the growth of cells xenotransplanted into immunocompromised mice, and reduces the level of pro-angiogenic and pro-proliferative markers (Gonzalez et al. 2009). However, none of the site I and site II-derived leptin derivative peptides is a pure antagonist, i.e., lacks agonistic properties without the presence of leptin (Otvos et al. 2008).

We developed a family of 9–10-amino acid long peptide analogs of ObR-binding site III of leptin that acts as selective ObR inhibitors without any partial agonistic activity within a 1,000-fold concentration increase range (Otvos et al. 2011a). The lead peptide-based therapeutic called Allo-aca (Table 1) reduces leptin-dependent growth and signaling in hormone-positive and -negative breast cancer cell lines with IC_{50} values of 50–200 pM. Allo-aca is orexigenic in mice when added intraperitoneally (ip) or subcutaneously (sc) (Otvos et al. 2011b). In immunocompromised mice Allo-aca suppresses the growth of established hormone-sensitive orthotopic human breast cancer xenografts by 45–51 % when administered either ip or sc for 38 days at a dose as low as 0.1 mg/kg/day (Otvos et al. 2011a). In human triple negative breast cancer (TNBC) xenografts, Allo-aca administered sc significantly extends the average survival time from 15.4 days (untreated controls) to 24 and 28.1 days at 0.1 and 1 mg/kg/day doses, respectively (Otvos et al. 2011b). Particularly attractive with Allo-aca, and probably other ObR antagonists, is its ability to interfere with cancer development at multiple stages. In the TNBC model, the survival figures are more impressive than the primary tumor growth inhibition data. This can be due to at least partial inhibition of metastasis and angiogenesis and perhaps delaying systemic inflammatory processes (Otvos and Surmacz 2011). Indeed, Allo-aca reduces the levels of rheumatoid arthritis (RA) development markers in murine models, which is quite understandable as many molecular mechanisms in RA and cancer are dramatically similar (Otvos et al. 2011c). Most recently, leptin-dependent proliferation of monkey retinal and bovine corneal endothelial cells was inhibited with mid-nanomolar concentrations of peptide Allo-aca suggesting potential therapy opportunities in ophthalmic diseases (Scolaro et al. 2013). As a target validation tool, Allo-aca had a major role to document that leptin protein protects hippocampal neurons from amyloid β protein (Aβ)-induced cell

Table 1 Peptides used in this study and their relationship to the native leptin site III sequence and other ObR-binding site III leptin receptor response modifier peptides

Peptide	Sequence
Human leptin 121–129[a]	Thr-Glu-Val–Val-Ala-Leu-Ser-Arg-Leu
E1/Aca pM agonist	H-Tyr(I_2)-Ser-Thrα(GalNAc)-Glu-Val-Val-Ala-Leu-Ser-Arg-Aca-NH_2
Aca1 nM antagonist	H-Thr-Glu-Nva-Val-Ala-Leu-Ser-Arg-Aca-NH_2
Allo-aca pM antagonist	H-*allo*Thr-Glu-Nva-Val-Ala-Leu-Ser-Arg-Aca-NH_2
Allo-aca (biotin)	H-*allo*Thr-Glu-Nva-Val-Ala-Leu-Ser-Arg-Aca-Lys(biotin)-NH_2

Tyr(I_2): 3,5-diiodo-tyrosine; Thrα(GalNAc): *O*-α-*N*-acetyl galactosamine linked to threonine; the abbreviations for other non-natural amino acids are detailed in the text

[a] Numbering is from Peelman et al. (2004)

death through ObR activation (Martins et al. 2013). As far as we know, in addition to our laboratories, the peptide is currently under study as a therapeutic option or as a target validation reagent internationally in over 10 institutions.

What missing from the complete evaluation of the pharmaceutical utility of Allo-aca was the characterization of two basic biochemical properties: the quantitation of molecular interactions (as opposed to the heavily studied cellular activity in ObR-expressing cells) between the peptide and isolated ObR and stability of the peptide in various biological media where the activity in vivo is manifested. Here we show that the driving force behind the remarkable animal efficacy data of Allo-aca is its extremely strong binding to ObR.

Materials and methods

Peptide synthesis

The Allo-aca and Allo-aca(biotin) peptides were synthesized by solid-phase methods. The peptide chain assembly was made on a CEM Liberty microwave-assisted (Allo-aca) or a Protein Technologies PS3 (biotin-labeled analog) automated synthesizers using TentaGel S-Ram-Fmoc resin with an initial load of 0.3 mol/g (Rapp Polymere). Standard Fmoc-chemistry was used throughout (Fields and Noble 1990) with a 4-molar excess of the acylating amino acids. For biotin labeling, the C-terminal lysine was incorporated as Fmoc-Lys(Dde)-OH, the side-chain protecting group was removed with 2 % hydrazine, and biotin was coupled overnight as a preformed *N*-hydroxy succinimide ester in *N*-methyl morpholine and diisopropyl ethylamine. Both unlabeled and labeled peptides were cleaved from the solid support with trifluoroacetic acid (TFA) in the presence of thioanisole (5 %), and water (5 %) as scavengers. After cleavage, the peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) and the final products were characterized by RP-HPLC and matrix-assisted laser ionization/desorption time-of-flight mass spectrometry (MALDI TOF–MS). Allo-aca: H-*allo*Thr-Glu-Nva-Val-Ala-Leu-Ser-Arg-Aca-NH_2; Allo-aca(biotin): H-*allo*Thr-Glu-Nva-Val-Ala-Leu-Ser-Arg-Aca-Lys(biotin)-NH_2, where *allo*Thr is *allo*-threonine, Nva is norvaline, and Aca is 6-amino-caproic acid.

Cancer cell proliferation

Although MCF-7 cells are estrogen-dependent for growth in vivo, in vitro the cells proliferate well without estrogen added (Otvos et al. 2008). Thus, MCF-7 breast cancer and K-562 chronic myeloid leukemia (CML) cells were grown in standard media. Seventy-five thousand MCF-7 and 100,000 K-562 cells grown in RPMI 1640 medium containing 10 % FBS (MCF-7) or 0.5 % bovine serum albumin, BSA (K-562) were seeded into wells of 24-well culture plates. Twenty-four hours later, the medium for MCF-7 cells was replaced by that used for K-562 cells. After an additional 24 h, the wells were treated with 6 nM leptin (R&D Systems), various concentrations of peptides or a mixture of 6 nM leptin and a dilution series of peptides. Cell numbers were counted under a microscope 72 h after ObR ligand addition. The assays were run in triplicates and repeated once.

Serum stability

For serum stability studies (Powell et al. 1993), 250 μL of an aqueous Allo-aca stock solution (0.8 mg/mL) was added to 2.5 mL 25 % aqueous pooled human serum in triplicate. The peptide-serum mixtures were thermostated at 37 °C. After 0, 45, 90, 120, 240, and 480 min, serum proteins from aliquots (210 μL) were precipitated by the addition of 40 μL 15 % aqueous trichloroacetic acid (TCA). The samples were centrifuged and 220 μL aliquots of the supernatants were analyzed on RP-HPLC. This well-established protocol was satisfactory for in vitro serum stability studies without the modifications needed for quantitative analysis of vitreous fluid and tear in vitro and mouse plasma in vivo (*vide infra*).

Vitreous fluid and tear stability assay

Extension of serum stability studies to other tissues was inspired by an earlier LC–MS study on peptide hormones in rat kidney, lung and liver homogenates (Liao et al. 2010). Vitreous fluid was extracted from fresh bovine eyes obtained at a slaughterhouse in Budapest, Hungary. The two eyes of the same cow were sterilized (Octenisept spray, Schulke) on the surface and the vitreous fluid (approximately 1 mL from each eye) was extracted with a 20 gauge needle. Tear production of a laboratory volunteer was stimulated by inhalation of freshly cut onion vapors. Up to 200 μL tear fluid was collected with a sterile transfer pipette and stored at −18 °C until use. Aqueous Allo-aca solutions (1 μg in 10 μL) were mixed with 93 μL vitreous or tear fluid in 0.5 mL Eppendorf tubes and incubated at 37 °C at 500 rpm. After 0, 1, 4 and 8 h 34 μL aliquots was mixed with 114 μL 89 % aqueous acetonitrile containing 1 % formic acid and incubated for 10–30 min on ice. The precipitated proteins were separated by centrifugation (10 min, 12,000 × *g*, Eppendorf MiniSpin). The supernatant (140 μL) was dried by vacuum centrifugation (SpeedVac, Eppendorf) and the residue was dissolved in 3 % aqueous acetonitrile containing 0.1 % TFA (120 μL). After centrifugation (5 min, 12,000 × *g*, Eppendorf MiniSpin), a 100 μL solution was analyzed by RP-HPLC using a Jupiter C-18 column (2.0 mm inner diameter, 150 mm

length, 5 μm particle size, 30 nm pore size; Phenomenex, Torrance) on a Beckman Gold HPLC System. The gradient from 0.1 % TFA in water (eluent A) to 60 % acetonitrile in 0.1 % aqueous TFA (eluent B) was developed using 5–56 % B in 17 min. The assays were run in triplicates in two independent assays using vitreous fluids obtained from different eyes, and tears separately collected from the same donor.

Mouse pharmacokinetics (PK)

Eight female CD-1 mice of 20 g were each inoculated sc with 40 μg peptide Allo-aca (2 mg/kg total body weight) dissolved in sterile saline solution at 0.2 mg/mL. Blood (approximately 50 μL) was taken from the tail vein at 0, 2, 5, 10, 30, 60, 120 and 240 min after drug administration into serum separator tubes containing EDTA. Each mouse was used three times, allowing sample collection in triplicates in each time point. Animals were maintained and handled in accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of Semmelweis University (permission no.: 22.1/1159/3/2010).

The tubes were centrifuged for 3 min at 8,000 rpm and frozen until analysis. Before Allo-aca concentration determination, the proteins were precipitated by mixing the plasma samples with aqueous acetonitrile (89 %) containing aqueous formic acid (1 %) (1:4, v/v). The samples were filtrated on 96-well Strata Plate (Phenomenex), dried under vacuum (room temperature) and dissolved in 3 % aqueous acetonitrile containing 0.1 % formic acid. Allo-aca was quantified by a combination of MS and RP-HPLC. MS was run on a QSTAR instrument using an extracted ion chromatogram of $[M + 2H]^{2+}$ (m/z 493.76 ± 0.25). Internal standard was the analog ObR agonist peptide E1/Aca with m/z 846.22 ± 0.25 of $[M + 2H]^{2+}$. For RPC, peptides were separated on an Ultimate nanoHPLC operated at a flow rate of 200 μL/min using an Aqua C_{18}-column (2 mm internal diameter, 150 mm length, 3 μm particle diameter, 12.5 nm pore size). The gradient was developed from 3 to 95 % aqueous acetonitrile containing 0.1 % formic acid. The analysis relied on a single calibration curve and 2 sets of sample analyses. Before MS and HPLC, the samples from identical time points were combined and re-divided into two parts. Figure 2 shows the results of the first assay; the second assay yielded similar results albeit with slightly lower peptide concentrations due to the repeated freezing-thawing and sample manipulation processes.

Surface plasmon resonance (SPR)

The leptin binding domain (LBD, residues 428-635) of human ObR was cloned into a pEX vector (OriGene), expressed in *Escherichia coli* SHuffle T7 Express cells (NEB Biolabs) and purified following previously published methods (Sandowski et al. 2002; Carpenter et al. 2012). SPR measurements were performed using a SR7500DC instrument from Reichert Technologies (Buffalo, New York). NeutrAvidin surface sensors (Reichert) were used to immobilize biotinylated Allo-aca on the left flow channel L. The right flow channel (R) of the sensor chip was used as reference channel. TBST (50 mM Tris, 150 mM NaCl, 0.002 % Tween20, pH 7.4) was used as running and dilution buffer for ObR-LBD in all experiments. SPR traces were acquired at a flow rate of 25 μL/min at a constant temperature of 25.0 °C using the Integrated SPR Autolink software (Reichert) and were analyzed using Scrubber 2.0 (BioLogic Software, Campbell, Australia) and ClampXP to calculate kinetic rate constants. The experiment was repeated ten times with very consistent results.

Results

Anti-cancer cell proliferation

To verify that the C-terminally biotin-labeled Allo-aca analog retains ObR antagonist properties and such can be used in the SPR assay to study receptor-peptide ligand interactions, the ability of the labeled peptide to inhibit MCF-7 cell growth was compared to that of the unmodified and previously heavily studied parent analog Allo-aca. As the main theme of the stability/PK studies is the concentration level of Allo-aca in blood preparations, we also wanted to see if the peptides can be developed as drugs to hematological malignancies and what concentrations are needed to inhibit the growth of leukemia cells. For this latter purpose we selected the K-562 CML cell line that produces ObR mRNA, albeit the expression level of various ObR forms is greatly reduced in CML patients (Ozturk et al. 2012). The 500 pM IC_{50} value of Allo-aca against MCF-7 cells, as we detected here (Table 2), is in the range of data we published earlier (200 pM). Similar to earlier results (Otvos et al. 2011a), the peptide did not induce cell proliferation without leptin added (no agonistic activity) up to the 100 nM maximal peptide concentration studied. Addition of the biotin label increased the IC_{50} figure to 100 nM (a 200-fold decrease in activity) indicating that Allo-aca has a close-to-optimal size as an ObR antagonist (see "Discussion" for design of the SPR probe). In the first K-562 cell assay, leptin induced a 10 % additional cell growth during the 3-day examination period, that is approximately half of its effects on MCF-7 cells (data not shown). This finding confirms the low ObR expression level in K-562 cells (Ozturk et al. 2012). Allo-aca was more active against the K-562 cell line than against MCF-7, exhibiting IC_{50} values at the low pM range (Table 2). Biotin labeling reduced the activity figure by

Table 2 Stability of leptin receptor antagonist in various biological media and in vitro activity of analogs in cellular tumor models

Peptide	Half-life in mouse serum	IC_{50}/EC_{50} in KL562 model (nM)	Control IC_{50}/EC_{50} in MCF-7 model (nM)	Half-life in bovine vitreous fluid	Half-life in human tear
Allo-aca	9 min[a]	0.1/10	0.5/ > 100	2.2–2.8 h	10.2 h
Allo-aca(biotin)		20/ > 100	100/ > 1,000	n.d.[b]	n.d.

[a] Calculated from 36 min $t_{1/2}$ measured in 25 % aqueous mouse serum based on Powell et al. (1992)

[b] Not determined

200-fold, similar to those observed for MCF-7 proliferation inhibition. However, as opposed to MCF-7 cells, unlabeled Allo-aca was mitogenic to the CML cell line at 10 nM concentration with and without 6 nM leptin present. This suggests that not only the activity improved from solid to hematologic tumors, but also the antagonist → agonist switch occurred at lower concentrations. K-562 cells express low and variable level of ObR and unknown amounts of endogenous leptin protein. In our second assay identical IC_{50} values were measured for both peptides [Allo-aca and Allo-aca(biotin)], but the cell growth inhibitory effects were retained even if leptin was not added to the cell culture indicating that the peptide is a strong enough ObR antagonist to counteract the growth-promoting effects of leptin produced by K-562 cells themselves. Alternatively, similar to many other leptin-derived peptides against MCF-7 cells (Otvos et al. 2008), Allo-aca acts as an inverse ObR agonist in K-562 cells.

Stability in various biological fluids

Up to today, serum stability represented the most important secondary screening assay in drug development. Serum stability identified drug leads that were unstable in the circulation and the assay had a predictive value to the success of ensuing human and veterinary efficacy trials (Powell et al. 1992). Due to the desirable activity of Allo-aca in ophthalmic disease models, we also characterized the stability in bovine vitreous fluid and human tears. The altered experimental conditions were implemented to better quantify the presence of the peptide in these fluids and the degradation kinetics. In spite of the positioning of non-natural amino acids into terminal positions, Allo-aca was rapidly degraded in human serum. The half-life in 25 % pooled aqueous serum was measured to 36 min, representing $t_{1/2}$ of 9 min in full serum (Table 2). These results are in agreement with the generally observed minutes-to-hours peptide stability in human plasma in general (Werle and Bernkop-Schnurch 2006). Allo-aca stability in bovine vitreous fluid was significantly increased and the $t_{1/2}$ figure was extended to over 2 h (Table 2; Fig. 1). Even further stability was detected in human tears when the calculated half-life was extended to over 10 h (Table 2). It has to be mentioned that irritant-induced tears contain less peptide- or protein-like molecules than emotional tears (Frey et al. 1981) although the level of proteolytic enzymes is unlikely to be significantly different.

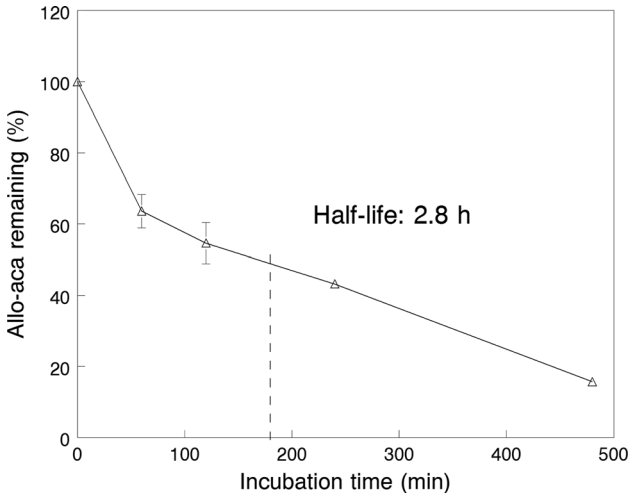

Fig. 1 Stability of peptide Allo-aca in bovine vitreous fluid extracted from fresh bovine eyes. The figure shows the degradation kinetics of the peptide from the vitreous fluid from one eye, taken from triplicate samples. Identical assays from the second eye showed very similar kinetics albeit with somewhat faster degradation times. The *error bars* indicate the standard deviation of triplicate parallels

Pharmacokinetics in mice

When administered to CD-1 mice at a 2 mg/kg bolus sc dose, Allo-aca appeared in low concentration (34 ng/mL) in the plasma at 2 min, peaked at 5 min, dropped again considerably at 10 min (63 ng/mL), further to 27 ng/mL at 30 min, and was undetectable at 1 h and beyond (Fig. 2). Calculating with a 1 mL total peripheral blood volume (Joslin 2009), the C_{max} of 8.9 µg/mL corresponds to approximately 22 % injected peptide present in the circulation. The further reduced $t_{1/2}$ in live animals (4 min, starting at the 2 min time point) compared to the stability in serum in vitro (9 min) is probably due to the very fast renal clearance of peptide drugs (Lister-James et al. 1996). Having said this, with a 1 min whole body blood circulation time in humans and less in mice, high concentrations of Allo-aca are able to reach the cell surface ObR in most

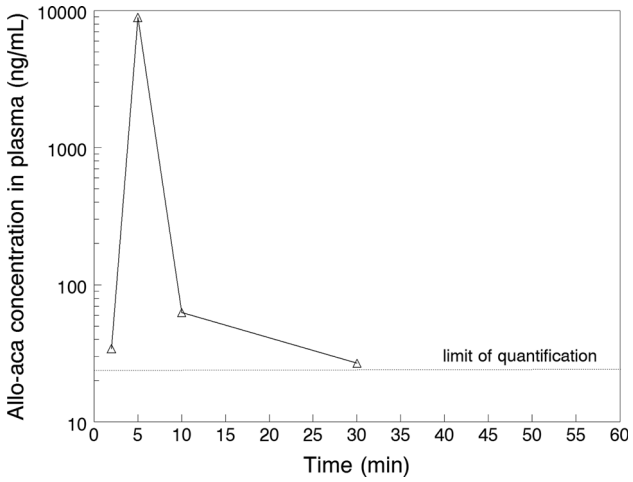

Fig. 2 Pharmacokinetics of peptide Allo-aca injected into CD-1 mice at 2 mg/kg subcutaneously. The peptide concentrations from samples taken from the tail vein were quantified by a combination of nano-high performance liquid chromatography and mass spectrometry. The peptide amount peaked at 5 min with very small quantities detectable at 2 min and 10–30 min. Peptide amounts at 0 min and beyond 1 h were below the detection limit of 25 ng/mL. Each time point represents the peptide amounts detected in a mixture of blood taken from three animals. The plasma peptide concentration determination was repeated from frozen samples with very similar kinetic results

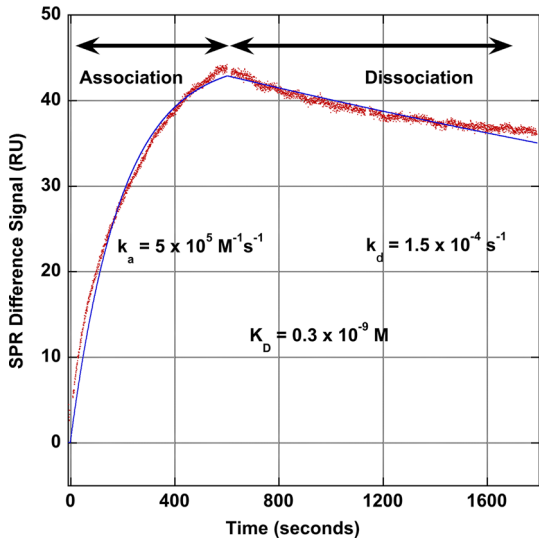

Fig. 3 Representative surface plasmon resonance (SPR) blank corrected difference trace (*left* channel–*right* channel) showing the association and dissociation phase of the interaction between the leptin binding domain of the human leptin receptor and the Allo-aca peptide immobilized via a C-terminal biotin-lysine modification onto a NeutrAvidin-coated SPR sensor chip. The estimated binding affinity K_d for the protein–peptide complex is 0.3 nM and the dissociation rate constant (k_{diss}) of 1.5×10^{-4} s^{-1} corresponds to a half-life of the complex of close to 2 h (111 min)

organs before it is eliminated in vivo. Indeed, near infrared fluorophor-labeled Allo-aca was found to be uniformly distributed in mice 15 min after intraperitoneal administration (Beccari et al. 2013). Nevertheless, the in vivo distribution data do not reveal total peptide concentration in the tissues, just the presence of the labeled peptide (or a shortened peptide carrying the Alexa-680 label) in any quantity that triggers fluorophor detector response.

Allo-aca binding to ObR

A biotin group was utilized to immobilize the Allo-aca peptide on NeutrAvidin SPR sensor surfaces. NeutrAvidin is a streptavidin derivative with reduced non-specific binding properties, while retaining the extremely high affinity for biotin. Injection of ObR-LBD showed tight binding to the immobilized Allo-aca peptide (Fig. 3). The obtained estimated dissociation rate constants k_{diss} of $1.5 \pm 0.5 \times 10^{-4}$ s^{-1} and association rate constants k_a of $5.0 \pm 1.0 \times 10^5$ M^{-1} s^{-1} were calculated from computational fitting of SPR traces using a simple biomolecular binding model. The calculated binding affinity $K_d = k_{diss}/k_a$ is $0.3 \pm 0.15 \times 10^{-9}$ M (300 pM). Although the data could be fitted acceptably to a 1:1 binding model as suggested earlier to leptin protein and ObR (Mistrik et al. 2004), based on our SPR curves a two-step binding process in which the initial binding complex undergoes a slight rearrangement to form a final, more stable complex cannot be excluded either. Attempts to dissociate the ObR-LBD–Allo-aca complex on the sensor chip by injection of an acidic regeneration buffer (20 mM glycine, pH 2.0) did not result in complete dissociation on the protein–peptide complex confirming the extremely tight Allo-aca–ObR binding.

Discussion

To maximize the signal strength during SPR, we immobilized the Allo-aca peptide ligand to the chip surface. The SPR signal is proportional to the mass of the binding partner applied in the mobile phase, and when peptide-protein interactions are studied, usually the peptide component is immobilized (Kilko et al. 1993). In our case, the ObR-LBD is about 25 times larger than Allo-aca. The biotin-labeled peptide analog was designed to maintain as much activity of the parent analog as possible and for optimal surface binding on the sensor chip. The first generation leptin receptor antagonist peptide Aca1 contains an N-terminal Thr instead of *allo*Thr and displays a 20-fold reduction in cellular activities suggesting that the N-terminal residue is involved in receptor binding (Otvos et al. 2011a). The N-terminal extension in peptide E1/Aca (Table 1) endows agonistic properties to the peptide further confirming the importance of the N-terminal region. Thus, we placed the biotin label to the C-terminus. The biotin

moiety was attached to the side-chain of an extra lysine to provide a spacer between the NeutrAvidin-coated chip and the peptide active site. Based on the cellular assay results of Table 2, biotin labeling still interfered with receptor binding, decreasing the activity figure by approximately 200-fold. While the precise IC_{50} values rely on the cell line of leptin signaling for growth (although for us biotin labeling reduced the efficacy of Allo-aca against two completely different cell lines to identical degree), the number of receptors on the cell surface and other parameters, in ballpark, the K_d of the unlabeled peptide to ObR is estimated to be 200-fold less than that we measured by SPR, i.e., at the single digit picomolar range. This value is exactly what was anticipated based on all cellular activity figures we collected so far.

To obtain accurate quantification of peptides in vitreous fluid and tear, we modified the protein precipitation step during the stability studies. Instead of 6–15 % TCA, proteins were precipitated with 89 % acetonitrile and 1 % formic acid. For antimicrobial peptides, this strategy improved the recovery yield from 1 to 100 % (Knappe et al. 2010), and the protocol was also useful for pegylated peptides that are more hydrophobic than regular analogs, elute later on RP-HPLC and co-elute with serum components. In an earlier study using TCA as a protein precipitating agent, we could not recover any Allo-aca from mouse plasma when we tried to characterize the pharmacokinetic parameters (Otvos et al. 2011b). Then we hypothesized that the peptide binds to serum components in vivo (as opposed to the stability assay in pooled serum in vitro) and remains unseen later with the analytical protocol used. Applying the new protein precipitation technique to the PK assay as shown in the current report, we not only could recover high amounts of Allo-aca from mouse plasma and quantify the PK parameters, but taking into consideration the improvement of peptide yields in the above-listed examples, obtained further support for the Allo-aca serum binding process. In support, 8–20 % of exogenously added human leptin protein is found serum-bound in human blood ex vivo (Landt 2000).

During the calibration stage of the PK analysis, the limit of quantification (LOQ) was 0.5 ng Allo-aca injected to the column (20 µL injection volume) and the linear range extended until at least 200 ng ($R^2 = 0.9949$) analyte; that is the LOQ was around 25 ng/mL serum and the linear range extended to 10 µg/mL. At the measurement stage signals from 2 to 30 min were within the linear range, whereas the quantities detected at time points 0, 60, 120 and 240 min were below the LOQ. With the used MS-HPLC pharmacokinetic measurement protocol, our C_{max} was 5 min with approximately 10 µM peptide after 2 mg/kg bolus peptide administration, and these figures compare very well to a 10-min C_{max} of 37 µM at a 6 mg/kg dose of a modified peptide prodrug determined by very similar analytical techniques (Janssen et al. 2006).

Our LOQ corresponds to approximately 25 nM Allo-aca concentration in plasma. This sensitivity is approximately in the dynamic range of RP-HPLC-based peptide PK that has a lower limit of 10 ng/mL (in our case this is equivalent of 10 nM Allo-aca) in validated pharmaceutical protocols (Zannikos et al. 2000). However, receptor agonist and antagonist peptide drugs act in the pM range, and expectedly Allo-aca was present in the blood at a concentration higher than the 100–500 pM IC_{50} value well beyond the 30 min mark that roughly corresponded to our LOQ. The situation is less problematic in humans where higher blood volumes are available than in rodents, although the required drug doses in humans are about 12-fold lower than in mice due to differences in the body surface area/weight ratio (Reagan-Shaw et al. 2008). Human serum concentrations of triptorelin, a 10-residue agonist of the gonadotropin-releasing hormone receptor, of 8 ng/mL are already associated with activation of 90 % of the receptor population, and higher peptide concentrations are not expected to induce any stronger biological responses (Romero et al. 2012). In turn, even if we can push the sensitivity of the murine PK quantification protocol down by a magnitude, we will still miss later time points when Allo-aca, or similar highly active peptide drugs, are present in the circulation above their IC_{50}/EC_{50} figures. Taken together, current RP-HPLC peptide PK protocols, where the analytes are not radioactively labeled, cannot accurately predict the real time while peptide drugs are active in rodent models.

While various peptides exhibit up to threefold species-dependent stability half-life differences in mammalian sera in vitro (McDermott et al. 1981), in our hands and protocols used by us, these are not significant between pooled commercial human and mouse serum preparations (Hoffmann et al. 1999). Allo-aca degraded in diluted human serum in <1 h with improved stability in bovine vitreous fluid. The highest stability of Allo-aca (>10 h half-life) was observed in human tears. Understanding that the tear turnover in vivo is 11 %/min in healthy people (Sorbara et al. 2004) and <5 % of topically applied protein-based drug penetrates the cornea and reaches intraocular compartments (Ottiger et al. 2009), Allo-aca stability in ophthalmic fluids is not the bottleneck in potential therapeutic applications. Although the drugs instilled into the eye as eye drops are available for activity only for a limited time period, intravitreal injection of bevacizumab, an angiogenesis inhibitor monoclonal antibody drug allows systemic distribution as much as higher plasma than eye content is observed between 4 and 8 weeks after administration (Miyake et al. 2010), indicating activity longer than any ocular turnover would warrant it.

Most leptin receptor antagonist peptides and some mutant proteins exhibit partial agonist properties depending upon their test concentrations and other receptor ligands available

Table 3 A comparison of surface plasmon resonance-based binding data of leptin receptor–ligand complexes

Ligand	Receptor form	Immobilized/ derivatization	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_d (pM)	References
Allo-aca peptide	ObR-LBD	Ligand/biotin	5×10^5	1.5×10^{-4}	300	Herein
Leptin protein	ObR extracellular	Receptor/Fc	1.9×10^6	4.4×10^{-4}	230	[a]
Leptin protein	ObR-LBD	Ligand/amino groups	1.2×10^5	1.9×10^{-3}	15,000	[b]

[a] Mistrik et al. (2004)

[b] Sandowski et al. (2002)

in the assay milieu (Otvos et al. 2008). In all earlier cell systems, Allo-aca distinguished itself from other ObR antagonists with its large concentration window (>1,000-fold) from going from antagonist to agonist. Against the K-562 CML cell line as presented here, the peptide either retained anti-proliferative activity without exogenous leptin added, or exhibited a narrower concentration window (~100-fold) before it stimulated rather than inhibited cell growth. The low concentration agonistic contribution has to be taken into account when designing Allo-aca doses in hematological animal models. In different breast cancer and arthritis systems, Allo-aca shows ObR antagonist properties at 0.1 and 1 mg/kg daily doses with little dose-dependence (Otvos et al. 2011a, b, c). In vivo, the Allo-aca antagonist → agonist switch takes place above 10 mg/kg in the orexigenic test (Otvos et al. 2011b) and is more observable (at 1 mg/kg) in close analog peptides containing D-amino acid replacements that exhibit low pM cellular activity and are shown to bind ObR in 3-dimensional cultures (Beccari et al. 2013). When calculating from the approximately 9 μg/mL, i.e., 9 μM peak plasma concentration of the PK assay run at a 2 mg/kg dose, even at 0.1 mg/kg the peak Allo-aca plasma concentration should be around 500 nM, which falls into the antagonist range against MCF-7 breast cancer cells, but is in the agonist range for K-562 leukemia cells. Accordingly, the planned antagonist doses in hematological cancers (and likely other cardiovascular applications) have to be lowered compared to those against solid tumors.

The most significant finding of the current report is the extreme stability of the complex between the leptin antagonist peptide and the ligand binding domain of the receptor. The half-life of the Allo-aca–ObR-LBD complex was almost 2 h, indicating that Allo-aca keeps ObR deactivated for about 4 h, and due to the high homology between Allo-aca our ObR antagonist peptide and E1/Aca the ObR agonist analog (Kovalszky et al. 2010), we expect similar values for the agonist as well. As compared with earlier SPR studies between different ObR forms and the full leptin protein ligand, Allo-aca dissociates from the complex slower than leptin itself, in spite of utilizing only 1 of the 3 ObR-binding sites (Table 3). Moreover, the C-terminal biotin label on the peptide is expected to decrease the binding efficiency more than derivatization through some of amino groups on the ligand (Sandowski et al. 2002) or incorporation an IgG Fc-fragment to the receptor (Mistrik et al. 2004). Apparently, the non-natural amino acid replacements in Allo-aca (Thr → *allo*Thr, Val → Nva and Leu → Aca) (Otvos et al. 2011a) endow the peptide with increased ability to bind the receptor compared to the natural counterpart (Table 1). The pharmaceutical relevance for this very strong binding for example is underlined by the observation that Allo-aca remains bound to ObR for a significantly longer period of time measured by SPR than a longer endostatin peptide, considered for further drug development, to its target vascular endothelial growth factor receptor type 3 (Han et al. 2010).

The very strong binding between Allo-aca and ObR explains all the remarkable in vivo efficacy data (Otvos et al. 2011a, b, c) in spite of the relatively fast degradation in human serum in vitro and in mouse plasma in vivo. In general, for Allo-aca and probably for many other peptide drug leads, stability data (as we measure them today) in various biological fluids have less predictive value for in vivo efficacy than previously thought. Peptides excel in terms of high activity and selectivity to their targets (Albericio and Kruger 2012) and thus, may activate or inactivate receptor functions considerably longer than molecular and cellular turnovers (including their own degradation and elimination) that take place in experimental animals or human and veterinary patients.

Acknowledgments This work was partially supported by the Novo Nordisk Diabetes Innovation Award to E. S. Another part of this work was supported by Australian Research Council Discovery grant DP120101963 to J. D. W. who is also an NHMRC (Australia) Principal Research Fellow.

Conflict of interest The authors declare no competing financial interests.

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