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Screening of synthetic phage display scFv libraries yields competitive ligands of human leptin receptor

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ABSTRACT

Initially considered the main endogenous anorexigenic factor, fat-derived leptin turned out to be a markedly pleiotropic hormone, influencing diverse physiological processes. Moreover, hyperleptinemia in obese individuals has been linked to the onset or progression of serious disorders, such as cancer, autoimmune diseases, and atherosclerosis, and antagonizing peripheral leptin's signalization has been shown to improve these conditions. To develop an antibody-based leptin antagonist we have devised a tailored panning procedure and screened two phage display libraries of single chain variable antibody fragments (scFvs) against recombinant leptin receptor. One of the scFvs was expressed in *Escherichia coli* and its interaction with leptin receptor was characterized in more detail. It was found to recognize a discontinuous epitope and to compete with leptin for receptor binding with IC50 and K_d values in the nanomolar range. The reported scFv represents a lead for development of leptin antagonists that may ultimately find use in therapy of various hyperleptinemia-related disorders.

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1. Introduction

Leptin is a 16 kDa adipocyte-derived protein hormone (adipokine) structurally resembling members of the long-chain helical cytokine family [1]. Its cognate receptor is a single spanning membrane receptor belonging to the class I cytokine receptor family. To date, six isoforms of leptin receptor generated as a result of alternative splicing and ectodomain shedding, all sharing the same N-terminal structure, have been identified. Only the long isoform (LRb) is fully capable of signal transduction [2]. Through higher order clustering of LRb monomers on specific hypothalamic neurons, leptin reduces appetite and food intake by promoting the expression of anorexigenic melanocortins while inhibiting secretion of orexigenic peptides [1,3]. Besides its role in energy homeostasis, leptin as a pleiotropic hormone acting centrally as well as peripherally oversees many other physiological processes, such as control of blood glucose levels, reproductive function, hematopoiesis, innate and adaptive immune responses, bone remodeling, angiogenesis, wound healing, and tissue regeneration (reviewed in [4–6]).

Although plasma leptin concentrations normally correlate with body fat percentage, central leptin resistance typically accompanied with hyperleptinemia in obese individuals has been described [7]. Numerous studies show that over-activation of *peripheral* leptin receptors may negatively contribute to the outbreak or progression of diseases such as cardiovascular defects [8], some forms of cancer [9,10] as well as chronic inflammatory and autoimmune diseases [11,12] and, in this respect, hyperleptinemia is considered one of the main factors linking obesity with these pathologies [9,13]. Potential leptin antagonists thus hold promise as primary or at least additive treatment in the therapy of diverse spectrum of obesity-related conditions discussed above [14]. On the other hand, leptin antagonists are useful for studying biological activity of leptin [14,15] as well as leptin–leptin receptor interaction at the molecular level [3].

Leptin antagonists developed to date can be classified into four groups: (i) leptin muteins [15,16], (ii) antagonistic LR-targeting antibodies and their fragments [17,18], (iii) neutralizing anti-leptin antibodies and soluble leptin receptor variants [19,20], and (iv) antagonistic short peptides binding to the LR [21,22]. In addition, PPARγ agonists (e.g., rosiglitazone) have been shown to indirectly inhibit leptin signaling by reversing leptin-induced upregulation

Abbreviations: B-hLEP, biotinylated recombinant human leptin; hLR-Fc, extracellular region of human leptin receptor fused to the human gamma Fc-region; LR, leptin receptor; scFv, single-chain variable fragment.

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of leptin and LR expression, and blocking intracellular pathways mediated by MAPK, STAT3 and Akt [23], however their actions are not selective. If predominantly peripheral activity is desired, LR-targeting antibodies and antibody-based constructs of large molecular size (e.g., pegylated or fused to large protein domains) seem most attractive [17,18]. Still, they may interfere with leptin transport across the blood–brain barrier (BBB) which is most probably mediated by the short receptor isoform LRa, and may thus exert some central effects. In contrast, short peptides and leptin muteins were shown to readily cross the blood–brain barrier, likely via receptor-mediated transcytosis, and thus significantly block central leptin effects as well [22,24].

Aiming to develop leptin antagonists, we screened two phage display libraries of single chain variable fragments (scFvs) for binding to the human leptin receptor-Fc fusion (hLR-Fc). A tailored panning procedure was devised to preferentially enrich scFvs interacting with the leptin receptor ectodomain. Selected scFvs competed with leptin for receptor binding, thereby representing leads for development of leptin antagonists with therapeutic potential in various hyperleptinemia-related disorders.

2. Materials and methods

2.1. Screening of phage-displayed scFv libraries

Phage display libraries Tomlinson I and J (Source BioScience) were subjected to separate panning processes using hLR-Fc (R&D Systems) as the target following a modified protocol of Lee et al. [25]. The selection was carried out in solution by capturing phage-target complexes onto protein G-coated paramagnetic beads (Invitrogen Dynal AS). Stringency was gradually enhanced by reducing the amount of target from 2.5 μg in the first to 0.1 μg in the third selection cycle. Subtractive selection (incubation of amplified phages from preceding round with protein G-coated beads) was carried out in the second and third selection rounds to avoid enrichment of protein G binders. Phages were eluted by trypsinization, only in the last screening round elution with recombinant leptin (R&D Systems) was also employed to favor selection of scFvs that compete with native ligand for receptor binding.

2.2. Polyclonal phage ELISA

Wells of the MaxiSorp microtiter plate (Nunc) were coated with either hLR-Fc, human gamma Fc-region (Jackson ImmunoResearch), or BSA. Wells were blocked and 100 μL of 30-fold diluted amplified eluates in 0.5% milk/0.05% PBST were added and incubated 1 h with gentle agitation. After extensive washing, bound phages were detected using horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibodies (GE Healthcare) and chromogenic substrate (3,3′,5,5′-tetramethylbenzidine (TMB)). Finally, reaction was stopped with 2 M H_2SO_4 and absorbance was recorded at 450 nm.

2.3. Monoclonal phage ELISA

Fourteen phage clones from library J were randomly chosen from the second selection round, along with 24 and 8 clones collected after the third round by specific (competitive) and non-specific (trypsin-mediated) elution, respectively. Monoclonal ELISA was performed as described for polyclonal ELISA, except that each clone from clear culture supernatants was incubated only in hLR-Fc- and human gamma Fc-region-coated wells.

2.4. Sequencing of positive clones

Phagemid DNA of eight clones giving highest ELISA signals was isolated using GenElute™ HP Plasmid Miniprep Kit (Sigma–Aldrich). The inserts encoding scFvs were sequenced using the pHEN primer (5′-CTATGCGGCCCCATTCA-3′) by GATC Biotech AG (Konstanz, Germany).

2.5. Competition assay using recombinant leptin

The ability of leptin to prevent binding of scFv-bearing phages to hLR-Fc was tested by competitive phage ELISA. Equal titers of each clone (5×10^9 phages per well) were incubated with increasing concentrations of recombinant leptin (0, 0.0625, 0.625, 6.25, 62.5 and 625 nM) in hLR-Fc-coated wells. After one hour the content was discarded and the wells were washed. Bound phages were detected as described above.

2.6. Epitope characterization

hLR-Fc was covalently bound to the wells of Nunc Amino Immobilizer strips (Thermo Scientific) which were afterward blocked with ethanolamine. For each tested phage clone, target protein in one well was denatured by adding PBS supplemented with 150 mM DTT and 1.3% SDS for one hour, while PBS alone was added to the second hLR-Fc-coated well to preserve the integrity of bound protein. The wells were washed and filled with suspensions of individual phage clones diluted in 0.5% milk/0.1% PBST. After one hour the wells were washed and bound phages were detected as described above.

2.7. Large scale expression and purification of soluble recombinant scFv

Recombinant scFv 3L5 was produced as a soluble protein in *Escherichia coli* HB2151 following the protocol of Kipriyanov [26].

2.8. Determination of IC50

Half maximal inhibitory concentration (IC50) for 3L5 scFv-mediated inhibition of the leptin–LR interaction was determined by a competitive ELISA. Wells of the Nunc Maxisorp microtiter plate were coated with hLR-Fc, blocked and washed. Seven serial dilutions of 3L5 scFv (0, 0.1, 1, 10, 100 nM, 1 and 10 μM), and infinitely high scFv concentration (simulated by 0.77 μM hLEP) were prepared in PBS containing 2.5 nM biotinylated leptin (B-hLEP), 0.5% milk and 0.1% Tween 20, and 100 μL of each were transferred to hLR-Fc-coated wells of the microtiter plate. After 90 min, the wells were washed and streptavidin-horseradish peroxidase conjugate (SA-HRP, GenScript) was used for detection of bound B-hLEP in each well followed by the addition of TMB substrate. The experiment was performed in triplicate. Absorbances measured at 450 nm were used to plot a competition diagram using GraphPad Prism 6 (GraphPad Software).

2.9. Estimation of dissociation constant

To estimate the dissociation constant (K_d) for 3L5 scFv–hLR-Fc interaction, a non-conventional ELISA-based assay was performed as described by Friguet et al. [27] and Martineau [28] with the main modification being a reverted interpretation – i.e., in our experiment hLR-Fc represented an antibody and scFv represented an antigen. Ten nanomolar hLR-Fc was incubated with increasing concentrations (0, 31.3, 62.5, 125, 250, 500, 1000 nM and infinite – mimicked by 1.1 μM human leptin) of 3L5 scFv for 1 h and 100 μL of each equilibrated mixture were transferred to the lep-

tin-coated wells of Nunc Maxisorp microtiter plate for 10 min to capture hLR-Fc molecules with unoccupied binding sites. After washing, the amount of bound hLR-Fc molecules, which was proportional to their free binding sites in equilibrium, was detected using anti-human IgG antibody–HRP conjugate (Bio-Rad, Richmond, CA, USA) and TMB. Absorbances recorded at 450 nm were used for construction of binding curve and calculation of K_d by using GraphPad Prism 6 software.

3. Results and discussion

Elevated plasma leptin levels are known to contribute to the outbreak or progression of diverse pathologies. Consequently, there is a growing interest in discovery and development of clinically useful leptin antagonists. In this context, we have screened two commercially available phage display libraries of scFvs for binding the extracellular part of human leptin receptor under tailored biopanning conditions and characterized interaction of identified ligands with the receptor.

3.1. Selection of leptin receptor binders

Biopanning procedure was based on interacting library phages with the recombinant hLR-Fc fusion in solution, followed by capture of target molecule-phage complexes onto the protein G-coated paramagnetic beads. Selection progress was assessed by monitoring the enrichment ratio (fold increase in the number of eluted virions relative to the number of phage particles introduced in successive selection rounds) and polyclonal phage ELISA using amplified eluates. LR-binders were only enriched from library J. More than 200-fold rise in percent of eluted phages was observed after specific leptin-mediated elution in the third selection round as compared to the second round (Fig. 1A). The following non-specific elution of residual phages still resulted in a relative enrichment factor of ~50 (dashed line in Fig. 1A). On the other hand, the proportion of eluted phages from the third round of screening library I was much lower. Moreover, subsequent trypsin elution in the third round was somewhat more efficient compared to leptin displacement. This suggests poor enrichment of library I phages in leptin receptor binders. The results are in agreement with polyclonal phage ELISA (Fig. 1B) which indicated prevalent enrichment of LR-binders from library J. However, phage pools, especially the one collected by trypsinization in the third round (3.AE-J (TRYP)), still contained a significant proportion of phage virions targeting Fc-region, a fusion partner in hLR-Fc. This is not surprising since no measures were carried out to eliminate Fc-binders in contrast to the attempts to avoid potential BSA- or protein G-binders; we included albumin in the washing buffer and performed negative selections against naked protein G beads, respectively. No significant binding of phages from the enriched pools of library I to any of the potential targets was observed, therefore individual clones from this library were not analyzed further. The reason for unsuccessful panning of library I may lie in its bias against many amino-acids in the randomized CDR positions. The NNK-randomization type in library J allows for incorporation of any amino acid at each of the 18 randomized residues of scFv, while library I was constructed by DVT diversification encoding mostly hydrophilic amino acids which likely represented too strong constraint to isolate binders in this case.

3.2. Identification of scFvs competing with leptin for receptor binding

Binding of individual phage clones selected from library J after rounds 2 and 3 to the hLR-Fc was evaluated by a monoclonal phage ELISA. According to arbitrary criterion (absorbance higher than 0.2 and at least three-times that of respective negative control, i.e. human gamma Fc-region binding), a total of 21 of 46 randomly picked phage clones (46%) showed specific binding to the recombinant leptin receptor ectodomain (see Supplementary Fig. 1). As expected, the majority of specific binders (18 out of 24) came from the third eluate obtained by specific elution using recombinant human leptin and only a single LR-binder was detected among 14 clones from non-specific trypsin elution in the second round. Eight binders producing the highest ELISA signals were sequenced and among them four unique clones were identified (termed 3L2, 3L5, 3L7, and 3L18; see Supplementary Fig. 2 for details).

We next checked whether the four phage clones compete with leptin for receptor binding. As shown on Fig. 2, leptin inhibited binding of all four clones to the hLR-Fc in a concentration dependent manner, confirming that phage-displayed scFvs interact specifically with either the leptin-binding site on the leptin receptor or regions in close proximity, thereby sterically hindering leptin-binding site. The fact that the four clones were obtained by competitive elution in the final selection round demonstrates that this

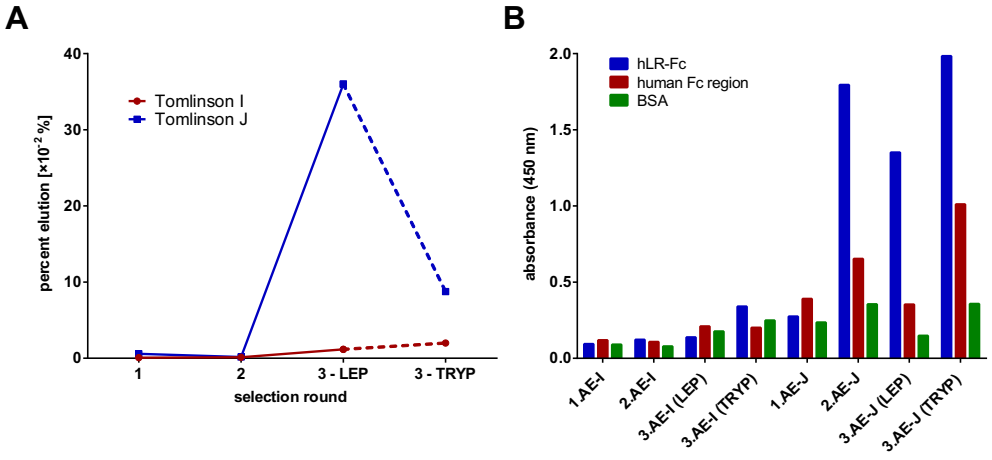

Fig. 1. (A) The proportion of eluted phages after individual selection rounds relative to the respective input phage numbers. Dashed lines connect consecutive competitive and non-specific elution steps in the final selection round. (B) Results of the polyclonal phage ELISA showing binding of enriched phage pools to different proteins encountered by library phages during affinity selection. The names under the axis are composed of a number (representing the selection round), abbreviation AE (stands for "amplified eluate"), the phage library name (either Tomlinson I or J), and the elution strategy in round 3 (either LEP for leptin displacement or TRYP for trypsin-mediated detachment).

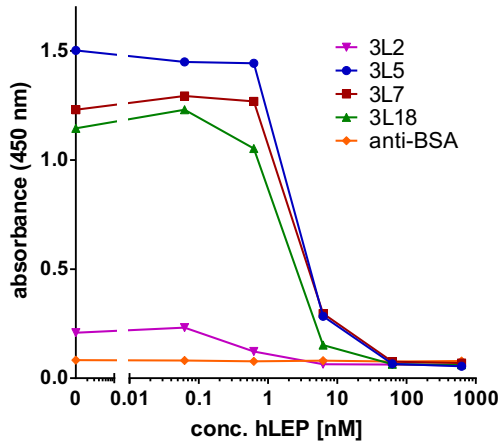

Fig. 2. Competition between phage clones and leptin for receptor binding. Equal titer of each phage clone was incubated in hLR-Fc-coated wells either alone or in presence of increasing concentrations of leptin.

strategy is very efficient for guiding selection to the desired site on target molecule. Notably, non-specific elution resulted in a more diverse set of binders targeting receptor ectodomain as well as the Fc-region (Fig. 1B). Similarly, Zabeau and co-workers isolated nanobodies interacting with different domains of LR ectodomain by using a non-specific elution approach [18].

3.3. Characterization of scFv-leptin receptor binding

To determine whether scFvs recognize linear or discontinuous epitopes on LR ectodomain we subjected scFv-bearing phage clones to ELISA against receptor in its native or denatured state (see Supplementary Fig. 3). All four tested phage clones bound exclusively to the native form of recombinant human leptin receptor, suggesting that respective scFvs recognize discontinuous epitopes on hLR-Fc molecules. Leptin-binding interface on LR is relatively large and extends over at least two subdomains of the cytokine receptor homology domain 2 (CRH2), the main leptin-binding part of the LR [29]. This is in line with the apparent complexity of epitopes to which the leptin-competing scFvs bind. Of note, our previous attempts to identify leptin-receptor ligands from random peptide libraries were unproductive, likely because short peptides were unable to form strong interactions with large flat surfaces. Three out of four LR-binders (clones 3L2, 3L7 and 3L18) contained at least one amber stop codon in the randomized regions, but 3L5 (also producing the highest ELISA signals in competition ELISA assay; Fig. 2) was amber-free and could therefore be directly expressed for further characterization in an amber non-suppressor strain of *E. coli* transformed with the phagemid harboring *scFv-[amber codon]-coat protein III* fusion gene. Purified 3L5 scFv migrated as a single band corresponding to ~28 kDa in SDS PAGE (not shown). Typical yield was 0.5–1 mg of soluble scFv from 1 L bacterial culture.

In competitive ELISA, mixtures of biotinylated recombinant human leptin (B-hLEP) and increasing concentrations of recombinant 3L5 scFv were incubated in hLR-Fc-coated wells, followed by detection of bound B-hLEP. From resulting competition diagram (Fig. 3A), an IC50 was estimated to be 815 nM (assuming that receptor ectodomain has one binding site for both, leptin and 3L5 scFv).

A modified ELISA assay was performed to construct the binding curve and estimate dissociation constant for the 3L5 scFv–hLR-Fc complex in solution. In the procedure, we monitored the amount of hLR-Fc molecules captured to the leptin-coated microtiter plate depending on the total concentration of scFv added to the hLR-Fc solution. The concentration of hLR-Fc in the assay was much below the saturation point. Therefore, measured absorbance directly correlates to the concentration of unoccupied leptin-binding sites on hLR-Fc molecules at a certain concentration of 3L5 scFv present in the mixture. Since the total concentration of hLR-Fc was known, we calculated individual concentrations of occupied binding sites on hLR-Fc molecules, which were equal to the concentrations of bound scFv molecules ($[\mathrm{scFv}]_{\mathrm{bound}}$) in the same mixtures, according to the formula:

$$[\mathrm{scFv}]_{\mathrm{bound}} = \frac{A_0 - A_i}{A_0 - A_\infty} \cdot [x]_{\mathrm{tot}},$$

where A_0 represents absorbance measured in wells with no scFv added to hLR-Fc, A_i represents individual absorbances measured in wells with different concentrations of scFvs, A_∞ represents background absorbance where specific binding is inhibited by a large excess of hLEP, and $[x]_{\mathrm{tot}}$ represents the total concentration of scFv-binding sites on hLR-Fc and equals twice the total concentration of hLR-Fc homodimer. The concentrations of bound scFv were then plotted against the total concentration of scFv to construct a binding diagram (Fig. 3B). After a non-linear fit of the binding curve (assuming one binding site per receptor ectodomain) the dissociation constant for 3L5 scFv–hLR-Fc interaction was calculated to be 110 nM.

3.4. Therapeutic potential of scFvs interfering with leptin binding to its cognate receptor

In an attempt to develop antibody-based leptin receptor antagonists we decided to start with screening synthetic antibody repertoires. Because of the human framework of library scFvs it should

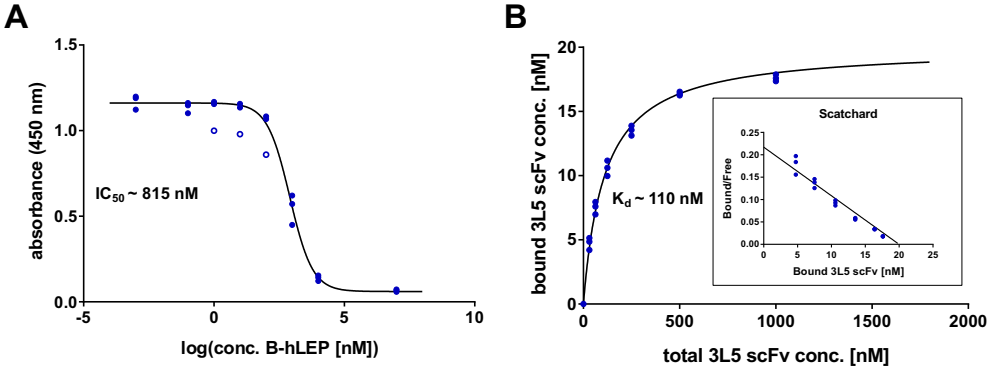

Fig. 3. (A) Competitive binding of leptin and 3L5 scFv to the immobilized leptin receptor as determined by competitive ELISA. Open circles represent outliers. (B) Concentration dependent binding of 3L5 scFv to the hLR-Fc in solution. The inner diagram represents Scatchard plot constructed from the same measurements.

take less effort to turn isolated binders into safe, low-immunogenic antibody drug candidates in contrast to immune libraries of animal origin where excessive optimization (i.e., back-mutation of specific framework residues after CDR-grafting) might be required in humanization process [30].

However, synthetic libraries typically yield antibody fragments of lower affinity due to lack of affinity maturation. This contrasts the situation in immune libraries where animal's immune system is exploited to pre-enrich antibody gene pool for specific antigen prior to phage library construction. This explains the relatively high K_d value of 3L5 scFv for receptor binding; the determined dissociation constant is approximately two orders of magnitude higher compared to the typical K_d of therapeutic antibodies.

The dissociation constant of 3L5 scFv for binding leptin receptor is also considerably higher than that of leptin (ranging from 0.2 to 15 nM) [29], which is in accordance with relatively high IC50 of 3L5 scFv. Due to insufficient affinity, the scFvs reported here do not represent suitable drug candidates since they would need to be administered in doses generally unacceptable for therapy in order to achieve a satisfactory biological response. However, the scFvs could be further developed for *in vivo* use as leptin antagonists. Specifically, grafting the selected scFv onto the antibody framework should contribute significantly to the greater functional affinity due to the introduction of avidity effect [31]. Moreover, compared to inhibitory leptin muteins and short peptide antagonists, antagonistic IgG molecules have longer plasma half-lifes and are expected to distribute mainly to peripheral tissues and exert less central effects [17,32] – the latter would perhaps emerge as a consequence of interfering with leptin transport across the blood–brain barrier. This is important since most of leptin's disease-related effects are actually due to excessive *peripheral* leptin signaling. In addition, pharmacokinetic profiles (plasma half-life and distribution) of scFvs may also be improved by pegylation. On the other hand, inhibition of *central* leptin activity might also be beneficial, if reversing decreased food intake is desired, for example in cancer-related cachexia.

Importantly, affinity of scFv molecules can be greatly improved to the level exceeding affinities of natural antibodies by *in vitro* affinity maturation of initially identified binders using mutagenesis methods, such as error-prone PCR or DNA shuffling, followed by additional screening of resulting libraries [33]. We believe that the scFvs targeting human leptin receptor ectodomain reported here may represent promising leads for development of new therapeutics for use in conditions where inhibition of leptin signaling is beneficial. In this scope, detailed assessment of biological activity of the scFvs reported here together with improvement of their binding properties remains the main goal of our future work.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.087.

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