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(54) **Title:** PEPTIDES BINDING TO LDL-RECEPTOR AS CARRIERS FOR CROSSING THE BLOOD BRAIN BARRIER

(57) **Abstract:** The current invention concerns peptides capable of binding to LDL-receptor, wherein said peptide has at least 90% sequence identity to an amino acid sequence chosen from SEQ ID 1 to 12, and it concerns peptides capable of binding to LDL-receptor, wherein said peptide has an amino acid sequence that differs maximally two amino acids from SEQ ID 1 to 12, as well as conjugate compounds comprising such peptides, as well as the use of such peptides in compositions.

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PEPTIDES BINDING TO LDL-RECEPTOR AS CARRIERS FOR CROSSING THE BLOOD BRAIN BARRIER

FIELD OF THE INVENTION

The present invention relates to peptides and their use as vectors of molecules of interest. The invention also relates to conjugate compounds comprising said peptide of the invention. The peptides and conjugates of the invention can be used as a vehicle for molecules of pharmaceutical or diagnostic interest, such as, for example, therapeutic molecules, imaging or diagnostic agents, or molecular probes, through cell membranes, and in particular to promote their transport across the blood-brain barrier (BBB).

BACKGROUND

The blood-brain barrier (BBB) is a structure at the interface between the brain and the blood that strictly controls the brain homeostasis in association with the blood-cerebrospinal fluid barrier (BCSFB) and the ependymal barrier. BBB is composed of a monolayer of endothelial cells joined by tight junctions, limiting the paracellular crossing, and surrounded by astrocytes and pericytes. In addition to the brain's physical protection, BBB exhibits biological characteristics improving its efficacy, such as a well-developed enzymatic function, a low number of pinocytosis vesicles and a high proportion of mitochondria, reflecting its important metabolic activity. Due to the presence of the BBB, most drugs are not able to passively access the brain if they do not meet certain characteristics, such as the lipophilicity and a size smaller than 400 Daltons.

The development of new BBB crossing strategies is a real challenge and some invasive and non-invasive methods are available. The first group (i.e., ultrasounds, microwaves, osmotic opening, etc.) leads to the BBB disruption (transient or not), which precludes their routine clinical implementation due to the crucial role played by this barrier in brain protection and homeostasis. The second group shows more interest by employing natural pathways to allow brain access while the BBB's integrity is preserved. If the use of the nasal pathway and of non-specific pathways such as the passive diffusion (lipidization) or the adsorptive-mediated transcytosis (cationization) is possible, the receptor-mediated transcytosis (RMT) offers the advantage to be specific. It involves the binding of a vector (i.e. endogenous ligand, antibody or peptide), coupled with the molecule of interest, to a receptor that

initiates the endocytosis of this receptor and leads to the transcytosis of the complex across the endothelial cells. The most studied receptors used for this purpose are the transferrin receptor (TfR), the insulin receptor (IR) and the Low-Density Lipoprotein receptor (LDLR) and its related proteins (LRP1 and LRP2).

The LDLR is an ubiquitary transmembrane receptor of a large family of receptors involved in lipid metabolism. It recognizes a variety of ligands among which apolipoproteins B (apoB) and E (apoE) are present in lipoprotein particles. The binding of these particles leads to their endocytosis through clathrin-coated pits and their transfer to the lysosome degradation pathway that delivers cholesterol for cellular exploitation. On the other hand, the caveolae-mediated endocytosis is employed for the LDLR crossing over the BBB, whereas the lysosomal degradation is shown to be bypassed. These modifications of endocytosis mechanisms between peripheral tissues and the central nervous system (CNS) make the LDLR an attractive target for BBB crossing.

EP 2350117 discloses peptide derivatives (peptides and pseudo-peptides) and prodrug conjugates, comprising a peptide derivative, which are capable of binding to human LDLR at the surface of cell membranes and having a MPR motive (or analogue), and can be used to vector molecules of pharmaceutical or diagnostic interest, such as, for example, therapeutic molecules, imaging or diagnostic agents, or molecular probes, through cell membranes, and in particular to promote their transport across the BBB.

However, EP 2350117 further describes that these known peptides are selected against human LDLR expressed by transfected CHO cells, which does not guarantee binding to the extracellular domain of the receptor to which natural ligands, as ApoB and ApoE, bind. Moreover, EP 2350117 especially mentions that said peptides bind LDLR in absence of competition with the natural ligands, indicating binding to a different binding site. Binding of peptides to the LDL binding site would show competition with natural ligands. This is favourable for drug delivery, as said peptides can, in a similar manner as natural ligands, associate with LDLR at the cell surface and dissociate after endocytosis.

The peptides known from EP 2350117 were also found in lysosomes, which leads to degradation of the peptides, however, to ensure intact delivery of molecules of pharmaceutical or diagnostic interest, peptide vectors should follow the caveolae-mediated endocytosis pathway in endothelial cells, and not the pathway of

lysosomes. To be considered efficient, the general scientific consensus is that at least 2-2.5 % of the injected dose should be delivered to the brain. In EP 2350117, only a low portion of the injected dose of peptides (0.095 %) was delivered to the brain.

Similar peptides are also disclosed in CN 110330550 and Michel Demeule *et al.*, 2008.

In view of the prior art, there remains a need for an improved vector that could be used to improve brain access of therapeutic or diagnostic molecules.

The present invention aims to resolve at least some of the problems and disadvantages mentioned above.

SUMMARY OF THE INVENTION

The present invention and embodiments thereof serve to provide a solution to one or more of the above-mentioned disadvantages. To this end, the present invention provides for peptides according to claim 1 or a conjugate compound according to claim 12. These peptides or conjugates are capable of binding to extracellular domain of LDLR (ED-LDLR).

Preferred embodiments of said peptide are shown in any of the claims 2-10 and 13-15. Preferred embodiments of said conjugates are shown in any of the claims 13-15.

In a second aspect, the current invention also relates to the use of a peptide according to claim 11.

In a third aspect, the current invention relates to a pharmaceutical composition according to claim 16.

DESCRIPTION OF FIGURES

Figure 1. (A-B) Specific binding of the 50 clones isolated from the pool of the 3rd round of panning, determined by the ratio between the binding to ED-LDLR over that to BSA (see **Fig. 11**). **(A)** Clones 1 to 24. **(B)** Clones 25 to 50. **(C)** Frequency of amino acids in the 13 different peptide sequences obtained after DNA sequencing.

Figure 2. Binding of selected clones to ED-LDLR in various experimental conditions. **(A)** Apparent dissociation constants (K^*_d) of the 12 representative clones selected from the phage display experiments. Clones with low K^*_d, revealing high affinities and selected for further characterization, are in light gray. **(B-D)** K^*_d of the clones 40, 41 and 47 in normal conditions, at acidic pH (pH 6.0, pH 5.0) and in absence of calcium (Ca^{2+} free). **(E-G)** Ratios between K^*_d in modified conditions and K^*_d of the clones, reflecting the inhibitory effects of these modifications on the clones binding.

Figure 3. Three-dimensional structure **(A, F)** and spatial conformation **(B, G)** of peptides LRPep1 **(A, B)** and LRPep2 **(F, G)**. The three-dimensional structures of peptides were drawn with ACD/ChemSketch 2.0 software. The peptides are represented with disulfide bridges that could occur in oxidizing conditions between the pairs of Cys (in **A and F**). Their spatial conformations were obtained with MarvinSketch 19.2 software (2019, http://www.chemaxon.com). Interaction of LRPep1 **(C-E)** and LRPep2 **(H-J)** with ED-LDLR was predicted using the HPEPDOCK program of blind peptide-protein docking (http://huanglab.phys.hust.edu.cn/hpepdock/). The binding regions of peptides to ED-LDLR are zoomed in figures **D-E** (LRPep1) and **I-J** (LRPep2) to better observe the docking models. LRPep1 and LRPep2 appear lighter, the ED-LDLR appears darker.

Figure 4. Endocytosis of the peptides LRPep1-rho and LRPep2-rho (stained with rhodamine: rho) in **(A)** HUVEC and HepaRG cells and **(B)** ACBRI376 and 1321N1 cells. Nuclei are stained with Hoechst. **(C-F)** The endocytosis of peptides as well as the expression of LDLR were semi-quantitatively evaluated by the measurement of fluorescent labelling using the ImageJ software and was normalized to the number of cells and to the background, giving the Relative Ratio of Fluorescent Labelling (RRFL); *: $p < 0.05$, **: $p < 0.001$. **(G)** Correlation coefficient between the expression of LDLR and the endocytosis of peptides.

Figure 5. Colocalization of LRPep2-rho with LDLR on mouse brain slices **(A)** and ACBRI376 cells **(B)**. LRPep2 is stained due to the coupled rhodamine, LDLR is stained with fluorescein and nuclei with DAPI. White arrows highlight examples of colocalization areas.

Figure 6. Semi-quantitative analysis of fluorescent labelling of caveolae and lysosomes when ACBRI376 **(A)**, HepaRG **(B)**, N18(H) **(C)** and 1321N1 **(D)** cells are

incubated or not with peptide LRPep2 by the measurement of the RRFL. *: $p < 0.05$, **: $p < 0.001$.

Figure 7. (A) K^*_d value of USPIO-LRPep2 for LDLR. **(B-D)** *In vivo* evaluation of the BBB crossing ability of USPIO-LRPep2 by MRI. **(B)** Raw images of representative MRI images of the brains of NMRI mice acquired with RARE protocol (spatial resolution = 48µm) before (pre-contrast) and 52 min after injection of USPIO derivatives (post-contrast). **(C)** Overlays of the raw images. **(D)** Analysis of the contrast (Δ%SNR) observed on MRI images, measured by the ImageJ software. *: $p < 0.05$.

Figure 8. Biodistribution of USPIO-LRPep2 and USPIO-NSP at 55 minutes post-injection. **(A)** R_2^{Norm} ($=1/T_2^{Sample} - 1/T_2^{Control}$) for each USPIO derivative in the kidney, the spleen and the liver. * $p < 0.05$. **(B)** and **(C)** Iron concentration (µmol/L) in urine and plasma respectively. **(D)** $R_{2(1)}^{Norm}$ for each USPIO derivative in the brain. **(E)** Iron concentration (µmol/g of dried tissue) in brains after digestion in acidic conditions. **(F)** Correlation coefficients between the $R_{2(1)}^{Norm}$ of USPIO derivatives in the brains and their concentrations in the blood.

Figure 9. Perls'-DAB staining of USPIO derivatives in mouse brains collected at 55 minutes post-injection. Mice injected with PBS were used as negative control. USPIO derivatives are stained by DAB.

Figure 10. (A) FLI images of the brains of nude mice before (pre-iv) and after injection of CF770-LRPep2 or CF770 (post-iv). **(B)** Analysis of the fluorescence observed on FLI images, measured by the M3Vision software in the brain area, and normalized to the pre-iv signal. * $p < 0.05$. **(C)** FLI images of mouse brains *ex vivo*. **(D)** Analysis of the fluorescence of the brains *ex vivo* normalized to the brain signal of a non-injected mouse. § $p = 0.05$.

Figure 11. Individual affinities of the 50 clones isolated from the 3rd round of panning evaluated against the ED-LDLR and the BSA. **(A)** Clones 1 to 24. **(B)** Clones 25 to 50. The blank corresponds to the non-specific binding of anti-M13 antibody to the ELISA plate.

Figure 12. Fluorescent immunostaining of LDLR (stained with Texas Red) in **(A)** HUVEC, **(B)** HepaRG, **(C)** ACBRI376 and **(D)** 1321N1 cells. Nuclei are stained with DAPI.

Figure 13. Colocalization of LRPep2-rho with caveolae **(A)** and lysosomes **(B)** in ACBRI376 human brain microvascular endothelial cells. LRPep2 is stained due to its rhodamine, caveolae and lysosomes are stained with fluorescein and nuclei with DAPI. White arrows show accumulation of LRPep2-rho that colocalize with large spots of caveolae, but not with lysosomes.

Figure 14. Whole body FLI images of 3 mice before (pre-iv) and after CF770 injection (50 min post-iv).

Figure 15. Whole body FLI images of 4 mice before (pre-iv) and after CF770-LRPep2 injection (50 min post-iv).

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns peptides and conjugates capable of binding to LDLR and their (therapeutic and diagnostic) use.

The human LDLR is a transmembrane protein composed of 839 amino acids (Ala^{22}-Ala^{860}) whose the N-terminal region spans almost the entire molecule (Ala^{22}-Arg^{788}) and is extracellular. The seven Cys-rich type A repeats (R1-R7) of the extracellular domain (ED) are responsible for ligand binding. The brain has a strong need for LDL for its proper functioning. The natural ligands to LDLR are LDL and more particularly apolipoprotein B (ApoB) and apolipoprotein E (ApoE) constitutive of LDL particles thus allowing the transport of cholesterol, contained in these particles, through cell membranes and more especially through the BBB.

Unless otherwise defined, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. By means of further guidance, term definitions are included to better appreciate the teaching of the present invention.

As used herein, the following terms have the following meanings:

"A", "an", and "the" as used herein refers to both singular and plural referents unless the context clearly dictates otherwise. By way of example, "a compartment" refers to one or more than one compartment.

"About" as used herein referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, is meant to encompass variations of +/-20% or less, preferably +/-10% or less, more preferably +/-5% or less, even more preferably +/-1% or less, and still more preferably +/-0.1% or less of and from the specified value, in so far such variations are appropriate to perform in the disclosed invention. However, it is to be understood that the value to which the modifier "about" refers is itself also specifically disclosed.

"Comprise", "comprising", and "comprises" and "comprised of" as used herein are synonymous with "include", "including", "includes" or "contain", "containing", "contains" and are inclusive or open-ended terms that specifies the presence of what follows e.g. component and do not exclude or preclude the presence of additional,

non-recited components, features, elements, members, steps, known in the art or disclosed therein.

Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order, unless specified. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within that range, as well as the recited endpoints.

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, in the following claims, any of the claimed embodiments can be used in any combination.

In a first aspect, the invention relates to a peptide capable of binding to human LDLR.

The term "peptide," as used herein and in the appended claims, refers to any compound containing two or more amino acid residues joined by an amide bond formed from the carboxyl group of one amino acid residue and the amino group of the adjacent amino acid residue. The amino acid residues may have the L-form as well as the D-form, and may be naturally occurring or synthetic, linear as well as cyclic. Also included within the term "peptide" as used herein and in the claims are polypeptides and peptide dimers which can be peptides linked C-terminus to N-terminus (tandem repeats), peptides linked C-terminus to C-terminus or N-terminus

to N-terminus (parallel repeats) or peptides linked via their C-terminus or N-terminus to a macromolecular scaffold or macromolecular moiety, such as, but not limited to, iron oxide nanoparticles, liposomes, micelles or dendrimers.

In an embodiment, said peptide is a synthetic peptide. By the term "synthetic peptide" it is understood to refer to a peptide which has been artificially synthesized. Such synthesis methodologies are readily known in the art. The terms "therapeutic molecule" or "therapeutic agent" as used herein denotes a bioactive molecule or agent that has therapeutic utility. The terms "diagnostic molecule" or "diagnostic agent" as used herein denotes a bioactive molecule or agent that has diagnostic utility.

Another method for the preparation of the peptides according to embodiments disclosed herein is the use of peptide mimetics. Mimetics are peptide-like molecules which mimic elements of protein or peptide secondary structure. The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule.

The peptide according to the embodiments of the current invention may be purified. Generally, "purified" will refer to a protein or peptide composition which has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this will refer to a composition in which the peptide forms the major component of the composition, such as constituting about 50% or more of the peptides in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the number of polypeptides within a fraction by SDS/PAGE analysis. Actual units used to represent activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulfate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydro

xylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the peptide always be provided in the most purified state. It is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater - fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

The peptides of the invention can be synthesized by any technique known *per se* to those skilled in the art (chemical, biological, genetic synthesis, etc.). They can be stored as is, or formulated in the presence of a substance of interest or any acceptable excipient.

In an embodiment, the peptides will have a length of 6 to 25 amino acids, more preferably between 10 to 15 amino acids, even more preferably twelve amino acids.

In another or further embodiment, said peptides according to the current invention share at least 90%, more preferably at least 95%, even more preferably at least 99% sequence identity to an amino acid sequence chosen from SEQ:ID n° 1 to 12, preferably SEQ:ID n° 10 or 12.

In another embodiment, said peptides have a sequence which differs maximally 3, more preferably maximally 2, even more preferably maximally 1 amino acid from one of the sequences chosen from SEQ:ID n° 1 to 12, preferably SEQ:ID n° 10 or 12.

In another embodiment, said peptides have an amino acid sequence which incorporates one of the amino acid sequences chosen from SEQ:ID n° 1 to 12, or a sequence which has at least 90%, more preferably 95%, even more preferably 99% sequence identity with the amino acid sequences chosen from SEQ:ID n° 1 to 12, preferably SEQ:ID n° 10 or 12.

The term "sequence identity" as used herein refers to the extent that sequences are identical on an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical amino acid occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. A gap, i.e., a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. Determining the percentage of sequence identity can be done manually, or by making use of computer programs that are available in the art. Examples of useful algorithms are PILEUP. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Amino acid sequence variants of a peptide contemplated herein may be substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the peptide which may not be critical for function. Substitutional variants typically contain an alternative amino acid at one or more sites within the peptide and may be designed to modulate one or more properties of the polypeptide such as stability against proteolytic cleavage. Substitutions preferably are conservative, that is, one amino acid is replaced with one of similar size and side chain or functional group. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to glycine, valine or leucine; arginine to lysine; asparagine to glutamine; aspartate to glutamate; cysteine to methionine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to glutamine, tyrosine, arginine, lysine, asparagine or cysteine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to phenylalanine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

In a most preferred embodiment, said peptide has an amino acid sequence which is identical to a sequence chosen from SEQ:ID n° 1 to 12, more preferably, said sequence is SEQ:ID n° 10 or 12.

The current invention equally relates to a synthetic sequence encoding for a peptide according to SEQ ID n° 1 – 12 and according to one of the embodiments above.

The peptides described in the present invention possess the ability to target cellular receptors/transporters, particular cell types and/or to pass cell membranes including those physiological barriers of the brain and more particularly the BBB.

The peptides described in the present invention possess the capacity to target cellular receptors/transporters, particular cell types and/or to pass cell membranes, in particular those of physiological barriers of the central nervous system (CNS). Said peptides may therefore be used in the treatment of more particularly infectious diseases, cerebral or other, bacterial, viral, parasitic or fungal, or in the treatment or prevention of a neurologic, neuropsychiatric or neurodegenerative condition, or a cerebral tumor.

The peptides described in the present invention possess the ability to bind to human LDLR, preferably LDLR of the cell membrane and to cross said membrane through this receptor by transcytosis.

In an embodiment, the peptides as described above bind to the extracellular domain of LDLR, preferably at the interface between R4 and the β-Propeller (βP) domain of the extracellular domain of LDLR.

Said interface between R4 and the β-propeller (βP) domain of the extracellular domain of LDLR corresponds to the binding site of natural ligands LDL, ApoB and ApoE. This is advantageous as it indicates that said peptide will associate with LDLR at the cell surface and dissociate after endocytosis, in a similar manner as natural ligands, which is favourable for drug delivery.

The invention therefore relates more particularly to peptides which have an affinity for LDLR. Suitable peptides can be identified via ligand binding assays, phage display, computational modeling or other high throughput screenings.

In a preferred embodiment, said peptides follow the same pathway as LDL, the natural ligand of LDLR upon binding to this receptor. More specifically, said peptides are able to activate the LDLR signaling upon binding at the cell surface. More preferably, said peptides will subsequently be endocytosed and will dissociate after endocytosis. Even more preferably, subsequent dissociation of peptides is favoured by mild low pH and low Ca^{2+} concentration (similar as conditions in endocytic compartment).

A preferred aspect of the invention resides in a peptide as defined above, characterized in that it has the ability to pass the BBB.

The present invention also pertains to a conjugate compound comprising a peptide as described above. In an embodiment, this conjugate compound comprises a peptide as described above, coupled to a substance of interest.

The term "conjugate" or "conjugate compound" refers to a molecule resulting from the combination of one or more peptides of the invention and one or more molecules of interest. The conjugation may be of a chemical nature, such as by means of a spacer arm (linker or spacer), or of a genetic nature, such as genetic recombination technology, as in a fusion protein with for example a marker or tracer molecule (for example, GFP, β-galactosidase, etc.) or a therapeutic molecule (for example a growth factor, neurotrophic factor, etc.), or by independently coupling one or more peptides of the invention and one or more molecules of interest to a macromolecular scaffold or macromolecular moiety, such as, but not limited to, iron oxide nanoparticles, liposomes, micelles or dendrimers.
The coupling is advantageously covalent and can be made to dissociate after the passage of cell membranes, in order to release the substance of interest in a site of interest. Depending on the nature of the coupling, the release of the substance can take place for example passively or under the action of enzymes or specific physiological conditions.

This conjugate compound, comprising a peptide as described above coupled to a substance of interest, enables or improves the passage of a molecule through the BBB.

The peptides of the invention make it possible to cross the BBB to an active substance that does not pass this barrier. They can therefore be used in the treatment, prevention or diagnosis of any disease affecting the CNS, but also as carriers of biological material (biotransporters) in studies conducted on various families of molecules with cell membrane models and more particularly of BBB.

The active substance or molecule of interest may be any molecule of pharmaceutical interest, in particular therapeutic, a diagnostic or medical imaging agent, or a molecular probe. It may be in particular any chemical entity having a biological interest such as a small chemical molecule (antibiotic, antiviral, immunomodulator, anticancer, anti-inflammatory, etc.), a peptide or a polypeptide, a protein (enzyme,

hormone, cytokine, apolipoprotein, growth factor, antigen, antibody or part of antibody), a nucleic acid (ribonucleic acid or deoxyribonucleic acid of human, viral, animal, eukaryotic or prokaryotic origin, vegetable, synthetic, etc., may have a variable size, ranging from simple oligonucleotide to genome or genome fragment), a viral genome or a plasmid, a ribozyme, a marker or tracer. Generally speaking, the "substance of interest" can be any active ingredient of a drug, be it a chemical, biochemical, natural or synthetic. The term "small chemical molecule" denotes a molecule of pharmaceutical interest having a molecular weight of up to 1000 Daltons, typically between 300 and 700 Daltons.

It is preferable that the interaction between the peptide and the active substance is sufficiently solid so that the peptide does not dissociate from the active substance until it has reached its site of action. For this reason, the preferred coupling according to the invention is a covalent coupling, but it could however be a non-covalent coupling. The substance of interest can be coupled directly to the peptide (tandem synthesis) at one of these terminal ends (N-term or C-term). The substance of interest can also be coupled indirectly via a linker or spacer arm (synthesis via linker) at one of the terminal ends of the peptides. In addition to this, the substance of interest can also be coupled to a macromolecular scaffold or macromolecular moiety, such as, but not limited to, iron oxide nanoparticles, liposomes, micelles or dendrimers, and said scaffold or moiety is also coupled to the peptide. Thus, both the substance of interest and the peptide, which is a vector, are coupled independently on the same macromolecular scaffold or macromolecular moiety.

Mention may be made, as covalent chemical coupling means, with or without a spacer arm, of those chosen from bi- or multifunctional agents containing alkyl, aryl or peptide groups by esters, aldehydes or alkyl or aryl acids, anhydride, sulfhydryl, amine or carboxyl groups, groups derived from cyanogen bromide or chloride, carbonyldiimidazole, succinimide esters, sulphonic halides, or others readily known in the art.

In a preferred embodiment, peptides of the invention and conjugate compounds comprising peptides of the invention follow the same pathway as LDL, the natural ligand of LDLR upon binding to this receptor. More specifically, said peptides and conjugate compounds are able to activate the LDLR signaling upon binding at the cell surface. More preferably, said peptides will subsequently be endocytosed and will dissociate after endocytosis. Even more preferably, subsequent dissociation of peptides is favoured by mild low pH and low Ca^{2+} concentrations (similar as conditions in endocytic compartment).

As a consequence, a binding mechanism similar to the binding of natural ligands to LDLR will be favourable for the delivery of the compound of interest as it will be endocytosed and will dissociate when it has reached its site of action, in this embodiment upon meeting the conditions of the endocytic compartment, more specifically this is mild low pH and a low Ca^{2+} concentration.

In a further preferred embodiment, peptides and conjugates of the invention will follow the pathway of caveolae and not that of lysosomes (which leads to degradation). This ensures the intact delivery of the carried compound of interest.

In a further preferred embodiment, peptides and conjugates of the invention will follow the pathway of caveolae mainly by endothelial cells. By contrast, in hepatocytes, for example, peptides will follow the lysosomal pathway, which is the classical pathway for intracellular trafficking of LDLR. This allows intact delivery of carried compounds of interest to the brain, while these compounds of interest will be inactivated by proteolysis in other tissues, such as hepatic tissue. This limits possible secondary non-desirable effects in other tissues.

In another preferred embodiment, peptides of the invention are competitors of natural ligands, such as ApoB and ApoE, which means that said peptides will not be destabilized by the natural ligands *in vivo*, allowing delivery of the compound of interest.

The present invention also pertains to a peptide or conjugate compound as described above for the use in the treatment or prevention of medical conditions or diseases which required crossing the blood-brain barrier of pharmaceutical agents or compounds. In one embodiment, said condition or disease is a brain disease, neurologic or neurodegenerative condition such as Alzheimer disease, or a cerebral tumor.

Present invention also relates to a peptide or conjugate compound according to the embodiments described above, for use in diagnostics.

In one embodiment, said use in diagnostics might pertain to imaging and/or diagnosis of neurological pathologies, as well as infectious or cancerous pathologies, cerebral or otherwise.

Present invention also pertains to a composition, preferably a pharmaceutical composition comprising at least one conjugate compound according to the embodiments described above and one or more pharmaceutically acceptable excipients.

In one embodiment, this composition can be a diagnostic composition which comprises a diagnostic or medical imaging agent consisting of a conjugated compound as defined above.

The conjugate can be used in the form of any pharmaceutically acceptable salt. By pharmaceutically acceptable salts is meant, for example and without limitation, pharmaceutically acceptable basic or acid addition salts, hydrates, esters, solvates, precursors, metabolites or stereoisomers, said vectors or conjugates charged with at least one substance of interest.

The term pharmaceutically acceptable salts refers to non-toxic salts, which can be generally prepared by reacting a free base with a suitable organic or inorganic acid. These salts retain the biological efficacy and properties of free bases. Representative examples of such salts are water-soluble and water-insoluble salts, such as acetates, N-methylglucamine ammonium, ansonates (4,4-diaminostilbene-2,2'-disulfonates), benzenesulfonates, benzonates, bicarbonates, bisulfates, bitartrates, borates, hydrobromides, bromides, buryates, camsylates, carbonates, hydrochlorides, chlorides, citrates, clavulariates, dihydrochlorides, diphosphates, edetates, calcium edetates, edisylates, estolates, esylates, fumarates, gluceptates, gluconates, glutamates, glycolylarsanylates, hexafluorophosphates, hexylresorcinates, hydrabamines, hydroxynaphthoates, iodides, isothionates, lactates, lactobionates, laurates, malates, maleates, mandelates, mesylates, methylbromides, methylnitrates, methylsulfates, mucates, napsylates, nitrates, 3-hydroxy-2-naphthoates, oleates, oxalates, palmitates, pamoates (1,1-methylene-bis-2-hydroxy-3-naphthoates, or emboates), pantothenates, phosphates, picrates, polygalact uronates, propionates, p-toluenesulfonates, salicylates, stearates, subacetates, succinates, sulfates, sulfosalicylates, suramates, tannates, tartrates, teoclates, tosylates, triethiodides, trifluoroacetates, valerate.

The compositions of the invention advantageously comprise a pharmaceutically acceptable carrier or excipient. The pharmaceutically acceptable vehicle may be chosen from the vehicles conventionally used according to each of the modes of administration. Depending on the intended mode of administration, the compounds

may be in solid, semi-solid or liquid form. For solid compositions, such as tablets, pills, powders or granules in the free state or included in capsules, the active substance may be combined with: a) diluents, for example lactose, dextrose, sucrose, mannitol, sorbitol, cellulose and/or glycine; b) lubricants, for example silica, talc, stearic acid, its magnesium or calcium salt and / or polyethylene glycol; c) binders, for example magnesium aluminum silicate, starch paste, gelatin, gum tragacanth, methylcellulose, sodium carboxymethylcellulose and/or polyvinylpyrrolidone; d) disintegrants, for example starch, agar, alginic acid or its sodium salt, or effervescent mixtures; and/or e) absorbents, dyes, flavoring agents and sweeteners. The excipients may be, for example, mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate and pharmaceutical grade analogs. For semi-solid compositions, such as suppositories, the excipient may, for example, be a fat emulsion or suspension, or based on polyalkylene glycol, such as polypropylene glycol. Liquid compositions, in particular injectable or to be included in a soft capsule, can be prepared for example by dissolution, dispersion, etc. of the active substance in a pharmaceutically pure solvent such as, for example, water, physiological saline, aqueous dextrose, glycerol, ethanol, an oil and its analogues.

Embodiments herein provide for administration of compositions to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the active agent (e.g., pharmaceutical chemical, protein, gene, antibody, aptamers etc. of the embodiments) to be administered in which any toxic effects are outweighed by the diagnostic or therapeutic effects of the active agent.

The compositions or conjugates of the invention may be administered by any suitable route and, in a non-limiting manner by the parenteral route, such as, for example, in the form of injectable preparations subcutaneously, intravenously, intracardiac, intracoronary, intraperitoneally or intramuscularly; orally (or *per os*), such as, for example, in the form of coated tablets or not, capsules, powders, liposomes, granules, suspensions or oral solutions (such a form for oral administration can be either immediate release, either prolonged or delayed release); - rectal route, such as, for example, in the form of suppositories; - topical route, particularly transdermal, such as, for example, in the form of patches, ointments or gels; - intranasal route, as for example in the form of aerosols and sprays; - perlingual route; - intraocular route. Depending on the route of administration, the active compound may be coated in a material to protect the active agent from the

degradation by enzymes, acids and other natural conditions that may inactivate the compound.

Pharmaceutical compositions suitable for injectable use may be administered by means known in the art. For example, sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion may be used.

Sterile injectable solutions can be prepared by incorporating the active agent in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.

Aqueous compositions can include an effective amount of the active agent, being one or more peptides according to the current invention dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Compounds and biological materials disclosed herein can be purified by means known in the art. Solutions of the active compounds as free-base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration.

The pharmaceutical compositions typically comprise an effective dose of a peptide or conjugate of the invention. A "therapeutically effective dose" as herein described is understood to be the dose that provides a therapeutic effect for a given condition and administration regimen. This is typically the average dose of an active substance to be administered to substantially improve some of the symptoms associated with a disease or condition. For example, in the treatment of cerebral or non-brain cancer, a pathology, a lesion or a disorder of the CNS, the dose of an active substance which decreases, prevents, delays, suppresses or stops the any of the causes or symptoms of the disease or disorder would be therapeutically effective.

A "therapeutically effective dose" of an active substance is not required to cure a disease or disorder but will provide treatment for that disease or disorder so that its onset is delayed, hindered or prevented, or that its symptoms are alleviated, or the term is modified or, for example, less severe or the patient's recovery is accelerated.

It is understood that the "therapeutically effective dose" for a particular individual will depend on various factors, including the activity/efficacy of the active substance, its time of administration, route of administration, rate of excretion and metabolism, associations/drug interactions and the severity of the disease (or disorder) treated as a preventative or curative, as well as age, genetic background, body weight, overall health status, sex, lifestyle, exposure to risk factors and/or diet of the patient.

Depending on the coupled substance, the peptides, conjugates and compositions of the invention may be used for the treatment, prevention, diagnosis or imaging of numerous pathologies, in particular pathologies affecting the CNS, infectious pathologies or cancers.

In this regard, the invention relates to the use of conjugates or pharmaceutical compositions as described above for the treatment or the prevention of CNS pathologies or disorders, of cerebral tumors or of other cancerous cells, and of infectious pathologies, cerebral or otherwise, bacterial, viral, parasitic or fungal.

The invention also relates to the use of conjugates or pharmaceutical compositions as described above for the diagnosis or imaging of CNS pathologies or disorders, of cerebral tumors or of other cancerous cells, and of cerebral infectious pathologies or others, bacterial, viral, parasitic or fungal.

The invention also relates to the use of a conjugate or a composition as defined above for the treatment, imaging and/or diagnosis of a cerebral tumor or of another type of cancer cells such as cancers of the liver, pancreas, ovary, lung, stomach, etc.

The invention also relates to the use of a conjugate or a composition as defined above for the treatment, imaging and/or diagnosis of infectious diseases of the brain or the like, of the bacterial, viral, parasitic or fungal, such as, and not limited to, AIDS, even meningitis, etc.

The invention also relates to the use of a conjugate or a composition as defined above for the treatment, imaging and/or diagnosis of neurodegenerative pathologies such as but not limited to Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jacob disease, stroke, bovine spongiform encephalitis, multiple sclerosis, amyotrophic lateral sclerosis, etc.

The invention also relates to the use of a conjugate or a composition as defined above for the treatment, imaging and/or diagnosis of neurological pathologies such as, but not limited to, epilepsy, migraine, encephalitis, CNS pain, etc.

The invention also relates to the use of a conjugate or a composition as defined above for the treatment, imaging and/or diagnosis of neuropsychiatric pathologies such as, but not limited to, depression, autism, anxiety, schizophrenia, etc.

The terms "treatment," "treating," and other similar expressions mean achieving a pharmacological and/or physiological effect, for example, inhibition of cancer cell growth, cancer cell death, or amelioration of cancer cells, a disease or neurological disorder. The effect may be prophylactic or preventative in order to totally or partially prevent the aggravation of a disease or symptom thereof, in a sick person, or its spread, in healthy subjects, and/or may be therapeutic in order to treat totally or partially a disease and/or its related harmful effects. The term "treatment" as used herein includes any treatment of a disease in a mammal, and more particularly a human, and includes: (a) the prevention of a disease (for example, the prevention of cancer) or a condition that may occur in a person who is prone to this condition or disorder, but has not yet been diagnosed as having it, (b) slowing down a disease (for example, stopping its development) , or (c) the relief of a disease (for example, by reducing the symptoms associated with a disease). This term "treatment" also includes any administration of an active substance to treat, cure, relieve, ameliorate, diminish or inhibit a condition in an individual or patient, including but not limited to, administering to a person in need of a drug composed of a vector or conjugate as described herein.

The invention is further described by the following non-limiting examples which further illustrate the invention, and are not intended to, nor should they be interpreted to, limit the scope of the invention.

EXAMPLES

The present invention will now be further exemplified with reference to the following examples. The present invention is in no way limited to the given examples or to the embodiments presented in the figures.

EXAMPLE 1

The human LDLR is a transmembrane protein composed of 839 amino acids (Ala^{22}-Ala^{860}) whose the N-terminal region spans almost the entire molecule (Ala^{22}-Arg^{788}) and is extracellular. The seven Cys-rich type A repeats (R1-R7) of the extracellular domain (ED) are responsible for ligand binding, i.e., ApoB100 and ApoE-comprising lipoprotein particles.

Selection of the LDLR-targeted peptides

A randomized library of linear dodecapeptides (Ph.D.-12, New England Biolabs Inc., Bioké, Leiden, The Netherlands) expressed at the N-terminus of pIII minor coat protein of M13 bacteriophage was screened against the ED-LDLR (Ala^{22}-Arg^{788}) (Recombinant Human LDLR, R&D Systems, Abingdon, Oxon, UK). Three rounds of selection were performed to obtain a pool of phages with an increasing affinity to the target. This selection of the hits was based on (a) the K^*_d; (b) the IC_{50} of natural ligands; and (c) the influence of pH and calcium on ED-LDLR binding. These evaluations were specific to the target.

For this selection procedure, a biopanning assay was performed in which the target was immobilized in a well of a 96-well plate at a concentration of 25 µg/ml in $NaHCO_3$ 0.1M, pH 8.6 (immobilization buffer). After overnight incubation at 4°C and elimination of the target solution, this well and another one, used for negative selection, were blocked for 2 hours at 4°C using immobilization buffer completed with 0.5 % of Bovine Serum Albumin (BSA). Wells were emptied and rinsed 6 times with TBSC-T (Tris-HCl 50 mM, NaCl 150 mM, $CaCl_2$ 2 mM, pH 7.4, completed with Tween-20 at the concentration of the round, see below). Non-specific phages were removed by the library pre-incubation ($2x10^{11}$ phages in 200µl of TBSC-T) with the BSA-coated well for 60 minutes before the transfer of the solution in the ED-LDLR-coated well for 120 minutes. In order to enhance the selection pressure, the conditions of incubation were modified gradually at each panning round: (i) Tween-20 concentration was increased from 0.1% for the 1st round to 0.3% for the 2nd round and 0.5% for the 3rd round; (ii) the incubation times of the library were increased for the BSA-coated well (90 and 120 minutes respectively) and decreased for the ED-LDLR-coated well (90 and 60 minutes respectively).

The phages-containing solution was discarded, and the well was rinsed 10 times with TBSC-T. Phages bound to the target were eluted by addition of glycine-HCl buffer 0.2 M (pH 2.2) completed with 0.1% BSA and stirring for 20 minutes. The output

was recovered in a tube and the acidic solution was neutralized by Tris-HCl buffer 1M ($C_4H_{11}NO_3$ · HCl 1M, pH 9.1).

After each biopanning, phages were amplified in *E. coli* ER2738 (New England Biolabs). Phage Outputs were added to a bacteria culture diluted 1:100 from an overnight culture (LB 25g/L, Luria Bertani Broth Miller, Sigma-Aldrich, Bornem, Belgium; completed with 40 µg/mL of tetracyclin [stock prepared at 200 mg in 50 mL of ethanol 50%]). After 4.5 hours of stirring (160 rpm) at 37°C, the culture was centrifuged (15 minutes, 10000 rpm, 4°C) and the supernatant was added to Polyethylen Glycol 8000 20% - NaCl 2.5 M (1/6 of the supernatant volume) to precipitate phages overnight at 4°C. The solution was centrifuged (20 minutes, 10000 rpm, 4°C) allowing to obtain a pellet containing phages, this one being resuspended in 1mL of TBS (Tris-HCl 50mM, NaCl 150 mM, pH 7.5) and transferred in a tube before a new centrifugation (5 minutes, 7000 rpm, 4°C). The supernatant was added to 1/6 of PEG-NaCl and incubated on ice for one hour. Once more, the solution was centrifuged (10 minutes, 14000 rpm, 4°C) before resuspending the pellet in 20-200µL of TBS (depending on the pellet size). A final centrifugation (1 minute) allowed to obtain the amplified Output in the supernatant. This one was used for the subsequent biopanning.

The third Output (not amplified) was used for the isolation and amplification of 50 individual clones. For this purpose, a culture of *E. coli* at mid-log (optical density read at 600nm [OD_{600}] around 0.5) was incubated for 5 minutes with dilutions of the output (10^1 to 10^5 in LB). Then, the solutions were transferred to agarose (25g/mL LB, $MgCl_2 \cdot 6H_2O$ 5mM, 7g/L agarose) maintained at 45°C, quickly mixed and spread on LB/agar/IPTG/XGal petri dishes (1L of LB supplemented with 15g of agar and 1mL of IPTG/XGal solution [1.25g isopropyl-beta-d-thiogalactoside IPTG, 1g 5-bromo-4-chloro-3-indolyl-beta-d-galactopyranoside XGal, 25mL dimethylformamide DMF]). After overnight incubation (upside down, maximum 18 hours), 50 isolated blue colonies were collected and amplified in microculture plates. Each colony was placed in one well containing 1.5 mL of *E. coli* overnight culture diluted 1:100 in LB and plates were stirred for 4.5 hours at 37°C (600 rpm). Plates were centrifuged (20 minutes, 3000 rpm, 4°C) and the supernatant of each well was transferred in tubes containing PEG-NaCl for overnight precipitation. After centrifugation (10 minutes, 14000 rpm, 4°C), the pellets were resuspended in TBS/glycerol (1:1) for long storage at -20°C.

The selection of the hits among the 50 isolated clones was performed by the evaluation of their affinity, and thus specific binding to the target (ED-LDLR) as

compared to the non-specific binding to BSA, using an ELISA assay (Fig. 11, 1A and 1B). The target was immobilized at 10µg/mL in immobilization buffer (one well per clone + one blank) and wells were blocked (all LDLR-coated wells and an equal number of empty wells) as previously described. After each incubation step, the wells were rinsed 3 times with TBSC-T (0.5% Tween-20) using an automatic microplate washer (Beckman Coulter, Analis, Suarlée, Belgium).

Each clone was then incubated with LDLR and BSA-coated wells at a concentration of $5x10^{11}$ phages in 100µl of buffer for 2 hours under stirring (350 rpm). The blank was incubated with the buffer. Then the HRP-conjugated monoclonal anti-M13 antibody (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) was incubated for 1 hour (dilution 1:5000 in TBSC with 0.5% BSA) to detect bound phages. The revelation was performed by the addition of ABTS-H_2O_2 0.05% solution (22mg 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid diammonium salt], ABTS, in 100mL sodium citrate 50mM pH 4). The OD_{405} (differential filter: 630nm) was measured using a microplate reader (StatFax-2100, Awareness Technology, Fisher Bioblock Scientific, Tournai, Belgium).

The same protocol is used below for the evaluation of the apparent dissociation constant (K^*_d) using a range of 10 dilutions (1:2) of selected clones, starting at 2 x 10^{12} phages.

Among the 50 clones isolated from the 3rd pool of phages, 25 clones presenting a specific binding ≥ to the mean (7.93 ± 3.32) were selected as hit candidates.

The DNA of each selected clone was extracted and purified using the phenol/chloroform method. The sequencing was performed (Beckman Coulter Genomics, Grenoble, France) using a 20-base primer (5´- CCC TCA TAG TTA GCG TAA CG –3´), named SEQ ID 13, complementary to a sequence located 96 bp downstream of the inserted sequence. DNA sequences were analysed to identify the sequence coding for the inserted peptide located between the pIII leader sequence and the N-terminus of the mature pIII protein of M13 bacteriophage (flanking sequences: CACTCT – X – GGTGGAGGTTCG) before translating it into the coded amino acid sequence (JaMBW 1.1 software, http://bioinformatics.org/JaMBW).

The 25 hit clones revealed 12 different peptide sequences (Table 1). Seven peptides (expressed by 18 clones) have a probability to be expressed (P) of > 90%, meaning that their selection may have been promoted by their high representation in the library.

Table 1. Amino acid sequences obtained from the 25 selected clones and their probability to be expressed in the phage display library (P), and their corresponding SEQ ID. Consensus motifs are underlined, whereas Cys pairs are shown in bold

SEQ ID	Clones	Sequences	P (k>0) (%)
1	1	GHIPT**C**LTPM**C**R	40.9
2	9	HSIRDGFRSTPV	99.9
3	10	TGQTVTGLSYIF	99.4
4	16	KVVSLSALQSMT	100
5	21	WTSQPHLQHVDD	91.5
6	17, 23	KVWSLVNQGGQF	39.3
7	2, 7, 8, 12, 14, 18, 22, 24, 35, 36, 44, 48	AHLPTSMLKGQG	99.9
8	38	GHLAVNMPRASL	100
9	40	HHTG**C**LSPLS**C**S	99.9
10	34, 41	YHFNG**C**EDPL**C**R	6.1
11	42	HWKVTTWNSSTV	89.8
12	47	HPW**CC**GLRLDLR	38.3

The analysis of these peptide sequences shows different consensus motifs (GH, PT, QGGQ, KV) as well as Cys pairs present in 4 sequences, probably participating in the tridimensional conformation of peptides through disulfide bridge constrains. It is known that LDLR, ApoE and ApoB present intramolecular disulfide bonds, which are crucial for molecule stabilization and ligand binding (LDLR), or for dimerization (ApoE), assembly and secretion of hepatic lipoproteins (ApoB). Moreover, the analysis of amino acid frequencies (Fig. 1C) reveals that three amino acids are more frequent (L, S and G) with a percentage above the mean ± SD. Leu and Gly are important in the tertiary conformation of proteins, the side chain of Leu being relatively rigid whereas Gly allows a high flexibility.

The apparent dissociation constants (K^*_d) of the clones representing these 12 sequences (one clone per sequence) were evaluated, using the same protocol as discussed above for evaluation of binding to ED-LDLR of the 50 isolated phage clones (Fig. 2A). Based on these results, 6 clones (clones 1, 36, 38, 40, 41, 47, highlighted in light gray in Fig. 2A) were selected for further characterizations, their K^*_d being in the order of 10^{-10} – 10^{-12} M.

The following selection of the hits was based on their ability to bind the target in presence of the natural ligands of LDLR, ApoB and ApoE respectively. The 50%

inhibitory concentration (IC_{50}) of ApoB and ApoE reflects the concentration of competitor required to block 50% of the clone's binding. The IC_{50} value is thus directly proportional to the strength of the clone's binding to ED-LDLR, meaning that a high concentration of competitor is needed to destabilize the clone from its binding site.

For this evaluation, the IC_{50} of natural ligands (ApoB and ApoE, Abcam, Cambridge, UK) was determined using a constant concentration of phages (corresponding to the K^*_d, prepared at 2x K^*_d in TBSC-Tween 0.5%) and a range of 8 dilutions (1:4) of competitors starting at 5 nM in TBSC complemented with 0.1% BSA and 0.5% Tween-20. LDLR-coated wells were pre-incubated with 50 µL of competitors for 30 minutes (ambient temperature, 350 rpm) before adding 50 µL of phages in each well and incubating for 1.5 hour. Blanks were incubated with TBSC-T whereas the non-inhibited binding was determined by the incubation of the target with phages (pre-incubation with TBSC-T). The detection of bound phages was performed as previously described.

The ratios IC_{50}/ K^*_d (Table 2) indicate the power of inhibition of these natural ligands. Clones 1 and 36 are highly destabilized by competitors, whereas the clones 38, 40 and 47 seem to be more stable, e.g. the binding of the clone 40 is inhibited at 50% in presence of ApoE at a concentration 1537-fold higher than the K^*_d of this clone. Clones 40 and 47 were thus selected because of the highest ratios. Furthermore, we arbitrarily decided to select the clone 41 because of its lower K^*_d than the clone 38, even if ratios IC_{50}/ K^*_d show that this clone may be destabilized by ApoB and ApoE.

Table 2. K^*_d of selected clones, IC_{50} of ApoB and ApoE and ratios IC_{50}/ K^*_d, reflecting the inhibitory effects of ApoB and ApoE on the clones' binding. The IC_{50}/ K^*_d value is directly proportional to the strength of the clones' binding to ED-LDLR

Clones	K^*_d	IC_{50} ApoB	IC_{50} ApoE	Ratio ApoB	Ratio ApoE
1	$1{,}36 \times 10^{-10}$	$6{,}23 \times 10^{-19}$	$1{,}67 \times 10^{-20}$	$4{,}55 \times 10^{-9}$	$1{,}22 \times 10^{-10}$
36	$2{,}23 \times 10^{-11}$	$8{,}31 \times 10^{-19}$	$1{,}71 \times 10^{-8}$	$3{,}71 \times 10^{-8}$	763,97
38	$2{,}41 \times 10^{-10}$	$3{,}49 \times 10^{-9}$	$2{,}57 \times 10^{-9}$	14,48	10,66
40	$2{,}17 \times 10^{-12}$	$2{,}95 \times 10^{-9}$	$3{,}33 \times 10^{-9}$	1361,51	1536,90
41	$7{,}12 \times 10^{-11}$	$2{,}32 \times 10^{-11}$	$1{,}90 \times 10^{-10}$	0,33	2,67
47	$8{,}73 \times 10^{-11}$	$3{,}54 \times 10^{-9}$	$1{,}26 \times 10^{-7}$	40,54	1443,01

The final selection was performed by the evaluation of the clones' behavior in absence of calcium and at acidic pH, following a protocol adapted from Huang et al.

(2010) Indeed, the calcium is necessary for the binding of ApoB and ApoE to the receptor, while a modification of pH induces different conformations of the receptor, an acidic pH in endosomes being responsible for the dissociation of ligands from the LDLR.

Concerning the influence of pH, after the blocking step, LDLR coated wells were incubated with TBSC 3 x 10 minutes before the incubation with dilutions of phages (range of the K^*_d) for 2 hours (ambient temperature, 350 rpm). After discarding the phage solutions and rinsing the plate, wells were incubated with TBSC (pH 5 or 6, supplemented with 0.5% BSA) for 30 minutes under stirring. The detection of bound phages was performed as previously described.

Concerning the influence of calcium, the rinsing buffer was TBSM-T (Tris-HCl 20 mM, NaCl 100 mM, pH 8, supplemented with 0.5% Tween-20) free of calcium. Phages were diluted (range of the K^*_d) in TBSM supplemented with EDTA 20 mM and 1% BSA (TBSM-EDTA-BSA). After the blocking step, wells were incubated 3 x 10 minutes with TBSM-EDTA-BSA before the incubation with phages (2 hours, ambient temperature, 350rpm). The anti-M13 antibody was prepared in TBS completed with 0.5% BSA.

The K^*_d of clones 40, 41 and 47 at acidic pH and in absence of calcium are shown in Fig. 2B, 2C and 2D respectively. We observe that their K^*_d value increases in these conditions, revealing the dissociation from ED-LDLR. In order to quantify the effect of these modifications, the ratios between these values and the K^*_d in normal conditions were calculated (Fig. 2E, 2F and 2G), a high ratio reflecting a high inhibition of the binding in modified conditions. All three clones lose affinity at pH 6.0 characteristic to endosomes, the clones 41 and 47 being mostly affected.

These results suggest that peptides could dissociate from LDLR once inside the endosomes, the same as LDL particles. Moreover, the absence of calcium seems to decrease the binding of our clones to the target, similarly to the natural ligands, suggesting their binding to the same epitope.

Taken together, the clones 41 and 47 seem to be the most promising because of their interesting characteristics and their peptides were synthetized. They show low K^*_d (7.12×10^{-11} and 8.73×10^{-11} M respectively), are relatively stable against natural ligands (mainly the clone 47), whereas acidic pH as well as the absence of calcium promote their dissociation from LDLR. Even if the clone 40 seems promising too, the lower binding destabilization at pH 6.0 suggests that this peptide could not be able to detach the receptor in endosomes and would instead return to the plasma membrane together with LDLR.

Analysis of selected peptides

By facility, the peptide carried by clone 41 (**YHFNGCEDPLCR**) was named LRPep1 (LDL Receptor-Peptide 1), whereas the one carried by clone 47 (**HPWCCGLRLDLR**) was named LRPep2 (LDL Receptor-Peptide 2). Their probability (P) to be expressed in the phage display library is of 6.1% and 38.3% respectively, which could be considered low compared to other identified sequences with P > 90%. This suggests that these peptides were mostly selected due to their affinity to the LDLR and not because of their high frequency in the library.

Their sequences present some interesting amino acids and shared motifs. They both present a pair of Cys, supporting the hypothesis that these peptides bind the LDLR by a mechanism that may be similar to that of natural ligands, as already suggested above. The presence of Pro and Leu also suggests the importance of the tertiary structure in the binding of peptides to the LDLR. Moreover, the peptide LRPep2 presents the pattern "Leu-Arg" in two copies, whereas the peptide LRPep1 shows this motif separated by a Cys. Interestingly, this pattern is present 32 times in ApoB100 and 6 times in ApoE, while Arg residues are involved in the binding of ApoB and ApoE to LDLR, the mutations in these residues being responsible for the loss of affinity.

The binding of LRPep1 and LRPep2 to ED-LDLR has been then investigated using the HPEPDOCK web server program (http://huanglab.phys.hust.edu.cn/hpepdock/). This program allows the blind docking of peptides to proteins, by employing a docking algorithm which considers that linear peptides can adopt a wide range of spatial conformations. Among the generated docking models, 10 of them are proposed as the top binding prediction models. The crystallographic structure of ED-LDLR can be either uploaded as a PDB file or is provided by the server after introducing the sequence in a FASTA format or the PDB ID of the protein, i.e., 3M0C chain C for the sequence 4-788 of LDLR. The quality of docking is evaluated based on the root-mean-square deviation (RMSD) that takes into account the atoms of the peptide and protein residues located within 10 Å of distance. A successful docking prediction is indicated by a RMSD ≤ 2.0 Å.

The results shown in Fig. 3 predict that LRPep1 binds to the linker between R4 and R5 of LDLR, whereas LRPep2 is docked at the interface between R4 and the β-propeller (βP) domain of ED-LDLR, which is much closer to the binding mechanism of LDL. At neutral pH, negatively charged residues of R1-R7 (but mainly R4 and R5) in ED-LDLR interact with positively charged regions in LDL, this interaction being furthermore stabilized by the Trp and His residues in βP domain. At acidic pH, the loss of Ca^{2+} ions (that stabilize the loops in R1-R7 together with disulfide bridges)

promotes the LDL release, in addition to the repulsive forces developed by positive charges acquired in these conditions by His^{562} and His^{568} in βP. The interaction of R4 and R5 with βP triggers moreover the allosteric release of LDL. As our studies have shown above, LRPep1 and mainly LRPep2 dissociate from ED-LDLR at low pH and in the absence of Ca^{2+}, which pleads for a binding/release from the receptor in a similar manner as the LDL.

In vitro evaluation of the endocytosis of peptides LRPep1 and LRPep2

ACBRI376, HepaRG, HUVEC, N18(H) and 1321N1 cell lines, used in the protocols of subsequent analyses, were cultured under following conditions:
ACBRI376 cells (primary human brain microvascular endothelial cells) were cultured in complete CSC medium (Cell Systems, Kirkland, WA, USA) supplemented with 1% Antibiotic-Antimycotic (Fisher Scientific, Brussels, Belgium) and 2% CultureBoost (Cell Systems). HepaRG (hepatocytes cell line) were maintained in William'E medium supplemented with 10% FBS, 13% Thaw, Plate & General Purpose Medium Supplement and 1% GlutaMAX (all from Fisher Scientific). HUVEC (human umbilical vein endothelial cells) were cultured in MCDB131 medium supplemented with 20% FBS, 1% L-glutamine, 1% antibiotic-antimycotic and 0.14% heparin 5000 U/mL (all from Fisher Scientific). N18(H) (neuroblastoma cell line) and 1321N1 (astrocytoma cell line) cells were cultured in DMEM (4.5g/L glucose, L-glutamine, sodium pyruvate) supplemented with 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin 1% for N18(H) or 2% for 1321N1 (all from Fisher Scientific). Experiments on N18(H) cells were performed after differentiation. Cells were immobilized on the appropriate support and the differentiation was induced the second day using medium containing 0.2% FBS for 48h.

In order to evaluate the potential of LDLR-targeted peptides to penetrate cells, the endocytosis of peptides coupled to rhodamine (LRPep1-rho and LRPep2-rho) was evaluated in a first stage on HUVEC and HepaRG at 200µM (Fig. 4A), and in a second stage on ACBRI376 and 1321N1 at 25µM (Fig. 4B). The endocytosis of peptides and the expression of LDLR were semi-quantitatively evaluated by measuring the fluorescent labeling of cells (endocytosis of peptides: Fig. 4C and 4E; LDLR expression: Fig. 4D and 4F, Supplementary Fig. 12). For this, following protocol was used:
Cells were seeded onto coverslips coated with collagen (0.2mg/mL, Sigma-Aldrich, Overijse, Belgium) at a density of $8x10^5$ cells/well and grown for 3 days. Then, they were incubated for 2h at 37°C in the dark with LRPep1-rho or LRPep2-rho (200 µM

in culture medium for HepaRG and HUVEC, 25µM for ACBRI376 and 1321N1); the negative control was incubated with culture medium. Cells were rinsed two times with PBS. The Hoechst solution (Hoechst 33342 trihydrochloride, Fisher Scientific) prepared at 2µg/mL in HBSS (per liter: 0.140g $CaCl_2$, 0.1g $MgCl_2 \cdot 6H_2O$, 0.4g KCl, 0.06g KH_2PO_4, 0.35g $NaHCO_3$, 8g NaCl, 0.121g $Na_2HPO_4 \cdot 12H_2O$, pH 7.4) was incubated for 5 minutes to stain nuclei. Cells were rinsed two times and mounted with HBSS. Fluorescence was observed using a Leica DM2000 microscope equipped with a light source EL 6000 and a DFC 425C camera (Leica Microsystems, Groot Bijgaarden, Belgium).

With the aim to select the most promising peptide, we calculated the correlation coefficients between the endocytosis of peptides and the LDLR expression (Fig. 4G). LDLR expression was determined using following protocol:

Cells seeded onto coverslips were rinsed two times with PBS and fixed using 4% buffered paraformaldehyde for 15 minutes. Then, cells were permeabilized with methanol 100% for 10 minutes at -20°C. Between each step, cells were rinsed two times with PBS (per liter: 8 g NaCl, 0.2 g KCl, 2.31 g $Na_2HPO_4 \cdot 12\ H_2O$, 0.2 g KH_2PO_4, pH 7.4). Finally, cells were blocked with PBS supplemented with 5% normal goat serum (NGS, Cell Signaling Technology, BIOKE, Leiden, The Netherlands) and 0.3% Triton X-100 (Sigma-Aldrich). LDLR was detected using the anti-LDLR antibody made in rabbit (Thermo Fisher Scientific, Erembodegem, Belgium) incubated overnight at 7µg/mL in PBS and the anti-rabbit IgG made in goat coupled to Texas Red (Vector Labconsult, Brussels, Belgium) incubated 1h at 20µg/mL in phosphate buffer ($Na_2HPO_4 \cdot 12\ H_2O$ 10mM, $NaH_2PO_4 \cdot H_2O$ 10 mM, NaCl 150 mM, pH 7.8) supplemented with 0.5% Bovine Serum Albumin (BSA). After a final rinsing step, they were mounted using Vectashield Mounting Medium with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI, Vector Labconsult).

For the colocalization of LRPep2-rho with LDLR (7µg/mL of anti-LDLR antibody made in rabbit) on endothelial cells, the peptide LRPep2-rho was incubated at 25µM during the incubation with the secondary antibody (20µg/mL of anti-rabbit IgG antibody made in goat and coupled to Texas Red, Vector Labconsult).

Because the liver is the predominant organ metabolizing the cholesterol in the body, the expression of LDLR in HepaRG is higher than in HUVEC (Fig. 4D, $p < 0.05$). In parallel, we observe a better endocytosis of both peptides in HUVEC than in HepaRG, as shown by the higher Relative Ratio of Fluorescent Labelling (RRFL, Fig. 4C, LRPep1: $p < 0.01$, LRPep2: $p < 0.05$). On the other hand, a better endocytosis of both peptides was found in ACBRI376 than in 1321N1 cells (Fig. 4E, $p < 0.05$), even

if the LDLR expression in these cells shows no statistical differences (Fig. 4F). However, in all studied cell models, the endocytosis of peptide LRPep2 was more important than that of peptide LRPep1 ($p < 0.001$ in HepaRG, $p < 0.05$ in HUVEC, ACBRI376 and 1321N1).

The correlation coefficients revealed that LRPep2 endocytosis was positively correlated with LDLR expression ($r = 0.734$) contrariwise to peptide LRPep1 ($r = 0.093$). Based on these results, we concluded that peptide LRPep2 has the most promising potential to be used as vector and its evaluation has been pursued in additional experiments.

Colocalization of LRPep2 with LDLR on mouse brain slices and endothelial cells

The binding of peptide LRPep2 to LDLR expressed in mouse brain slices (79% identity to human LDLR using the Basic Local Alignment Search Tool - BLAST) was verified by immunofluorescence (Fig. 5A) by following protocol:

Slices (5 µm thickness) were obtained from the brain of a healthy NMRI mouse (Naval Medical Research Institute, Harlan, Horst, The Netherlands) fixed in 4% paraformaldehyde solution (Sigma-Aldrich, Bornem, Belgium) and paraffin embedded. Slices were rehydrated, and an antigen retrieval was performed using citrate buffer ($C_6H_5Na_3O_7 \cdot 2H_2O$ 10mM, Tween 20 0.05%, pH 6.0). Slices were rinsed with PBS (3 x 5min) and blocked for one hour with PBS supplemented with 1% BSA. After rinsing in PBS-0.1% Tween-20 and PBS, the LDLR was detected using the antibody anti-LDLR made in rabbit prepared at 3.5µg/mL in PBS and incubated overnight at 4°C. Slices were rinsed 3 times in PBS-0.1% Tween-20. Then, the peptide LRPep2-rho (10 µM) and the anti-rabbit IgG made in goat and coupled to fluorescein (5 µg/mL, Vector Labconsult) were prepared in phosphate buffer supplemented with 0.05% BSA and 0.5% Tween-20 and incubated with slices for 2 hours. After rinsing again with PBS-0.1% Tween-20, slices were mounted using Vectashield Mounting Medium with DAPI.

For the colocalization of LRPep2-rho with LDLR (7µg/mL of anti-LDLR antibody made in rabbit) on endothelial cells, the peptide LRPep2-rho was incubated at 25µM during the incubation with the secondary antibody (20µg/mL of anti-rabbit IgG antibody made in goat and coupled to Texas Red, Vector Labconsult).

The colocalization of peptide LRPep2 and LDLR was quantified using the JACoP plugin of the ImageJ software (National Institute of Health, USA), the Mander's coefficient (M) reflecting the percentage of colocalization of the peptide LRPep2 with LDLR.

Interestingly, a good colocalization between LRPep2-rho and LDLR (highlighted by fluorescein) was observed at the level of blood vessels (on merged microphotographs, M = 76.97%), corresponding to the BBB. Also, a large colocalization in the cortex (M = 89.04%, some colocalization areas are highlighted by white arrows), and in a lower proportion in the hippocampus (M = 67.95%), was observed. These results suggest the ability of LRPep2 to target LDLR expressed by cerebral cells. The binding of peptide LRPep2 to the LDLR was moreover confirmed by immunofluorescence on ACBRI376 cells (Fig. 5B), where the peptide LRPep2-rho colocalizes with the fluorescein staining of LDLR (M = 74.35%), as shown by white arrows on the figure.

In vitro study of the endocytosis mechanism of peptide LRPep2

The endocytosis mechanism of peptide LRPep2 has been studied on the cell models described above and on differentiated N18(H) neuroblastoma cell line by the colocalization of LRPep2-rho with caveolae and lysosomes. Following protocol was used:

Cells were first incubated with LRPep2-rho as described above (cf. evaluation of the endocytosis of peptides LRPep1 and LRPep2) before being fixed with paraformaldehyde 4%. Then, cells were blocked with PBS supplemented with 5% NGS and 0.3% Triton X-100. Caveolae and lysosomes were detected using anti-caveolin 1 and anti-LAMP1 antibodies respectively, made in rabbit (both from Santa Cruz, Heidelberg, Germany) and incubated overnight at 4µg/mL in PBS. Finally, cells were incubated for 1h with an anti-rabbit IgG made in goat coupled to fluorescein (Vector Labconsult) at 20µg/mL in phosphate buffer pH 7.8 supplemented with 0.5% BSA. Cells were mounted with Vectashield Mounting Medium with DAPI.

In ACBRI376 cells (Fig. 6A), the peptide LRPep2 is endocytosed by the non-degradation pathway involving caveolae, as revealed by the increased fluorescence observed for caveolin-1 ($p < 0.05$), whereas the fluorescence of lysosomes, highlighted by the detection of LAMP1, is decreased in the presence of peptide LRPep2 ($p < 0.05$). Moreover, we observe on microphotographs some large spots of caveolin-1 that colocalize with packs of rhodamine (white arrows on Fig. 13, M = 82.7%), suggesting the presence of endocytosis vesicles comprising the peptide LRPep2. The bypass of the lysosome pathway in ACBRI376 is in accordance with previous studies revealing the localization of LDLR in membrane fraction rich in caveolin, where its colocalization with the transferrin receptor is observed.

On the contrary, in HepaRG cells, a significant increase of fluorescence was observed for lysosomes (Fig. 6B, $p < 0.001$), being in accordance with the involvement of hepatocytes in the metabolism of cholesterol. Finally, both pathways seem to be borrowed by peptide LRPep2 to penetrate in N18(H) and 1321N1 cells (Fig. 6C and 6D respectively).

In vivo MRI evaluation of USPIO-LRPep2

The peptide LRPep2 was grafted to USPIO (USPIO-LRPep2) in order to evaluate by MRI its ability to cross over the BBB. Subsequent steps were followed to synthesize USPIO derivatives:

The peptide LRPep2 or the peptide NSP (non-specific peptide: HSCNKNSCT, named SEQ ID 14; both synthesized by Eurogentec, Seraing, Belgium), presenting a molecule of PEG (8-amino-3,6-dioxaoctanoyl) at their N-terminus, were covalently grafted to the carboxylic groups of the USPIO as previously described. Then a coating of PEG [O-(2-aminoethyl)-O-methyl-polyethyleneglycol, MW ~ 750g/mol, Sigma-Aldrich] was added in order to saturate free carboxyl groups. The non-conjugated peptides in USPIO suspensions were removed by extensive dialysis (MWCO: 30 kDa, Millipore, USA), whereas the concentration of peptides coupled to USPIO was determined using the Coomassie (Bradford) protein assay kit (Thermo Scientific). Based on this measurement, it has been estimated that 1-2 peptides are bound per particle by considering that each particle contains ~11.000 Fe atoms.

Using the USPIO-derivatives, the affinity of USPIO-LRPep2 for ED-LDLR was evaluated by determining its K^*_d by ELISA (Fig. 7A). The K^*_d of USPIO-LRPep2 was evaluated using a protocol similar to that used for phage clones. Briefly, after the target immobilization, wells were blocked with Protein-Free (TBS) Blocking Buffer (PFBB, Pierce, Fisher Scientific) and incubated with a range of 12 dilutions 1:1 of USPIO-LRPep2 starting at 7.36×10^{-6} M in TBSC (Tris-Buffered Saline containing Calcium: Tris-HCl 50 mM, NaCl 150 mM, $CaCl_2$ 2 mM, pH 7.4). Rinsing buffer was TBSC supplemented with 0.05% Tween-20 (TBSC-T). USPIO-LRPep2 was detected using a rabbit anti-PEG antibody at 2 µg/mL (Abcam) in TBSC-T supplemented with 0.5% BSA, a biotinylated anti-rabbit IgG made in goat at 5 µg/mL (Vector Labconsult) in phosphate buffer pH 7.8 and Vectastain ABC kit (Vector Labconsult).

The results showed a good affinity in the range of nanomolar ($K^*_d = 7.25 \times 10^{-8}$ M) and proved the ability of USPIO-LRPep2 to target LDLR. However, its lower affinity as compared to that of LRPep2 displayed by phages ($K^*_d = 8.73 \times 10^{-11}$ M) can be

attributed to different number of peptides exposed by each supramolecular entity, i.e. 1-2 peptides/USPIO and 5 peptides/phage respectively.

The ability of USPIO-LRPep2 to cross the BBB was explored first by MRI on NMRI mice injected with USPIO-LRPep2 or USPIO-NSP (grafted with a non-specific peptide, used as control). MRI experiments and contrast analysis measurement were performed using following steps:

All *in vivo* experiments are in accordance with UMONS Animal Care and Use Committee (protocol MU-10-01 for the period July 2010 – July 2014 and MU-10-02 for the period September 2014 – September 2019). Molecular imaging by MRI was performed on 6 NMRI mice (RjHan:NMRI, Janvier laboratories, St Berthevin, France) anesthetized with Nembutal 50 mg/kg b.w. (Sanofi, Brussels, Belgium) during MRI acquisitions. A small-animal monitoring and gating system was used to monitor the animal respiration rate and the body temperature was maintained at 37°C.

MRI images were acquired at the level of the head with T_2-weighted RARE (Rapid Acquisition with Relaxation Enhancement) imaging protocol (TR/TE = 3000/60 ms, RARE factor = 4, NEX = 4, matrix = 512x512, FOV = 2.5cm, slice thickness 1mm, 20 axial slices, spatial resolution = 48µm, TA = 25 min 36 sec) on a 300 MHz (7T) Bruker Pharmascan imaging system (Bruker, Ettlingen, Germany) equipped with a horizontal magnet and a circular polarized MRI transceiver coil (55 mm x 23mm, frequency of 3MHz, maximum RF of 5ms). After pre-contrast acquisitions, USPIO derivatives were injected in the tail vein (3 mice/USPIO derivative) at a dose of 200 µmol Fe/kg b.w and a follow-up until approximatively 4 hours post-injection was performed as well as an acquisition at 22h.

The contrast was analyzed using the ImageJ software. The whole brain was selected manually and the signal intensity (SI) of this region of interest (ROI) was measured on pre- (SI_{pre}) and post-contrast (SI_{post}) images. The standard deviation (SD) of the noise was measured in a region outside of the animal's head. The Percentage change of Signal-to-noise Ratio (Δ%SNR) on post-contrast images is calculated as follows:

$$\Delta\%\mathrm{SNR} = \left[\frac{(\mathrm{SI_{post}}/\mathrm{Noise\ SD}) - (\mathrm{SI_{pre}}/\mathrm{Noise\ SD})}{(\mathrm{SI_{pre}}/\mathrm{Noise\ SD})}\right] \times 100$$

A global darkening is observed on 52 min (average timing) post-contrast images of the brain of mice injected with USPIO-LRPep2, whereas mice injected with USPIO-NSP do not present this contrast (Fig. 7B, overlays in Fig. 7C). The analysis of this contrast, measured on MRI images and normalized to the noise and to the pre-contrast signal, confirms these results with the increase of the negative contrast

(Fig. 7D). A notable negative contrast is present until more than 2 hours post-injection for USPIO-LRPep2 ($p < 0.05$ at 22 minutes and 108 minutes) whereas no negative contrast is observed for USPIO-NSP. Then, the negative contrast progressively returns to the basal level and disappears after approximately 3 hours.

In vivo biodistribution of USPIO derivatives

The biodistribution of our USPIO derivatives was studied by the measurement of the transversal relaxation times (T_2) of organs collected at the optimal timing observed by MRI (55 minutes post-injection). For this, NMRI mice were injected with USPIO derivatives or with PBS (n = 3/experimental group) and euthanized 55 minutes later by injection of a lethal dose of Nembutal (500mg/kg b.w) corresponding to the optimal timing of contrast enhancement determined by MRI. The blood and the urine were harvested, and the circulatory system was rinsed with PBS by a transcardial perfusion. Then, the brains as well as the kidneys, the liver and the spleen were also collected for immunohistochemistry (IHC) and biodistribution studies. For IHC, brains were fixed in 4% paraformaldehyde, followed by dehydration in successive baths of alcohol and butanol and paraffin embedding. Plasma was isolated by centrifugation at 7000 rpm for 30 minutes. The organs, plasma and urines were conserved at -20°C before using them in biodistribution studies.

To evaluate the biodistribution of UPSIO derivatives by NMR relaxometry, organs, plasma and urine conserved at -20°C were placed in pyrex tubes for NMR analysis. Transversal relaxation time of water protons (T_2) of each sample was measured on a Minispec Mq60 analyzer (60MHz, 37°C, Bruker, Karlsruhe, Germany). The relaxation rate of each organ ($R_2 = 1/T_2$) was calculated and normalized by the subtraction of the mean R_2 of control mice ($R_2^{Norm} = R_2^{Sample} - R_2^{Control}$). For plasma and urine, the normalized R_2 of each sample was related to the r_2 (relaxivity) obtained for each USPIO derivative at 1mM in plasma or urine controls, allowing to calculate the concentration of USPIO derivatives in plasma and urines of injected mice.

The passage of USPIO derivatives in brains was also evaluated by the dosage of the iron contained in USPIO. Brains were recovered from pyrex tubes and dried in Eppendorf at 65°C for 48 hours. The weight of each sample was measured before digestion in 2 mL of HNO_3-H_2O_2 (ratio 3:1) by microwaves (2 cycles: 5 min 250W, 5 min 400W, 5 min 650W, 5 min 250W; Milestone MSL-1200, Sorisole, Italy). The longitudinal relaxation time (T_1) of each sample was measured on the Minispec Mq60 analyzer, the R_1 was calculated ($R_1=1/T_1$), and the R_1 of the blank (digestion solution) was subtracted. The iron concentration was obtained using a standard

curve of iron. Concentrations were normalized to the final volume of digestion and to the weight of each sample, and finally to control mice.

The normalized relaxation rates obtained ($R_2^{Norm} = 1/T_2^{Sample} - 1/T_2^{Control}$) are shown in the Fig. 8. Both USPIO derivatives, but mainly USPIO-NSP, are massively taken up by the spleen and the liver (Fig. 8A), these organs containing macrophages involved in the clearance of many molecules, and especially in iron recycling. A low contribution of kidneys in their elimination is observable, in accordance with the low R_2 in urine (Fig. 9B). This distribution is characteristic for USPIO. By contrast, the high plasma concentration of both USPIO derivatives confirms that they are still circulating in the blood stream (31% of the injected dose, ID, for USPIO-LRPep2 and 34% of ID for USPIO-NSP respectively) at this time after injection (Fig. 8C).

Concerning the brain, the fast ($R_{2(1)}^{Norm}$) component of the relaxation rate obtained after the biexponential fitting of the T_2 relaxation curve is higher in mice injected with USPIO-LRPep2, suggesting the crossing of the BBB (Fig. 8D). The dosage of iron in the brains of injected mice confirms these results (Fig. 8E), the concentrations being normalized to non-injected mice in order to selectively reflect the iron contained in USPIO. Interestingly, the $R_{2(1)}^{Norm}$ of USPIO-LRPep2 is not linked to the plasma concentration of USPIO-LRPep2, as shown by a low correlation coefficient ($r = 0.365$, Fig. 8F), meaning that the free fraction in the blood stream does not influence this parameter and suggesting once more the BBB crossing by USPIO-LRPep2. On the contrary, USPIO-NSP shows a high positive correlation coefficient between $R^{2(1)Norm}$ and its concentration in the plasma ($r = 0.984$, Fig. 8F), meaning that $R_{2(1)}^{Norm}$ is directly influenced by the free fraction of USPIO-NSP present in the blood stream.

Based on the iron concentrations in brains, the percentage of injected dose per gram of dried tissue (%ID/g) of present USPIO derivatives was calculated. Other groups have estimated the %ID/g of different molecules in the brain, ranging between 0.02% and 1%. Concerning present USPIO derivatives, higher %ID/g values (USPIO-LRPep2 = 13.5%; USPIO-NSP = 6.6%) are obtained. However, it is difficult to compare these results to the other ones because of the different expression of the initial injected dose, the time point analyzed, the type of the injected agent and the region of the brain. The ability to cross the BBB is also dependent to the coating used. Indeed, USPIO injected by Shanehsazzadeh et al. (2013), being the most similar contrast agent to our USPIO derivatives, are coated with dextran whereas present example used polyethylene glycol (PEG). Interestingly, even if it is generally admitted that USPIO cannot cross the BBB without functionalization, it is known that PEG facilitates brain penetration, already shown previously by our group. This

property could explain the relatively high %ID/g of USPIO-NSP. Nevertheless, the ratio USPIO-LRPep2/USPIO-NSP for %ID/g reveals that USPIO-LRPep2 is 2 times more concentrated in mouse brains than USPIO-NSP.

Detection of USPIO derivatives in mouse brains by Perls'-DAB staining

USPIO-LRPep2 and USPIO-NSP were detected on mouse brain slices by the Perls'-DAB staining, highlighting the iron present within these nanoparticles (Fig. 9). For this, brain slices (5 µm thickness) from injected mice were dewaxed, rehydrated and blocked with 1% H_2O_2 in PBS for 15 minutes. After the rinsing steps (3 x 5 minutes in distilled water), slices were incubated in Perls' solution (5% potassium ferrocyanide and 5% HCl in equal proportions) for 30 minutes. After rinsing 3 times (10 minutes) in distilled water, a solution of 0.05% 3,3'-Diaminobenzidine (DAB) tetrachlorhydrate (Sigma-Aldrich) was added for 10 minutes. Finally, the revelation was performed using 0.05% DAB supplemented with 0.033% H_2O_2 prepared in PBS, pH 7.4. After staining, slices were rinsed, counterstained using Mayer's Hemalun (VWR International, Leuven, Belgium) and Luxol Fast Blue, and mounted in a permanent medium (Leica Microsystems). Images were acquired using the Leica DM2000 microscope equipped with a DFC 425C camera.

A brown staining for mice injected with USPIO-LRPep2 was observed in different areas of brain slices (hippocampus, choroid plexus, cortex and parenchyma in the hippocampus area), whereas no staining was visible on brain slices of mice injected with USPIO-NSP. This last result, identical to that obtained for mice injected with PBS used as negative control, suggests that USPIO-NSP quantity that penetrates the brain tissue is inferior to the detection limit of this method. Moreover, the iron detected by NMR as shown above in Fig. 8 could probably be attributed to the residual USPIO-NSP present in capillaries.

In vivo fluorescence evaluation of CF770-LRPep2

Finally, in order to support the MRI results by a different in vivo imaging method, the LDLR-targeted peptide was coupled to a fluorescent dye (CF770), and the BBB crossing of CF770-LRPep2 was evaluated by Fluorescence Lifetime Imaging (FLI).

Molecular imaging by FLI was performed on 9 nude mice (NU(NCr)-Foxn1<nu>, Charles River Laboratories, L'Arbresle, France). Mice were anesthetized with 4% isoflurane in O_2 at a rate of 2 L/min then maintained with 2% isoflurane at 0.3 L/min. FLI images were acquired with the PhotonIMAGER Optima (Ex = 737 nm, Em = 797 nm, TA = 5 sec; BioSpace Lab, Nesles la Vallée, France). After pre-injection

acquisitions, CF770-LRPep2 (Biosynthesis, Lewisville, USA; n = 6 mice) and CF770 (VWR International; n = 3 mice) were injected in the tail vein at a dose of 800 nmol per kg b.w. and images were acquired at 25 minutes and 50 minutes post-injection. Mice were finally euthanized, and brains were collected (n = 3 for each compound) for ex vivo acquisitions (lens = 65 mm with f = 2.8, distance to lens = 279 mm). The fluorescence analysis was performed using the M3Vision software (BioSpace Lab). On whole mouse images, the brain area was selected, and the fluorescent signal of this ROI was measured on pre- and post-injection images in photons by second, square centimeter and steradian (ph/s/cm^2/sr). The ratio "signal post-iv/signal pre-iv" was calculated for both compounds. On *ex vivo* images, a ROI was drawn in order to select the entire signal emitted by the brain, including the signal "outside" the brains. This signal was normalized to the signal emitted by the brain of a non-injected mouse.

Control mice were injected with the dye alone. A higher fluorescence is observed in the area of the brain of mice injected with CF770-LRPep2 as compared to the dye alone (Fig. 10A, whole body images in Fig. 14 and 15), this observation being confirmed by the analysis of the fluorescence (normalized to the signal before the injection) at both studied post-injection times ($p < 0.05$, Fig. 10B). After brain collection, the signal of CF770 *ex vivo* seems to be identical to that of the non-injected mouse, whereas CF770-LRPep2 allows to observe a better fluorescence (Fig. 10C). The fluorescence measured on *ex vivo* brains and normalized to the signal of the non-injected mouse shows a difference ($p = 0.05$) between both compounds (Fig. 10D). Moreover, these compounds are massively taken by the liver (CF770-LRPep2) and the kidneys (both CF770 and CF770-LRPep2) (fig. 14 and 15), contrarily to USPIO derivatives that were taken by the liver and the spleen for iron metabolism.

Statistical analysis

The results are expressed as means ± standard deviation (SD). The statistical analysis between experimental groups was performed using One-Way ANOVA with SigmaPlot 11.0 software when data showed a normal distribution. For non-normal distribution of the data, the Mann-Whitney test (non-parametric test) was used.

Sequence IDs

SEQ ID	Sequence	Molecule
1	GHIPTCLTPMCR	Peptide
2	HSIRDGFRSTPV	Peptide
3	TGQTVTGLSYIF	Peptide
4	KVVSLSALQSMT	Peptide
5	WTSQPHLQHVDD	Peptide
6	KVWSLVNQGGQF	Peptide
7	AHLPTSMLKGQG	Peptide
8	GHLAVNMPRASL	Peptide
9	HHTGCLSPLSCS	Peptide
10	YHFNGCEDPLCR	Peptide
11	HWKVTTWNSSTV	Peptide
12	HPWCCGLRLDLR	Peptide
13	CCCTCATAGTTAGCGTAACG	Primer
14	HSCNKNSCT	Peptide

The present invention is in no way limited to the embodiments described in the example and/or shown in the figures. On the contrary, methods according to the present invention may be realized in many different ways without departing from the scope of the invention.

CLAIMS

1. A peptide capable of binding to LDLR, characterized in that said peptide has at least 90% sequence identity to an amino acid sequence chosen from SEQ ID 1 to 12.
2. A peptide capable of binding to LDLR, characterized in that said peptide has an amino acid sequence that differs maximally two amino acids from SEQ ID 1 to 12.
3. Peptide according to claim 1 or 2, characterized in that said peptide has an amino acid sequence chosen from SEQ ID 1 to 12.
4. Peptide according to any of the previous claims 1-3, characterized in that said peptide has an amino acid sequence chosen from SEQ ID 10 or SEQ ID 12.
5. A peptide capable of binding to LDLR, characterized in that said peptide has an amino acid sequence which is identical to SEQ ID 12.
6. Peptide according to any of the previous claims 1-5, characterized in that said peptide is able to bind to the extracellular domain of LDLR, preferably at the interface between R4 and the β-Propeller domain of the extracellular domain of LDLR.
7. Peptide according to claims 4-6, characterized in that said peptide is endocytosed upon binding to the extracellular domain of LDLR.
8. Peptide according to claim 4-7, characterized in that said peptide, upon binding to the LDLR is able to cross the blood-brain barrier.
9. Peptide according to any of the previous claims 1-8, characterized in that said peptide is coupled or linked to a pharmaceutical agent or compound.
10. Peptide according to claim 9, characterized in that said pharmaceutical agent or compound is considered a treatment for a brain disease or neurologic or neurodegenerative condition, or a cerebral tumor.
11. Use of a peptide according to any of the claims 1 to 10 as a vehicle for crossing the blood brain barrier.
12. A conjugate compound, characterized in that said compound comprises a peptide according to claims 1-11.
13. Peptide according to any of the previous claims 1-10 or conjugate compound according to claim 12, for use in the treatment or prevention of medical conditions or diseases which require crossing the blood-brain barrier of pharmaceutical agents or compounds.
14. Peptide or conjugate compound according to claim 13, characterized in that said condition or disease is a brain disease, neurologic, neuropsychiatric or neurodegenerative condition such as Alzheimer disease, or a cerebral tumor.

15. Peptide according to any of the previous claims or conjugate compound according to claims 12-14, for use as a diagnostic agent.

16. Pharmaceutical composition, characterized in that said composition comprises at least one conjugate compound according to any of the claims 12-15 and one or more pharmaceutically acceptable excipients.

FIGURES

FIG. 1

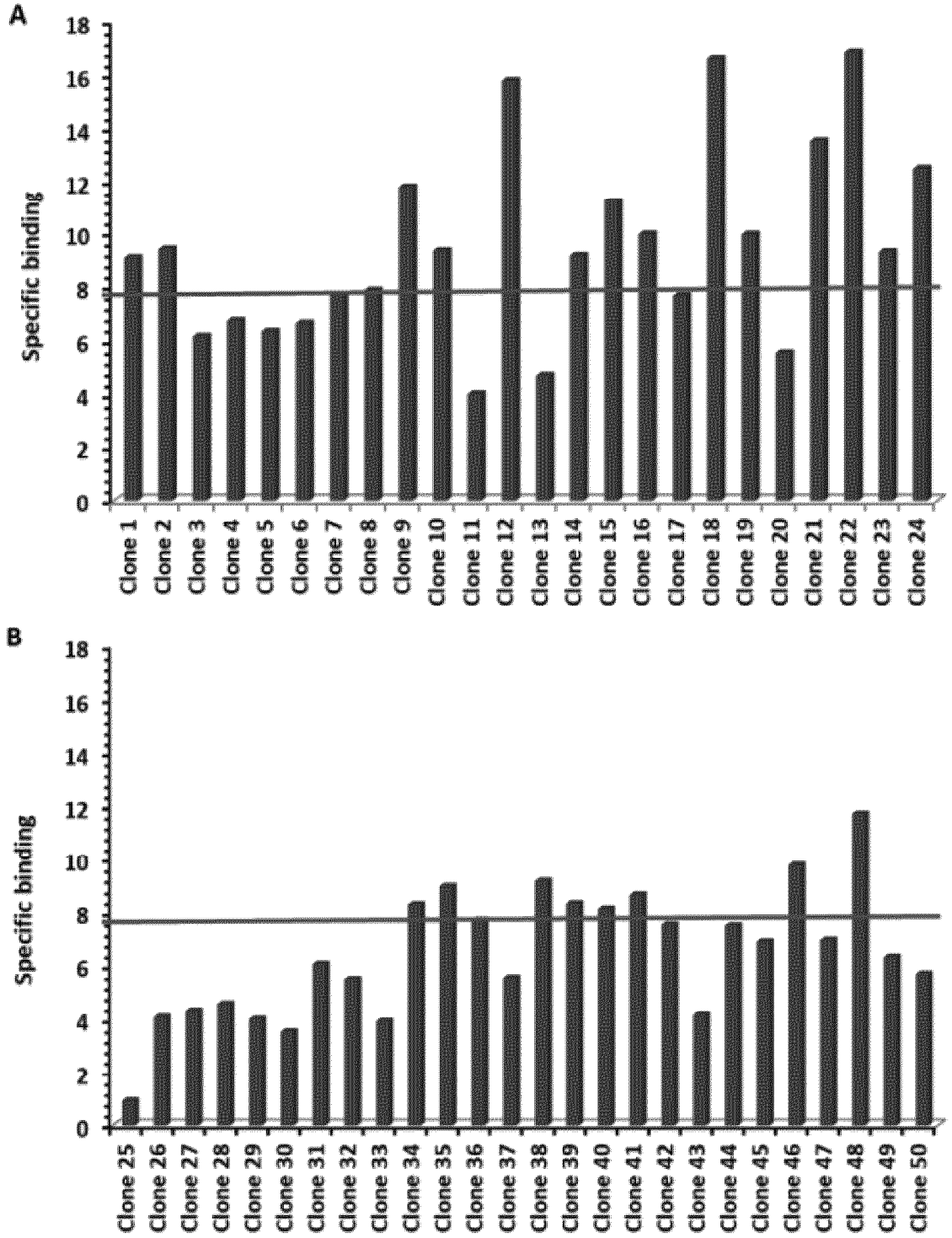


FIG. 1

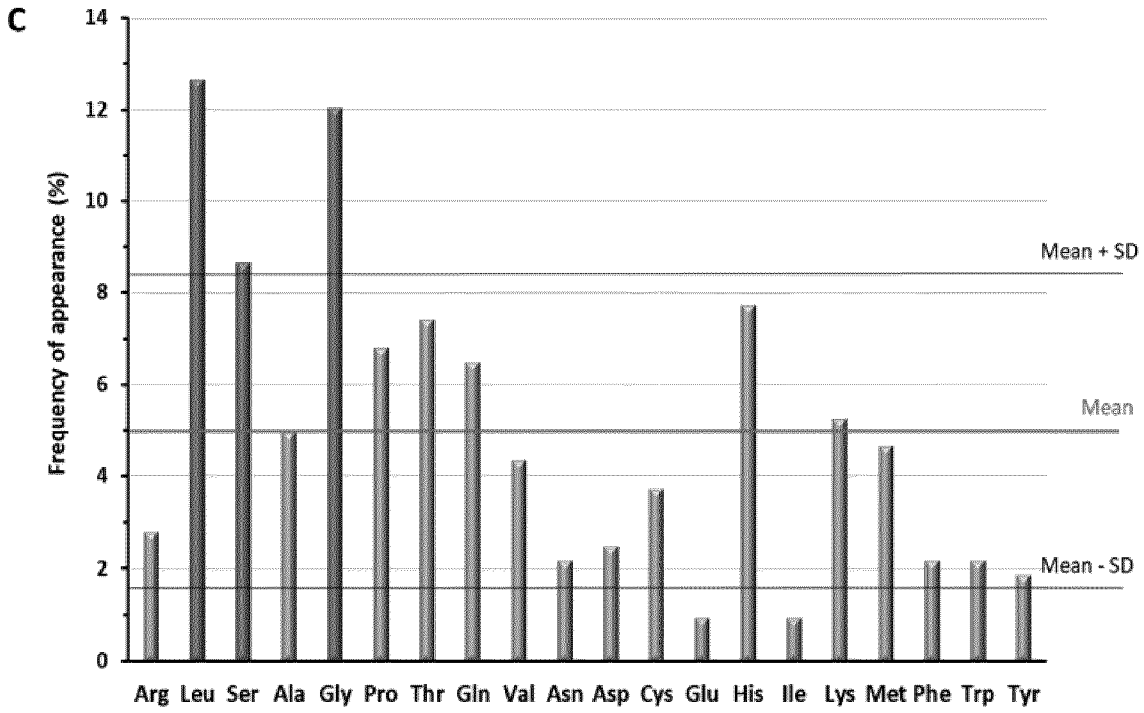

C
Frequency of appearance (%)
14
12
10
8
6
4
2
0
Mean + SD
Mean
Mean - SD
Arg Leu Ser Ala Gly Pro Thr Gln Val Asn Asp Cys Glu His Ile Lys Met Phe Trp Tyr

FIG. 2

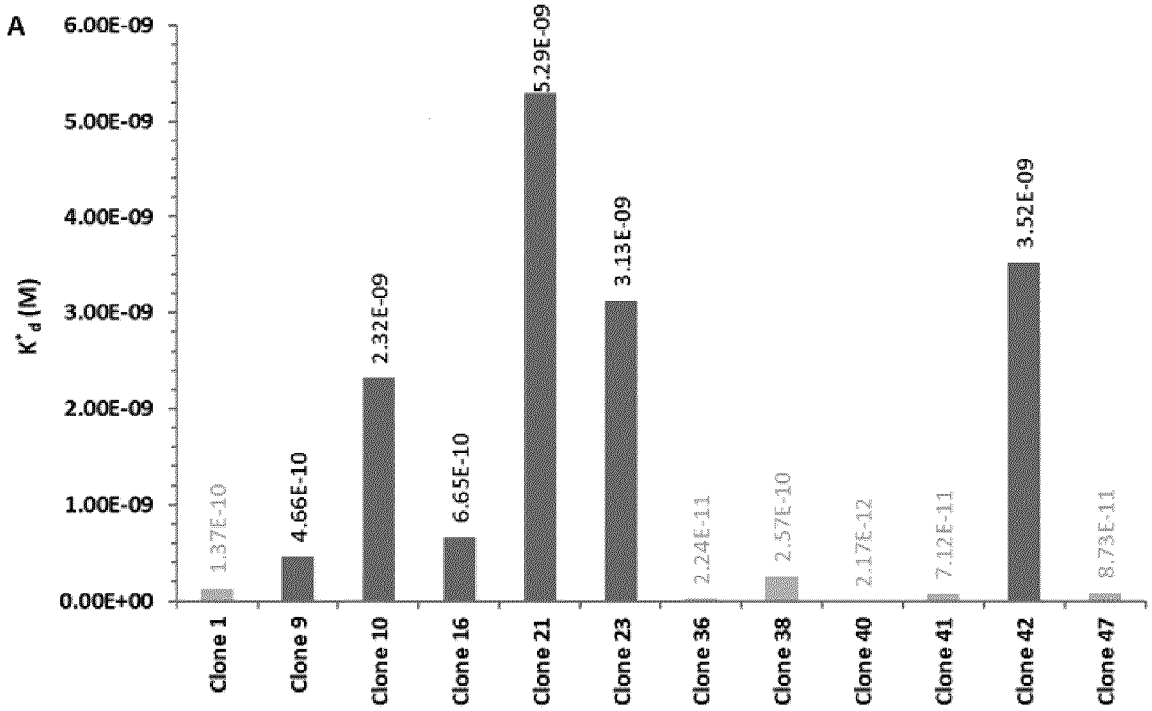

A
K*d (M)
6.00E-09
5.00E-09
4.00E-09
3.00E-09
2.00E-09
1.00E-09
0.00E+00
1.37E-10
4.66E-10
2.32E-09
6.65E-10
5.29E-09
3.13E-09
2.24E-11
2.57E-10
2.17E-12
7.12E-11
3.52E-09
8.73E-11
Clone 1
Clone 9
Clone 10
Clone 16
Clone 21
Clone 23
Clone 36
Clone 38
Clone 40
Clone 41
Clone 42
Clone 47

FIG. 2

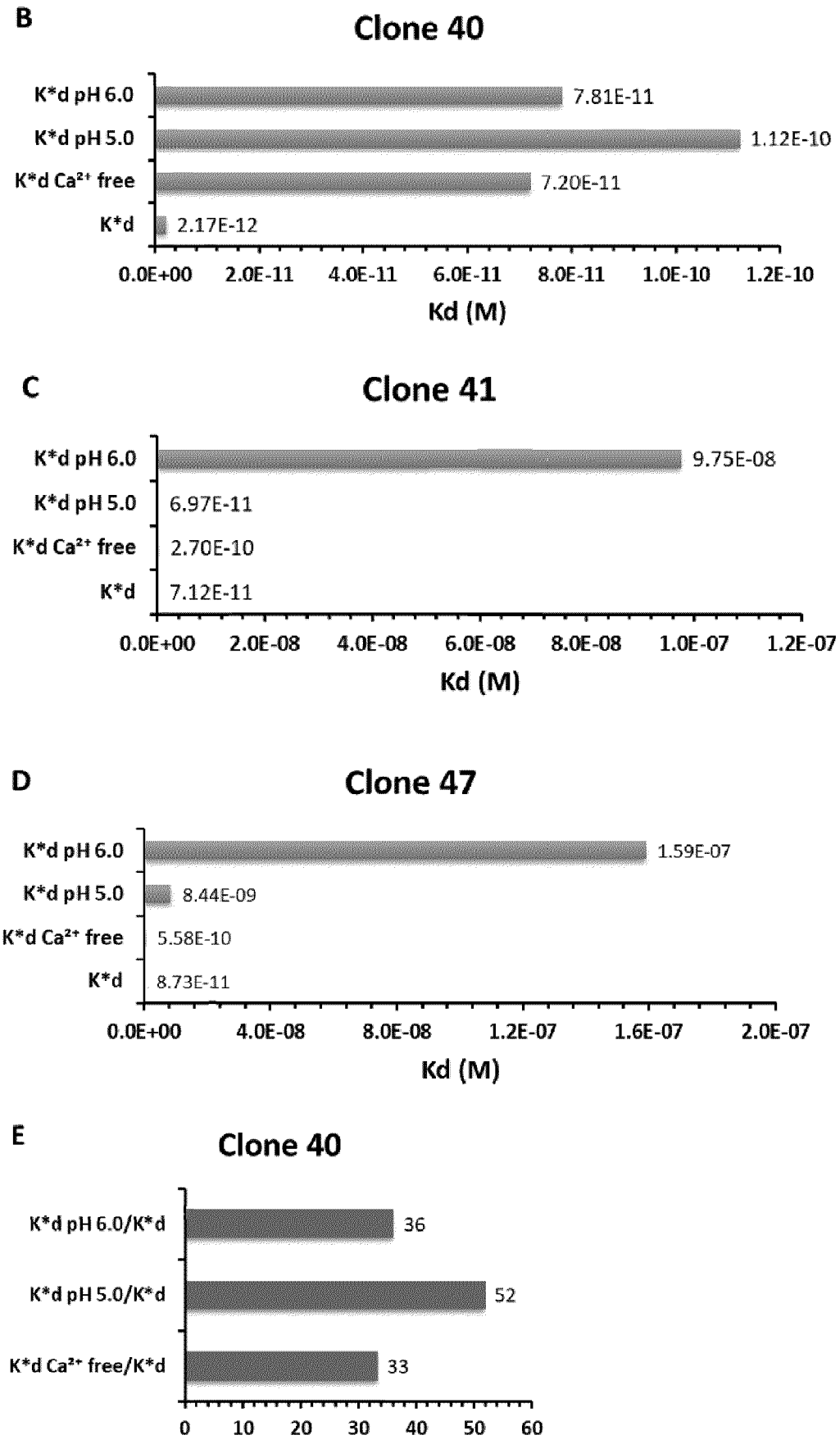
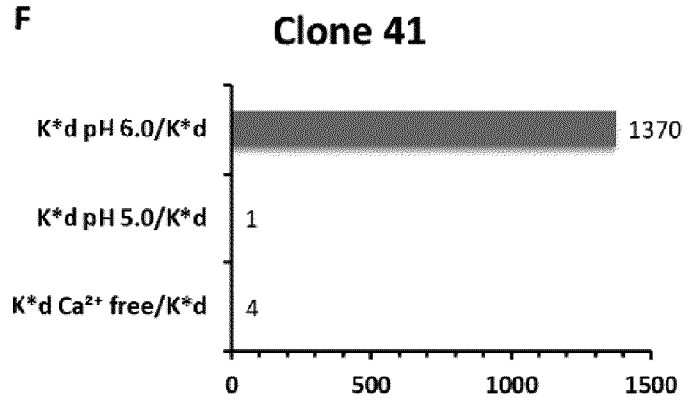

B
Clone 40
K*d pH 6.0
K*d pH 5.0
K*d Ca²⁺ free
K*d
7.81E-11
1.12E-10
7.20E-11
2.17E-12
0.0E+00
2.0E-11
4.0E-11
6.0E-11
8.0E-11
1.0E-10
1.2E-10
Kd (M)
C
Clone 41
K*d pH 6.0
K*d pH 5.0
K*d Ca²⁺ free
K*d
9.75E-08
6.97E-11
2.70E-10
7.12E-11
0.0E+00
2.0E-08
4.0E-08
6.0E-08
8.0E-08
1.0E-07
1.2E-07
Kd (M)
D
Clone 47
K*d pH 6.0
K*d pH 5.0
K*d Ca²⁺ free
K*d
1.59E-07
8.44E-09
5.58E-10
8.73E-11
0.0E+00
4.0E-08
8.0E-08
1.2E-07
1.6E-07
2.0E-07
Kd (M)
E
Clone 40
K*d pH 6.0/K*d
K*d pH 5.0/K*d
K*d Ca²⁺ free/K*d
36
52
33
0
10
20
30
40
50
60

FIG. 2

F


Clone 41
K*d pH 6.0/K*d
1370
K*d pH 5.0/K*d
1
K*d Ca²⁺ free/K*d
4
0
500
1000
1500

G

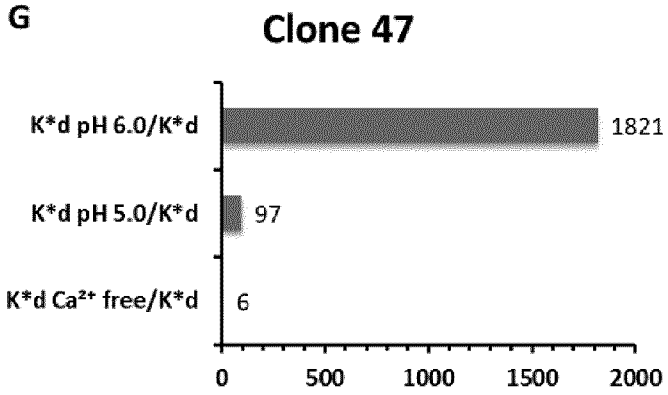

Clone 47
K*d pH 6.0/K*d
1821
K*d pH 5.0/K*d
97
K*d Ca²⁺ free/K*d
6
0
500
1000
1500
2000

FIG. 3

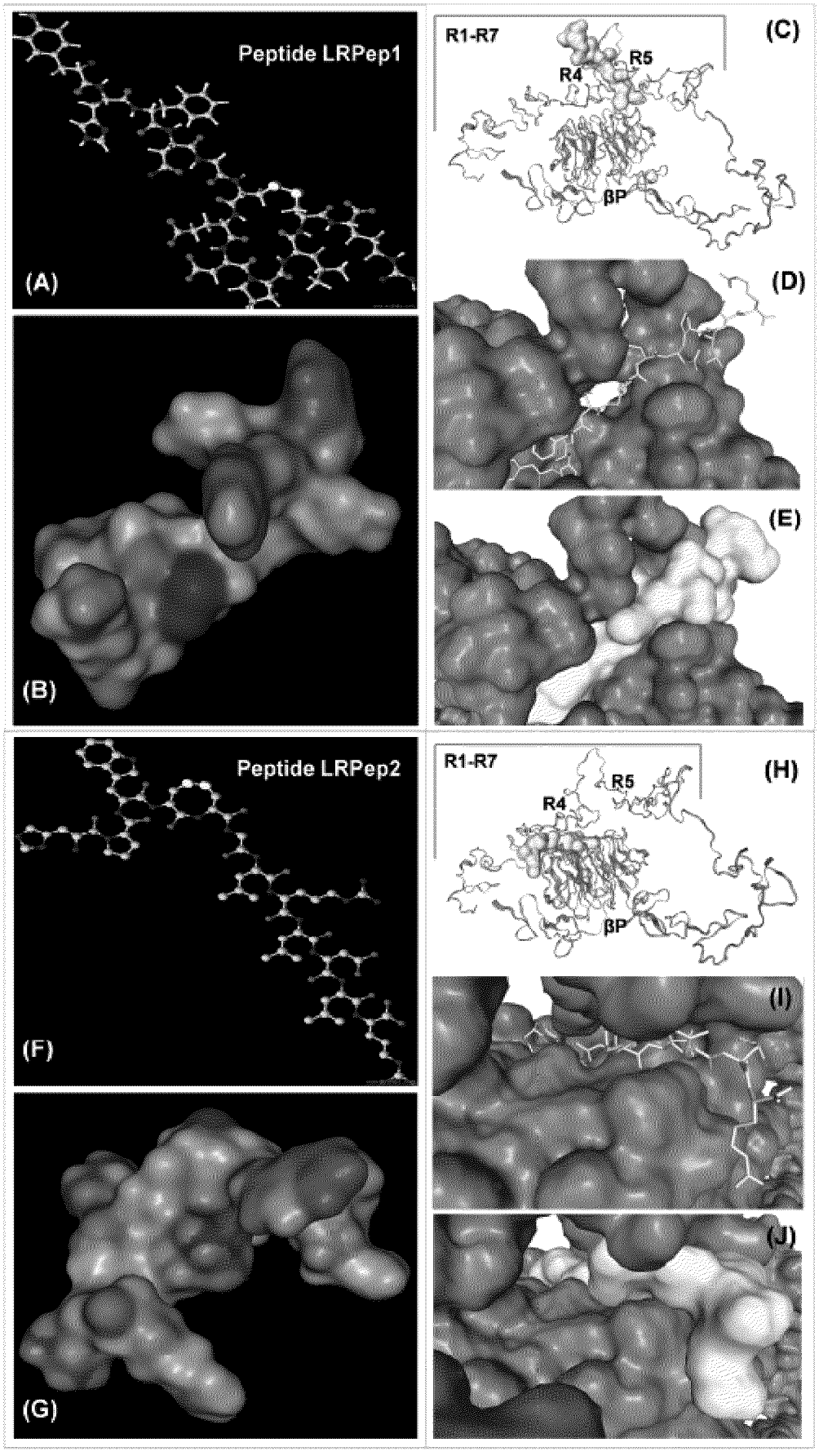
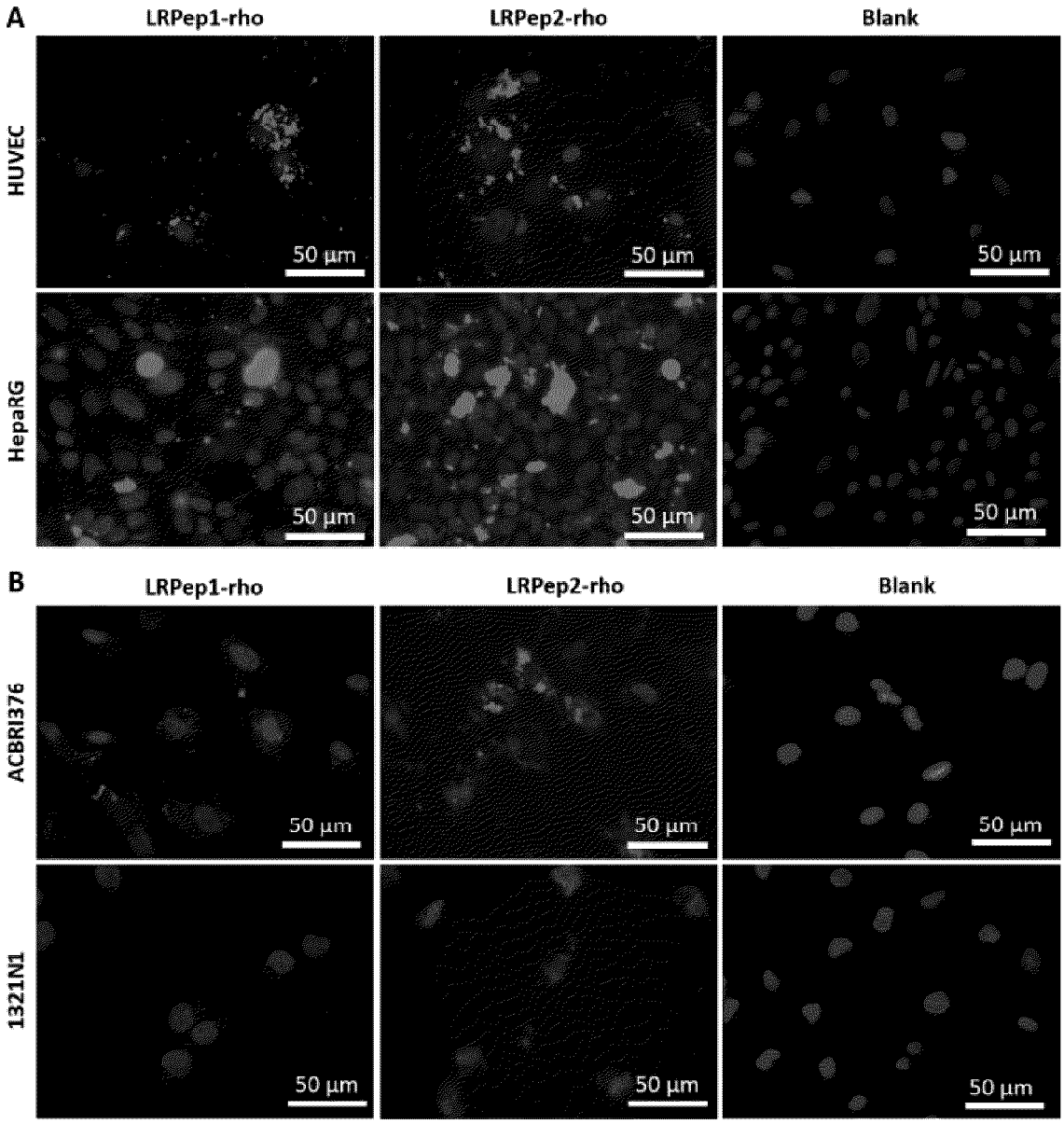

Peptide LRPep1
(A)
(B)
R1-R7
R5
R4
βP
(C)
(D)
(E)
Peptide LRPep2
(F)
(G)
R1-R7
R5
R4
βP
(H)
(I)
(J)

FIG. 4


A
LRPep1-rho
LRPep2-rho
Blank
HUVEC
HepaRG
50 µm
B
LRPep1-rho
LRPep2-rho
Blank
ACBRI376
1321N1
50 µm

FI G. 4

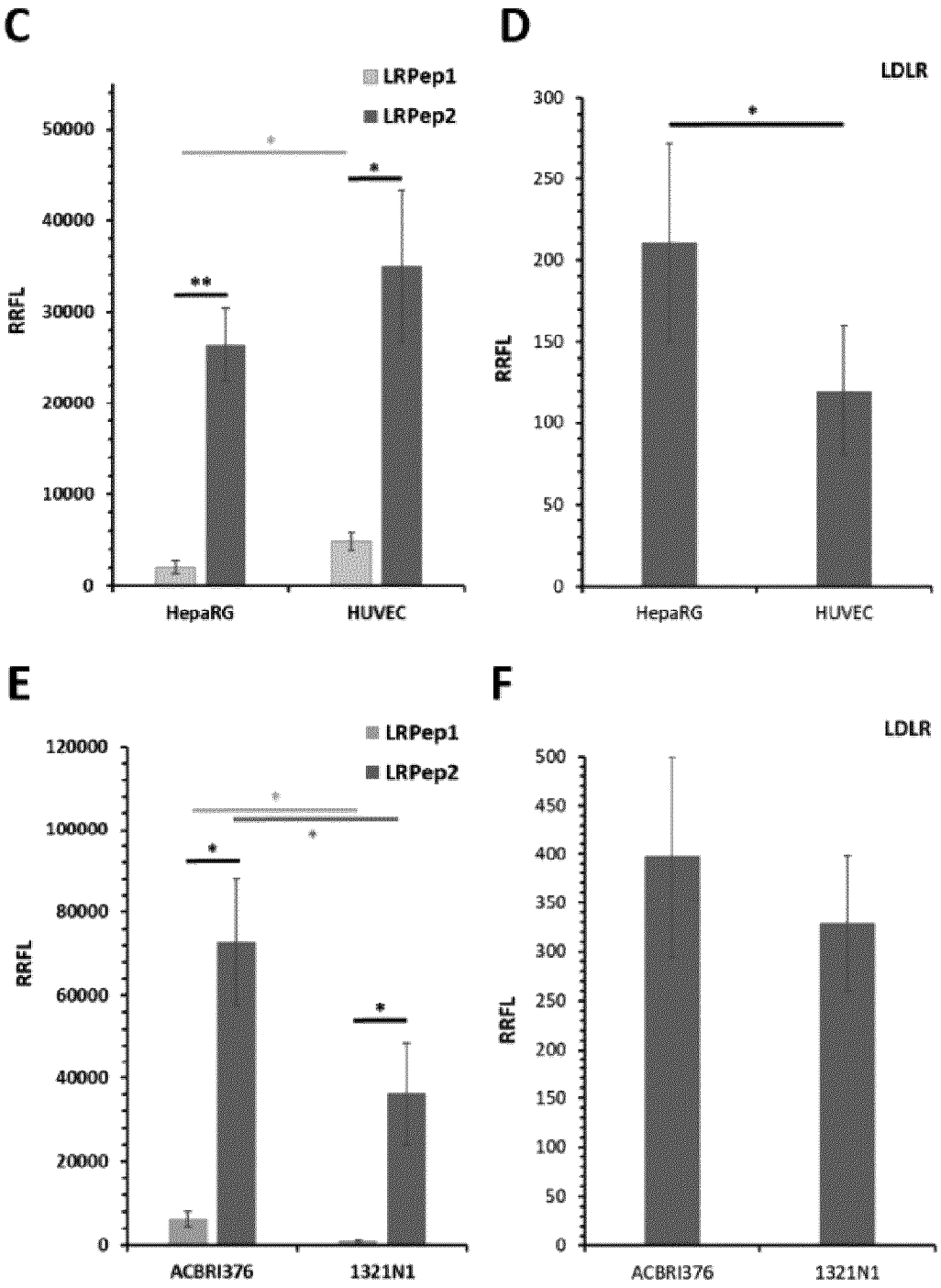

C
LRPep1
LRPep2
RRFL
50000
40000
30000
20000
10000
0
HepaRG
HUVEC
D
LDLR
RRFL
300
250
200
150
100
50
0
HepaRG
HUVEC
E
LRPep1
LRPep2
RRFL
120000
100000
80000
60000
40000
20000
0
ACBRI376
1321N1
F
LDLR
RRFL
500
450
400
350
300
250
200
150
100
50
0
ACBRI376
1321N1

FIG. 4

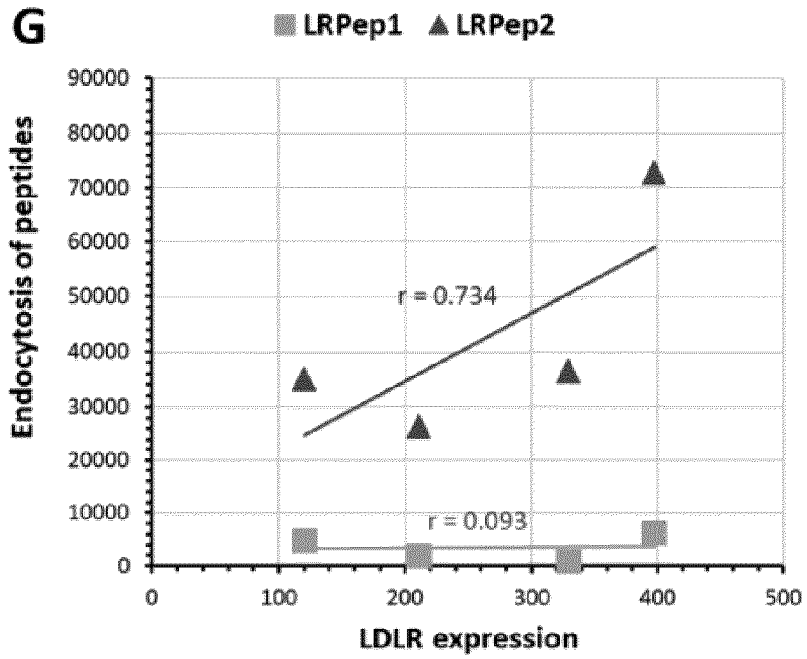

G
LRPep1
LRPep2
Endocytosis of peptides
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
r = 0.734
r = 0.093
0
100
200
300
400
500
LDLR expression

FIG. 5

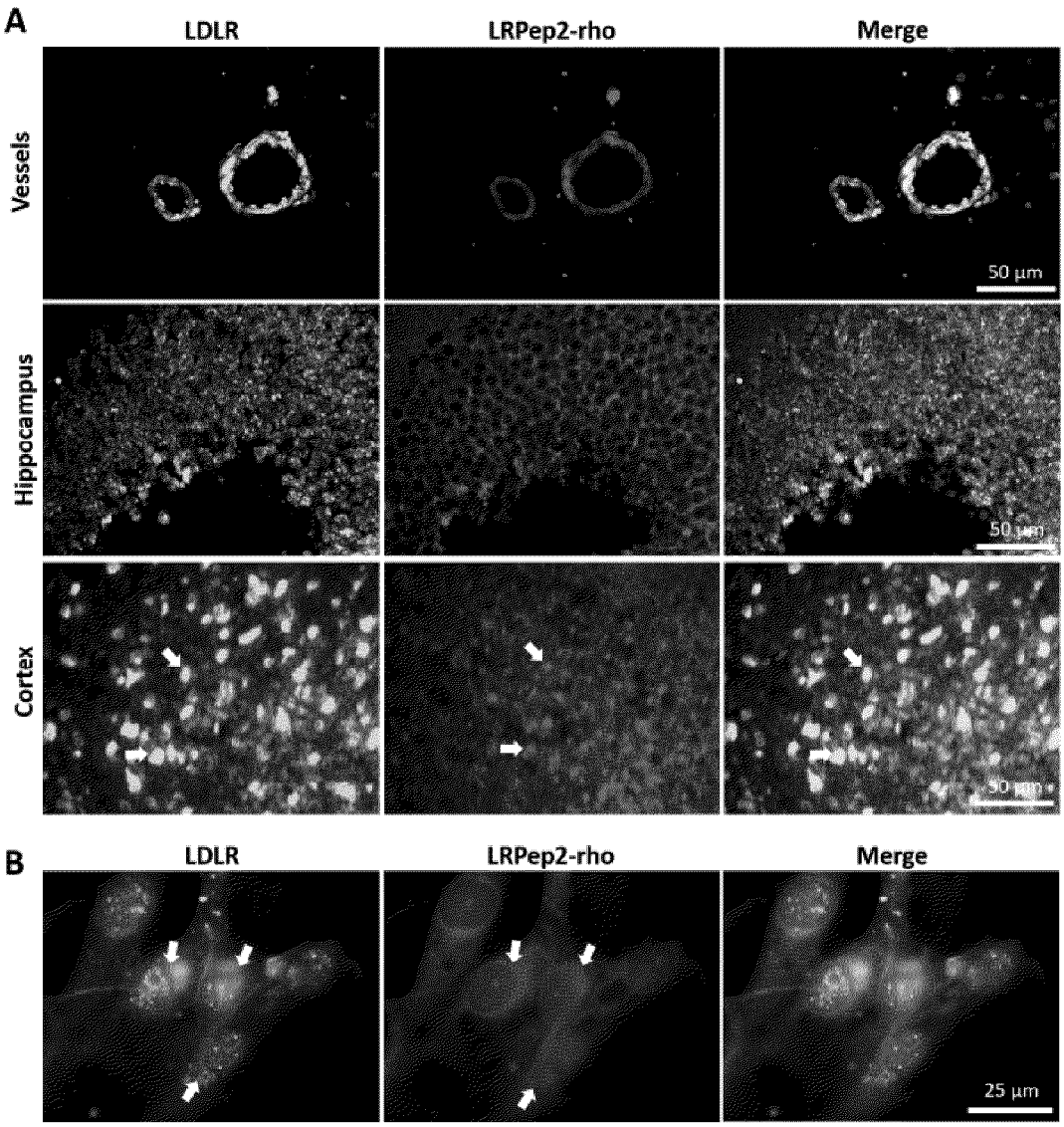

A
LDLR
LRPep2-rho
Merge
Vessels
Hippocampus
Cortex
50 µm
50 µm
50 µm
B
LDLR
LRPep2-rho
Merge
25 µm

FIG. 6

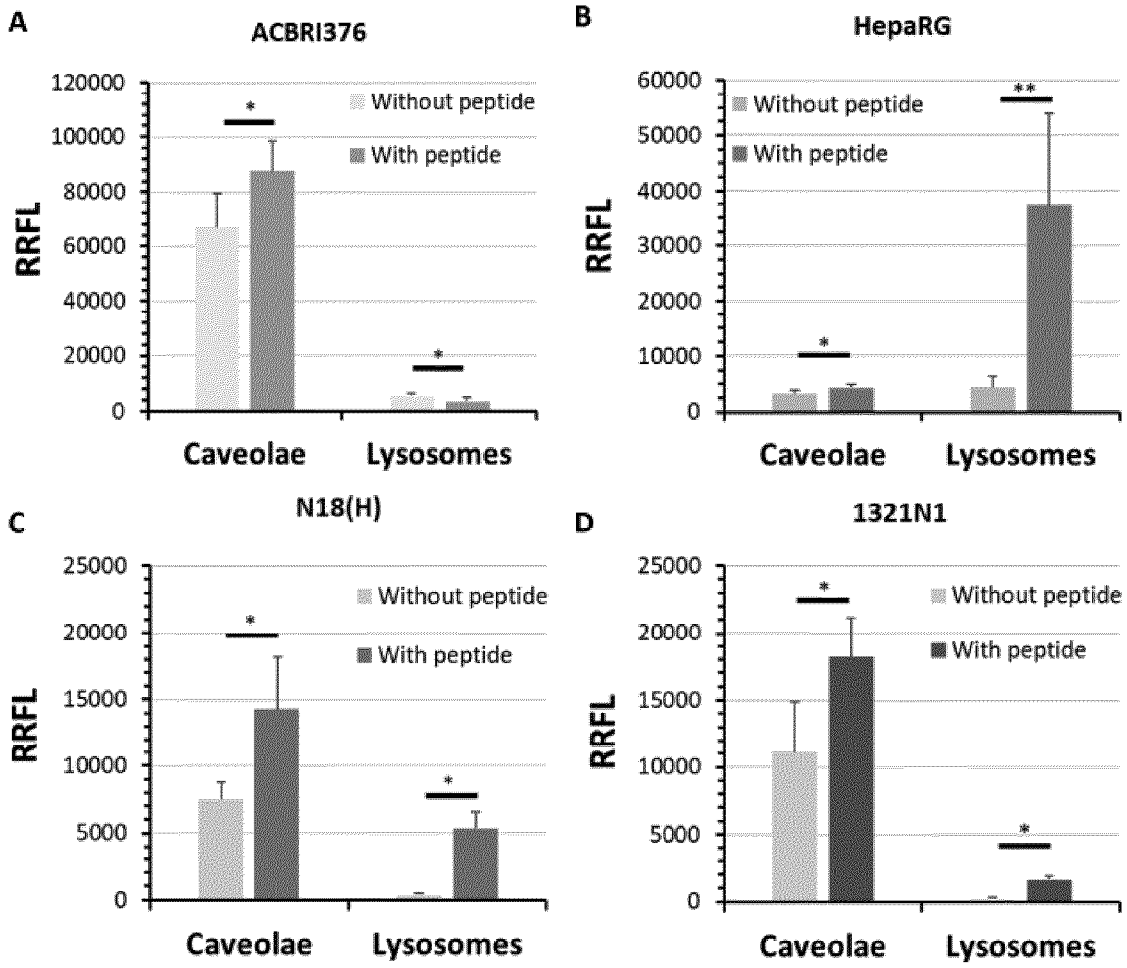

A
ACBRI376
B
HepaRG
C
N18(H)
D
1321N1
RRFL
Without peptide
With peptide
Caveolae
Lysosomes
120000
100000
80000
60000
40000
20000
0
50000
30000
10000
25000
15000
5000
*
**

FIG. 7

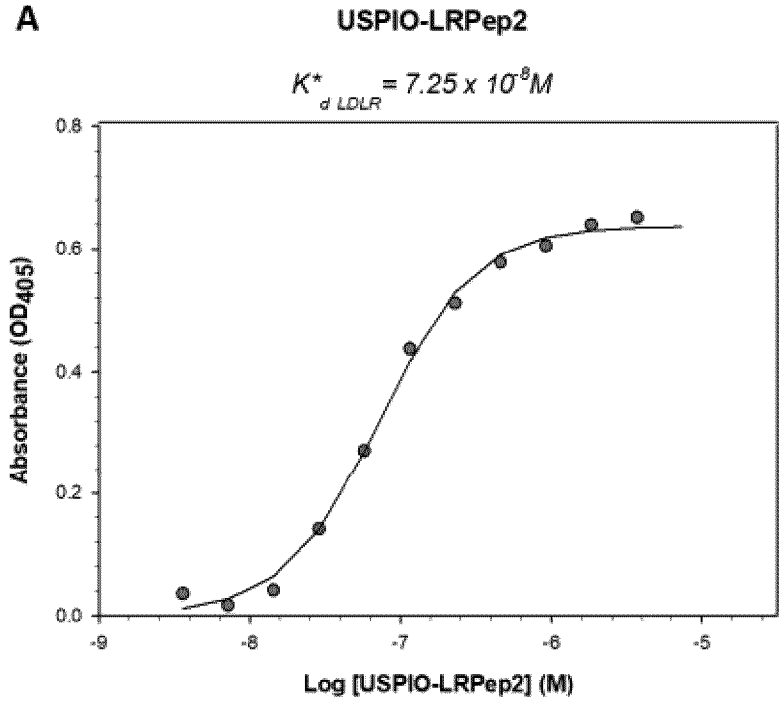

A
USPIO-LRPep2
$K^*_{d\ LDLR} = 7.25 \times 10^{-8} M$
Absorbance (OD_{405})
0.8
0.6
0.4
0.2
0.0
-9
-8
-7
-6
-5
Log [USPIO-LRPep2] (M)

FIG. 7

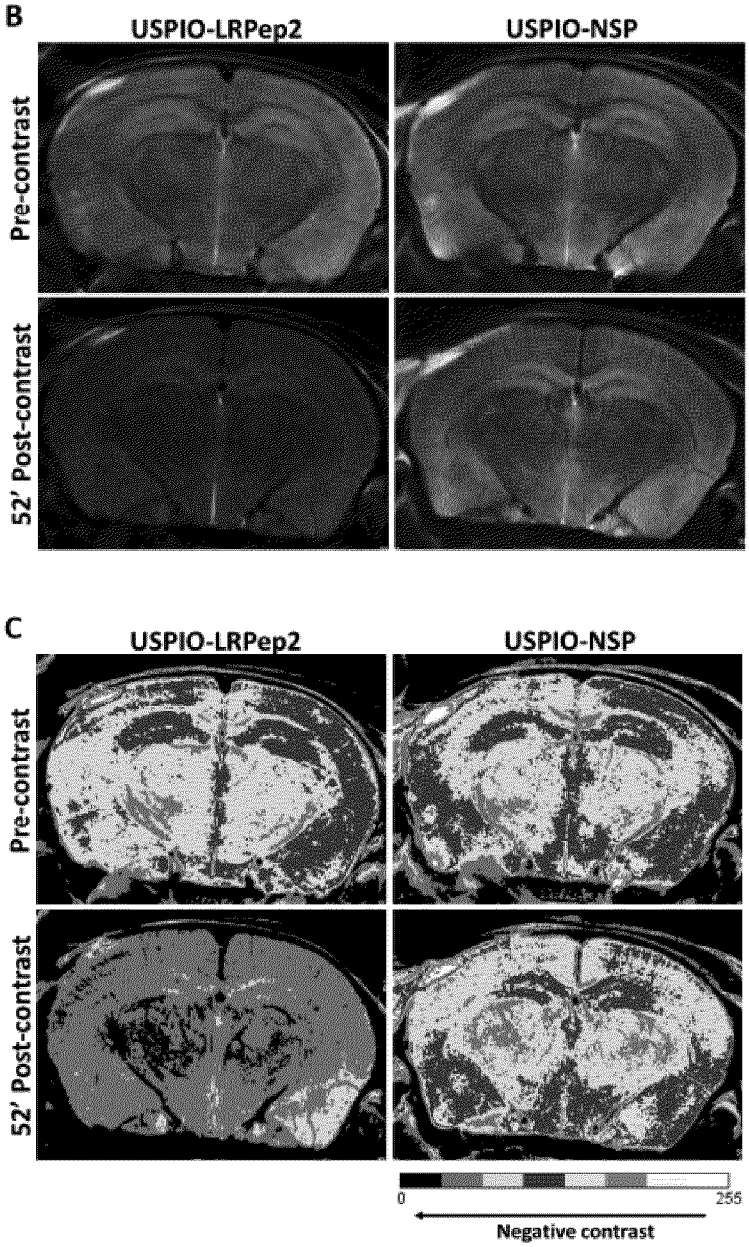

B
USPIO-LRPep2
USPIO-NSP
Pre-contrast
52' Post-contrast
C
USPIO-LRPep2
USPIO-NSP
Pre-contrast
52' Post-contrast
0
255
Negative contrast
D
20
10
0
-10
-20
-30
-40
Δ%SNR
Time (min)
0
22
52
78
108
136
166
191
219
1,330
*
*
USPIO-LRPep2
USPIO-NSP

FIG. 8

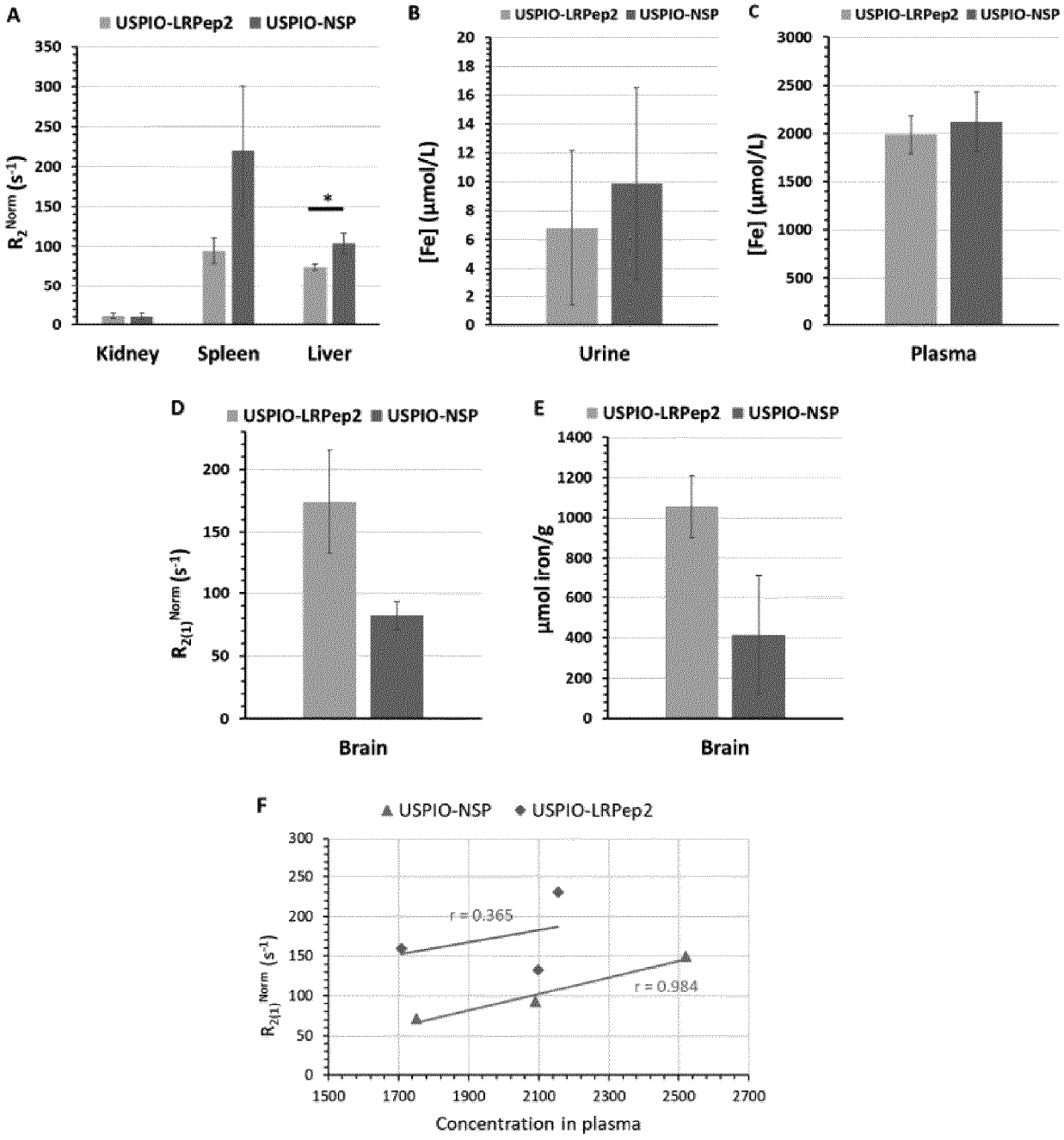

A
USPIO-LRPep2
USPIO-NSP
R_2^{Norm} (s^{-1})
Kidney
Spleen
Liver
*
B
[Fe] (µmol/L)
Urine
C
[Fe] (µmol/L)
Plasma
D
$R_{2(1)}^{Norm}$ (s^{-1})
Brain
E
µmol iron/g
Brain
F
USPIO-NSP
USPIO-LRPep2
r = 0.365
r = 0.984
$R_{2(1)}^{Norm}$ (s^{-1})
Concentration in plasma

FIG. 9

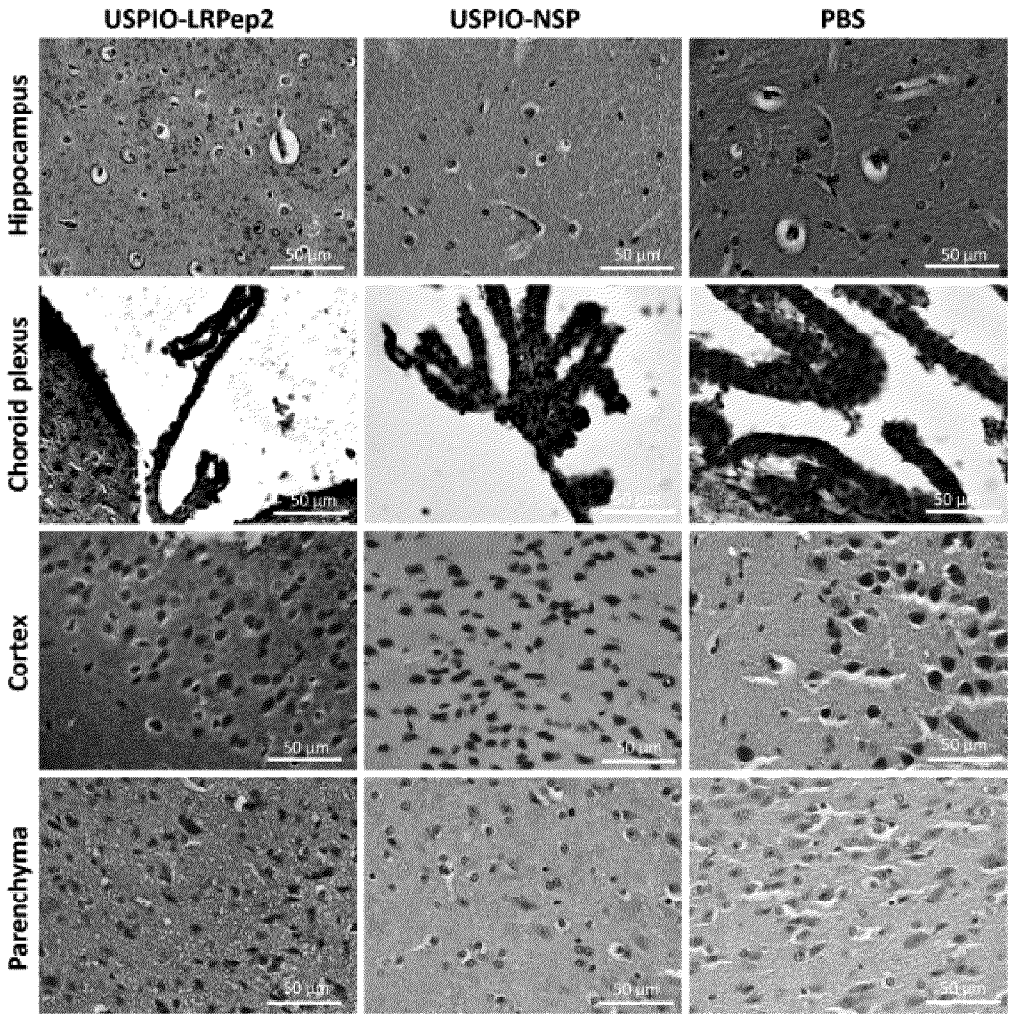
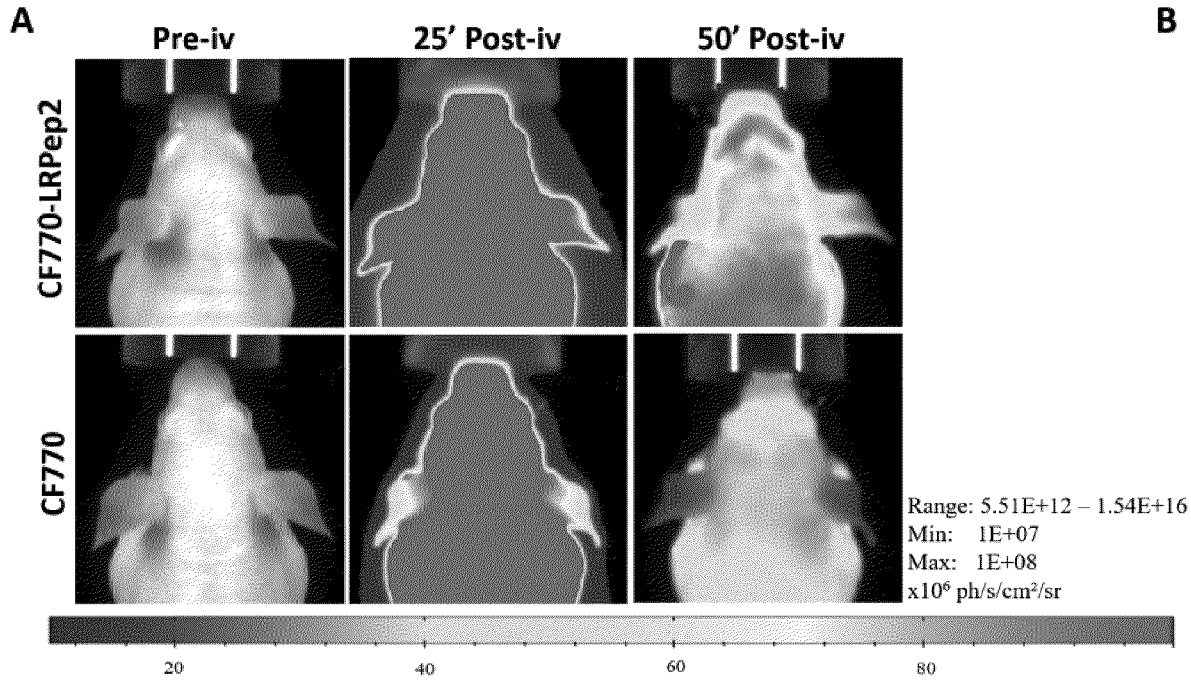

USPIO-LRPep2
USPIO-NSP
PBS
Hippocampus
Choroid plexus
Cortex
Parenchyma
50 µm

FIG. 10


A
Pre-iv
25' Post-iv
50' Post-iv
B
CF770-LRPep2
CF770
Range: 5.51E+12 – 1.54E+16
Min: 1E+07
Max: 1E+08
x10⁶ ph/s/cm²/sr
20
40
60
80

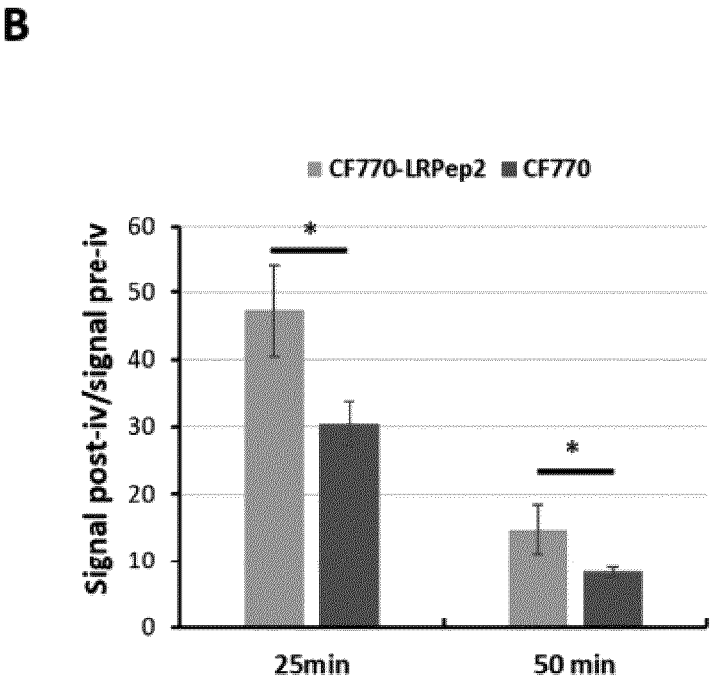
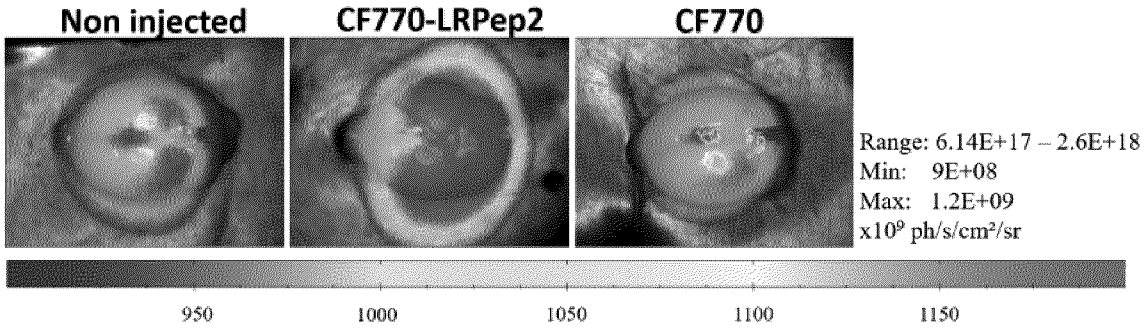

B
CF770-LRPep2
CF770
Signal post-iv/signal pre-iv
60
50
40
30
20
10
0
*
*
25min
50 min

FIG. 10

C


Non injected
CF770-LRPep2
CF770
Range: 6.14E+17 – 2.6E+18
Min: 9E+08
Max: 1.2E+09
x10⁹ ph/s/cm²/sr
950
1000
1050
1100
1150

D

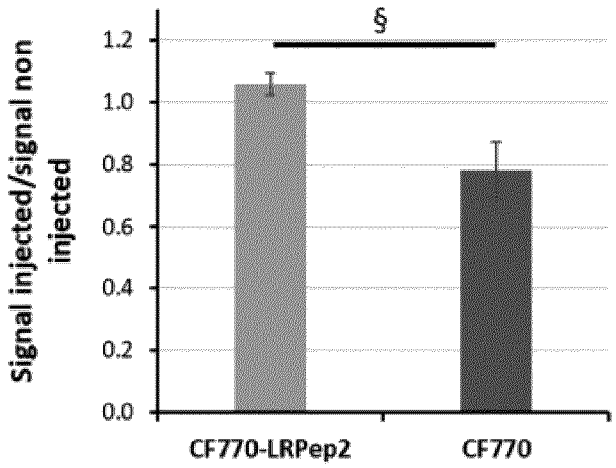

§
Signal injected/signal non injected
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CF770-LRPep2
CF770

FIG. 11

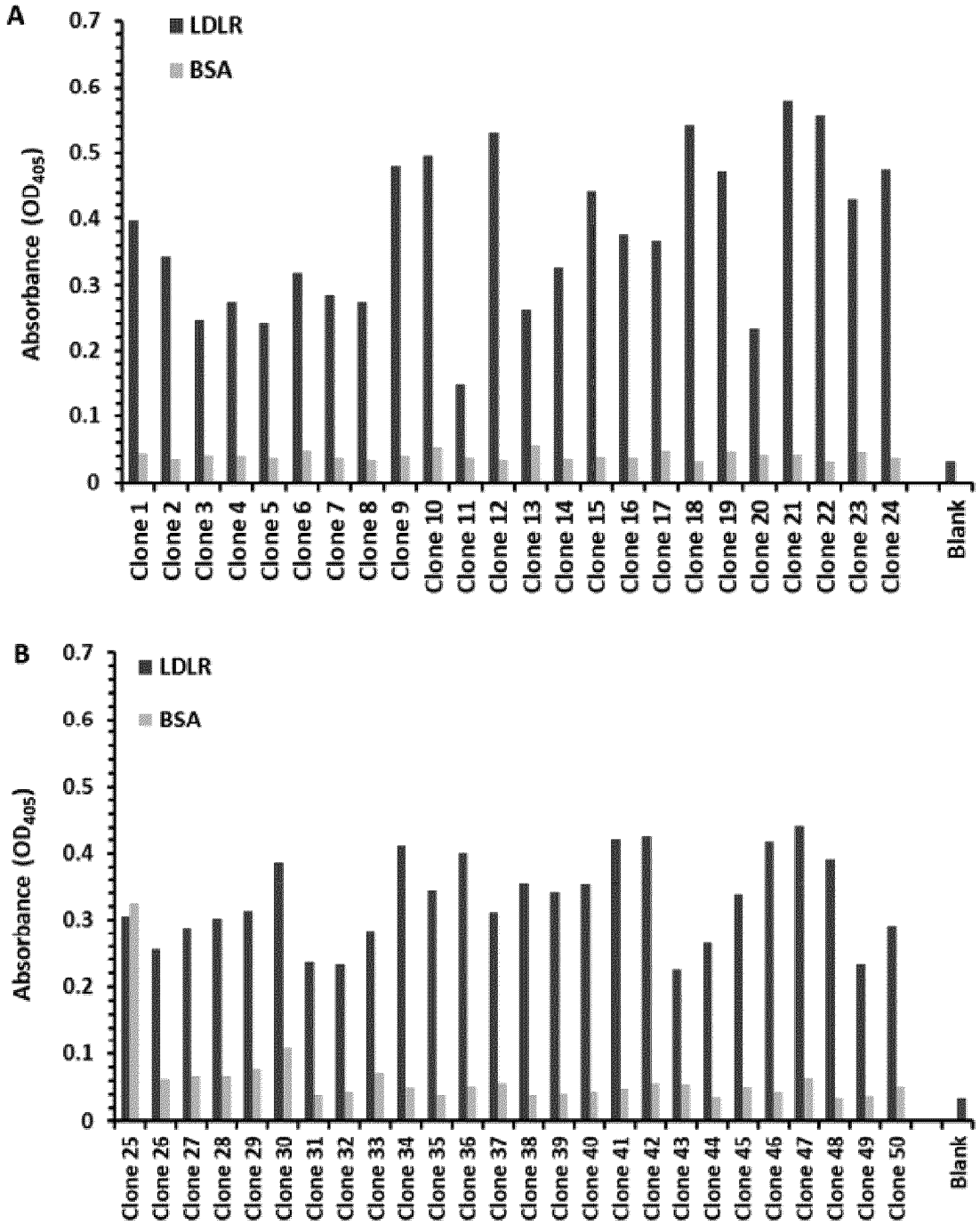

A
LDLR
BSA
Absorbance (OD405)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8
Clone 9
Clone 10
Clone 11
Clone 12
Clone 13
Clone 14
Clone 15
Clone 16
Clone 17
Clone 18
Clone 19
Clone 20
Clone 21
Clone 22
Clone 23
Clone 24
Blank
B
LDLR
BSA
Absorbance (OD405)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Clone 25
Clone 26
Clone 27
Clone 28
Clone 29
Clone 30
Clone 31
Clone 32
Clone 33
Clone 34
Clone 35
Clone 36
Clone 37
Clone 38
Clone 39
Clone 40
Clone 41
Clone 42
Clone 43
Clone 44
Clone 45
Clone 46
Clone 47
Clone 48
Clone 49
Clone 50
Blank

FIG. 12

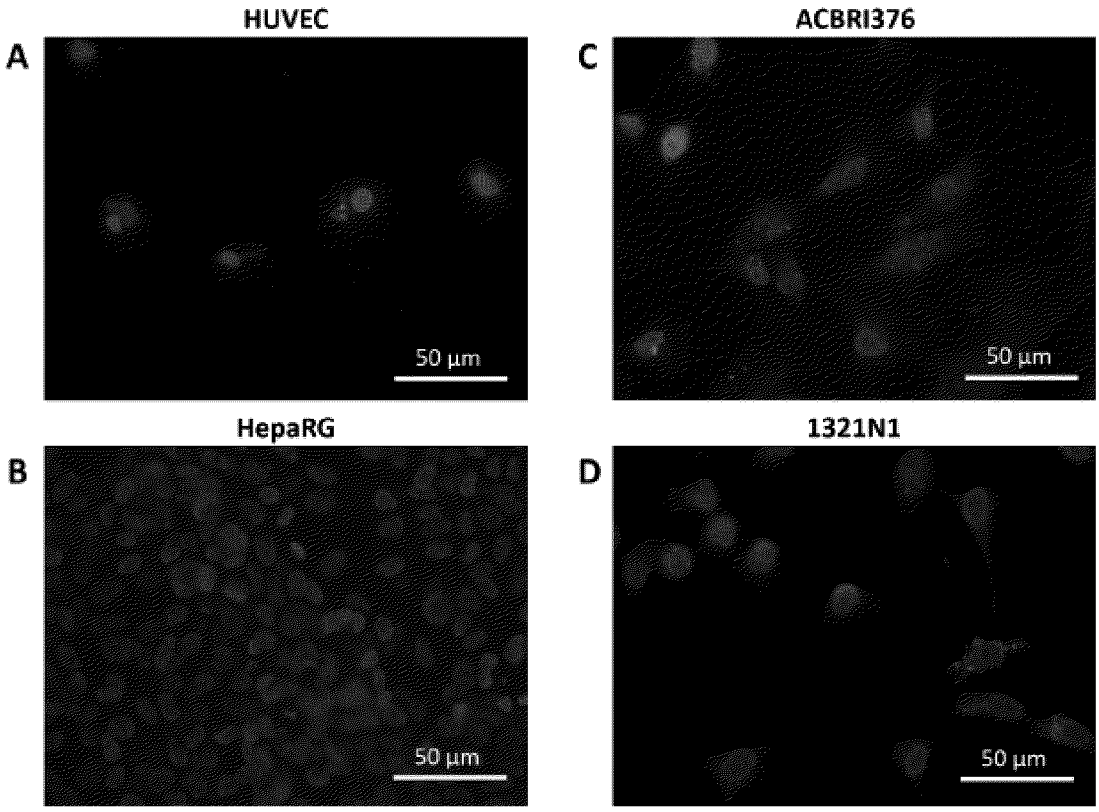


FIG. 13

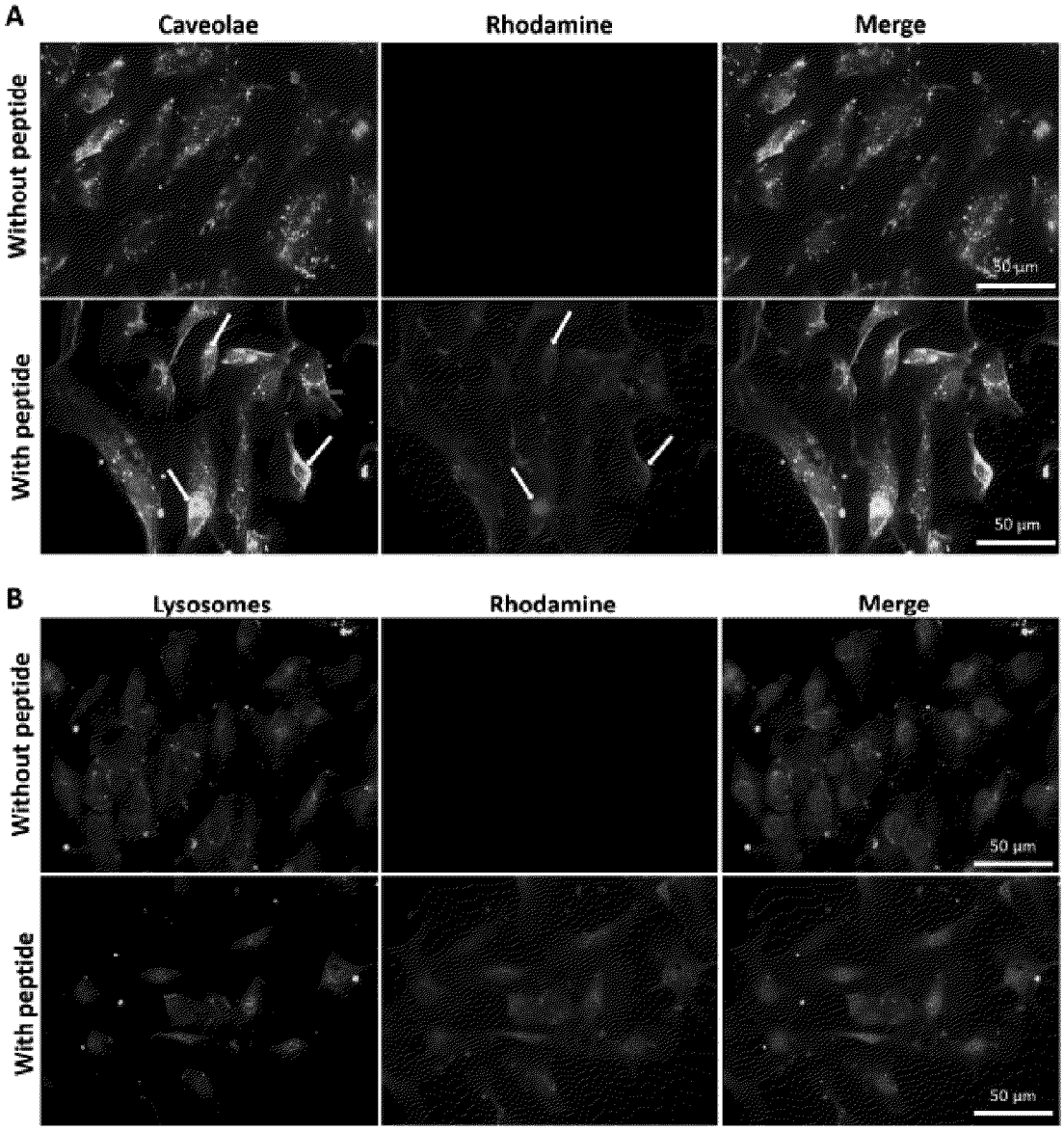

A
Caveolae
Rhodamine
Merge
Without peptide
With peptide
50 µm
50 µm
B
Lysosomes
Rhodamine
Merge
Without peptide
With peptide
50 µm
50 µm

FIG. 14

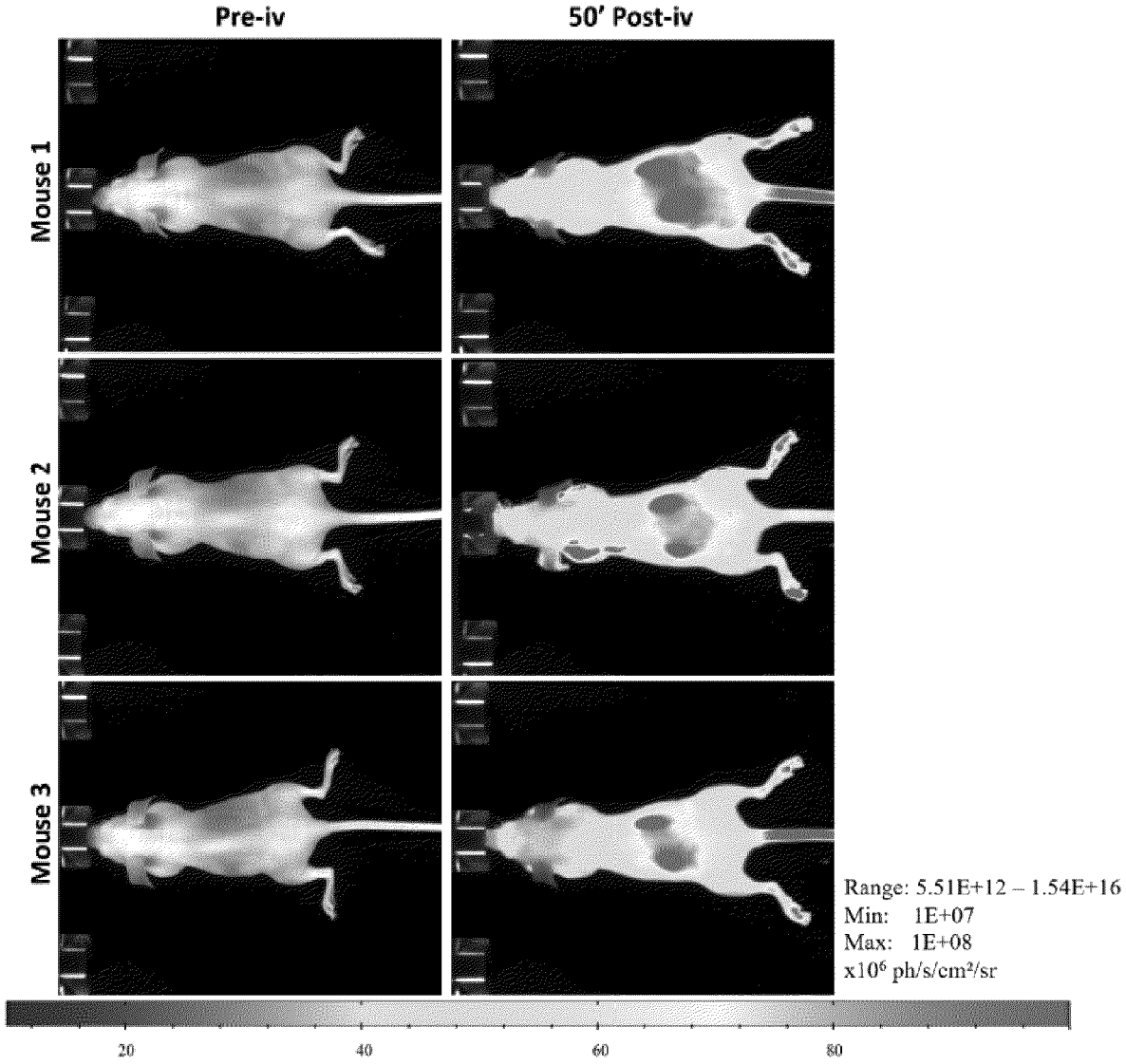
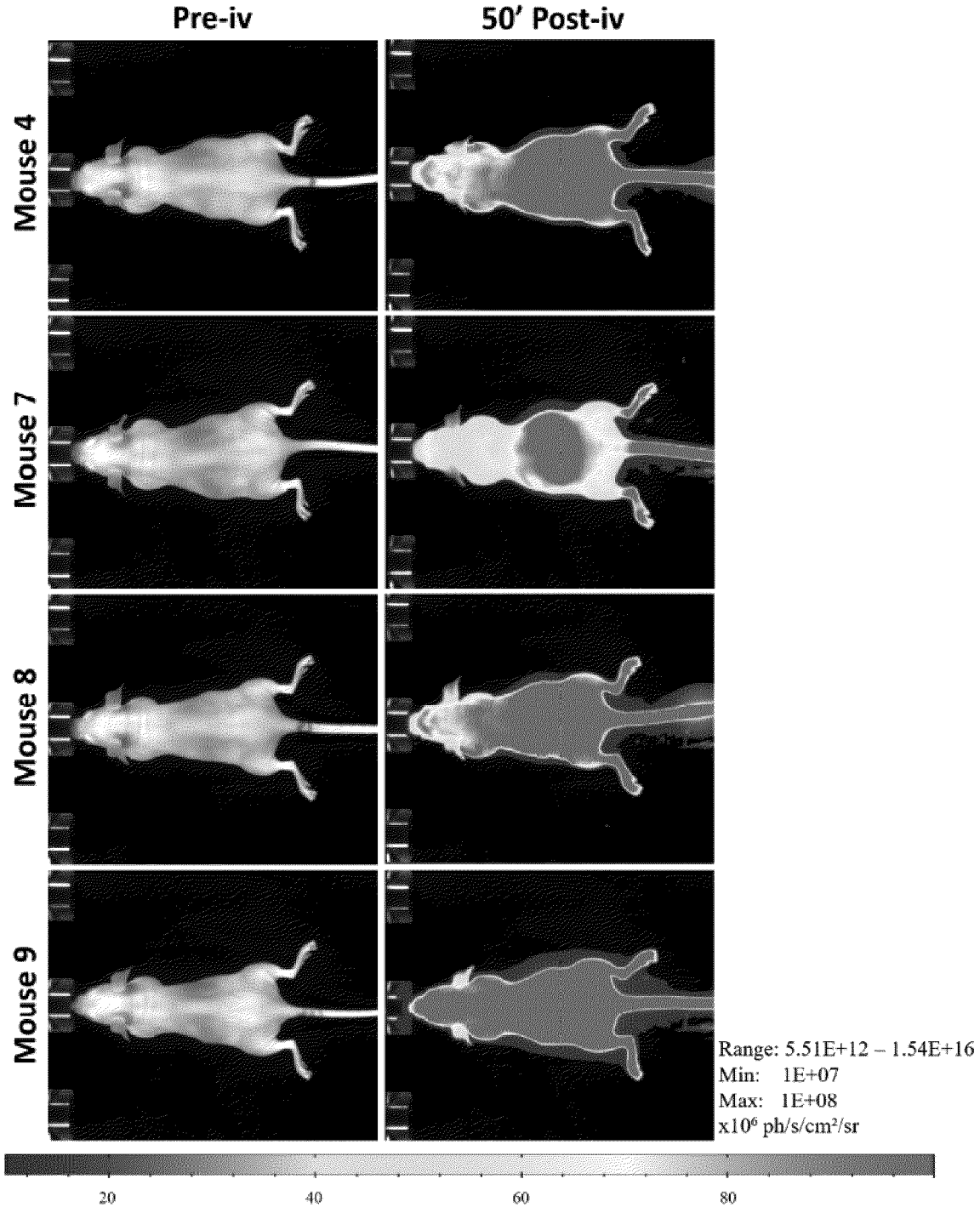

Pre-iv
50' Post-iv
Mouse 1
Mouse 2
Mouse 3
Range: 5.51E+12 – 1.54E+16
Min: 1E+07
Max: 1E+08
x10⁶ ph/s/cm²/sr
20
40
60
80

FIG. 15


Pre-iv
50' Post-iv
Mouse 4
Mouse 7
Mouse 8
Mouse 9
Range: 5.51E+12 – 1.54E+16
Min: 1E+07
Max: 1E+08
x10⁶ ph/s/cm²/sr
20
40
60
80

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/085025

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/08 A61K49/00 A61K49/18
ADD. A61K38/00 G01N33/68 A61K47/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 2 350 117 A1 (VECT HORUS [FR]; CENTRE NAT RECH SCIENT [FR] ET AL.) 3 August 2011 (2011-08-03) cited in the application	1,2,6-16
A	paragraphs [0023] - [0031]; claims 1-18; examples I-XI; sequences 1-72	3-5

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 January 2021	19/04/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schmidt-Yodlee, H

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MICHEL DEMEULE ET AL: "Involvement of the low-density lipoprotein receptor-related protein in the transcytosis of the brain delivery vector Angiopep-2", JOURNAL OF NEUROCHEMISTRY, vol. 106, no. 4, 1 August 2008 (2008-08-01), pages 1534-1544, XP055053569, ISSN: 0022-3042, DOI: 10.1111/j.1471-4159.2008.05492.x	1,2,6-16
A	abstract page 1536; figures 1-8	3-5

A	HO CHUL KANG ET AL: "Identification and characterization of four novel peptide motifs that recognize distinct regions of the transcription factor CP2", FEBS JOURNAL, vol. 272, no. 5, 17 February 2005 (2005-02-17), pages 1265-1277, XP055316727, GB ISSN: 1742-464X, DOI: 10.1111/j.1742-4658.2005.04564.x abstract; figure 1; table 1; compound clone number 50	1-16

X	CN 110 330 550 A (UNIV ZHENGZHOU) 15 October 2019 (2019-10-15)	2,6-10, 12-16
Y	claims 1-10; sequence 1	1,11
A		3-5

X,P	SÉVERINE ANDRÉ ET AL: "Development of an LDL Receptor-Targeted Peptide Susceptible to Facilitate the Brain Access of Diagnostic or Therapeutic Agents", BIOLOGY, vol. 9, no. 7, 11 July 2020 (2020-07-11), page 161, XP055768184, ISSN: 2079-7737, DOI: 10.3390/biology9070161 the whole document	1-16

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/085025

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2350117 A1	03-08-2011	AU 2009306237 A1	29-04-2010
		BR PI0919777 A2	20-09-2016
		CA 2741098 A1	29-04-2010
		CN 102282159 A	14-12-2011
		DK 2350117 T3	08-12-2014
		EA 201170587 A1	30-05-2012
		EP 2350117 A1	03-08-2011
		ES 2525649 T3	26-12-2014
		FR 2937322 A1	23-04-2010
		HR P20141139 T1	13-03-2015
		JP 5808250 B2	10-11-2015
		JP 6232396 B2	15-11-2017
		JP 2012506407 A	15-03-2012
		JP 2015212264 A	26-11-2015
		PL 2350117 T3	30-04-2015
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2020/085025

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

5(completely); 1-4, 6-16(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5(completely); 1-4, 6-16(partially)

peptide capable of binding to LDLR, wherein said peptide has at least 90% identity to the amino acid sequence set forth in SEQ ID 1 or SEQ ID 12, peptide capable of binding to LDLR, wherein said peptide has an amino acid sequence that differs maximally two amino acids from SEQ ID 1 or SEQ ID 12, its use, conjugate and pharmaceutical composition

1.1. claims: 1-4, 6-16(all partially)

peptide capable of binding to LDLR, wherein said peptide has at least 90% identity to the amino acid sequence set forth in SEQ ID 1, peptide capable of binding to LDLR, wherein said peptide has an amino acid sequence that differs maximally two amino acids from SEQ ID 1, its use, conjugate and pharmaceutical composition

1.2. claims: 5(completely); 1-4, 6-16(partially)

peptide capable of binding to LDLR, wherein said peptide has at least 90% identity to the amino acid sequence set forth in SEQ ID 12, peptide capable of binding to LDLR, wherein said peptide has an amino acid sequence that differs maximally two amino acids from SEQ ID 12, its use, conjugate and pharmaceutical composition

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2-11. claims: 1-4, 6-16(all partially)

peptide capable of binding to LDLR, wherein said peptide has at least 90% identity to the amino acid sequence set forth in SEQ ID 2 (Invention 2), SEQ ID 3 (Invention 3), ..., or SEQ ID 11 (Invention 11) peptide capable of binding to LDLR, wherein said peptide has an amino acid sequence that differs maximally two amino acids from SEQ ID 2, 3, .., or 11, its use, conjugate and pharmaceutical composition

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