



(86) Date de dépôt PCT/PCT Filing Date: 2011/04/18
(87) Date publication PCT/PCT Publication Date: 2011/10/27
(45) Date de délivrance/Issue Date: 2018/07/17
(85) Entrée phase nationale/National Entry: 2012/10/11
(86) N° demande PCT/PCT Application No.: FR 2011/050883
(87) N° publication PCT/PCT Publication No.: 2011/131896
(30) Priorité/Priority: 2010/04/21 (FR1053036)

(51) Cl.Int./Int.Cl. *C07K 7/06* (2006.01),
A61K 38/08 (2006.01), *A61K 38/12* (2006.01),
A61K 38/17 (2006.01), *A61K 49/00* (2006.01),
A61P 25/28 (2006.01), *C07K 14/775* (2006.01),
C07K 7/02 (2006.01), *C07K 7/50* (2006.01),
C07K 7/64 (2006.01), *G01N 33/68* (2006.01)

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(54) Titre : DERIVES PEPTIDIQUES, LEUR PREPARATION ET LEURS UTILISATIONS
(54) Title: PEPTIDE DERIVATIVES, PREPARATION THEREOF AND USES THEREOF

(57) **Abrégé/Abstract:**

The invention relates to peptide derivatives (peptides and pseudo-peptides) and use thereof as vectors for molecules of interest. The invention also relates to conjugates containing a peptide derivative of the invention bound to a molecule of interest. The peptides of the invention can be used to vectorize in the form of conjugates generally prodrugs of molecules of pharmaceutical or diagnostic interest, such as, for example, therapeutic molecules, imaging or diagnostic agents, or molecular probes, across cell membranes, and notably to enable their transport across the blood-brain barrier (BBB).

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ABSTRACT**PEPTIDE DERIVATIVES, PREPARATION THEREOF AND USE THEREOF**

The invention relates to peptide derivatives (peptides and pseudo-peptides) and use thereof as vectors for molecules of interest. The invention also relates to conjugates containing a peptide derivative of the invention bound to a molecule of interest. The peptides of the invention can be used to vectorize in the form of conjugates generally prodrugs of molecules of pharmaceutical or diagnostic interest, such as, for example, therapeutic molecules, imaging or diagnostic agents, or molecular probes, across cell membranes, and notably to enable their transport across the blood-brain barrier (BBB).

PEPTIDE DERIVATIVES, PREPARATION AND USES THEREOF

The invention relates to peptide derivatives (peptides and pseudo-peptides) and the use thereof as vectors for molecules of interest. The invention also relates to conjugates containing a peptide derivative of the invention bound to a molecule of interest. The peptides of the invention can be used, in particular, to vectorize, generally in the form of prodrug conjugates, molecules of pharmaceutical or diagnostic interest such as, for example, therapeutic molecules, imaging or diagnostic agents, or molecular probes, across cell membranes, and notably to enable their transport across the blood-brain barrier (BBB).

CONTEXT OF THE INVENTION

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According to *IMS Health*, the global market for drugs for treating central nervous system (CNS, brain and spinal cord) pathologies was approximately 70 billion dollars in 2007, with nearly 9 billion dollars of this amount representing products arising from drug delivery technologies (*Jain, 2008, Jain PharmaBiotech Report, Drug Delivery in CNS disorders*). Thus, neurology is today one of the three largest therapeutic areas, along with cardiovascular medicine and oncology. Although the number of people suffering from CNS disorders and pathologies throughout the world is larger than that of people with cardiovascular diseases or cancers, neurology remains an under-developed market. This is explained by the fact that 98% of potential drugs for treating

CNS pathologies do not cross the blood-brain barrier or BBB (*Pardridge, 2003, Mol. Interv., 3, 90-105*).

Indeed, the brain is protected from potentially toxic substances by the presence of two principal physiological barrier systems: the BBB, and the blood-cerebrospinal fluid barrier (BCSFB). The BBB is regarded as the principal route for the uptake of plasma ligands. Its surface area is approximately 5000 times larger than that of the BCSFB. The overall length of the constitutive blood vessels of the BBB is approximately 600 km. Each cm³ of cerebral cortex contains the equivalent of 1 km of blood vessels. The total surface area of the BBB is estimated at 20 m² (*De Boer et al., 2007, Clin. Pharmacokinet., 46(7), 553-576*). Thus, the cerebral endothelium, which constitutes the BBB, represents a major obstacle to the use of potential drugs against many CNS disorders, but also a large surface of potential exchange between the blood and nervous tissue.

As a general rule, only a few small lipophilic molecules of approximately 450 to 600 Daltons can pass through the BBB (only 2% of drug candidates), that is to say, pass from the blood to the brain. The molecular weight and the size of many drug candidates, which show promising results in *in vitro* studies and in animal studies for treating CNS disorders, are considerably larger. Thus, most molecules such as therapeutic peptides or proteins are generally excluded from passage/transport from the blood to the brain, because of the low transcellular permeability of brain capillary endothelial cells (BCECs) for this type of drug candidate. The BCECs organized in vessels are surrounded by a basal lamina, astrocyte end-feet, pericytes and microglial and neuronal cells. The tight association of endothelial cells with astrocyte end-feet is responsible for the development and maintenance of properties of BBB impermeability to most molecules, thus ensuring strict and effective control of molecular exchanges between the blood and the brain in order to maintain brain homeostasis. BCECs are closely bound by tight junctions, compared to other endothelial cells of other organs, which are fenestrated. These tight junctions thus prevent any paracellular transport across the BBB.

The BBB is regarded as the major obstacle to overcome in the development of novel therapies for treating brain pathologies, and notably for the use of molecules for treating CNS disorders (*Neuwelt et al., 2008, Lancet Neurol., 7, 84-96*).

5 One of the reasons that may explain why no effective treatment is currently available for the principal brain pathologies (brain cancer, Parkinson's and Alzheimer's diseases, cerebrovascular accidents (CVA), etc.) is that the drug developers for treating brain pathologies carry out in-house research programs (brain drug-discovery programs) while investing little effort in the problems of passing the BBB and in the preferential
10 targeting of the CNS, and notably of the brain (brain drug-targeting programs), (*Pardridge, 2003, Mol. Interv., 3, 90-105*). A drug candidate must follow certain structural, physicochemical, pharmacochemical and pharmacological rules in order to have the best chances of becoming a drug for treating a CNS pathology or disorder (*Pajouhesh et al., 2005, NeuroRx, 2(4), 541-553*). Thus, in the development of a drug
15 candidate, the selectivity and specificity (pharmacological profiling) of a molecule for its target are essential to its therapeutic activity (effectiveness). The bioavailability and potential toxicity (pharmaceutical profiling) of a molecule are crucial for its future as a drug. In other words, any molecule likely to become a drug for treating a CNS pathology or disorder must move across the BBB, maintain its biological activity, and
20 exhibit suitable properties of pharmacokinetics (PK), absorption, distribution, metabolism and excretion/elimination (ADME) and pharmacodynamics (PD), with low toxicity (Tox). In fact, the hydrophilic/lipophilic balance of the molecule under development is particularly difficult to find for medicinal chemists in this field of CNS therapeutics.

25

The major problem in treating CNS disorders and pathologies thus lies in the fact that the molecules administered do not pass the BBB and thus cannot reach their target(s) in the CNS. The endothelial cells of the vessels and capillaries of the CNS which are constitutive of the BBB are an obstacle to molecules that cannot pass from the blood to
30 the nervous tissue. Indeed, as mentioned above, these endothelial cells and the astrocyte end-feet which surround them constitute a physical barrier related notably to the existence of tight junctions between endothelial cells which limit/prevent any

passage/transport by the paracellular route, and also a physiological barrier, since these cells have effective efflux systems which restrict any passage/transport by the transcellular route. These properties thus strongly limit the passage of substances from the blood plasma towards the brain extracellular space.

5

Indeed, certain molecules capable of crossing the BBB are actively expelled from the brain towards the blood system by multidrug resistant (MDR) transport proteins. These active efflux transport (AET) systems generally control the active efflux of small molecules from the brain towards the blood system. The model AET system at the BBB is the ATP binding cassette (ABC) transporter, namely P-glycoprotein (P-gp); however, other AET systems are present at the BBB such as MDR-associated protein 1 (MRP1). P-gp, which is principally located on the luminal surface of BCECs, is an essential element in the function of the physiological barrier of the BBB preventing entry into the brain of most xenobiotics but also drug candidates and other molecules of therapeutic interest capable of being active in the CNS.

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One of the priorities of research in the discovery of molecules for treating, diagnosing or imaging brain disorders or pathologies is thus to find means for increasing the effectiveness of passage of active substances across the BBB.

20

In this respect, strategies for the vectorization of molecules across the BBB, currently studied and used by developers of drug candidates in order to enable a molecule of therapeutic interest to reach the CNS, can be divided into two principal strategies: pharmacological approaches and physiological approaches (Figure 1), (*Pardridge, 2007, Pharm. Res., 24(9), 1733-1744; De Boer et al., 2007, Clin. Pharmacokinet., 46(7), 553-576; De Boer et al., 2007, Annu. Rev. Pharmacol. Toxicol., 47, 327-355; Jones et al., 2007, Pharm. Res., 24(9), 1759-1771*).

25

Invasive approaches

Invasive approaches can be implemented by direct intraventricular injection in the brain, intracerebral injection or intrathecal infusion of the active substance, or by disruption of the BBB (temporary rupture of the integrity of the BBB).

30

The principal problem of neurosurgical approaches by intraventricular injection, apart from the costs relating to the neurosurgical procedure, is that the drug is not delivered directly in the brain parenchyma but in the cerebrospinal fluid. Intraventricular infusion involves placing a catheter in the ventricles (*Aird, 1984, Exp. Neurol., 86, 342-358*).

5 This highly invasive technique is not effective for the transport of active substances in the brain parenchyma. Indeed, the flow volume from the cerebrospinal fluid to the brain parenchyma during delivery of a drug by intraventricular infusion is governed by an abnormally slow diffusion of its convection (transport), because the brain does not have intraparenchymal volumetric flow.

10 Similarly for intracerebral injection, the diffusion of an active substance in the brain decreases very quickly from the injection site to the site of the lesion. Indeed, the cerebral concentration of an active substance decreases by 90% at a distance of 500 μm from its injection site.

Intrathecal infusion involves placing a catheter in the brain. This catheter is connected
15 to a pump that delivers the active substance at a predefined flow rate. Owing to the fact that the brain is the only organ that does not have a lymphatic system, normally serving to transport extracellular fluids back to general circulation, the distribution of an active substance by intrathecal infusion in the brain is very slow. This decreases the concentration of the active substance at the site of the lesion.

20 Moreover, risks of infection are significant during such neurosurgical procedures, notably by the presence of the catheter. Under these conditions, patient comfort is not optimal.

The temporary rupture of the impermeability of the BBB is associated with transitory
25 opening of the tight junctions of BCECs. This is the case for vasoactive substances such as leukotrienes or bradykinins (*Baba et al., 1991, J. Cereb. Blood Flow Metab., 11, 638-643*). This strategy is equally invasive and requires arterial access to the carotid in sedated subjects/patients. The major problem encountered by the temporary rupture of the integrity of the BBB, besides expenses relating to the radiological procedure for
30 access to the carotid, is that the BBB only remains open for a short period of time, thus limiting the possibility of delivering a drug over an extended period. Moreover, the temporary rupture of the BBB allows plasma proteins to enter the brain (whereas these

proteins can be toxic for the brain) and can also facilitate the entry of infectious agents. This type of rupture of the BBB can thus lead to chronic neuropathologic disruptions and is associated with high risks of infection (*Salahuddin et al., 1988, Acta Neuropathol., 76, 1-10*).

5

Pharmacological approaches to vectorization

Pharmacological strategies for transporting molecules include transcellular diffusion of molecules made more hydrophobic by the addition of lipid or lipophilic groups on the active substance (transcellular lipophilic diffusion, TLD) or the use of liposomes (*Zhou et al., 1992, J. Control. Release, 19, 459-486*), and transport by ionic adsorption via positively charged vector molecules or by cationization of the active molecule (adsorptive-mediated transport or AMT).

The addition of a lipid or lipophilic group enables the chemical conversion of hydrophilic molecules into more hydrophobic molecules notably through prodrug approaches. However, the synthesis of such compounds leads to molecules that exceed the optimal transport threshold to cross the BBB, notably with regard to molecular weight (MW) which becomes greater than the optimal limit of 450 Daltons (*Pajouhesh et al., 2005, NeuroRx, 2(4), 541-553*). For the same reason, liposomes or even small vesicles (micelles, etc.) or nanoparticles (nanospheres, nanocapsules) are generally too large, are not specific enough for the BBB, and consequently are relatively less effective for transporting molecules of therapeutic interest (or imaging or diagnostic agents, or any other molecule such as a molecular probe) across the BBB (*Levin, 1980, J. Med. Chem., 23, 682-684; Schackert et al., 1989, Selective Cancer Ther., 5, 73-79*). Moreover, this type of vesicle system generally has non-negligible toxic effects in the cerebrum. Thus, the principal problems encountered by lipidization technologies are their low specificity for specifically targeting and crossing the BBB compared to other cell membranes, the decrease in the plasma values of the area under the curve (AUC) of the drug, and their generally limited use for vectorization of small molecules.

In AMT approaches (addition of a cationic group via covalent bonding or direct cationization of the drug), the principal encountered problem is the low specificity for targeting and crossing specifically the BBB compared to other cell membranes. Indeed, AMT is based on cationic molecules adsorbing on cells whose membrane is negatively

30

charged, which is the case for most cells. The decrease in plasma values of the AUC of the drug, their generally limited use for vectorization of small molecules, and their cytotoxicity are additional factors which penalize the AMT vectorization approach.

5 *Physiological approaches to vectorization*

Strategies based on physiological approaches to vectorization consist in exploiting the various natural transport mechanisms of the BBB. These mechanisms of active transport of molecules across the BBB work either via coupling with a specific receptor substrate or by molecular mimicry with a specific receptor substrate (carrier-mediated transport
10 or CMT), or via coupling or fusion with a ligand specifically targeting a receptor (receptor-mediated transport or RMT).

As an example, molecules such as L-DOPA (Parkinson's disease), melphalan (brain cancer), α -methyl-DOPA (arterial hypertension) and gabapentin (epilepsy) pass into the
15 brain by CMT via large neutral amino-acid transporters 1 and 2 (LAT1 and LAT2), (*Pardridge, 2003, Mol. Interv., 3, 90-105*). These molecules have chemical structures close to phenylalanine, one of the natural substrates of LAT1. However, the principal problems encountered by CMT approaches are their broad selectivity/specificity for conjugates that closely imitate/mimic the substrate of the endogenous
20 receptor/transporter, and consequently their use which remains limited to vectorization of small molecules.

RMT calls upon a receptor-dependent transport system. Vectorization is carried out via mechanisms of endocytosis by targeting the endogenous receptors/transporters present in brain capillaries. Notable examples of the various human BBB receptors involved in
25 RMT include: transferrin receptor (TfR), insulin receptor (IR), low-density lipoprotein (LDL) receptors, which enable cholesterol transport, including LDL receptor (LDLR) and members of the family of low-density lipoprotein receptor-related protein (LRP), or insulin-like growth factor receptor (IGFR), diphtheria toxin receptor (DTR) or heparin binding epidermal growth factor-like growth factor (HB-EGF), as well as scavenger
30 receptors (SCAV-Rs) including scavenger receptor class B type I (SR-BI). In RMT, the receptors on the membrane of a BBB endothelial cell bind their ligand, which leads to endocytosis of the complex composed of the receptor/transporter and its ligand in a

vesicle which forms on the cell surface and then penetrates the BBB endothelial cell. The ligand/receptor complex can pass through the endothelial cell (transcytosis), and thus can consequently cross the BBB to act in nervous tissue. This RMT process does not depend on the size of the molecule engulfed by endocytosis. Thus, RMT is a mechanism that enables transport from the blood to the brain of molecules such as insulin, iron transport proteins, cholesterol, various peptide derivatives and proteins, etc. For example, transferrin is used as a ligand vector of TfR present on the BBB (*Jefferies et al., 1984, Nature, 312, 162-163; Friden et al., 1983, Science, 259, 373-377; Friden, 1994, Neurosurgery, 35, 294-298*), and the molecule to be transported (active substance) is coupled/conjugated with transferrin (ligand vector). Although this vectorization strategy using a macromolecule enables an increase in the passage of the conjugate molecules of interest across the BBB, it has several disadvantages. First, the molecule is generally coupled/conjugated to the vector by gene expression methods (fusion) thus limiting the number of molecules to be transported to only polypeptides or proteins. Second, the system for coupling/conjugating the molecule with the vector is rather complex; traditional chemical or biochemical coupling does not yield well-defined macromolecular systems from a structural and molecular point of view. Moreover, potential competition between the conjugates and the endogenous ligands for the targeted receptor can lead either to an inhibition of the physiological process of RMT or to a decrease in the concentration of the endogenous ligands required for the proper functioning of the brain. RMT receptors are involved in cell signaling processes in the cerebrum and the conjugates could potentially interfere with these processes.

International patent application WO2010/046588 describes for the first time peptides or pseudo-peptides that bind human LDLR and are able to carry across the BBB substances that can be of high MW and/or large volume.

The present application relates to novel peptides binding human LDLR, optimized for the transport of molecules.

The present invention provides optimized peptides or pseudo-peptides capable of transporting across cell membranes, and more specifically across the BBB, substances that can be of high MW and/or high volume. The invention thus makes it possible to improve the biodistribution or the bioavailability of molecules of interest, and notably to
5 improve their access (targeting) to the CNS.

In application WO2010/046588, the inventors have developed peptide derivatives capable of binding human LDLR. The inventors have shown that these derivatives are capable of crossing the BBB. The inventors have also shown that these derivatives can
10 transport, in BBB cells, molecules of therapeutic or diagnostic interest.

Continuing their research, the inventors succeeded in designing and testing novel peptides exhibiting advantageous properties for the transport of molecules. These novel peptides, capable of binding LDLR without competition with the natural ligand, and
15 thus without interference with the transport of endogenous LDL, represent novel products (vectors) that are particularly advantageous for the design and vectorization of diagnostic or imaging drugs or agents, notably to reach the CNS.

One object of the invention is thus a peptide or pseudo-peptide, characterized in that it
20 is of the following general formula (I):



wherein A1 represents cysteine or an analogue thereof or an isostere thereof, A2
25 represents proline or an analogue thereof or an isostere thereof, and A3 represents glycine or an analogue thereof or an isostere thereof.

According to preferred embodiments, A1 represents cysteine (Cys) or an analogue thereof selected from (D)-cysteine, penicillamine (Pen) and (D)-penicillamine ((D)-pen); A2 represents proline (Pro) or an analogue thereof selected from pipecolic acid (Pip) and thiazolidine-4-carboxylic acid (Thz); and/or A3 represents glycine (Gly), or sarcosine (Sar).
30

A preferred object of the invention is a peptide or pseudo-peptide of the following general formula (I'):



- 5 wherein A1 represents cysteine or an analogue thereof preferably selected from (D)-cys, Pen and (D)-pen; and A2 represents proline or an analogue thereof preferably selected from Pip and Thz.

Another particular object of the invention is a peptide or pseudo-peptide of the following general formula (I''):



wherein A1 represents cysteine or an analogue thereof preferably selected from (D)-cys, Pen and (D)-Pen; and A2 represents proline or an analogue thereof preferably selected from Pip and Thz. The alanine (Ala) can be of L or D configuration.

- 15 The peptides of the invention advantageously have the ability to bind human LDL receptor (hLDLR), with a high affinity. Furthermore, they are small, typically fewer than 10 amino acids, which is particularly advantageous. Particular examples of such peptides are peptides of sequences SEQ ID NOs: 1 to 10.

- 20 Another object of the invention relates to the use of a peptide or pseudo-peptide such as defined above for preparing a pharmaceutical or diagnostic composition to vectorize an active substance or a substance of diagnostic, imaging or therapeutic interest.

Another object of the invention relates to the use of a peptide or pseudo-peptide such as defined above for increasing the biological activity or for decreasing the toxicity of an active substance or of a substance of interest to which it is coupled.

- 25 Another object of the invention relates to a peptide or pseudo-peptide such as defined above for use to vectorize an active substance or a substance of diagnostic, imaging or therapeutic interest.

Another object of the invention relates to a peptide or pseudo-peptide such as defined above for use to increase the biological activity or to decrease the toxicity of an active substance or a substance of interest to which it is coupled.

Another object of the invention relates to any conjugated compound of the following
5 formula (II):



wherein V represents a peptide or pseudo-peptide such as defined above, D represents an active substance or a substance of interest, and x and y are integers between 1 and 5.

The invention also relates to any conjugated compound of the following formula (III):



wherein V represents a peptide or pseudo-peptide such as defined above, L represents a spacer, D represents an active substance or a substance of interest, x and y are integers between 1 and 5 and z is an integer between 1 and 10.

Another object of the invention relates to a pharmaceutical composition containing at
15 least one conjugated compound such as defined above and one or more pharmaceutically acceptable excipients.

Another object of the invention relates to a diagnostic composition characterized in that it contains a medical diagnostics or imaging agent comprised of a conjugated compound such as defined above.

20 Another object of the invention relates to a method for improving or enabling the passage of a molecule across the BBB, comprising the coupling of said molecule to a peptide or pseudo-peptide such as defined above.

Another object of the invention is an improved method for treating with a drug a pathology in a subject, the improvement consisting in coupling said drug with a peptide
25 or pseudo-peptide such as defined above.

The invention can be used in any mammal, in particular any human being.

DESCRIPTION OF FIGURES

5

Figure 1

Diagram illustrating the various modes of passage of natural or pharmacological molecules across the BBB, adapted from *Abbott and Romero, 1996, Mol. Med. Today, 2(3), 106-113.*

10

Figure 2

Comparative diagram of synthesis in tandem and synthesis via a linker of a vector/molecule of therapeutic interest conjugate.

15 Figure 3

A – Diagram of the plasmid used for cloning hLDLR mLDLR.

B – Diagram representing the fusion protein expressed by transfected cells.

Figure 4

20 Western blot performed on CHO cell lines expressing constitutively hLDLR-GFP or GFP (control) fusion proteins. A 190 kDa band corresponding to the size of the hLDLR-GFP fusion protein is detected with anti-hLDLR antibody.

Figure 5

25 Immunocytochemistry on non-permeabilized CHO cells, stably expressing either A – GFP alone (control), or B – the hLDLR-GFP construction. Cell nuclei are stained with Hoechst (blue, A1 and B1). GFP fluorescence is visible in green (A2 and B2), that of the staining of the extracellular domain of hLDLR by anti-hLDLR antibody is visible in red (A3 and B3) and the superimposing of red and green stains is visible in
30 yellow/orange (A4 and B4). Note that only the cells stably transfected with the hLDLR-GFP construction express the membrane receptor (B3).

Figure 6

- A – Cells of the CHO-hLDLR-GFP cell line expressing hLDLR-GFP (green) incubated with DiI-LDL (red), the superimposing of red and green stains are visible in yellow/orange: note the strong staining of the cells.
- 5 B – Cells of a CHO-TfR-GFP cell line expressing hTfR-GFP (green) incubated with DiI-LDL (red): absence of DiI-LDL binding and endocytosis.
- C – Cells of the CHO-hLDLR-GFP cell line expressing hLDLR-GFP (green) incubated with transferrin coupled to Texas Red (red): absence of Tf ligand binding and endocytosis.
- 10 Note the difference in intensity of staining between A on the one hand, and B and C on the other hand, where only the staining of hTfR and hLDLR receptors fused with GFP is detected.

Figure 7

- 15 General scheme of synthesized peptides conjugated with rhodamine or S-Tag, with a C-terminus (C-term) spacer.

DETAILED DESCRIPTION OF THE INVENTION

20

The invention relates to peptide derivatives capable of binding human LDLR and the use thereof in the field of pharmaceuticals, notably to transport, in BBB cells, molecules of therapeutic or diagnostic interest.

- 25 Human LDLR is a transmembrane protein of 839 amino acids comprising three regions: the extracellular region (1-768), the transmembrane region (768-790) and the cytoplasmic region (790-839). The extracellular region is divided into two subregions: that of LDL binding (1-322) and that outside the LDL binding region (322-768) (see *WO2007/014992*).

30

The brain has a great need for LDL to function properly. The natural ligands of LDLR are LDL and more particularly the apolipoprotein B (ApoB) and apolipoprotein E

(ApoE) components of LDL particles which thus enable the transport of cholesterol contained in these particles across cell membranes and more particularly across the BBB.

5 It has thus been shown that LDLR enabled the transcytosis of LDL particles across the BBB (*Dehouck et al., 1997, J. Cell Biol., 138(4), 877-889*), via an RMT process, in specific endosomal vesicles that prevent fusion with the lysosome. These lipoproteins, having crossed the BBB by transcytosis, are then taken up by neurons and/or astrocytes (*Spencer et al., 2007, Proc. Natl. Acad. Sci. USA, 104(18), 7594-7599*). This property
10 was used to vectorize molecules of therapeutic interest by nanoparticles on which are conjugated whole apolipoproteins (LDL components) (*Kreuter et al., 2007, J. Control. Release, 118, 54-58*). In this study, however, a whole apolipoprotein was used, and in a form coupled to a nanoparticle in order to mimic LDL particle structure.

International patent application No WO2010/046588 describes for the first time
15 peptides or pseudo-peptides that bind human LDLR and are able to carry across the BBB substances that can be of high MW and/or large volume.

The present application relates to novel peptides binding human LDLR, optimized for the transport of molecules. Peptide or pseudo-peptide vectors can be easily synthesized chemically, and most molecules of therapeutic interest or imaging or diagnostic agents
20 can be coupled with the peptide or pseudo-peptide vector simply and effectively through a prodrug strategy via a spacer (synthesis via a linker) or by direct coupling (synthesis in tandem) between the two entities (Figure 2). The inventive peptides and pseudo-peptides are designed to adopt a cyclic configuration, thus more resistant to proteolysis. Moreover, the inventive peptides and pseudo-peptides bind LDLR without competition
25 with the natural ligand.

The inventive peptides or pseudo-peptides can be used as vectors for molecules of therapeutic interest, or for imaging or diagnostic agents, or for any other molecule such as a molecular probe, in the treatment, imaging and/or diagnosis of neurological
30 pathologies, as well as infectious or cancerous pathologies of the brain or other tissues.

The peptides or pseudo-peptides described in the present invention have the capacity to target cell receptors/transporters and particular cell types and/or to cross cell membranes, notably those of the physiological barriers of the brain and more particularly the BBB or the blood-retinal barrier (BRB).

5

The peptides or pseudo-peptides described in the present invention have the capacity to target cell receptors/transporters and particular cell types, notably cancer cells, nervous or non-nervous tissue and/or to cross cell membranes, notably those of the physiological barriers of the CNS and more particularly the blood-tumor barrier (BTB) of cancerous nervous tissue.

10

The peptides or pseudo-peptides described in the present invention have the capacity to target cell receptors/transporters and particular cell types and/or to cross cell membranes, notably those of the physiological barriers of the CNS, to treat more particularly infectious brain pathologies or other pathologies of a bacterial, viral, parasitic or fungal nature/origin.

15

The peptides or pseudo-peptides described in the present invention have the capacity to bind to a murine or human LDLR of the cell membrane and to cross the aforesaid membrane via this receptor by transcytosis.

20

The peptides or pseudo-peptides described in the present invention have the capacity to bind to LDLR at the surface of cell membranes of physiological barriers of the murine and human brain and to cross the aforesaid physiological barrier via LDLR by RMT.

25

One object of the invention thus relates to peptides or pseudo-peptides, characterized in that they are of the following general formula (I):



30

wherein:

- A1 represents cysteine or an analogue thereof or an isostere thereof,

- A2 represents proline or an analogue thereof or an isostere thereof, and
- A3 represents glycine or an analogue thereof or an isostere thereof,

More particularly, group A1 represents a residue selected from cysteine (Cys, C), of D
 5 or L configuration, or a derivative thereof selected from 2-amino-3-mercaptopropanoic
 acid and S-substituted derivatives thereof, S-acetylcysteine or 2-amino-3-
 (acetylthio)propanoic acid, selenocysteine (Sec, U) or 2-amino-3-(seleno)propanoic
 acid, cysteinol, 3-mercaptopropanoic acid (Mpa) or penicillamine (Pen), of L or D
 configuration.

10

Group A2 represents more preferentially a residue selected from proline (Pro, P) or
 pyrrolidine-2-carboxylic acid, homoproline or 2-(2-pyrrolidinyl)ethanoic acid, 3-
 hydroxyproline (3Hyp), 4-hydroxyproline (4Hyp), 3-methylproline, 3,4-dehydroproline,
 3,4-methanoproline, 4-aminoproline, 4-oxoproline, thioproline or thiazolidine-4-
 15 carboxylic acid (Thz), 2-oxothiazolidine-4-carboxylic acid, indolin-2-carboxylic acid
 (Idc), pipecolic acid (Pip) or piperidin-2-carboxylic acid, nipecotic acid (Nip) or
 piperidin-3-carboxylic acid, 4-oxopipecolic acid, 4-hydroxypipecolic acid, amino-1-
 cyclohexanecarboxylic acid, prolinol.

In a preferred embodiment, group A2 is selected from proline, pipecolic acid (Pip) or
 20 thiazolidine-4-carboxylic acid (Thz).

Group A3 represents more preferentially a residue selected from glycine (Gly, G) or 2-
 aminoethanoic acid, sarcosine (Sar) or N-methylglycine (MeGly), N-ethylglycine
 (EtGly), allylglycine (allylGly) or 2-aminopent-4-enoic acid, 2-cyclopentylglycine
 25 (Cpg), 2-cyclohexylglycine (Chg), 2,2-dipropylglycine (Dpg), 2-(3-indolyl)glycine
 (IndGly), 2-indanylglycine (Igl), 2-neopentylglycine (NptGly), 2-octylglycine (OctGly),
 2-propargylglycine (Pra) or 2-amino pent-4-ynoic acid, 2-phenylglycine (Phg), 2-(4-
 chlorophenyl)glycine, azaglycine (AzGly), or glycinol or 2-aminoethanol.

In a preferred embodiment, group A3 is selected from glycine and sarcosine.

30 Among the peptides or pseudo-peptides of formula (I), particularly preferable are the
 peptides in which group A3 is glycine. Thus, a particular object of the invention relates
 to peptides of the following formula (I'):

A1-Met-A2-Arg-Leu-Arg-Gly-Cys (I')

wherein A1 represents cysteine or an analogue thereof or an isostere thereof, preferably A1 represents Cys, (D)-cys, Pen, or (D)-Pen; and A2 represents a proline analogue, preferably A2 represents Pip or Thz.

5 Particular examples of peptides according to the invention are described below:

SEQ ID NO: 1, (D)-cys-Met-Pip-Arg-Leu-Arg-Gly-Cys;

SEQ ID NO: 2, (D)-pen-Met-Pip-Arg-Leu-Arg-Gly-Cys;

SEQ ID NO: 3, Pen-Met-Pip-Arg-Leu-Arg-Gly-Cys;

10 SEQ ID NO: 4, (D)-cys-Met-Thz-Arg-Leu-Arg-Gly-Cys;

SEQ ID NO: 5, (D)-pen-Met-Thz-Arg-Leu-Arg-Gly-Cys;

SEQ ID NO: 6, Pen-Met-Thz-Arg-Leu-Arg-Gly-Cys;

SEQ ID NO: 7, (D)-cys-Met-Thz-Arg-Leu-Arg-Sar-Cys;

SEQ ID NO: 8, (D)-pen-Met-Thz-Arg-Leu-Arg-Sar-Cys;

15 SEQ ID NO: 9, Pen-Met-Thz-Arg-Leu-Arg-Sar-Cys;

SEQ ID NO: 10, (D)-cys-Met-Pro-Arg-Leu-Arg-(D)-ala-Cys.

The results obtained by the applicant show that the peptides of the invention have an improved affinity for LDLR. Thus, compared to the reference peptide SEQ ID NO: 17,
 20 all the peptides of formula (I) tested exhibit improved affinity, as seen in Table 1 (EXAMPLE III). These results are all the more remarkable as the modification of other residues in the sequence, such as for example the replacement of arginine residues, led to a substantial drop in affinity (see reference peptides SEQ ID NO: 11-16).

25 SEQ ID NO: 11, (D)-Cys-Met-Pro-hArg-Leu-Arg-Gly-Cys;

SEQ ID NO: 12, (D)-Cys-Met-Pro-Agb-Leu-Arg-Gly-Cys;

SEQ ID NO: 13, (D)-Cys-Met-Pro-Agp-Leu-Arg-Gly-Cys;

SEQ ID NO: 14, (D)-Cys-Met-Pro-Cit-Leu-Arg-Gly-Cys;

SEQ ID NO: 15, (D)-Cys-Met-Pro-Arg-Leu-Cit-Gly-Cys;

30 SEQ ID NO: 16, (D)-Cys-Met-Pro-Arg-Leu-Arg(NO₂)-Gly-Cys;

SEQ ID NO: 17, (D)-Cys-Met-Pro-Arg-Leu-Arg-Gly-Cys.

These results are also particularly remarkable taking into account the small size of the peptides (the peptides above contain 8 amino acids), which constitutes an additional advantage in their industrial use.

5

As indicated above, the cyclic peptides or pseudo-peptides of the invention can comprise peptide, non-peptide and/or modified peptide bonds. In a preferred embodiment, the peptides or pseudo-peptides comprise at least one peptidomimetic bond, chosen preferably among intercalation of a methylene (-CH₂-) or phosphate (-PO₂-) group, secondary amine (-NH-) or oxygen (-O-), alpha-azapeptides, alpha-alkylpeptides, N-alkylpeptides, phosphoramidates, depsipeptides, hydroxymethylenes, hydroxyethylenes, dihydroxyethylenes, hydroxyethylamines, retro-inverso peptides, methyleneoxy, cetomethylene, esters, phosphinates, phosphinics, phosphonamides and carba analogues.

15

Furthermore, in a particular embodiment, the peptides or pseudo-peptides of the invention comprise an N-term and/or C-term function respectively protected, for example, by acylation, or amidation or esterification.

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

25 For chemical syntheses, commercial apparatuses that can incorporate natural as well as non-natural amino acids, such as D enantiomers and residues with side chains with hydrophobicities and steric obstructions different from those of their natural homologues (so-called exotic, *i.e.*, non-coded, amino acids), or a peptide sequence containing one or more peptidomimetic bonds that can include notably intercalation of a
30 methylene (-CH₂-) or phosphate (-PO₂-) group, a secondary amine (-NH-) or an oxygen (-O-) or an N-alkylpeptide, are used.

During synthesis, it is possible to introduce various chemical modifications, such as for example, putting in the N-term or C-term position or on a side chain a lipid (or phospholipid) derivative or a constituent of a liposome or a nanoparticle, in order to be able to incorporate the peptide or pseudo-peptide of the invention within a lipid
5 membrane such as that of a liposome composed of one or more lipid layers or bilayers, or of a nanoparticle.

The peptides of the invention, or a proteic part thereof, can also be obtained from a nucleic acid sequence coding for the same. The present invention also relates to a
10 nucleic acid molecule comprising, or consisting of, a nucleic sequence coding for a peptide such as defined above. More particularly, the invention relates to a nucleic acid molecule comprising at least one sequence coding for a peptide of general formula (I). These nucleic acid sequences can be DNA or RNA and be combined with control sequences and/or be inserted in biological expression vectors.

15

The biological expression vector used is selected according to the host in which it will be transferred. It can be, for example, a plasmid, cosmid, virus, etc. The invention relates in particular to these nucleic acids and biological expression vectors, which can be used to produce the peptides of the invention, or proteic parts thereof, in a host cell.
20 These biological expression vectors can be prepared and the peptides can be produced or expressed in a host by molecular biology and genetic engineering techniques well known to those persons skilled in the art.

Another object of the invention relates to the use of a cyclic peptide or pseudo-peptide
25 such as defined above, as a vector for the transfer/transport of molecules of therapeutic interest, or of imaging or diagnostic agents, or of any other molecule.

The invention also relates to the use of a cyclic peptide or pseudo-peptide such as defined above for preparing a drug capable of crossing the BBB.

30

The invention also relates to a method for enabling or improving the passage of a molecule across the BBB, comprising the coupling of the molecule to a peptide or pseudo-peptide of the invention.

- 5 Thus, the invention relates in particular to a conjugate compound of formula (II) as follows:



- wherein V represents an peptide or pseudo-peptide of the invention, D represents an active substance or a substance of interest, and x and y are integers between 1 and 5. In
10 a particular embodiment, x and y are equal to 1, x is greater than y, or y is greater than x.

The invention also relates to a conjugate compound of formula (III) as follows:



- 15 wherein V represents an peptide or pseudo-peptide of the invention, L represents a spacer, D represents an active substance or a substance of interest, x and y are integers between 1 and 5 and z is an integer between 1 and 10. In a particular embodiment, $x=z=y=1$ or $x=z>y$ or $y=z>x$ or $z>x>y$.

- 20 The active substance or substance of interest can be any molecule of pharmaceutical interest, notably therapeutic, a diagnostic or medical imaging agent, or a molecular probe. It can be in particular any chemical entity of biological interest such as a small chemical molecule (antibiotic, antiviral, immunomodulator, antineoplastic, anti-inflammatory, etc.), a peptide or polypeptide, a protein (enzyme, hormone, cytokine,
25 apolipoprotein, growth factor, antigen, antibody or part of an antibody), a nucleic acid (ribonucleic acid or deoxyribonucleic acid of human, viral, animal, eukaryotic or prokaryotic, plant or synthetic origin, etc., whose size can range from that of a single oligonucleotide to that of the genome or a genome fragment), a viral genome or a plasmid, a ribozyme, a marker or a tracer. Generally, the "substance of interest" can be
30 any drug active ingredient, whether a chemical, biochemical, natural or synthetic compound. The expression "small chemical molecule" designates a molecule of

pharmaceutical interest with a maximum molecular weight of 1000 Daltons, typically between 300 Daltons and 700 Daltons.

- 5 The invention also relates to a compound of following formula (IV):



wherein V represents an peptide or pseudo-peptide of the invention, L represents a spacer, x is an integer between 1 and 5 and z is an integer between 1 and 10. In a particular embodiment, $x=z=1$ or $z>x$.

10

In the conjugate compounds of the invention, coupling between V and D, or between V and L on the one hand and L and D on the other hand, can be carried out by any acceptable means of bonding taking into account the chemical nature, obstruction and number of associated active substances and peptides or pseudo-peptides. Coupling can thus be carried out by one or more covalent, ionic, hydrogen, hydrophobic or Van der
 15 Waals bonds, cleavable or non-cleavable in physiological medium or within cells. Furthermore, D can be coupled with V, if need be via L, at various reactive groups, and notably at one or more N-term and/or C-term ends of V and/or at one or more reactive groups carried by the natural or non-natural amino acid side chains constitutive of V.

20

Coupling can be carried out at any site of the peptide or pseudo-peptide where functional groups such as -OH, -SH, -CO₂H, -NH₂, -SO₃H or -PO₂H are naturally present or have been introduced. Thus, a therapeutic molecule of interest, or a diagnostic (or a medical imaging) agent or any other molecule such as a molecular
 25 probe can be linked (coupled) to the peptide or pseudo-peptide vector by a covalent bond either at the N-term or C-term ends, or at the reactive groups carried by the natural or non-natural amino acid side chains of this peptide sequence.

Similarly, coupling can be carried out at any site of the active substance or substance of
 30 interest (molecule of therapeutic interest, diagnostic or medical imaging agent, any other molecule such as a molecular probe) where, for example, functional groups such

as -OH, -SH, -CO₂H, -NH₂, -SO₃H, -PO₂H are naturally present or have been introduced.

It is preferable that the interaction is sufficiently strong so that the peptide is not dissociated from the active substance before having reached its site of action. For this reason, the preferred coupling of the invention is covalent coupling, but non-covalent coupling could be used, however. The substance of interest can be coupled directly with the peptide (synthesis in tandem) either at one of these terminal ends (N-term or C-term), or at a side chain of one of the constitutive amino acids of the sequence (*Majumdar and Siahaan, Med Res Rev., Epub ahead of print*). The substance of interest can also be coupled indirectly by means of a linker or spacer, either at one of the terminal ends of the peptides, or at a side chain of one of the constitutive amino acids of the sequence (Figure 2). Means of covalent chemical coupling, calling upon a spacer or not, include those selected from bi- or multifunctional agents containing alkyl, aryl or peptide groups by esters, aldehydes or alkyl or aryl acids, anhydride, sulfhydryl or carboxyl groups, groups derived from cyanogen bromide or chloride, carbonyldiimidazole, succinimide esters or sulfonic halides.

In this respect, the invention also relates to a method for preparing a conjugate compound such as defined above, characterized in that it comprises a step of coupling between a peptide or pseudo-peptide V and a substance D, if need be via L, preferably by a chemical, biochemical or enzymatic pathway, or by genetic engineering.

The invention also relates to a pharmaceutical composition characterized in that it comprises at least one conjugate compound such as defined above and one or more pharmaceutically acceptable excipients.

The invention also relates to a diagnostic composition characterized in that it comprises a diagnostic or medical imaging agent composed of a conjugate compound such as defined above.

The conjugate can be used in the form of any pharmaceutically acceptable salt. The expression “pharmaceutically acceptable salts” refers to, for example and in a non-restrictive way, pharmaceutically acceptable base or acid addition salts, hydrates, esters, solvates, precursors, metabolites or stereoisomers, said vectors or conjugates loaded
 5 with at least one substance of interest.

The expression “pharmaceutically acceptable salts” refers to nontoxic salts, which can be generally prepared by reacting a free base with a suitable organic or inorganic acid. These salts preserve the biological effectiveness and the properties of free bases.
 10 Representative examples of such salts include water-soluble and water-insoluble salts such as acetates, N-methylglucamine ammonium, amsonates (4,4-diaminostilbene-2,2'-disulphonates), benzenesulphonates, benzonates, bicarbonates, bisulphates, bitartrates, borates, hydrobromides, bromides, butyrates, camsylates, carbonates, hydrochlorates, chlorides, citrates, clavulanates, dichlorhydrates, diphosphates, edetates, calcium
 15 edetates, edisylates, estolates, esylates, fumarates, gluceptates, gluconates, glutamates, glycolylarsanylates, hexafluorophosphates, hexylresorcinates, hydrabamines, hydroxynaphthoates, iodides, isothionates, lactates, lactobionates, laurates, malates, maleates, mandelates, mesylates, methylbromides, methylnitrates, methylsulphates, mucates, napsylates, nitrates, 3-hydroxy-2-naphthoates, oleates, oxalates, palmitates, pamoates (1,1-methylene-bis-2-hydroxy-3-naphthoates, or emboates), pantothenates,
 20 phosphates, picrates, polygalacturonates, propionates, p-toluenesulphonates, salicylates, stearates, subacetates, succinates, sulphates, sulphosalicylates, suramates, tannates, tartrates, teoclates, tosylates, triethiodides, trifluoroacetates and valerianates.

25 The compositions of the invention advantageously comprise a pharmaceutically acceptable vector or excipient. The pharmaceutically acceptable vector can be selected from the vectors classically used according to each mode of administration. According to the mode of administration envisaged, the compounds can be in solid, semi-solid or liquid form. For solid compositions such as tablets, pills, powders, or granules that are
 30 free or are included in gelatin capsules, the active substance can 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 and aluminum silicate, starch paste, gelatin, tragacanth, methylcellulose, sodium carboxymethyl cellulose and/or polyvinylpyrrolidone; d) disintegrants, for example starch, agar, alginic acid or its sodium salt, or effervescent mixtures; and/or d) absorbents, dyes, flavoring agents and sweeteners. The excipients can be, for example, mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate and analogues of pharmaceutical quality. For semi-solid compositions such as suppositories, the excipient can, for example, be an emulsion or oily suspension, or polyalkylene glycol-based, such as polypropylene glycol. Liquid compositions, in particular injectables or those 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 solution, aqueous dextrose, glycerol, ethanol, oil and analogues thereof.

The compositions or conjugates of the invention can be administered by any suitable route and, in a non-restrictive way, by parenteral route, such as, for example, in the form of preparations that can be injected by subcutaneous, intravenous or intramuscular route; by oral route (or *per os*), such as, for example, in the form of coated or uncoated tablets, gelatin capsules, powders, pellets, suspensions or oral solutions (one such form for oral administration can be either with immediate release or with extended or delayed release); by rectal route such as, for example, in the form of suppositories; by topical route, in particular by transdermal route, such as, for example, in the form of patches, pomades or gels; by intranasal route such as, for example, in aerosol and spray form; by perlingual route; or by intraocular route.

The pharmaceutical compositions typically comprise an effective dose of a peptide or pseudo-peptide or conjugate of the invention. A “therapeutically effective dose” as described herein refers to the dose that gives a therapeutic effect for a given condition and administration schedule. It is typically the average dose of an active substance to administer to appreciably improve some of the symptoms associated with a disease or a pathological state. For example, in treating a cancer of the brain or of other tissue, a pathology, a lesion or a disorder of the CNS, the dose of an active substance that

decreases, prevents, delays, eliminates or stops one of the causes or symptoms of the disease or disorder would be therapeutically effective.

5 A “therapeutically effective dose” of an active substance does not necessarily cure a disease or disorder but will provide a treatment for this disease or disorder so that its appearance is delayed, impeded or prevented, or its symptoms are attenuated, or its term is modified or, for example, is less severe, or the recovery of the patient is accelerated.

10 It is understood that the “therapeutically effective dose” for a person in particular will depend on various factors, including the activity/effectiveness of the active substance, its time of administration, its route of administration, its rate of elimination and its metabolism, drug combinations/interactions and the severity of the disease (or disorder) treated on a preventive or curative basis, as well as the age, weight, overall health, sex and/or diet of the patient.

15

Depending on the substance coupled, the conjugates and compositions of the invention can be used for treating, preventing, diagnosing or imaging numerous pathologies, notably pathologies affecting the CNS, infectious pathologies or cancers.

20 In this respect, the invention relates to the use of pharmaceutical conjugates or compositions as described above for treating or preventing CNS pathologies or disorders, brain tumors or other cancer cells, and bacterial, viral, parasitic or fungal infectious pathologies of the brain or other tissues.

25 The invention also relates to the use of the pharmaceutical conjugates or compositions as described above for diagnosing or imaging CNS pathologies or disorders, brain tumors or other cancer cells, and bacterial, viral, parasitic or fungal infectious pathologies of the brain or other tissues.

30 The invention also relates to the use of a conjugate or composition such as defined above for treating, imaging and/or diagnosing a brain tumor or other types of cancer cells. Studies have indeed shown that patients with certain cancers have

hypocholesterolemia. This hypocholesterolemia is the consequence of an overuse of cholesterol by cancer cells. The latter to survive induce an increase in the level of LDLR expression within organs with tumors (*Henricksson et al., 1989, Lancet, 2(8673), 1178-1180*). There is thus a correlation between the increase in the level of LDLR expression
 5 by cells and certain cancers. It has also been recently shown that the number of LDLR is very high on the surface of certain pathological cells such as cancer cells. It is generally accepted that 1000 to 3000 LDLR are present at the surface of a non-pathological cell. Similarly, non-pathological neurons have only few LDLR (*Pitas et al., 1987, J. Biol. Chem., 262, 14352-14360*). In the case of the glioblastoma, LDLR overexpression has
 10 been shown. Thus, on the surface of brain tumor cells, 125,000 (for U-251 cells) to 900,000 (for SF-767 cells) LDLR have been counted (*Malentiska et al., 2000, Cancer Res., 60, 2300-2303; Nikanjam et al., 2007, Int. J. Pharm., 328, 86-94*). It should also be noted that many tumor cells overexpress LDLR, such as those of prostate cancer (*Chen et al., 2001, Int. J. Cancer, 91, 41-45*), colon cancer (*Niendorf et al., 1995, Int. J. Cancer, 61, 461-464*), leukemia (*Tatidis et al., 2002, Biochem. Pharmacol., 63, 2169-2180*), colorectal cancer (*Caruso et al., 2001, Anticancer Res., 21, 429-433*), breast cancer (*Graziani et al., 2002, Gynecol. Oncol., 85, 493-497*), as well as cancers of the liver, pancreas, ovaries, lung and stomach, etc.

20 The invention also relates to the use of a conjugate or composition such as defined above for treating, imaging and/or diagnosing bacterial, viral, parasitic or fungal infectious pathologies of the brain or other tissues, such as, and in a non-restrictive way, AIDS or meningitis, etc. LDLR is also present on hepatic cells. It is now known that endocytosis of the hepatitis C virus (HCV) can occur via LDLR. LDLR could serve as a
 25 viral receptor at an early stage of infection of human hepatocytes by HCV (*Molina et al., 2007, J. Hepatol., 46(3), 411-419*). The inventive conjugates can thus be used to specifically target pathological cells, infected by viruses such as those of hepatitis B and hepatitis C that express LDLR and/or to modulate via LDLR the viral infection process of healthy cells.

30

The invention also relates to the use of a conjugate or composition such as defined above for treating, imaging and/or diagnosing neurodegenerative pathologies such as, in

a non-restrictive manner, Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jakob disease, cerebrovascular accidents (CVA), bovine spongiform encephalopathy, multiple sclerosis, amyotrophic lateral sclerosis, etc.

- 5 The invention also relates to the use of a conjugate or composition such as defined above for treating, imaging and/or diagnosing neurological pathologies such as, in a non-restrictive manner, epilepsy, migraine, encephalitis, CNS pain, etc.

- 10 The invention also relates to the use of a conjugate or composition such as defined above for treating, imaging and/or diagnosing neuropsychiatric pathologies such as, in a non-restrictive manner, depression, autism, anxiety, schizophrenia, etc.

- The terms "treatment," "treating," "treat" and other similar expressions refer to obtaining a pharmacological and/or physiological effect, for example, inhibition of
15 cancer cell growth, cancer cell death or improvement of a disease or neurological disorder. The effect can be prophylactic or preventive in order to completely or partially prevent the aggravation of a disease or a symptom such a disease, in an ill person, or its propagation, in healthy subjects, and/or can be therapeutic in order to completely or partially treat a disease and/or its related harmful effects. The term "treatment" as used
20 in the present document covers any treatment of a disease in a mammal, and more particularly in man, and comprises: (a) prevention of a disease (for example, prevention of cancer) or a condition that can arise in a person predisposed to this pathology or disorder, but who has not yet been positively diagnosed, (b) the slowing of a disease (for example, by stopping its development), or (c) relief from a disease (for example, by
25 reducing the symptoms associated with a disease). This term "treatment" also covers any administration of an active substance in order to tend, cure, relieve, improve, decrease or inhibit a condition in an individual or patient, including, but not limited to, the administration to a person in need of a drug composed of a vector or conjugate as described in the present document.

30

The present invention also relates to the use of a peptide or pseudo-peptide of the invention to increase the biological activity of an active substance or of a substance of

interest (therapeutic molecule of interest, diagnostic or medical imaging agent, or any other molecule such as a molecular probe) to which it is coupled.

5 The present invention also relates to the use of a peptide or pseudo-peptide of the invention to decrease the toxicity of an active substance or of a substance of interest (therapeutic molecule of interest, diagnostic or medical imaging agent, or any other molecule such as a molecular probe) to which it is coupled.

10 Other aspects and advantages of the present invention will become apparent upon consideration of the examples below, which are only illustrative in nature and which do not limit the scope of the present application.

EXAMPLES

EXAMPLE I

Construction of CHO cell lines stably expressing human and murine LDLR.

5

The inventors identified peptides on the basis of their interaction with and affinity for human and murine low-density lipoproteins receptors (hLDLR and mLDLR), which are notably involved in the endocytosis and transcytosis (transcellular transport, notably across the BBB) of cholesterol. The prerequisite to the characterization of these peptides

10 was the establishment in eukaryotic cells (Chinese hamster ovary cells, CHO) of stable cell lines expressing hLDLR and mLDLR constitutively and at high rates. These cell lines are used: I) for the characterization of peptides binding to hLDLR expressed at the cell surface, in its native configuration; II) to verify that hLDLR and mLDLR can internalize the selected peptides by endocytosis.

15 The construction of these cell lines has been described in application WO2010/046588. Briefly, the messenger RNA sequences coding for hLDLR and mLDLR are available in databases (accession numbers: NM_000527 and NM_010700, respectively). The primers necessary for cDNA amplification by PCR were selected, comprising at their end (in bold type) the restriction sites necessary (HindIII and SalI for human LDLR and

20 HindIII and KpnI for murine LDLR) for cloning in the pEGFP-N1 expression vector (Clontech):

hLDLR

Forward primer: ATATATA**AAGCTTC**GAGGACACAGCAGGTCGTGAT (SEQ ID

25 NO: 18)

Reverse primer: TTAATT**GTCGACC**ACGCCACGTCATCCTCCAGACT (SEQ ID

NO: 19)

mLDLR

30 Forward primer: ATATATA**AAGCTT**GACGCAACGCAGAAGCTAAG (SEQ ID

NO: 20)

Reverse primer: TTAATTGGTACCGTTGCCACATCGTCCTCCAG (SEQ ID NO: 21)

- Total RNA prepared from human and murine brains were transformed into cDNA by reverse transcription for PCR amplification of DNA fragments coding for hLDLR and mLDLR. After amplification, the PCR products were digested by HindIII-Sall and HindIII-KpnI restriction enzymes, respectively, and ligated in the pEGFP-N1 expression vector (Clontech), digested by the same restriction enzymes. After transfection in eukaryotic cells, this vector enables the expression, under control of the CMV promoter, of LDLR fused with GFP at their C-term end, *i.e.*, at the end of their intracellular domains (Figure 3). After transforming competent *E. coli* DH5a bacteria, obtaining isolated colonies and preparing plasmid DNA, both strands of the constructions were sequenced in their entirety for verification.
- Transitory transfections in various cell lines (CHO, COS, N2A and HEK293) were carried out to determine on living or fixed cells the expression levels and membrane location of hLDLR and mLDLR. The receptor is directly visible on living cells, under fluorescence microscopy, without the need for immunostaining, by virtue of green fluorescence emitted by GFP fused at the C-term of these receptors. Stable transfectants were selected by limit dilution and by the geneticin resistance gene (G418) carried by the expression vector. These cell lines were amplified while maintaining selective pressure. In the example cited here, the expression of hLDLR-GFP of the expected size was verified by Western blot on cell lysates with antibodies directed against human LDLR and against GFP. A protein corresponding to the combined sizes of GFP and hLDLR (190 kDa) is recognized by anti-hLDLR (Figure 4) and anti-GFP antibodies in cell extracts prepared from stable cell lines. A CHO cell line expressing GFP constitutively was used as negative control. The anti-hLDLR antibody detects no protein in the GFP cell line.
- Immunocytochemistry with anti-hLDLR antibody on fixed (PFA) cells of the CHO-GFP (control) and CHO-hLDLR-GFP cell lines shows that hLDLR-GFP fusion is well expressed in the transfected cells. Immunocytochemistry experiments on non-

permeabilized cells with Triton X100 show that the extracellular domain of LDLR is well detected at the extracellular level (Figure 5).

Co-location between hLDLR and its natural ligand LDL, made fluorescent by adsorption of DiI, a fluorescent marker, is shown. This natural fluorescent ligand (DiI-LDL) quickly is internalized (endocytosis) as visualized under fluorescence microscopy on fixed cells (Figure 6-A). In contrast, DiI-LDL is not incorporated by endocytosis by the control CHO-GFP cell line, or by another control CHO cell line, which overexpresses, for example, human transferrin receptor (hTfR, Figure 6-B), another receptor involved in transcytosis. Moreover, endocytosis activity of the CHO-LDLR-GFP cell line is specific to LDLR ligand since in this cell line endocytosis of ligand not specific for it, for example transferrin (Tf) stained with red fluorochrome (Texas Red, Figure 6-C), is not observed.

Receptor functionality (endocytosis capacity) is confirmed by real-time experiments, under video fluorescence microscopy showing that LDL, natural ligand of hLDLR, stained with DiI, is actually transported rapidly and very effectively in cells expressing hLDLR-GFP compared with cells expressing GFP alone or cells expressing another receptor involved in endocytosis such as hTfR (negative control). Conversely, video microscopy experiments carried out with Tf stained with Texas Red, which is very efficiently incorporated by endocytosis by cells expressing hTfR-GFP, confirm that transferrin is not incorporated by endocytosis by cells of a control GFP cell line or by cells of an hLDLR cell line.

In spite of a high hLDLR expression level in CHO-hLDLR-GFP cell lines, the endocytosis system is not only effective, but it preserved its selectivity. The presence of GFP fusion altered neither the membrane insertion properties of hLDLR, exposure outside the cell of the hLDLR extracellular domain, nor the functionality of the receptor in endocytosis processes.

30

EXAMPLE II

Synthesis of peptides and coupling with a tracer molecule (biotin, fluorescein, or S-Tag with enzymatic activity).

Peptides were synthesized by the solid phase peptide synthesis (SPPS) method on an
 5 Advanced ChemTech Apex396 (AAPPTec) synthesizer, or a Liberty™ (CEM) microwave synthesizer, using an Fmoc/tBu strategy on Rink Amide AM resin on polystyrene-1% DVB, Wang on polystyrene-1% DVB, Barlos (2-chlorotrityl chloride) on polystyrene-1% DVB, or Sieber Amide on polystyrene-1% DVB. The load (or substitution) is between 0.25 mmol/g and 1.6 mmol/g according to the resin used.

10 The amino acids N-protected by Fmoc (or Boc for certain N-term ends) and/or protected by orthogonal functions (notably acid labile functions) at their side chains, the chemical coupling and deprotection reagents and the solvents are bought from specialised companies and are used as-is.

The Rink Amide and Wang resins make it possible to synthesize peptide sequences
 15 completely deprotected on their side chains and their C-term ends. This is thus 2-dimension (Fmoc/tBu) orthogonal SPPS.

Barlos and Sieber hypersensitive acid labile (HAL) resins respectively enable the release of the terminal (C-term) acid or amide function while preserving the orthogonal side protections of the various amino acids of the synthesized peptide as well as the
 20 terminal (N-term) amine protection of the amine function of its last amino acid (for example, N-acetylation for questions of stability of the neosynthesized peptide sequence). These type of resins, via an Fmoc (Prot₁) synthesis strategy, makes it possible to use acid-labile orthogonal side protections (Prot₂: Boc, tBu, OtBu, Trt, Mmt, Acn, etc.) cleavable only in strongly acid medium, whereas the protected peptide is
 25 cleaved in very weak acid conditions. This type of cleavage makes it possible to recover the peptide sequence completely protected on its side functions (Prot₂) with a view in particular to coupling a therapeutic molecule of interest with the peptide. This is thus three-dimension (Barlos or Sieber/Fmoc/tBu) orthogonal SPPS.

The standard orthogonal side protections (Prot₂) used for each amino acid during
 30 peptide synthesis are: Arg(N-Pbf), Arg(N-Pmc), Asn(N-Trt), Asp(O-tBu), Cys(S-Acn), Cys(S-Mmt), Cys(S-4MeBn), Cys(S-tBu), Cys(S-Tmob), Cys(S-Trt), Glu(O-tBu), Gln(N-Trt), His(N-Trt), Lys(N-Boc), Pen(S-Acn), Pen(S-Trt), Ser(O-tBu), Thr(O-tBu),

Trp(N-Boc), Tyr(O-tBu) (*Applied Biosystems, 1998, Cleavage, Deprotection, and Isolation of Peptides after Fmoc Synthesis. Technical Bulletin*). Gly, Sar, Ala, Val, Leu, Ile, Phe, Met, Pro, Pip and Thz do not have side protections, since their respective chemical structures do not require it.

- 5 Amino acids are coupled via activation of the acid function of the n+1 amino acid using DIEA/HBTU/HOBt or DIPC/HOBt in DMF.

Deprotection of the Fmoc (Prot₁) group of a new amino acid thus coupled is carried out using 20% piperidin in DMF.

- 10 The last amino acid coupled during peptide sequencing will either be protected by a Boc function (with a view to releasing its free terminal amine function at the end of synthesis), or acetylated (in order to stabilize the synthesized neopeptide but also to reduce the risks of secondary reactions during covalent coupling of the therapeutic molecule of interest in the C-term position, for example) or propionylated.

- 15 According to the peptide synthesized, disulfide bridges are obtained by intramolecular cyclisation from two thiol functions of two suitably protected Cys (Acm, Trt, tBu, etc.), either in solution or on resin, using reagents classically used by those persons skilled in the art: H₂O/AcOH/,(NH₄)₂CO₃/DMSO, H₂O/AcOH/,(NH₄)₂CO₃, I₂/DMF, I₂/HFIP/DCM, TFA/DMSO/anisole, I₂/DCM/MeOH/H₂O, etc. A Cys in the N-term position can advantageously be replaced by Pen or Mpa for cyclisation via a disulfide
20 bridge. Lanthionine bridges (by cyclisation via dehydroalanine) or dicarba (by cyclisation via allylGly) can also be obtained by synthesis pathways known to those persons skilled in the art. A lactam bridge can be created between the side acid function of a Glu (or Asp) residue and a side amine function on a Lys or an N-term amine. Similarly, cyclisation between the N-term amine function and the C-term acid function
25 (head/tail) can be carried out via an amide bond, just as cyclisation between the side amine function of Lys and the C-term acid function of the peptide (*Majumdar and Siahhaan, Med Res Rev., Epub ahead of print*).

- Peptides from Barlos or Sieber resins are cleaved by methods classically used by those persons skilled in the art, either with 0.5% TFA (v/v) in DCM, or with
30 AcOH/TFE/DCM (1/1/3), or with HFIP (30%) in DCM, or with TFE (30%) in DCM, etc.

Deprotection of side chains, and cleavage of peptides from Rink Amide or Wang resins, are carried out by methods classically used by those persons skilled in the art: either with TFA/H₂O/TIS or TIPS (95/2.5/2.5), or with TFA/H₂O/EDT/TIS or TIPS (94/2.5/2.5/1), or with TFA/thioanisole/H₂O (94/5/1), or with TFA/TIS/H₂O/thioanisole
 5 (90/5/3/2), or with TFA/H₂O/phenol/thioanisole/EDT (82.5/5/5/5/2.5), etc.

Biotins, fluoresceins, or S-Tag (see EXAMPLE IV, below) are generally introduced in the C-term position, these tracers are sometimes couples coupled in the N-term position, according to classic synthesis and coupling methods known to those persons skilled in the art.

10 Peptides are isolated and purified by HPLC on a Beckman System Gold 126 apparatus with a Chromolith C18 (4.6 mm x 50 mm) or Nucleosil C18 (10 mm x 250 mm) column with, for example, a 0% to 100% acetonitrile gradient in an aqueous phase (H₂O + 0.1% TFA) in 3.5 min then 100% to 0% in 1.5 min (flow rate: 1 ml/min to 5 ml/min), or on a Waters 1525 system with a Chromolith Speed ROD RP-18 (4.6 mm x 50 mm) column
 15 (stationary phase) with detection by a Waters 996 PDA detector (190 nm – 400 nm), or on a Waters Alliance 2690 system with a Chromolith Performance RP-18 (3 mm x 100 mm) column (stationary phase) with detection by a Waters 996 PDA detector (190 nm – 400 nm). UV detection is carried out at 214 nm and 254 nm.

Preparative purifications are carried out with a Waters Prep LC 4000 system with a
 20 Guard-Pak[™] column (stationary phase) with Delta-Pak[™] C18 cartridges (25 mm x 10 mm) with detection by a Waters 2487 Dual Wavelength Absorbance Detector.

Molecular weights are determined using an electrospray ionisation (ESI) mass spectrometer in positive mode. The spectra are obtained by using a Waters Micromass Quattro Micro (quadrupole analyzer) equipped with a Waters Alliance 2690 HPLC
 25 system enabling LC-MS coupling.

The LC-MS analysis conditions used are as follows:

- Chromolith Flash C18 column (4.6 mm x 25 mm),
- 3 ml/min flow rate,
- linear gradient from 0% to 100% of B in 2.5 min (A: 0.1% H₂O/HCO₂H; B: 0.1%
 30 ACN/HCO₂H).

Mass spectra in positive electrospray mode are acquired at a flow rate of 100-200 µl/min. The data are obtained in scan mode from 200-1700 *m/z* with 0.1 s intervals.

EXAMPLE III

Peptide vectors design.

- 5 The peptide of sequence SEQ ID NO: 17/S-Tag, described in application WO2010/046588, was used as the reference.

From this peptide, various approaches were used to try to improve binding, synthesis and vectorization properties. Many peptides were thus synthesized, differing from SEQ
10 ID NO: 17 by the deletion and/or the replacement and/or the chemical modification of one or more amino acid residues.

The capacity of the peptides thus synthesized to bind hLDLR was determined.

- 15 To this end, adherent and confluent CHO-hLDLR-RFP cells were cultivated in 6-well plates. Three wells of cells are used per condition.

A solution containing 10 μ M of peptide SEQ ID NO: 17/S-Tag was prepared in HamF12-1% BSA culture medium. To this solution was added 10 μ M of the peptide to evaluate (competition).

- 20 Several control solutions are also prepared:

- (i) HamF12-1% BSA medium.
- (ii) HamF12-1% BSA medium + 10 μ M control peptide CTRL-S-Tag (evaluation of nonspecific binding of any peptide comprising an S-Tag).
- (iii) HamF12-1% BSA medium + 10 μ M peptide SEQ ID NO: 17/S-Tag + 10 μ M
25 control peptide CTRL (evaluation of “nonspecific” competition between the peptide of interest and the control peptide CTRL).

The FRET approaches used are those described in EXAMPLE IV.

- 30 The results obtained for the best peptides are presented in table 1 below. The table gives the results of the competitions in % of reference peptide vector (SEQ ID NO: 17/S-Tag), affinity for hLDLR, displaced by the peptides of the invention. The larger the

displacement value, the more affinity the peptide has for hLDLR. When this value is greater than 50%, the peptide has an affinity greater than that of the reference peptide (SEQ ID NO: 17).

5 Table 1

Test peptides	Displacement of the control peptide
SEQ ID NO: 1	66%
SEQ ID NO: 2	70%
SEQ ID NO: 3	75%
SEQ ID NO: 4	88%
SEQ ID NO: 5	91%
SEQ ID NO: 6	77%
SEQ ID NO: 7	74%
SEQ ID NO: 8	84%
SEQ ID NO: 9	80%
SEQ ID NO: 10	54%
SEQ ID NO: 11	22%
SEQ ID NO: 12	23%
SEQ ID NO: 13	8%
SEQ ID NO: 14	9%
SEQ ID NO: 15	< 5%
SEQ ID NO: 16	< 5%
SEQ ID NO: 17	50%

The results presented show that the peptides of sequences SEQ ID NOs: 1 to 10 have improved affinity for human HDL receptor. These results are particularly interesting considering the small size of the peptides, and are particularly remarkable and surprising considering the negative effects on affinity observed with peptides SEQ ID NO: 11-16.

Thus, while being based on a medicinal chemistry optimization approach, the inventors progressed in the understanding of the structure/activity (affinity) relationships of the reference peptide vector (SEQ ID NO: 17), and in particular:

- 5 (i) the importance of each amino acid residue (Ala-scan, D-scan), *i.e.*, the alternative replacement (one by one) of each original residue either by an alanine, or by their non-natural analogue of D configuration;
 - (ii) the importance of N- and C-term ends (acetylation, amidation, etc.);
 - (iii) the importance/advantage of cyclization of the peptide.
- 10 The principal limitations generally attributed to peptide derivatives used in therapeutics are:
- (i) their low oral bioavailability (their administration by intravenous route is generally necessary);
 - (ii) a short half-life due to their rapid breakdown by proteolytic enzymes (in
15 particular peptidases or proteases) of the digestive system and blood plasma;
 - (iii) their rapid elimination from circulating blood by the liver (hepatic clearance) and the kidneys (renal clearance);
 - (iv) their low capacity to pass through biological or physiological membranes because of their generally hydrophilic character;
 - 20 (v) their great conformational flexibility, which may involve a lack of selectivity leading to interactions with different receptors or targets (resulting in weak specific biodistribution), which causes the activation of several targets and leads to side effects or undesirable effects;
 - (vi) the high costs of synthesis and industrial production (the production cost of a
25 5 kDa peptide are greater than the production cost of a 500 Da organic molecule).

In a rather remarkable way, the peptides or pseudo-peptides of the invention have the following pharmacological prerequisites:

- 30 (i) a high affinity for hLDLR;

- (ii) physicochemical characteristics (cyclization via a disulfide bridge; up to 3 non-natural amino acids for some; a size reduced to 8 amino acids) promoting their specificity for this receptor;
- (iii) a greater resistance to enzymatic proteolysis because of their cyclization, the introduction of non-natural amino acids and for some a pseudo-peptide bond, but also by modifying/blocking the N- and C-term ends;
- (iv) a small size and a molecular weight near 1 Da making it possible to reduce the costs of synthesis and of future production on an industrial scale.

10 EXAMPLE IV

Binding and endocytosis of synthesized peptides with affinity for hLDLR in CHO-LDLR-GFP cell lines.

Inventive peptides with affinity for hLDLR-GFP are coupled/conjugated in the C-term position with various tracer molecules, either rhodamine or S-Tag, separated by a spacer generally composed of three Gly residues (Figure 7). S-Tag (a 15 amino acid peptide derived from sequence 1-15 of bovine pancreatic ribonuclease A) on the one hand can be recognised by an anti-S-Tag antibody for immunocytochemistry or FACS approaches, and on the other hand can reconstitute enzymatic activity by binding with ribonuclease S-protein (C-term portion, amino acids 21-124) in tests of activity *in vitro* using the FRETWorks S-Tag assay kit (Novagen 70724-3). The ribonuclease thus activated digests an RNA substrate releasing a masked fluorescent agent visualised by FRET (fluorescence resonance energy transfer) and quantified in a 96-well plate in a Beckmann spectrofluorimeter. For these FRET experiments, control CHO cells were used and GFP fused in the C-term position with hLDLR and mLDLR, which generates strong background noise at the wavelengths used for FRET, was replaced by red fluorescent protein (RFP). The stable cell lines generated for the FRET experiments are thus CHO-RFP and CHO-hLDLR-RFP.

30 For the FRET approaches, cells are washed twice with 2 ml PBS and then incubated for 1 h at 37 °C with 250 µl peptide solution. They are again washed twice with 2 ml PBS and then twice with 1 ml PBS, and then scraped in 1 ml PBS and centrifuged for 5 min

at 1250 rpm. The supernatant is then aspirated and the cell pellet is lysed in 80 μ l PBS + 0.1% Triton X100. Twenty μ l of each cell lysate is analyzed by measuring fluorescence emission after the FRET reaction.

- 5 Thus, experiments involving the incubation of peptides on various cells expressing hLDLR are performed demonstrating that the peptides bind well to CHO-LDLR-GFP cells and that they are incorporated by endocytosis to accumulate in the cells of the cell line that expresses hLDLR, which is not the case for the control peptides. In these experiments, preliminary incubation of peptides conjugated with S-Tag, with a primary
10 antibody (primary Ab) directed against S-Tag, and a secondary antibody (secondary Ab) directed against the primary antibody, show that the complex between a peptide/S-Tag, a primary Ab and a secondary Ab binds to cells expressing hLDLR and is internalized by endocytosis. These results indicate that the cyclic peptides of this family can bind to cells expressing hLDL and vectorize large loads (two antibodies), i.e., these loads are
15 internalized by endocytosis.

EXAMPLE V

Toxicity, endocytosis and transcytosis of synthesized peptides with affinity for hLDLR on endothelial cells in *in vitro* BBB models.

20

- The potential toxic effects of peptides on endothelial cells, the binding/accumulation of peptides in these cells, and the passage by transcytosis of peptides are evaluated on *in vitro* BBB models. The cells necessary to set up the model (co-culture of endothelial cells from brain microvessels and astrocytes) are bovine cells (bovine brain
25 microvascular endothelial cells, BBMECs) sold by Cellial Technologies (Lens, France) or rat cells (rat brain capillary endothelial cells, rat BCECs) having made it possible to develop an internal murine model. This type of *in vitro* BBB model is used to evaluate the passive passage or active transport of numerous molecules, notably pharmacological agents, across BCECs and thus by extrapolation their capacity to reach CNS tissue *in*
30 *vivo*. The bovine and murine models have ultrastructural properties characteristic of the brain endothelium, notably tight junctions, absence of pores, absence of transendothelial channels, low permeability to hydrophilic molecules and high electrical resistance.

Moreover, these models have shown solid correlations between the results of measurements taken on various molecules evaluated *in vitro* and *in vivo* for their property of passing across the BBB. To date, all the data obtained show that these *in vitro* BBB models closely imitate the situation *in vivo* by reproducing some of the complexities of the cell environment that exist *in vivo*, while preserving the advantages associated with cell culture experimentation.

For example, the *in vitro* bovine BBB model brings into play a co-culture of BBMECs and astrocytes. Prior to cell culture, membrane inserts (Millicell-PC 3.0 μm ; 30 mm diameter) are treated on the upper part with rat tail collagen in order to enable optimal adhesion of BBMECs and to create the conditions of a basal lamina. Primary cultures of mixed astrocytes are established from neonatal rat cerebral cortex (*Dehouck et al., 1990, J. Neurochem., 54, 1798-1801*). Briefly, after the meninges are removed, the brain tissue is passed through an 82 μm nylon sieve. The astrocytes are distributed in microplate wells at a concentration of 1.2×10^5 cells/ml with 2 ml optimal culture medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum. The medium is changed twice per week. The BBMECs obtained from Cellial Technologies are grown in the presence of DMEM medium supplemented with 10% (v/v) horse serum and 10% heat-inactivated calf serum, 2 mm glutamine, 50 $\mu\text{g/ml}$ gentamicin and 1 ng/ml basic fibroblast growth factor, added every two days. The BBMECs are then distributed on the upper surface of the filters in 2 ml co-culture. This BBMEC medium is changed three times per week. Under these conditions, differentiated BBMECs form a monolayer of confluent cells seven days later.

To test their toxicity, the inventive peptides coupled to rhodamine are incubated in the upper chamber of the culture system, in contact with endothelial cells for 1 h, 5 h and 24 h. The culture medium of the lower chamber is collected at various times and fluorescence quantified by fluorimetric analysis. The results are expressed as endothelial surface permeability (Pe) in units of 10^{-3} cm/min. Lucifer Yellow (LY), a small fluorescent molecule which crosses the BBB very little, is used first to evaluate the integrity of the BBB *in vitro*, in all the wells analyzed, and second for peptide co-incubation in order to evaluate the absence of toxicity of the peptides for the endothelial

cells that form this BBB. The *in vitro* barrier is considered “permeable” or “open” if the Pe value of LY is greater than 1×10^{-3} cm/min. Transendothelial electrical resistance (TEER), measured with an ohmmeter and expressed in ohm.cm², also makes it possible to measure BBB integrity *in vitro* during tests of passage across the BBB. The quality
5 threshold value is set at >500 ohm.cm².

The experiments carried out show an absence of toxicity of the peptides, as well as for the control peptide used, and an absence of deleterious effects on the permeability properties of the BBB.

10

The passage across the BBB of an inventive peptide conjugated to rhodamine is verified on the *in vitro* bovine model described above. This analysis is carried out by measuring by fluorimetry the quantity of fluorescence accumulated in the receiver wells at various times (1 h, 4 h, 24 h). The integrity of the BBB in the various wells analyzed is
15 evaluated by simultaneous measurement of the level of LY that passes from one compartment to the other as a function of time.

EXAMPLE VI

20 Protocols for chemical synthesis of conjugates comprised of a vector and a therapeutic molecule of interest or an imaging (or diagnostic) agent or any other molecule such as a molecular probe.

A therapeutic molecule of interest or an imaging or diagnostic agent or any other
25 molecule such as a molecular probe can be cleaved/released/salted out from the vector after transport and passage across cell membranes, more particularly the BBB, for example through a prodrug strategy by hydrolysis or enzymatic cleavage of a chemical bond between the vector and the active substance.

Covalent coupling between the peptide vector completely protected on its reactive side
30 functions (coupling at the C-term and N-term) or partially protected (coupling on a reactive function of a side chain) and the therapeutic molecule of interest is carried out via two general strategies (Figure 2):

- synthesis in tandem (*i.e.*, direct coupling with no intermediate between the two entities),
- synthesis via a linker (*Temsamani et al.*, 2004, *Drug Discov. Today*, 23, 1012-1019).

5

According to the peptide vector and molecule of therapeutic interest selected, one or the other of the various strategies is applied either on the C-term, or on the N-term, or on a side chain reactive function of this peptide vector (*Majumdar and Siahaan, Med Res Rev., Epub ahead of print*). Ideally, in a prodrug strategy, the spacers selected should enable suitable release of the active substance and improvement of the solubility of the conjugate (*Molema et al.*, 2001, *Vectorization, organ-specific strategies. In: Methods and principles in medicinal chemistry, vol. 12*). Various labile covalent chemical bonds can thus be generated between the two entities (vector and active substance) via or not via a spacer: amides, carbamates, esters, thioester, disulfide, etc. For example, it has been shown in the literature that disulfide bonds, relatively stable in plasma, can be cleaved, by enzymes such as protein-disulfide reductase, inside the intracerebral compartment to restore a free thiol function (*Saito et al.*, 2003, *Adv. Drug Deliv. Rev.*, 55, 199-215).

Others compounds of interest are those wherein the spacer is a polymer such as polyethylene glycol (PEG). Indeed, it has been shown in the literature that the conjugation of an organic molecule of biological interest with PEG made it possible to increase the plasma half-life of this molecule (*Greenwald et al.*, 2003, *Adv. Drug Deliv. Rev.*, 55, 217-250) and to decrease its clearance.

Vectors conjugated with an active substance or a substance of interest can be used in the diagnosis, imaging or therapy of a pathology, lesion or disorder of the CNS for preparing a drug capable of crossing the BBB, a brain tumour or another type of cancer cell for preparing a drug capable of crossing cancer cell membranes, and/or infectious pathologies for preparing a drug capable of crossing cell membranes and to target the infected cells of bacterial, viral, parasitic or fungal infectious pathologies of the brain or other tissues.

30

EXAMPLE VII

Brain perfusion *in situ* for vectors alone and vectors conjugated with a therapeutic molecule of interest or an imaging (or diagnosis) agent or any other molecule such as a molecular probe, and study of their transport kinetics across the BBB and their
5 accumulation in the brain of mouse.

The *in situ* brain perfusion technique (in adult male OF1 mouse) is used to illustrate passage in the brain across the BBB.

- 10 Beforehand, the peptide vectors are radiolabelled with tritium (^3H), an element that offers the strongest sensitivity for the detection of radioactive compounds, notably on tissue sections. Radioactive peptides with high specific radioactivity (RAS, up to 100 Ci/mmol) are prepared by a strategy of acylation of the N-term amine function by tritiated propionic (or propanoic) anhydride or tritiated N-propionyl-succinimide (NPS).
- 15 This tritiation method can be applied to all peptides (vectors, or conjugates between a therapeutic peptide and a peptide vector in tandem or via a linker (of peptide or organic nature)), provided that modification of the N-term does not affect the affinity of the peptides for the targeted receptor (*i.e.*, LDLR) or their biological activity in the case of therapeutic peptides.
- 20 The tritiation reaction of the peptide vector in the N-term position by propionylation is carried out in DMF (1 mg peptide in 100 μl to 450 μl according to solubility) by adding 0.1 equivalent of tritiated NPS for 5 min at room temperature, then 0.9 equivalent of cold NPS (non-tritiated) for 1 h, and then a new equivalent of cold NPS for 5 h. The reaction medium is then left at 4 $^{\circ}\text{C}$ overnight and purified the following day by HPLC.
- 25 The RAS for each tritiated peptide is typically between 5 Ci/mmol and 10 Ci/mmol. The total quantity of radioactivity prepared by synthesis is generally between 600 μCi and 950 μCi .

Radiolabelled peptides (radiolabelled with ^3H , for example) are coupled covalently with
30 a radiolabelled active substance (radiolabelled with ^{14}C , for example) as described for example in EXAMPLE VI. As previously mentioned, this covalent coupling is carried out according to the structure and physicochemical properties of the active substance, in

particular the presence of functional chemical groups that can be modified without decreasing the biological activity of this substance. Radiolabelled conjugates are synthesized by extrapolation from synthetic pathways developed for non-radiolabelled conjugates.

5

The techniques briefly summarized below were developed previously to study the distribution in the brain of active substances and, in particular, the role of the BBB and, more particularly, of LDLR in the penetration of these molecules in the brain. *In situ* brain perfusion techniques are among the most technically demanding and the most
10 difficult to perform in the mouse. However, *in situ* brain perfusion (like *in vitro* models) enables total control of the composition of the artificial perfusate in order to maintain the cells and vascularisation of the brain under normal physiological and anatomical conditions within the animal, without the disrupting factor of systemic distribution.

15 This strategy of *in situ* brain perfusion normally carried out in the rat was adapted for mouse (*Dagenais et al., 2000, J Cereb Blood Flow Metab., 20(2), 381-6*) in order to broaden its application for evaluating the parameters of transport kinetics at the BBB and the blood-retinal barrier, and this also in transgenic and KO mutant mice for the receptors, enzymes or transporters of active substances. It involves catheterization of a
20 carotid artery in anaesthetized mice (typically OF1) and ligation of certain branches of this carotid artery (external, thyroïdal, occipital) in order to specifically perfuse the internal carotid and pterygopalatine arteries, which are used to evaluate the uptake in the brain of the vectors and conjugates. The catheter makes it possible to replace general circulation by infusion with a well-controlled perfusate (bicarbonate buffer, plasma or
25 blood) by passing by the carotid. Oxygenated Krebs bicarbonate buffer is first used to evaluate the capacities of the vectors and conjugates to pass in the brain. After catheterisation of the carotid, endogenous blood flow is stopped by sectioning the ventricles of the heart in order to avoid the mixture of buffer with blood and elevation in blood pressure. The duration of the fixed flow-rate perfusion is monitored. The buffer
30 perfusion can be extended up to 20 min, or up to 1 h in the presence of oxygen transporters (washed erythrocytes) for studies of receptor-mediated transport (RMT).

The experiments carried out made it possible to establish cerebral transport, or the transfer coefficient (K_{in} : relationship between distribution volume and cerebral perfusion time), of several peptide vectors of the invention. The duration of brain perfusion for these experiments is 5 min with a perfusate flow rate of 2 ml/min. Thus, the K_{in} of the peptide vectors of SEQ ID NO: 2 and SEQ ID NO: 4, radiolabeled with a RAS of 2 Ci/mmol, are respectively 3.3 and 3.0×10^{-4} ml/s/g.

In comparison, transferrin (Tf) has a K_{in} of 3.0×10^{-4} ml/s/g (*Demeule et al., 2008, J. Neurochem., 106(4), 1534-1544*), and the reference peptide, SEQ ID NO: 17 has, under the same experimental conditions, a K_{in} of $3.2 \pm 0.4 \times 10^{-4}$ ml/s/g.

These results thus show that the peptide vectors of the invention, due to their small size and advantageous configuration, have a very high cerebral transfer coefficient, greater than that of transferrin.

15

In addition, this type of *in situ* brain perfusion experiment also makes it possible to establish a distinction between compounds that remain in the cerebral vascular compartment and those having crossed the abluminal endothelial membrane to enter the brain parenchyma. This technique, called post-perfusion capillary depletion, makes it possible to measure if the molecule actually crosses the endothelium in order to enter the brain parenchyma. The use of this technique makes it possible to show that specific peptide vectors (or conjugates) accumulate in the brain parenchyma. Thus, this technique (*Triguero et al., 1990, J Neurochem., 54(6), 1882-8*) is used in order to make a distinction between the fraction of vectors (or conjugates) that transited by the endothelium and entered the brain via the extracellular space or brain cells, and the remaining fraction associated with endothelial cells.

The key steps of the *in situ* brain perfusion studies in mouse can be summarized as follows for the vectors and conjugates studied:

- i) Evaluation of tolerance (non-toxicity) of the vectors and conjugates at the BBB and preservation of the integrity of this physiological barrier.
- ii) Study of kinetics and linearity of brain uptake via RMT by LDLR.

- iii) Study of brain uptake rate as a function of vector or conjugate concentration ($T_{\max}$, K_m).
- iv) Study of transport mechanisms using substrates that inhibit or modulate LDLR.
- v) Distribution in the compartments of the brain: capillary depletion (*Triguero et al., 1990, J Neurochem., 54(6), 1882-8*).
- vi) Evaluation of binding rate of vectors and conjugates with plasma proteins (albumins, etc.) and study of their influence on brain uptake of these molecules.
- vii) Intravenous administration and evaluation of tissue distribution (brain and other organs) as a function of time.

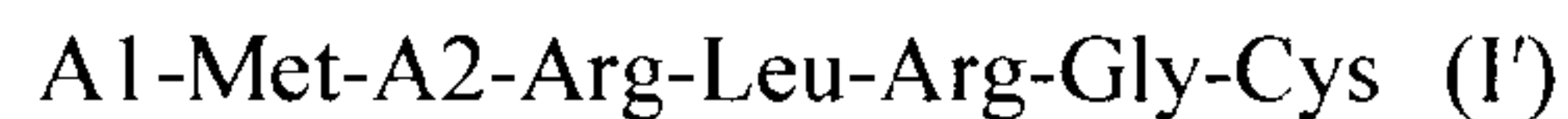
CLAIMS

1. A peptide or pseudo-peptide, characterized in that it is of the following general
5 formula (I):



- wherein A1 represents cysteine (Cys), (D)-cys, penicillamine (Pen) or (D)-penicillamine ((D)-Pen), A2 represents proline (Pro), pipecolic acid (Pip) or thiazolidine-4-carboxylic acid (Thz), and A3 represents glycine (Gly) or sarcosine (Sar), and wherein the peptide
10 or pseudopeptide binds human low-density lipoprotein receptor (hLDLR).

2. The peptide or pseudo-peptide of claim 1, characterized in that it is of the following general formula (I'):



- 15 wherein A1 represents Cys, (D)-cys, Pen, or (D)-Pen; and A2 represents Pro, Pip or Thz.

3. A peptide or pseudo-peptide selected from peptides of sequence SEQ ID NOs: 1 to 10.

4. The peptide or pseudo-peptide of any one of claims 1 to 3, characterized in that
20 it has a cyclic configuration.

5. The peptide or pseudo-peptide of any one of claims 1 to 4, characterized in that it contains at least one peptidomimetic bond.

6. The peptide or pseudo-peptide of claim 5, characterized in that the at least one peptidomimetic bond is chosen from intercalation of a methylene (-CH₂-) or phosphate
25 (-PO₂-) group, secondary amine (-NH-) or oxygen (-O-), alpha-azapeptides, alpha-alkylpeptides, N-alkylpeptides, phosphonamidates, depsipeptides, hydroxymethylenes,

hydroxyethylenes, dihydroxyethylenes, hydroxyethylamines, retro-inverso peptides, methyleneoxy, cetomethylene, esters, phosphinates, phosphinics, phosphonamides and carba analogues.

7. The peptide or pseudo-peptide of any one of claims 1 to 6, characterized in that
5 the N-terminal (N-term) function is protected by acylation and/or in that the C-terminal (C-term) function is protected by amidation or esterification.

8. The peptide or pseudo-peptide of any one of claims 1 to 7, for use to vectorize an active substance or a substance of interest in diagnostics, imaging or therapeutics.

9. The peptide or pseudo-peptide of any one of claims 1 to 8, for use to increase the
10 biological activity or to decrease the toxicity of an active substance or of a substance of interest to which it is coupled.

10. A conjugated compound of following formula (III):



wherein V represents a peptide or pseudo-peptide according to any one of claims 1 to 7,
15 L represents a spacer, D represents an active substance or a substance of interest, x and y are integers between 1 and 5 and z is an integer between 0 and 10.

11. The conjugated compound of claim 10, characterized in that $x=z=y=1$; $x=z>y$, $y=z>x$ or $z>x>y$; or in that z is equal to 0 and x and y are equal to 1 or y is greater than x.

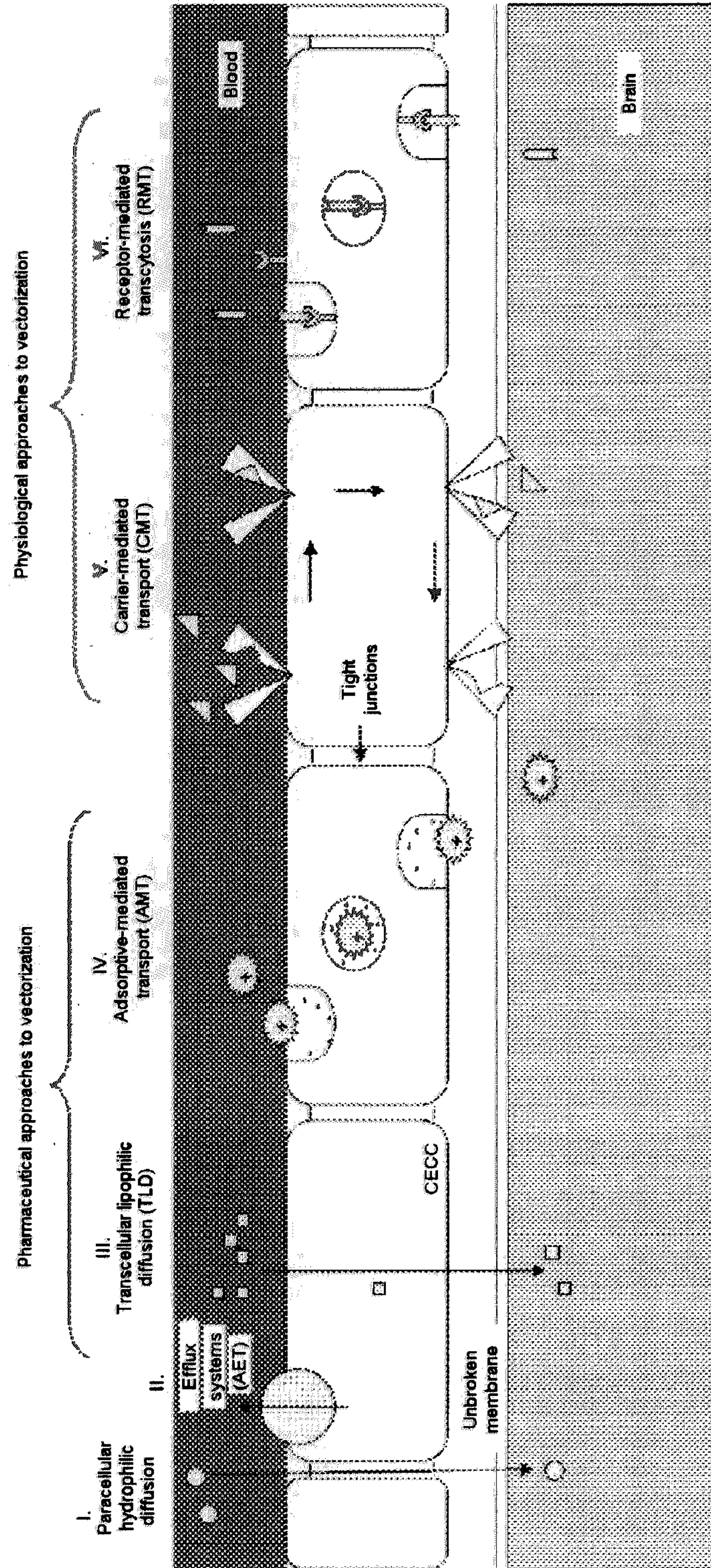
20 12. The conjugated compound of claim 10 or claim 11, characterized in that the active substance or substance of interest is a molecule of therapeutic interest, a diagnostic or medical imaging agent, or a molecular probe.

13. The conjugated compound of claim 12, characterized in that the active substance or substance of interest is selected among a small chemical molecule, a peptide or
25 polypeptide, a protein, an antigen, an antibody or part of an antibody, a nucleic acid or an oligonucleotide, a ribozyme, and a marker or a tracer.

14. The conjugated compound of any one of claims 10 to 13, characterized in that the coupling between V and D, or between V and L on the one hand and L and D on the other, is carried out by one or more covalent, ionic, hydrogen, hydrophobic or Van der Waals bonds, cleavable or not in physiological medium or within cells.
- 5 15. The conjugated compound of any one of claims 10 to 14, characterized in that D is coupled with V, if necessary via L, at one or both of the N-term and/or C-term ends of V and/or at one or more reactive groups carried by the side chains of the natural or non-natural amino acids constitutive of V.
16. A method for preparing a conjugated compound of any one of claims 10 to 15,
10 characterized in that it comprises a coupling step between a peptide or pseudo-peptide V and a substance D, if necessary via L.
17. The method of claim 16, characterized in that the coupling step is performed by chemical, biochemical or enzymatic route or by genetic engineering.
18. A pharmaceutical composition characterized in that it comprises at least one
15 conjugated compound of any one of claims 10 to 15 and one or several pharmaceutically acceptable excipients.
19. A diagnostic composition characterized in that it comprises a diagnostic or a medical imaging agent comprised of a conjugated compound of any one of claims 10 to 15.

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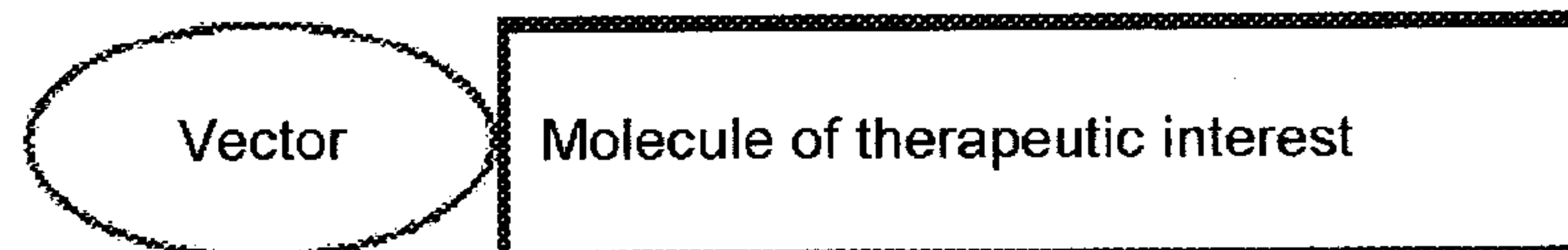
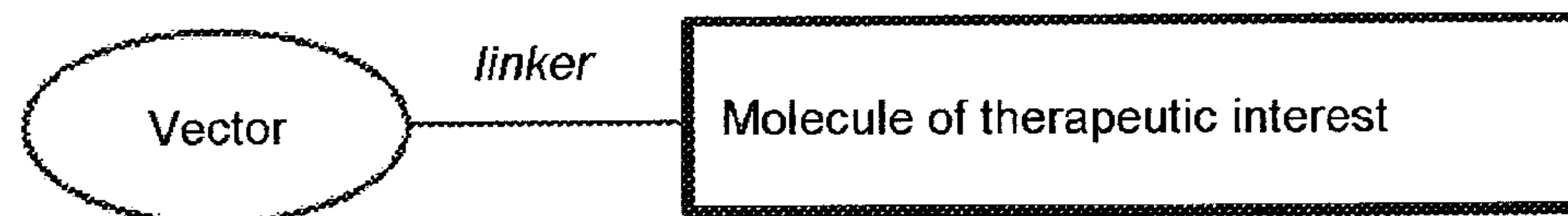
Figure 1



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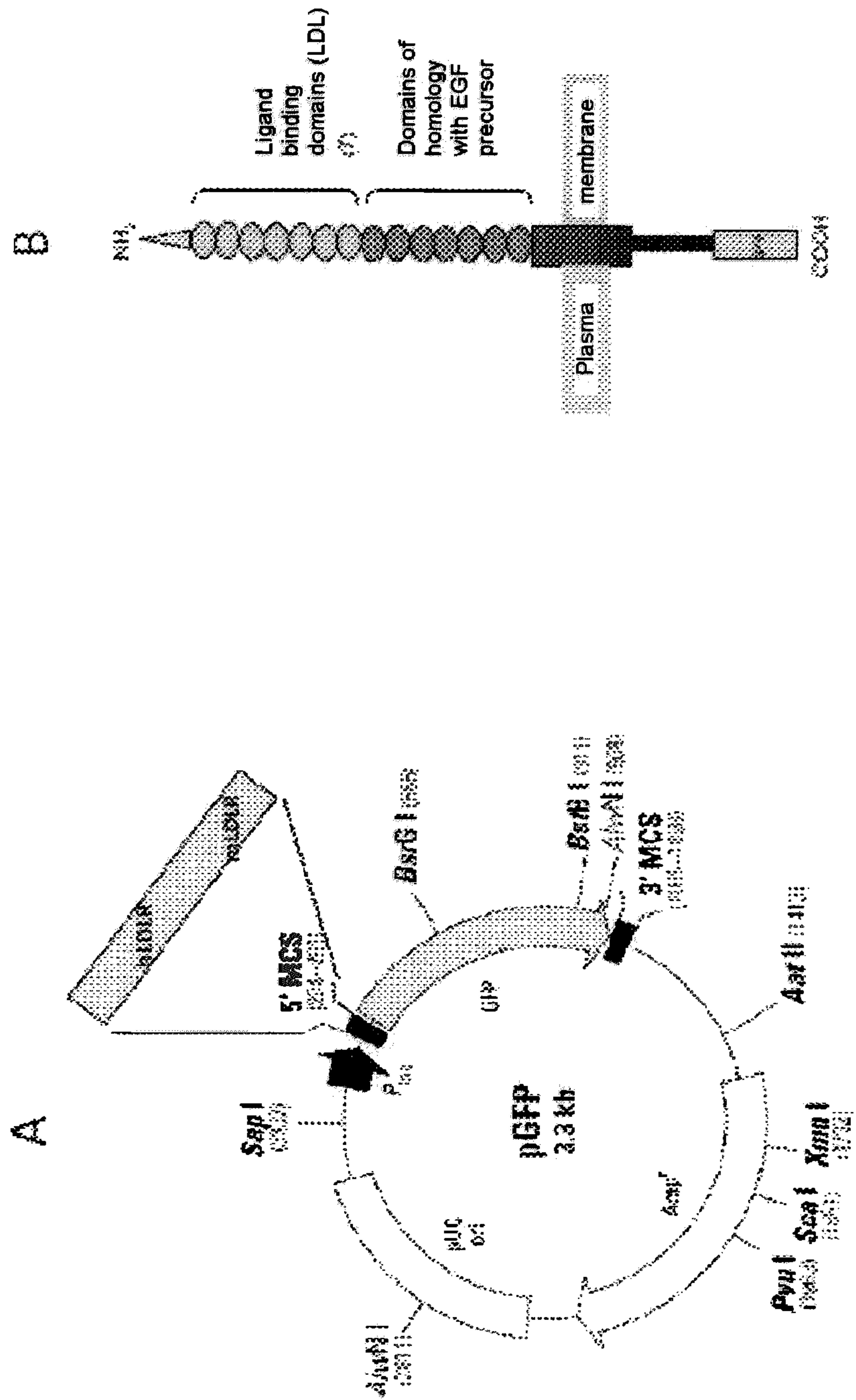
Figure 2

a) Tandem synthesis

b) Synthesis via a *linker*

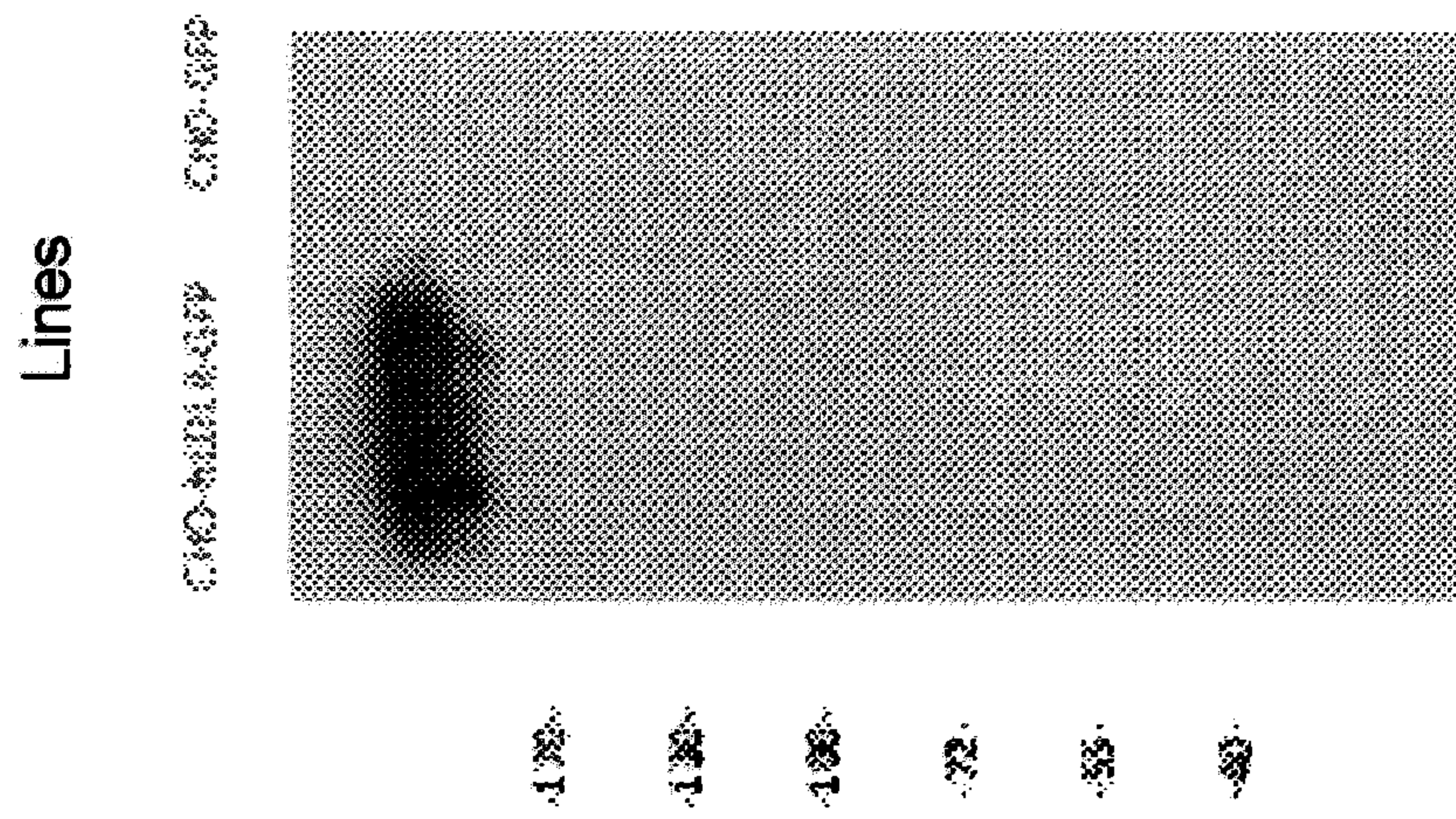
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Figure 3



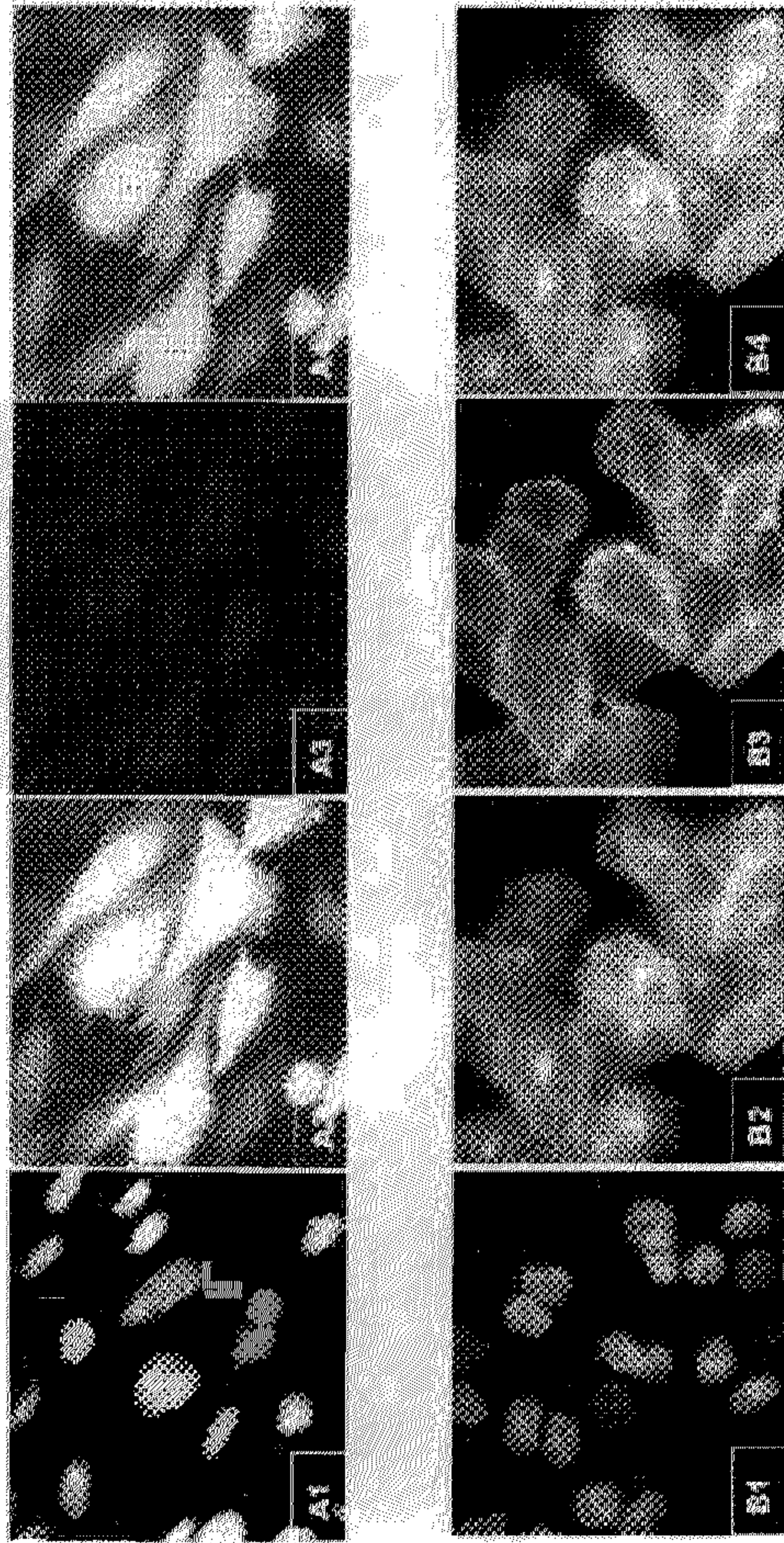
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Figure 4



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Figure 5



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Figure 6

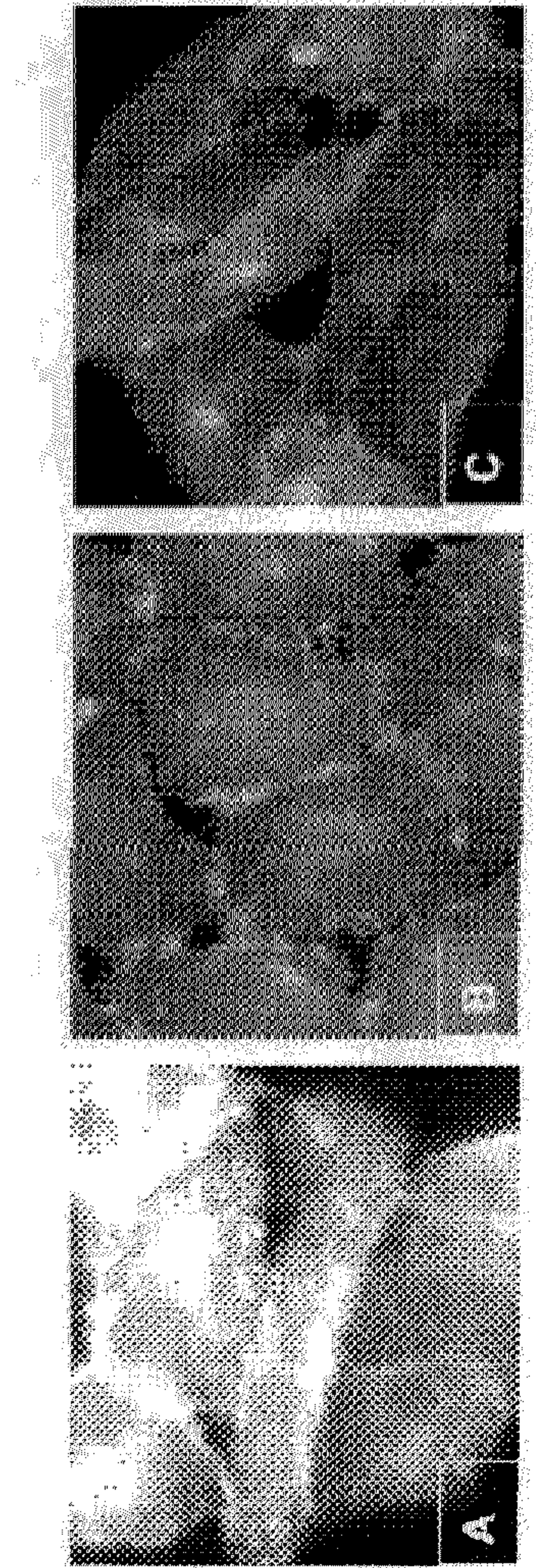


Figure 7

