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(71) Applicant *(for all designated States except US)*: **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE** [FR/FR]; 3, rue Michel Ange, F-75016 Paris (FR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **DIVITA, Gilles** [FR/FR]; 476 rue de la Rave, F-34130 Mauguio (FR). **KONATE, Karidia** [FR/FR]; Résidence Sébastien Barton 2 rue des Abeilles, F-34090 Montpellier (FR). **MORRIS, May Catherine** [GB/FR]; 476 rue de la Rave, F-34130 Mauguio (FR). **DESHAYES, Sébastien** [FR/FR]; Résidence Le Mirador 51 rue de la Cavalerie, F-34000 Montpellier (FR).

(74) Agents: **MARCADÉ, Véronique** et al.; Cabinet Ores, 36, rue de St Pétersbourg, F-75008 Paris (FR).

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(54) **Title**: CELL PENETRATING PEPTIDES FOR INTRACELLULAR DELIVERY OF MOLECULES

(57) **Abstract**: The present invention relates to a new family of cell-penetrating peptides, comprising an amino acid sequence $X_1LX_2RALWRLX_3RX_4LWRLX_5X_6X_7X_8$, wherein X_1 is beta-A or S, X_2 is F or W, X_3 is L, W, C or I, X_4 is S, A, N or T, X_5 is L or W, X_6 is W or R, X_7 is K or R, and X_8 is A or none. These peptides exhibit high efficiency, low toxicity and a natural tropism for lung tissues, and can be used either in simple complex with the cargo to be vectorised, or in nanoparticles comprising two layers of cell-pentrating peptides around the cargo.

CELL PENETRATING PEPTIDES FOR INTRACELLULAR DELIVERY OF MOLECULES

The present invention pertains to the field of intracellular delivery of molecules such as nucleic acids and small hydrophobic molecules. In particular, the invention relates to a new cell-penetrating peptide (CPP) family, which exhibits high efficiency, low toxicity and a natural tropism for lung tissues.

Although small molecules remain the major drugs used in clinic, in numerous cases, their therapeutic impact has reached limitations such as insufficient capability to reach targets, lack of specificity, requirement for high doses leading to toxicity and major side effects. Over the past ten years, in order to circumvent limitations of small molecules and of gene-based therapies, we have witnessed a dramatic acceleration in the discovery of larger therapeutic molecules such as proteins, peptides and nucleic acids which present a high specificity for their target but do not follow Lipinski's rules. Pharmaceutical potency of these molecules remains restricted by their poor stability *in vivo* and by their low uptake in cells. Therefore, "delivery" has become a central piece of the therapeutic puzzle and new milestones have been established to validate delivery strategies: (a) lack of toxicity, (b) efficiency at low doses *in vivo*, (c) easy to handle for therapeutic applications (d) rapid endosomal release and (e) ability to reach the target. Although viral delivery strategies had given much hope for gene and cellular therapies, their clinical application has suffered from side- and toxicity- effects [1,2]. Researches were mainly focused on the development of non-viral strategies, and different methods have been proposed including lipid, polycationic nanoparticles and peptide-based formulations, but only few of these technologies have been efficient *in vivo* and have reached the clinic. Cell Penetrating Peptides (CPP) are one of the most promising non-viral strategies. Although definition of CPPs is constantly evolving, they are generally described as short peptides of less than 30 amino acids either derived from proteins or from chimeric sequences. They are usually amphipathic and possess a net positive charge [3-5]. CPPs are able to penetrate biological membranes, to trigger the movement of various biomolecules across cell membranes into the cytoplasm and to improve their intracellular routing, thereby facilitating interactions with the target. CPPs can be subdivided into two main classes, the first requiring chemical linkage with the cargo and the second involving the formation of stable, non-covalent complexes. CPPs from both strategies have been reported to favour the delivery of a large panel of cargos (plasmid DNA, oligonucleotide, siRNA, PNA, protein, peptide, liposome, nanoparticle...) into a wide variety of cell types and in vivo models [3-7].

Twenty years ago, the concept of protein transduction domain (PTD) was proposed based on the observation that some proteins, mainly transcription factors, could

shuttle within cells and from one cell to another [for review see ref 3,4]. The first observation was made in 1988, by Frankel and Pabo. They showed that the transcription-transactivating (Tat) protein of HIV-1 could enter cells and translocate into the nucleus. In 1991, the group of Prochiantz reached the same conclusions with the Drosophila Antennapedia homeodomain and demonstrated that this domain was internalized by neuronal cells. These works were at the origin of the discovery in 1994 of the first Protein Transduction Domain: a 16 mer-peptide derived from the third helix of the homeodomain of Antennapedia named Penetratin . In 1997, the group of Lebleu identified the minimal sequence of Tat required for cellular uptake and the first proofs-of-concept of the application of PTD *in vivo*, were reported by the group of Dowdy, for the delivery of small peptides and large proteins . Historically, the notion of Cell Penetrating Peptide (CPP) was introduced by the group of Langel, in 1998, with the design of the first chimeric peptide carrier, the Transportan, which derived from the N-terminal fragment of the neuropeptide galanin, linked to mastoparan, a wasp venom peptide. Transportan has been originally reported to improve the delivery of PNAs both in cultured cells and in vivo . In 1997, the group of Heitz and Divita proposed a new strategy involving CPP in the formation of stable but non-covalent complexes with their cargo [7]. The strategy was first based on the short peptide carrier (MPG) consisting of two domains: a hydrophilic (polar) domain and a hydrophobic (apolar) domain. MPG was designed for the delivery of nucleic acids [7]. The primary amphipathic peptide Pep-1 was then proposed for non-covalent delivery of proteins and peptides [8]. Then the groups of Wender and of Futaki demonstrated that polyarginine sequences (Arg8) are sufficient to drive small and large molecules into cells and *in vivo*. Ever since, many CPPs derived from natural or unnatural sequences have been identified and the list is constantly increasing. Peptides have been derived from VP22 protein of Herpes Simplex Virus, from calcitonin, from antimicrobial or toxin peptides, from proteins involved in cell cycle regulation, as well as from polyproline-rich peptides [reviews 4-6].

The inventors have now designed a new family of cell-penetrating peptides for the delivery of charged and hydrophobic molecules, named VEPEP-6. Delivery strategies using VEPEP-6 peptides as the outer layer of nanoparticles are referred to as NANOPEP-6.

VEPEP-6 are short secondary amphipathic peptides forming stable nanoparticles with molecules such as nucleic-acids (in particular siRNA), DNA mimic and small hydrophobic molecules, hereafter designated as "SHM". VEPEP-6 vectors comprise the following amino acid sequence:

$X_1LX_2RALWRLX_3RX_4LWRLX_5X_6X_7X_8$ (SEQ ID No: 13),

wherein

X_1 = beta-A or S

X_2 = F or W
X_3 = L, W, C or I
X_4 = S, A, N or T
X_5 = L or W
X_6 = W or R
X_7 = K or R
X_8 = A or none.

VEPEP-6 strategy improves both *ex-vivo* and *in vivo* delivery and efficiency of nucleic acids and small hydrophobic molecules, without activating the innate immune response or inducing toxic side effects.

Particular cell-penetrating peptides according to the present invention comprise the following amino acid sequence:

$X_1LX_2RALWRLX_3RX_4LWRLX_5X_6KX_7$ (SEQ ID No: 14),

wherein X_1 is beta-A or S, X_2 is F or W, X_3 is L or W, X_4 is S, A or N, X_5 is L or W, X_6 is W or R, X_7 is A or none.

According to a preferred embodiment of the present invention, illustrated in the experimental part below, the amino acid sequence of the cell-penetrating peptide is selected in the group consisting of:

- $X_1LFRALWRLLRX_2LWRLLWX_3$ (SEQ ID No: 7)
- $X_1LWRALWRLWRX_2LWRLLWX_3A$ (SEQ ID No: 8)
- $X_1LWRALWRLX_4RX_2LWRLWRX_3A$ (SEQ ID No: 9)
- $X_1LWRALWRLWRX_2LWRLWRX_3A$ (SEQ ID No: 10)
- $X_1LWRALWRLX_5RALWRLLWX_3A$ (SEQ ID No: 11) and
- $X_1LWRALWRLX_4RNLWRLLWX_3A$ (SEQ ID No: 12),

wherein X_1 is beta-A or S, X_2 is S or T, X_3 is K or R, X_4 is L, C or I and X_5 is L or I.

According to a preferred embodiment, a cell-penetrating peptide of the present invention further comprises, covalently linked to the N-terminal end of the amino acid sequence, one or several chemical entities selected in the group consisting of an acetyl, a fatty acid, a cholesterol, a poly-ethylene glycol, a nuclear localization signal, a nuclear export signal, an antibody, a polysaccharide and a targeting molecule.

In addition or alternatively, a cell-penetrating peptide according to the invention can comprise, covalently linked to the C-terminal end of its amino acid sequence, one or several groups selected in the group consisting of a cysteamide, a cysteine, a thiol, an amide, a nitrilotriacetic acid optionally substituted, a carboxyl, a linear or ramified C1-C6 alkyl optionally substituted, a primary or secondary amine, an osidic derivative, a lipid, a phospholipid, a fatty acid, a cholesterol, a poly-ethylene glycol, a

nuclear localization signal, nuclear export signal, an antibody, a polysaccharide and a targeting molecule.

Particular examples of cell-penetrating peptides according to the invention are the following:

- VEPEP-6a: Ac-X_1LFRALWRLLRSLWRLLWK-cysteamide (SEQ ID No: 1)
- VEPEP-6b: Ac-X_1LWRALWRLWRSLWRLLWKA-cysteamide (SEQ ID No: 2)
- VEPEP-6c: Ac-X_1LWRALWRLLRSLWRLWRKA-cysteamide (SEQ ID No: 3)
- VEPEP-6d: Ac-X_1LWRALWRLWRSLWRLWRKA-cysteamide (SEQ ID No: 4)
- VEPEP-6e: Ac-X_1LWRALWRLLRALWRLLWKA-cysteamide (SEQ ID No: 5) and
- VEPEP-6f: Ac-X_1LWRALWRLLRNLWRLLWKA-cysteamide (SEQ ID No: 6),

wherein X_1 is beta-A or S, and Ac represents an acetyl.

Another aspect of the present invention is a complex comprising a cell-penetrating peptide as described above and a cargo selected amongst nucleic acids and hydrophobic molecules. Examples of nucleic acid cargoes are small single stranded RNA or DNA (size between 2 to 40 base pair) and double stranded RNA or DNA (size up to 100 base pairs), in particular siRNA selected to silence a target mRNA. In a preferred embodiment of the complex according to the invention, the cargo is a small molecule (size lower that 1.5 kDa), preferably hydrophobic. Non-limitative examples of small hydrophobic molecules which can be used include daunomycin, Paclitaxel, doxorubicin, AZT, porphyrin, fluorescently-labelled-nucleosides or nucleotides (FAM-Guanosine), hydrophobic maghemite (contrast agents or magnetic nanoparticles Fe_2O_3) and fluorescent dyes.

The size of the complexes described above is preferably between 50 and 300 nm, more preferably between 50 and 200 nm (the size of the complex herein designates its mean diameter).

In the complexes according to the invention, the VEPEP-6/cargo molar ratio depends on the nature and size of the cargo, but is generally comprised between 1/1 and 50/1. For siRNA cargoes, the cargo/VEPEP-6 molar ratio preferably ranges from 10/1 to 30/1, more preferably from 15/1 to 25/1.

According to an advantageous embodiment of the complexes as described above, the VEPEP-6 peptides comprise an acetyl group covalently linked to their N-terminus, and/or a cysteamide group covalently linked to their C-terminus.

The above complexes can be advantageously used as "core shells" for obtaining bigger complexes, or nanoparticles, by an additional step of coating the VEPEP-6/cargo complex with another layer of cell-penetrating peptides, which can be different from the VEPEP-6 peptides described above. Examples of such nanoparticles are CADY/VEPEP-6/siRNA and MPG/VEPEP-6/siRNA nanoparticles described in example 5.4. Other examples of peptides which can be used for forming nanoparticles by coating a VEPEP-6/cargo complex are PEP-1, ppTG1 and polyarginine such as Arg8.

Another aspect of the present invention pertains to nanoparticles made of a "core shell" comprising a cargo and a first carrier molecule, surrounded by VEPEP-6 peptides. These are herein referred to as "NANOPEP-6" particles. NANOPEP-6 technology constitutes a "custom-built" delivery system containing a common core particle, trapping therapeutic molecule, with surface VEPEP-6 peptides which are preferably functionalized for tumour or tissue targeting *in vivo*. From a structural point of view, NANOPEP-6 particles are constituted by a "core" which is coated by a layer of VEPEP-6 peptides. The "core" corresponds to a complex comprising a cargo and a vector or carrier such as a first cell-penetrating peptide, a liposome, a polycationic structure, a carbon nanoparticle, *etc.* In NANOPEP-6 particles, the layer of VEPEP-6 peptides (peripheral peptide) stabilizes the particle and can be functionalized. Functionalizing NANOPEP-6 particle surface with either cholesterol or PEG-molecules improves particles stability *in vivo*, favours their administration by either systemic or topical route and allows rapid liberation of active cargoes within tumor cells or tissues. Functionalization of the surface of NANOPEP-6 particles with small FAB fragments, peptides, antibodies and lipids has been shown to favour *in vivo* tissue or tumor targeting (examples 5.2 and 5.3 below).

NANOPEP-6 technology improves both cellular and *in vivo* delivery of biologically active cargoes and has been validated on a large set of cell lines including adherent and suspension cell lines, hard to transfect cell lines. NANOPEP-6 particles strongly interact with cell membranes and enter the cell independently of the endosomal pathway or rapidly escape from early endosomes. NANOPEP-6 technology presents several advantages including rapid delivery with very high efficiency, stability in physiological buffers, lack of toxicity and of sensitivity to serum, ability of forming mix nanoparticles, can be functionalized and have been successfully applied to the delivery of different types of cargoes into a large variety of cell lines as well as in animal models, thereby constituting powerful tools for basic research and therapeutic applications. NANOPEP-6 technology can be applied both at therapeutic and diagnostic/theragnostic levels.

In a particular embodiment of NANOPEP-6 particles according to the present invention, the cargo is complexed to a first cell-penetrating peptide, which can be,

for example, selected amongst CADY (described in US 7,579,318, herein incorporated by reference), MPG (SEQ ID No: 23, N-acetyl-GALFLGFLGAAGSTMGAWSQPKKKRKV-Cysteamide) or its variants described in US 7,514,530, PEP-1 (SEQ ID No: 24, N-acetyl-KETWWETWWTEWSQPKKKRKV-cysteamide), PPTG1 (SEQ ID No: 25, GLFKALLKLLKSLWKLLLKA), poly Arginine motif such as Arg8, VEPEP-6 peptides described above, or any other known CPP. This cargo/CPP complex is then coated with a layer of VEPEP-6 peptides. According to this embodiment, the skilled artisan will advantageously choose the first CPP depending on the nature of the cargo, so that the complex of cargo and first CPP is stable. Hence, a wide diversity of cargoes can be included in NANOPEP-6 particles (See example: 5.3 using PEP-1/peptide complex associated to VEPEP-6, as short peptide are not cargo of VEPEP-6 peptide).

In the nanoparticles as above-described, the VEPEP-6/core molar ratio depends on the nature and size of the core, but is generally comprised between 1/1 and 50/1. For CPP/siRNA cores, the peripheral VEPEP-6/cargo molar ratio preferably ranges from 10/1 to 30/1, more preferably from 15/1 to 25/1. In what follows, when a ratio of peripheral peptide/core shell particle is indicated for nanoparticles according to the invention, this ratio in fact represents the peripheral peptide/cargo molar ratio.

In a preferred embodiment of the nanoparticles according to the invention, the size of the nanoparticle is between 80 and 400 nm.

According to an advantageous embodiment of the NANOPEP-6 particles according to the invention, the VEPEP-6 peptides forming the peripheral layer of the nanoparticles comprise an acetyl group covalently linked to their N-terminus, and/or a cysteamide group covalently linked to their C-terminus.

According to another preferred embodiment, described in example 4 and illustrated in Example 5.2 below, the core shell of the particles is coated with a VEPEP-6 peptide functionalized with NTA (for example, a VEPEP-6 peptide with nitrilotriacetic acid covalently linked to its C-terminus). This allows the subsequent attachment to the surface of the particle, of any protein (or other molecule) harboring a histidine tag. This strategy offers the major advantage of having a common two-layers particles "NANOPEP-6-HIS" that can be associated to any His-tagged molecule.

In the present text, the following notations will be used to describe the nanoparticles according to the present invention:

As already described, "NANOPEP-6" designates a particle comprising a core shell made of at least a cargo and a first carrier molecule, surrounded by a VEPEP-6 layer. To further specify the nature of the core shell carrier and of the cargo, the notation "NANOPEP-6/core shell carrier/cargo" notation will be used. For example, the nanoparticles formed by CADY/siRNA particles surrounded by a layer of VEPEP-6

peptides (Example 5.3 below) will be noted "NANOPEP-6/CADY/siRNA". In order to simplify the notations, nanoparticles formed with two layers of VEPEP-6 peptides around a cargo can be noted "NANOPEP-6/cargo".

The same principles are used to designate the functionalized nanoparticles, with the specificity that NTA-functionalized NANOPEP particles are noted NANOPEPHIS. Hence, in Example 5.2.2, "NANOPEP-6-HIS/PEP1/PC4" designates nanoparticles in which the PC4 cargo is complexed to PEP1 as first cell-penetrating peptide and then surrounded by a layer of NTA-functionalized VEPEP-6 peptides, and, in example 5.2.1, "MUC-NANOPEP-6-HIS/siRNA" designates nanoparticles with siRNA as a cargo, complexed to a first layer of VEPEP-6 petides, and surrounded by a second layer of VEPEP-6 peptides which are functionalized and bound to a molecule targeting MUC1.

In particular embodiments of the complexes and nanoparticles according to the invention, at least part of the VEPEP-6 cell-penetrating peptides are bound to a targeting molecule. In the case of NANOPEP-6 particles, at least part of the cell-penetrating peptides which are at the periphery of the nanoparticle are preferentially bound to a targeting molecule. Examples of targeting molecules include: antibodies, Fc and FAB fragments, and ligands, especially targeting receptors which are over-expressed at the surface of certain cell-types, *etc.* Among the numerous molecules which can be targeted by antibodies, Fc and FAB fragments, one can cite the receptor tyrosine kinase HEK2 receptor, MUC1, the EGF receptor and CXCR4. Non-limitative examples of other ligands *which can be used are: RGD-peptide, homing targeting peptides (brain NT1 peptide,* Ganglion GM peptide), folic acid, polysaccharides, Matrix metalloprotease targeting peptide motif (MMP-9).

Another aspect of the present invention is a therapeutic composition comprising a complex or a nanoparticle as described above. For example, a composition comprising a complex or nanoparticle having an anti-cyclin B1 siRNA as a cargo, and a targeting molecule specific for tumor cells (for example: RGD-peptide, folic acid, MUC-1 or HEK2 receptor antobodies ...), is part of the present invention. It is to be noted that complexes and nanoparticles according to the invention can also be advantageously used for formulating non-therapeutic compositions (for example, for research, imaging and/or diagnosis purposes).

Compositions according to the invention (therapeutic or non-therapeutic) can be formulated, for example, for intravenous, topical, intrarectal, intranasal or intradermal administration, as well as for administration *via* a mouth spray. Due to their specifically efficient delivery to the lung, particular compositions according to the present invention can target lung cells, for example for treating or diagnosing lung cancers or for treating lung diseases such as asthma. Any formulation known in the pharmacologic field can be used, such as suppository, solutions, sprays, ointments, *etc.* Of course, another

object of the present invention is a method for delivering a molecule to a patient in need thereof, comprising administrating to said patient a complex or nanoparticle according to the present invention, in particular through intrarectal, intranasal (or oral, with a spray) or intradermal routes.

The present invention also pertains to a method for delivering a molecule into a cell *in vitro*, comprising a step of putting said cell into contact with a complex comprising said molecule and VEPEP-6 cell-penetrating peptides as described above.

The invention is further illustrated by the following figures and examples.

LEGENDS TO THE FIGURES

Figure 1: Structure of VEPEP-6 in the presence of double stranded oligonucleotide or phospholipid as monitored by circular dichroïsm (a) and molecular modeling calculation (using Peplook program, ref 9) or NMR measurement (b).

Figure 2: Binding of nucleic acid to VEPEP-6a as monitored by TRP-intrinsic fluorescence spectroscopy. Dissociation constants were calculated from data fitting. Nucleic acids used: double stranded DNA (45/35 mer), siRNA (19/19 or 25/25) and single stranded DNA (19 or 36 mer)

Figure 3: Binding of small hydrophobic molecules to VEPEP-6 as monitored by fluorescence spectroscopy. The binding of Doxorubicin, AZT and Porphyrin to VEPEP-6 was monitored by following the quenching of tryptophan- intrinsic VEPEP-6 fluorescence and that of fluoresceine-labelled-Guanosine (FAM-G) by monitoring FAM-fluorescence. Dissociation constants were calculated from data fitting.

Figure 4: Impact of VEPEP-6 particle size on silencing efficiency. A fixed concentration of 20 nM of siRNA (Cyc-B1) was associated with different molar ratios of VEPEP-6/siRNA ranging from 1/1 to 50/1. The size of the VEPEP-6/siRNA particles were measured by light scattering (grey bars) and the biological response associated with siRNA internalization was evaluated in cultured cells by measuring reduction of cyclin B1 protein levels 24 hrs after transfection (black bars).

Figure 5: Structural analysis ofVEPEP-6/siRNA complexes by scanning electron microscopy (a) and Atomic Force Microscopy (AFM) (b).

Figure 6: VEPEP-6 dose response of Cyclin B1 silencing at the protein and mRNA levels on Hela (panel A) and HS68 (panel B) cells. Stock solution of VEPEP-6/siRNA (100 nM) particles were prepared at a molar ratio of 1/20 (siRNA/VEPEP-6), and lower concentrations (from 200 nM to 0.125 nM) were obtained by serial dilution of the stock solution in PBS. HeLa (Panel A) and HS-68 (Panel B) cells (60 % confluency) were overlaid with preformed complexes for 30 min, then fresh DMEM supplemented with 10% FCS was added directly to the cells, which were then returned to the incubator for 24 hrs.

Cyclin B1 protein levels were determined by Western Blotting using Cdk2 as a control for quantification (white bars). Cyclin B1 mRNA levels were measured 12 hrs after transfection using Quantigen technology (black bars). Mismatched Cyc-B3 siRNA associated with VEPEP-6 (200 nM) and empty VEPEP-6 particles (20μM) were used as a control. Panel C: Dose-response of G2-arrest associated with Cyclin B1 silencing . HeLa (black bars) and HS68 (white bars) cells were treated with increasing concentrations of VEPEP-6/siRNA-Cyc-B1 from 0.25 nM to 20 nM. The cell cycle status was evaluated by FACS analysis. Mismatched Cyc-B3 siRNA (100 nM) and GAPDH siRNA (100 nM) associated to VEPEP-6 as well as VEPEP-6 carrier alone (20 μM) were used as controls. Results are the means ± of 4 separate experiments.

Figure 7: Toxicity of VEPEP-6 particles: **(A)** The toxicity of VEPEP-6 particles was investigated by MTT assay (grey bars) and by monitoring the level of cyclophilin mRNA measured by quantigenTM technology (Affymetrix) (white bars). HeLa cells were treated with increasing concentrations of VEPEP-6/siRNA particles ranging from 1 to 100 μM and toxicity was then evaluated 12 hr (Cyclophilin mRNA) or 24hr (MTT) after treatment. (B) VEPEP-6 particle does not induce non-specific immune or interferon responses *in vivo*. VEPEP-6/siRNA (set 2), NANOPEP-6/siRNA (set 3) (1 mg/kg of siRNA), and VEPEP-6 carrier (Set 4) were intravenously injected into mice and cytokines induction were measured in the serum 6 (set A), 12 (set B), 24 (set C) and 48 (set D) hrs after injection using 10 plex Invitrogen kit (LMC 10001). This kit allowed the simultaneous measurement of mouse IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-gamma, TNF-α, and GM-CSF in serum, or tissue culture supernatant. Data were compared to control molecule LPS (set 1). Reported data are the average of 3 separate experiments.

Figure 8: (A) Binding of small hydrophobic fluorescent molecules (SFM) to VEPEP-6 as monitored by fluorescence spectroscopy. In the present case, a fluorescently labeled nucleotide (fluorescein-labeled-Guanine) was used. Dissociation constants were calculated from data fitting. (B) Effect of VEPEP-6 mediated doxorubicin on cancer cell viability. Dose responses of free (white bars) and VEPEP-6 mediated (grey bars) doxorubicin have been evaluated on the viability of U2OS and MCF7 cancer cells.

Figure 9: VEPEP-6 mediated *in cellulo* delivery of SFM. A dose response of VEPEP-6 mediated SFM delivery was evaluated on Hela cell line. Membranes were labeled in green with WGA, nucleus in blue with Hoescht and SFM (fluorescein-

labelled guanine) molecules are reported in red. The measurements were performed after 20 and 135 min incubation.

Figure 10: Protocol of particle formation using VEPEP-6 (panel A) and functionalized-VEPEP-6 or NANOPEPHIS-6 (Panel B).

Figure 11: NANOPEP-6 Cyclin B1 siRNA delivery upon topical and systemic injection. Athymic female nude mice were subcutaneously inoculated into the flank with 1 x 10^6 PC3 (panels A&B) or HT29 (panels C&D). Two to three weeks after tumour implant, when tumour size reached about 100 mm^3, animals were treated by intratumoral (panels A&C) injections, every 3 days, with a solution either saline buffer solution (closed triangle), free Cyc-B1 siRNA (open triangle), control siRNA Cyc-B3 (closed box) or Cyc-B1 siRNA at 5µg (open circle) and 10µg (closed circle) complexed with NANOPEP-6 at a 1/20 molar ratio. By intravenous (panels B&D) injections, every 3 days, with a solution either free Cyc-B1 siRNA (closed triangle), control siRNA Cyc-B3 (open triangle) or Cyc-B1 siRNA at 5µg (open circle) and 10 µg (closed circle) complexed with NANOPEP-6 at a 1/20 molar ratio. Tumour diameter was measured in two directions at regular intervals using a digital calliper and tumour volume was calculated as length x width x height x 0.52. Curves show the mean value of tumour size in a cohort of six animals and neither animal death nor any sign of toxicity were observed.

Figure 12: NANOPEPHIS mediated Cyclin B1 siRNA or PEP1/PC4 delivery upon systemic injection. Athymic female nude mice were subcutaneously inoculated into the flank with 1 x 10^6 PC3 or A549 tumor cells. Panel A :Two weeks after tumour implant, when tumour size reached about 100 mm^3, animals were treated by a single intravenous injection of NANOPEP-6/siRNA (in white), MUC-NANOPEP-6-HIS/siRNA (in grey) or (HEK-NANOPE-6-PHIS/siRNA (in black). In all the cases, 5 µg of $Alexa^{700}$ labelled siRNA were used. At 24 hr, mice were euthanized and different organs were removed for quantification of Alexa fluorescence in the different tissues and normalized to the protein level in the sample. Panel B : Two weeks after A549 tumour implant, when tumour size reached about 100 mm^3, animals were treated every 3 days, by intravenous injection of either NANOPEP-6/siRNA (open circle), MUC-NANOPEP-6-HIS/siRNA (open triangle), HEK-NANOPEP-6-HIS/siRNA (closed triangle) or saline buffer solution (closed circle). In all cases, siRNA are complexed with NANOPEP-6 or NANOPEPHIS at a 1/20 molar ratio. Tumour diameter was measured in two directions at regular intervals using a digital calliper and tumour volume was calculated as length x

width x height x 0.52. Curves show the mean value of tumour size in a cohort of six animals and neither animal death nor any sign of toxicity were observed. Panel C: Two weeks after tumour implant, when tumour size reached about 100 mm^3, animals were treated by a single intravenous injection of PEP1/PC4 (in white), MUC-NANOPEP-6-HIS/PEP1/PC4 (in grey) or HEK-NANOPEP-6-HIS/PEP1/PC4 (in black). In all the cases, 10 µg of CY5-labelled PC4 were used. At 24 hr, mice were euthanized and different organs were removed for quantification of CY5 fluorescence in the different tissues and normalized to the protein level in the sample.

Figure 13: NANOPEP-6-mediated *in vivo* targeted delivery in the lung. siRNA targeting GAPDH, Alexa^{700} labelled or not, was complexed with CADY-peptide at ratio 1/20, then the CADY/siRNA "core shell" was coated by VEPEP-6 peptide at a 1/10 molar ratio (Panels A,B). A short 15-mer peptide labelled with CY5 dye was associated to PEP-1 peptide at a molar ratio 1/10, then PEP1/peptide "core shell" was coated by VEPEP-6 peptide at a 1/10 molar ratio (Pane C). In all the cases, 5 µg /of cargoes complexed with peptide carrier were injected intravenously. *In vivo* biodistribution of the core shell particles (in black) and of VEPEP-6 coated core shell particles (in grey) were monitored using live fluorescence animal imaging 48hr after injection. Panel B: The biodistribution of CADY/siRNA complex coated or not with VEPEP-6 was also validated by monitoring the level of housekeeping gene mRNA GAPDH in the different tissues. A single injection of naked siRNA (in white: 100 µg), CADY/siRNA (in black: 5µg) and NANOPEP-6/CADY/siRNA (in grey: 5µg) was performed. The level of GAPDH mRNA was quantified in all the tissues using Quantigen technology. Reported data are the average of 3 separate experiments.

Figure 14 : CPP-mediated *in vivo* targeted delivery of VEPEP-6/siRNA complexes. siRNA targeting GAPDH, Alexa^{700} labelled or not, was complexed with VEPEP-6-peptide at ratio 1/20, then the VEPEP6/siRNA "core shell" was coated by MPG or CADY peptide at a 1/10 molar ratio. In both cases, 5 µg of siRNA complexed with VEPEP-6 carrier were injected intravenously. Panel A : *In vivo* biodistribution of MPG/VEPEP6/siRNA (in black) and of CADY/VEPEP-6/siRNA particles (in grey) were monitored using live fluorescence animal imaging 48hr after injection. Panel B: The biodistribution of VEPEP6/siRNA complex coated with MPG (In black) or CADY (in grey) was also validated by monitoring the level of housekeeping gene mRNA GAPDH in the different tissues. A single injection of naked siRNA (in white: 100 µg),

MPG/VEPEP6/siRNA (in black: 5μg) and MPG/VEPEP6/siRNA (in grey: 5μg) was performed. The level of GAPDH mRNA was quantified in all the tissues using Quantigen technology. Reported data are the average of 3 separate experiments.

Figure 15: NANOPEP-6 mediated siRNA delivery by dermal/intranasal and intrarectal administration. A fluorescently labelled siRNA with Alexa700 or a siRNA targeting the GAPDH was associated to VEPEP-6 as a "core shell" surrounded by VEPEP-6 molecules. Biodistribution of the fluorescently labelled siRNA was evaluated *in vivo* on Balb6 Mouse, 5 hr after a single intrarectal (Panel A), intranasal (Panel B) or intradermal (Panel C) administration of 10 μg of either naked siRNA or siRNA in NANOPEP-6 particles. Panel D : The siRNA associated GAPDH knock out was evaluated after 48 hr on different tissues, by monitoring the level of GAPDH mRNA using Quantigen technology (Panomics inc). Intravenous (ligth grey), intrarectal (in white), intranasal (in black) or intradermal (in light grey) administration. Reported data are the average of 3 separate experiments.

EXAMPLES

Example 1: Materials, Methods, chemistry ,and biophysic analysis

VEPEP-6 peptides

All peptides were synthesized by solid-phase peptide synthesis using AEDI-expensin resin with (fluorenylmethoxy)-carbonyl (Fmoc) on a Pioneer Peptide Synthesizer (PioneerTM, Applied Biosystems, Foster City, CA) starting from Fmoc-PAL-PEG-PS resin at a 0.2 mmol scale. The coupling reactions were performed with 0.5 M of HATU in the presence of 1 M of DIEA. Protecting group removal and final cleavage from the resin were carried out with TFA/Phenol/H_2O/Thioanisol/Ethanedithiol (82.5/5/5/5/2.5 %) for 3h30 min. All the peptides presented a cysteamide group at the C-terminus and were acetylated at the N-terminus. The peptide synthesis started by the C-terminus, using an AEDI-expensin resin starting with a cysteamide link, as described by Mery et al,1992 [10]. All the peptides contained a beta-Alanine or a serine at the N-terminus to favour any further functionalization without using the C-terminal cysteamide group.

The following peptides were synthesized:

VEPEP-6a: Ac-[βA/S]LFRALWRLLR[S/T]LWRLLW[K/R]-cysteamide (SEQ ID No: 7)

VEPEP-6b: Ac-[βA/S]LWRALWRLWR[S/T]LWRLLW[K/R]A-cysteamide (SEQ ID No: 8)

VEPEP-6c: Ac-[βA/S]LWRALWRL[L/C/I]R[S/T]LWRLWR[K/R]A-cysteamide (SEQ ID No: 9)

VEPEP-6d: Ac-[βA/S]LWRALWRLWR[S/T]LWRLWR[K/R]A-cysteamide (SEQ ID No: 10)

VEPEP-6e: Ac-[βA/S]LWRALWRL[L/I]RALWRLLW[K/R]A-cysteamide (SEQ ID No: 11)

VEPEP-6f: Ac-[βA/S]LWRALWRL[L/C/I]RNLWRLLW[K/R]A-cysteamide (SEQ ID No: 12),

wherein the notation [X/Y] means that both amino acids X and Y have been tested at this position, with identical results.

Functionalization of Vepep-6

Two approaches were used for peptide functionalization

(1) Peptide conjugations with peptide, antibody, pegylation, NTA, cholesterol, or stearylation were performed at the primary amino group of the N-terminal residue, through a beta alanine or serine. It is advantageous to maintain the C-terminal cysteamide free, since it is known to be required to stabilize the particle through disulfide bounds (SH-SH). Functionalized peptides were further purified by Reverse Phase-HPLC and analyzed by electro-spray ionization mass spectroscopy.

(2) Peptide conjugations were also performed *via* disulfide bound using the SH-group of the cysteamide moiety of the peptide.

VEPEP-6-Funct-1: X-ALFRALWRLLRSLWRLLWK-NH-CH_2-CH_2-SH (SEQ ID No: 15) (1)

VEPEP-6-Funct-2: Ac-ALFRALWRLLRSLWRLLWK-NH-CH_2-CH_2-S-S-X (SEQ ID No: 16) (2)

X: Cholesterol, Pegylation, stearyl, palmitoyl, small FC or FAB fragments, nitrilotriacetic acid (2 x NTA) or targeting peptide (for example, brain targeting peptide).

Oligonucleotides & siRNA

siRNAs and 5' $Alexa^{700}$ or fluorescein (5'-FAM) fluorescently labelled siRNA were synthesized by Eurogentec (Belgium) according to the following sequences.

Cyc-B1 sense 5'GGCGAAGAUCAACAUGGCATT3' (SEQ ID No: 17),

Cyc-B1 antisense	5'UGCCAUGUUGAUCUUCGCCTT3' (SEQ ID No: 18),
Cyc-B3 sense	5'GGUGAAGAUCAGCAUGGCATT3' (SEQ ID No: 19),
Cyc-B3 antisense	5'UGCCAUGUCGAUCUUCACCTT3' (SEQ ID No: 20),
GAPDH sense	5'CAUCAUCCCUGCCUCUACUTT-3' (SEQ ID No: 21), and
GAPDH antisense	5'AGUAGAGGCAGGGAUGAUG3' (SEQ ID No: 22).

Cyc-B1 siRNA targeting cyclin B1, and a derived siRNA harbouring two mismatches, Cyc-B3, was used as control. An siRNA targeting GAPDH was used as control to validate target specificity and to monitor associated non specific interferon response. The stock solution of siRNA was prepared in RNase free water.

VEPEP-6 structure

VEPEP-6 peptides are secondary amphipatic peptides, they are highly versatile and show a strong structural polymorphism. VEPEP-6 are unfolded in solution as a free form and adopted an alpha helical conformation in the presence of lipid or artificial cellular membranes as well as in the presence of cargos such as siRNA/single stranded and double stranded oligonucleotides (figure 1).

Characterization of peptide-based nanoparticles

Mean particle size distribution was determined with a Coulter N4 Plus (Coulter-Beckman) at 25 °C for 3 min per measurement and zeta potential was measured with Zetasizer 4 apparatus (Malvern Ltd,)

Cytotoxicity

Toxicity of VEPEP-6/siRNA complexes was investigated on Hela and HS-68 cell lines. 30,000 cells seeded in 24-well plated the day prior transfection, were incubated with increasing concentrations of siRNA complexed with VEPEP-6 at a 20/1 molar ratio ranging from 0.1 to 5 μM (100 μM VEPEP-6), for 30 min prior to addition of medium to reach a final 10% concentration of FCS. Cytotoxic response was measured 12hr or 24hr later by monitoring the housekeeping gene cyclophilin mRNA level (Quantigen, Panomic Inc.) and by colorimetric MTT assay (Sigma, Germany), respectively. For MTT assay, cell culture medium was removed and replaced with PBS containing 2.5 mg/ml of MTT for 4hr. Results correspond to the average of 3 separate experiments.

In vivo imaging of siRNA biodistribution

In vivo fluorescence imaging was performed as previously described [26, 27]. Mice were injected intravenously with 10 μg (200 μl) of Alexa700 fluorescently labelled siRNA either naked or complexed with different VEPEP-6-based formulations:

example 5.2.1 NANOPEP-6-HIS/siRNA; example 5.3 NANOPEP-6/CADY/siRNA and NANOPEP-6/PEP1/peptide; Example 5.4 CADY/VEPEP6/siRNA and MPG/VEPEP6/siRNA, Example 5.5 NANOPEP-6/siRNA (n= 3 animals per group). Anaesthetized mice, using 2% Isoflurane, were illuminated by 663 nm light emitting diodes equipped with interference filters and movies were acquired over the first 15 minutes and fluorescence images were taken every hour for 5 hrs and then after 24 hrs, with a back-thinned CCD cooled camera as previously described [11]. At 24 hr mice were euthanized and different organs were removed for quantification of Alexa fluorescence.

Example 2: VEPEP-6 peptides applications for molecules delivery

Example 2.1: VEPEP-6 peptides form stable nanostructures with nucleic acids

VEPEP-6 peptide form stable complexes with siRNA, single stranded and double stranded oligonucleotides. The binding of cargos to VEPEP-6 was monitored by fluorescence spectroscopy using the both intrinsic Trp group of VEPEP-6 (3 to 5 Trp-residues) and extrinsic fluorescently labeled cargoes (using Cy3, Cy5 or FITC). Fluorescence experiments were performed on a PTI spectrofluorimeter at 25°C in a NaCl 154 mM buffer. Intrinsic Trp-fluorescence of VEPEP-6 was excited at 290 nm and emission spectrum was recorded between 310 and 400 nm, with a spectral band-pass of 2 and 8 nm for excitation and emission, respectively. FITC-fluorescence of labelled-siRNA or DNA was excited at 492 nm and emission recorded between 500 and 580 nm. For VEPEP-6/siRNA interaction, 0.5μM of FITC-labelled siRNA was titrated by increasing concentrations of peptide. All measurements were corrected for the dilution and curve fitting were performed by using Grafit software (Erithacus). Curve fitting reveal that VEPEP-6 strongly binds the different cargoes with dissociation constant in the nanomolar range (Table 1 and Figure 2).

Example 2.2: VEPEP-6 peptides form stable nanostructures with small hydrophobic molecules

VEPEP-6 peptides also form stable particles with small aromatic and/or hydrophobic molecules including daunomycin, Paclitaxel, doxorubicin, AZT, porphyrin, FAM-G or fluorescent dyes (Figure 3). The dissociation constant for small hydrophobic

molecule ranges between 0.03 to 4 μM, depending on the nature of the dyes and of the peptides.

VEPEP-6	Cargoes					
	siRNA		SS-DNA		DD-DNA	
	Binding	Kd (nM)	Binding	Kd (nM)	Binding	Kd (nM)
VEPEP-6a:	yes	10-20	yes	50-100	yes	200
VEPEP-6b	yes	10-20	yes	50-100	yes	200
VEPEP-6c:	yes	10-20	yes	50-100	yes	200
VEPEP-6d:	yes	10-20	yes	50-100	yes	200
VEPEP-6e	yes	10-20	yes	50-100	yes	200
VEPEP-6f:	yes	10-20	yes	50-100	yes	200

Table 1: VEPEP-6/Cargo complexes characterization. SS-DNA: single-stranded DNA; DD-DNA: double-stranded DNA.

VEPEP-6	Cargoes							
	Doxorubicin		porphyrin		AZT		FAM-guanosine	
	Binding	Kd (μM)	Binding	Kd (μM)	Binding	Kd (μM)	Binding	Kd (μM)
VEPEP-6a:	yes	1	yes	4	no	--	no	--
VEPEP-6b	yes	0.7	yes	5	no	--	no	--
VEPEP-6c:	yes	0.03	yes	1.2	yes	0.3	yes	4
VEPEP-6d:	yes	0.5	yes	0.5	yes	0.1	yes	0.8
VEPEP-6e	yes	0.5	yes	0.2	yes	0.9	yes	2.3
VEPEP-6f:	yes	0.2	yes	0.2	yes	2.4	yes	2.8

Table 2: VEPEP-6/Cargo complexes characterization. SHM: small hydrophobic molecules (porphyrin, FAM-G, AZT, doxorubicin)

Similar results were obtained for Doxorubicin, paclitaxel and daunomycin.

Example 2.3: VEPEP-6 peptides form stable nanoparticles with their different cargoes

The size of the particles was monitored by dynamic light scattering. The optimal VEPEP-6 peptide/cargo molar ratio is ranging between 1/10 to 1/30 (Figure 4). The size of the particles is of about 100 to 200 nanometers in diameter.

The complex formation was also analyzed by dynamic light scattering, electron scan microscopy (Figure 5a) and atomic force microscopy (AFM) (Figure 5b). Size of the particles was of 100 to 300 nm when measured with these technologies.

Example 3: VEPEP-6 APPLICATIONS in CULTURED CELLS

Example 3.1: VEPEP-6 mediated delivery of siRNA in different cell lines

VEPEP-6 peptides have been applied for the delivery of different siRNA into different cell lines, including adherent, suspension and challenging cell lines such as primary (Jurkat, CEM-ss, HUVEC, THP1...) and stem cell lines (mES, CCM..). Cell lines (from American Type Culture Collection (ATCC)) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 2mM glutamine, 1% antibiotics (streptomycin 10'000 µg/ml, penicillin, 10,000 IU/ ml) and 10% (w/v) foetal calf serum (FCS), at 37°C in a humidified atmosphere containing 5% CO_2. Stock solutions of VEPEP-6/siRNA particles were prepared by complexing 100nM siRNA with VEPEP-6 peptides at a molar ratio of 1/20 for 30 min at 37°C. Lower concentrations of VEPEP-6-carrier / siRNA (from 20 nM to 0.125 nM) were obtained by serial dilution of the stock complexes in PBS, in order to preserve the same VEPEP-6-carrier/siRNA ratio. 150 000 cells seeded in a 35 mm dish the day prior transfection, were grown to 60 % confluence and overlaid with 200 µl of preformed complexes, incubated for 3-5 min, then 400 µl of DMEM were added. After 30 min. incubation at 37°C, 1 ml of fresh DMEM containing 16% foetal calf serum (FCS) was added in order to reach a final FCS concentration of 10%, without removing the overlay of VEPEP-6/siRNA complexes. Cells were returned to the incubator for 24 hrs. Dose-response experiments performed on different cultured cells revealed that VEPEP-6-mediated delivery of siRNA (GAPDH) induced a robust downregulation of both GAPDH protein and mRNA levels (Table 3). In most of the cell lines, knock out (KO) higher than 70% was obtained at the protein level. Table 4 provides another series of cell lines on which VEPEP-6 have been shown to induce more that 60% KO of GAPDH.

Cell lines	origin	efficiency
Hela	Human epithelial cervical cancer cells	75%
Jurkat	Human T lymphocyte	70%
HepG2	Human hepatocyte	80%
C2C12	Mouse myoblast	80%
MEF	Mouse fibroblast	90%
HS-68	Human fibroblast	90%
CEM-SS	Human macrophage	80%
U2OS	Human osteoblast	91%
MCF7	Human breast adenocarcinoma	68%
MT4	Human T lymphocyte	70%
HER2	Human breast cancer	90%
MDA-MB	Human breast cancer	85%

Table 3

Cells	**origin**
Balb /c3T3	differentiated mouse adipocytes
C2C12	differentiated mouse myotubes
C2C12	undifferentiated mouse myocytes
RAW 264.7	mouse macrophage cells
U87MG	Human brain glioblastoma astrocytoma cells
Astrocytoma cells	Human
NHEK-AD	primary human adult keratinocytes
THP -1	Human peripheral blood acute monocytic leukemia cells
HUVEC	Human umbilical vein endothelial cells
HT29	Human colon carcinoma cells
SWG20	Human colon carcinoma cells
BSMC	Human bronchial smooth muscle cells
A549	Human lung carcinoma cells
A2780	Human ovarian cancer cells
ASPC-1	Human pancreatic carcinoma
mES	Mouse embryonic stem cells
CCM	Primary chicken cardiac embryonic myocyte
MPN	Primary mouse neuronal cells

Table 4

Example 3.2: VEPEP6-mediated delivery of siRNA targeting cyclin B1 induces G2 arrest

Dose-response experiments performed on cultured cells revealed that VEPEP-6-mediated delivery of siRNA (Cyc-B1) induced a robust biological response associated with downregulation of both cyclin B1 protein and mRNA levels (Figure 6). Cyclin B1 mRNA and protein levels were determined 12 and 24 hrs following transduction, using Quantigen (Panomics Inc.) and Western blotting, respectively. Mouse monoclonal anti-Cyclin B1 antibodies (SC-245) and rabbit polyclonal anti-Cdk2 antibodies (SC-163) were obtained from Santa Cruz Biotechnology Inc. Data reported are an average of 3 or 4 distinct experiments. A siRNA concentration of 5 nM was sufficient to reduce cyclin B1 levels by more than 85% in Hela cells and IC_{50} of 1.7 ± 0.7 nM and 1.9 ± 0.3 nM were estimated for downregulation of protein levels, and of 0.7 ± 0.1 nM and 0.5 ± 0.1 nM for mRNA levels, for non-transformed HS68 fibroblasts (Figure 6) and HeLa cells, respectively.

Reduction of cyclin B1 protein levels was directly associated with accumulation of cells with a 4N content, consistent with downregulation of Cdk1-Cyclin B1 activity, and was optimally obtained with 2 nM siRNA and IC_{50} values estimated to 1.2 ± 0.2 nM and 1.7 ± 0.4 nM for HeLa and HS68 cells, respectively (Figure 6C). In contrast, no effect on cyclin B1 levels and cell cycle progression was observed with 200 nM of an unrelated siRNA (si-GAPDH), or of a mismatch siRNA harbouring two mutations (Cyc-B3) complexed with VEPEP-6 at a 1/20 ratio, or with VEPEP-6 carrier alone (100 µM).

Example 3.3: VEPEP6-mediated delivery of siRNA is not toxic.

As shown on Figure 7A, the toxicity of VEPEP-6 particles was investigated on HeLa cells by MTT assay and by monitoring the level of cyclophilin mRNA measured by quantigen™ technology (Affymetrix). No toxicity was detected at levels up to 10 nM, and only a mild toxicity was observed at the maximum concentration of 100 nM. VEPEP-6 and NANOPEP-6 (VEPEP6/VEPEP6/siRNA) did not induce immune response *in vivo*. As toxicity has been one of the major causes of failure of siRNA delivery systems, the impact of the VEPEP-6 and NANOPEP-6 particles on non specific cytokine induction has been investigated 6h and 24h after IV injection. Cytokine induction was monitored using the multiplex 10 cytokine KIT from invitrogen. As shown on Figure 7B, no induction of cytokine was observed after 6h and 24h, in contrast to control lipid-based formulation or Lipopolysaccharide (LPS).

Example 3.4: VEPEP-6 mediated delivery of small hydrophobic molecule in different cell lines

VEPEP-6 peptides have been applied for the delivery of different small fluorescent hydrophobic molecules and doxorubicin on different cell lines including primary cell lines and challenging cell lines. VEPEP-6 peptides also form stable particles with small aromatic molecules including doxorubicin or fluorescent dyes (Figure 8 and Figure 9). The dissociation constant for small hydrophobic molecules ranges between 0.1 to 4 μM, depending on the nature of the dyes and of the peptides.

Example 4: NANOPEP-6 formulations and applications for *in vivo* delivery

NANOPEP-6 particles contain a "peptide-core" or "core shell" corresponding to the association of either VEPEP-6 peptide or any other peptide forming non covalent complexes with its respective cargo, that is surrounded by additional VEPEP-6 "peripheral" peptides stabilizing the particle and favouring cell membrane association. The efficiency of NANOPEP-6 is mainly controlled by the size and the charge of the particles, which should be ranging between 100 -200 nm and +10 -+20 Volts, respectively. Several combinations can be used for the "core" and peripheral VEPEP-6 can be functionalized or not. The choice of the peptides in the "core" is dependent on the nature of the cargoes and can be, for example, VEPEP-6, CADY [12], MPG [11] or PEP-1 [8].

The NANOPEP-6 particles are formed in a two step process (Figure 10A): first the "core" at molar carrier/cargo ratio of 5/1 or 10/1, then the "peripheral" at a peripheral VEVEP-6/cargo molar ratio of 20/1 up to 80/1. The multilayer organization of the particle allows their oriented functionalization, that will be chosen depending on the nature of the cellular target/tissue and administration mode.

A three step protocol (Figure 10B) has been established when particle functionalization takes place *via* the nitrilotriacetic acid (NTA) linked to the VEPEP-6. NTA-group is well known as being able to chelate metal and to strongly interact with histidine tagged protein. Coating of the particles with NTA-functionalized VEPEP-6 allows the attachment any protein harboring a histidine tag to the particle. That strategy offers the major advantage of having a common 2 layers particles "NANOPEP-6-HIS" that can be associated to any His-tagged protein. The NANOPEP-6-HIS strategy has been applied to coat the particles with specific antibody targeting cell surface antigen (EGF,

HER-2 or MUC1) for targeted-delivery of siRNA and peptide (see example 5.2.1 for siRNA delivery and example 5.2.2 for peptide delivery using MUC1 and HER2-antibodies). NANOPEPHIS-6 strategy can be universally applied to any peptides and proteins harboring a Histidine cluster in their sequence.

Example 5: NANOPEP-6 strategy applications

NANOPEP-6 strategy has been applied for *in vivo* delivery and targeting of different cargos and different peptide-based nanoparticles. Four different examples of NANOPEP-6 and NANOPEPHIS-6 applications are reported thereafter.

Example 5.1: NANOPEP-6 mediated siRNA *in vivo* targeted delivery after systemic intravenous injection or topical injection

The therapeutic potential of the NANOPEP-6 technology has been evaluated *in vivo* with siRNA targeting either GAPDH and cyclin B1, a non-redundant mitotic cyclin. The potency of this technology has been validated *in vivo* with siRNA targeting cyclin B1, a non-redundant mitotic cyclin and established therapeutic target in several cancers, which forms together with Cdk1 kinase, the "Mitosis Promoting Factor" required for entry and progression through mitosis.

Athymic female nude mice (6-8 weeks of age) were subcutaneously inoculated into the flank with 1 x 10^6 PC3 (prostate cancer), A549 (lung cancer) or SCK-3-HEK2 (ovarian cancer) cells in 100 µl PBS. Two to three weeks after tumour implant, when tumour size reached about 100 mm^3, animals were treated by intratumoral or intravenous injection, every 3 days, with a solution of 0.1 ml of either free Cyc-B1 siRNA (50 or 100 µg), control siRNA Cyc-B3 or Cyc-B1 siRNA (1, 5, 10 µg) complexed within NANOPEP-6 particles. The "core" shell of the particles was formed using VEVEP-6 peptide at a molar ratio of 10/1 or 20/1 with a siRNA targeting cycline B1. siRNA stock solutions are in 50 mM Tris, 0.5 mM EDTA buffer or in RNase free water. VEPEP-6 peptides were solubilised in water and stock solution of peptide was sonicated for 10 min in a water bath before complex formation. Then VEPEP-6/siRNA complexes were formed by adding siRNA into the peptide solution and incubating at 37°C for 20-30 minutes to allow the carrier peptide/siRNA complexes to form. NANOPEP-6 particles contain a VEPEP-6/siRNA "core shell" surrounded by additional VEPEP-6 or Chol-VEPEP-6 at a 1/20 molar ratio. Formulations containing 15 % Chol-VEPEP-6 were prepared in a

stepwise fashion by first forming a precomplex of VEPEP-6/siRNA at molar ratio of 20/1, followed by addition of Chol-VEPEP-6 so as to increase the ratio of carrier/siRNA to 40/1.

Tumour diameter was measured in two directions at regular intervals using a digital calliper and tumour volume was calculated as length x width x height x 0.52. Curves show the mean value of tumour size in a cohort of six animals and neither animal death nor any sign of toxicity were observed. Experiments were performed according to national regulations and approved by the local animal experimentation ethical committee. The statistical significance of the results was calculated by Student's t test and $p<0.05$ considered to be statistically significant. The stock solutions of particles are performed in water and stable for at least three weeks at 4°C. Particles can be lyophilized for long time storage; in that case, 5 to 20% of glucose is added to the particle solution before lyophylization to stabilize the particles during the process. Before administration the particles are diluted in physiological conditions, in the presence: 0.9% NaCl and 5 to 20% glucose.

The inventors demonstrated that combining cyclin B1 siRNA with NANOPEP-6 prevents lung, ovarian and prostate tumour growth in xenografted mouse models, upon injection every three days of NANOPEP-6/siRNA complexes (Figure 11).

NANOPEP-6 Cyclin B1 siRNA delivery upon topical injection

The potential of NANOPEP-6 (VEPEP6/VEPEP6) to deliver cyclin B1 siRNA *in vivo* was first evaluated on human prostate carcinoma cell PC3-xenografted mice (Fig 11A). The effect of local intratumoral administration of NANOPEP-6/siRNA particles (molar ratio 20/1) on the growth of established subcutaneous tumours was evaluated. At day 50, tumor sizes in the control cohort, injected with PBS increased by about 3.5 fold. Reduction of tumor growth by 75% was observed using 1μg (0.05 mg/kg) of cyclin B1 siRNA in NANOPEP-6/siRNA and tumour growth was completely prevented with 5μg (0.25 mg/kg) of cyclin B1 siRNA in NANOPEP-6/siRNA (Figure 11A). At day 48, it was validated that the Cyc-B1 siRNA mediated inhibition of tumour growth was directly associated with a decrease in the level of cyclin B1 mRNA. As a control, the inventors showed that administration of 100 μg (intratumoral or intravenous) naked siRNA or NANOPEP-6 carrier alone had no significant effect on tumour growth. Moreover, inhibition of tumour growth was siRNA sequence-specific as a cyclin B1 siRNA

harbouring two mutations (Cyc-B3) complexed with NANOPEP-6 and injected into mice at 0.5mg/kg was unable to inhibit tumour growth.

NANOPEP-6 Cyclin B1 siRNA delivery upon systemic injection

NANOPEP-6 particles were used for systemic intravenous administration into two xenografted tumor mouse models: human prostate carcinoma (PC3) and ovarian cancer cells (HT29) injected subcutaneously into the flanks of NCR nude mice. NANOPEP-6/siRNA particles were obtained stepwise by complexing siRNA molecules with VEPEP-6 at a molar ratio of 10/1, followed by coating of particles with a second layer of VEPEP-6 at ratio 10/1. Five micrograms (0.25 mg/kg) and 10 μg (0.5 mg/kg) of Cyc B1 siRNA in NANOPEP-6 particles were injected intravenously every three days into mice bearing PC3 or HT29 xenografted tumors. A significant reduction in PC3 tumor size was observed at day 50, with 60% and 92% inhibition with 5μg and 10 μg of siRNA, respectively (Figure 11B). The reduction in tumour size was directly correlated to reduction of cyclin B1 protein levels, as evaluated by Western blotting, by 60% and 80% in animals treated with 5μg and 10 μg of siRNA complexed to NANOPEP-6, respectively. A significant reduction in HT29 tumor size was observed at day 50, with 35% and 70% inhibition with 5μg and 10 μg of siRNA, respectively (Figure 11C). These results together with the lack of anti-tumoral activity of the NANOPEP-6/mismatch siRNA (10μg) or of NANOPEP-6 carrier alone, underscores the robustness and specificity of the biological response associated with systemic delivery of cyclin B1 siRNA. The stability of drug-carrier formulations *in vivo* and in the blood circulation is a major issue for systemic administration of therapeutics. In order to improve the bioavailability and stability of the NANOPEP-6/siRNA (VEPEP-6 coating a VEPEP6/siRNA core shell) particles, thereby rendering them more suitable for systemic administration, the surface layer of NANOPEP-6 particles was functionalized with a cholesterol-moiety at the N- terminus of VEPEP-6 (Chol-VEPEP6), through activation of the N-terminal beta alanine amino group. Cholesterol-functionalized NANOPEP-6/siRNA particles were obtained stepwise by complexing VEPEP-6 molecules with siRNA at a molar ratio of 20/1, followed by coating of particles with a second layer of Chol-VEPEP6 at ratio 1/10. In order to analyze if increase in the distribution of siRNA associated to functionalized-NANOPEP-6 particles directly affects its potency to inhibit tumour growth, the particles were used for systemic intravenous administration into HT29 xenografted tumor mouse model. Two μg (0.25 mg/kg) of Cyc-B1 siRNA complexed with chol-NANOPEP-6 at a 1/30 ratio were injected

intravenously every three days into mice bearing HT29 xenografted tumor and a significant reduction in HT29 tumor size of 85% was observed at day 50 (Fig 11B), which is 5 fold more potent than the non functionalized-NANOPEP-6 nanoparticle, suggesting that cholesterol increases the biodistribution of siRNA in the tumour by maintaining siRNA in the plasma

Example 5.2: NANOPEPHIS-mediated *in vivo* targeted delivery in tumor after systemic intravenous injection.

5.2.1 NANOPEPHIS-mediated in vivo targeted delivery of siRNA in tumor after systemic intravenous injection

The VEPEP-6/siRNA core shell particles were coated with NTA functionalized VEPEP-6 peptide and then incubated with his-tagged MUC1 or HEK-2 antibodies, known to target tumour cells. The excess of antibodies was removed by filtration before *in vivo* IV injection. The NANOPEP-6-HIS/siRNA particles were administrated by systemic IV injection into xenografted human prostate (PC3) and lung (A549) carcinoma mouse models. PC3 and A549 cells were injected subcutaneously into the flanks of NCR nude mice. 1μg (0.05 mg/kg) and 5μg (0.25 mg/kg) of Cyc-B1 siRNA complexed with MUC-NANOPEP-6-HIS/siRNA or HEK-NANOPEP-6-HIS/siRNA at a 40/1 molar ratio were injected intravenously every three days into mice bearing PC3 or A549 xenografted tumour. The level of siRNA release in the tumor was determined by live fluorescence imaging using An Alexa-700 fluorescently-labelled siRNA. Live fluorescence imaging experiments demonstrated that the presence of MUC or HER-2 antibodies significantly increased the level of siRNA in the tumour (5 to 10 folds depending on the antibody and the tumors (see Fig 12 and table 5).

Formulations	siRNA delivery in PC3 (fold increase)	siRNA delivery in A549 (fold increase)
NANOPEP-6	1	1
MUC-NANOPEP-6-HIS	5	12
HEK-NANOPEP-6-HIS	7	8

Table 5: NANOPEP-6 mediated delivery of fluorescently labelled siRNA in PC3 and A549 xenografted tumour in mice.

A significant reduction in PC3 or A549 tumor size was observed at day 30, with 55% and 95% inhibition with 1μg and 5 μg of siRNA, respectively (Figure 12b). The reduction in tumour size is at least 5-fold more efficient with the MUC-NANOPEPHIS/siRNA or HEK-NANOPEPHIS/siRNA, than with the NANOPEP-6/siRNA.

Example 5.2.2: NANOPEPHIS-mediated in vivo targeted delivery of peptide in tumor after systemic intravenous injection.

The PEP1/peptide C4 (PC4: a 12 mer-peptide reported to block cancer cell proliferation: ref 13) core shell particles were coated with NTA functionalized VEPEP-6 peptide and then incubated with his-tagged MUC1 or HEK-2 antibodies. The excess of antibodies was removed by filtration before *in vivo* IV injection. The NANOPEP-6-HIS/PEP1/PC4 particles were administrated by systemic IV injection into xenografted human prostate (PC3) and lung (A549) carcinoma mouse models. PC3 and A549 cells were injected subcutaneously into the flanks of NCR nude mice. NTA-functionalized VEPEP-6 peptides were complexed with 10μg of PEP1/PC4 particles at a 20/1 VEPEP-6/PC4 molar ratio and then with HIS-tagged antibodies to form MUC-NANOPEP-6-HIS/PEP1/PC4 or HEK-NANOPEP-6-HIS/PEP1/PC4 particles, which were injected intravenously into mice bearing PC3 or A549 xenografted tumour. The level of PC4 release in the tumour was determined by live fluorescence imaging using a CY5-labelled PC4. Live fluorescence imaging experiments demonstrated that the presence of MUC or HER-2 antibodies significantly increased the level of PC4 in the tumours (15 to 40 folds depending on the antibody and the tumours (see Fig 12c and table 6).

Formulations	siRNA delivery in PC3 (fold increase)	siRNA delivery in A549 (fold increase)
PEP1/PC4	1	1
MUC-NANOPEP-6-HIS/PEP1/PC4	15	35
HEK-NANOPEP-6-HIS/PEP1/PC4	20	40

Table 6: NANOPEP-6-mediated delivery of fluorescently labelled peptide in PC3 and A549 xenografted tumour in mice.

Example 5.3: NANOPEP-6-mediated *in vivo* delivery of peptide based nanoparticles for therapeutic siRNA and Peptide and lung targeting.

VEPEP-6 peptides were used to promote lung targeting of peptide-based nanoparticles. VEPEP-6 peptide was added as a coating peptide on VEPEP-6/siRNA, CADY/siRNA and PEP-1/peptide "core shell particles" (Figures 13b and 13c). The cargos used were either a fluorescently-labelled siRNA or a peptide, or a siRNA targeting the housekeeping gene GAPDH. Particles were formed as reported in Fig 10. In all the cases, 5 µg of cargoes complexed with peptide carrier were injected intravenously. Then the *in vivo* biodistribution of the cargos was monitored using live fluorescence animal imaging and by monitoring the level of housekeeping gene mRNA 48hr after injection. The presence of VEPEP-6 coating induced an important increase of the cargoes delivery in the lung (Fig 13a and 13b). Monitoring fluorescence labelled cargoes showed a 5 to 10-fold increase in the lung, in comparison to control experiment in the absence of VEPEP-6 coating of the particle. The presence of VEPEP-6 coating also increased by a factor of 3 the knockdown of GAPDH in the lung (Fig 13c). Taken together, these results demonstrated that VEPEP-6 peptide improves significantly lung targeting of peptide-based nanoparticles.

Example 5.4: CPP-mediated *in vivo* targeted delivery of VEPEP-6/siRNA complexes

MPG and CADY cell penetrating peptides were used to promote different *in vivo* targeting of VEPEP-6/siRNA nanoparticles. The "core shell particles" VEPEP-6/siRNA were formed at 20/1 molar ratio and then MPG or CADY peptides were added as a coating peptide at a molar ratio 20/1, as reported in Fig 10. In both cases, 5 µg of siRNA targeting the housekeeping gene GAPDH complexed with peptide carrier were injected intravenously. Then the *in vivo* biodistribution of the siRNA was monitored using live fluorescence animal imaging and by monitoring the level of housekeeping gene mRNA 48hr after injection. Biodistribution of the fluorescently labelled siRNA was evaluated *in vivo* on Balb6 Mouse, 5 hr after a single administration of 5 µg siRNA in MPG/VEPEP-6 or CADY/VEPEP-6 particles. The presence of MPG or CADY coating induced an important increase of the siRNA delivery in most of the tissues. MPG specifically targets the brain, pancreas, lymph nodes and CADY the lung, muscle, heart and intestine (Fig 14a). The presence of CADY or MPG coating also promotes a marked knockdown of GAPDH in the selected tissues (Fig 14b). Taken together, these results

demonstrated that VEPEP-6/siRNA core shell can be coated by several other CPPs to improve *in vivo* tissue targeting of peptide-based nanoparticles.

Example 5.5: NANOPEP-6 mediated topical application of peptide-based nanoparticles: dermal/intranasal and intrarectal administration.

NANOPEP-6 (VEPEP-6/VEPEP-6) based particles have been evaluated using different administration routes including systemic intravenous, intrarectal, intranasal and transdermal administration. A fluorescently labelled siRNA with Alexa700 or a siRNA targeting the GAPDH was associated to VEPEP-6 as a "core shell", surrounded by VEPEP-6 molecules. Biodistribution of the fluorescently labelled siRNA was evaluated *in vivo* on Balb6 Mouse, 5 hr after a single administration of 10 µg siRNA in NANOPEP-6 particles. The siRNA associated GAPDH knock out was evaluated after 48 hr on different tissues, by monitoring the level of GAPDH mRNA using Quantigen technology (Panomics inc). Intravenous and intrarectal administrations of the NANOPEP-6/siRNA complex allowed the delivery of the siRNA in most of the analyzed tissues, excepted brain and ganglions (Fig 15a). These fluorescence results are directly correlated to a KO of the GAPDH mRNA, demonstrating that NANOPEP-6 delivers an active form of the siRNA in all the tissues and can be efficiently applied by topical administration (Fig 15b). Intranasal and intratracheal administration allowed the delivery of active siRNA mainly in the lung, liver and kidney and in the brain, with a significant KO of the GAPDH gene in these tissues. In contrast, transdermal administration is limited to the delivery of the siRNA into and through the skin and muscles, but not in the other tissues (Fig 15c).

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CLAIMS

1. A cell-penetrating peptide characterized in that it comprises an amino acid sequence $X_1LX_2RALWRLX_3RX_4LWRLX_5X_6X_7X_8$ (SEQ ID No: 13), wherein X_1 is beta-A or S, X_2 is F or W, X_3 is L, W, C or I, X_4 is S, A, N or T, X_5 is L or W, X_6 is W or R, X_7 is K or R, and X_8 is A or none.

2. The cell-penetrating peptide of claim 1, characterized in that it comprises an amino acid sequence of $X_1LX_2RALWRLX_3RX_4LWRLX_5X_6KX_7$ (SEQ ID No: 14), wherein X_1 is beta-A or S, X_2 is F or W, X_3 is L or W, X_4 is S, A or N, X_5 is L or W, X_6 is W or R, X_7 is A or none.

3. The cell-penetrating peptide of claim 1 or claim 2, wherein the amino acid sequence is selected in the group consisting of

- $X_1LFRALWRLLRX_2LWRLLWX_3$ (SEQ ID No: 7)
- $X_1LWRALWRLWRX_2LWRLLWX_3A$ (SEQ ID No: 8)
- $X_1LWRALWRLX_4RX_2LWRLWRX_3A$ (SEQ ID No: 9)
- $X_1LWRALWRLWRX_2LWRLWRX_3A$ (SEQ ID No: 10)
- $X_1LWRALWRLX_5RALWRLLWX_3A$ (SEQ ID No: 11) and
- $X_1LWRALWRLX_4RNLWRLLWX_3A$ (SEQ ID No: 12),

wherein X_1 is beta-A or S, X_2 is S or T, X_3 is K or R, X_4 is L, C or I and X_5 is L or I.

4. The cell-penetrating peptide of any of claims 1 to 3, further comprising, covalently linked to the N-terminal end of the amino acid sequence, one or several chemical entities selected in the group consisting of an acetyl, a fatty acid, a cholesterol, a poly-ethylene glycol, a nuclear localization signal, nuclear export signal, an antibody, a polysaccharide and a targeting molecule.

5. The cell-penetrating peptide of any of claims 1 to 4, further comprising, covalently linked to the C-terminal end of said amino acid sequence, one or several groups selected in the group consisting of a cysteamide, a cysteine, a thiol, an amide, a nitrilotriacetic acid optionally substituted, a carboxyl, a linear or ramified C_1-C_6 alkyl optionally substituted, a primary or secondary amine, an osidic derivative, a lipid, a phospholipid, a fatty acid, a cholesterol, a poly-ethylene glycol, a nuclear localization signal, nuclear export signal, an antibody, a polysaccharide and a targeting molecule.

6. The cell-penetrating peptide of any of claims 1 to 5, which is chosen in the group consisting of:

- VEPEP-6a: Ac-X_1LFRALWRLLRSLWRLLWK-cysteamide (SEQ ID No: 1)
- VEPEP-6b: Ac-X_1LWRALWRLWRSLWRLLWKA-cysteamide (SEQ ID No: 2)
- VEPEP-6c: Ac-X_1LWRALWRLLRSLWRLWRKA-cysteamide (SEQ ID No: 3)
- VEPEP-6d: Ac-X_1LWRALWRLWRSLWRLWRKA-cysteamide (SEQ ID No: 4)

- VEPEP-6e: Ac-X_1LWRALWRLLRALWRLLWKA-cysteamide (SEQ ID No: 5) and
- VEPEP-6f: Ac-X_1LWRALWRLLRNLWRLLWKA-cysteamide (SEQ ID No: 6),

wherein X_1 is beta-A or S.

7. A complex comprising a cell-penetrating peptide according to any of claims 1 to 6 and a cargo selected amongst nucleic acids and hydrophobic molecules.

8. The complex of claim 7, wherein said cargo is a siRNA selected to silence a target mRNA.

9. The complex of claim 7 or claim 8, wherein the size of the complex is between 50 and 300 nm.

10. A nanoparticle comprising a complex according to any of claims 7 to 9, coated by a layer of peripheral cell-penetrating peptides, wherein said cell-penetrating peptides have a peptide sequence different from SEQ ID No: 13.

11. A nanoparticle comprising a core which comprises a cargo complexed to a first entity selected in the group consisting of cell-penetrating peptides, liposomes, polycationic structures and carbon nanoparticles, wherein said core is coated by a cell-penetrating peptide according to any of claims 1 to 6.

12. The nanoparticle of claim 10, wherein said first entity is a cell-penetrating peptide selected in the group consisting of CADY, MPG, PEP-1, PPTG1, poly Arginine motif and cell-penetrating peptides according to any of claims 1 to 6.

13. The nanoparticle of any of claims 10 to 12, wherein the size of the nanoparticle is between 80 and 400 nm.

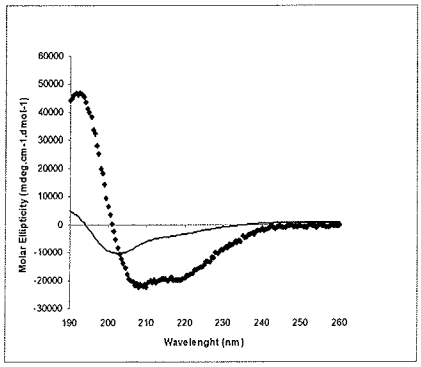
14. The complex of any of claims 7 to 9, or the nanoparticle of any of claims 10 to 13, wherein the cell-penetrating peptide according to any of claims 1 to 6 comprises an acetyl group covalently linked to its N-terminus, and/or a cysteamide group covalently linked to its C-terminus.

15. The complex or nanoparticle of any of claims 7 to 14, wherein at least part of the cell-penetrating peptides are bound to a targeting molecule.

16. A therapeutic composition comprising a complex or a nanoparticle according to any of claims 7 to 15.

17. A method for delivering a molecule into a cell *in vitro*, comprising a step of putting said cell into contact with a complex comprising said molecule and cell-penetrating peptides according to any of claims 1 to 6.

A



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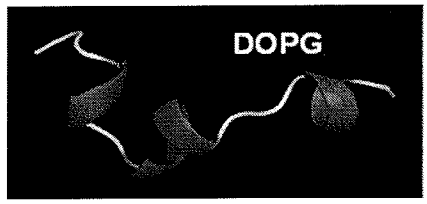

Figure 1

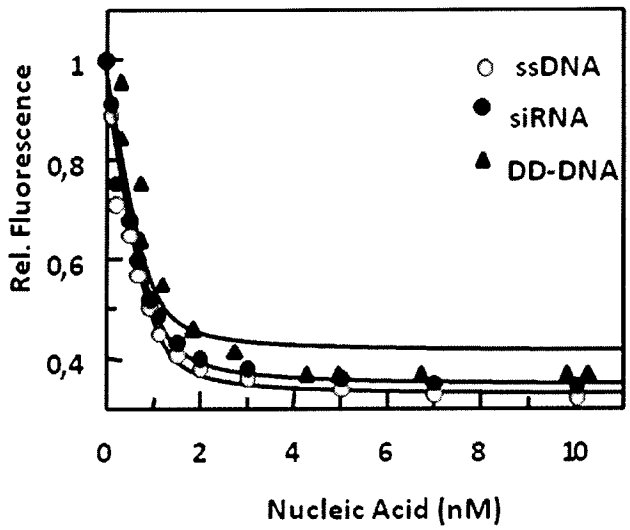

Figure 2

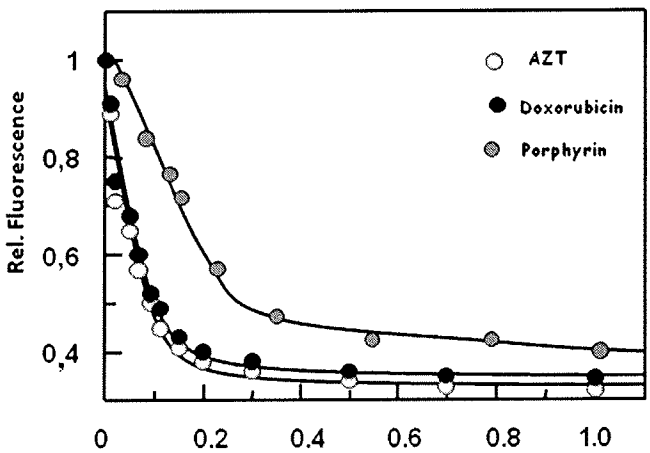

Figure 3

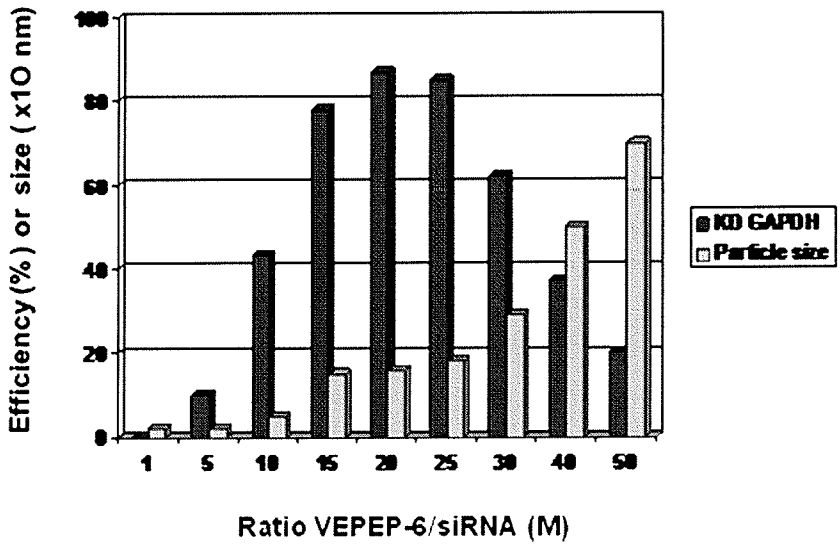

Figure 4

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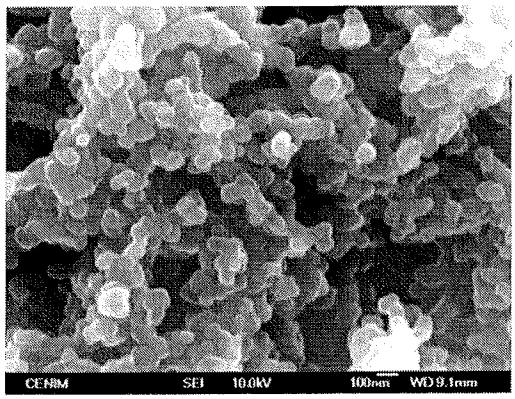

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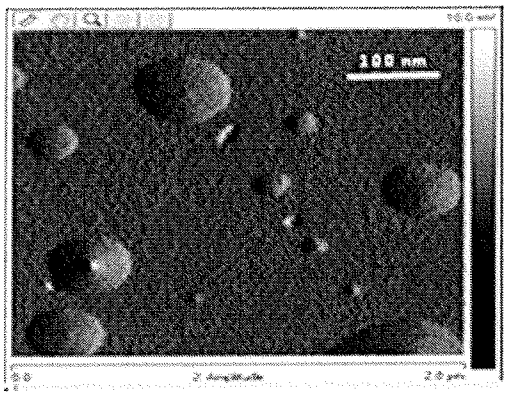
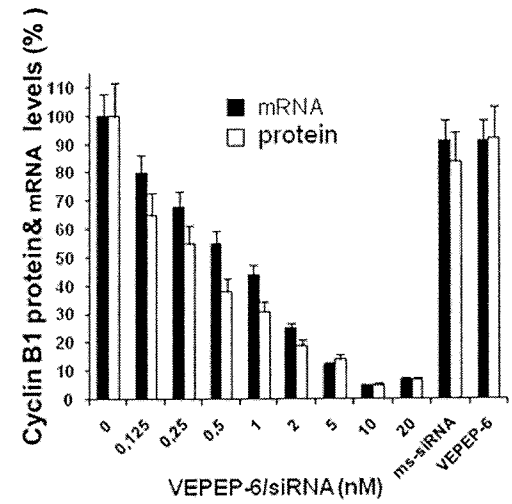


Figure 5


Cyclin B1 protein& mRNA levels (%)
mRNA
protein
0
10
20
30
40
50
60
70
80
90
100
110
0
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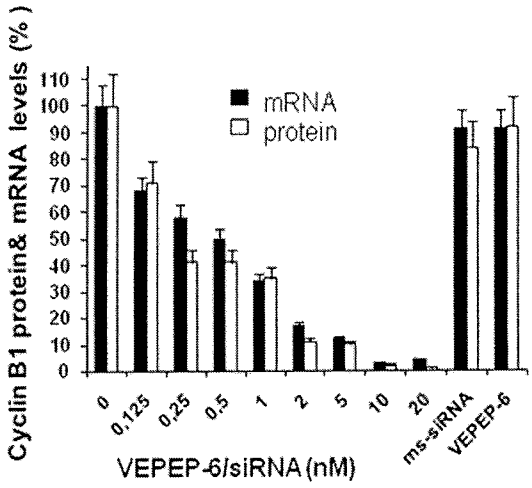
0,5
1
2
5
10
20
ms-siRNA
VEPEP-6
VEPEP-6/siRNA (nM)

A


Cyclin B1 protein& mRNA levels (%)
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protein
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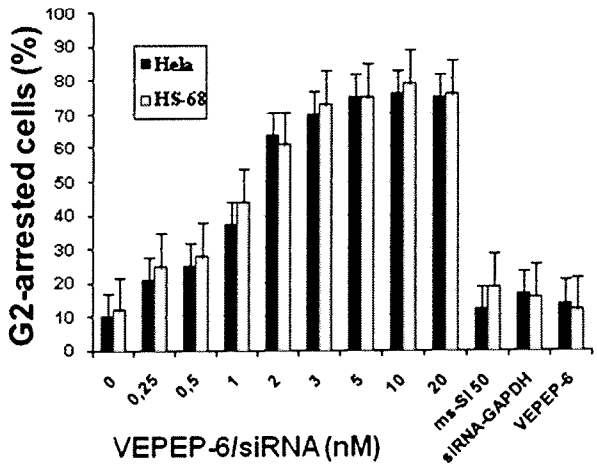
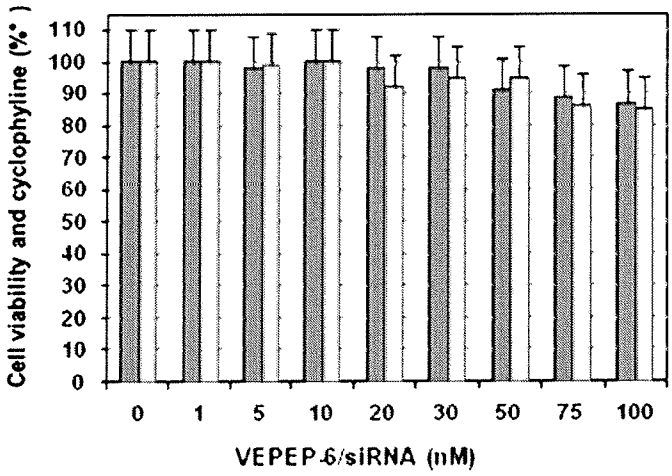
10
20
30
40
50
60
70
80
90
100
110
0
0,125
0,25
0,5
1
2
5
10
20
ms-siRNA
VEPEP-6
VEPEP-6/siRNA (nM)

B


G2-arrested cells (%)
Hela
HS-68
0
10
20
30
40
50
60
70
80
90
100
0
0,25
0,5
1
2
3
5
10
20
ms-SI50
siRNA-GAPDH
VEPEP-6
VEPEP-6/siRNA (nM)

C

Figure 6


Cell viability and cyclophyline (%*)
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100
90
80
70
60
50
40
30
20
10
0
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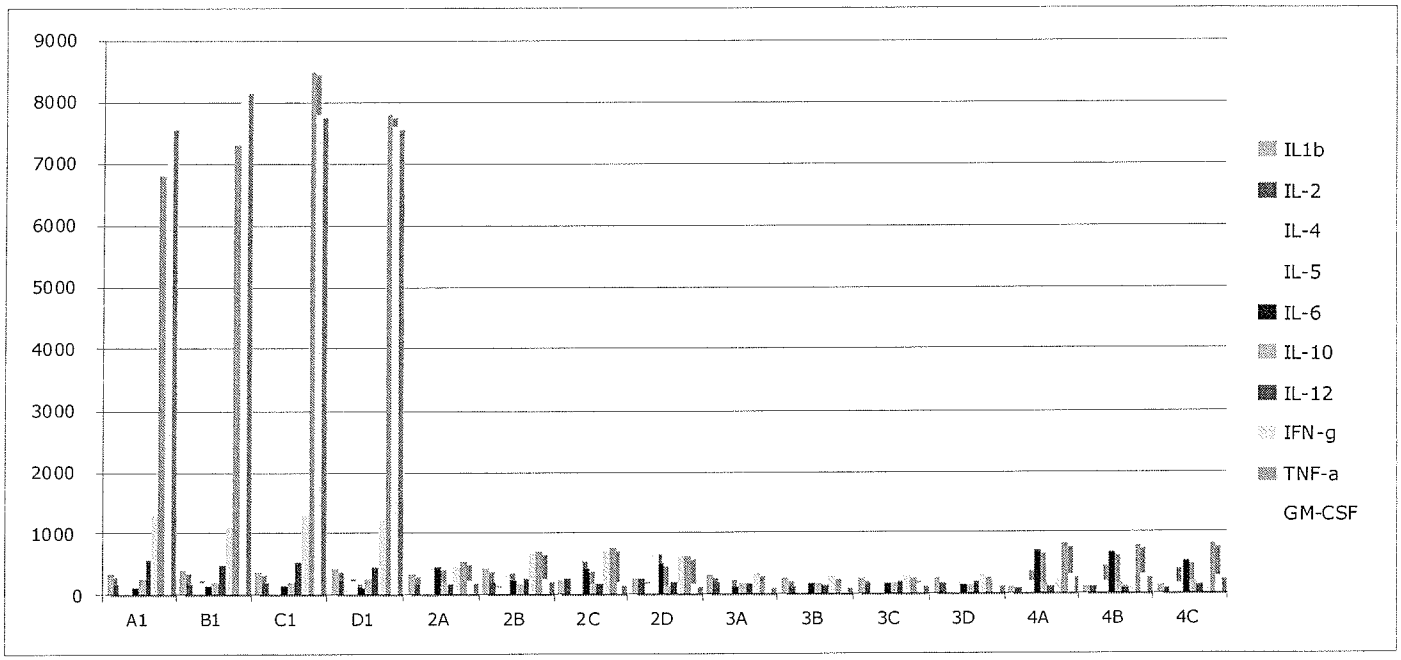
VEPEP-6/siRNA (nM)

A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7000
6000
5000
4000
3000
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1000
0
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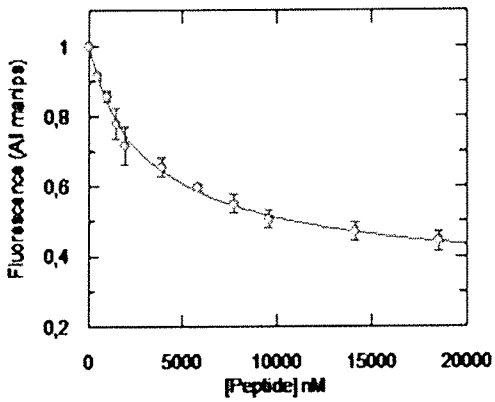
IL1b
IL-2
IL-4
IL-5
IL-6
IL-10
IL-12
IFN-g
TNF-a
GM-CSF

B

Figure 7


Fluorescence (AI units)
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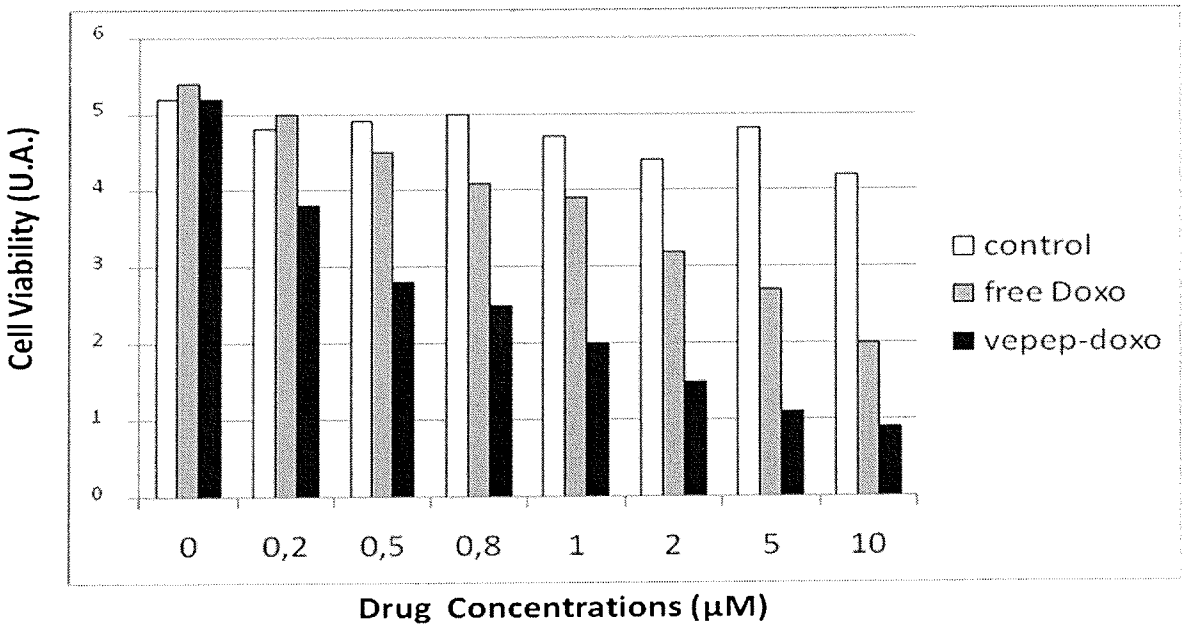
0,6
0,4
0,2
0
5000
10000
15000
20000
[Peptide] nM

A


Cell Viability (U.A.)
6
5
4
3
2
1
0
0
0,2
0,5
0,8
1
2
5
10
Drug Concentrations (µM)
control
free Doxo
vepep-doxo

B

Figure 8

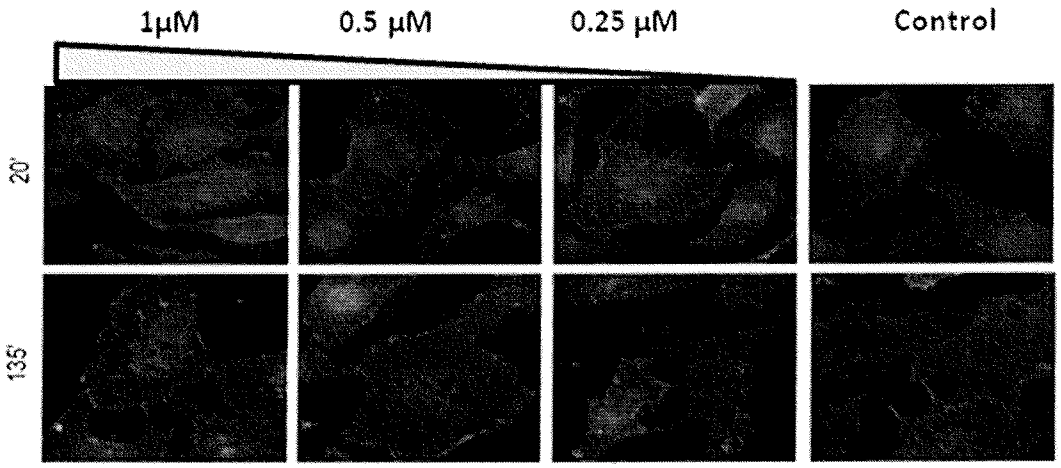
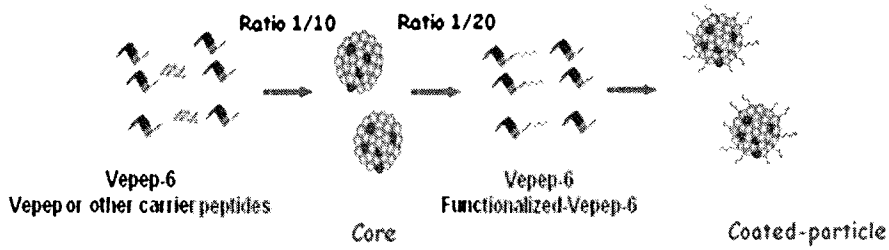


Figure 9



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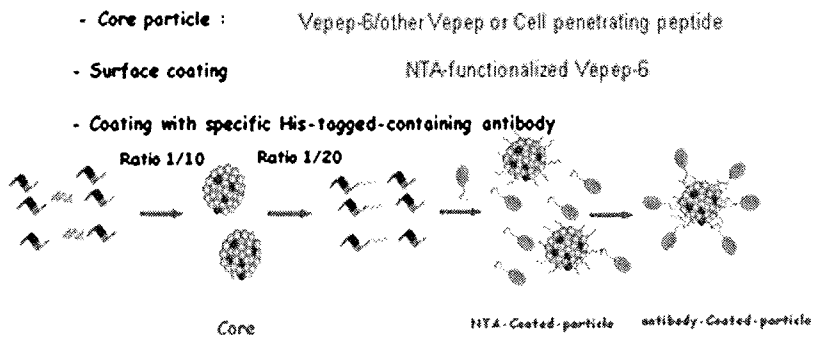
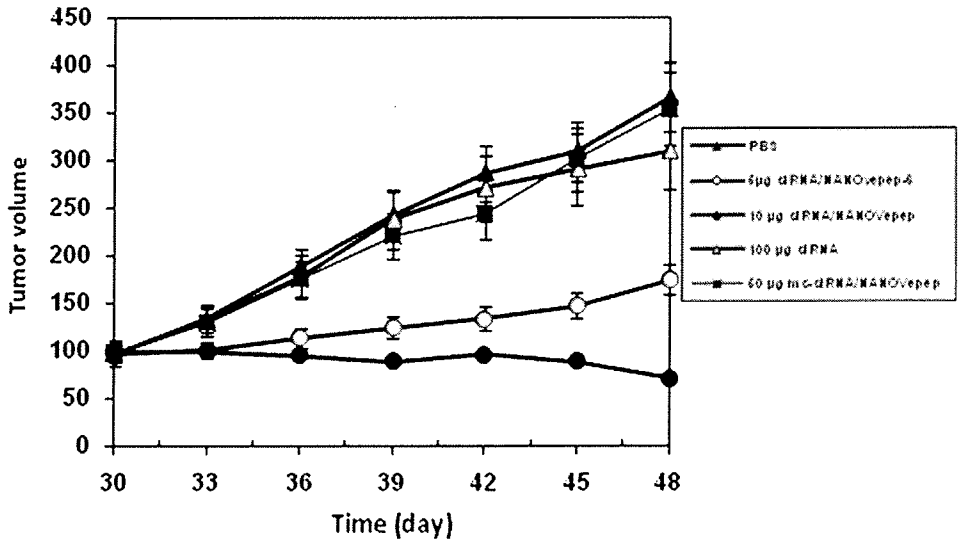


B

Figure 10

A



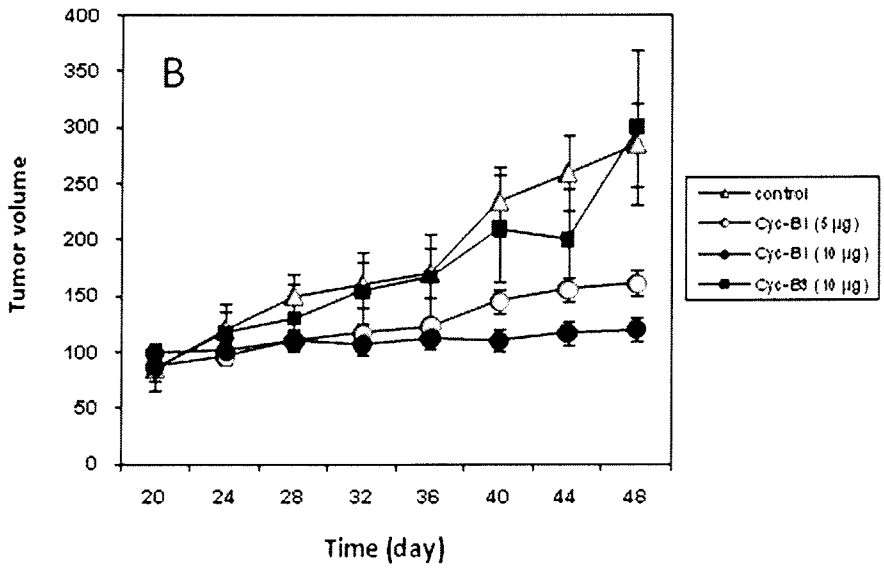
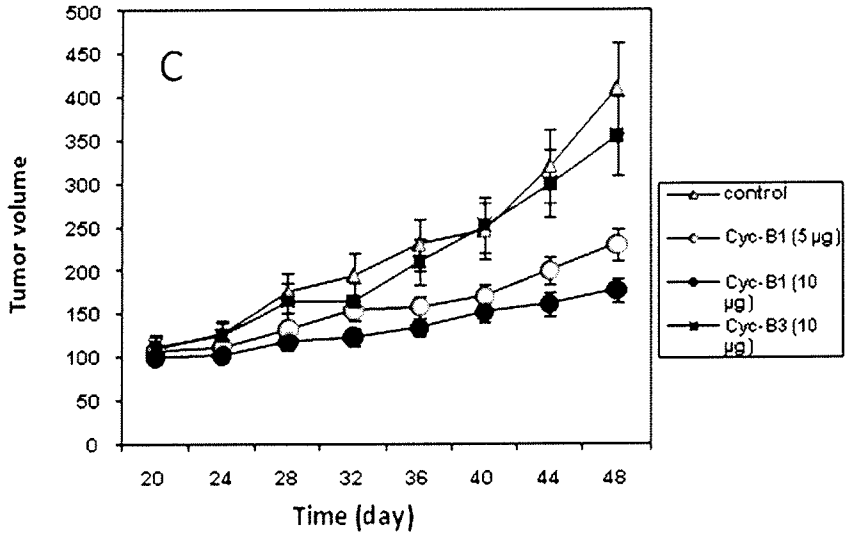


Figure 11 (A&B)


C
Tumor volume
Time (day)
control
Cyc-B1 (5 µg)
Cyc-B1 (10 µg)
Cyc-B3 (10 µg)

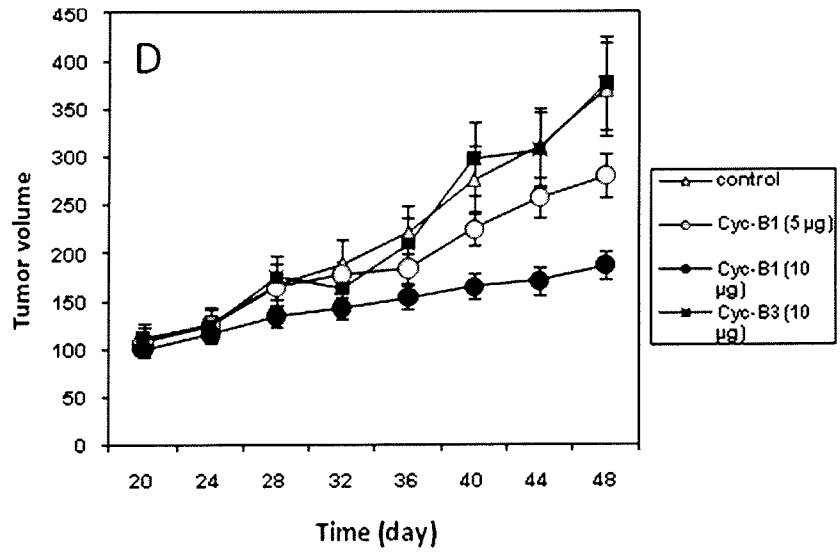
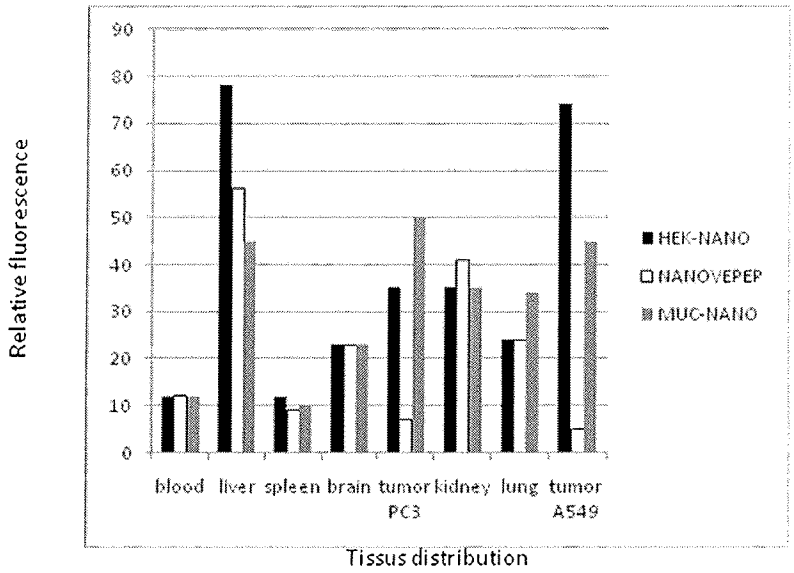
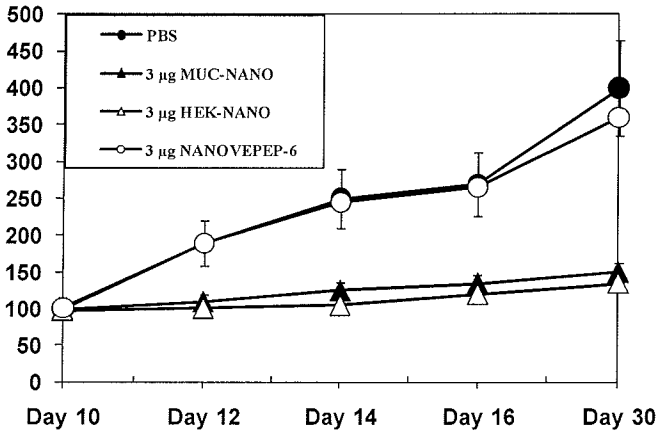

D
Tumor volume
Time (day)
control
Cyc-B1 (5 µg)
Cyc-B1 (10 µg)
Cyc-B3 (10 µg)

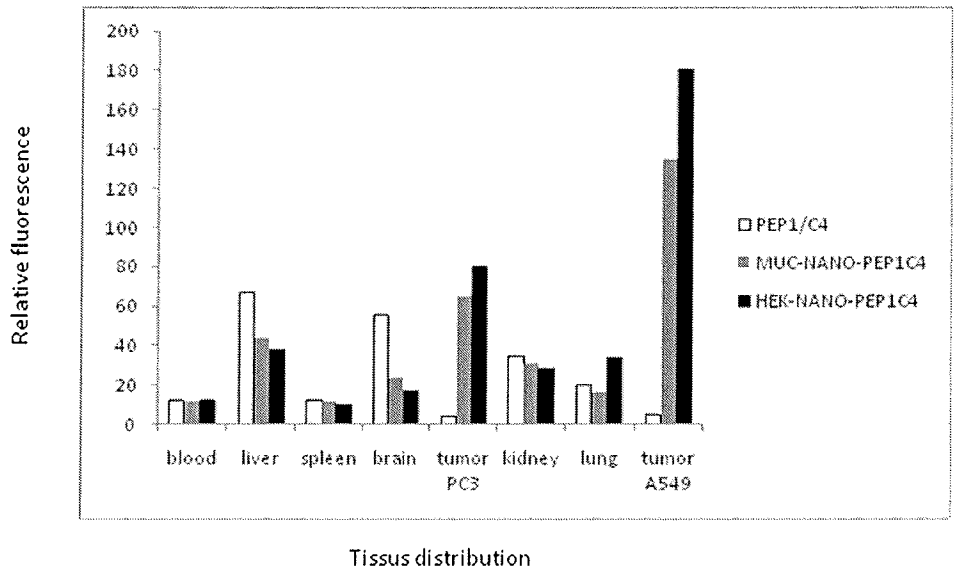
Figure 11 (C&D)



A

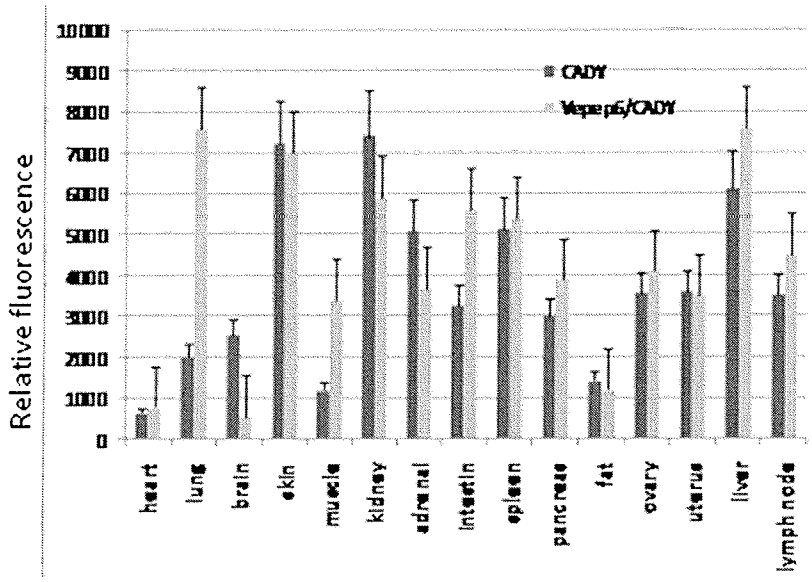


B

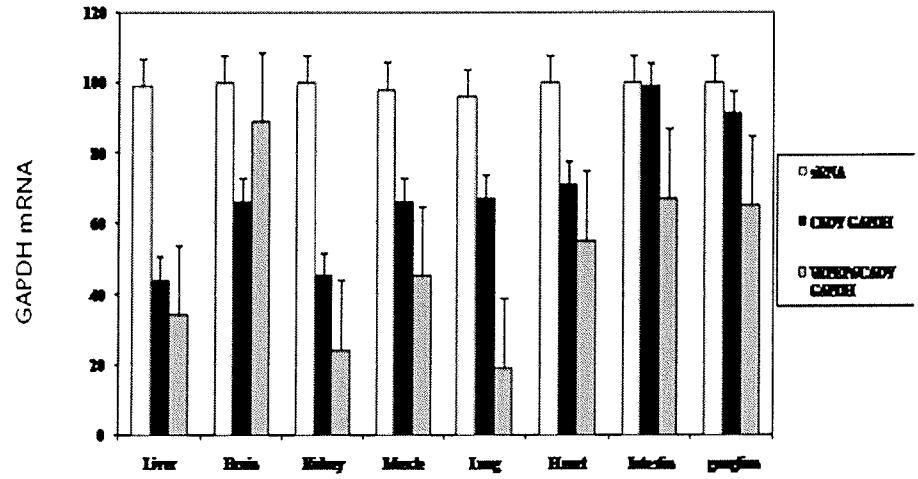


C

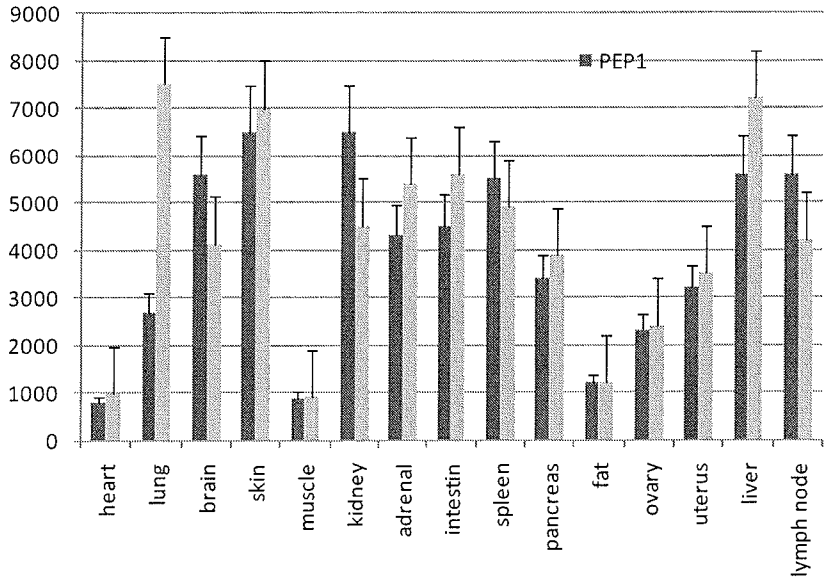
Figure 12



A

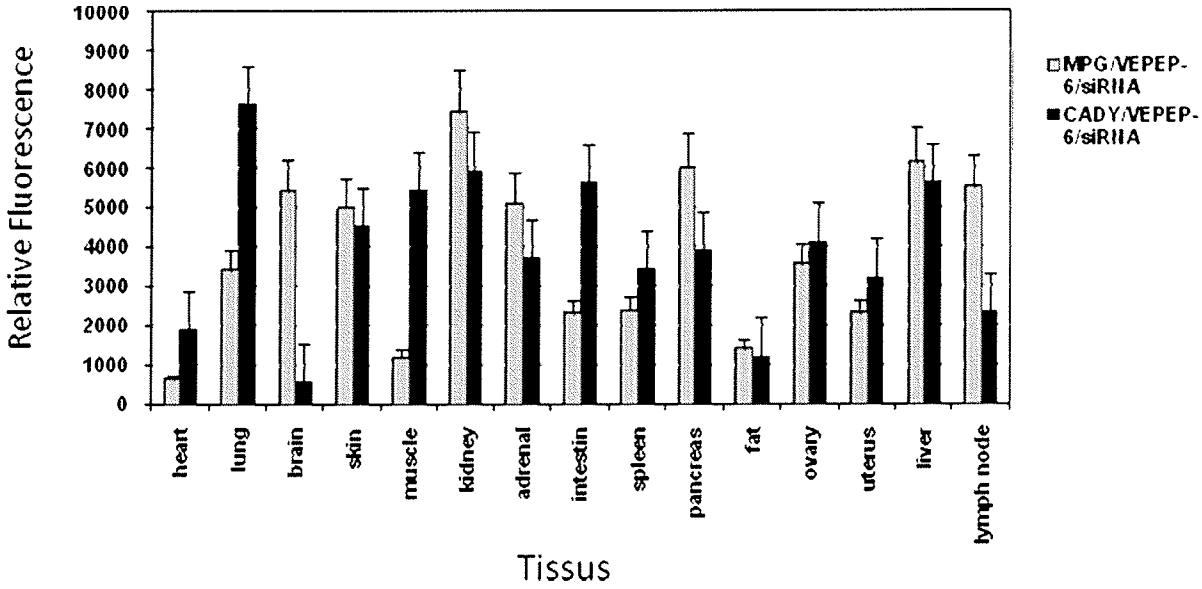


B

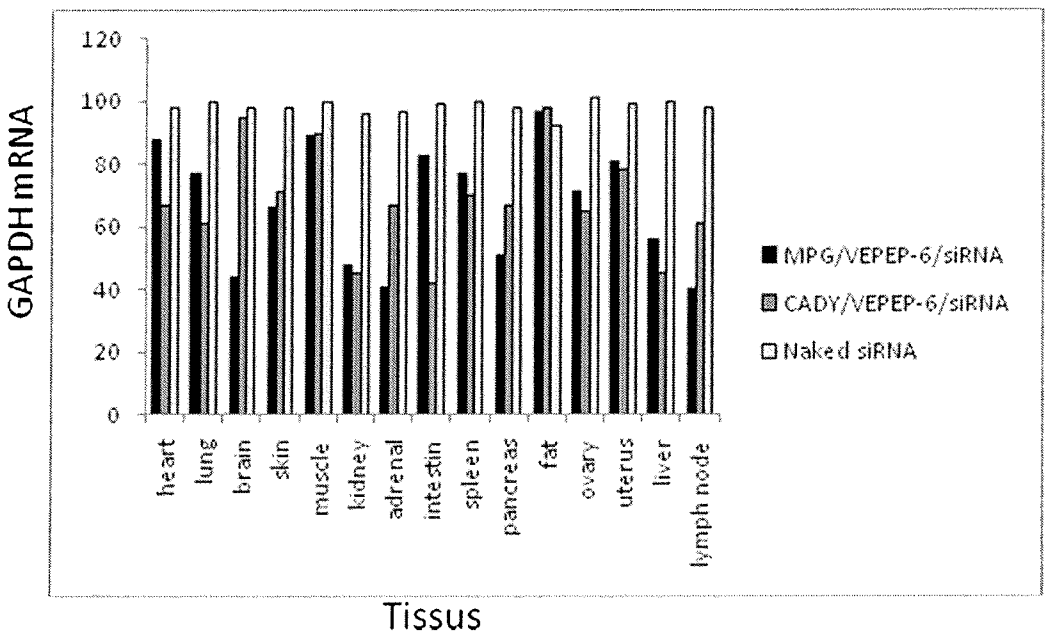


C

Figure 13

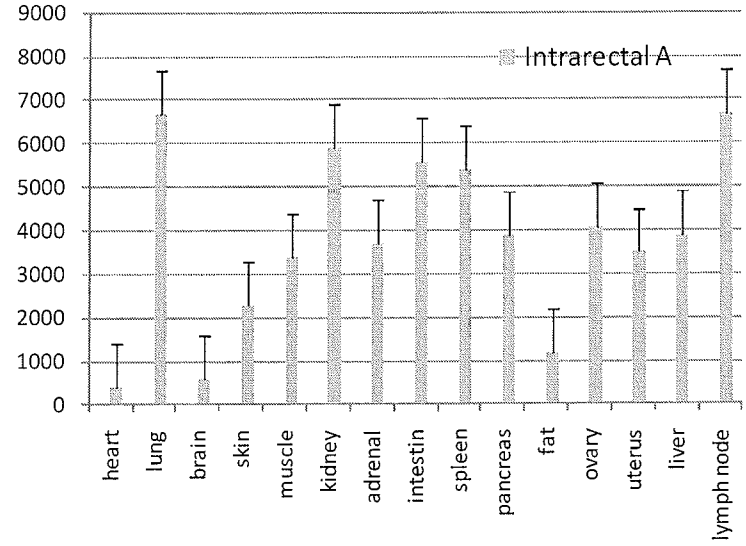

Relative Fluorescence
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
heart
lung
brain
skin
muscle
kidney
adrenal
intestin
spleen
pancreas
fat
ovary
uterus
liver
lymph node
Tissus
MPG/VEPEP-6/siRNA
CADY/VEPEP-6/siRNA

A

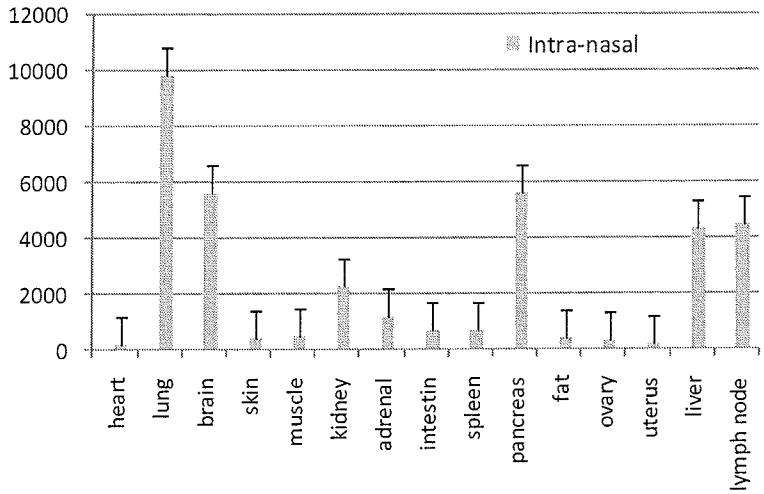

GAPDH mRNA
120
100
80
60
40
20
0
heart
lung
brain
skin
muscle
kidney
adrenal
intestin
spleen
pancreas
fat
ovary
uterus
liver
lymph node
Tissus
MPG/VEPEP-6/siRNA
CADY/VEPEP-6/siRNA
Naked siRNA

B

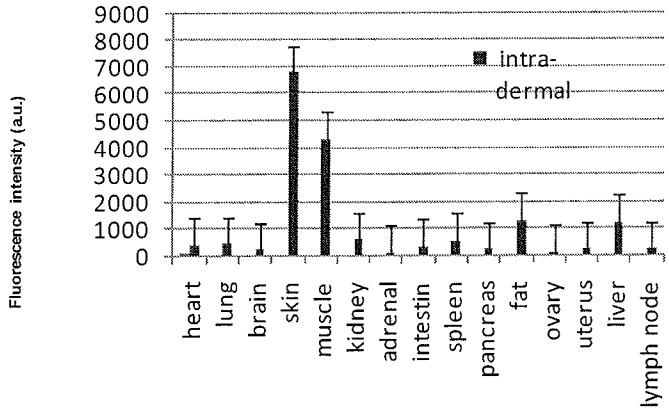
Figure 14



A



B



C

Figure 15

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/051435

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/00 A61K47/48 C12N15/113
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, Sequence Search, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/069090 A2 (CENTRE NAT RECH SCIENT [FR]; GENOSPECTRA INC [US]; DIVITA GILLES [FR];) 21 June 2007 (2007-06-21)	1-17
Y	the whole document -----	1-17
Y	WO 2008/036929 A2 (ALNYLAM PHARMACEUTICALS INC [US]; HADWIGER PHILIPP [DE]; GEICK ANKE [D) 27 March 2008 (2008-03-27) See particularly claims 20,21; Tables 6-8 -----	1-17

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 2011	07/12/2011

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2011/051435

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007069090 A2	21-06-2007	CA 2632451 A1	21-06-2007
		EP 1969000 A2	17-09-2008
		US 2009292003 A1	26-11-2009
		WO 2007069090 A2	21-06-2007
WO 2008036929 A2	27-03-2008	US 2009176710 A1	09-07-2009
		WO 2008036929 A2	27-03-2008