(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 September 2014 (25.09.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/147193 A1

(51) International Patent Classification:
G01N 33/58 (2006.01) *G01N 33/573* (2006.01)
C07K 7/00 (2006.01)

(21) International Application Number:
PCT/EP2014/055633

(22) International Filing Date:
20 March 2014 (20.03.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
13305335.5 20 March 2013 (20.03.2013) EP

(71) Applicants: **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3, rue Michel Ange, F-75016 Paris (FR). **UNIVERSITE MONTPELLIER 2 SCIENCES ET TECHNIQUES** [FR/FR]; Place Eugène Bataillon, F-34000 Montpellier (FR).

(72) Inventors: **MORRIS, May Catherine**; 476 Rue de la Rave, F-34130 Mauguio (FR). **DIVITA, Gilles**; 476 Rue de la Rave, F-34130 Mauguio (FR).

(74) Agent: **REGIMBEAU**; 20, rue de Chazelles, F-75847 Paris Cedex 17 (FR).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: NEW CELL-PENETRATING PEPTIDE MOTIFS

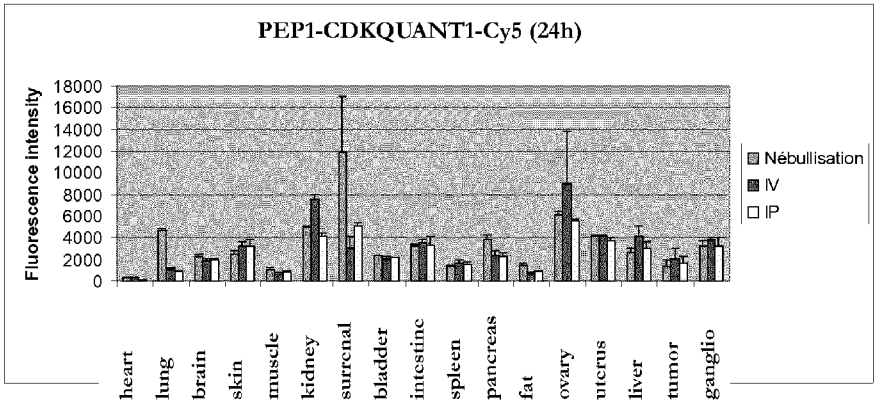


FIGURE 4

(57) Abstract: The present invention relates to new cell-penetrating peptide motifs which allow the intracellular delivery of products to which they are covalently linked or fused. It also relates to compounds comprising at least one of said cell-penetrating peptide motifs. The present invention also relates to a process for intracellular delivery of compounds and for targeting biological functions. The present invention furthermore relates to the use of said cell-penetrating peptide motifs for the intracellular delivery of products, in particular for detecting intracellular compounds, for interfering with biological function, for cellular imaging and for screening libraries of compounds to be delivered into a cell.

WO 2014/147193 A1

NEW CELL-PENETRATING PEPTIDE MOTIFS

The present invention relates to new cell-penetrating peptide motifs which allow the intracellular delivery of products to which they are covalently linked or fused. It also relates to compounds comprising at least one of said cell-penetrating peptide motifs. The present invention also relates to a process for intracellular delivery of compounds and for targeting biological functions. The present invention furthermore relates to the use of said cell-penetrating peptide motifs for the intracellular delivery of compounds, in particular for detecting intracellular compounds, for interfering with biological function, for cellular imaging and for screening libraries of compounds.

Cell membranes constitute barriers for the vast majority of biomolecules and therapeutic drugs. More often than not, molecules presenting potential for therapeutic intervention are limited by the lack of permeability and high selectivity of the cell membrane, and consequently never make it to the clinic due to poor delivery and low bioavailability. Several alternatives have been devised to facilitate the introduction of molecules which are refractory to cellular uptake, including physical, chemical or biological strategies such as microinjection, electro-permeabilisation, viral transformation or non-viral technologies such as facilitated delivery by so-called "smart drug carriers". Non-viral strategies that promote efficient delivery of bioactive macromolecules into living cells include lipid-based formulations, polycationic polymers and particles, carbon nanotubes and nanoparticles, as well as peptide-based formulations.

Cell-penetrating peptides are well known by the skilled person, and are described thoroughly in Grdisa *et al.*, (2011), Matjaz *et al.*, (2005), Morris *et al.* (2008), Fonseca *et al.* (2009) and Heitz *et al.* (2009). Protein transduction domains (PTDs) and cell-penetrating peptides or cell-penetrating peptide motifs (CPPs) constitute a family of peptides with the characteristic ability to cross biological membranes and deliver a molecular cargo into the intracellular milieu. Both covalent and non-covalent strategies based on PTDs/CPPs have been developed, the former involving chemical linkage, conjugation or genetic fusion to the cargo, the latter involving formation of stable yet non-covalent complexes between carrier and cargo.

The covalent strategy is best exemplified by TAT, the regulatory transactivating domain of HIV (Torchilin *et al.*, 2005), the third alpha helix of Antennapaedia homeodomain protein, also known as Penetratin (Derossi *et al.*, 1994) and Transportan (Pooga *et al.,* 1998), calcitonin- derived peptides (Rennert *et al.*, 2006) Herpes simplex viral protein VP22 (Elliot *et al.*, 1997) polyarginine peptides (Wender *et al.,* 2000) and polyproline sweet arrow peptide (Pujals *et al.,* 2006).

The first non-covalent CPP, published in 1997, was the short amphipathic peptide MPG shown to promote efficient delivery of nucleic acids into living cells (Morris *et al.*) This approach was then extended to the design of (non-covalent) Pep-1 in 2001, for delivery of proteins and peptides (Morris *et al.*, 2001), followed by the (non covalent) Pep-2 and Pep-3 derivatives (Morris *et al.*, 2007). The success of these approaches lead to the more recent design of a secondary amphipathic peptide named CADY which has proven efficient for delivery of siRNA into cell lines and in vivo (Crombez *et al.,* 2009) and its relative CADY2 for delivery of fluorescent peptides and proteins (Kurzawa *et al.,* 2011).

Over the last twenty years, the unique properties of PTDs and CPPs have been exploited to deliver a wide variety of biomolecules, into living cells and more recently in vivo, that have been successfully applied to treat hyperproliferative diseases such as cancer, asthma, ischemia, stimulating cytotoxic immunity and diabetes (Heitz *et al.*, 2009; Fonsecca *et al.*, 2009). Limitations of known covalent and non-covalent CPPs are well documented.

However, there is still a need for a carrier which is easy to handle, allowing for quick and efficient delivery, and ensuring a homogenous intracellular distribution and the preservation of the biological activity of the delivered product.

Kim *et al.* (2009) describes PEP1-CAT and PEP1-SOD fusion proteins. Shirong *et al.* (2007) describes a PEP1-P27mt fusion protein. Duk-Soo *et al.* (2012) describes a PEP1-p18 fusion protein. WO 2007/108749 describes chimeric constructs between cancer-homing peptides and a PEP1 cell-penetrating peptide. Kang Myung *et al.* (2011) describes a PEP-1 peptide-modified liposomal nanocarrier system for intracellular drug delivery. US 2006/035815 describes compounds comprising a 21 amino acid peptidic motif. These documents describe a « PEP1 » motif comprising 21 amino acids.

WO 02/061105 cites some peptides susceptible to be able to bind to a Permeability Transition Pore Complex. Kurzawa *et al.* (2011) describes a polypeptide non-covalently complexed to a cell-penetrating peptide carrier. The peptides differ from a peptide according to the invention.

The inventors have now developed a Tryptophan-rich peptide, named PEPCOV, which promotes delivery into living cells and in vivo, of compounds which are covalently coupled to it or directly synthesized/or produced as a single fusion compound. PEPCOV promotes rapid and efficient and homogeneous delivery of products such as peptide inhibitors and fluorescent biosensors, enzymes, cofactors into cells and leads to a related biological response. This technology has been validated with respect to delivery both in cellulo and in vivo, and has proven to be a useful tool for diagnostic and therapeutic approaches based on delivery of peptide or protein biosensors into cells, as well as a useful laboratory tool. These carriers can further be applied to high content, high throughput screening assays in the context of drug discovery programs, as well as in vivo for molecular imaging or for therapeutic strategies.

The advantages of this invention are numerous: (1) the peptide nature of the cell-penetrating sequence and its short size, which makes it easy to synthesize, handle and control (2) the simplicity of the approach which simply requires synthesis or conjugation of the auto-penetrating sequence with the sequence of the cargo (3) its applicability in cellulo and in vivo (4) its validation to enable self-penetration of peptide inhibitors and fluorescent biosensors with specific biological functions.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will become more fully understood from the detailed description given herein and from the accompanying drawings, which are given by way of illustration only and do not limit the intended scope of the invention.

The inventors have now designed compounds comprising at least one cell-penetrating peptide motif. Each amino acid is herein represented according to the IUPAC amino-acid abbreviation, such as follows:

Amino-acid or amino-acid residue	Abbreviation	Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid (Aspartate)	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid (Glutamate)	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Aspartic acid or Asparagine	Asx	B
Glutamine or Glutamic acid.	Glx	Z
Any amino acid.	Xaa	X

Table 1

The present invention first relates to a cell-penetrating peptide motif comprising at least the amino acid sequence: WW/FXXWW/F (SEQ ID N°1). More preferably, the present invention relates a to a cell-penetrating peptide motif comprising at least an amino acid sequence chosen in the group consisting of: WWXXWW (SEQ ID N°2), WFXXWW (SEQ ID N°3), WWXXWF (SEQ ID N°4) and WFXXWF (SEQ ID N°5).

In a particular embodiment, the invention relates to a compound comprising a cell-penetrating peptide motif, wherein said cell-penetrating peptide consists of an amino acid sequence chosen in the group consisting of: WWXXWW (SEQ ID N°2), WFXXWW (SEQ ID N°3), WWXXWF (SEQ ID N°4) and WFXXWF (SEQ ID N°5).

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer, as well as amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid. The terms "domain", "motif" and "moiety" are used to refer to parts of the peptide structure constitutive of an entity exhibiting a particular characteristic. A peptide comprising a cell-penetrating motif according to the present invention might also be referred as "PEPCOV".

The term "cell-penetrating peptide motif" refers to a short peptide, for example comprising from 5 to 50 amino acids, which can readily cross biological membranes and is capable of facilitating the cellular uptake of various molecular cargos, in vitro and/or in vivo. In a particular embodiment, the "cell-penetrating peptide motif" comprises a short polycationic or amphiphilic peptide. The terms "cell-penetrating motif", "self cell-penetrating domain", "cell-permeable peptide", "protein-transduction domain", and "peptide carrier" are equivalent.

The ability of a cell-penetrating peptide motif according to the invention to facilitate the intracellular uptake may be assessed, for example, by overlaying said peptide and its "molecular cargo" onto cells cultured to subconfluency, for example for 1 hour at 37°C. Methods for testing the ability of a peptide non-covalently linked to a molecular cargo to facilitate the intracellular uptake of said "molecular cargo" are described in Kurzawa *et al.* (2010). A person skilled in the art may easily adapt said protocol to assess the delivering capacities of a cell-penetrating peptide motif according to the present invention.

Preferably, the cell-penetrating peptide motif of a compound according to the invention is capable of facilitating cellular uptake of peptides of more than 5 amino-acids. In a particular embodiment the cell-penetrating peptide motif of a compound

according to the invention is capable of facilitating cellular uptake of a molecular cargo up to 500 kDa.

The inventors have found that the presence, within the amino acid sequence of a peptide, and from the N-terminal to the C-terminal extremity of said sequence, of two hydrophobic motifs, each of these hydrophobic motif comprising two hydrophobic amino acids being possibly two Tryptophan residues (WW) and/or a Tryptophan and a Phenylalanine (WF) residue, with said two motifs being separated by two amino acids, are associated with an ability for the peptide to cross the cellular membrane and to be detected within the cell. From these observations, the inventors have defined the consensus sequence: WW/FXXWW/F (SEQ ID N°1).

In a particular embodiment, the present invention relates to a compound comprising a peptide comprising at least two Tryptophan residue, preferably three Tryptophan residue and more preferably four Tryptophane residue.

In a particular embodiment, the present invention relates to a compound comprising a peptide comprising at least one cell-penetrating peptide motif having the consensus sequence: WW/FXXWW/F (SEQ ID N°1).

A compound of the invention may be prepared to allow its direct use in vitro, in cell extracts, in a cell, a cell culture, including tissue culture, or on animal and/or human tissues, originating for example from biopsies, or in living animal models. A compound according to the invention allows the delivery of a molecular cargo into a cell, more particularly a eukaryotic cell, and even more particularly an animal cell, including a human cell. A compound according to the invention allows the delivery of a molecular cargo into a healthy or non-healthy cell, and even more particularly it allows the delivery of an agent into a cancer cell. In a particular embodiment, and if optionally combined with appropriate targeting sequences, a compound according to the invention allows the delivery of a molecular cargo to the nucleus, to the mitochondrial compartment, to the plasma membrane or to any other organelle.

In a particular embodiment, a compound according to the invention comprises a peptide comprising a cell-penetrating peptide motif from 5 to 50 amino acids in length, preferably from 5 to 25 amino acids and more preferably from 6 to 13 amino acids. In a more particular embodiment, a compound according to the invention comprises a

peptide comprising a cell-penetrating motif comprising 13 amino acids. In another particular embodiment, a compound according to the invention comprises a peptide comprising a cell-penetrating motif comprising 6 amino acids. In a more particular embodiment, a compound according to the invention comprises 6, 7, 8, 9, 10, 11, 12 or 13 amino acids. In an even more particular embodiment, a compound according to the invention consists of 6, 7, 8, 9, 10, 11, 12 or 13 amino acids.

In a more particular embodiment, the invention relates to a compound comprising a peptide wherein said cell-penetrating peptide motif comprises an amino acid sequence chosen in the group consisting of: the sequence SEQ ID N°2, the sequence SEQ ID N°3, the sequence SEQ ID N°4 and the sequence SEQ ID N°5.

In a particular embodiment, the invention relates to a compound comprising a cell-penetrating peptide motif wherein the amino acids in position 3 and in position 4 of the sequence of said cell-penetrating motif sequence (SEQ ID N°1) are independently chosen in the group consisting of: K, R, E, D, T and G. In a more particular embodiment, the invention relates to a compound comprising a cell-penetrating peptide motif wherein the amino acids in position 3 and in position 4 of the sequence of said cell-penetrating motif sequence chosen in the group consisting of: SEQ ID N°2, SEQ ID N°3, SEQ ID N°4 and SEQ ID N°5 are independently chosen in the group consisting of: K, R, E, D, T and G.

In a particular embodiment, the invention relates to a compound comprising a cell-penetrating peptide motif wherein the amino acid in position 3 of the sequence of said cell-penetrating motif sequence is Glutamic acid (E).

In a particular embodiment, the invention relates to a compound comprising a cell-penetrating peptide motif wherein the amino acid in position 4 of the sequence of said cell-penetrating motif sequence is Threonine (T).

In a more particular embodiment, the invention relates to a compound comprising a cell-penetrating peptide motif wherein the amino acids in position 3 and in position 4 of the sequence of said cell-penetrating motif sequence are respectively Glutamic acid (E) and Threonine (T).

In another particular embodiment, the invention relates to a compound comprising a cell-penetrating peptide motif wherein the amino acid in position 3 and in position 4 of the sequence of said cell-penetrating motif sequence are Glycine (G).

In another particular embodiment, the invention relates to a compound comprising a cell-penetrating peptide motif, wherein said cell-penetrating peptide motif consists of 6, 7, 8, 9 or 10 amino acids and comprises at least an amino acid sequence chosen in the group consisting of: WWETWW (SEQ ID N°33), WFETWW (SEQ ID N°34), WWETWF (SEQ ID N°35) and WFETWF (SEQ ID N°36).

In a more particular embodiment, the invention relates to a compound comprising a cell-penetrating peptide motif, wherein said cell-penetrating peptide consists of an amino acid sequence chosen in the group consisting of: WWETWW (SEQ ID N°33), WFETWW (SEQ ID N°34), WWETWF (SEQ ID N°35) and WFETWF (SEQ ID N°36).In a more particular embodiment, the invention relates to a compound comprising a peptide wherein said cell-penetrating peptide motif comprises an amino acid sequence chosen in the group consisting of: the sequence SEQ ID N°7, the sequence SEQ ID N°8, the sequence SEQ ID N°9, the sequence SEQ ID N°10, the sequence SEQ ID N°11, the sequence SEQ ID N°12, the sequence SEQ ID N°13, the sequence SEQ ID N°14, the sequence SEQ ID N°15, the sequence SEQ ID N°16 and the sequence SEQ ID N°17.

In an even more particular embodiment, the invention relates to a compound wherein said cell-penetrating peptide motif comprises an amino acid sequence chosen in the group consisting of: the sequence KETWWETWWTEKK (SEQ ID N°7), the sequence KETWFETWFTEKK (SEQ ID N°8), the sequence KETWWETWFTEKK (SEQ ID N°9) and the sequence KETWFETWWTEKK (SEQ ID N°10).

In an even more particular embodiment, the invention relates to a compound wherein said cell-penetrating peptide motif has an amino acid sequence chosen in the group consisting of: the sequence KETWWETWWTEKK (SEQ ID N°7), the sequence KETWFETWWTEKK (SEQ ID N°8), the sequence KETWWETWFTEKK (SEQ ID N°9) and the sequence KETWFETWFTEKK (SEQ ID N°10).

In a more particular embodiment, the invention relates to a compound wherein said cell-penetrating peptide motif comprises the amino acid sequence SEQ ID N°7 (PEP1).

In another embodiment, the invention relates to a compound wherein said cell-penetrating peptide motif comprises an amino acid sequence having at least 80% identity, and preferably at least 90% identity with a sequence chosen in the group consisting of: SEQ ID N°1 to SEQ ID N°17, said compound comprising the amino acid sequence WW/FXXWW/F (SEQ ID N°1).

As applied to peptides, the term “identical” means that two peptide sequences, when optimally aligned, share 100 % sequence identity. As used herein the term "identity" herein means that two amino acid sequences are identical (i.e. at the amino acid by amino acid basis) over the window of comparison.

The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical amino acid residues occur in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e. the window size) and multiplying the result by 100 to yield the percentage of sequence identity. The percentage of sequence identity of an amino acid sequence can also be calculated using BLAST software with the default or user defined parameter.

Single amino acid substitutions, deletions, or insertions can be used, provided that the consensus motif is present. Substitutions may be conservative, regarding certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity), as known by a person skilled in the art biochemistry. The substituting amino acids are not limited to those naturally occurring in proteins, such as L-α-amino acids, or their D-isomers. The peptides can be substituted with a variety of moieties such as amino acid mimetics well known to those of skill in the art.

In a more particular embodiment, the invention relates to a compound comprising a peptide comprising two or more cell-penetrating peptide motifs according to the invention.

In another particular embodiment, the invention relates to an isolated nucleic acid molecule comprising a nucleotide sequence encoding for at least one cell-penetrating peptide motif having an amino acid sequence chosen in the group consisting of: the sequence SEQ ID N°2, the sequence SEQ ID N°3, the sequence SEQ ID N°4,

the sequence SEQ ID N°5, the sequence SEQ ID N°7, the sequence SEQ ID N°8, the sequence SEQ ID N°9, the sequence SEQ ID N°10, the sequence SEQ ID N°11, the sequence SEQ ID N°12, the sequence SEQ ID N°13, the sequence SEQ ID N°14, the sequence SEQ ID N°15, the sequence SEQ ID N°16 and the sequence SEQ ID N°17.

In another embodiment, the present invention relates to a nucleic acid vector containing a nucleic acid molecule according to the invention, said vector being preferably an expression vector comprising all means for the expression of polypeptides encoded by nucleic acid molecules.

In a more particular embodiment, the invention relates to a host cell transformed by a vector according to the invention. As an example, said host cell is chosen among eukaryotic cells, such as yeasts, or among prokaryotic cells, such as bacteria, and even more particularly said host cell is a bacteria adapted for the production of heterologous polypeptides, well known by a person skilled in the art. The methods classically used in molecular biology are well known to those skilled in the art and are fully described in the literature (Maniatis T. et al, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y, Edition 1999).

According to a particular aspect, the invention relates to a compound wherein said at least one cell-penetrating peptide motif is covalently linked or fused to at least one compound to be delivered into a cell.

As used herein, the terms “compound to be delivered into a cell” and “molecular cargo” refer to a molecule or a macromolecule which can be natural or synthetic, organic or inorganic, and which are chosen in the list consisting of: peptides, lipids, glucids, nucleic acids and macromolecules comprising thereof.

According to the invention, the cell-penetrating motif and the compound to be delivered into a cell are associated through covalent bonds, wherein the cell-penetrating peptide motif and the compound to be delivered can be associated in any order respectively to the peptide chain comprising the cell-penetrating peptide motif. In a preferred embodiment, the molecular cargo is located at the C-terminal extremity of the peptide chain comprising the at least one cell-penetrating peptide motif. In a more preferred embodiment, a compound according to the invention is a peptide comprising,

from its N-terminal extremity to its C-terminal extremity, a cell-penetrating peptide motif and a molecular cargo.

In a particular embodiment, in a compound according to the invention, at least one cell-penetrating peptide motif is linked or fused directly to a molecular cargo. In another embodiment, the cell-penetrating peptide motif is linked or fused to a molecular cargo via a linker.

In a more particular embodiment, the invention relates to a compound wherein said molecular cargo is a peptide chosen in the group consisting of: antibodies, enzymes, antigens, receptor ligands, and analogs, fragments and derivatives thereof. In a particular embodiment, said analogs, fragments and derivatives exhibit the functional activity of the original molecule.

In an even more particular embodiment, the invention relates to a compound comprising a cell-penetrating motif and a molecular cargo, wherein said molecular cargo is able to recognize and bind at least one CDK/Cyclin complex. In an even more particular embodiment, said compound comprises an amino acid sequence chosen in the group consisting of: the sequence SEQ ID N°20, the sequence SEQ ID N°21, the sequence SEQ ID N°22, the sequence SEQ ID N°23 and the sequence SEQ ID N°24. By "specifically binding", "specifically binds", "recognizing" or the like, it is intended herein that, the molecular cargo in a compound according to the invention forms a complex that is relatively stable under physiological conditions, with an intracellular product. An equilibrium dissociation constant, K_D, is commonly used in biological sciences to measure the affinity and to characterize the binding of a molecule for another molecule. Methods for determining whether two molecules bind specifically are well known in the art and include, for example, equilibrium dialysis, surface plasmon resonance, and the like. Typically, a smaller K_D means a greater affinity.

The specific binding of a compound according to the invention for at least one CDK can be characterized by a K_D comprised between about 100 nM and about 500 nM, and preferably of about 150 nM. It is intended herein that the term "about" is equivalent to "+/-10%". The specific binding of a compound according to the invention for at least one cyclin can be characterized by a K_D comprised between about 100 nM and about 500 nM, and preferably of about 150 nM.

Table 2 summarizes the description of peptides according to the invention and their amino acid sequences. Amino acids in bold belong to the cell-penetrating peptide motif of amino-acid sequences.

Name	Amino acid sequence	Sequence identification
Short consensus cell-penetrating peptide motif	WW/FXXWW/F	SEQ ID N°1
Short cell-penetrating peptide motif	WWXXWW WFXXWW WWXXWF WFXXWF	SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5
Long consensus cell-penetrating peptide motif	KETWW/FETWW/FTEKK	SEQ ID N°6
PEP1 cell-penetrating peptide motif	KETWWETWWTEKK	SEQ ID N°7
Long cell- penetrating peptide motif	KETWFETWWTEKK KETWWETWFTEKK	SEQ ID N°8 SEQ ID N°9
PEP2 cell-penetrating peptide motif	KETWFETWFTEKK	SEQ ID N°10
PEP3 cell-penetrating peptide motif	KWFETWFTEKK	SEQ ID N°11
PEP1b cell-penetrating peptide motif	KETWWETWW	SEQ ID N°12
PEP2b cell-penetrating peptide motif	KETWFETWF	SEQ ID N°13
PEP3b cell-penetrating peptide motif	KWFETWF	SEQ ID N°14
PEP1GW cell-penetrating peptide motif	GGWWGGWWGG	SEQ ID N°15
PEP2GW cell-penetrating peptide motif	GGWFGGWFGG	SEQ ID N°16
PEP3GW cell-penetrating peptide motif	GWFGGWFG	SEQ ID N°17
C4 peptide	TYTKKQVLRMAHLVLKVLTFDLC	SEQ ID N°18
PEP1-C4	**KETWWETWWTEKK**TYTKKQV LRMAHLVLKVLTFDLC	SEQ ID N°19
PEP1-CDKQUANT1	**KETWWETWWTEKK**HHAGPRK RVHERYCGPTAGSAKRRLFGED	SEQ ID N°20
PEP1-CDKQUANT2	**KETWWETWWTEKK**PEPILVDT ASPSPMETCGPTAGSAKRRLFGED	SEQ ID N°21
PEP1-CDKQUANT3	**KETWWETWWTEKK**RAGGPATP LSPTRLCGPTAGSAKRRLFGED	SEQ ID N°22

PEP1-CDKQUANT4	**KETWWETWWTEKK**YKFPSSPLRIPGCGPTAGSAKRRLFGED	SEQ ID N°23
PEP1-CDKQUANT5	**KETWWETWWTEKK**SGYSSPGSPGTPGSRCGPTAGSAKRRLFGED	SEQ ID N°24
PEP1-Control peptide	**KETWWETWWTEKK**VESSDTIDNVKSKIQDKEGC	SEQ ID N°25
TAT-C4	**GRKKRRQRRR**TYTKKQVLRMAHLVLKVLTFDLC	SEQ ID N°26
CADY1	GLWRALWRLLRSLWRLLWKV	SEQ ID N°27
CADY2	GLWWRLWWRLRSWFRLWFRV	SEQ ID N°28
CDKSENS1	HHAGPRKRVHERYCGPTAGSAKRRLFGED	SEQ ID N°29
Control peptide	VESSDTIDNVKSKIQDKEGC	SEQ ID N°30
PEP2b-C4	**KETWFETWF**TYTKKQVLRMAHLVLKVLTFDLC	SEQ ID N°31
PEP3b-C4	**KWFETWF**TYTKKQVLRMAHLVLKVLTFDLC	SEQ ID N°32
Short cell-penetrating peptide motif	WWETWW WFETWW WWETWF WFETWF	SEQ ID N°33 SEQ ID N°34 SEQ ID N°35 SEQ ID N°36

Table 2

In a particular embodiment, the invention relates to a compound wherein said peptide comprises an amino acid sequence chosen in the group consisting of the sequence SEQ ID N°19, the sequence SEQ ID N°20, the sequence SEQ ID N°21, the sequence SEQ ID N°22, the sequence SEQ ID N°23, the sequence SEQ ID N°24, the sequence SEQ ID N°31 and the sequence SEQ ID N°32.

In a more particular embodiment, the present invention relates to a compound comprising a peptide comprising a cell-penetrating peptide motif, a molecular cargo and a reporter molecule.

The term “reporter molecule” relates a molecule able to emit a signal or to allow its recovery, and therefore allowing the detection, and possibly the isolation, of a compound to which it is covalently or non-covalently bound. This includes a recoverable label such as a biotinyl moiety that can be recovered by avidin or streptavidin. Recoverable labels can include covalently linked polynucleotide sequences that can be recovered by hybridization to a complementary sequence

polynucleotide. Examples of labels include, but are not limited to, the following: radioisotopes (e.g., H, C, S, I, I), fluorescent or phosphorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, β-galactosidase, luciferase, alkaline phosphatase), biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for antibodies, transcriptional activator polypeptide, metal binding domains, epitope tags). Labels can also be attached by spacer arms of various lengths, e.g., to reduce potential steric hindrance.

In an embodiment of the invention, the reporter molecule is a fluorophore. By fluorophore, or fluorescent probe, it is herein meant a molecule capable of re-emitting light upon light excitation, or other electromagnetic light. In most cases, emitted light has a longer wavelength, and therefore lower energy, than the absorbed light. In a more particular embodiment, a compound according to the invention comprises an environmentally-sensitive dye or couples of fluorescent dyes capable of FRET.

The terms “environment-sensitive dye“, ”environment-sensitive probe”, “solvatochromic dye”, “solvatochromic probe” are herein interchangeable. By environment-sensitive dye, it is herein meant a fluorophore the properties of which change, for example intensity, half-life, and excitation or emission spectra, in a measureable manner upon a change in the fluorophore environment. Preferably, by environment-sensitive dye, it is herein meant a fluorophore the intensity or emission spectrum of which changes together with a change in its environment. According to the invention, the change in the fluorophore environment may be due to at least one of a variety of different environmental factors, such as polarity or hydrophobicity. Environment-sensitive dyes have been reviewed in Loving et al. (2010).

In a particular embodiment, the invention relates to a compound comprising a polypeptide and a unique fluorophore, wherein said fluorophore is coupled to a unique residue within the amino acid sequence of said polypeptide.

According to the invention, the fluorophore is coupled to specific functional groups, for example specific functional groups of amino-acid residues, such as amino, carboxyl, thiol or azide groups. Coupling the fluorophore to an amino acid functional group is a technique well known to the skilled person

In another particular embodiment, the invention relates to a compound according to the invention comprising at least one molecular cargo and at least one detectable moiety. In a particular embodiment, a compound according to the invention bears at least one site for coupling to a probe. In a more particular embodiment, a compound according to the invention bears a unique cysteine for coupling of a fluorescent probe.

As an example, a compound according to the invention comprises at least one cell-penetrating peptide motif, a compound to be delivered into a cell and at least one fluorophore, wherein said at least one fluorophore being coupled to an amino acid of said compound to be delivered.

In particular embodiment, the present invention relates to a compound comprising an amino acid sequence SEQ ID N°20 (PEP1-CDKQUANT1) and a fluorophore. In an even more particular embodiment, a compound according to the present invention comprises a peptide having an amino acid sequence SEQ ID N°20 (PEP1-CDKQUANT1) and a fluorophore FITC which is coupled to the unique cysteine residue of the peptide.

In another particular embodiment, the present invention relates to a compound comprising an amino acid sequence SEQ ID N°20 (PEP1-CDKQUANT1) and a fluorophore. In an even more particular embodiment, a compound according to the present invention comprises a peptide having an amino acid sequence SEQ ID N°20 (PEP1-CDKQUANT1) and a fluorophore Cy3 which is coupled to the unique cysteine residue of the peptide.

In another particular embodiment, the invention relates to a compound comprising at least one cell-penetrating peptide motif and a targeting motif. By "targeting motif" is intended a motif designed to target a defined compartment or molecule within the cell. As an example, said targeting motif could allow the targeting to a subcellular or extracellular compartment, or to a cell surface receptor or an antigen. In a particular embodiment, a motif designed to target a defined compartment or molecule within the cell, according to the invention comprises, is able to bind specifically to a ligand, being in particular a receptor or an antigen, within the cell.

In another particular embodiment, the invention relates to a compound comprising at least one cell-penetrating peptide motif and a protein tag. A motif defined

as a "peptide-Tag" or a "protein-Tag" (or tag) may be added, for example, to allow an easier purification of a protein to which it is linked, as an example, a polypeptide according to the invention may comprise a Glutathione-S-Transferase (GST) sequence. Protein Tags are well known by the skilled person and may for example be chosen in the list consisting of Isopeptag, BCCP, Myc-tag, Calmodulin-tag, FLAG-tag, HA-tag, His-tag, Maltose binding protein-tag, Nus-tag, Glutathione-S-transferase-tag, Green Fluorescent Protein-tag, Red Fluorescent Protein (RFP) tag and other genetically encoded autofluorescent proteins, Thioredoxin-tag, S-tag, Softag 1, Softag 3, Strep-tag, SBP-tag, Ty tag, V5 tag or TC tag.

In an even more particular embodiment, a compound according to the invention may comprise an "mRFP sequence" which provides an intramolecular protein fluorophore which allows to visualize cellular internalization through fluorescence microscopy or by FACS. The mRFP or any similar autofluorescent protein may also serve as a scaffold as well as an inert intramolecular fluorophore linked or fused to another molecular cargo of interest, or to a biosensor which bears an environmentally sensitive probe. This is of particular interest for imaging applications and high content screening where the RFP may be employed for standardized ratiometric quantification .

In another particular embodiment, the invention relates to a compound comprising at least one cell-penetrating peptide motif, a targeting motif and a protein tag.

In yet another embodiment of the invention, the compound of the invention may have such attributes as being non-hydrolyzable, thereby increasing the stability against proteases or other physiological conditions which degrade the corresponding peptide. For example, peptide analogs can be generated using benzodiazepines, substituted γ-lactam rings, C7 mimics, β-turn dipeptides cores, β-aminoalocohols, diaminoketones, and methylene amino-modified. Also, several surrogates of the amide bond, including in the group of trans-olefins, fluoroalkylene, methyleneamino, phosphonamides or sulfonamides can be used in order to increase the half-life of the polypeptide.

In another aspect, the invention relates to a process for preparing a compound comprising at least a cell-penetrating peptide motif of the invention. A compound of the invention may be obtained by standard methods known in the art.

In a first embodiment, a compound according to the invention is prepared by chemical synthesis, such as solid-phase peptide synthesis, without being limited to this particular method. A compound according to the invention may be chemically synthesized as a whole or as parts which are subsequently covalently linked or coupled.

In another embodiment, a compound according to the invention is prepared by recombinant technology. A compound according to the invention may be partly produced by recombinant technology, then covalently coupled to another part of the compound. A nucleic acid encoding for at least one polypeptide comprising a cell-penetrating motif is used for transforming a suitable host, then, by assessing suitable culture conditions, performing the production of a recombinant polypeptide comprising at least a cell-penetrating motif. Said polypeptide may then be coupled by covalent link to a compound to be delivered into a cell. A compound according to the invention may optionally be prepared as a fusion protein by recombinant technology, using a nucleic acid molecule encoding for, at least, a polypeptide comprising a cell-penetrating motif and optionally for a molecular cargo to be delivered into a cell.

In particular, polypeptides associated with, for example, an mRFP or a GST sequence may be prepared by genetic engineering, as fusion proteins. Production of the polypeptide according to the invention may be performed for example in expression systems derived from bacteria, yeast, baculovirus, insect, and mammalian cells, or in cell-free expression systems, reviewed in Higgins et al. (1999), Baneyx et al. (2004) and in Atherton et al. (1989).

A chemical synthesis or a recombinant preparation process of a compound according to the invention includes all needed purification steps.

In a particular embodiment, the invention relates to a process for preparing a compound according to the invention, said process comprising the steps of:

a) providing a peptide comprising a cell-penetrating peptide motif comprising a sequence chosen in the group consisting of: the sequence SEQ ID N°2, the sequence SEQ ID N°3, the sequence SEQ ID N°4, the sequence SEQ ID N°5, the sequence SEQ ID N°7, the sequence SEQ ID N°8, the sequence SEQ ID N°9, the sequence SEQ ID N°10, the sequence SEQ ID N°11, the sequence SEQ ID N°12, the sequence SEQ ID

N°13, the sequence SEQ ID N°14, the sequence SEQ ID N°15, the sequence SEQ ID N°16 and the sequence SEQ ID N°17.

b) contacting said peptide with a molecular cargo,

c) covalently coupling said peptide with said molecular cargo.

In another particular embodiment, the invention relates to a process for preparing a compound according to the invention, said process comprising the steps of:

a) providing a nucleic acid comprising at least a nucleic acid sequence encoding for a cell-penetrating peptide motif having a sequence chosen in the group consisting of: the sequence SEQ ID N°2, the sequence SEQ ID N°3, the sequence SEQ ID N°4, the sequence SEQ ID N°5, the sequence SEQ ID N°7, the sequence SEQ ID N°8, the sequence SEQ ID N°9, the sequence SEQ ID N°10, the sequence SEQ ID N°11, the sequence SEQ ID N°12, the sequence SEQ ID N°13, the sequence SEQ ID N°14, the sequence SEQ ID N°15, the sequence SEQ ID N°16 and the sequence SEQ ID N°17, and a nucleic acid sequence encoding for a compound to be delivered into a cell,

b) Contacting said nucleic acid with an appropriate host and producing as a recombinant fusion protein comprising said cell-penetrating peptide motif coupled to a compound to be delivered into a cell, and

c) Isolating said recombinant fusion protein by purification.

The present invention also relates to a compound such as directly obtained by a process according to the invention.

In another particular embodiment, the invention relates to a composition comprising at least one compound according to the invention and a pharmaceutically acceptable carrier.

As used herein, the term "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency or listed in a generally recognized pharmacopeia for use in animals, and more particularly in humans.

As used herein, the term "carrier" refers to a diluent, adjuvant, excipient, or vehicle. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions.

The compound of the invention may be solubilized in a buffer or water or incorporated in emulsions and microemulsions. Suitable buffers include, but are not limited to, phosphate buffered saline Ca++/Mg++ free (PBS), phosphate buffered saline (PBS), normal saline (150 mM NaCl in water), Tris buffer and surfactants.

There are numerous causes of peptide instability or degradation, including hydrolysis and denaturation. Hydrophobic interaction may cause clumping of molecules together (i.e. aggregation). Thus, in an embodiment, the composition according to the invention further comprises stabilizers.

Stabilizers according to the invention include cyclodextrine and derivatives thereof (see for reference US5730969). Suitable preservatives such as sucrose, mannitol, sorbitol, trehalose, dextran and glycerin can also be added to stabilize the final formulation. A stabilizer selected from ionic and non-ionic surfactants, D-glucose, D-galactose, D-xylose, D-galacturonic acid, trehalose, dextrans, hydroxyethyl starches, and mixtures thereof may be added to the formulation. Addition of alkali metal salt or magnesium chloride may stabilize the compound according to the invention. The peptide may also be stabilized by contacting it with a saccharide selected from the group consisting of dextran, chondroitin sulphuric acid, starch, glycogen, dextrin, and alginic acid salt. Other sugars that can be added include monosaccharides, disaccharides, sugar alcohols, and mixtures thereof (E.g., glucose, mannose, galactose, fructose, sucrose, maltose, lactose, mannitol, xylitol). Polyols may stabilize a peptide, and are water-miscible or water-soluble. Suitable polyols may be polyhydroxy alcohols, monosaccharides and disaccharides including mannitol, glycrol, ethylene glycol, propylene glycol, trimethyl glycol, vinyl pyrrolidone, glucose, fructose, arabinose, mannose, maltose, sucrose, and polymers thereof. Various excipients may also stabilize peptides, including serum albumin, amino acids, heparin, fatty acids and phospholipids,

surfactants, metals, polyols, reducing agents, metal chelating agents, polyvinyl pyrrolidone, hydrolysed gelatin, and ammonium sulfate.

The composition of the invention may be formulated according to standard pharmaceutical practice. The composition may be formulated in a form suitable for oral, enteral or parenteral administration, including the intravenous, intramuscular, intraperitoneal, subcutaneous, rectal, respiratory and topical routes of administration,

Ways to penetrate the cellular membrane may involve specific formulations of the compound according to the invention, preferentially formulations suitable for administration to cells or animal and/or human tissues. In particular, the compound of the invention may be encapsulated in liposomes to form pharmaceutical preparations suitable for administration to cells and animal and/or human tissues.

Other types of lipid aggregates may be used to formulate the compound of the invention. Such aggregates include liposomes, unilamellar vesicles, multilamellar vesicles, micelles and the like, having particle sizes in the nanometer to micrometer range. Methods of making lipid aggregates are by now well-known in the art.

In another aspect, a composition of the present invention is for in vivo diagnosis or for in vivo medical imaging.

In another aspect, the invention relates to a method for in vitro delivering a molecular cargo into a cell of a sample, comprising the steps of:

a) providing at least one compound according to the invention, said compound comprising at least one cell-penetrating peptide motif and at least one molecular cargo,

b) contacting said compound with said cell of said sample.

According to the invention, contacting the compound of the invention with a cell in a sample is performed for example by contacting the compound of the invention directly with a sample. As used herein, a “sample” might be living cells, preferably living cells in an *in vitro* culture, animal tissues, preferably animal tissues in an *in vitro* culture or in mouse models with tumour xenografts. Also, cells from the sample might be living or fixed on a support. Contacting said compound with said cell is performed in conditions allowing the delivery of the compound into the cell.

In a more particular embodiment, the invention relates to a method for detecting the presence of an intracellular compound in a sample, said method comprising the steps of:

a) providing a compound according to the invention, said compound comprising a molecular cargo able to bind to said intracellular compound, and a reporter molecule,

b) contacting said compound with said sample,

c) detecting the signal emitted by said reporter molecule,

d) comparing the signal of step c) with a reference signal, and

e) determining from the comparison of step d) if said intracellular product is present in said sample.

Contacting said compound with said cell is performed in conditions allowing the delivery of the compound into the cell and the formation of a complex between said molecular cargo and said intracellular product. According to the invention, the reference signal is a predetermined measure obtained from a biological sample with a known presence and/or known quantification of the intracellular element to be detected. This presence may be determined by conventional techniques known by the skilled person.

In a more particular aspect, the invention relates to a method for detecting the presence of an intracellular compound in a sample wherein said reporter molecule is a fluorescent probe. More preferably, said fluorescent probe is coupled to an amino acid of the peptide which is not an amino acid of the cell-penetrating peptide motif. Even more preferably, said fluorescent probe is coupled to the molecular cargo, and in particular is coupled to a unique site on the molecular cargo.

In another particular aspect, the invention relates to a method for determining the relative quantity of an intracellular compound in at least two different samples, said method comprising the steps of:

a) providing at least one compound according to the invention, wherein said compound comprises at least one cell-penetrating peptide motif, a molecular cargo able to bind to said intracellular compound, and a fluorescent probe,

b) contacting said compound with said sample,

c) illuminating said compound and said sample with an excitation light,

d) determining the fluorescent signal emitted by said compound,

e) comparing the fluorescent signal of step d) with a reference fluorescent signal, and

f) determining from the comparison of step e) the relative quantity of said intracellular product is said at least two different samples.

As used herein, the terms "fluorescence" and "fluorescent signal" are equivalent. According to the invention, determining the fluorescence in step d) can be achieved by any technique and using any appropriate apparatus known in the art. Any device adapted to measure the properties of emitted light, preferably fluorescence light may be used to determine the fluorescence of step d). The skilled person will easily adapt the intensity and wavelength of the excitation light of step c). As used herein, "determining the fluorescence emitted" refers to measuring the properties of the emitted fluorescence, such as for example measuring the wavelength spectrum, intensity or half-life of the emitted fluorescence. According to the invention, comparing the fluorescence in step e) means comparing the properties of the emitted fluorescence of step d) and the properties of the fluorescence reference, said fluorescence reference being a predetermined measurement of fluorescence obtained from a reference biological sample. As an example, a fluorescently labeled compound according to the invention emits a fluorescence that changes, for example in intensity or wavelength, depending on binding of the compound to its target.

In another aspect, the invention relates to a method for selecting a molecule able to affect the presence and/or the level of an intracellular compound within a sample, said method comprising the steps of:

a) providing a compound according to the invention, said compound comprising at least one cell-penetrating peptide motif, a molecular cargo able to bind to said intracellular compound within said sample and a reporter molecule,

b) contacting said compound according to the invention with said sample in the presence of said molecule,

c) detecting the signal emitted by the reporter molecule,

d) comparing the signal of step c) with a reference signal,

e) selecting, from the comparison of step d), a molecule able to affect the presence and/or the level of said intracellular compound.

In this aspect of the invention, said molecule may belong to a library of molecules. According to this particular embodiment of the invention, a method for selecting a molecule comprises the detection of the presence and/or the level of an intracellular compound and may be performed in the context of a high throughput and/or high content screening of a library of molecules. The reference signal is a predetermined measure obtained from a biological sample with a known presence and/or known quantification of the intracellular element to be detected. Said reference signal may be a signal obtained in the presence of a molecule with a known ability to affect the presence and/or the level of said intracellular compound. This presence may be determined by conventional techniques known by the skilled person.

In another particular aspect, the invention relates to a method for selecting a molecule able to affect the activity of an intracellular compound within a sample, said method comprising the steps of:

a) providing a compound according to the invention, said compound comprising at least one cell-penetrating peptide motif, a molecular cargo able to detect the activity of an intracellular compound within said sample, and a reporter molecule,

b) contacting said compound according to the invention with said sample, in the presence of said test molecule,

c) detecting the signal emitted by the reporter molecule,

d) comparing the signal of step c) with a reference signal,

e) selecting, from the comparison of step d), a molecule able to affect the activity and/or the level of said intracellular compound.

In this aspect of the invention, said test molecule may belong to a library of molecules. According to this particular embodiment, a method for selecting a molecule according to the invention comprises the detection of the activity of an intracellular compound and may be performed in the context of a high throughput and/or high content screening of a library of molecules.

According to another aspect, the invention relates to a method for inhibiting the growth of a cell of a sample, said method comprising the steps of:

a) providing a compound according to the invention, wherein said compound comprises at least one cell-penetrating peptide motif and a molecular cargo, with said molecular cargo being chosen in the group consisting of: inhibitors of enzymes of cell-proliferation, inhibitors of compounds of signaling pathways and in particular inhibitors of CDK/Cyclin complexes,

b) contacting said compound with said cell of said sample.

In a further aspect, said method comprises the measuring of cell growth and the detection of growth inhibition, in particular the inhibition of cell-proliferation and of signaling pathways.

In another aspect, the invention relates to a method for detecting the presence of an intracellular product in an organ.

By determining the “relative quantity”, the “level”, the “amount”, the “level of expression” or the “concentration” of an intracellular product, it is meant that the intensity of the signal emitted by the reporter molecule is compared between at least two samples, and the relative difference between the signals emitted is expressed as a percentage.

In a particular embodiment, the invention relates to a method for detecting the level of an intracellular product in living or fixed cells.

In a more particular embodiment, the invention relates to a process wherein a compound according to the invention is contacted with a cell which is present within a tissue or an organ.

The invention also relates to a method for medical imaging, especially endoscopic imaging, said method comprising the steps of:

a) administering to a subject a compound of the invention, wherein said compound comprises a molecular cargo and a reporter molecule,

b) obtaining an image with an apparatus detecting the signal emitted by said reporter molecule in at least one part of an organ of said subject.

The terms "individual", "subject" and "host" are used herein interchangeably and refer to any subject for whom diagnosis is desired, particularly humans. Other subjects

may include cattle, dogs, cats, guinea pigs, rabbits, rats, mice, horses, and the like. In some preferred embodiments the subject is a human.

As used herein, the term "biological sample" refers to biological material from a subject. The sample assayed by the present invention is not limited to any particular type. Samples include, as non-limiting examples, single cells, multiple cells, tissues, tumors, biological fluids, biological molecules, or supernatants and/or extracts of any of the foregoing. Examples include tissue removed for biopsy, tissue removed during resection, blood, serum, plasma, sputum, urine, lymph tissue, lymph fluid, cerebrospinal fluid, mucous, skin, saliva, gastric secretions, semen, seminal fluid, tears, spinal tissue or fluid, cerebral fluid, trigeminal ganglion sample, a sacral ganglion sample, adipose tissue, lymphoid tissue, placental tissue, upper reproductive tract tissue, gastrointestinal tract tissue, male genital tissue and fetal central nervous system tissue and stool samples.

The sample used will vary based on the assay format, the detection method and the nature of the tissues, cells or extracts to be assayed. Methods for preparing samples are well known in the art and can be readily adapted in order to obtain a sample that is compatible with the method utilized.

In another aspect, the present invention relates to the use of a compound according to the invention for the intracellular delivery of a molecular cargo.

In another particular embodiment, the present invention relates to the use of compounds according to the invention for detecting an intracellular compound in a sample.

In a particular embodiment, the invention relates to the use of a compound according to the invention for in vitro diagnostic of a disease characterized by the altered presence of the level of an intracellular ligand of said compound.

In another particular embodiment, the invention relates to the use of a compound according to the invention for medical imaging.

In another particular embodiment, the invention relates to the use of a compound according to the invention for high throughput and/or high content screening of libraries of compounds within cell samples.

In a particular embodiment, the invention relates to the use of a compound according to the invention for modulating a biological function. In a more particular embodiment, the invention relates to the use of a compound according to the invention for activating, or enhancing, a biological function. In another more particular embodiment, the invention relates to the use of a compound according to the invention for stopping or inhibiting a biological function.

In another particular embodiment, the invention relates to the use of a compound according to the invention for determining the relative quantity of at least one intracellular component in at least two different samples.

In another particular embodiment, the invention relates to the use of a compound according to the invention for screening a plurality of compounds for their ability to affect the presence and/or the quantity of a given a intracellular component, or to affect a given biological function.

In another particular embodiment, the invention relates to the use of a compound according to the invention for the in vitro diagnosis of a condition characterized by the presence or the absence of an intracellular component, or by the level of expression of a given intracellular component.

In another particular embodiment, the invention relates to the use of a compound according to the invention for the monitoring of the efficacy of a therapeutic treatment.

The invention also relates to the use of at least one compound and/or a composition of the invention for medical imaging.

In a particular embodiment, the invention also relates to the use of a compound and/or a composition of the invention for medical imaging, preferably endoscopic imaging. In another embodiment, the invention also relates to the use of at least one compound and/or a composition of the invention for *in vitro* imaging.

A compound according to the invention will be used in an effective amount, according to the nature of said use. According to the present invention, the term "effective amount" of a composition means the amount which is sufficient to allow for measurement of the fluorescence in the subject, particularly. It is understood that the effective dosage will be dependent upon the age, sex, health, and weight of the recipient, the nature of the disease or condition being investigated, and the nature of the

effect desired. The effective amount can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation.

In another particular embodiment, the invention relates to a kit of parts comprising at least one compound according to the invention and an acceptable solvent. In a particular embodiment, a kit according to the invention comprises at least one compound according to the invention and at least one component chosen among the group consisting of: buffer, positive control, phospholipids, cells to be transfected and instructions for use. In another particular embodiment, a kit according to the invention comprises a compound provided either in an aqueous or a lyophilized stock.

The following examples are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

LEGENDS OF THE FIGURES

Figures 1A to 1D: Internalization of C4 peptide into HeLa cells.

The C4 peptide is an inhibitor of CDK2/Cyclin A that blocks cancer cells proliferation (Gondeau *et al.,* 2005). Figure 1A shows the internalization of TAT-C4 biosensor (SEQ ID N°26) into HeLa cells. Figure 1B shows the internalization of PEP1-C4 biosensor (SEQ ID N°19) into HeLa cells. Figure 1C shows the internalization of PEP2b-C4 biosensor (SEQ ID N°31) into HeLa cells. Figure 1D shows the internalization of PEP3b-C4 biosensor (SEQ ID N°32) into HeLa cells. Peptides labeled with FITC were directly applied onto cultured HeLa cells, they penetrate readily and distribute homogeneously throughout the cytoplasm within less than 1 hour.

Figures 2A to 2D: Internalization of PEP1-CDKQUANT into HeLa cells.

Figure 2B represents the internalization of CDKSENS1 (SEQ ID N°29) into Hela cells when CDKSENS1 is labeled with Cy3 and is non covalently associated with CADY2 peptide (SEQ ID N°28). Figure 2D represents the internalization of PEP1-CDKQUANT1 (SEQ ID N°20) into Hela Cells, wherein PEP1-CDKQUANT1 is labeled with Cy3. Peptides were directly applied onto cultured HeLa cells. Figures 2A and 2C represent the respective Hoechst staining of Hela cells (control staining).

Figures 3A to 3D: Quantification of CDK/Cyclin levels in HeLa and HS68 cells through ratiometric quantification of PEP1-CDKQUANT1-Cy3/Ctrl-Cy5 fluorescence versus CADY2 formulations of CDKSENS1-Cy3/Ctrl-Cy5

In Figure 3A, the histograms represent the comparison of the fluorescence ratio of PEP1-CDKQUANT-Cy3 (SEQ ID N°20) /PEP1-control-Cy5 (SEQ ID N°25) in HeLa (left histogram) and in HS68 fibroblasts (right histogram), after normalization of cell extracts. In Figure 3B, the histograms represent the comparison of fluorescence ratio CADY2- CDKSENS1-Cy3 (SEQ ID N°29) /control-Cy5 (SEQ ID N°30) in HeLa (left histogram) and in HS68 fibroblasts (right histogram). Figure 3C shows a schematic representation of PEP1-CDKQUANT1. Figure 3D shows a schematic representation of the non-covalent formulation of CDKSENS1/ CADY2. The mean ratiometric value was

determined from 3-5 independent experiments in which PEP1-CDKQUANT1-Cy3 and PEP1-CTRLCy5 fluorescence was measured and the ratio of CDKSENS1-Cy3/CTRLCy5 determined in 3-4 fields of cells (n= 50 – 60).

Figure 4: Biodistribution of PEP1-CDKQUANT1-Cy5 in mouse tissues and organs

Figure 4 is a histogram representation of the distribution of PEP1-CDKQUANT1 through different tissues and organs, for each organ, respectively, after intratracheal/nebullisation (left column), intravenous (central column) and intraperitoneal (right column) administration.

Figures 5A and 5B: Ratiometric quantification of PEP1-CDKQUANT-Cy5/Ctrl-Alexa750 in tumour xenografts treated or not with si RNA targeting cyclin B.

Figure 5A represents the imaging of a mouse bearing a tumour and treated with PEP1-CDKQUANT1-Cy5 and PEP1-Ctrl-Alexa750. Figure 5B represents a histogram showing the differences in the fluorescence intensity ratio of PEP1/CDKQUANT1-Cy5 / PEP1-Ctrl-Alexa755 measured in ten mice treated with siRNA targeting cyclin B (left histogram) and ten mock-treated mice (right histogram).

Figures 6A to 6D: Application of PEP1-CDKQUANT1 to high content / high throughput screening.

Figure 6A: Schematic representation of PEP1-CDKQUANT expressed as a fusion protein with mRFP, so that an intramolecular fluorescence ratio could be used for quantification of CDK/Cyclin levels. Figure 6B: Internalization of PEP1-RFP-CDKQUANT-Cy5 in HeLa cells (left panel) and in HS68 (right panel). Figure 6C: Example of multiparametric high content screen of a small chemical compound library to identify compounds that affect CDK/Cyclin levels. The higher panel represents cytotoxicity, the middle panel represents PEP1-CDKQUANT fluorescence and the lower panel represents effect on cell cycle progression. Figure 6D: Histogram representation of the fluorescence distribution profiles of PEP1-CDKQUANT-Cy5 fluorescence/RFP from a field of 2000 HeLa cells either mock treated or treated with 50uM roscovitine for 7h.

EXAMPLES

Example 1: Internalization of PEP1-C4 inhibitor into Hela cells

Peptide design and synthesis, protein expression, purification and labeling

Cell-penetrating peptide according to the invention was linked to C4, an inhibitor of CDK2/cyclin complex, to generate PEP1-C4 peptide (SEQ ID N°19). PEP1-C4 biosensor bears a unique cysteine for coupling of an environmentally sensitive fluorescent probe.

Peptide synthesis, protein expression, purification and labeling:

PEP1-C4 compound was synthesized by solid-phase Fmoc strategy. PEP1-C4 was labeled on its unique cysteine with fluoresceinisothiocyanate (FITC) and further purified on NAP-5 columns (GE Healthcare).

Cell Culture and extract preparation:

Cell culture media, serum and antibiotics were purchased from Invitrogen. HeLa, HS68, A549, MCF-7 and U2OS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) + Glutamax supplemented with 10% Fetal Calf Serum (FCS), 1 mM penicillin and 1 mM streptomycin at 37°C in an atmosphere containing 5% CO2. Cell extracts were prepared in lysis buffer containing 50 mM TrisHCl, pH 7.4, 150 mM NaCl, 0.1% NP40, 0.1% Deoxycholate, 2 mM EDTA, 1 mM phenylmethlsulfonyl fluoride (PMSF), CompleteTM protease inhibitors (Roche), 50 mM NaF, 40 mM β-Glycero-phosphate, 1 mM Na3VO4 and normalized following spectrophometric dosage at 280nm.

Microscopy:

For fixed observations, cells were treated for 20 minutes with paraformaldehyde, nuclei were stained with Hoechst 33342 (Sigma), and cells were mounted with Prolong Gold AntiFade Reagent (Invitrogen). Epifluorescence images were acquired on a Leica DM6000 microscope (Leica Microsystems) piloted by the Metamorph software (Universal Imaging). For colocalization experiments, images were acquired on a Zeiss axioplan2/LSM510 META confocal microscope. Live-cell imaging was performed on a Zeiss Axiovert 200M piloted by the Metamorph software. Images were processed with ImageJ software.

Results

The entry of PEP1-C4-FITC into Hela cells is quick and efficient, as shown in Figure 1B. The covalent approach is much easier to apply than the non-covalent approach using CADY2 and yields significantly better results, most likely associated with a more homogenous distribution. The C4 peptide preserves its biological activity, which results in a blocade of cellular proliferation (Figure 1B). Results are compared with those obtained using a TAT-C4 compound comprising a TAT vector.

Example 2: Detection and quantification of CDK/cyclin level in different cell lines using PEP1-CDKQUANT1-Cy3 sensor.

Titration experiments were performed at 25°C in a Polarstar Spectrofluorimeter (BMG Labtech). 200nM FITC-labelled PEP1-CDKQUANT1 were titrated with increasing concentrations of purified, recombinant GST-tagged CDK1, CDK2, cyclins A, and E1, MBP-cyclin B, GST or MBP, from 25 nM to 1 μM in 200ul potassium phosphate buffer at pH 7.2, 150mM NaCl at 25°C in 96-well microplates, and changes in fluorescence emission were recorded at 520nm following excitation at 485nm. Data analysis and curve fitting were performed using the GraFit Software (Erathicus Ltd) and a standard quadratic equation, as described previously.

Antibodies for Western Blotting and Indirect Immunofluorescence:

Antibodies against Cyclin A (H432, sc-751), Cyclin B1 (GNS1, sc-245), Cyclin D1 (C20, sc-717), Cdk1 (C19, sc-954) Cdk2 (M2, sc-163), and Cdk4 (C22, sc-260) were purchased from Tebu-Bio (Santa-Cruz), anti-actin from Sigma (A2668), and used at 1:1000 dilution for Western blotting, except for anti-cyclin B1 at 1:500 dilution, 1:100 for indirect imunofluorescence. Secondary antibodies labelled with Alexa-488 were used for indirect immunofluorescence.

Fluorescence quantification and statistical analysis:

3uM PEP1-CDKQUANT1-Cy3 and Ctrl Peptide-Cy5 were overlaid onto cells for 1 hour then imaged by fluorescence microscopy. Live-cell imaging acquisitions were substracted for background signal corresponding to minimal fluorescence levels using Metamorph. Image J was then used for analysis and quantification of fluorescence values, as described previously (Kurzawa *et al.*, 2010).

Results:

PEP1-CDKQUANT1-Cy3 was applied to probe CDK/cyclin levels in living cells through ratiometric quantification of its fluorescence over that of a control peptide which is equally capable of penetrating cells without a carrier but which does not bear any CDK or cyclin-binding sequence and labeled with Cy5. Both self-penetrating peptides (PEP1-CDKQUANT1 and PEP-1-CTRL) were overlaid onto cells, and live-cell imaging of cells was performed to acquire PEP1-CDKQUANT1-Cy3 and PEP1-Ctrl-Cy5 fluorescence. Established ratiometric quantification strategy provides a means of standardizing PEP1-CDKQUANT1-Cy3 fluorescence with respect to the CTRL-Cy5 peptide (Figure 3B). Ratiometric quantification experiments were performed between normal diploid fibroblasts HS68 and HeLa cells. 45% difference in the PEP1-CDKQUANT1-Cy3/CTRL-Cy5 ratio was determined between HeLa cells and HS68 fibroblasts.

As a control, after prior incubation allowing non-covalent association, CADY2 and CDKSENS1-Cy3, or CADY2 and control peptide-Cy5, were overlaid onto Hela and HS68 cells, and live-cell imaging of cells was performed to acquire Cy3 and Cy5 fluorescence. Established ratiometric quantification strategy provides a means of standardizing Cy3 fluorescence with respect to Cy5 fluorescence (Figure 3A). Ratiometric quantification experiments were performed between normal diploid fibroblasts HS68 and HeLa cells. 15% difference in the CADY2/CDKSENS-Cy3/CTRL-Cy5 ratio was determined between HeLa cells and HS68 fibroblasts.

These results show that the PEP1-CDKQUANT biosensors according to the invention allow a detection which is more sensitive, quantitative, rapid and robust than the non-covalent formulations of CDKSENS delivered with CADY2.

Example 3: In vivo internalization of PEP1-CDKQUANT1 biosensor

PEP1-CDKQUANT1 (SEQ ID N°20) compound was synthesized by solid-phase Fmoc strategy. PEP1-CDKQUANT1 was labeled on its unique cysteine with Cy3 and further purified on NAP-5 columns (GE Healthcare). PEP1-CDKQUANT1 labeled with Cy3 was injected into mice xenografted with tumoral cells and its biodistribution and clearance were characterized over time (Figure 4). PEP1-CDKQUANT1 biosensors can be applied in vivo, and observed to distribute throughout different tissues and organs,

including tumors, following intravenous, intraperitoneal or intratracheal administration. These results show that, thanks to the PEP1 peptide according to the invention, the PEP1-CDKQUANT biosensor follows the biodistribution of CDK/Cyclin complexes. PEP1 allows the kinetic detection of the biosensor and its target, as well as the clearance of the biosensor.

Example 4: Monitoring of a response to siRNA treatment using PEP1-CDKQUANT1 biosensor

siRNA Transfections

siRNA targeting Cyclin B1 was a Smart Pool TM M003206-02 purchased from Dharmacon. siRNA transfections were performed for 72h with the cell-penetrating siRNA carrier CADY as described in Crombez *et al* (2009).

Results

Mice xenografted with tumoral cells were exposed to siRNA-mediated knockdown of cyclin B1 in tumour xenografts in mice. PEP1-CDKQUANT1-Cy5 and PEP1-Ctrl-Alexa750 were administered in vivo in mice, by intraveinous, intraperitoneal and intratracheal administration. CDK/Cyclin relative abundance in mice treated with siRNA targeting cyclin B compared to mock-treated mice was assessed through quantification of PEP1-CDKQUANT1-Cy5 / PEP1-Ctrl-Alexa750 fluorescence by in vivo fluorescence imaging (Figures 5A and 5B).

Example 5: Monitoring CDK/Cyclin levels by FACS analysis using PEP1-CDKQUANT

FACS analysis was performed as described in Crombez *et al* (2009). PEP1-CDKQUANT-Cy5 and PEP1-CTRL-Cy5 were added independently to cells and the intensity of Cy5 fluorescence in cells was measured independently by flow cytometry. FACS analysis revealed that HeLa cells exhibited 31% greater PEP1-CDKQUANT-Cy5 / CTRL-Cy5 fluorescence than HS68, reminiscent of the difference determined between these two cell lines by ratiometric quantification of the fluorescence acquired through live cell imaging. These results show that the PEP1-CDKQUANT biosensors according

to the invention may be implemented by FACS and allow a detection which is sensitive, quantitative, rapid and robust.

Example 6: Application of PEP1-CDKQUANT to High Throughput screening

Expression of PEP1-CDKQUANT as a fusion to mRFP, prepared such as described in Example 1 yields an ideal tool for high throughput screening. Indeed mRFP serves as a scaffold for presentation of the biosensor as well as an inert intramolecular fluorescent signal for standardized ratiometric quantification. Since the sequence of mRFP is devoid of cysteines (unlike other genetically-encoded fluorescent proteins), the cysteine within the peptide biosensor sequence remains unique, allowing for site-specific labeling with an environmentally-sensitive probe (Figures 6A to 6D). Note that roscovitine treatment leads to a shift of the fluorescence distribution profile by 18.5%, indicating that it leads to an increase in CDK/Cyclin abundance (Figure 6D)

BIBLIOGRAPHIC REFERENCES

Grdisa et al., *Curr. Med. Chem.,* 18(9): 1373-1379, 2011

Matjaz et al., *Adv. Drug Deliv. Rev.*, 57(4): 529-545, 2005

Morris et al. *Biol.Cell,* 100: 201-217, 2008

Fonseca et al. *Advanced Drug Delivery Reviews*, 61: 953-964, 2009

Heitz et al. *British J.Pharmacol.* 157:195-206, 2009.

Torchilin et al., *Adv.Drug.Deliv.Rev.* 60, 548-558, 2008

Derossi et al., *J.Biol.Chem.*, 269, 10444-450, 1994

Pooga et al., *FASEB J.,* 15, 1451-53, 1998

Rennert et al., *Biochim.Biophys.Acta,* 1758, 264-279, 2006

Elliot et al., *Cell,* 88, 223-233, 1997

Wender et al., *P.N.A.S. USA*, 13003-008, 2000

Pujals et al., *Biochim.Biophys.Acta*, 1758, 264-279, 2006

Morris et al., *Nucl.Acids.Res.,* 25, 2730-36, 1997

Morris et al. *Nat. Biotechnol.* 19: 1173-1176, 2001

Morris et al., *Nucl.Acids.Res.*, 35, e49-e59, 2007

Crombez et al., *Mol. Ther. 2009*, 17, 95-103

Kurzawa et al., 4559-69, 2011

Kurzawa et al. *Biochim. Biophys. Acta* 1798:2274-2285, 2010

Kim et al., *Free Radical Biology & Medicine*, 47, 941-952, 2009

Shirong et al., *Journal of Huazhong University of Science and Technology*, 27(3), 225-229, 2007

Duk-Soo *et al., BMB Reports*, 45(9), 2012

Kang *et al.*, *Journal of Drug Targeting*, 19(7), 497-505, 2011

Maniatis T. et al, *Molecular cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.*, Edition 1989.

Loving et al., *Trends Biotechnol.*, 28(2):73-83, 2010

Higgins et al, *"Protein Expression: A Practical Approach"*, Oxford, UK, Oxford University Press

Baneyx F et al, *"Protein Expression Technologies: Current Status and Future Trends"*, Norfolk, UK: Horizon Bioscience

Atherton et al., *"Solid Phase peptide synthesis: a practical approach"*, Oxford, England.

Gondeau et al., *J.Biol.Chem.*, 280, 13793-800, 2005

Morris et al. *J Biol Chem.,* 277:23847–23853, 2002

CLAIMS

1. Cell-penetrating peptide motif comprising at least the amino acid sequence: WW/FXXWW/F (SEQ ID N°1).

2. Compound comprising a peptide comprising at least one cell-penetrating peptide motif according to claim 1.

3. Compound according to claim 2, wherein said cell-penetrating peptide motif comprises an amino acid sequence chosen in the group consisting of the sequence SEQ ID N°2 to the sequence SEQ ID N°5 and the sequence SEQ ID N°7 to the sequence SEQ ID N°17.

4. Isolated nucleic acid molecule comprising a nucleotide sequence encoding for at least one cell-penetrating peptide motif having an amino acid sequence chosen in the group consisting of: the sequence SEQ ID N°2 to the sequence SEQ ID N°5 and the sequence SEQ ID N°7 to the sequence SEQ ID N°17.

5. Compound according to any one of claims 1 to 3, wherein said at least one cell-penetrating peptide motif is covalently linked to at least one molecular cargo.

6. Compound according to claim 5, wherein said at least one molecular cargo is chosen in the group consisting of: peptides, lipids, glucids, nucleic acids, and macromolecules comprising thereof.

7. Compound according to claim 6, wherein said molecular cargo is a peptide chosen in the group consisting of: antibodies, enzymes, ligands, and fragments, analogs and derivatives thereof.

8. Compound according to claim 8, wherein said compound comprises an amino-acid sequence chosen in the group consisting of the sequence SEQ ID N°19 to

the sequence SEQ ID N°24 and the sequence SEQ ID N°31 to the sequence SEQ ID N°32.

9. Compound according to any one of claims 5 to 8, said compound comprising a molecular cargo and a reporter molecule.

10. Composition comprising at least one compound according to any one of claims 2 to 3 and 5 to 9, and a pharmaceutically acceptable carrier.

11. Method for in vitro delivering a molecular cargo into a cell of a sample, comprising the steps of:

a) providing a compound according to any one of claims 5 to 9, or a composition according to claim 10, said compound comprising a molecular cargo, and

b) contacting said compound with said cell of said sample.

12. Method for in vitro determining the presence of an intracellular compound into a cell of a sample, comprising the steps of:

a) providing a compound according to claim 9, or a composition according to claim 10, said compound comprising a molecular cargo able to form a complex with said intracellular compound, and a reporter molecule,

b) contacting said compound with said cell of said sample,

c) detecting the signal emitted by said reporter molecule, and

d) determining the presence of said intracellular compound from the results of the detection of step c).

13. Method for determining the relative quantity of an intracellular compound in at least two different samples, said method comprising the steps of:

a) providing at least one compound according claim 9, said compound comprising a molecular cargo able to form a complex with said intracellular compound, and a reporter molecule,

b) contacting said compound with one of said samples,

c) detecting the signal emitted by said reporter molecule,

d) comparing said signal of step c) with a reference signal,

e) performing steps b), c), and d) with at least another of said samples,

f) comparing said signals of steps d) and e) to determine the relative quantity of said intracellular compound in said samples.

14. Use of a cell-penetrating peptide motif according to claim 1 or of a compound according to any of claims 2 to 9 for the delivery of a molecular cargo into a cell.

15. Kit comprising at least one compound according to any one of claims 2 to 9, and a product chosen in the group consisting of: buffer, positive control, cells to be transfected and instructions for use.

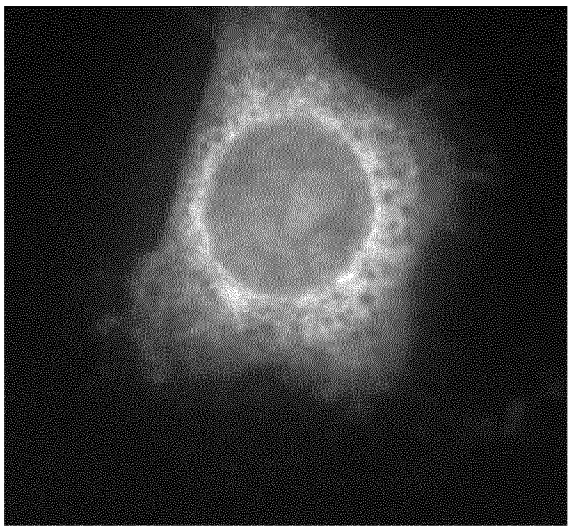


FIGURE 1A

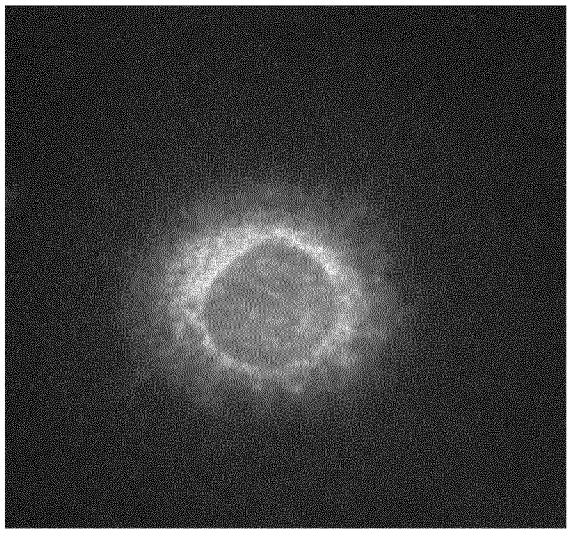


FIGURE 1B

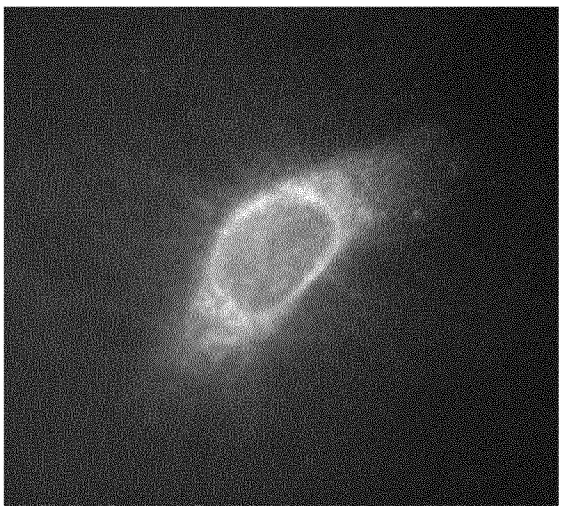


FIGURE 1C

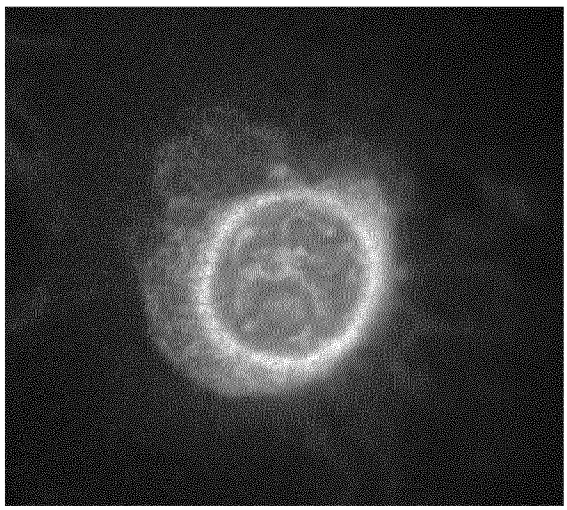


FIGURE 1D

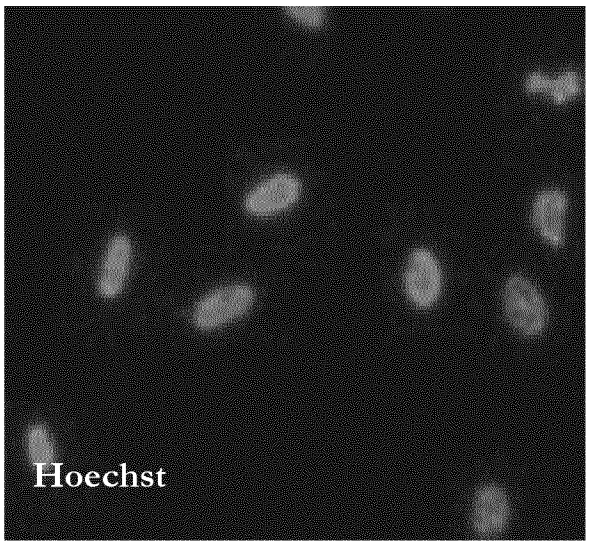


FIGURE 2A

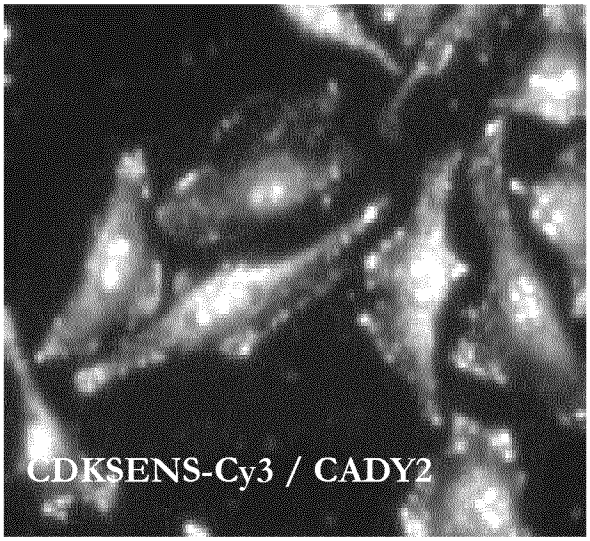


FIGURE 2B

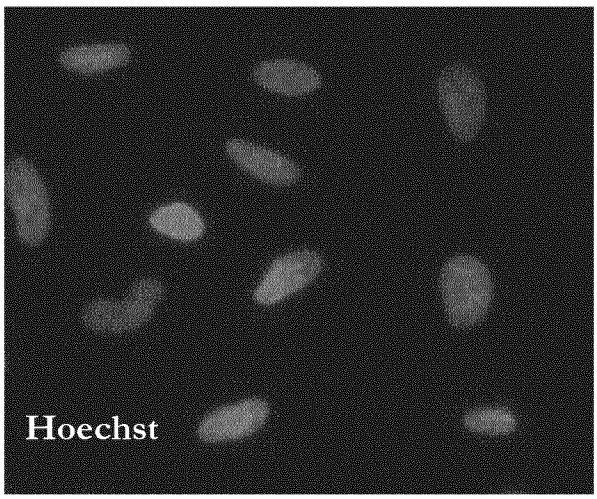


FIGURE 2C

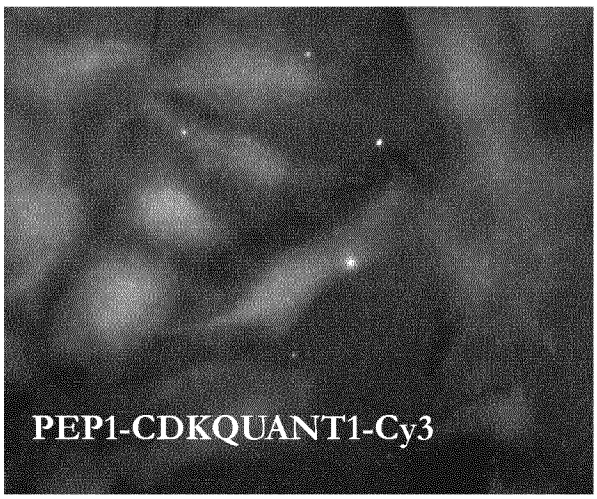


FIGURE 2D

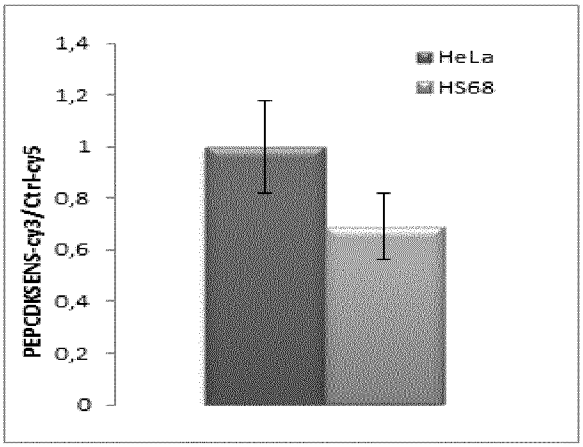


FIGURE 3A

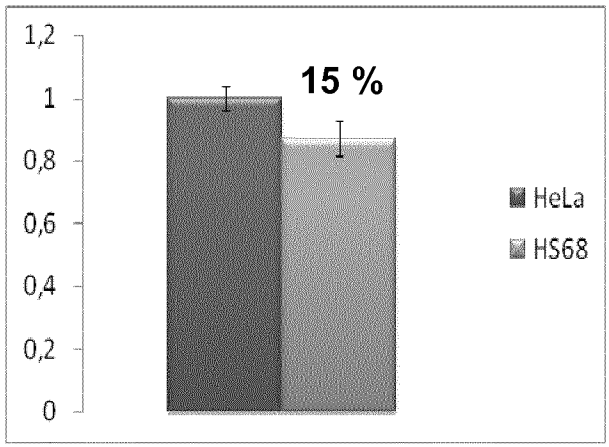


FIGURE 3B

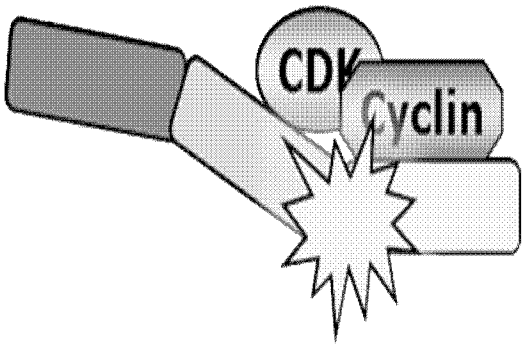


FIGURE 3C

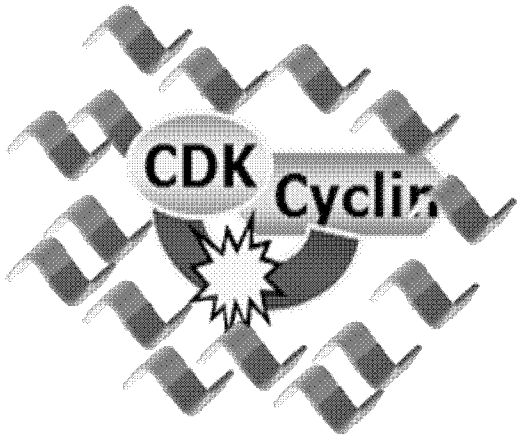


FIGURE 3D

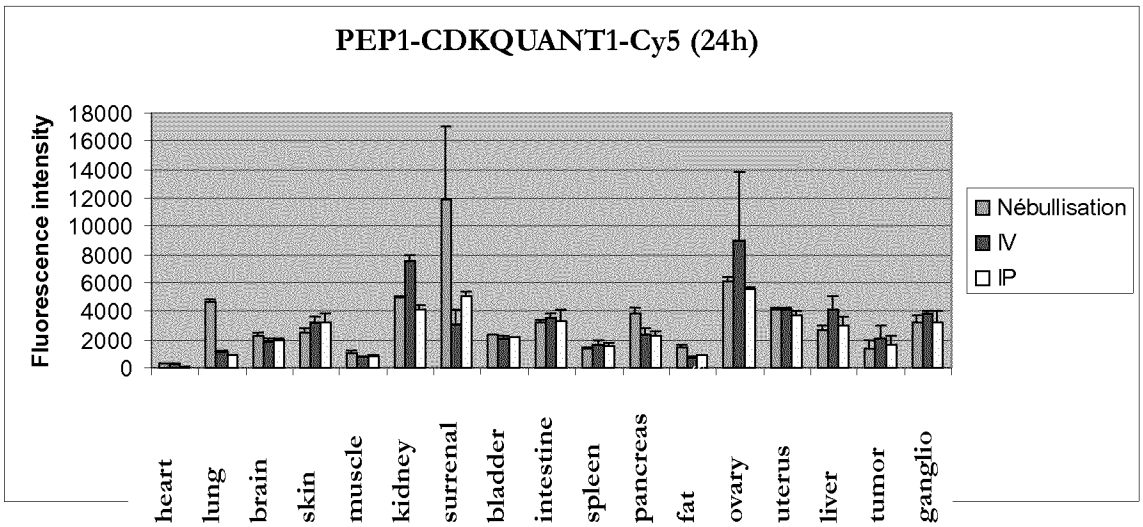


FIGURE 4

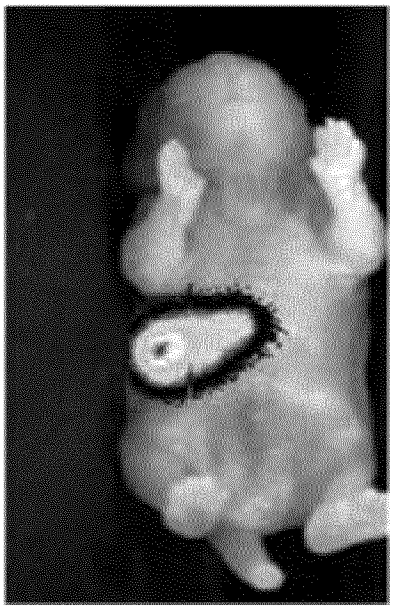


FIGURE 5A

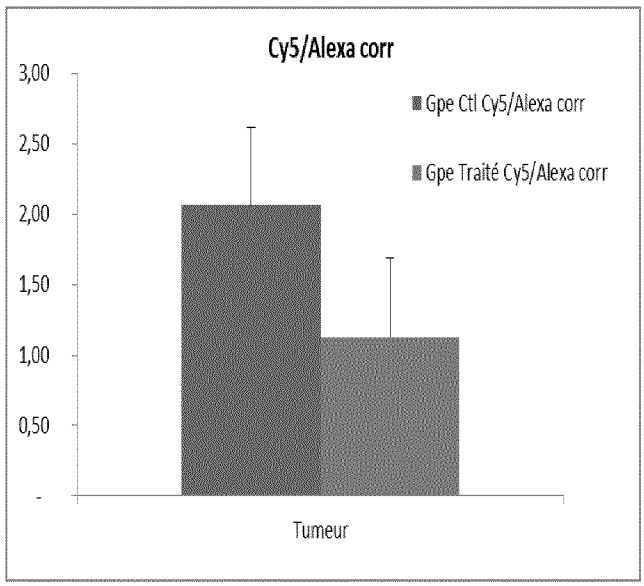


FIGURE 5B

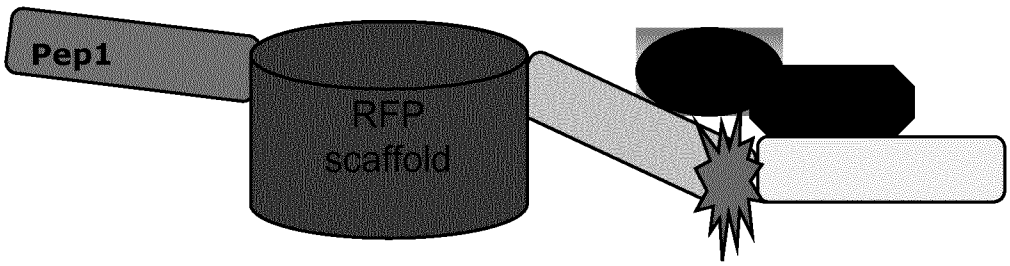


FIGURE 6A

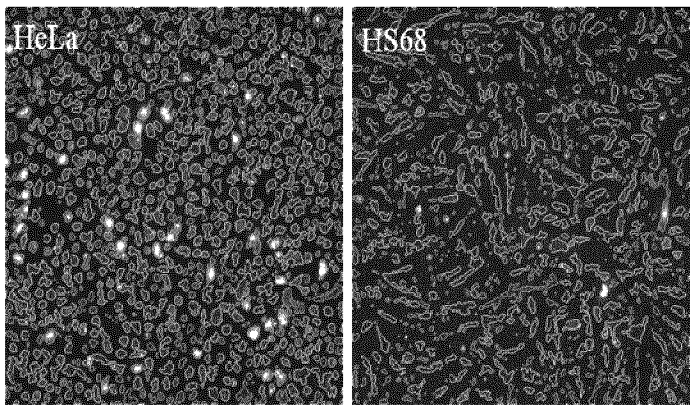


FIGURE 6B

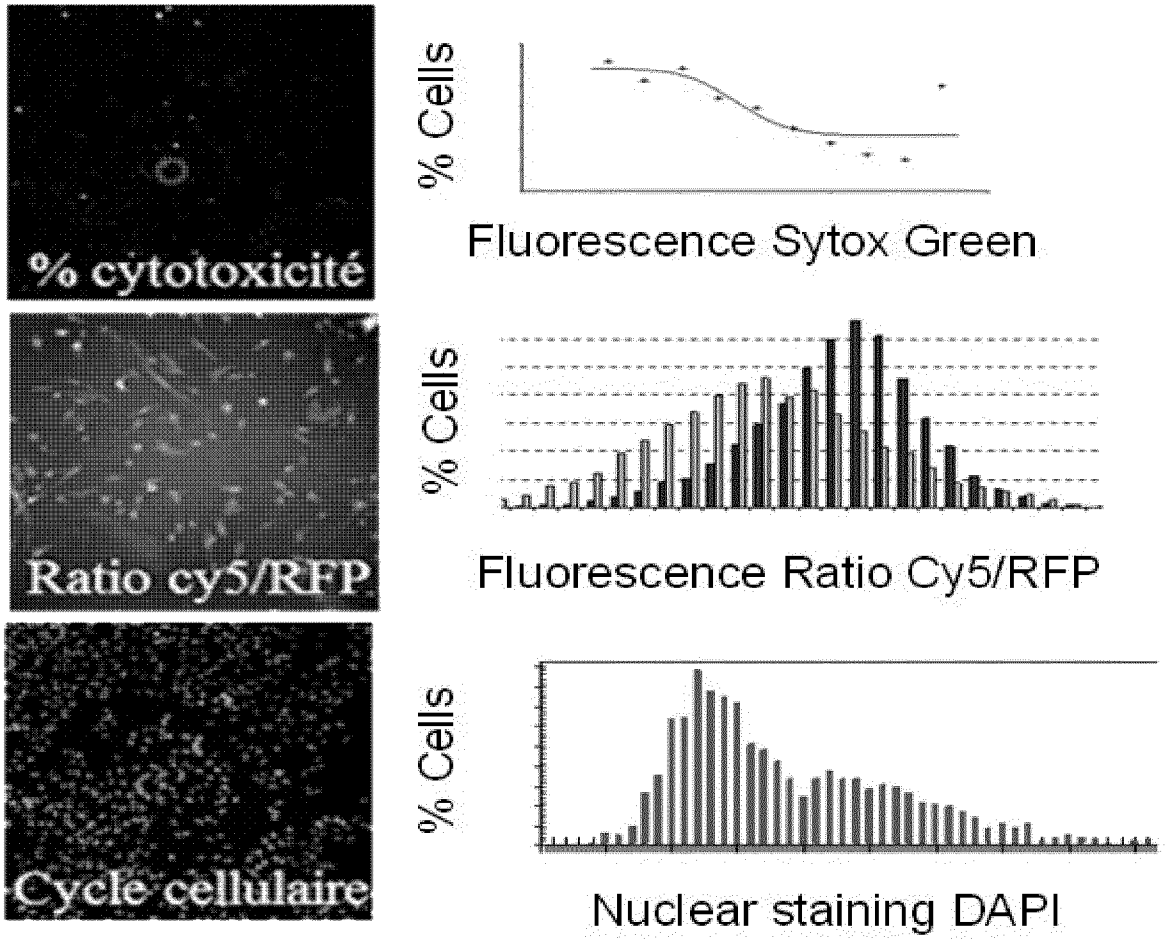


FIGURE 6C

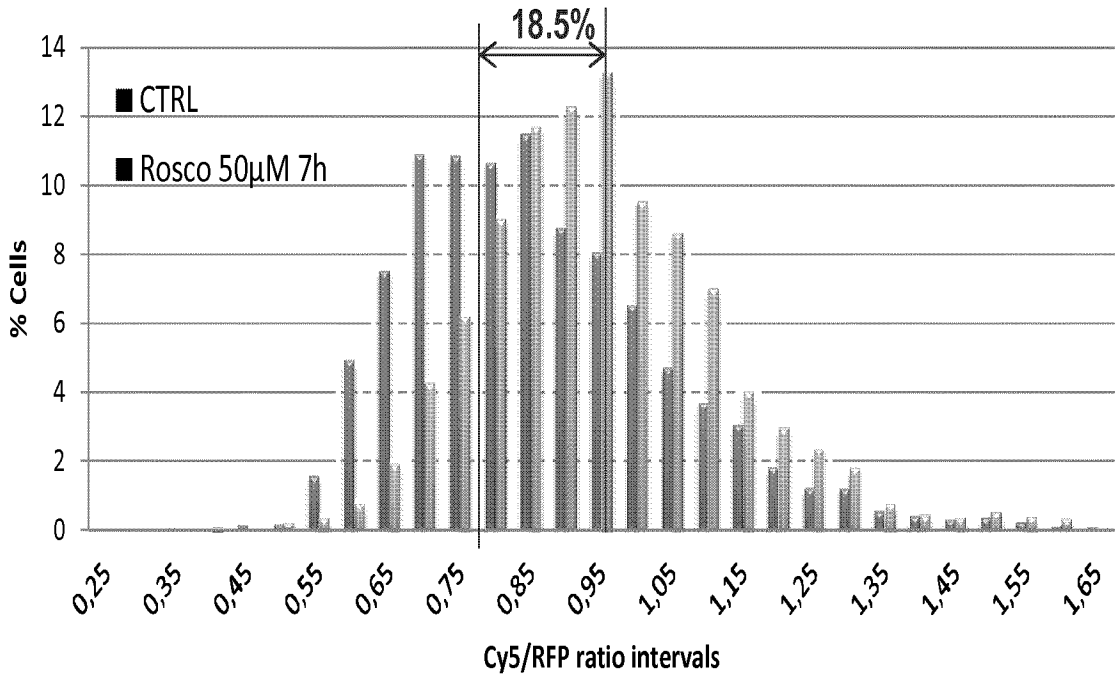


FIGURE 6D

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/055633

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/58 C07K7/00 G01N33/573
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)
G01N C07K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIM D W ET AL: "Transduced human PEP-1-catalase fusion protein attenuates ischemic neuronal damage", FREE RADICAL BIOLOGY AND MEDICINE, ELSEVIER SCIENCE, US, vol. 47, no. 7, 1 October 2009 (2009-10-01), pages 941-952, XP026542081, ISSN: 0891-5849, DOI: 10.1016/J.FREERADBIOMED.2009.06.036 [retrieved on 2009-07-03]	1-7, 9-11,14, 15
Y	the whole document, in particular abstract; page 942, sections "expression and purification of ...", "transduction of ...", "fluorescence microscopic analysis"; page 943, column 1, lines 22-27; figures 1 and 2	8,12,13

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 June 2014	08/07/2014

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	Weber, Peter

1

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHIRONG YAN ET AL: "Study on the penetrability of PEP-1-P27mt for cell membranes in Vitro", JOURNAL OF HUAZHONG UNIVERSITY OF SCIENCE AND TECHNOLOGY, vol. 27, no. 3, 1 June 2007 (2007-06-01), pages 225-229, XP055076672, ISSN: 1672-0733, DOI: 10.1007/s11596-007-0302-2	1-7, 9-11,14, 15
Y	the whole document, in particular abstract; page 225, sentence spanning columns; paragraphs 1.8 and 2.6; figure 5	8,12,13
X	DUK-SOO KIM ET AL: "PEP-1-p18 prevents neuronal cell death by inhibiting oxidative stress and Bax expression", BMB REPORTS, vol. 45, no. 9, 30 September 2012 (2012-09-30), pages 532-537, XP055076817, ISSN: 1976-6696, DOI: 10.5483/BMBRep.2012.45.9.083	1-7, 9-11,14, 15
Y	the whole document, in particular abstract; page 535, sections "expression and purification of ..." and "transduction of the ..."; page 536, section "experimental animals and ..."; figure 1	8,12,13
X	WO 2007/108749 A1 (CEPEP III AB [SE]; BURNHAM INST MEDICAL RESEARCH [US]; LANGEL UELO [SE]) 27 September 2007 (2007-09-27)	1-7, 9-11,14, 15
Y	the whole document, in particular claims 1, 4, 7, 8, 11; SEQ ID NOs: 15 and 16	8,12,13
X	US 2006/035815 A1 (CHEN LISHAN [US] ET AL) 16 February 2006 (2006-02-16) the whole document, in particular examples 2 and 5; table 8 (row 2: "dsCoP317nfR320"); SEQ ID NO: 104	1-3,5,6, 9-11,14, 15

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KANG MYUNG J ET AL: "Design of a Pep-1 peptide-modified liposomal nanocarrier system for intracellular drug delivery: Conformational characterization and cellular uptake evaluation", JOURNAL OF DRUG TARGETING, HARWOOD ACADEMIC PUBLISHERS GMBH, DE, vol. 19, no. 7, 1 January 2011 (2011-01-01), pages 497-505, XP009172102, ISSN: 1061-186X, DOI: 10.3109/1061186X.2010.511226 the whole document, in particular abstract; page 499, section "pep-1 peptide conjugation ..."; figure 2	1-3,5,6, 9-11,14, 15
X	WO 02/061105 A2 (PASTEUR INSTITUT [FR]; CENTRE NAT RECHERCHE [FR]; EDELMAN LENA [FR]; J) 8 August 2002 (2002-08-08) the whole document, in particular page 33, compound "Ctr5Tox1"	1-3,5-7, 9-11,14, 15
X	KURZAWA L ET AL: "PEP and CADY-mediated delivery of fluorescent peptides and proteins into living cells", BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - BIOMEMBRANES, ELSEVIER, AMSTERDAM, NL, vol. 1798, no. 12, 1 December 2010 (2010-12-01), pages 2274-2285, XP027430150, ISSN: 0005-2736, DOI: 10.1016/J.BBAMEM.2010.02.027 [retrieved on 2010-02-25] cited in the application the whole document, in particular abstract; page 2274, column 2, lines 12-18; figures 1, 3-6; tables 1, 3	1-3,10, 14,15
X	C. GONDEAU: "Design of a Novel Class of Peptide Inhibitors of Cyclin-dependent Kinase/Cyclin Activation", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 280, no. 14, 1 January 2005 (2005-01-01), pages 13793-13800, XP055057298, ISSN: 0021-9258, DOI: 10.1074/jbc.M413690200 cited in the application the whole document, in particular abstract; page 13796, section "mechanism of inhibition of cdk2 ..."; table 1, compound C4-Tat	8

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KURZAWA L ET AL: "Fluorescent peptide biosensor for probing the relative abundance of cyclin-dependent kinases in living cells", PLOS ONE, PUBLIC LIBRARY OF SCIENCE, US, vol. 6, 1 January 2011 (2011-01-01), pages e26555,1-11, XP009168416, ISSN: 1932-6203, DOI: 10.1371/JOURNAL.PONE.0026555 [retrieved on 2011-10-18] the whole document, in particular abstract; page 2, section "design and in vitro ..."; page 3f, section "cellular internalization and ..."; page 4f, section "CDKSENS resports on the ..."; page 5f, section "CDKSENS reports differences ..."; figures 1 and 3-6; tables 2 and 3	8,12,13
A	HEITZ FREDERIC ET AL: "Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics", BRITISH JOURNAL OF PHARMACOLOGY, NATURE PUBLISHING GROUP, BASINGSTOKE, HANTS; GB, vol. 157, no. 2, 1 May 2009 (2009-05-01), pages 195-206, XP008118202, ISSN: 0007-1188, DOI: 10.1111/J.1476-5381.2009.00057.X [retrieved on 2009-03-20] cited in the application the whole document	1-15
A	NHU NGOC VAN THI ET AL: "Fluorescent sensors of protein kinases: from basics to biomedical applications", PROGRESS IN NUCLEIC ACID RESEARCH AND MOLECULAR BIOLOGY, ACADEMIC PRESS, US, vol. 113, 13 December 2012 (2012-12-13), pages 217-274, XP009168168, ISSN: 0079-6603, DOI: 10.1016/B978-0-12-386932-6.00006-5 [retrieved on 2013-03-20] the whole document, in particular section 3.6	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/055633

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007108749 A1	27-09-2007	AT 495197 T	15-01-2011
		EP 1991586 A1	19-11-2008
		US 2010279918 A1	04-11-2010
		WO 2007108749 A1	27-09-2007
US 2006035815 A1	16-02-2006	CN 101263230 A	10-09-2008
		US 2006035815 A1	16-02-2006
WO 02061105 A2	08-08-2002	CA 2436281 A1	08-08-2002
		EP 1379672 A2	14-01-2004
		JP 2004532005 A	21-10-2004
		JP 2009060891 A	26-03-2009
		US 2003077826 A1	24-04-2003
		US 2004265300 A1	30-12-2004
		WO 02061105 A2	08-08-2002