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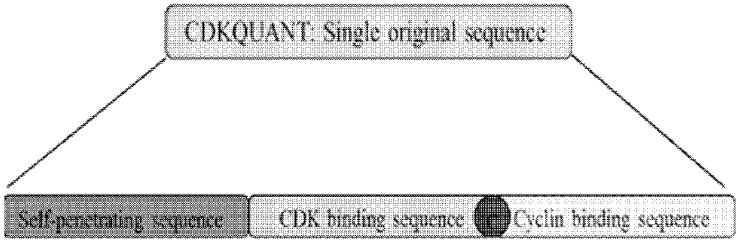

FIGURE 1A

FIGURE 1B

(57) Abstract: The present invention relates to compounds comprising a polypeptide and a fluorophore, said said compounds being able to penetrate directly into living cells and to bind to CDK/Cyclin complexes. Compounds according to the invention are therefore able to probe the relative abundance of these protein complexes. The present invention also relates to a method for determining the presence of CDK/Cyclin complexes in cell samples through a ratiometric quantitation strategy using compounds according to the invention. The invention finally relates to the use of said compounds and/or of compositions comprising said compounds for in vitro detecting the presence and/or the level of expression of CDK/Cyclin complexes, for high throughput and high content screening of libraries of compounds and for medical imaging.

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SELF-CELL PENETRATING FLUORESCENT PEPTIDE BIOSENSORS TO PROBE AND QUANTIFY CDK/CYCLIN KINASES

The present invention relates to compounds comprising a polypeptide and a fluorophore, said compounds being able to penetrate directly into living cells and to bind to CDK/Cyclin complexes. Compounds according to the invention are therefore able to probe the relative abundance of these protein complexes. The present invention also relates to a method for determining the presence of CDK/Cyclin complexes in cell samples through a ratiometric quantitation strategy using compounds according to the invention. The invention finally relates to the use of said compounds and/or of compositions comprising said compounds for in vitro detecting the presence and/or the level of expression of CDK/Cyclin complexes, for high throughput and high content screening of libraries of compounds and for medical imaging.

Cell cycle progression is driven by a family of serine/threonine protein kinases named Cyclin-Dependent Kinases (CDK), whose sequential activities promote phosphorylation of key substrates involved in cell growth and division (Malumbres *et al.*, 2005; Obaya *et al.*, 2002; Satyanarayana *et al.*, 2009; Merrick *et al.*, 2010). CDK/Cyclin complexes are formed through association of a CDK with a Cyclin partner, which plays a major role in promoting activation of the CDK by inducing significant conformational changes, in defining substrate specificity, and in targeting the heterodimeric complex to well-defined subcellular locations (Jeffrey *et al.*, 1995; Morgan *et al.*, 1997; Morris *et al.,* 2002; Lolli *et al.,* 2010). The kinase activity of CDK is primarily conditioned by formation of the CDK/Cyclin complex, and thus expression of either counterpart. This heterodimeric complex is then further regulated by several phosphorylations on the CDK that either inhibit or promote its complete activation (Morgan *et al.,* 1997). Additional regulatory proteins are known to regulate CDK and/or CDK/Cyclin complex

activity, such as the INK4 family and the Cip/Kip family of CKI (Cyclin-dependant Kinase Inhibitors).

CDK activities are frequently altered in human cancers, and contribute to sustain abnormal proliferation in cancer cells (Lapenna & Giordano, 2009; Malumbres & Barbacid, 2009). More particularly, aberrant CDK activities have been reported in a wide range of cancers including breast, ovarian, prostate, colorectal and lung cancer, lymphoma, myeloma and sarcoma (Harwell *et al.*, 2004; Ekberg *et al.*, 2005; Husdal *et al.*, 2006; Suzuki *et al.*, 2007; Kim *et al.,* 2009). CDK and/or CDK/Cyclin complex aberrant activity may result from many different causes, such as gene amplification, protein overexpression, mislocalization, expression of truncated variants, or posttranslational modifications affecting either Cyclins, CDK or regulatory proteins such as the INK4 family and the Cip/Kip family of CKI (Stivala *et al.*, 2012; Nozoe *et al.*, 2006). For example, a subset of mutations of CDK4 and CDK6 are known to confer a selective growth advantage through loss of natural inhibitor (CKI) binding, whilst other mutations have been reported to promote CDK1, CDK2 or CDK4 overexpression (Malumbres *et al.,* 2001; Malumbres *et al.,* 2007). Because of this diversity of causes, and despite its oncological relevance, there are no direct means of assessing the relative abundance of CDK/Cyclin complexes, particularly in real-time and/or in living cells.

Indeed, the measure of the presence and/or of the level of expression of CDKs and cyclins remains essentially limited to antigenic approaches. Antibodies can be used to probe individual CDKs or cyclins by Western blotting of cell lysates, or by indirect immunofluorescence of fixed cells, but do not provide a readout of CDK/Cyclin complex levels. Moreover these approaches do no not allow for real-time analysis of CDK/Cyclin complexes, or for studies relative to dynamic changes in their concentration, expression or stability. They require cell lysis or cell fixation and extraction and are not reversible.

Diagnosis approaches for detecting alterations in the concentrations of these intracellular targets in a standardized fashion have not been developed. Hence,

there is currently no means of quantifying the levels of these kinase complexes directly in living cells, and in vivo, within tissues and tumors, which stems from a lack of reliable and sensitive tools for their direct visualization, together with the lack of efficient and non-invasive approaches for their intracellular delivery. Today, there is an urgent need for innovative sensing yet non-invasive technologies, which allow for sensitive, rapid and non-invasive detection of biomarkers in situ.

Together with the development of molecular imaging technologies, fluorescent biosensors constitute powerful and sensitive tools to detect and monitor activity of intracellular targets. Such bioprobes or biosensors that can detect and report on specific molecular anomalies in malignant cells are of major interest for development of diagnostic strategies and associated therapeutic strategies. Moreover, the development of biosensor technologies to probe intracellular biomarkers in a non-invasive yet sensitive and direct fashion remains a major challenge for medical imaging diagnostics and therapeutics.

Kurzawa *et al.* (2011) describe a fluorescent peptide biosensor for probing the relative abundance of cyclin-dependent kinases in living cells, wherein said 29-mer biosensor was introduced into living cells through non-covalent complexation with the cell-penetrating carrier CADY2. Shirong *et al.* (2007) describe a fusion peptide PEP-1-P27mt, wherein PEP-1 is a 21 amino acids peptide and p27 is a mutated anti-oncogene. Duk-Soo *et al.* (2012) describe a PEP1-p18 fusion peptide, wherein p18 is a tumor suppressor protein. These documents do not describe a compound comprising a peptide and a fluorophore covalently coupled to said peptide, wherein said peptide comprises a cell-penetrating motif, a CDK binding motif and a cyclin binding motif,

With the aim of proposing new strategies for detection and quantification of intracellular cancer biomarkers, the inventors have developed the family of self-cell-penetrating compounds, named “CDKQUANT biosensor” or “CDKQUANT”, which can report on the relative abundance of CDK/Cyclin complexes in vitro, in living cells and in vivo with high sensitivity and specificity, in a non-invasive

fashion, by fluorescence imaging or by FACS. CDKQUANT biosensors can further be applied to high content cell-based automated screening assays. CDKQUANT technology provides information on the overall status of CDK/Cyclin complexes which cannot be obtained through antigenic detection of individual subunits. Furthermore this technology does not call for cell fixation or extraction procedures.

The inventors have therefore designed a compound comprising a peptide and a fluorophore whose fluorescence increases in a sensitive fashion upon recognition of the peptide by CDK/Cyclin complexes, in a sensitive and reversible fashion. The sensitivity of detection and specificity of these biosensors for CDK/cyclin targets is directly related to their sequence and to the position onto which the fluorescent probe is coupled or incorporated. The compound can be used to assess the presence of CDK/Cyclin complexes through fluorescence imaging. Additionally, the inventors have designed the compound so that it may be used in living cells or tissues, or in vivo on mouse models. The inventors have set up methods that allow for the detection of subtle differences in CDK/Cyclin complex levels between different cell lines in a standardized, sensitive and quantitative, yet non-destructive fashion. Those compounds and methods afford direct readout and real-time monitoring of CDK/Cyclin complex levels in extracts, in living cells and in tumour xenografts in animal models, thus providing tools to identify cells or tissues in which CDK/Cyclin complexes are overexpressed, for cancer diagnostics, for monitoring response to therapeutics, and for cell-based drug discovery strategies. The advantages of this invention are numerous: (1) the peptide nature of the biosensor makes it easy to synthesize and handle (2) the presence of a unique cysteine in the sequence allows for straightforward labeling with any fluorescent probe (3) the efficiency and specificity of target recognition (4) its applicability in vitro, in cellulo and in vivo, thanks to its self-cell-penetrating properties.

The compound of the invention is based on the strong fluorescence enhancement exhibited by fluorophores, particularly environmentally-sensitive dyes when their exposure to their immediate environment is modified. Upon

recognition by CDK/Cyclin complexes, there is a modification in the immediate environment of the fluorophore of the compound of the invention. The emitted fluorescence of the compound thus varies with its interaction with CDK/Cyclin complexes, and particularly increases substantially when it is recognized by CDK/Cyclin complexes. These variations are reversible, and thus afford for real-time analysis of CDK/Cyclin complex levels, for example changes in their expression or in their stability.

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 present invention first relates to a compound comprising a polypeptide and at least one fluorophore, wherein

a) said polypeptide comprises:
   - a cell-penetrating motif
   - a CDK-binding motif comprising an amino acid sequence having at least 70% identity with the amino acid sequence of a substrate of a CDK,
   - a Cyclin-binding motif being 10 to 20 amino acids long, said Cyclin-binding motif comprising at least an amino acid sequence RXL and having at least 80% identity, preferably at least 90% identity, more preferably at least 95% identity and even more preferably at least 98% identity with the sequence of the RXL motif of p107 (SEQ ID N°1), and

b) said at least one fluorophore is coupled to an amino acid from said polypeptide.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein 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 substituting amino acids, however, 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. The terms "motif", "peptide motif", "domain" and "moiety" are used to refer to parts of the peptide structure constitutive of an entity exhibiting a particular characteristic. The terms "CDKQUANT" and "CDKQUANT biosensor" used in the present description refer to a compound according to the present invention.

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

According to the invention, the cell penetrating motif, the CDK-binding motif and the Cyclin-binding motif are associated through covalent bonds. In a polypeptide according to the invention, the cell penetrating motif, the CDK-binding motif and the Cyclin-binding motif may be present in any order respectively along the polypeptidic chain. However, in a preferred embodiment, the cell penetrating motif is located at the N-terminal extremity of a polypeptide according to the invention. In a more preferred embodiment, a polypeptide according to the invention comprises, from its N-terminal extremity to its C-terminal extremity, a cell penetrating motif, a CDK-binding motif and a Cyclin-binding motif.

The terms "Cell penetrating motif", "Self cell penetrating motif", "cell-permeable peptide" (CPP), "protein-transduction domain" (PTD), "membrane-translocation sequences" (MTS), are equivalent and refer to a short polycationic or amphiphilic peptide, for example comprising 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*. As used herein, the term "molecular cargo" refers to a molecule or a macromolecule which can be natural or synthetic, organic or inorganic, and which is chosen in the group consisting of: peptides, lipids, glucids, nucleic acids and macromolecules comprising thereof. Cell penetrating peptides are described thoroughly in Grdisa *et al.*, (2011), Matjaz *et al.*, (2005), Morris *et al.* (2008), Fonseca *et al.* (2009), Heitz *et al.* (2009). Methods for testing the ability of a peptide to facilitate the intracellular uptake of a molecular cargo to which it is associated are described in Kurzawa et al. (2010). These methods include the overlaying of said peptide and its molecular cargo onto cells cultured to subconfluency, for example for 1 hour at 37°C. A person skilled in the

art may easily adapt said method to assess the delivering capacities of a cell-penetrating peptide motif of a compound according to the present invention.

Preferably, the cell-penetrating peptide motif of a compound according to the invention is capable of facilitating the 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 the cellular uptake of a molecular cargo of up to 500 kDa.

As used herein, the "CDK-binding motif" is a motif capable of binding to a CDK. In a polypeptide of the invention, a CDK-binding motif comprises an amino acid sequence derived from the sequence of any protein capable of binding specifically to at least one CDK. The amino acid sequence of a CDK-binding motif is possibly, but not exclusively, an amino acid sequence derived from the sequence of substrate of at least one CDK. At the time of filing of the present patent application, the amino acid sequences identified for substrates for at least one CDK are for example described in Chi *et al.* (2008); Blethrow *et al.,* (2008); Holt *et al.*, (2009); Brown *et al.*, (1999); Holmes & Solomon, (1996); Dhavan R. *et al.*, (2001); Pan *et al.,* (1998).

The selection of a CDK-binding motif according to the invention can be performed by any method known by the person skilled in the art. This can be done for example by incubating *in vitro* a labeled CDK with said polypeptide, then by detecting the formation of the CDK/polypeptide complex. The affinity of a polypeptide for a protein such as a CDK can be determined either by direct or indirect in vitro detection methods. Several reporter systems can possibly be used, including for example colorimetric, radioactive or fluorometric detection. The skilled person may decide to select a substrate motif which is capable of binding to at least one CDK, to several CDKs, or to only one CDK.

By "specifically binding", "specifically binds", "binding motif" "recognizing" and the like, it is intended herein that, the peptide according to the invention forms a complex that is relatively stable under physiological conditions,

with a CDK, via its CDK substrate motif, and with a cyclin, via its cyclin-binding motif. 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 a 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 a cyclin can be characterized by a K_D comprised between about 100 nM and about 500 nM, and preferably of about 150 nM.

The specific binding of a compound according to the invention for a CDK/cyclin complex can be characterized by a K_D comprised between about 0,2 nM and 50 nM, more preferably between about 1 nM and 10 nM, and even more preferably of about 5 nM.

A compound according to the present invention comprises a CDK-binding motif comprising an amino acid sequence having at least 70% identity with the amino acid sequence of a substrate of a CDK, more preferably at least 80% identity, even more preferably at least least about 90 % identity, more preferably at least about 95 % identity or even more preferably at least 98% identity with the amino acid sequence of a substrate of a CDK.

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 occurs in both sequences to yield the number of matched positions, dividing the

number of matched positions by the total number of positions in the window of comparison (i.e. the window size) and multiplying the result by 100 to yield the percentage of sequence identity. The percentage of sequence identity of an amino acid sequence can also be calculated using BLAST software with the default or user defined parameter. As used herein, a "derivative" or "sequence derived from" refers to an amino acid sequence having at least 70 % identity with the reference amino acid sequence. The term "substantial identity" means that two peptide sequences, when optimally aligned, share at least about 80 % sequence identity, preferably at least about 90 % sequence identity, more preferably at least about 95 % sequence identity or even more preferably at least 98% identity with the reference amino acid sequence.

As used herein, the "Cyclin-binding motif" is a motif capable of binding to a cyclin. In a polypeptide of the invention, a Cyclin-binding motif comprises at least an amino acid sequence RXL and has at least 80% identity and more preferably 100% identity with the RXL motif of p107 (SEQ ID N°1).

A compound according to the invention comprises a polypeptide and at least one fluorophore, said at least one fluorophore being coupled to an amino acid of the polypeptide. The coupling site of said at least one fluorophore is defined within the amino acid sequence of said polypeptide. The coupling site of said at least one fluorophore is chosen among the amino acids of the polypeptide sequence with the exception of the amino acids of the cell penetrating motif. In a particular embodiment, said at least fluorophore binds to an amino acid chosen among the amino acids of the CDK-binding motif and the amino acids of the Cyclin-binding motif. In a preferred embodiment of the invention, said at least one fluorophore is coupled to an amino acid of the CDK-binding motif. In another embodiment, said at least one fluorophore is coupled to an amino acid of the cyclin-binding motif.

In a particular embodiment, the polypeptide of a compound of the present invention comprises a cell-penetrating motif comprising at least an amino acid sequence having the sequence WW/FXXWW/F (SEQ ID N°2). 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 of the peptide to cross the cellular membrane. In a preferred embodiment, said peptide may be detected within the cytoplasm. From these observations, the inventors have defined the consensus sequence: WW/FXXWW/F (SEQ ID N°2), wherein the amino acid residues in position 2 and in position 6 are independently chosen among Tryptophan (W) and Phenylalanine (F), and amino acid residues in position 3 and in position 4 can be any amino acid residue. Accordingly, said amino acid sequences belong to one of the following sequences: the sequence WWXXWW (SEQ ID N°3), the sequence WFXXWW (SEQ ID N°4), the sequence WWXXWF (SEQ ID N°5) and the sequence WFXXWF (SEQ ID N°6).

In a particular embodiment, the cell penetrating motif comprises an amino acid sequence having at least 70% identity, preferably at least 80% identity, preferably at least 90% identity, more preferably at least 95% identity and even more preferably at least 98% identity with the sequence KETWWETWWTEKK (SEQ ID N°7). In a more preferred embodiment, a polypeptide according to the invention comprises a cell penetrating motif comprising the following amino-acid sequence: KETWWETWWTEKK (SEQ ID N°7). In an even more preferred embodiment, the cell penetrating motif of a polypeptide according to the invention has the following amino-acid sequence: KETWWETWWTEKK (SEQ ID N°7).

In an embodiment of the invention, the CDK-binding motif is capable of binding to at least one CDK chosen in the list consisting of: CDK1, CDK2, CDK3, CDK4, CDK5, CDK6, CDK7, CDK8, CDK9 and CDK10. The substrate specificity of a CDK is regulated in part by its cyclin counterpart, and it is known by the person skilled in the art that different CDK/Cyclin complexes comprising the same

CDK may have different ligands or substrates. The skilled person may decide, for example to provide more specificity to the compound of the invention, to select a CDK-binding motif which is capable of binding to at least one CDK wherein said CDK is complexed with a defined cyclin. In a particular embodiment, the CDK-binding motif is capable of binding to at least one CDK/Cyclin complex. In a preferred embodiment, the CDK-binding motif is capable of binding to at least one of the human CDK/Cyclin complex chosen in the list consisting of: Cyclin A - CDK1, Cyclin B - CDK1, Cyclin A – CDK2, Cyclin E - CDK2, Cyclin C - CDK3, Cyclin D1 - CDK4, Cyclin D2 - CDK4, Cyclin D3 - CDK4,p25– CDK5, Cyclin D1 - CDK6, Cyclin D2 – CDK6, Cyclin D3 – CDK6, Cyclin H - CDK7, Cyclin C – CDK8, Cyclin T1 - CDK9, Cyclin T2a - CDK9, Cyclin T2b - CDK9 and Cyclin K - CDK9. The skilled person may decide to choose specifically a substrate motif which is capable of binding to only one CDK/Cyclin complex. In a particular embodiment, the CDK-binding motif is capable of binding to only one of the CDK/Cyclin complex chosen in the list consisting of: Cyclin A - CDK1, Cyclin B - CDK1, Cyclin A – CDK2, Cyclin E - CDK2, Cyclin C - CDK3, Cyclin D1 - CDK4, Cyclin D2 - CDK4, Cyclin D3 - CDK4,p25– CDK5, Cyclin D1 - CDK6, Cyclin D2 – CDK6, Cyclin D3 – CDK6, Cyclin H - CDK7, Cyclin C – CDK8, Cyclin T1 - CDK9, Cyclin T2a - CDK9, Cyclin T2b - CDK9 and Cyclin K - CDK9.

In an embodiment, the amino acid sequence substrate motif according to the invention has a sequence derived from the peptide sequence of at least one the protein chosen from: histone H1, CDC6, Cyclin B1, lamin B2, Rb (Retinoblastoma protein) and Tau. In a more particular embodiment, the CDK-binding motif of a polypeptide according to the invention comprises an amino acid sequence having at least 80% identity, preferably at least 90% identity, more preferably at least 95% identity and even more preferably at least 98% identity with a sequence chosen in the group consisting of: the sequence SEQ ID N°8 (HHAGPRK from CDC6), the sequence SEQ ID N°9 (PEPILVDCSSPSPMET from S126S128 of cyclinB1), the sequence SEQ ID N°10 (RAGGPATCSSPTRL from S17 of LB2), the sequence

SEQ ID N°11 (YKFCSSPLRIPG from S795 of Rb), the sequence SEQ ID N°12 (SGYSSPGSCSTPGSR from S202T205 of Tau) and the sequence SEQ ID N°13 (GGCSTPKKAKKL from Histone H1).

According to the invention, the CDK-binding motif has a sequence that comprises at least 5 amino-acid residues. In an embodiment, the CDK-binding motif of a peptide of the invention has a sequence comprising less than 100 amino-acid residues, preferably less than 50 amino-acid residues, even more preferably less than 25 amino-acid residues.

In another embodiment, the present invention relates to a compound wherein said Cyclin-binding motif comprises an amino acid sequence having the sequence SEQ ID N°1 (RXL motif of p107). In a more preferred embodiment, the present invention relates to a compound wherein said Cyclin-binding motif has the amino acid sequence SEQ ID N°1 (RXL motif of p107).

In a more particular embodiment, the present invention relates to a compound wherein said polypeptide comprises an amino acid sequence identical to a sequence chosen among the group consisting of: the sequence SEQ ID N°14 (CDKQUANT1), the sequence SEQ ID N°15 (CDKQUANT2), the sequence SEQ ID N°16 (CDKQUANT3), the sequence SEQ ID N°17 (CDKQUANT4), the sequence SEQ ID N°18 (CDKQUANT5). These polypeptides are described in table 2.

Name	Amino acid sequence	Identification
RXL motif of p107	RRLFGED	SEQ ID N°1
Consensus cell-penetrating peptide motif	WW/FXXWW/F	SEQ ID N°2
Cell-penetrating peptide motif	WWXXWW WFXXWW WWXXWF WFXXWF	SEQ ID N°3 SEQ ID N°4 SEQ ID N°5 SEQ ID N°6
PEP1 cell-penetrating peptide motif	KETWWETWWTEKK	SEQ ID N°7
CDK-binding motif of CDC6	HHAGPRK	SEQ ID N°8
CDK-binding motif S126S128 of cyclinB1 (human)	PEPILVDCSSPSPMET	SEQ ID N°9

CDK-binding motif S17 of LB2 (human)	RAGGPATCSSPTRL	SEQ ID N°10
CDK-binding motif S795of Rb (human)	YKFCSSPLRIPG	SEQ ID N°11
CDK-binding motif S202T205 of Tau (human)	SGYSSPGSCSTPGSR	SEQ ID N°12
CDK-binding motif of Histone H1	GGCSTPKKAKKL	SEQ ID N°13
CDKQUANT1 (42 mer derived from CDC6, binds to all CDKs)	**KETWWETWWTEKK**HHAGPRKRVHERYCGPTAGSAKRRLFGED	SEQ ID N°14
CDKQUANT2 (45 mer derived from S126S128 cyclin B1, binds to CDK1)	**KETWWETWWTEKK**PEPILVDTASPSPMETCGPTAGSAKRRLFGED	SEQ ID N°15
CDKQUANT3 (43 mer 4862 derived from S17 of LB2, binds to CDK2)	**KETWWETWWTEKK**RAGGPATPLSPTRLCGPTAGSAKRRLFGED	SEQ ID N°16
CDKQUANT4 (41 mer 4830 derived from S795Rb, binds to CDK4)	**KETWWETWWTEKK**YKFPSSPLRIPGCGPTAGSAKRRLFGED	SEQ ID N°17
CDKQUANT5 (44 mer 4861 derived from S202T205 of tau, binds to CDK5)	**KETWWETWWTEKK**SGYSSPGSPGTPGSRCGPTAGSAKRRLFGED	SEQ ID N°18
CTRL: Control peptide (no substrate)	**KETWWETWWTEKK**VESSDTIDNVKSKIQDKEGC	SEQ ID N°19
CDKSENS1 (29 mer derived from CDC6)	HHAGPRKRVHERYCGPTAGSAKRRLFGED	SEQ ID N°20
CADY2	GLWWRLWWRLRSWFRLWFRV	SEQ ID N°21
CTRL2: Control peptide (no cell penetrating motif, no substrate)	VESSDTIDNVKSKIQDKEGC	SEQ ID N°22

Table 2

According to the invention, the fluorophore is an environmentally-sensitive dye. 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. Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds. The inventors have found that the change in fluorescence emission upon binding of the peptide to a CDK/Cyclin complex is more easily monitored and allows for a more accurate and sensitive

measure of said binding when using fluorophores that are environment-sensitive dyes 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).

Environment-sensitive dyes are well known by the skilled person and may include for example any dye that contains an electron-donating and an electron-accepting group at opposite ends of the aromatic system. According to the invention, the environment-sensitive dye is for exemple, without restriction to those examples, Cascade Yellow, prodan, Dansyl, Dapoxyl sulfonic acid, NBD, PyMPO, Pyrene, diethylaminocoumarin, SYPRO Orange dye, SYPRO Red dye, nile red, CPM (7-Diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin), DCDHF (2,7-Dichlorodihydrofluorescein diacetate), fluorophore from the BODIPY family of dyes (boron-dipyrromethene family of dyes).

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. In a preferred embodiment, the fluorophore is coupled to a thiol group of an amino-acid residue. In a more prefered

embodiment, the fluorophore is coupled to a thiol group of a cysteine residue. Coupling the fluorophore to an amino acid functional group is a technique well known to the skilled person, and may involve chemical reactions such as, for example, amine coupling of lysine amino acid residues (typically through amine-reactive) or sulfhydryl coupling of cysteine residues (via a sulfhydryl-reactive or photochemically initiated free radical reactions).

In a more particular embodiment, the invention relates to a compound wherein said unique residue within the amino acid sequence of the peptide is a cysteine.

In a particular embodiment, the present invention relates to a compound comprising a polypeptide and at least one fluorophore coupled to an amino acid of said polypeptide, wherein the compound comprises two fluorophores, with each of the fluorophores being coupled to an amino acid residue of said peptide.

In particular embodiment, the present invention relates to a compound wherein said peptide comprises an amino acid sequence identical to the sequence SEQ ID N°14 (CDKQUANT1), the fluorophore is Cy3 and is coupled to the unique cysteine residue of the polypeptide.

In an even more particular embodiment, the present invention relates to a compound wherein said polypeptide has an amino acid sequence identical to the sequence SEQ ID N°14 (CDKQUANT1), the fluorophore is Cy3 and is coupled to the cysteine residue of the polypeptide.

In another particular embodiment, the invention relates to a compound wherein said polypeptide further comprises a motif defined as a “peptide-Tag” or a “protein-Tag” (or tag). A protein 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 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 polypeptide according to the invention may comprise an "mRFP sequence", which provides an intramolecular fluorophore which is insensitive to environmental changes. The mRFP or any similar autofluorescent protein serves as a scaffold for presentation of the biosensor as well as an inert intramolecular signal for standardized ratiometric quantification. This is of particular interest for imaging applications and high content screening.

Preferably, the sequence of the mRFP (or an equivalent protein Tag) is devoid of any amino acid susceptible to be coupled to the fluorophore according to the invention. In a particular embodiment, mRFP is devoid of any cysteine residue and the fluorophore is coupled to a unique cysteine residue of the peptide according to the invention.

A compound of the invention may be obtained by standard methods known in the art. As an example, a polypeptide according to the invention may be prepared by chemical synthesis, such as solid-phase peptide synthesis, without being limited to this particular method.

A polypeptide in a compound according to the invention may be obtained by recombinant protein engineering. In particular, polypeptides associated with, for example, a mRFP or a GST sequence may be prepared by genetic engineering, as fusion proteins. Production of the polypeptide according to the invention may be done 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).

The 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.

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.

The invention also relates to compositions comprising said compound 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.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,

Administration to animals and/or human tissues may require specific formulations of the compound. 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, the invention relates to a method for detecting the presence of at least one CDK/Cyclin complex in a sample, said method comprising the steps of:

a) providing at least one compound according to the invention,
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 fluorescent signal of step d) with a reference fluorescent signal, and
f) determining from the comparison of step e) if at least one CDK/Cyclin complex is present in said sample.

According to the invention, contacting the compound of the invention with a CDK/Cyclin complex is performed, for example by contacting the compound of the invention directly with a sample. As used herein, a "sample" might be a recombinant form of CDK/Cyclin or solutions, extracts, particularly cell extracts, living cells, preferably living cells in an *in vitro* culture, animal tissues, preferably animal tissues in an *in vitro* culture, in mouse models with tumour xenografts, or any type of sample containing a CDK/Cyclin complex whose presence is to be determined. Within cellular extracts, cells may be in suspension, or adhering, for

example to a plate. Also, cells from the sample might be living or fixed on a support.

The skilled person will easily adapt the intensity and wavelength of the excitation light of step c), for example depending on the fluorophore of the compound according to the invention of step a). In an embodiment, the illumination of step c) is performed at a wavelength corresponding to the excitation wavelength of the fluorophore of the compound according to the invention of step a). Various light sources may be used to provide for the excitation light, including lasers, photodiodes, and lamps, preferably xenon arcs lamps and mercury-vapor lamps.

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). Said devices are, for example, a fluorimeter, a fluorescence-activated cell sorter (FACS) (for cells in suspension), or a microscope (for adhering cells).

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. In an embodiment, determining the fluorescence emitted is achieved by measuring the wavelength spectrum of the emitted fluorescence. In another embodiment, determining the fluorescence emitted is achieved by measuring the intensity of the emitted fluorescence at a given wavelength (generally its maximum).

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. In an embodiment, comparing the fluorescence in step e) means comparing the wavelength spectrum of the emitted fluorescence of step d) and the wavelength spectrum of the fluorescence reference. In another embodiment, comparing the fluorescence in step e) means comparing the intensity of the emitted

fluorescence of step d) to the intensity of the fluorescence reference at a chosen wavelength.

According to the invention, the reference fluorescence is a predetermined measurement of fluorescence, obtained from a biological sample with a known CDK/Cyclin complex present. In an embodiment, the reference fluorescence is a predetermined measure of fluorescence obtained from a reference biological sample wherein said CDK/Cyclin complex is known to be present and this presence has been quantified. This presence may be determined by conventional techniques known by the skilled person.

The 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, a CDK/Cyclin complex.

In another aspect, the invention relates to a method for detecting the presence of at least one CDK/Cyclin complex in an organ.

In another embodiment, the present invention relates to a method for determining the relative quantity of at least one CDK/Cyclin complex in at least two different samples, comprising the steps of:

a) providing at least one compound according to the invention,
b) contacting said compound with one of said samples,
c) illuminating said compound and said sample with an excitation light,
d) determining the fluorescent signal emitted by said compound,
e) comparing said fluorescent signal with a reference fluorescent signal,
f) performing steps b), c), d) and e) with at least another of said samples,
g) comparing said fluorescent signals obtained in steps e) and f) to determine the relative quantity of said at least one CDK/Cyclin complex in said samples.

By determining the "relative quantity", the "level", the "amount", the "level of expression" or the "concentration" of a CDK/Cyclin complex, it is meant that the intensity of the fluorescent signal is compared between at least two samples, and

the relative difference between the fluorescent signals emitted is expressed as a percentage.

In a particular embodiment, the invention relates to a method for detecting the level of expression of a CDK/Cyclin complex in living or fixed cells.

In another particular embodiment, the invention further relates to a method for monitoring the response to a treatment intended to affect the level of a CDK/Cyclin complex in a cell. Said method comprises the comparison of the fluorescent signal detected in at least two different samples taken from a subject, with the first sample being taken before the treatment and the following samples being taken during and/or after the treatment. Said method comprises the following steps:

a) providing at least one compound according to the invention,
b) contacting said compound with one of said samples,
c) illuminating said compound and said sample with an excitation light,
d) determining the fluorescent signal emitted by said compound,
e) comparing said fluorescent signal with a reference fluorescent signal,
f) performing steps b), c), d) and e) with at least another of said at least two different samples,
g) comparing said fluorescent signals obtained in steps e) and f) to monitor the response to said treatment.

The invention also relates to methods for screening a plurality of products for their ability to modulate the quantity of at least one CDK/Cyclin complex in a sample, said method comprising the following steps:

a) providing at least one compound according to the invention ,
b) contacting said sample with at least one of said products,
c) contacting said compound with said sample,
d) illuminating said compound and said sample with an excitation light,
e) determining the fluorescence signal emitted by said compound,
f) performing steps b), c), d) and e) with at least another product,

g) comparing said fluorescence signals with a reference fluorescence signal, and

h) determining from the comparison of step g) if a product is able to affect the level of at least one CDK/Cyclin complex.

According to the invention, a product able to modulate the quantity of at least one CDK/Cyclin complex is a CDK/Cyclin modulator able affect the level of expression of said proteins, more precisely, it can be an activator or an inhibitor of the level of at least one CDK/Cyclin complex. Preferably, the modulator is an inhibitor of the level of at least one CDK/Cyclin complex.

Thus, a compound according to the invention may be useful for example for screening, particularly high content high throughput screening.

In another embodiment, the present invention also relates to a method for the in vitro diagnostic of the altered expression, and preferably the overexpression of at least one CDK/Cyclin complex in a subject, comprising the steps of:

a) providing at least one compound according to the invention ,

b) contacting said compound with a biological sample from said subject,

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

d) determining the fluorescent signal emitted by said compound,

e) comparing said fluorescent signal with a reference fluorescent signal, and

f) determining from the comparison of step e) if at least one CDK/Cyclin complex is overexpressed.

As used herein, the term “CDK/Cyclin complex overexpression” means that at least one CDK/Cyclin complex is overexpressed, e.g. is present in an above normal quantity. According to the invention, the above normal quantity is determined by comparison of the test value with a reference value. The reference value according to the invention is for example a value obtained by the present method with a biological sample wherein the CDK/Cyclin complex quantity is

normal, such as for example non transformed cell lines, for example normal diploid fibroblast, preferably the HS68 cell line (ATCC code HTB-138).

The terms "individual", "subject", "host" and “subject" 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.

According to the invention, the reference fluorescence is a predetermined measure of fluorescence, obtained from a biological sample where the CDK/Cyclin complex of interest is known to be normally present. In an embodiment, the reference fluorescence is a predetermined measure of fluorescence obtained from a reference biological sample from the subject according to the invention, wherein the reference biological sample is known to have CDK/Cyclin complex of interest normally present. Preferably, the reference fluorescence is a predetermined measure

of fluorescence obtained from a biological sample from a subject known to have CDK/Cyclin complex of interest normally present.

In another general embodiment, the present invention relates to a method for in vitro diagnosis of a disease characterized by the overexpression of at least one CDK/Cyclin complex in a subject, comprising the steps of:

a) providing at least one compound according to the invention,
b) contacting said compound with a biological sample from said subject,
c) illuminating said biological sample and compound with an excitation light,
d) determining the fluorescent signal emitted by said compound,
e) comparing said fluorescent signal with a reference fluorescent signal, and
f) determining from the comparison of step e) if said subject is affected by a disease characterized by the overexpression of at least one CDK/Cyclin complex.

The disregulation of Cyclin-dependent kinase level or activation is suspected to contribute to the observed sustained aberrant proliferation of cancer cells, and as such is considered a hallmark of several diseases. Indeed, the levels of either Cyclin or of CDKs are frequently altered in human cancers. CDK and/or cyclin overexpression has been reported in a wide range of cancers including breast, ovarian, prostate, colorectal, and lung cancers, as well as lymphoma, myeloma, sarcoma, and glioblastoma.

The invention thus discloses a method for the *in vitro* diagnostic of cancer in a subject, comprising the steps of:

a) providing at least one compound according to the invention ,
b) contacting said compound with a biological sample from said subject,
c) illuminating said biological sample and compound with an excitation light,
d) determining the fluorescent signal emitted by said compound,

e) comparing said fluorescent signal with a reference fluorescent signal, and

f) diagnosing a cancer in said subject from the comparison of step e) if the fluorescence of step d) is above the fluorescent signal reference.

As used herein, "cancer" refers to primary or metastatic cancers, leukemia, or lymphomas, colon cancer, liver cancer, testicular cancer, thymus cancer, breast cancer, skin cancer, esophageal cancer, pancreatic cancer, prostatic cancer, uterine cancer, cervical cancer, lung cancer, bladder cancer, ovarian cancer, multiple myeloma, melanoma and glioblastoma. Preferably, the cancer according to the invention is a CDK/Cyclin complex-associated cancer. By "CDK/Cyclin complex associated cancer" it is herein referred to cancers associated with an altered expression, and preferably an overexpression of at least one CDK/Cyclin complex.

In an embodiment, the reference fluorescent signal, or fluorescence threshold reference, is a predetermined measure of fluorescence, obtained from one or several biological sample from subjects known to have cancer. In a particular embodiment, the fluorescence threshold reference is a statistically relevant data obtained from predetermined measures of fluorescence, obtained several biological samples from subjects known to have cancer.

The invention also discloses a method for the *in vitro* monitoring of the efficacy of a therapeutic treatment of a disease characterized by at least one CDK/Cyclin complex hyperactivation in a subject, comprising the steps of:

a) providing at least one compound according to the invention,

b) contacting said compound with a first biological sample from said subject,

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

d) determining the fluorescent signal emitted by said compound,

e) comparing said fluorescent signal with a reference fluorescent signal,

f) performing steps c), d) and e) on at least a second sample, said second sample being taken later than said first sample,

g) comparing said fluorescent signal of step e) and step f), and

h) monitoring, from the comparison of step g), the efficacy of said therapeutic treatment.

In a particular embodiment, the invention also discloses a method for evaluating *in vitro* the therapeutic efficiency of a cancer treatment for a subject. In another particular embodiment, the invention relates to a method for monitoring a therapeutic response in mouse tumor models. In another particular embodiment, the invention relates to a method for developing a diagnostic assay for blood pathologies, comprising the steps of:

a) providing at least one compound according to the invention,

b) contacting said compound with one of said cell samples,

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

d) determining the fluorescent signal emitted by said compound,

e) comparing said fluorescent signal with a reference fluorescent signal,

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

g) comparing said fluorescent signals obtained in step e) with the at least two different cell samples to develop a diagnostic assay for blood pathologies.

In another particular embodiment, the invention relates to a kit comprising at least one compound according to the invention and an acceptable carrier and/or an acceptable solvent.

In another particular embodiment, the invention relates to the use of a compound according to the invention for detecting the presence of at least one CDK/Cyclin complex in a sample. In another particular embodiment, the invention relates to the use of said compound for determining the relative quantity of at least

one CDK/Cyclin complex in at least two different samples. In another particular embodiment, the invention relates to the use of said compound for screening a plurality of products for their ability to affect the quantity of at least one CDK/Cyclin complex in a sample. In another particular embodiment, the invention relates to the use of said compound for the in vitro diagnostic of a CDK/Cyclin complex alterered expression and preferably overexpression. In another particular embodiment, the invention relates to the use of said compound 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 fluorescence imaging. In a particular embodiment, the invention also relates to the use of at least one compound and/or a composition of the invention for fluorescence 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* fluorescence imaging.

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

a) administering to a subject a compound of the invention,

b) illuminating at least one part of an organ of said subject at a wavelength corresponding to the wavelength of the fluorophore of the compound of the invention, and

c) obtaining an image with an apparatus detecting the fluorescence emitted by said administered compound.

The skilled person may select the appropriate imaging apparatus depending on the fluorophore of the compound according to the invention.

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.

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

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B: Schematic representation of an example of CDKQUANT

Figure 1A schematically represents the three motifs of an example of a CDKQUANT peptide, CDKQUANT1, which has the amino acid sequence SEQ ID N°14, including a unique cysteine for coupling of fluorescent probe. The self-penetrating sequence and the cyclin-binding sequences are conserved sequences. The CDK-binding sequence is a variable sequence. Figure 1B represents the fluorescence enhancement of environmentally-sensitive probe observed upon recognition of CDK/Cyclin complexes by CDKQUANT1.

Figures 2A to 2C: Cell internalization of CDKQUANT1-Cy3 sensor.

Figure 2A: Hoechst staining of the cells. Figure 2B: CDKQUANT1-Cy3 fluorescence of cells. Figure 2C: Overlay. CDKQUANT1 labeled with Cy3 was directly applied onto cultured HeLa cells.

Figures 3A to 3I: Detection and quantification of CDK/cyclin level in different cell lines using CDKQUANT1-Cy3 sensor.

Figure 3A: Western blot of CDKs and Cyclins in HeLa cells and in HS68 cells, after normalization of cell extracts. Figures 3B to 3E: Histogram representation of the comparison of the CDKQUANT1-Cy3/CTRL-Cy5 fluorescence ratio in different healthy and cancer cell lines, namely HeLa cells and HS68 fibroblasts (respectively left and right side of Fig. 3B), HeLa cells and U2OS fibroblasts (left and right side of Fig. 3C), HeLa cells and A549 cells (left and right side of Fig. 3D), HeLa cells and MCF7 fibroblasts (left and right side of Fig. 3E). The mean ratiometric value was determined from 3-5 independent experiments in which CDKQUANT1-Cy3 and CTRLCy5 fluorescence was measured and the ratio of CDKQUANT1-Cy3/CTRLCy5 determined in 3-4 fields of cells (n= 50 – 60). Figures 3F to 3I: fluorescence of CDKQUANT1-Cy3 following internalization respectively in HeLa, U20S, A549 and MCF7 cells.

Figures 4A to 4C: Detection of alteration of a single CDK/Cyclin level using CDKQUANT1 probe

Figure 4A: Western blot of CDK1 in HT2-19 cells induced to express or not CDK1 and histogram showing differences in CDKQUANT1-Cy3/CTRL-Cy5 fluorescence ratio. HT2-19 cells were cultured with IPTG for 4 days and expressing CDK1 (left panels, IPTG), or without IPTG and not expressing CDK1 (right panels, WO). Figure 4B: Detection of CDK2 in Hela cells treated with siRNA targeting luciferase (siLuc) or with siRNA targeting CDK2 (siK2). Figure 4C: Detection of Cyclin B in HeLa cells treated with siRNA targeting luciferase (siLuc) or with siRNA targeting CyclinB (siB). Representative examples of mean CDKQUANT1-Cy3 and CTRL-Cy5 fluorescence are shown among measure on 25-30 cells in each cell type.

Figures 5A to 5F: Application of CDKQUANT to FACS analysis of CDK/cyclin levels in HeLa cells.

HeLa cells and HS68 cells were treated, incubated with CDKQUANT1-Cy5 or with the Ctrl-Cy5 and analyzed by FACS. FACS fluorescence profiles are shown together with the mean and median values of fluorescence for CDKQUANT1-Cy5 and Ctrl-Cy5 (Figures 5A to 5D). The ratio of the mean CDKQUANT1/Ctrl fluorescence and the ratio of the median CDKQUANT1/Ctrl fluorescence values were calculated for HeLa cells and for HS68 cells (Figures 5E and 5F). The relative difference between HeLa and HS68 cells is between 27 and 31%.

Figures 6A to 6D: In vivo application of CDKQUANT

Figure 6A: Schematic representation of the experiment on mice. Figure 6B: Kinetics of the biodistribution and clearance of CDKQUANT1. Figure 6C: Histogram representation of the distribution of CDKQUANT1 through different tissues and organs, respectively after intratracheal/nebullisation (left column), intravenous (central column) and intraperitoneal (right column) administration. Figure 6D: Observation of Hoechst (right panels), CDKQUANT-Cy5 (middle) and

Merged (right) coloration of tissues, including kidney (upper panels), liver (middle panels) and tumour (lower panels).

Figures 7A and 7B: Monitoring siRNA-mediated knockdown of cyclin B1 in tumour xenografts in mice.

Figure 7A: siRNA targeting cyclin B1 was injected into TS/A adenocarcinoma tumour xenografts implanted subcutaneously in mice according to the protocol described in Example 5. Figure 7B: Following the protocol of siRNA injection at day 18 (J18) after tumour implantation, CDKQUANT1-Cy5 and Ctrl-Alexa-750 were co-injected into the tumours and their respective fluorescence was imaged and quantified. The ratio of CDKQUANT1/Ctrl fluorescence is represented for the group of control/ mock treated mice, and for the group of mice treated with siRNA targeting cyclin B (5 mice in each group).

Figures 8A to 8D: Application of CDKQUANT to high content / high throughput screening.

Figure 8A: schematic representation of CDKQUANT1 expressed as a fusion protein with mRFP so that an intramolecular fluorescence ratio could be used for quantification of CDK/Cyclin levels. Figure 8B: Applied RFP-CDKQUANT1-Cy5 onto Hela Cells. 8C: Example of multiparametric high content screen of a small chemical compound library to identify compounds that affect CDK/Cyclin levels. Three parameters can be monitored simultaneously: cytotoxicity, CDKQUANT1 fluorescence and effect on cell cycle progression. Figure 8D: Example of the fluorescence distribution profiles of CDKQUANT1-Cy5 fluorescence/RFP from a field of 2000 HeLa cells either mock treated or treated with 50uM roscovitine for 7h.

Figures 9A to 9D: Quantification of CDK/Cyclin levels in HeLa and HS68 cells through ratiometric quantification of CDKQUANT1-Cy3 / CTRL-Cy5 fluorescence versus CADY2 formulations of CDKSENS1-Cy3 / CTRL2-Cy5

In Figure 9A, the histograms represent the comparison of the fluorescence ratio of a biosensor according to the invention CDKQUANT1-Cy3 (CDKQUANT1: SEQ ID

N°14) /CTRL-Cy5 (CTRL: SEQ ID N°19) in HeLa (left histogram) and in HS68 fibroblasts (right histogram), after normalization of cell extracts. In Figure 9B, the histograms represent the comparison of fluorescence ratio of a non-covalent formulation of a biosensor CADY2- CDKSENS1-Cy3 (CDKSENS1: SEQ ID N°20, CADY2: SEQ ID N°28) / CTRL2-Cy5 (CTRL2: SEQ ID N°30) in HeLa (left histogram) and in HS68 fibroblasts (right histogram). Figure 9C shows a schematic representation of CDKQUANT1. Figure 9D shows a schematic representation of the non-covalent formulation of CADY2 - CDKSENS1. The mean ratiometric value was determined from 3-5 independent experiments in which CDKQUANT1-Cy3 and CTRL-Cy5 fluorescence was measured and the ratio of CDKSENS1-Cy3 / CTRL-Cy5 determined in 3-4 fields of cells (n= 50 – 60).

EXAMPLES

Example 1: Design and in vitro characterization of CDKQUANT

Self-cell-penetrating fluorescent peptide biosensors termed CDKQUANT are designed to recognize different subsets of CDK/Cyclin complexes. These peptide biosensors bear a sequence which ensures their introduction through cell membranes, a CDK-binding motif derived from a consensus peptide substrate (see Table 2, page 13) and a cyclin-binding motif derived from the RXL motif of p107. In a particular example, CDKQUANT biosensor bears a unique cysteine for coupling of an environmentally sensitive fluorescent probe between the CDK- and the cyclin-binding moieties (See Figures 1A and 1B).

Peptide synthesis, protein expression, purification and labeling:

Peptide biosensors and control peptide according to the invention were synthesized by solid-phase Fmoc strategy. GST or mRFP serve as a scaffold for presentation of the biosensor as well as a fluorescent probe for intramolecular ratiometric quantification. Since the sequence of mRFP is devoid of cysteine, 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.

CDKQUANT1 (SEQ ID N°14) and CTRL peptide (SEQ ID N°19) were labeled on their unique cysteine with fluorescein isothiocyanate (FITC), Cy3- or Cy5-maleimide, and further purified on NAP-5 columns (GE Healthcare).

Results:

The fluorescence of CDKQUANT1 increases in a sensitive fashion upon recognition of their targets thanks to enhancement of the environmentally-sensitive fluorescent probe which docks onto the CDK/Cyclin complex. The sensitivity of detection and specificity of these biosensors for CDK/Cyclin targets is directly related to their sequence and to the position onto which the fluorescent probe is incorporated.

Example 2: Self-cell-penetration of CDKQUANT1

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. HT2-19 cells, provided by Dr. A.Porter, were cultured in DMEM supplemented with non-essential amino acids, antibiotics, sodium pyruvate , glutamine, and 10% FCS and grown with 50uM isopropylthiogalactoside (IPTG) for maximal induction of CDK1 (Itzhaki et al, Nat. Genet. 1997). 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:

CDKQUANT1 labelled with Cy3 and directly applied onto HeLa enters cells promptly without requiring a carrier/vehicle. Following cell internalization, CDKQUANT1-Cy3 distributes through the cellular cytoplasm homogeneously and

rapidly, within less than one hour. CDKQUANT1 biosensor penetrates readily into cultured mammalian cells, without requiring cell fixation or permeabilization steps (Figures 2A to 2C).

Example 3: Determination of the relative abundance of CDK/cyclins in living cells, steady-state fluorescence titration experiments:

Titration experiments were performed at 25°C in a Polarstar Spectrofluorimeter (BMG Labtech). 200nM FITC-labelled 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.

siRNA Transfections:

siRNA targeting Cyclin B was a Smart PoolTM 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).

Fluorescence quantification and statistical analysis:

3uM CDKQUANT1-Cy3 and Ctrl Peptide-Cy5 were overlaid onto cells for 1hour 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:

CDKQUANT1 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. Both self-penetrating peptides (CDKQUANT1 and CTRL) were overlaid onto cells, and live-cell imaging of cells was performed to acquire CDKQUANT1-Cy3 and Ctrl-Cy5 fluorescence. Established ratiometric quantification strategy provides a means of standardizing CDKQUANT1-Cy3 fluorescence with respect to the CTRL-Cy5 peptide (Figures 3A to 3E). The fluorescence of CDKQUANT1-Cy3 following internalization respectively in HeLa, U20S, A549 and MCF7 cells was detected. Ratiometric quantification experiments were performed between normal diploid fibroblasts and HeLa cells, as well as between different cancer lines (HeLa, U2OS, MCF7, A549). 45% difference in the CDKQUANT1-Cy3/CTRL-Cy5 ratio was determined between HeLa cells and HS68 fibroblasts. Moreover CDKQUANT1 can be applied to quantify differences in a single CDK or cyclin (Figures 4A to 4C), through siRNA knockdown or through ectopic expression. These results show that the CDKQUANT biosensors according to the invention allow a detection which is sensitive, quantitative, rapid and robust.

Example 4: Application of CDKQUANT to monitor CDK/Cyclin levels by FACS analysis

FACS analysis was performed as described in Crombez et al (2009).

Results:

CDKQUANT1 technology was successfully applied to compare the relative CDK/Cyclin abundance between different cell lines by FACS. In these experiments

CDKQUANT1-Cy5 or CTRLCy3 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 CDKQUANT1-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 (Figures 5A to 5F). These results show that that the CDKQUANT1 biosensors according to the invention may be implemented by FACS and allow a detection which is sensitive, quantitative, rapid and robust.

Example 5: In vivo application of CDKQUANT1

CDKQUANT1 was injected into mice and its biodistribution and clearance were characterized over time (Figures 6A to 6D). CDKQUANT1 biosensors can be applied in vivo, and observed to distribute throughout different tissues and organs, including tumours, following intravenous, intraperitoneal or intratracheal administration. These results show that CDKQUANT1 biosensors according to the invention allow the biodistribution of CDK/Cyclin complexes, it also allows the kinetic detection of the biosensor and its target, as well as the clearance of the biosensor. The coloration of tissues, including kidney, liver and tumour, was observed via detection by Hoechst coloration, fluorescence of CDKQUANT1-Cy5 and merged signals. This technology was further applied to monitor response to siRNA-mediated knockdown of cyclin B1 in tumour xenografts in mice (Figures 7A and 7B). The protocol was the following: J1: sub-cutaneous implantation of 5 M cells in 10 mice, J7: cyclin B siRNA treatment and measurement of tumoral volumes, J10: cyclin B siRNA treatment and measurement of tumoral volumes, J14: measurement of tumoral volumes, J16: cyclin B siRNA treatment, J17: cyclin B siRNA treatment and measurement of tumoral volumes, J18: measurement of tumoral volumes, injection of biosensor, image detection at 1h, 2h, 3h and 5h.

Example 6: Application of CDKQUANT1 to High Throughput screening

As shown in Figures 8A to 8D, expression of CDKQUANT1 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 cysteine (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. RFP-CDKQUANT1-Cy5 was applied onto HeLa cells for automated acquisition of Cy5 and RFP fluorescence in fields of 2000 cells in 96 well plates on Cellomics ArrayScan Robot. 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 8D).

Example 7: Detection and quantification of CDK/cyclin level in different cell lines using CDKQUANT1-Cy3 sensor, as compared with a CDKSENS1 biosensor.

Titration experiments, antibodies, fluorescence quantification and statistical analysis were performed according to Example 3. As a control, CADY2 (CADY2: SEQ ID N°21) and CDKSENS1-Cy3 (CDKSENS1: SEQ ID N°20), or CADY2 and CTRL2-Cy5 (CTRL2: SEQ ID N°22) were preincubated, respectively, in order to allow non-covalent association, CDKSENS1/Cys3 was complexed with CADY at 40:1 ratio.

Results:

CDKQUANT1-Cy3 (CDKQUANT1: SEQ ID N°14) 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 CDKQUANT1-Cy3 and

CTRL-Cy5 (CTRL : SEQ ID N°19) were overlaid onto cells, and live-cell imaging of cells was performed to acquire CDKQUANT1-Cy3 and CTRL-Cy5 fluorescence. Established ratiometric quantification strategy provides a means of standardizing CDKQUANT1-Cy3 fluorescence with respect to the CTRL-Cy5 peptide (Figure 9A). Ratiometric quantification experiments were performed between normal diploid fibroblasts HS68 and HeLa cells. 45% difference in the 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 CTRL2-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 9B). Ratiometric quantification experiments were performed between normal diploid fibroblasts HS68 and HeLa cells. 15% difference in the CADY2 - CDKSENS-Cy3 / CTRL2-Cy5 ratio was determined between HeLa cells and HS68 fibroblasts.

These results show that the CDKQUANT1 biosensors according to the invention allow a detection which is more sensitive, quantitative, rapid and robust than the non-covalent formulations of CDKSENS1 delivered with CADY2.

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CLAIMS

1. Compound comprising a polypeptide and at least one fluorophore, wherein:
   a) said polypeptide comprises:
      - a cell-penetrating peptide motif
      - a CDK-binding motif comprising an amino acid sequence having at least 70% identity with the sequence of a substrate of a CDK,
      - a cyclin-binding motif being 10 to 20 amino acids long, said Cyclin-binding motif comprising at least an amino acid sequence RXL and having at least 80% identity with the sequence of the RXL motif of p107 (SEQ ID N°1).
   b) said at least one fluorophore is coupled to an amino acid chosen in the group consisting of the amino acids of said CDK-binding motif and the amino acids of said cyclin-binding motif.
2. Compound according to claim 1, wherein said cell-penetrating peptide motif comprises at least an amino acid sequence chosen in the group consisting of the sequence SEQ ID N°3 to the sequence SEQ ID N°6.
3. Compound according to claim 1 or 2, wherein said cell-penetrating peptide motif comprises an amino acid sequence having at least 80% identity with the sequence SEQ ID N°7 (KETWWETWWTEKK).
4. Compound according to any one of claims 1 to 3, wherein said CDK-binding motif comprises an amino acid sequence having at least 80% identity with a sequence chosen among the group consisting of: the sequence SEQ ID N°8 (CDC6), the sequence SEQ ID N°9 (S126S128 cyclinB1), the sequence SEQ ID N°10 (S17 of LB2), the sequence SEQ ID N°11 (S795Rb), the sequence SEQ ID N°12 (S202T205 of Tau) and the sequence SEQ ID No.13 (Histone H1).
5. Compound according to any one of claim 1 to 4, wherein said cyclin-binding motif comprises the amino acid sequence SEQ ID N°1 (RXL motif of p107).

6. Compound according to any one of claims 1 to 5, wherein said polypeptide comprises an amino acid sequence chosen in the group consisting of: the sequence SEQ ID N°14 (CDKQUANT1), the sequence SEQ ID N°15 (CDKQUANT2), the sequence SEQ ID N°16 (CDKQUANT3), the sequence SEQ ID N°17 (CDKQUANT4) and the sequence SEQ ID N°18 (CDKQUANT5).

7. Compound according to any one of claims 1 to 6, wherein said at least one fluorophore is unique and is coupled to a unique residue within the amino acid sequence of said polypeptide.

8. Compound according to any one of claims 1 to 7, wherein said polypeptide comprises the amino acid sequence SEQ ID N°14 (CDKQUANT1) and said fluorophore is Cy3.

9. Composition comprising at least one compound according to any of the claims 1 to 8, and a pharmaceutically acceptable carrier.

10. Method for detecting the presence of at least one CDK/Cyclin complex in a sample, comprising the steps of:

a) providing at least one compound according to any one of claims 1 to 8,

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 said fluorescent signal with a reference fluorescent signal, and

f) determining from the comparison of step e) if at least one CDK/Cyclin complex is present in said sample.

11. Method according to claim 10, for determining the relative quantity of at least one CDK/Cyclin complex in at least two different samples, comprising the steps of:

a) providing at least one compound according to any one of claims 1 to 8,

b) contacting said compound with one of said samples,

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

d) determining the fluorescent signal emitted by said compound,

e) comparing said fluorescent signal with a reference fluorescent signal,

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

g) comparing said fluorescent signals obtained in step e) and f) to determine the relative quantity of said at least one CDK/Cyclin complex in said samples.

12. Method according to claim 11, which is a method for screening a plurality of products for their ability to modulate the quantity of at least one CDK/Cyclin complex in a sample, said method comprising the following steps:

a) providing at least one compound according to any of claims 1 to 8,

b) contacting said sample with one of said products,

c) contacting said sample and product from step b) with said compound,

d) illuminating said sample, product and compound from step c) with an excitation light,

e) determining the fluorescent signal emitted by said compound,

f) comparing said fluorescent signal with a reference fluorescent signal,

g) performing steps b), c), d), e) and f) with at least another of said products,

h) comparing said fluorescent signals obtained in step f) and g) to determine the ability of said at least one product to affect the quantity of at least one CDK/Cyclin complex.

13. Method for the *in vitro* diagnosis of a CDK/Cyclin complex presence and/or level of expression in a subject, comprising the steps of:

a) providing at least one compound according to any one of claims 1 to 8,

b) contacting said compound with a biological sample from said subject,

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

d) determining the fluorescent signal emitted by said compound,

e) comparing said fluorescent signal with a reference fluorescent signal, and

f) determining from the comparison of step e) the presence and/or the level of expression of at least one CDK/Cyclin complex.

14. Kit comprising at least one compound according to any of claims 1 to 8, and an acceptable carrier and/or an acceptable solvent.

15. Use of a compound according to any of claims 1 to 8, for detecting the presence of at least one CDK/Cyclin complex in a sample or for determining the relative quantity at least one CDK/Cyclin complex in at least two different samples.

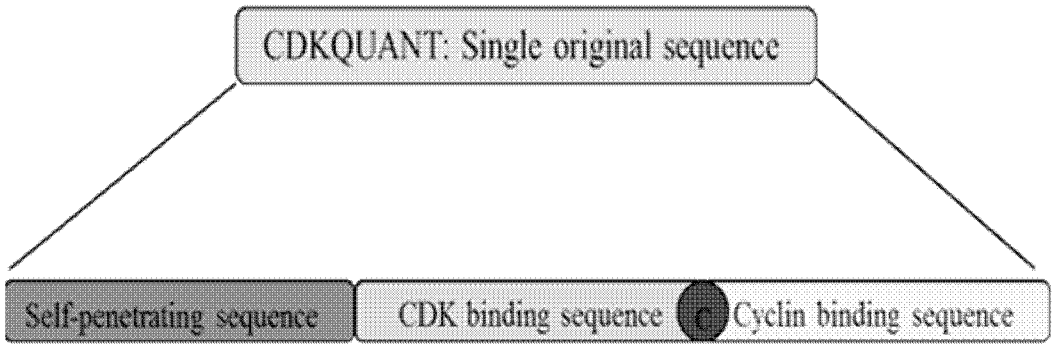

FIGURE 1A

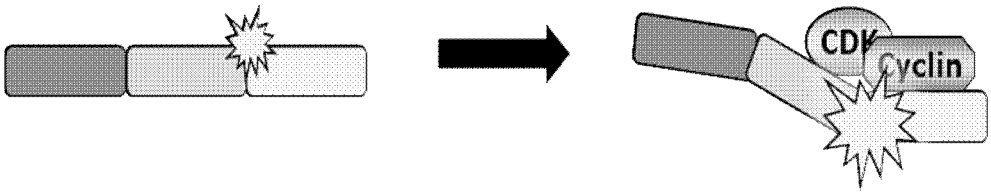

FIGURE 1B

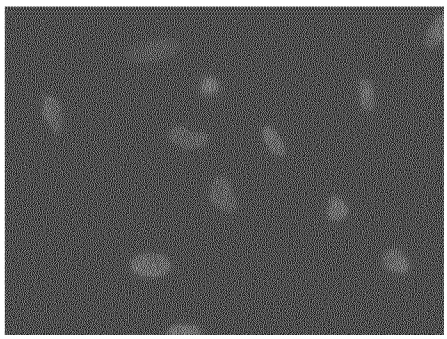

FIGURE 2A

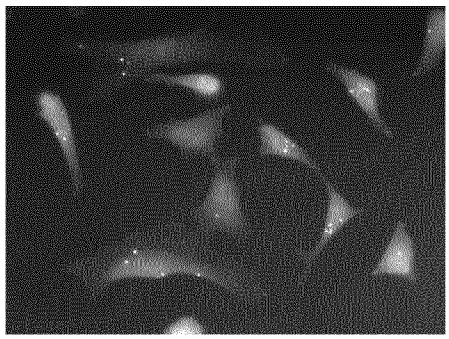

FIGURE 2B

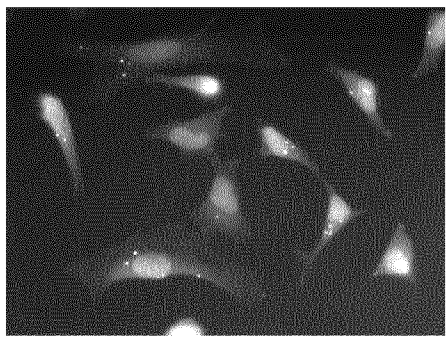

FIGURE 2C

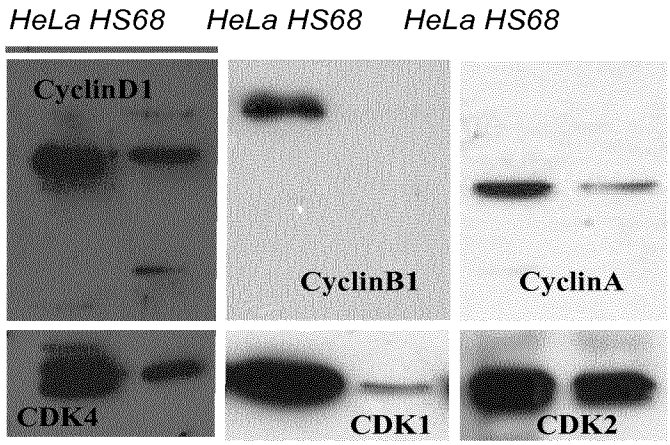

FIGURE 3A

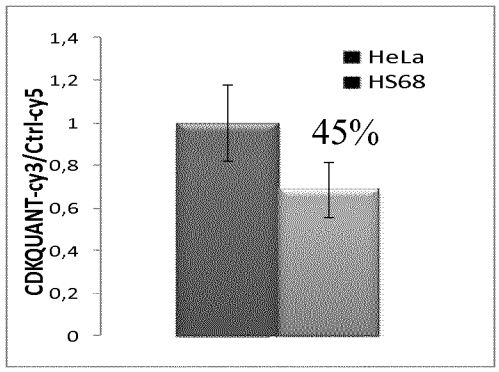

FIGURE 3B

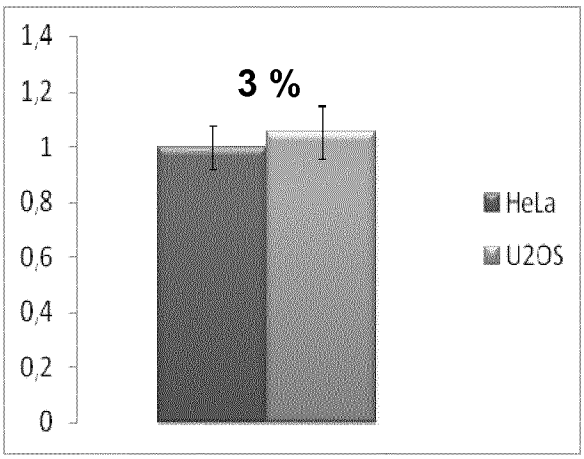

FIGURE 3C

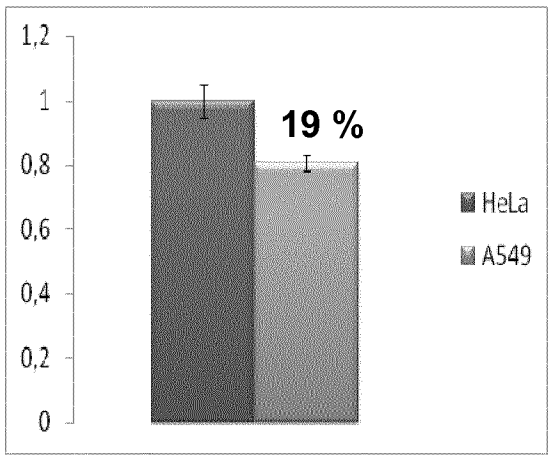

FIGURE 3D

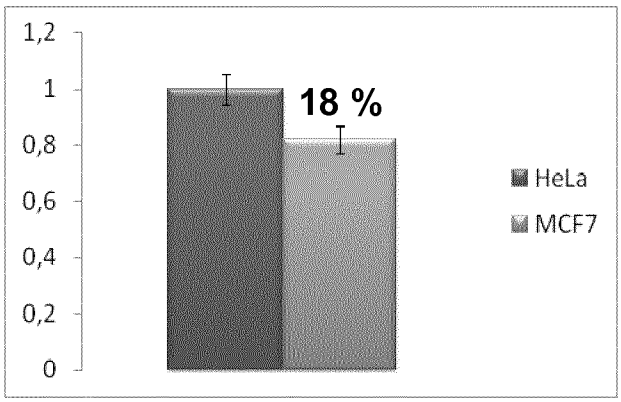

FIGURE 3E

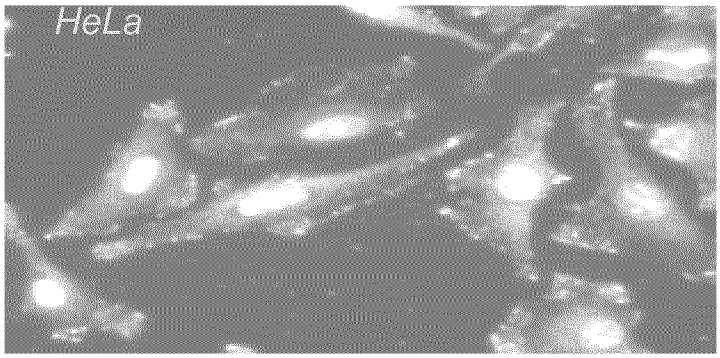

FIGURE 3F

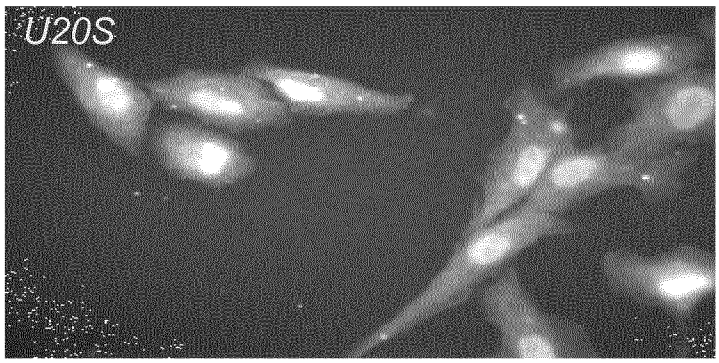

FIGURE 3G

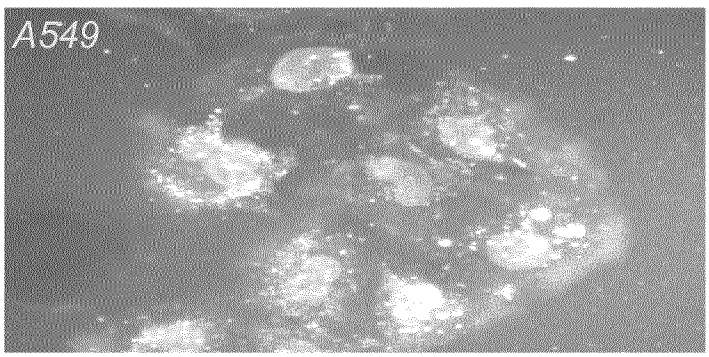

FIGURE 3H

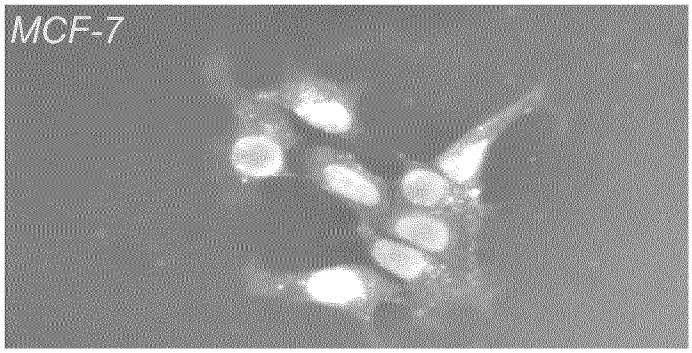

FIGURE 3I

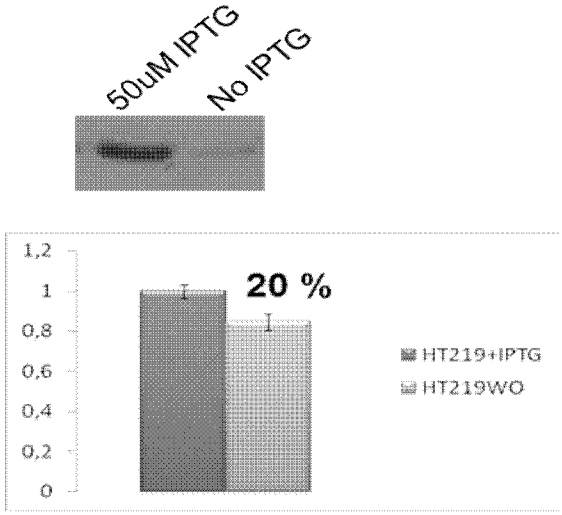

FIGURE 4A

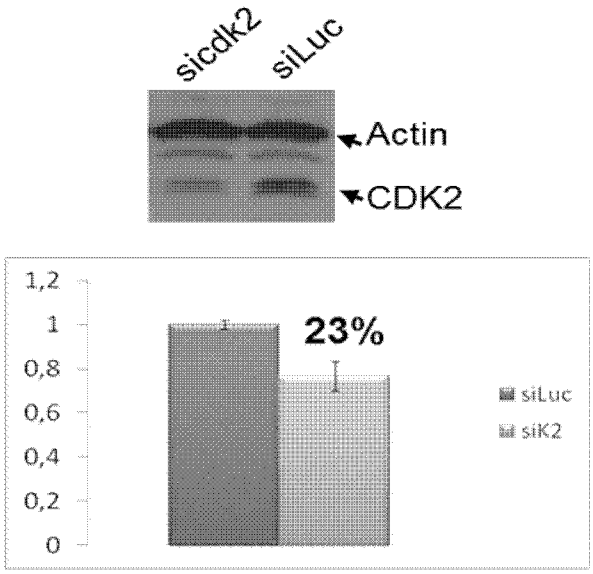

FIGURE 4B

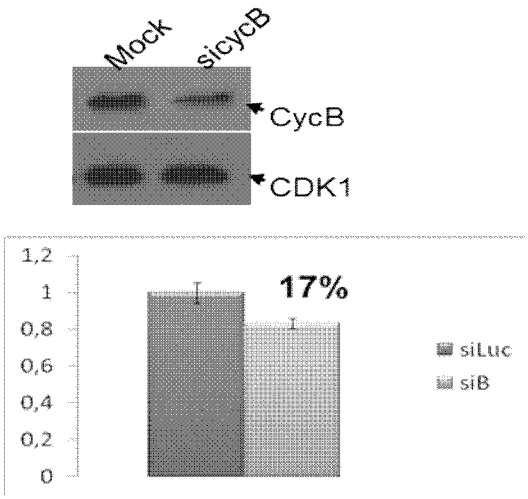

FIGURE 4C

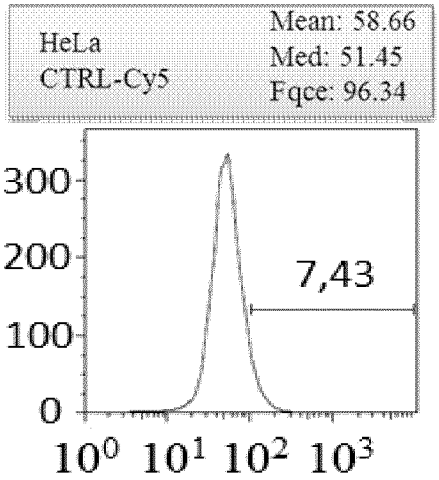

FIGURE 5A

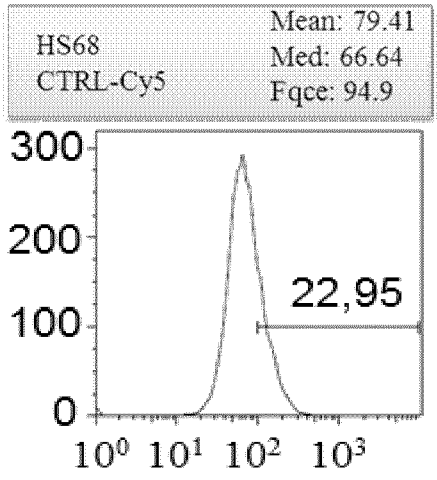

FIGURE 5B

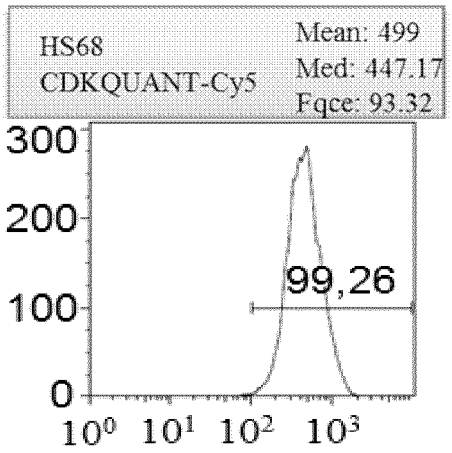

FIGURE 5C

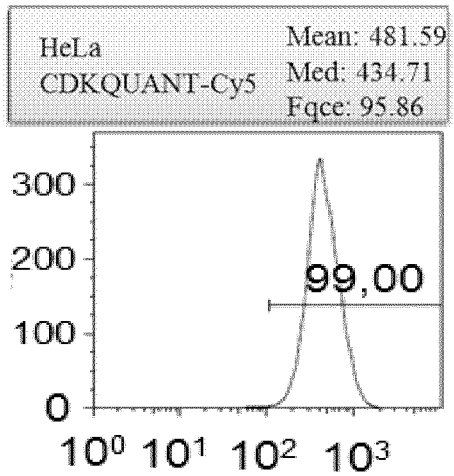

FIGURE 5D

Parameter/Cell line	HeLa		HS68	
	CDKQUANT Cy5	CTRL Cy5	CDKQUANT Cy5	CDKQUANT Cy5
Mean fluo	481.59	58.66	499	79.41
Med fluo	434.71	51.45	444.17	66.64

FIGURE 5E

	HeLa	HS68	Difference
Mean (CDKQUANT/CTRL)-Cy5	8.21	6.28	1.31
Med (CDKQUANT/CTRL)-Cy5	8.45	6.67	1.27

FIGURE 5F

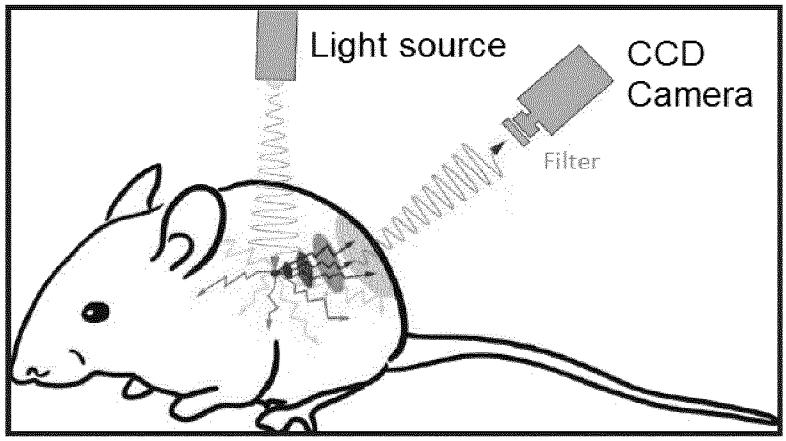

FIGURE 6A

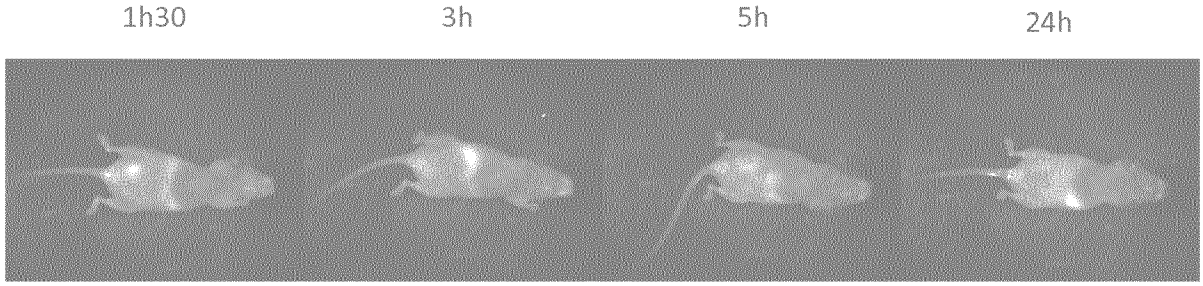

FIGURE 6B

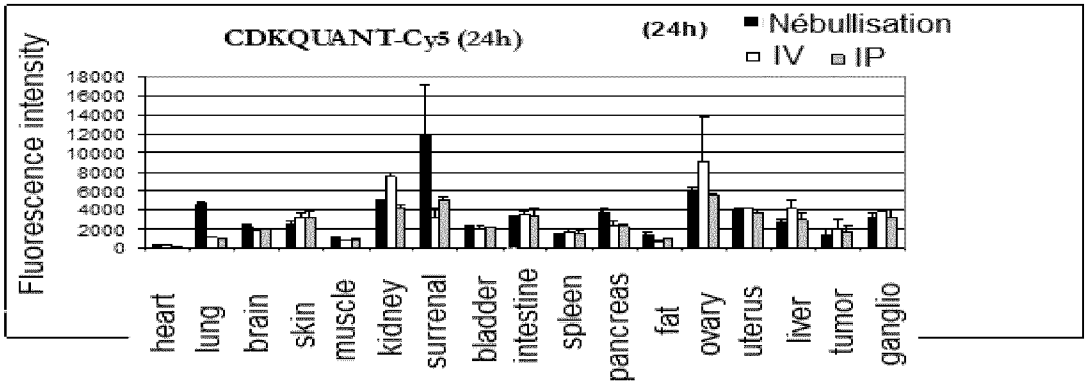

FIGURE 6C

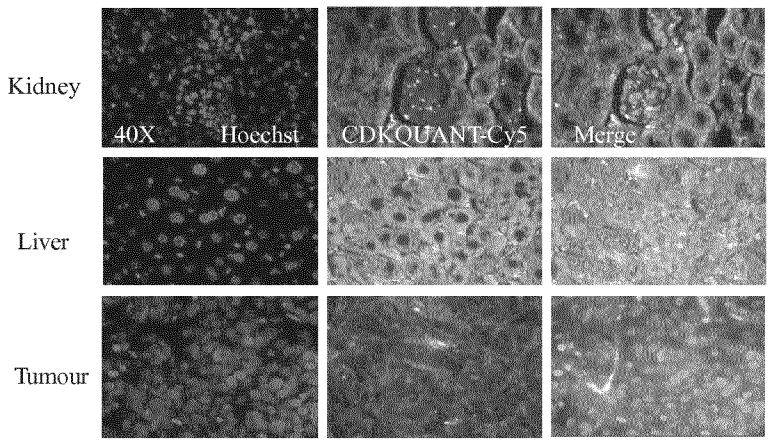

FIGURE 6D

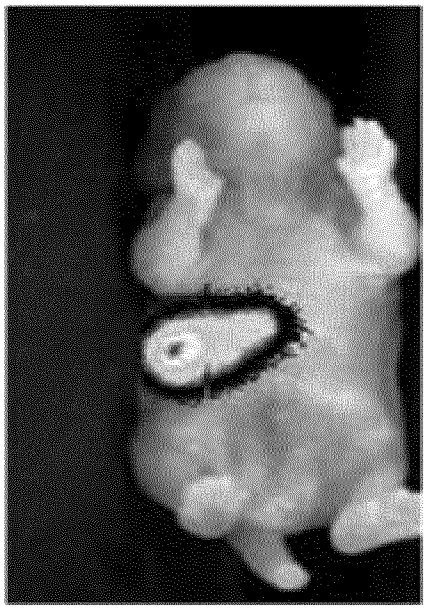

FIGURE 7A

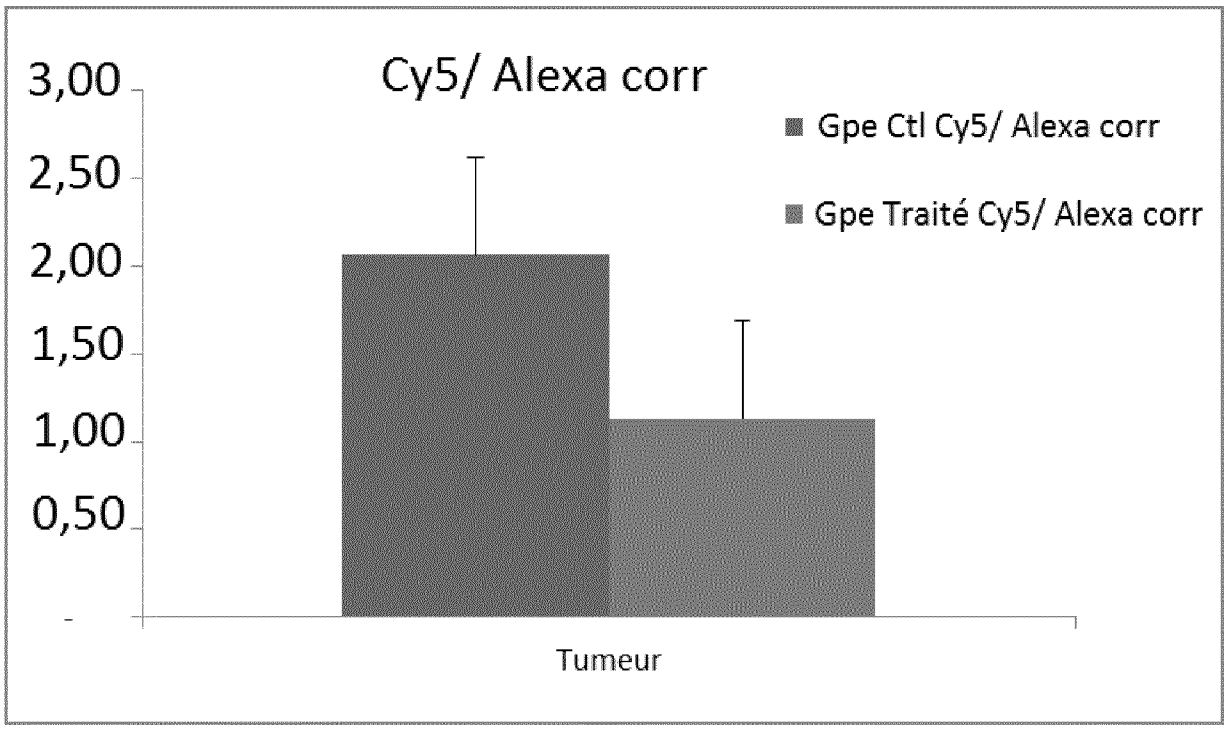

FIGURE 7B

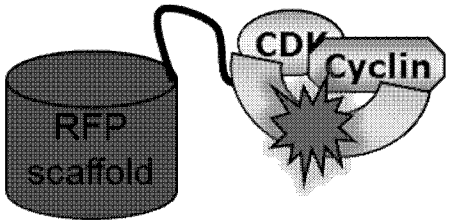

FIGURE 8A

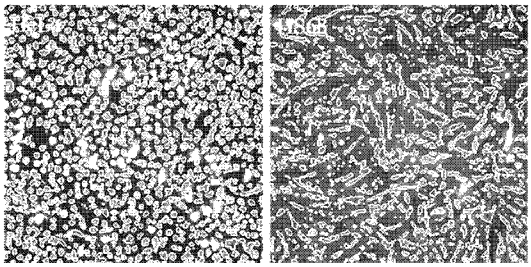

FIGURE 8B

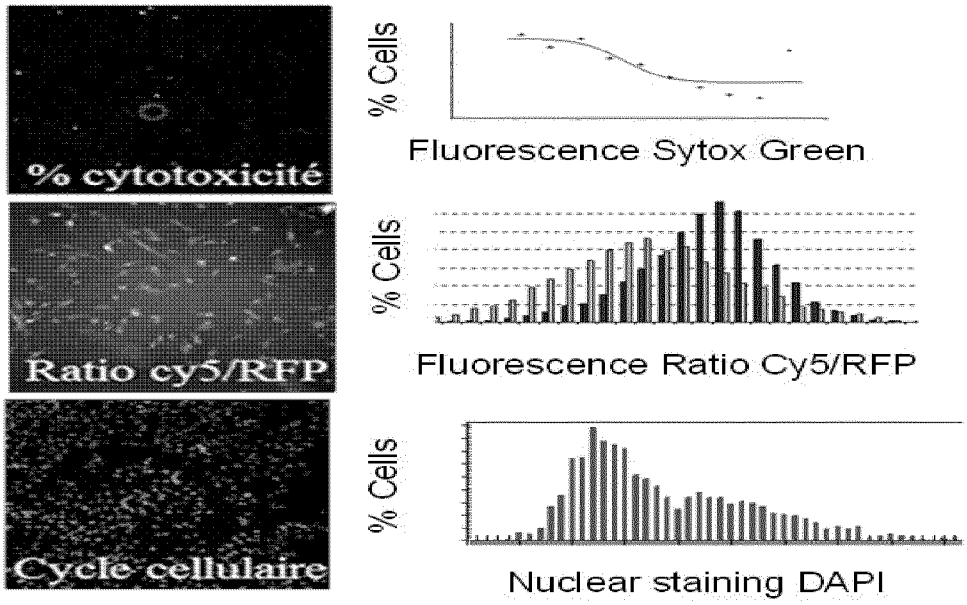

FIGURE 8C

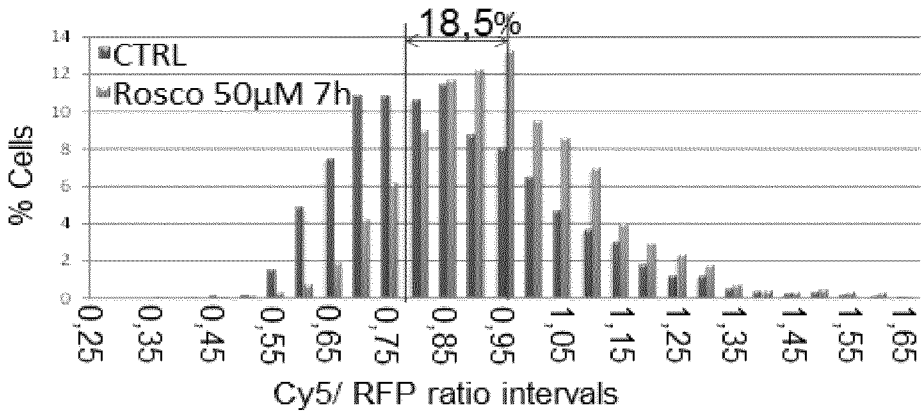

FIGURE 8D

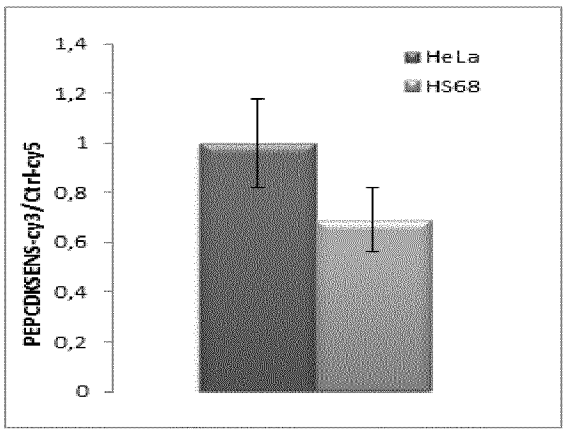

FIGURE 9A

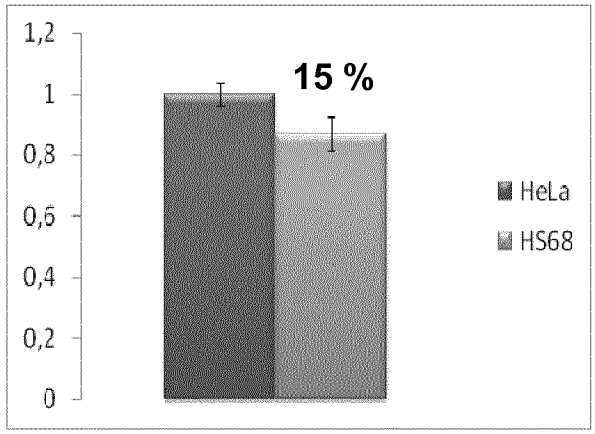

FIGURE 9B

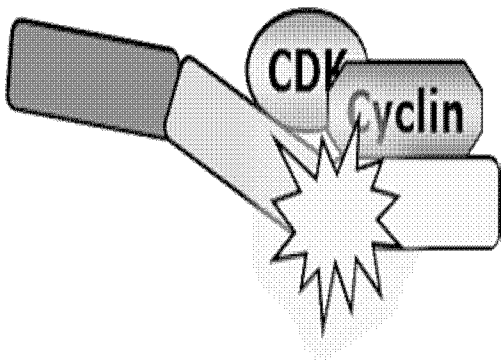

FIGURE 9C

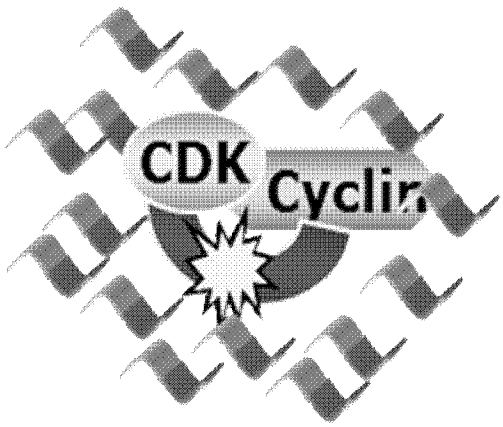

FIGURE 9D

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/055631

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/573 C07K7/00 G01N33/58
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.
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 ----- -/--	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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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.
Y	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	1-15
Y	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 the whole document, in particular abstract; page 225, sentence spanning columns; paragraphs 1.8 and 2.6; figure 5	1-15
Y	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 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	1-15
A	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 the whole document, in particular abstract; table 1, compound C4-Tat	1-15

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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
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