NEW PROGNOSTIC METHOD FOR PATIENTS SUFFERING OF A CANCER

The present invention relates to an in vitro method for classifying a patient suffering from cancer comprising the steps consisting of i) determining the expression level of PLCy1 in a sample from said patient, ii) comparing said expression level with a predetermined reference value and iii) providing the stage of the patient according to the expression level of PLCy1. The invention also relates to an agonist of CD47 receptor for use in the treatment of patient suffering of a cancer with a high expression level of PLCy.
NEW PROGNOSTIC METHOD FOR PATIENTS SUFFERING OF A CANCER

FIELD OF THE INVENTION:

The present invention relates to an in vitro method for classifying a patient suffering from cancer comprising the steps consisting of i) determining the expression level of PLCy1 in a sample from said patient, ii) comparing said expression level with a predetermined reference value and iii) providing the stage of the patient according to the expression level of PLCy1.

The invention also relates to a agonist of the CD47 receptor for use in the treatment of patient suffering of a cancer with a high expression level of PLCy.

BACKGROUND OF THE INVENTION:

Chronic lymphocytic leukemia (CLL), a human malignancy caused by an imbalance between proliferation and programmed cell death (PCD) [N. Chiorazzi et al, 2003], is the most common form of leukemia in adults. CLL is characterized by an accumulation of monoclonal B cells (CD20+, CD5+, and CD23+) in the peripheral blood, bone marrow, and secondary lymphoid organs that leads to the progressive failure of the immune and hematopoietic systems. CLL prognosis is dependent on clinical staging and biological markers like the IGHV status, cytogenetic abnormalities, expression of proteins such as CD38 or ZAP-70, and NOTCH 1 or SF3B1 mutations. Despite intense research and pharmaceutical development, there remains an unmet medical need for CLL. Indeed, 15-25% of patients remain or become refractory to the current chemotherapeutics [J. G. Gribben, 2010]. Moreover, patients with dysfunction relevant to TP53 gene (-7-10%) require a specific aggressive therapy that often yields a negative issue [H. Dohner et al, 2000]. It is therefore of highest interest to find original approaches in order to overcome CLL refractoriness.

From this perspective, CD47 appears to be a target of high therapeutic potential. CD47, a cell surface receptor that binds to signal regulatory protein a (SIRPa) and thrombospondin-1 (TSP1), serves as a marker of self and participates in the regulation of the cellular responses to stress. The binding of CD47 either to SIRPa or TSP1 provides two anticancer strategies. On the one hand, antibodies targeting CD47 or SIRPa, recombinant SIRPa or TSP1-derived proteins promotes phagocytosis and tumor cell elimination by
disruption of the CD47-SIRPa interaction and/or induction of Fc-dependent mechanisms (e.g., ADCC or CDC) or PCD [X. W. Zhao et al, 2012]. On the other hand, the binding of CD47 to 4N1K, a decapeptide derived from the TSPl C-terminal domain, directly kills tumor cells [V. Mateo et al, 1999]. The latter strategy is underused but appears especially relevant.

**SUMMARY OF THE INVENTION:**

For a therapeutic purpose against CLL, the inventors have developed new peptides like PKHB1, a novel human serum-stable TSPl -derived decapeptide that links CD47 and kills primary CLL cells, including those from drug-resistant patients. During their investigations, the inventors show that the cytotoxic effect of the developed peptides and especially PKHB1 passes through the induction of a caspase-independent PCD pathway mediated by the sustained activation of phospholipase C gamma-1 (PLCyl), a signal transduction protein that they found over-expressed in CLL. PLCyl over-activation provoked endoplasmic reticulum (ER)-stress, Ca2+ overload, mitochondrial damage, calreticulin exposure, and PCD in CLL. The inventors concluded that the measure of the expression level of the PLCyl protein could be used in the prognostic of the gravity of patient suffering a cancer and especially CLL. The measurement of the expression level of the PLCyl protein may also be used as a therapeutic companion test. Moreover, the inventors show that inhibition of the PLCyl protein and its down-regulation abolishes CD47-mediated killing.

Thus, the invention relates to an in vitro method for classifying a patient suffering from cancer comprising the steps consisting of i) determining the expression level of PLCyl in a sample from said patient, ii) comparing said expression level with a predetermined reference value and iii) providing the stage of the patient according to the expression level of PLCyl.

The invention also relates to an agonist of CD47 receptor for use in the treatment of patient suffering of a cancer with a high expression level of PLCy.

The invention also relates to a compound which active the PLCyl for use in the treatment of cancer.

**DETAILED DESCRIPTION OF THE INVENTION:**

*Classifying and Predicting methods*
The invention relates to an in vitro method for classifying a patient suffering from cancer comprising the steps consisting of i) determining the expression level of PLCyl in a sample from said patient, ii) comparing said expression level with a predetermined reference value and iii) providing the stage of the patient according to the expression level of PLCyl.

The invention also relates to an in vitro method for the prognosis of the survival time of a patient suffering from cancer comprising the steps consisting of i) determining the expression level of PLCyl in a sample from said patient, ii) comparing said expression level with a predetermined reference value and iii) providing a good outcome prognosis when the expression level is lower than the predetermined reference value and a poor outcome prognosis when the expression level is higher than the predetermined reference value.

The invention also relates to an in vitro method for the diagnosis of the severity of a cancer comprising the steps consisting of i) determining the expression level of PLCyl in a sample from said patient, ii) comparing said expression level with a predetermined reference value and iii) providing a good diagnosis when the expression level is lower than the predetermined reference value and a poor diagnosis when the expression level is higher than the predetermined reference value.

As used herein, the term "PLCyl" has its general meaning in the art and denotes an enzyme, which cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). An exemplary sequence for human PLCyl protein is deposited in NCBI (NM_002660.2).

In a one embodiment, the cancer is selected form the group consisting of adrenal cortical cancer, anal cancer, bile duct cancer, bladder cancer, bone cancer, brain and central nervous system cancer, breast cancer, Castleman disease, cervical cancer, colorectal cancer, endometrial cancer, esophagus cancer, gallbladder cancer, gastrointestinal carcinoid tumors, Hodgkin's disease, non-Hodgkin's lymphoma, Kaposi's sarcoma, kidney cancer, laryngeal and hypopharyngeal cancer, liver cancer, lung cancer, mesothelioma, plasmacytoma, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, oral cavity and oropharyngeal cancer, ovarian cancer, pancreatic cancer, penile cancer, pituitary cancer, prostate cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, skin cancer,
stomach cancer, testicular cancer, thymus cancer, thyroid cancer, vaginal cancer, vulvar cancer, and uterine cancer.

In another particular embodiment, cancer is selected from the group consisting of leukemia, acute lymphoblastic leukemia, B-chronic lymphocytic leukemia, hairy-cell leukemia, adult T-cell leukemia, prolymphocytic leukaemia of T-cell type or myeloid leukaemia.

In one embodiment, the leukemia is a B-chronic lymphocytic leukemia (CLL).

In a particular embodiment, the CLL is a refractory CLL.

In a particular embodiment, the invention relates to an in vitro method for classifying a patient suffering from a CLL comprising the steps consisting of i) determining the expression level of PLCyl in a sample from said patient, ii) comparing said expression level with a predetermined reference value and iii) providing the stage of the patient according to the expression level of PLCyl.

In this case, according to the expression level of PLCyl, the patient suffering from a CLL can be classified in CLL Binet Stage A (indolent CLL) or in CLL Binet Stages B/C (advanced disease, see for review Binet JL et al, 1981).

A patient with a CLL may be classified according to the Binet classification Nomenclature and criteria about this classification can be find in the National Cancer Institute website (http://www.cancer.gov/cancertopics/pdq/treatment/CLL/HealthProfessional/page2).

Typically, the sample according to the invention may be a blood, plasma, serum sample. In a particular embodiment, said sample is blood.

The term "detecting" as used above includes qualitative and/or quantitative detection (measuring levels) with or without reference to a control. Typically PLCyl expression may be measured for example by immunoblotting, enzyme-labeled and mediated immunoassays (such as ELISA), flow cytometry assessment or qRT-PCR performed on the sample. In these case, PLCyl mRNA or PLCyl protein can be detected.

Particularly, the methods of the invention further comprise a step of comparing the PLCyl expression level obtained in step a) to a threshold value.

The "control" may be a healthy subject, i.e. a subject who does not suffer from any cancer. Particularly, said control is a healthy subject.
Detection of PLCyl expression in the sample may be performed by measuring the level of PLCyl protein. In the present application, the "level of PLCyl protein" means the quantity or concentration of said PLCyl protein.

Such methods comprise contacting a sample with a binding partner capable of selectively interacting with PLCyl protein present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, particularly monoclonal.

The presence of the protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; Immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies against the proteins to be tested. A sample containing or suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule is added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate is washed and the presence of the secondary binding molecule is detected using methods well known in the art.

Various immunoenzymatic staining methods are known in the art for detecting a protein of interest. For example, immunoenzymatic interactions can be visualized using different enzymes such as peroxidase, alkaline phosphatase, or different chromogens such as DAB, AEC, or Fast Red; or fluorescent labels such as FITC, Cy3, Cy5, Cy7, Alexafluors, etc. Counterstains may include H&E, DAPI, Hoechst, so long as such stains are compatible with
other detection reagents and the visualization strategy used. As known in the art, amplification reagents may be used to intensify staining signal. For example, tyramide reagents may be used. The staining methods of the present invention may be accomplished using any suitable method or system as would be apparent to one of skill in the art, including automated, semi-automated or manual systems.

The method of the invention may comprise a further step consisting of comparing PLCyl expression with a control reference.

As used herein, "expression level of PLCyl" could also refer to an amount or a concentration of a transcription product, for instance mRNA coding for PLCyl, or of a translation product, for instance the protein PLCyl. Typically, a level of mRNA expression can be expressed in units such as transcripts per cell or nanograms per microgram of tissue. A level of protein can be expressed as nanograms per microgram of tissue or nanograms per milliliter of a culture medium, for example. Alternatively, relative units can be employed to describe an expression level.

In a one embodiment, when the measure of PLCyl protein is performed by quantitative RT-PCR (qPCR), immunoblotting or Flow cytometry the expression level of PLCyl protein in a patient suffering of a CLL Binet Stage A is increased by at least 100%, particularly by at least 110%; particularly by at least 120%, particularly by at least 130%, particularly by at least 140%, more particularly by at least 150% compared to a control reference obtained in normal B cells. In other words, particularly, when PLCyl protein is measured, the quantity of PLCyl protein in a patient suffering of a CLL Binet Stage A is by at least 100%, particularly by at least 110%; particularly by at least 120%, particularly by at least 130%, particularly by at least 140%, more particularly by at least 150% increased compared to a control reference obtained in normal B cells.

In another embodiment, when the measure of PLCyl protein is performed by qPCR or Flow cytometry, the expression level of PLCyl protein in a patient suffering of a CLL Binet Stage B/C is increased by at least 350%, particularly by at least 360%; particularly by at least 370%, particularly by at least 380%, particularly by at least 390%, more particularly by at least 400% compared to a control reference in normal B cells. In other words, particularly, when PLCyl protein is measured, the quantity of PLCyl protein in a patient suffering of a CLL Binet Stage B/C is by at least 350%, particularly by at least 360%; particularly by at least 370%, particularly by at least 380%, particularly by at least 390%, more particularly by at least 400% increased compared to a control reference obtained in normal B cells.
Typically, a "threshold value", "threshold level" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. Particularly, the person skilled in the art may compare the expression levels of PLCyl obtained according to the method of the invention with a defined threshold value.

Particularly, said threshold value is the mean expression level of PLCyl of a population of healthy individuals. As used herein, the term "healthy individual" denotes a human which is known to be healthy, i.e. which does not suffer from a cancer and in particular from a CLL and does not need any medical care.

Typically, the skilled person in the art may determine the expression level of PLCyl in a biological sample, particularly blood, of 100 individuals known to be healthy. The mean value of the obtained expression levels is then determined, according to well known statistical analysis, so as to obtain the mean expression level of PLCyl. Said value is then considered as being normal and thus constitutes a threshold value. By comparing the expression levels of PLCyl to this threshold value, the physician is then able to classify and prognostic the cancer. Indeed, by comparing the expression level of PLCyl obtained in a biological sample, particularly blood, of a given subject to a threshold value, one can easily determines the cancer stage of the subject or its prognostic.

Accordingly, the physician would be able to adapt and optimize appropriate medical care of a subject in a critical and life-threatening condition suffering from cancer. The determination of said prognosis is highly appropriate for follow-up care and clinical decision making.

Therefore, the invention is drawn to an in vitro method for classifying a patient suffering from cancer comprising the following steps:

a) determining the level of expression of PLCyl in a sample obtained from said patient;

b) determining the mean expression level of PLCyl in a biological sample of a population of healthy individuals, particularly 100 healthy individuals; and

c) a step of comparing the expression level of PLCyl obtained of a) to the mean expression level of PLCyl obtained in b).
The present invention also relates to kits useful for the methods of the invention, comprising means for detecting PLCyl expression.

According to the invention, the kits of the invention may comprise an anti-PLCyl protein antibody; and another molecule coupled with a signalling system which binds to said PLCyl protein antibody.

Typically, the antibodies or combination of antibodies are in the form of solutions ready for use. In one embodiment, the kit comprises containers with the solutions ready for use. Any other forms are encompassed by the present invention and the man skilled in the art can routinely adapt the form to the use in immunohistochemistry.

In another embodiment, the invention relates to an in vitro method for monitoring a patient's response to cancer treatment which comprises a step of measuring the level of PLCyl protein, in a sample from a patient.

Thus, the present invention relates to the use of PLCyl protein as a biomarker for the monitoring of anti cancer therapies.

According to the invention, the expression level of PLCyl protein may be determined to monitor a patient's response to cancer treatment and especially to monitor a patient's response to a CLL treatment.

In another embodiment, the expression of PLCyl is a biomarker of cancer severity and especially in CLL severity.

In still another embodiment, the expression of PLCyl mRNA is a biomarker of cancer severity and especially in CLL severity.

In another embodiment, the measurement of the expression level of the PLCyl protein may be used as a therapeutic companion test.

**Therapeutic methods of patient with high expression level of PLCy**

A second aspect of the invention relates to an agonist of CD47 receptor for use in the treatment of patient suffering of a cancer with a high expression level of PLCy.
In another embodiment, the invention also relates to an agonist of CD47 receptor for use in the treatment of patients suffering from a cancer with a poor outcome prognosis according to the prognostic method of the invention.


In another particular embodiment, cancer is selected from the group consisting of leukemia, acute lymphoblastic leukemia, B-chronic lymphocytic leukemia, hairy-cell leukemia, adult T-cell leukemia, prolymphocytic leukaemia of T-cell type or myeloid leukaemia.

In one embodiment, the leukemia is a B-chronic lymphocytic leukemia (CLL).
In a particular embodiment, the CLL is a refractory CLL (high-risk patients).

In one embodiment, the invention relates to an agonist of CD47 receptor for use in the treatment of CLL's patient with a high expression level of PLCy.

In another embodiment, the invention also relates to an agonist of CD47 receptor for use in the treatment of CLL's patient classified in CLL Binet Stage A or in CLL Binet Stages B/C).

To determine the expression level of PLCy or the stage of the patient, means as explained above in the invention may be used.

Typically, the agonist according to the invention includes but is not limited to a small organic molecule, an antibody, and a peptide/polypeptide.
In one embodiment, the agonist according to the invention is a soluble peptide comprising the amino acids sequence: KRFYVVMWKK (SEQ ID NO:1) or a function-conservative variant thereof for use in the treatment of cancer.

The agonist also encompasses peptides that are function-conservative variants of the soluble peptide comprising SEQ ID NO: 1 as described here above.

In one embodiment, the soluble peptide according to the invention may differ from 1, 2 or 3 amino acids to the SEQ ID NO: 1.

In another embodiment, the soluble peptide according to the invention may differ from 4 or 5 amino acids to the SEQ ID NO:1.

In one embodiment, the soluble peptide of the invention comprises at least 75% identity over said the SEQ ID NO: 1, even more particularly at least 80%, at least 85%, at least 90%, at least 95%, at least 97% and is still able to decrease tumor cell proliferation or still able to induce PCD in tumor cell.

In one embodiment, the soluble peptide of the invention consists in the amino acid sequence as set forth in SEQ ID NO:1 or a variant thereof comprising at least 75%, particularly at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5% or 99.9% identity with SEQ ID NO:1 and is still able to decrease tumor cell proliferation or still able to induce PCD in tumor cells.

To verify whether the newly generated soluble peptides induce the same type of caspase-independent PCD than the initially characterized peptide 4N1K a flow cytometry analysis may be performed with each peptide. A comparison of the results obtained in treatments with/without the caspase inhibitor Z-VAD.fmk will corroborate that the mode of cell death induced by the 4NIK-derived peptides is caspase-independent. Additionally, a time-course and a dose-response performed in different tumor cells will determine the optimal conditions for each peptide and each malignant cell type.

In one embodiment of the invention, said soluble peptide is an amino acid sequence of less than 50 amino acids long that comprises the amino acid sequence SEQ ID NO:1 as defined here above.

In another embodiment of the invention, said soluble peptide is an amino acid sequence of less than 45 amino acids long that comprises the amino acid sequence SEQ ID NO:1 as defined here above.
In another embodiment of the invention, said soluble peptide is an amino acid sequence of less than 40 amino acids long that comprises the amino acid sequence SEQ ID NO:1 as defined here above.

In another embodiment of the invention, said soluble peptide is an amino acid sequence of less than 30 amino acids long that comprises the amino acid sequence SEQ ID NO:1 as defined here above.

In another embodiment of the invention, said soluble peptide is an amino acid sequence of less than 20 amino acids long that comprises the amino acid sequence SEQ ID NO:1 as defined here above.

In another embodiment of the invention, said soluble peptide is an amino acid sequence of less than 15 amino acids long that comprises the amino acid sequence SEQ ID NO:1 as defined here above.

As used herein, the term "Function-conservative variants" refer to those in which a given amino acid residue in a protein or enzyme has been changed (inserted, deleted or substituted) without altering the overall conformation and function of the peptide. Such variants include protein having amino acid alterations such as deletions, insertions and/or substitutions. A "deletion" refers to the absence of one or more amino acids in the protein. An "insertion" refers to the addition of one or more of amino acids in the protein. A "substitution" refers to the replacement of one or more amino acids by another amino acid residue in the protein. Typically, a given amino acid is replaced by an amino acid having similar properties (such as, for example, polarity, hydrogen bonding potential, acidic, basic, hydrophobic, aromatic, and the like). This given amino acid can be a natural amino acid or a non natural amino acid. Amino acids other than those indicated as conserved may differ in a protein so that the percent protein or amino acid sequence similarity between any two proteins of similar function may vary and may be, for example, from 70 % to 99 % as determined according to an alignment scheme such as by the Cluster Method, wherein similarity is based on the MEGALIGN algorithm. A "function-conservative variant" also includes a polypeptide which has at least 60 % amino acid identity as determined by BLAST or FASTA algorithms, particularly at least 75 %, more particularly at least 85%, still particularly at least 90 %, and even more particularly at least 95%, and which has the same or substantially similar properties or functions as the native or parent protein to which it is compared. Two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 80 %, particularly greater than 85 %, particularly greater than 90 % of the amino acids are
identical, or greater than about 90%, particularly greater than 95%, are similar (functionally identical) over the whole length of the shorter sequence. Particularly, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin) pileup program, or any of sequence comparison algorithms such as BLAST, FASTA, etc.

Typically, the invention encompasses soluble peptides substantially identical to the soluble peptide comprising SEQ ID NO:1 in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the functional aspects of the soluble peptides comprising SEQ ID NO:1 as described here above, i.e. being still able to decrease tumor cell proliferation in substantially the same way as a peptide consisting of the given amino acid sequence.

Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid or another.

The term "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue. "Chemical derivative" refers to a subject peptide having one or more residues chemically derivatized by reaction of a functional side group. Examples of such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzyllhistidine. Chemical derivatives also include peptides that contain one or more naturally-occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxyllysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. The term "conservative substitution" also includes the use of non natural amino acids aimed to control and stabilize peptides or proteins secondary structures. These non natural amino acids are chemically modified amino acids
such as prolinoamino acids, beta-amino acids, N-methylamino acids, cyclopropylamino acids, alpha,alpha-substituted amino acids as described here below. These non natural amino acids may include also fluorinated, chlorinated, brominated- or iodinated modified amino acids.

In one embodiment, soluble peptides of the invention may be as described in example 3.

In another embodiment, the soluble peptide of the invention is the PKHB1 peptide as described in example 3.

In another embodiment, the soluble peptide of the invention is the PKHB3 peptide as described in example 3.

In another embodiment, the soluble peptide of the invention is the PKHB4 peptide as described in example 3.

In another embodiment, the soluble peptide of the invention is the PKHB9 peptide as described in example 3.

In another embodiment, the soluble peptide of the invention is the PKHB10 peptide as described in example 3.

In another embodiment, the soluble peptide of the invention is the PKHB11 peptide as described in example 3.

In one embodiment, soluble peptides of the invention may comprise a tag. A tag is an epitope-containing sequence which can be useful for the purification of the soluble peptides. It is attached to by a variety of techniques such as affinity chromatography, for the localization of said peptide or polypeptide within a cell or a tissue sample using immunolabeling techniques, the detection of said peptide or polypeptide by immunoblotting etc. Examples of tags commonly employed in the art are the GST (glutathion-S-transferase)-tag, the FLAG™-tag, the Strep-tag™, V5 tag, myc tag, His tag etc.

In one embodiment, soluble peptides of the invention may be labelled by a fluorescent dye. Dye-labelled fluorescent peptides are important tools in cellular studies. Peptides can be labelled on the N-terminal side or on the C-terminal side.

N-Terminal Peptide Labeling Using Amine-Reactive Fluorescent Dyes:

Amine-reactive fluorescent probes are widely used to modify peptides at the N-terminal or lysine residue. A number of fluorescent amino-reactive dyes have been developed
to label various peptides, and the resultant conjugates are widely used in biological applications. Three major classes of amine-reactive fluorescent reagents are currently used to label peptides: succinimidyl esters (SE), isothiocyanates and sulfonyl chlorides.

C-Terminal Labeling Using Amine-Containing Fluorescent Dyes:

Amine-containing dyes are used to modify peptides using water-soluble carbodiimides (such as EDC) to convert the carboxy groups of the peptides into amide groups. Either NHS or NHSS may be used to improve the coupling efficiency of EDC-mediated protein-carboxylic acid conjugations.

Labelled peptides derived from 4N1K have the following general formula:

![Diagram]

Where X and/or Y can be nothing or hydrogen and/or spacers and/or fluorescent dyes.

PKHB8 (formula (VII)) is an example of peptide from this series where a spacer formed by two beta-alanine residues and a fluorescent dye (fluorescein) have been introduced on the N-terminal side of the peptide, on the alpha-amino group of the lysine residue:

![Diagram]

In another embodiment, the soluble peptide of the invention is the PKHB8 peptide.
In specific embodiments, it is contemplated that soluble peptides used in the therapeutic methods of the present invention may be modified in order to improve their therapeutic efficacy. Such modification of therapeutic compounds may be used to decrease toxicity, increase circulatory time, or modify biodistribution. For example, the toxicity of potentially important therapeutic compounds can be decreased significantly by combination with a variety of drug carrier vehicles that modify biodistribution.

A strategy for improving drug viability is the utilization of water-soluble polymers. Various water-soluble polymers have been shown to modify biodistribution, improve the mode of cellular uptake, change the permeability through physiological barriers; and modify the rate of clearance from the body. To achieve either a targeting or sustained-release effect, water-soluble polymers have been synthesized that contain drug moieties as terminal groups, as part of the backbone, or as pendent groups on the polymer chain.

Polyethylene glycol (PEG) has been widely used as a drug carrier, given its high degree of biocompatibility and ease of modification. Attachment to various drugs, proteins, and liposomes has been shown to improve residence time and decrease toxicity. PEG can be coupled to active agents through the hydroxyl groups at the ends of the chain and via other chemical methods; however, PEG itself is limited to at most two active agents per molecule. In a different approach, copolymers of PEG and amino acids were explored as novel biomaterials which would retain the biocompatibility properties of PEG, but which would have the added advantage of numerous attachment points per molecule (providing greater drug loading), and which could be synthetically designed to suit a variety of applications.

Those of skill in the art are aware of PEGylation techniques for the effective modification of drugs. For example, drug delivery polymers that consist of alternating polymers of PEG and tri-functional monomers such as lysine have been used by VectraMed (Plainsboro, N.J.). The PEG chains (typically 2000 daltons or less) are linked to the α- and ε-amino groups of lysine through stable urethane linkages. Such copolymers retain the desirable properties of PEG, while providing reactive pendent groups (the carboxylic acid groups of lysine) at strictly controlled and predetermined intervals along the polymer chain. The reactive pendent groups can be used for derivatization, cross-linking, or conjugation with other molecules. These polymers are useful in producing stable, long-circulating pro-drugs by varying the molecular weight of the polymer, the molecular weight of the PEG segments, and the cleavable linkage between the drug and the polymer. The molecular weight of the PEG segments affects the spacing of the drug/linking group complex and the amount of drug per molecular weight of conjugate (smaller PEG segments provides greater drug loading).
general, increasing the overall molecular weight of the block co-polymer conjugate will increase the circulatory half-life of the conjugate. Nevertheless, the conjugate must either be readily degradable or have a molecular weight below the threshold-limiting glomular filtration (e.g., less than 45 kDa).

In addition, to the polymer backbone being important in maintaining circulatory half-life, and biodistribution, linkers may be used to maintain the therapeutic agent in a pro-drug form until released from the backbone polymer by a specific trigger, typically enzyme activity in the targeted tissue. For example, this type of tissue activated drug delivery is particularly useful where delivery to a specific site of biodistribution is required and the therapeutic agent is released at or near the site of pathology. Linking group libraries for use in activated drug delivery are known to those of skill in the art and may be based on enzyme kinetics, prevalence of active enzyme, and cleavage specificity of the selected disease-specific enzymes (see e.g., technologies of established by VectraMed, Plainsboro, N.J.). Such linkers may be used in modifying the soluble peptides-derived described herein for therapeutic delivery.

According to the invention, soluble peptides may be produced by conventional automated peptide synthesis methods or by recombinant expression. General principles for designing and making proteins are well known to those of skill in the art.

Soluble peptides of the invention may be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols as described in Stewart and Young; Tarn et al., 1983; Merrifield, 1986 and Barany and Merrifield, Gross and Meienhofer, 1979. Soluble peptides of the invention may also be synthesized by solid-phase technology employing an exemplary peptide synthesizer such as a Model 433A from Applied Biosystems Inc. The purity of any given protein; generated through automated peptide synthesis or through recombinant methods may be determined using reverse phase HPLC analysis. Chemical authenticity of each peptide may be established by any method well known to those of skill in the art.

As an alternative to automated peptide synthesis, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a protein of choice is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression as described herein below. Recombinant methods are especially preferred for producing longer polypeptides.
A variety of expression vector/host systems may be utilized to contain and express the peptide or protein coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors (Giga-Hama et al., 1999); insect cell systems infected with virus expression vectors (e.g., baculovirus, see Ghosh et al., 2002); plant cell systems transfected with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with bacterial expression vectors (e.g., Ti or pBR322 plasmid; see e.g., Babe et al., 2000); or animal cell systems. Those of skill in the art are aware of various techniques for optimizing mammalian expression of proteins, see e.g., Kaufman, 2000; Colosimo et al., 2000. Mammalian cells that are useful in recombinant protein productions include but are not limited to VERO cells, HeLa cells, Chinese hamster ovary (CHO) cell lines, COS cells (such as COS-7), W138, BHK, HepG2, 3T3, RIN, MDCK, A549, PC12, K562 and 293 cells. Exemplary protocols for the recombinant expression of the peptide substrates or fusion polypeptides in bacteria, yeast and other invertebrates are known to those of skill in the art and a briefly described herein below. U.S. Pat. No. 6,569,645; U.S. Pat. No. 6,043,344; U.S. Pat. No. 6,074,849; and U.S. Pat. No. 6,579,520 provide specific examples for the recombinant production of soluble peptides and these patents are expressly incorporated herein by reference for those teachings. Mammalian host systems for the expression of recombinant proteins also are well known to those of skill in the art. Host cell strains may be chosen for a particular ability to process the expressed protein or produce certain post-translation modifications that will be useful in providing protein activity. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be important for correct insertion, folding and/or function. Different host cells such as CHO, HeLa, MDCK, 293, WI38, and the like have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the introduced, foreign protein.

In the recombinant production of the soluble peptides-derived of the invention, it would be necessary to employ vectors comprising polynucleotide molecules for encoding the soluble peptides-derived. Methods of preparing such vectors as well as producing host cells transformed with such vectors are well known to those skilled in the art. The polynucleotide molecules used in such an endeavor may be joined to a vector, which generally includes a selectable marker and an origin of replication, for propagation in a host. These elements of the
expression constructs are well known to those of skill in the art. Generally, the expression vectors include DNA encoding the given protein being operably linked to suitable transcriptional or translational regulatory sequences, such as those derived from a mammalian, microbial, viral, or insect genes. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation.

The terms "expression vector," "expression construct" or "expression cassette" are used interchangeably throughout this specification and are meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed.

The choice of a suitable expression vector for expression of the peptides or polypeptides of the invention will of course depend upon the specific host cell to be used, and is within the skill of the ordinary artisan. Methods for the construction of mammalian expression vectors are disclosed, for example, in Okayama and Berg, 1983; Cosman et al, 1986; Cosman et al, 1984; EP-A-0367566; and WO 91/18982. Other considerations for producing expression vectors are detailed in e.g., Makrides et al, 1999; Kost et al, 1999. Wurm et al., 1999 is incorporated herein as teaching factors for consideration in the large-scale transient expression in mammalian cells for recombinant protein production.

Expression requires that appropriate signals be provided in the vectors, such as enhancers/promoters from both viral and mammalian sources that may be used to drive expression of the nucleic acids of interest in host cells. Usually, the nucleic acid being expressed is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the DNA encoding the peptide of interest (i.e., 4N1K, a variant and the like). Thus, a promoter nucleotide sequence is operably linked to a given DNA sequence if the promoter nucleotide sequence directs the transcription of the sequence.

Similarly, the phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene. Any promoter that will drive the expression of the nucleic acid may be used. The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is
targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter. Common promoters include, e.g., the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, [beta]-actin, rat insulin promoter, the phosphoglycerol kinase promoter and glyceraldehyde-3-phosphate dehydrogenase promoter, all of which are promoters well known and readily available to those of skill in the art, can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient to produce a recoverable yield of protein of interest. By employing a promoter with well known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized. Inducible promoters also may be used.

Another regulatory element that is used in protein expression is an enhancer. These are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Where an expression construct employs a cDNA insert, one will typically desire to include a polyadenylation signal sequence to effect proper polyadenylation of the gene transcript. Any polyadenylation signal sequence recognized by cells of the selected transgenic animal species is suitable for the practice of the invention, such as human or bovine growth hormone and SV40 polyadenylation signals.

**Acids nucleic, Vectors, recombinant host cells and uses thereof**

A third object of the invention relates to a nucleic acid encoding an amino acids sequence comprising SEQ ID NO: 1 or a function-conservative variant thereof as described here above for use in the treatment of patient suffering of a cancer with a high expression level of PLCy.

In one embodiment, the invention also relates to a nucleic acid encoding an amino acids sequence comprising SEQ ID NO: 1 or a function-conservative variant thereof as described here above for use in the treatment of patient suffering of a cancer with a poor outcome prognosis according to the prognostic method of the invention.

In another particular embodiment, cancer is selected from the group consisting of leukemia, acute lymphoblastic leukemia, B-chronic lymphocytic leukemia, hairy-cell leukemia, adult T-cell leukemia, prolymphocytic leukaemia of T-cell type or myeloid leukaemia.

In one embodiment, the leukemia is a B-chronic lymphocytic leukemia (CLL).

In a particular embodiment, the CLL is a refractory CLL.

In one embodiment, the invention relates to a nucleic acid encoding an amino acids sequence comprising SEQ ID NO: 1 or a function-conservative variant thereof as described here above for use in the treatment of CLL's patient with a high expression level of PLCy.

In another embodiment, the invention relates to a nucleic acid encoding an amino acids sequence comprising SEQ ID NO: 1 or a function-conservative variant thereof as described here above for use in the treatment of CLL's patient classified in CLL Binet Stage A or in CLL Binet Stages B/C).

In one embodiment, said nucleic acid encoding an amino acids sequence consisting on SEQ ID NO: 1.

Nucleic acids of the invention may be produced by any technique known per se in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination(s).
Another object of the invention is an expression vector comprising a nucleic acid sequence encoding an amino sequence comprising SEQ ID NO: 1 or a function-conservative variant thereof as described here above for use in the prevention or treatment of cancer.

According to the invention, expression vectors suitable for use in the invention may comprise at least one expression control element operationally linked to the nucleic acid sequence. The expression control elements are inserted in the vector to control and regulate the expression of the nucleic acid sequence. Examples of expression control elements include, but are not limited to, lac system, operator and promoter regions of phage lambda, yeast promoters and promoters derived from polyoma, adenovirus, retrovirus, lentivirus or SV40. Additional preferred or required operational elements include, but are not limited to, leader sequence, termination codons, polyadenylation signals and any other sequences necessary or preferred for the appropriate transcription and subsequent translation of the nucleic acid sequence in the host system. It will be understood by one skilled in the art that the correct combination of required or preferred expression control elements will depend on the host system chosen. It will further be understood that the expression vector should contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the host system. Examples of such elements include, but are not limited to, origins of replication and selectable markers. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods or commercially available.

Another object of the invention is a host cell comprising an expression vector as described here above for use in the prevention or treatment of cancer.

According to the invention, examples of host cells that may be used are eukaryote cells, such as animal, plant, insect and yeast cells and prokaryotes cells, such as E. coli. The means by which the vector carrying the gene may be introduced into the cells include, but are not limited to, microinjection, electroporation, transduction, or transfection using DEAE-dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art.

In another embodiment, eukaryotic expression vectors that function in eukaryotic cells are used. Examples of such vectors include, but are not limited to, viral vectors such as retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, poxvirus, poliovirus; lentivirus, bacterial expression vectors, plasmids, such as pcDNA3 or the baculovirus transfer vectors. Preferred eukaryotic cell lines include, but are not limited to,
COS cells, CHO cells, HeLa cells, NIH/3T3 cells, 293 cells (ATCC# CRL1573), T2 cells, dendritic cells, or monocytes.

**Therapeutic methods**

A fourth aspect of the invention relates to a compound which active the PLCyl for use in the treatment of cancer.

As used herein, the term "active PLCyl" denotes the activation of the PLCyl by the phosphorylation of PLCyl in the tyrosine 783 (Y783). Once activated, PLCyl-Y783 catalyzes the formation of inositol 1,4,5-triphosphate (IP3), which, in turn, generates the endoplasmic reticulum-mediated Ca2+ deregulation that provokes caspase-independent PCD.


In another particular embodiment, cancer is selected from the group consisting of leukemia, acute lymphoblastic leukemia, B-chronic lymphocytic leukemia, hairy-cell leukemia, adult T-cell leukemia, prolymphocytic leukaemia of T-cell type or myeloid leukaemia.

In one embodiment, the leukemia is a B-chronic lymphocytic leukemia (CLL).

In a particular embodiment, the CLL is a refractory CLL.

Typically, the compound according to the invention includes but is not limited to a small organic molecule, an antibody, and a polypeptide.
In one embodiment, the compound according to the invention may be a low molecular weight compound, e.g. a small organic molecule (natural or not).

The term "small organic molecule" refers to a molecule (natural or not) of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e.g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 10000 Da, more particularly up to 5000 Da, more particularly up to 2000 Da and most particularly up to about 1000 Da.

In one embodiment, the compound according to the invention may be a kinase, which will activate the PLCγ1 that is to say which will phosphorylate the PLCγ1 in the tyrosine 783 (Y783). Kinases of the invention can be SRC (LCK, FynT, LYN), SYK (SYK, ZAP70), and TEC (ITK, RLK, TEC, BTK) kinases (for review see: Wilde JI et al, 2011).

In another embodiment, the compound according to the invention may be an inhibitor of a phosphatase which will dephosphorylate the PLCγ1 in the tyrosine 783 (Y783). Phosphatases of the invention can be PTP1B, PTPRM (see for review: Phillips-Mason PJ et al, 2011).

Thus, in another embodiment, the invention relates to a compound which active the PLCγ1 by phosphorylation of the tyrosine 783 (Y783) for use in the treatment of cancer.

In one embodiment, the compound according to the invention is an antibody linked to a kinase which activate the PLCγ1 that is to say which will phosphorylate the PLCγ1 in the tyrosine 783 (Y783) may be used. Accordingly, the antibody will be directed against PLCγ1.

Antibody against PLCγ1 can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred. Monoclonal antibodies against PLCγ1 can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975); the human B-cell hybridoma technique (Cote et al., 1983); and the EBV-hybridoma
technique (Cole et al. 1985). Alternatively, techniques described for the production of single chain antibodies (see e.g., U.S. Pat. No. 4,946,778) can be adapted to produce anti-PLCyl single chain antibodies. Compounds useful in practicing the present invention also include anti-PLCyl antibody fragments including but not limited to F(ab')2 fragments, which can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab and/or scFv expression libraries can be constructed to allow rapid identification of fragments having the desired specificity to PLCyl.

Humanized anti-PLCyl antibodies and antibody fragments thereof can also be prepared according to known techniques. "Humanized antibodies" are forms of non-human (e.g., rodent) chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (CDRs) of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Methods for making humanized antibodies are described, for example, by Winter (U.S. Pat. No. 5,225,539) and Boss (Celltech, U.S. Pat. No. 4,816,397).

In another embodiment, the compound according to the invention is an antibody linked to an inhibitor of a phosphatase which will deactivate the PLCyl that is to say which will dephosphorylate the PLCyl in the tyrosine 783 (Y783). Accordingly, the antibody will be directed against PLCyl or against the phosphatase.

Another object of the invention relates to a method for treating cancer comprising administering to a subject in need thereof a therapeutically effective amount of a compound which active the PLCyl.
In one embodiment, said compound is a kinase.
In another embodiment, the cancer is a CLL.
In another embodiment, the CLL is a refractory CLL.

The invention relates to a method for treating patient who has been considered as a poor outcome prognosis for the survival time of a cancer according to the invention comprising administering to a subject in need thereof a compound which active the PLCy1.

In another embodiment, the compound of the invention may be a peptide or a polypeptide derived from the PLCy comprising the tyrosine 783 (Y783) phosphorylated for use in the treatment of cancer.

The peptide or the polypeptide derived from the PLCy comprising the tyrosine 783 (Y783) phosphorylated is able to treat cancer through its properties of decoy receptor.

By "decoy receptor", is meant that the peptides or the polypeptides according to the invention mime the phosphorylated PLCy and is dephosphorylated instead of the PLCy.

Therapeutic composition

A fifth aspect of the invention relates to a therapeutic composition comprising a compound which active the PLCy1 for use in the treatment of cancer.

In another embodiment, the invention relates to a therapeutic composition comprising an agonist as described above for use in the treatment of cancer.

In a one embodiment, the cancer is selected from the group consisting of adrenal cortical cancer, anal cancer, bile duct cancer, bladder cancer, bone cancer, brain and central nervous system cancer, breast cancer, Castleman disease, cervical cancer, colorectal cancer, endometrial cancer, oesophagus cancer, gallbladder cancer, gastrointestinal carcinoid tumors, Hodgkin's disease, non-Hodgkin's lymphoma, Kaposi's sarcoma, kidney cancer, laryngeal and hypopharyngeal cancer, liver cancer, lung cancer, mesothelioma, plasmacytoma, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, oral cavity and oropharyngeal cancer, ovarian cancer, pancreatic cancer, penile cancer, pituitary cancer, prostate cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, skin cancer,
stomach cancer, testicular cancer, thymus cancer, thyroid cancer, vaginal cancer, vulvar
cancer, and uterine cancer.

In another particular embodiment, cancer is selected from the group consisting of
leukemia, acute lymphoblastic leukemia, B-chronic lymphocytic leukemia, hairy-cell
leukemia, adult T-cell leukemia, prolymphocytic leukaemia of T-cell type or myeloid
leukaemia.

In one embodiment, the leukemia is a B-chronic lymphocytic leukemia (CLL).

In a particular embodiment, the CLL is a refractory CLL.

Any therapeutic agent of the invention may be combined with pharmaceutically
acceptable excipients, and optionally sustained-release matrices, such as biodegradable
polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and
compositions that do not produce an adverse, allergic or other untoward reaction when
administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable
carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent,
encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage
and the regimen naturally depend upon the condition to be treated, the severity of the illness,
the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical,
oral, intranasal, parenteral, intraocular, intravenous, intramuscular or subcutaneous
administration and the like.

Particularly, the pharmaceutical compositions contain vehicles, which are
pharmaceutically acceptable for a formulation capable of being injected. These may be in
particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium,
potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry,
especially freeze-dried compositions which upon addition, depending on the case, of sterilized
water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various
parameters, and in particular as a function of the mode of administration used, of the relevant
pathology, or alternatively of the desired duration of treatment.

In addition, other pharmaceutically acceptable forms include, e.g. tablets or other
solids for oral administration; time release capsules; and any other form currently can be used.
Pharmaceutical compositions of the present invention may comprise a further therapeutic active agent.

The present invention also relates to a kit comprising a compound according to the invention and further a therapeutic active agent.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

**FIGURES:**

**Figure 1: PLCyl is over-expressed in CLL.**

(A) PLCGl mRNA levels of normal B-cells (from healthy donors n=11) and B-lymphocytes from CLL patients (n=50). The numbers refer to the mean PLCGl transcript expression. GUSB mRNA level was used to normalize the data. (B) The PLCGl mRNA expression level measured in the panel of B-lymphocytes used in (A) was represented by discriminating the CLL patients by their clinical Binet Stage. The numbers refer to the mean PLCGl transcript expression. (C) The PLCGl mRNA expression level measured in the panel of normal B-cells and in CLL B-lymphocytes used in (A) was represented by discriminating the CLL patients by their TP53 and ATM gene status (functional or dysfunctional). GUSB mRNA level was used to normalize the data. (D) The PLCGl mRNA expression level measured in the panel of normal B-cells and in CLL B-lymphocytes used in (A) was represented by discriminating the CLL patients by their ZAP70 levels.

**Figure 2: PLCyl controls PKHBl-induced PCD.**

(A) IP1 production was quantified in PKHB1-treated normal and CLL B-cells. The histogram depicts the mean ± s.d. (n=7). (B) IP1 generation was determined in PKHB1-treated CLL B-lymphocytes pre-incubated with vehicle or the PLC inhibitor U73122. Data in the plot are the mean ± s.d. (n=5 patients). Immunoblotting confirmed that U73122 inhibited PLCyl-Y783 phosphorylation. Equal loading was confirmed by PLCyl probing. (C) Cell death was measured in untreated (Control) or PKHB1-treated CLL cells incubated with vehicle or U73122. Data are presented as the mean ± s.d. (n=10). (D) The effects of down-
regulating PLCyl by shRNA on CD47-mediated PCD were assessed in leukemic B-cells transduced with scrambled shRNA (Scr) or two shRNAs targeting PLCyl (shRNA PLCylA and B). Data are presented as the mean ± s.d. (n=6). Changes in PLCyl expression were observed by immunoblotting. Equal loading was confirmed by α-tubulin detection.

Figure: 3. PKHBl reduced in vivo CLL tumor burden by inducing PLCyl activation.

PLCyl-Y783 phosphorylation was assessed in tumors obtained from the vehicle (Control) and PKHBl-treated mice. Phospho-PLCyl was quantified using flow cytometry by the mean fluorescence intensity. The data are presented as the mean ± s.d. (n= 6).

EXAMPLE:

Material & Methods

Patients, B-cell purification, and culture conditions.

CLL patients were diagnosed according to classical morphological and immunophenotypic criteria. This includes clinical Binet staging and the biological parameters IGVH mutational status, CD38, and ZAP-70 levels. Deletions of 17pl3, 1lq22 andl3ql4 and trisomy 12 were detected by fluorescence in situ hybridization (FISH) using the Vysis LSI p53 / LSI ATM and LSI D13S319 / LSI 13q34 / CEP 12 Multi-color Probe kits (Abbott Molecular). The functional status of TP53 was determined by flow cytometry as previously published (Le Garff Tavernier et al. Functional assessment of p53 in chronic lymphocytic leukemia. Blood Cancer J. 2011 Feb;1(2):e5. doi: 10.1038/bcj.2011.3). TP53 mutations were detected using high resolution melting (HRM) and confirmed by sequencing the amplicons with an abnormal HRM profile. Mononuclear cells were purified and selected using magnetic microbeads (Miltenyi Biotec). B-lymphocytes, MEC-1 and M210B4 bone marrow stromal cells (ATCC) were cultured in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin- streptomycin). This CLL work was approved by the Institutional Ethics Committee at Pitie-Salpetriere Hospital.

Peptide synthesis.

Peptides were synthesized by SPPS on a preloaded Fmoc-Lys(Boc)-Wang or Fmoc-D-Lys(Boc)-Wang resins (Merck Chemicals). The syntheses were performed on an ABI 433A...
peptide synthesizer (Applied Biosystems) at the 0.25 mmol scale. Peptides were purified by reverse phase HPLC on an ACE (C8, 5 μm, 300 A, 10 mm x 250 mm) column (Waters) with a gradient elution [0.1% (v/v) TFA in acetonitrile] in aqueous 0.1% (v/v) TFA. Homogeneous fractions were pooled and lyophilized after confirming a purity of greater than 95% by analytical HPLC.

PKHB1, 4N1K, and 4NGG: All Na-Fmoc-amino acids (10 equiv.) were coupled after activation with HBTU (0.45 M in NMP) in the presence of DIEA (6 equiv., 2 M in NMP) (10 equiv., 1:1). N-Fmoc deprotection was performed using piperidine (20%> in NMP) and was monitored by measuring the UV absorbance of the released N-(9-fluorenylmethyl)-piperidine group at 301 nm. PKHB1, MALDI-TOF MS (4N1K (SEQ ID NO:1) with first lysine amino acid in (D) form and last lysine amino acid in form (D)) (MH+) calculated: 1383.8, (MH+) actual: 1385.3. 4N1K, MALDI-TOF MS: K-R-F-Y-V-V-M-W-K-K (SEQ ID NO: 1) (MH+) calculated: 1383.8, (MH+) actual: 1385.0. 4NGG, MALDI-TOF MS: K-R-F-Y-G-G-M-W-K-K (SEQ ID NO: 2) (MH+) calculated: 1300.70, (MH+) actual: 1300.4.

Peptide degradation assays.

4N1K or PKHB1 (10μg/mL), diluted in a mixture 1/4 human serum/RPMI 1640, were incubated at 37°C at different times. Then, mixed with ethanol and 5 ml of 1M NaOH and incubated at 4 °C for at least 15 min to precipitate serum proteins. The supernatant was collected, injected in an HPLC and the soluble peptide was eluted by a linear gradient 5 to 50% ACN [0.1% (v/v) TFA in acetonitrile] in aqueous 0.1% (v/v) TFA. The concentration of the peptide was calculated by the integration of the absorbance at 220 nm as a function of retention time.

Flow cytometry.

 Annexin V-APC (0.1 μg/μl) was used for the assessment of PS exposure, propidium iodide (PI, 0.5 μg/ml) for PCD analysis, and tetramethylrhodamine ethyl ester (TMRE, 20 nM) for ΔΨm quantification. PCD was recorded in a FACSCanto II (BD Biosciences) in the total population (10,000 cells) and data were analyzed using FlowJo software. CD5+/CD5- discrimination was determined with anti-CD5-PE-Cy7 mAb (clone L17F12; BD Biosciences), calreticulin cell surface exposure was recorded with anti-calreticulin-PE (clone FMC75; Assay Designs), CD47 analysis was assessed with conjugated anti-CD47-PE (clone B6H12; BD Biosciences), p21 with anti-p21-FITC (clone EA10; Calbiochem), and p53 with
anti-p53-PE (clone DO-7; BD Biosciences). QuantiBrite flow cytometry System (BD Biosciences) was used to assess the number of CD47 molecules expressed on B-lymphocytes.

Cell death induction and inhibition.

To induce death, cells were treated for 2 h with PKHB1 (200 µM), 4N1K (300 µM), or 4NGG (300 µM). To control for caspase-dependent apoptosis, cells were incubated for 24 h with etoposide (250 µM). For the inhibition assays, BAPTA-AM (20 µM), BAPTA (5 mM), Ru360 (750 nM), dantrolene (40 µM), 2-minoethoxydiphenyl borate (2-APB, 60 µM), U73122 (200 nM), the broad spectrum caspase inhibitors Q-VD-OPh (QVD, 10 µM) and z-VAD-FMK (50 µM) or the specific caspase inhibitors z-DEVD-FMK (50 µM, caspase 3/7), z-LEHD-FMK (50 µM, caspase-8) and z-IETD-FMK (50 µM, caspase-9) were added 30 min before inducing PCD.

Protein extraction and immunoblotting.

Cell fractions were lysed in 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA supplemented with anti-protease and anti-phosphatase cocktails (Roche). The protein concentration was determined using the Bio-Rad DC kit, and 70 µg of protein was loaded in linear SDS-PAGE gels. After blotting, nitrocellulose filters were probed with primary antibodies against CD47 (48), activated caspase-3, PLCyl, and PLCyl-Y783 (Cell Signaling) and a-tubulin. Immunoreactive proteins were detected using HRP-conjugated secondary antibodies and visualised with the ECL system. Immunoblot images were acquired on a Bio-Imaging System MF-ChemiBIS 4.2 (DNR Bio-Imaging Systems). PLCyl-Y783 was quantified using Multi Gauge 3.0 software (Fujifilm Life Sciences). The optical density was normalized to the background and was expressed relative to the untreated cells (set at 1.0).

Electron microscopy.

CLL cells were fixed with 2% glutaraldehyde in phosphate buffer (pH 7.4) for 2 h at RT, washed, and postfixed in 2% Os04 before being embedded in Durcupan® resin. Ultrathin sections stained with uranyl acetate and lead citrate were analyzed with a transmission electron microscope (Carl Zeiss Microimaging).

Ca2+ mobilization assessment. B-cells were loaded for 30 min at 37°C with Fura2-AM and pluronic acid (Invitrogen) in glass bottom dishes (MatTek Corporation), washed in Ringer solution, and activated with PKHB1 or ionomycin (positive control) at the indicated concentrations. Cells were excited at wavelengths 340 and 380 nm. The fluorescence
emissions of several cells were simultaneously recorded at a frequency of 1 Hz using a dual excitation fluorometric imaging system (TILL-Photonics). Fluorescence values were analyzed using Origin software (OriginLab Corporation) and were normalized to the first value according to the equation (F/F0)-1, where 'F' is the fluorescence at a specific timepoint and 'F0' is the fluorescence at time 0. Ca2+ curves were normalized to the maximal response elicited by ionomycin. The area under the curve, representing the extent of Ca2+ mobilization, was calculated for each cell and graphed.

**qRT-PCR.**
10 Total RNA was extracted from normal or CLL B-lymphocytes using the Nucleospin® RNA II kit (Macherey-Nagel). cDNA was prepared using Superscript® II reverse transcriptase (Life Technologies). Quantitative RT-PCR was performed using TaqMan® Gene Expression Assays (Life Technologies) for CD47, PLCB1 (phospholipase C, beta 1), PLCB2 (phospholipase C, beta 2), PLCB3 (phospholipase C, beta 3), PLCB4 (phospholipase C, beta 4), PLCG1 (phospholipase C, gamma 1), PLCG2 (phospholipase C, gamma 2), ITPR1 (inositol 1,4,5-trisphosphate receptor, type 1), ITPR2 (inositol 1,4,5-trisphosphate receptor, type 2), ITPR3 (inositol 1,4,5-trisphosphate receptor, type 3), RYR1 (ryanodine receptor 1), RYR2 (ryanodine receptor 2), RYR3 (ryanodine receptor 3), STIM1 (stromal interaction molecule 1), STIM2 (stromal interaction molecule 2), ORAI1 (calcium release-activated calcium modulator 1), ORAI2 (calcium release-activated calcium modulator 2) and ORAI3 (calcium release-activated calcium modulator 3). PCR reactions were performed in triplicate using TaqMan® Universal PCR Master Mix (Life Technologies). The products were amplified in a ViiA7 Real-time PCR System (Life Technologies) at 60°C for 40 cycles. Data were analyzed using the comparative threshold cycle method. The expression of GUSB or ABL (49) was utilized to normalize the data.

**PLCyl-Y783 flow cytometry.**
At various time points after PKH21 treatment, 5x105 cells were fixed in ethanol and permeabilized in Triton X-100. Then, cells were saturated in PBS+Triton X-100+10% FCS, incubated with anti-PLCyl-Y783 (Cell Signaling), and detected using an anti-rabbit IgG conjugated with Alexa Fluor 488. The data on the entire cell population were collected on a FACSCanto II flow cytometer. PLCyl-Y783 was quantified based on the mean fluorescence intensity (MFI) for each sample.
IP3 quantification.

IP3 was measured using IP1 as a surrogate (E. Trinquet et al, 2006) with an HTRF assay (Cisbio). The assays were performed in triplicate in 96-well plates, and the signal was quantified on an M1000 plate-reader (TECAN).

Vectors and lentiviral transduction.

To down-regulate PLCy1, we utilized two shRNAs (A: CTGAGAAATACGTGAACAA, SEQ ID NO: 3; B: AGTGAATGCTAGCACAGAAA, SEQ ID NO: 4) and a control scrambled shRNA (ACGATAGTCGGTCGATAAA, SEQ ID NO: 5). Forward and reverse oligonucleotides (Invitrogen) were annealed and cloned into the pLVTHM lentiviral vector (Addgene 12247). Virus was produced in 293T cells after the CaCl2-mediated transient transfection of the lentiviral constructs and the packaging plasmids pMD2.G and psPAX-2 (Addgene 12259 and 12260, respectively). Forty-eight hours after the transfection, the lentiviral supernatants were harvested, clarified by filtration and immediately used to transduce 5x106 primary CLL or MEC-1 cells. At 72 h post-infection, GFP-positive cells were sorted on a FACSVantage cytofluorimeter (BD Biosciences) to analyze PCD.

CLL xenograft model.

MEC-1 cells (3x106) were injected subcutaneously into NOD scid gamma (NSG) mice (Charles River). When the tumor volume reached 100 mm3, the mice received intraperitoneal injections of PBS, PKHB1, or 4N1K (400 µg in 200 µl PBS) once a week. Tumor size was measured with a caliper, and tumor volume was calculated using the formula (length x width2)/2 and expressed in mm3. Alternatively, XenoLight RediJect 2-DG-750 Probe (Caliper) was injected into the sinus retro-orbital to visualize tumor glucose uptake, which reflects cell proliferation. Fluorescence was measured using an in vivo imaging system FX Pro (Kodak), and pictures were analyzed with Carestream MI software. These in vivo studies were approved by the Institutional Animal Care and Use Committee at Bichat hospital.

To determine hemoglobin levels, tumors were excised from euthanized mice, weighed, and homogenized in PBS. After centrifugation, formic acid was added to the supernatant, and the hemoglobin concentration was calculated based on the absorbance at 405 nm.

To assess PCD, calreticulin exposure and PLCy1-Y783, dissociated tumors were digested in medium containing Liberase-TL (Roche), DNase-I (Calbiochem), and
Collagenase-IV (Life technologies). Single-cell suspension, obtained by filtering through a 70 µM cell strainer, was analyzed by flow cytometry.

**Immunohistochemistry.**

Tumor vessels were observed on 5 µη cryo-sections by targeting mouse CD31 protein. Briefly, sections were pre-treated by blocking endogenous peroxidase with 0.3% H202, and endogenous Avidin/Biotin (Vector labs). Then, sections were incubated with a rat anti-mouse CD31 (BD Biosciences) followed by a goat F(ab')2 anti-rat IgG(H+L)-Biotin (Southern Biotech). mCD31 was visualised with Vectastain Elite ABC kit (Vector labs) plus DAB chromogenic substrate (Interchim). Sections were counterstained with hematoxylin and mounted with ImmunoMount (Thermo Scientific).

**Statistical analysis.**

Mann-Whitney tests and Student's t-tests were performed using GraphPad Prism software. Except otherwise noted, our assessments included an equal number of stage A and stage B/C CLL patients.

**Results**

**Example 1: Efficacy of TSPI-derived peptides that binds CD47 to kill leukemic CD5+ B-lymphocytes and associated mechanism.**

PKHB1, a human-serum stable TSPI-derived peptide that binds CD47, selectively kills leukemic CD5+ B-lymphocytes, including those from individuals with dysfunctional TP53.

To explore the potential of CD47 activation by peptide targeting in primary CLL B-lymphocytes, we first analyzed the triggering with 4N1K. We observed that after only 2 h of treatment with 4N1K, 43% of CLL cells obtained from a cohort of 80 patients were AnnexinV/PI positive (data not shown). This cohort of patients included individuals with positive and adverse prognostic features, including those with CLL Binet Stages B and C classifications, unmutated IGHV, positive ZAP70 and CD38 expression, 11q, 13q, 17p deletions, and trisomy 12 (data not shown). In contrast to 4N1K, the negative control analogue 4NGG was ineffective at inducing cytotoxicity in CLL. Moreover, 4N1K incubation had no effect on the normal B-lymphocytes isolated from healthy donors (data not shown).
The specificity of 4N1K to induce PCD in leukemic cells, but not in normal B-lymphocytes, led us to further investigate the therapeutic potential of 4N1K.

The major weakness in the use of peptides as therapeutic agents is their short in vivo half-life due to protease degradation. Using an HPLC approach, we observed that a 1 h incubation in human serum resulted in more than 90% of the 4N1K peptide being degraded (data not shown). For that reason, we sought to improve 4N1K stability in human serum by replacing, at selected positions, natural L-amino acids by their D-counterparts. The N- and C-terminal lysines of 4N1K, which were introduced to improve solubility, are not related to the CD47 interaction site of the peptide (VVM motif). Therefore, we replaced these two terminal residues with their D-analogues (data not shown). The novel decapeptide, named hereafter PKHB1, was not degraded after long-time incubation in human serum (data not shown), but maintained the solubility and the CD47 link core of 4N1K. Moreover, the induction of PCD in CLL cells with PKHB1 was more potent than with 4N1K, both in terms of concentration level and incubation times (data not shown).

Consequently, we assessed the effect of PKHB1 on B-cells from 20 healthy donors and in our entire cohort of 80 CLL patients. We used PKHB1 at 200 µM, a concentration that resulted in similar PCD responsiveness in CLL cells as 300 µM of 4N1K. PKHB1-treated CLL cells underwent a rapid cell viability loss (~49% in 2 h). Similarly to 4N1K, PKHB1 had no effect on B-lymphocytes from healthy donors. More significant from a therapeutic perspective, PKHB1 treatment significantly killed the CD5+ tumor B-cells while sparing the residual CD5− B-lymphocytes of the CLL patient (data not shown). These findings reveal that PKHB1 selectively kills the leukemic B-cells.

Because no adapted therapeutic is currently being used to treat CLL refractory patients with dysfunctional TP53, we compared the response of the functional and dysfunctional TP53 CLL cells (data not shown) to PKHB1 and the P53-dependent PCD inducer etoposide. In cells with a functional TP53, a 24 h etoposide treatment was required to provoke a PCD comparable to that obtained after 2 h incubation with PKHB1. B-lymphocytes with a dysfunctional TP53, which are resistant to etoposide, were killed after 2 h incubation with PKHB1 (data not shown). Thus, targeting CD47 with PKHB1 efficiently killed CLL cells, including those from dysfunctional TP53 individuals.

Next, given that the microenvironment plays a critical role in the progression and drug resistance of tumors, we analyzed whether PKHB1-mediated PCD is modulated by the presence of either bone marrow stromal cells or sCD40L and IL-4, two anti-apoptotic cytokines generated by lymphoid tissue. Under these conditions, we observed that the
responsiveness of CLL cells to PKHBl remained unchanged (data not shown). In contrast, the induction of PCD by etoposide was significantly diminished. These data confirm that, contrary to other forms of cell death, the PKHBl-mediated killing is not down-modulated by the survival stimuli provided by the lymphocyte microenvironment.

PKHBl-induced caspase-independent PCD in CLL cells.

We next assessed the mechanism regulating PKHBl-induced PCD in CLL. In contrast to etoposide-induced caspase-dependent PCD, the features of PKHBl-induced killing, including phosphatidylserine (PS) exposure, cell viability loss, and mitochondrial transmembrane potential (ΔΨm) disruption, were not prevented by pre-incubation with broad spectrum or specific caspase inhibitors (data not shown). The main effector caspase-3 remained as an inactive pro-enzyme after treatment of CLL cells with PKHBl (data not shown), demonstrating that this CD47 peptide agonist induces caspase-independent PCD. Moreover, PKHBl also provoked in CLL cells a caspase-independent exposure of calreticulin, a pro-phagocytic protein that promotes cell removal. Note that, in contrast to the constitutive expression of calreticulin in acute leukemia cells and solid tumors, this protein was not detected on the surface of untreated CLL cells (data not shown). Thus, PKHBl activated a caspase-independent PCD that enabled dying CLL cells to be recognized and engulfed.

PKHBl provoked PCD in CLL via ER-stress, Ca2+ overload, and mitochondrial damage.

From the above experiments, we learned that PKHBl treatment triggered PCD in leukemic but not in normal B-lymphocytes. As demonstrated by qRT-PCR, immunoblot, and flow cytometry approaches, this different response to PKHBl is not correlated with a different expression level of CD47 (data not shown). Therefore, we searched for a PCD mechanism specifically activated by PKHBl in CLL, but not in normal B-lymphocytes. Because morphological alterations in intracellular organelles correlate with the different forms of PCD, we first performed an ultra-structural analysis of PKHBl-treated normal and CLL B-cells. This approach revealed that PKHBl treatment induced a significant dilation of the ER in leukemic B-lymphocytes (data not shown). The ER plays a key role in modulating Ca2+ mobilization, we therefore considered a potential role of Ca2+ in the PKHBl-mediated PCD. This role was confirmed by pre-treating the CLL cells with the Ca2+ chelator BAPTA prior to incubation with PKHBl. This pre-treatment abolished the PKHBl-mediated killing.
Moreover, pre-treatment with BAPTA-AM (intracellular Ca$^{2+}$ chelator), 2-APB (inositol-1,4,5-triphosphate ER receptor (IP3R) inhibitor) or dantrolene (ryanodine ER receptor (RyR) inhibitor) significantly decreased PKHB1-mediated death (data not shown). Altogether, these findings revealed that PKHB1 treatment induces an ER stress, which provokes Ca$^{2+}$-mediated PCD in the CLL cells.

Next, we compared Ca$^{2+}$ mobilization in normal and CLL B-lymphocytes. In normal B-cells, PKHB1 triggered a classical Ca$^{2+}$ signal with a rapid transient increase in intracellular Ca$^{2+}$ ([Ca$^{2+}$]) that returned to baseline (data not shown). In CLL cells, PKHB1 incubation triggered a strong and sustained Ca$^{2+}$ mobilization, which did not return to basal level (data not shown). Thus, PKHB1 provoked a different Ca$^{2+}$ mobilization in normal and leukemic cells. Moreover, Ca$^{2+}$ mobilization and PCD increased in PKHB1-treated CLL cells in a dose-dependent manner (data not shown). These results provided a direct link between Ca$^{2+}$ overload and induction of PKHB1-mediated PCD in CLL.

An important question arising from our above data is how the PKHB1-induced sustained Ca$^{2+}$ mobilization affects CLL cell viability. Because regulated Ca$^{2+}$ entry into mitochondria is required to maintain intracellular Ca$^{2+}$ homeostasis and avoid PCD, we analyzed whether the sustained Ca$^{2+}$ mobilization induced by PKHB1-treatment in CLL affected mitochondria. The pharmacological blockade of the Ca$^{2+}$ entry into mitochondria with the mitochondrial Ca$^{2+}$ uniporter (MCU) inhibitor Ru360 moderated the ΔΨm loss induced by PKHB1-treatment, precluding PCD. Thus, it seems that the treatment of CLL cells with PKHB1 induced a Ca$^{2+}$ overload that provoked PCD via mitochondrial damage.

**Example 2: Identification of the PLCy1 as a signal transduction protein in the mechanism of the TSPI-derived peptides and its usefulness as a biomarker.**

PLCy1 is over-expressed in CLL with increased expression correlated to disease progression.

To unravel the molecular mechanism responsible for the sustained Ca$^{2+}$ mobilization leading to PCD in CLL, we performed a quantitative RT-PCR analysis of the major genes involved in the regulation of cellular Ca$^{2+}$ homeostasis. Out of 17 different genes tested, only PLCy1 mRNA was highly expressed in CLL cells compared to normal B-lymphocytes (data not shown). Taking advantage of our important cohort of CLL patients we confirmed that, compared to normal B-cells, the level of PLCy1 mRNA was on average more than three times higher in CLL cells compared to normal B-cells (Figure 1A). This result was fully supported
by protein expression data (data not shown). Because the panel of PLCyl mRNA expression in CLL cells was quite scattered (Figure 1A, right panel), we wondered if there was a correlation between the expression of the PLCyl mRNA and the progression of the disease. The clinical analysis of the CLL patients corroborated that the PLCyl mRNA is more expressed in patients with advanced disease (Binet Stages B/C), than in patients with indolent CLL (Binet Stage A) (Figure 1B). This result revealed that the expression of the PLCyl mRNA could be considered as a marker of CLL severity.

Moreover, in the high-risk CLL patients with a deleted ATM gene (del11q) or a dysfunctional TP53 gene (placed in the 17p region), the PLCyl mRNA is more expressed than in patients with functional ATM and TP53 genes (Figure 1C). Thus, the expression of PLCyl mRNA could be considered a marker of CLL severity. This is fully supported by the results obtained by measuring the levels of the surrogate CLL marker ZAP70. In the CLL patients with high levels of ZAP70 (bad prognosis), the PLCyl mRNA is more expressed than in patients with low ZAP70 levels (good prognosis) (Figure 1D).

ZAP70 (70-kDa zeta-associated protein) is an intracellular tyrosine kinase associated with the B-cell receptor in CLL. The expression of ZAP-70 (> or =20% of B cells) has been associated with an increased risk for an adverse outcome in B-cell CLL and is considered an important risk factor in these patients. ZAP-70 expression, if present, is constant throughout the patient's clinical course and thus is a valid risk marker regardless of when it is evaluated.

The sustained activation of PLCyl controls PKHBl-mediated PCD in CLL.

PLCyl catalyzed the formation of IP3, which binds to its receptors on the ER and, subsequently, triggers store-operated Ca2+ release. PLCyl is over-expressed in CLL; therefore, the disparate Ca2+ mobilization recorded in the PKHBl-treated normal and CLL B-cells could be related to the differential activation of PLCyl. Measuring PLCyl activation by its phosphorylation at Y783, we observed that PLCyl was rapidly phosphorylated before returning to basal levels in the PKHBl-treated normal B-cells. However, PLCyl Y783 phosphorylation in the PKHBl-treated CLL cells remained high for at least 2 h (data not shown). Note that the kinetic of PLCyl phosphorylation matched the kinetic of Ca2+ response measured in normal and CLL B-cells. Next, by measuring IP1 production in PKHBl-treated CLL cells, we validated that the sustained PLCyl phosphorylation correlated with the increased catalytic activity of the protein in the CLL B-cells (Figure 2A). Moreover, the IP1 production following PKHBl incubation was reduced using the PLC inhibitor U73122
(Figure 2B), emphasizing the correlation between the over-activation of PLCy1 triggered by PKHBl and Ca2+ overload measured in CLL cells. Consequently, the pre-incubation of CLL cells with U73122 prior to PKHBl treatment significantly decreased PCD (Figure 2C).

In addition to the above pharmacological approach, we assessed the role of PLCy1 in PKHBl-mediated PCD in primary CLL by using lentiviral down-regulation of PLCy1 with two independent shRNAs. As shown in Figure 2D, the down-regulation of PLCy1 desensitized the primary CLL lymphocytes to PKHBl-induced PCD. Overall, these findings confirm the key role of PLCy1 in the CD47 peptide targeting-mediated PCD in CLL.

Treatment with PKHBl reduced CLL tumor burden in vivo.

Finally, from a therapeutic perspective, we analyzed the in vivo effect of PKHBl on tumor growth of MEC-1 cells in NOD scid gamma (NSG) mice. MEC-1 is an established CLL cell line with dysfunctional TP53 that is resistant to etoposide but responds to PKHB1 in exactly the same way as primary CLL cells (data not shown). NSG mice were subcutaneously injected with MEC-1 cells, and the tumors were allowed to grow for 14 days until they reached 100 mm³. The mice were then treated intraperitoneally once a week with the vehicle or PKHBl. After two weeks of treatment, PKHBl-treated, but not vehicle-treated, mice had a significantly decreased tumor growth rate (-50% of tumor volume diminution). However, as could be expected by the instability of the peptide, the 4N1K treatment was unable to reduce tumor volume (data not shown). The analysis of the tumors from the PKHBl- and vehicle-treated mice revealed similar hemoglobin levels within the engrafted tumors, indicating that PKHBl did not provoke anemia (data not shown). Moreover, anti-CD31 labeling performed in tumors from PKHBl- and vehicle-treated mice revealed that the decreased tumor growth rate induced by PKHBl was not a consequence of anti-angiogenic effects (data not shown).

Indeed, compared to the vehicle, the mice receiving PKHBl showed increased levels of PLCy1-Y783 phosphorylation (Figure 3), as well as an enhanced calreticulin exposure (data not shown) and a loss of cell viability loss (data not shown) within the engrafted tumors. Together, these findings confirmed that the treatment with PKHBl eliminates CLL cells in vivo by inducing PLCy1-mediated PCD.

Example 3: Soluble peptides agonist of CD47

The structure of 4N1K peptide is as followed:
4N1K is a undecapeptide with the following sequence: K-R-F-Y-V-V-M-W-K-K (SEQ ID NO:1).

To improve peptide solubility, stability and pharmacological properties, the 4N1K is modified by chemical modifications that are established on the following 2 models:

Model 1:
A1-A2-A3-A4-A5-A6-A7-A8-A9-A10

Model 2:

In model peptide 1, A (1 to 10) correspond to nothing and/or natural amino acids and/or non natural amino acids or amino acids derivatives as for example prolinoamino acids [Mothes C et al, 2008], beta-amino acids [Moumne R et al, 2007], cyclopropylamino acids [Joosten A et al, 2009], N-methylamino acids [Sagan S et al, 2004], and/or alpha-alpha disubstituted amino acids, and/or disubstituted beta-amino acids (Beta2,2, beta3,3 ou beta2,3) and or aza-amino acids (Proulx, C et al, 2001) or azidolysine [Larregola M et al, 2001]. The absolute configurations of the stereogenic centers are not indicated since all the amino acids are natural amino acids or synthetically obtained amino acids, enantiomerically pur from (L) or (D) series or used as racemate.
For model 2, the absolute configurations of the stereogenic centers are not indicated since all the amino acids are natural amino acids or synthetically obtained amino acids, enantiomerically pur from (L) or (D) series or used as racemate.

The R groups (1 to 10) correspond to amino acids side chains that can be natural or synthetic amino acids side chains. For the glycine residu, this side chain correspond to a hydrogen atom.

P1 and P2 correspond to functions respectively on the N-terminal and C-terminal sides of the peptides.

Thus, P1 can be an amine function (P1 = -NR11, where R11 is a hydrogen atom or an alkyle chain (similar to methyl, ethyl or benzyl), a carboxylic function (P1 = -C02H), a carboxamide function (P1 = -CONR12R13, where R12 and R13 are hydrogen atoms and/or alkyle chains (similar to methyl, ethyl or benzyl) or an amine function, or an azido function (N3).

Thus, P2 can be an amine function (P2 = -NR11, where R11 is a hydrogen atom or an alkyle chain (similar to methyl, ethyl or benzyl), a carboxylic function (P2 = -C02H), a carboxamide function (P2 = -CONR12R13, where R12 and R13 are hydrogen atoms and/or alkyle chains (similar to methyl, ethyl or benzyl) or an amine function, or an azido function (N3).

Y groups (1 to 10) correspond to hydrogen atoms and/or methyl groups, and/or natural amino acids side chains.

Y1 can also be a protecting group such as an acetamide, a benzamide, a benzyloxy carbonyl, a tertbutyloxycarbonyl, a phenylfluorenylmethoxycarbonyl protecting groups.

Peptides and analogues are synthesized by SPPS or LPPS with Boc, Fmoc or Z strategies. The protonation states of all functions (amino groups, carboxylic functions, guanidinium,...) is depending on the syntheses and purifications procedures and can be different from the one indicated on the schemes.

I- Short analogues based on model 1 : A1-A2-A3-A4-A5-A6-A7-A8-A9-A10

To improve the solubility and the stability of the octapeptide R-F-Y-V-V-M-W-K (SEQ ID NO:3), the following chemical modifications are realized:

a) Mixed salts:
N-methylmorpholine and chlorhydrate or trifluoroacetylcarboxylate mixed salts:

R7 refers to methionine, methionine sulfoxide, methionine sulfone or alanine or butylglycine or lysine side chain.

A2 refers to lysine or azidolysine or arginine or bis-ornithine [Aussedat B et al, 2006] or bis-arginine or beta-2-homo lysine, or beta-2-homoarginine or beta-2-bis-homoornithine or beta-2-bis-homoarginine. The N-terminal amine function can be free or protected by Boc, Fmoc or Cbz groups, and/or can be N-methylated.

A9 refers to a lysine or arginine or bis-ornithine or bis-arginine or a beta-3-homolysine, or a beta-3-homoarginine or a beta-3-bis-homoornithine or a beta-3-bis-homoarginine. The A9 carboxylic function is free or protected as a carboxamide (P2 = \(-\text{CONR}_{12}\text{R}_{13}\), where R12 and R13 are hydrogen atoms and/or alkyls groups such as methyl, ethyle, benzyl groups and derivatives).

Free or N-methylated amine functions can be obtained as Chlorhydrate or TFA salts (n is variable, depending on the nature of A2, R7 or A9).

Free carboxylic function can be obtained (but not necessarily) as N-methylmorpholine salts (p = 1).

PKHB3 is an example of peptide from these series where A2 and A9 correspond respectively to beta-2-homoarginine and beta-3-homolysine:
b) Tetraphenylborate salts:

R7 corresponds to methionine, methionine sulfoxide, methionine sulfone or alanine or butylglycine or lysine side chains.

A2 corresponds to lysine or azidolysine or arginine or bis-ornithine or bis-arginine or a beta-2-homo-lysine, or a beta-2-homoarginine or a beta-2-bis-homoornithine or a beta-2-bis-homoarginine. The free N-terminal amine function can also be protected by a Boc, Fmoc or Cbz groups, and/or N-methylated.

A9 corresponds to lysine or arginine or bis-ornithine or bis-arginine or beta-3-homo-lysine, or beta-3-homoarginine or beta-3-bis-homoornithine or beta-3-bis-homoarginine. The carboxylic function of A9 is free or protected as a carboxamide (PI = -CONR12R13, where R12 and R13 are hydrogen atoms and/or alkyl groups such as methyl, ethyl, benzyl derivatives).

The free or N-methylated amine functions are obtained as tetraphenylborate salts, m being variable, depending on A2, R7 or A9.

The structure and the syntheses of the peptides below are given as examples:
c) **N and C- termini protections:**

The peptides described above are also prepared with N- and C-termini protecting groups.

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**d) Various chemical modifications:**

A2-A3-A4-A5-A6-A7-A8-A9

N-methyl amino acids are introduced on various positions as described here below:
R7 is described above.

e) (D)-amino acids:

The introduction of (D)-amino acids in peptide sequences stabilize peptides towards proteolytic degradations and thus enhance its pharmacological properties. (D)-amino acids are introduced on the N terminus and/or on the C-terminus and/or instead of one, two, three, four, five, six or seven residues in peptide sequence:

(D)R-F-Y-V-V-M-W-K
R-(D)F-Y-V-V-M-W-K
R-F-(D)Y-V-V-M-W-K
R-F-Y-(D)V-V-M-W-K
R-F-Y-V-(D)V-M-W-K
R-F-Y-V-V-(D)M-W-K
R-F-Y-V-V-M-(D)W-K
R-F-Y-V-M-(D)W-K
**Retro-inverso sequences:**

The retro-inverso sequences led to peptides partially or fully modified with (D)-amino acids keeping however the spatial orientations of crucial amino acids side chains. The following peptides are prepared, incorporating some chemical modifications in order to maintain peptide polarity, around diamine and diacid, introduced respectively on the C-terminus and N-terminus sides:

**Inverso Peptides:**

\[(D)R-(D)F-(D)Y-(D)/(D)\] W-(D)K

**Retro-inverso peptides:**


\[Az ido(D)K-(D)W-(D)M-(D)/(D)\] Y-(D)R-(D)F-(D)R

R4 correspond to methionine or alanine or butylglycine or lysine side chains.

g) Some analogues based on model 1: A1-A2-A3-A4-A5-A6-A7-A8-A9-A10 incorporating modified amino acids:
A1 and A10 can correspond to (D)-lysine residus or (D)-arginine residus or beta-2 and/or beta-3 homolysine and/or beta-2 and/or beta-3 homoarginine or beta-2,2 or beta-3,3-homolysine or beta-2,2 or beta-3,3 homoarginine. PKHB4 (formula III) is an example from this series where A10 have been replaced by a (D)-Lysine residue:

![Formula III](image)

PKHB11 is another example of peptide from this series where both A1 and A2 residues have been replaced by the beta-2,2 homolysine and a D-lysine has been introduced in C-terminal position of the peptide (see for example PKHB1 1 (formula IV)):

![Formula IV](image)

A2 can correspond to arginine or lysine residus.

N-methylated amino acids or cyclopropylamino acids can be introduced in the sequence in position A2 and/or A3 and/or A4 and/or A5 and/or A6 and/or A7 and/or A8 and/or A9. A prolinovaline can be introduced in position A5 and/or A6. PKHB9 (formula (V)) and PKHB10 (formula (VI)) are both example of peptide from this series:
A prolinomethionine can be introduced in position A7.
A prolinotryptophane or prolmohomotryptophane can be introduced in position A8.
A prolinolysine can be introduced in position A9.

**II- Analogues based on model 2 :**

a) **Protocols of N- and C- termini of the peptide**

PI is an amine function (PI = -NR1, where R1 is a hydrogen atom or an alkyl chain such as a methyl group for example) or a carboxylic acid (PI = -CO₂H) or an azido group (N₃).
P2 can be an amine function (P2 = -NR1, where R1 is a hydrogen atom or an alkyl chain such as a methyl group for example) or a carboxylic acid (P1 = -CO₂H) or an azido group (N₃).

The following peptide is given as an example:

An azido group is present on the N-terminal side. An azido-lysine with S or R configuration is introduced. The R2 group corresponds to lysine or arginine side chains. R7 group corresponds to methionine side chain, methionine sulfoxide side chain, methionine sulfone side chain or a methyl, or an n-butyl or lysine side chain.

b) **Amino acids sequence modifications**

- **(D) residus:**

  The introduction of (D)-amino acids in peptide sequences stabilize peptides towards proteolytic degradations and thus enhance its pharmacological properties. (D)-amino acids are introduced on the N terminus and/or on the C terminus and/or instead of one, two, three, four, five, six, seven, eight or nine residus in peptide sequence:

  (D)K-R-F-Y-V-V-M-W-K-K
  K-(D)R-F-Y-V-V-M-W-K-K
  K-R-(D)F-Y-V-V-M-W-K-K
  K-R-F-(D)Y-V-V-M-W-K-K
  K-R-F-Y-(D)V-V-M-W-K-K
  K-R-F-Y-V-(D)V-M-W-K-K
  K-R-F-Y-V-V-(D)M-W-K-K
  R-R-F-Y-V-V-M-(D)W-K-K
  K-R-F-Y-V-V-M-W-(D)K-K
  K-R-F-Y-V-V-M-W-(D)K-K
The retro-inverso sequences lead to peptides partially or fully modified with (D)-amino acids keeping however the spatial orientations of crucial amino acids side chains. The following peptides are prepared, incorporating some chemical modifications in order to maintain peptide polarity, around diamine and diacid, introduced respectively on the C-terminus and N-terminus sides:

**Inverso Peptide:**

\[(D)K-(D)R-(D)F-(D)Y-(D)\]

**Retro-inverso peptide:**

\[(D)K-(D)W-(D)M-(D)Y-(D)F-(D)R-(D)K\] (SEQ ID NO:5)

Specific chemical modifications are introduced in retro-inverso peptide sequence in order to keep peptide polarity. The following peptides are prepared:

**Retro-inverso modified peptide**
In this peptide, (D)-amino acids are introduced instead of (Z)-amino acids, excepted on the N-terminus and C-terminus where diacide (N-terminus) and diamine (C-terminus) are introduced keeping 4N1K peptide polarity.

III- Polymer analogues

Polymers (dimers or trimers) analogues are obtained by reacting the azidopeptides (short or long analogues) through Huisgen type cycloaddition reactions:

For example, trimer analogues are obtained by reacting the azidopeptides (short or long analogues) with tripropargylamine as followed:

The R group corresponds to sequences of azidopeptides described here above.

REFERENCES:
Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.


CLAIMS:

1. An in vitro method for classifying a patient suffering from cancer comprising the steps consisting of i) determining the expression level of PLCyl in a sample from said patient, ii) comparing said expression level with a predetermined reference value and iii) providing the stage of the patient according to the expression level of PLCyl.

2. An in vitro method for the prognosis of the survival time of a patient suffering from cancer comprising the steps consisting of i) determining the expression level of PLCyl in a sample from said patient, ii) comparing said expression level with a predetermined reference value and iii) providing a good outcome prognosis when the expression level is lower than the predetermined reference value and a poor outcome prognosis when the expression level is higher than the predetermined reference value.


4. An agonist according to claim 3 wherein the agonist is a soluble peptide comprising the amino acids sequence: KRFYVVMWKK (SEQ ID NO:1) or a function-conservative variant thereof.

5. A compound which active the PLCyl for use in the treatment of cancer.


7. An in vitro method according to claim 1 or 2 or an agonist according to claim 3 to 4 or a compound according to claim 5 or a therapeutic composition according to claim 6 wherein the cancer is a B-chronic lymphocytic leukemia (CLL).

8. A method for treating cancer comprising administering to a subject in need thereof a therapeutically effective amount of a compound which active the PLCyl.
**Figure 1 A and B**

**Panel A**
- Normal B Cells: PLCγ1 expression level = 1.6
- CLL Cells: PLCγ1 expression level = 5.3
- Statistical significance: \( p < 0.0001 \)

**Panel B**
- Normal B Cells: PLCγ1 expression level = 1.6
- CLL Cells Binet Stage A: PLCγ1 expression level = 3.9
- CLL Cells Binet Stage B/C: PLCγ1 expression level = 6.7
- Statistical significance: \( p < 0.0001 \)

Note: The graphs illustrate the comparison of PLCγ1 expression levels between Normal B Cells and CLL Cells, with Binet staging B/C adding additional data points.
Figure 1 C and D
Figure 2 A and B
Figure 2 C and D
Figure 3