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(71) Applicants: CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE [FR/FR]; 3 rue Michel Ange, 75016 PARIS (FR). UNIVERSITÉ DE MONTPELLIER [FR/FR]; 163 rue Auguste Broussonnet, 34090 MONTPELLIER (FR).

(72) Inventors: ROGOWSKI, Krzysztof; 12B rue de l'Abbé de l'Épée, 34070 MONTPELLIER (FR). VAN DER LAAN, Siem; 66 rue du Claous, 34190 CAZILHAC (FR).

(74) Agent: CABINET BECKER ET ASSOCIES; 25, rue Louis le Grand, 75002 PARIS (FR).

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(54) Title: METHODS FOR PURIFYING PROTEINS HAVING A TUBULIN CARBOXYPEPTIDASE ACTIVITY AND PEPTIDIC BASED INHIBITORS THEREOF

(57) Abstract: The invention relates to a method for purifying proteins having a tubulin carboxypeptidase activity from a biological extract, comprising polymerization/depolymerization cycle performed on a biological extract in presence of microtubules. The invention further relates to a peptidic based inhibitor for use in the treatment of a disorder involving altered microtubule detyrosination in an animal, wherein the peptidic based inhibitor comprises a peptidic moiety constituted of 1 to 20 amino acids, said peptidic moiety having an amino acid selected from Y or F at the C-terminal position, and wherein the peptidic based inhibitor inhibits at least partially a tubulin carboxypeptidase activity.



Methods for purifying proteins having a tubulin carboxypeptidase activity and peptidic based inhibitors thereof

FIELD OF THE INVENTION

The present invention relates to a method for purifying a protein having a tubulin carboxypeptidase activity in a biological extract. The invention further relates to a method for identifying a peptidic based inhibitor suitable for inhibiting a tubulin carboxypeptidase activity. The present invention also relates to the use of such peptidic based inhibitors for use in the treatment of a disorder involving defects in microtubule detyrosination in an animal, preferably a mammal.

BACKGROUND OF THE INVENTION

Microtubules (MTs) are the major types of filaments that constitute the eukaryotic cytoskeleton. They are formed by the polymerization of a dimer of two globular proteins, α - and β -tubulin heterodimers. They are involved in many different functions including intracellular transport (cargo transport), cell motility, cell division, cell morphogenesis and convey mechanical signals to intracellular effectors (mechanotransduction). The intrinsic dynamic instability of the cytoskeletal microtubular system is essential for neuronal remodelling, plasticity and adaptation. Each particular MT function requires the recruitment of a specific set of MT-associated proteins (MAPs) and molecular motors. Many MAPs and motors interact with the C-terminal tails of tubulins, which protrude from the MT surface (Ciferri et al, 2008; Mizuno et al, 2004; Roll-Mecak & Vale, 2008; Skiniotis et al, 2004). Thus, one way to adapt MTs to different functions is to change the properties of the tubulin C-terminal tails through posttranslational modifications.

Among said post-translational modifications of the tubulin C-terminal tails, two polymodifications, namely polyglutamylation and polyglycylation, occur on both α - and β -tubulin (Edde et al, 1992; Redeker et al, 1994). Polyglutamylation and polyglycylation consist of the addition of side chains composed of either glutamate or glycine residues to the primary sequence glutamates present at the C-terminus of both tubulins. Enzymes that catalyze these modifications have recently been identified (Janke et al, 2005; Rogowski et al, 2009; van Dijk et al, 2007) as well as the enzymes that remove polyglutamylation (Rogowski et al, 2010). Apart from polymodifications, also detyrosination occurs on the C-terminus but it is specific to α -tubulin (Arce et al, 1975). Detyrosination consists of the removal of the very C-terminal tyrosine from α -tubulin and it results in generation of so-called $\Delta 1$ -tubulin (Figure 1).

Up to now, the enzymes responsible for detyrosination that possess tubulin carboxypeptidase (TCP) activity are unknown. Of note, native TCP activity contained in protein extracts obtained from different tissues and/or organisms likely differ in their set of enzymes responsible for detyrosination.

- 5 The identification of specific inhibitors of enzymes with TCP activity is of particular interest for treating disorders involving microtubule detyrosination, such as neurodegenerative diseases, neuronal regeneration disorders, cancers, muscular dystrophies, heart diseases, vascular disorders, retinal degeneration, infertility or ciliopathies.

Accordingly, there is thus a need for a method allowing identification of enzymes with TCP
10 activity and a method to allow design and identification of molecules that act on native TCP enzymatic activity.

SUMMARY OF THE INVENTION

Interestingly, the present invention proposes methods for identifying both, the enzymes that possess TCP activity and peptidic based inhibitors regardless of the original tissue and/or
15 organism. To this end, the present invention proposes to purify TCP activity from a biological extract and to use such purified biological extract, which exhibits native TCP activity to test and identify peptidic based inhibitors. More particularly, the inventors surprisingly discovered that it is possible to exploit the natural substrate of tubulin carboxypeptidases, i.e. the very C-terminal amino acid(s) of alpha-tubulins, as moieties or backbone for modifications, to inhibit
20 TCP activity. The peptides that share sequence homology with the C-terminal amino acid sequence of alpha-tubulins could mimic the natural substrate of enzymes that possess TCP activity and consequently inhibit its activity. More particularly, the inventors have inventively used peptides composed of variable lengths (1 to 20 amino acids) of the very C-terminal sequence of alpha-tubulin that protrudes out of the hollow tube comprised by the MT structures,
25 to inhibit native TCP activity contained in biological extracts. Highly selective, cell permeable, reversible or irreversible (suicide ligands) modified peptides with undetectable cytotoxicity that specifically modulate TCP activity were generated. The present invention further proposes a group of chemically modified peptides that pharmacologically act on microtubule dynamics by specifically modulating TCP activity.

30 Since the enzymes responsible for detyrosination that possess tubulin carboxypeptidase (TCP) activity may be valuable pharmacological targets, the present invention now proposes a method for identifying enzymes responsible for detyrosination that possess tubulin carboxypeptidase (TCP) activity. To do so, the inventors originally set up a biochemical approach to isolate TCP

activity contained in a biological extract followed by a specific enrichment of all proteins that could bind to microtubules. As such, the set of microtubule associated proteins (MAPs) was identified before and after the biochemical purification steps. It is thus an object of the present invention to provide a method for purifying proteins having a tubulin carboxypeptidase activity
5 from a biological extract, comprising:

(a) centrifuging the biological extract at a temperature comprised between 0 and 10°C, preferably between 2 and 5°C, more preferably at 2°C;

(b) recovering the supernatant from step (a) and proceeding to a first microtubule polymerization cycle by adding GTP and incubating the mixture at a temperature between 35
10 and 40°C, preferably at 37°C, +/- 2°C, then centrifuging;

(c) recovering the pellets of step (b), resuspending in ice-cold buffer, incubating at 4°C +/- 1°C, and proceeding to a second microtubule polymerization cycle by adding GTP and incubating the mixture at 37°C, +/- 2°C, then centrifuging;

(d) recovering the pellets of step (c) resuspending in ice-cold buffer, incubating at 4°C +/- 1°C,
15 and proceeding to a third microtubule polymerization cycle by adding GTP and incubating the mixture at 37°C, +/- 2°C, then centrifuging;

(e) resuspending the pellets of step (d) and submitting the mixture to an ion exchange chromatography and recovering the flow through;

(f) precipitating the proteins of the flow through with a 60% saturated ammonium sulphate
20 solution;

(g) submitting the precipitated fraction of step (f) to an hydrophobic chromatography and eluting by gradually decreasing ammonium sulphate concentration up to zero to recover the fraction of proteins with a tubulin carboxypeptidase activity.

It is a further object of the invention to provide a method for selecting a peptidic based inhibitor
25 able to inhibit a tubulin carboxypeptidase activity among peptidic based inhibitor candidates that comprise a peptidic moiety constituted of 1 to 20 amino acids, said modified peptide having at the C-terminal position an amino acid selected from Y or F, wherein the method comprises
(a) contacting the peptidic based inhibitor candidate with a mixture containing both a fraction of native or recombinant proteins with a tubulin carboxypeptidase activity and microtubules,
30 which preferably comprise synthetic microtubules and/or α -tubulins, with labeled C-terminal Y; and (b) measuring the level of isolated Y and/or detyrosinated microtubules.

Advantageously, the fraction of proteins with a tubulin carboxypeptidase activity is obtained with the method for purifying proteins as exposed above.

In an embodiment, the microtubules comprise synthetic microtubules and/or α -tubulins, with labeled C-terminal Y.

- 5 In an embodiment, the level of isolated Y in the reaction sample is compared to the level of isolated Y in a control sample comprising solely a fraction of proteins with a tubulin carboxypeptidase activity and microtubules.

10 In a particular embodiment, the peptidic moiety of the peptidic based inhibitor candidate is constituted of between 1 and 20 amino acids of the most C-terminal amino acids of an alpha-tubulin.

In a particular embodiment, the peptidic moiety of the peptidic based inhibitor candidate is constituted of between 1 and 16 of the most C-terminal amino acids of the amino acid sequence Nter-X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-Cter, wherein

- 15 - X1, X2, X5, X7, X9 and X13 are hydrophobic amino acids, preferably selected from G, A or V,
- X3, X6, X8, X10, X11, X12, X14 and X15 are negatively charged amino acids, preferably selected from E or D,
- X4 is a polar uncharged side chains, preferably selected from S, T, N or Q, and
- X16 is a large hydrophobic amino acid, selected from Y or F.

- 20 For instance, the peptidic moiety of the peptidic based inhibitor candidate has the amino acid sequence selected from Y, EAY, EDY and EEY.

25 In a particular embodiment, the peptidic based inhibitor candidate further comprises a reactive moiety, preferably selected from epoxysuccinyl, acyloxymethyl, aldehydes and ketones. In an embodiment, the reactive group is incorporated within the peptidic sequence. For instance, the reactive group is an epoxyde, which replaces the glutamate residue adjacent to the very C-terminal aromatic residue, preferably F or Y.

It is another object of the invention to provide a peptidic based inhibitor for use in the treatment of a disorder involving altered microtubule detyrosination in an animal, wherein the peptidic

based inhibitor comprises or a peptidic moiety constituted of 1 to 20 amino acids, said peptidic moiety having an amino acid selected from Y or F at the C-terminal position, and wherein the peptidic based inhibitor inhibits at least partially a tubulin carboxypeptidase activity.

According to the invention, the peptidic based inhibitor inhibits irreversibly or reversibly a tubulin carboxypeptidase activity.

The disorder is preferably selected from neurodegenerative diseases, preferably selected from Alzheimer disease, Parkinson disease, psychiatric disorders, and neural disorders, neuronal regeneration disorders, cancers, preferably selected from colon cancer and neuroblastoma, muscular dystrophies, heart diseases, vascular disorders, infertility, retinal degeneration, and ciliopathies.

It is another object of the present invention to provide a pharmaceutical composition comprising a therapeutically effective amount of such peptidic based inhibitors.

FIGURES

Figure 1: schematic overview of detyrosination and tyrosination cycle of microtubule, which consists of the removal of the very C-terminal tyrosine from α -tubulin by use of Tubulin CarboxyPeptidase (TCP) and results in generation of so-called $\Delta 1$ -tubulin. Incorporation of tyrosine (Y) at the very C-terminus of the detyrosinated soluble tubulin is obtained by Tubulin tyrosine ligase (TTL).

Figure 2: isolation of native TCP activity from a brain extract by way of cycles of depolymerization/polymerization to isolate and purify Microtubule Associated Proteins (MAPs).

Figure 3: biochemical isolation of MAPs before (MAPs) and after (Enriched fraction) enrichment by biochemical enrichment including ammonium sulphate precipitation and hydrophobic chromatography. (The polymerization/depolymerization method is used to get the initial MAPs. The enriched fraction is after the biochemical procedure involving ammonium sulphate and hydrophobic chromatography)

Figure 4: (A) schematic representation of detyrosination assay wherein ^3H Tyrosine is incorporated into soluble Tubulin by use of TTL. Following polymerization cycles ^3H Tyr-Tubulin is incorporated in microtubules to obtain ^3H Tyr-microtubule; (B) validation of the TCP activity contained in the isolated MAPs fraction.

Figure 5: inhibition of native TCP activity in brain extract by treatment with different Aspartic, Cysteine, Metallo and Serine proteases inhibitors (ASP= aspartic protease inhibitor, CYS= cysteine protease inhibitors, METALLO= metalloprotease inhibitors, SER= serine protease inhibitors).

- 5 Figure 6: Immunoblot analysis of protein extract obtained from HEK293 ectopically expressing individual CRMP family members. Δ 1-tubulin represents the amounts of deetyrosinated tubulin. HA displays the level of ectopically expressed CRMP members. The α -tubulin labeling served as a loading control and allows to compare the ratio of deetyrosinated tubulin to total tubulin.

- 10 Figure 7: Immunofluorescence analysis of U2OS cells ectopically expressing the five members of CRMP family. The left panel displays the immunofluorescence (IF) signal for the ectopically expressed CRMPs. In the right panel the signal can be observed for deetyrosinated MTs.

Figure 8: Immunoblot showing specific knockdown of endogenous CRMP1 expression in U2OS cells by siRNA interference. The α -tubulin labeling serves as a loading control and allows to compare the ratio of deetyrosinated tubulin to total tubulin.

- 15 Figure 9: Immunofluorescence analysis of U2OS cells depleted for CRMP1 protein. The left panel displays the total tubulin level per cell. On the right panel only microtubules labeled for deetyrosination are staining positively.

- 20 Figure 10: (A) Immunoblot analysis of U2OS cells depleted for CRMP1 protein. Cell cycle arrest is demonstrated by accumulation of cyclin dependent kinase inhibitor p21 and its downstream effector p53 as well as decrease in the amount of phosphorylated histone 3 (serine - Ser10P). (B) Flow cytometry analysis of bulk DNA content of CRMP1 depleted U2OS cells. (C) Graphical representation of the relative number of cells in different cell cycle stages from control (Luciferase) and CRMP1 depleted U2OS cells.

- 25 Figure 11: (A) inhibition of native TCP activity in brain-derived MAPs resulting from incubation with various peptides inspired by the C-terminal sequence of tubulin (EDY, EEY). (B) Dose response curve of TCP activity in presence of increasing EEY concentrations.

- 30 Figure 12: C2C12 muscle differentiation model. Time-course of C2C12 cells mimicking muscle differentiation in presence or absence of the TCP inhibitor EEY. Immunoblotting analysis of protein extracts obtained from myogenic differentiation of C2C12 cells. Incubation with EEY led to decrease tubulin deetyrosination levels (Δ 1-tubulin).

Figure 13: SH-SY5Y neural differentiation process. (A) Phase contrast microscopy pictures of SH-SY5Y cells at Day 0 and Day 8 after neural differentiation. (B) Gene expression analysis of DDC (Aromatic-L-amino-acid decarboxylase), a marker of dopaminergic neurons and of CRMP1 (C1) expression during the neuronal differentiation process.

- 5 Figure 14: Immunoblot analysis of detyrosinated tubulin (deTyr-tub) of Control and DMD cells that contain a causal genetic mutation for Duchenne Muscular Dystrophy.

Figure 15: Immunoblot analysis of detyrosinated tubulin (deTyr-tub) of Control and SH-SY5Y cells.

- 10 Figure 16: Immunoblot analysis of detyrosinated tubulin (deTyr-tub) of Control and iPSC generated from skin fibroblasts from patients carrying two different familial Alzheimer's Disease mutations (Mut_1 and Mut_2).

Figure 17: Immunoblot analysis of detyrosinated tubulin (deTyr-tub) of Control and CHL-1 cells and HEK cells, in presence or absence of TCPase inhibitor (Eps-Y).

- 15 Figure 18: Examples of peptide-based inhibitors comprising a peptidic moiety and a reactive group composed of? for example? an epoxyde group. A first general example illustrates a subtype of different inhibitors composed of a very C-terminal tyrosine (Y) attached to an epoxyde group and the C-terminal sequence of alpha tubulin such as GEepoxydeY (inh1). In the chemical formula, R1 represents the amino acid sequence of human alpha tubulin and R2 a wide variety of C-terminal modifications such as, but not limited to, COOH; CONH₂, NH₂,
20 aldehyde, pNA, Amc, hydrazide, hydroxamic acid, CMK). Those modifications may contribute to preventing enzyme degradation, to mimic native proteins, and in some cases to remove hydrogen bonding at the C-terminal of the peptides, tools for studying structure-activity relationship (SAR), and more. The second molecule (inh2) represent the formula of a shorter version of the peptidic inhibitor.

25 DETAILED DESCRIPTION OF THE INVENTION

- The present invention originally proposes to exploit the natural substrate of proteins with TCP activity, i.e., the very C-terminal sequence of α -tubulin, as pharmacological tool for inhibiting TCP activity. The inventors discovered several families of proteins possessing TCP activity in a controlled purified biochemical assay as well as in human cell cultures. In turn, the inventors
30 have developed a method to design pharmacological compounds that specifically inhibit (either partially or irreversibly) detyrosinase activity *in cellulo*. A plethora of compounds that

selectively act on TCP activity can be designed according to the invention, for applications as research tools and most promisingly in treatment for several disorders such as neurodegenerative diseases and psychiatric disorders.

Method for purifying proteins having a tubulin carboxypeptidase activity

- 5 The present invention proposes a method suitable for purifying proteins having a tubulin carboxypeptidase activity from a biological extract.

In the context of the present invention, the terms “*protein having a tubulin carboxypeptidase activity*” or “*protein having a TCP activity*” or “*TCPase protein*” or “*TCP*” are used for referring to a class of proteins that are able to cleave off the Glu-Tyr bond to release the C-
10 terminal tyrosine residue from a native tyrosinated tubulin (Figure 1).

The term “*biological sample*” means any sample derived from an animal, including multi- or uni-cellular organisms, which contains microtubules. Preferably, the biological sample derived from a mammal, preferably selected from pig, monkey, human, rat or mouse. Examples of such biological samples include fluids, tissues, cell samples, organs, biopsies, etc. Most preferred
15 samples are brain extract, testis extract, and lung extract.

The biological sample may be treated prior to its use, *e.g.* in order to render the microtubules available. Techniques of cell lysis, concentration or dilution of microtubules, are known by the skilled person.

According to the invention, the method for purifying proteins having a tubulin
20 carboxypeptidase activity from a biological extract, comprises:

- (a) centrifuging the biological extract at a temperature comprised between 0 and 10°C, preferably between 2 and 5°C, more preferably at 2°C;
- (b) recovering the supernatant from step (a) and proceeding to a first microtubule polymerization cycle by adding GTP and incubating the mixture at a temperature between 35
25 and 40°C, preferably at 37°C, +/- 2°C, then centrifuging;
- (c) recovering the pellets of step (b), resuspending in ice-cold buffer, incubating at 4°C, +/- 1°C, and proceeding to a second microtubule polymerization cycle by adding GTP and incubating the mixture at 37°C, +/- 2°C, then centrifuging;

(d) recovering the pellets of step (c) resuspending in ice-cold buffer, incubating at 4°C +/- 1°C, and proceeding to a third microtubule polymerization cycle by adding GTP and incubating the mixture at 37°C, +/- 2°C, then centrifuging;

5 (e) resuspending the pellets of step (d) and submitting the mixture to an ion exchange chromatography and recovering the flow through;

(f) precipitating the proteins of the flow through with a 60% saturated ammonium sulphate solution;

10 (g) submitting the precipitated fraction of step (f) to an hydrophobic chromatography and eluting by gradually decreasing ammonium sulphate concentration up to zero to recover the fraction of proteins with a tubulin carboxypeptidase activity.

In a particular embodiment, the first polymerization cycle comprises (i) adding GTP and incubating the mixture at 37°C, +/- 2°C, for 30 minutes, +/- 10 minutes; (ii) centrifuging at 22,000 g, +/- 1,000 g, at 37°C, +/- 2°C, for 45 minutes, +/- 10 minutes.

15 Alternatively or in addition the second polymerization cycle may comprise (i) incubating the mixture on ice for 30 minutes, +/- 10 minutes; (ii) centrifuging at 150,000g +/- 10,000 g, 30 minutes, +/- 10 minutes; (iii) recovering the supernatant and adding GTP; (iv) incubating the mixture at 37°C, +/- 2°C, for at 30 minutes, +/- 10 minutes; (v) centrifuging at 50,000 g, +/- 1,000 g at a temperature comprised between 30°C and 37°C, for 30 minutes, +/- 10 minutes.

20 Alternatively or in addition the third polymerization cycle may comprise (i) incubating the mixture on ice for 30 minutes, +/- 10 minutes; (ii) centrifuging at 150,000 g, +/- 10,000 g, 30 minutes, +/- 10 minutes; (iii) recovering the supernatant and adding GTP; (iv) incubating the mixture at 37°C, +/- 2°C, for at 30 minutes, +/- 10 minutes; (v) centrifuging at 50,000 g, +/- 1,000 g at a temperature comprised between 30°C and 37°C, for 30 minutes, +/- 10 minutes.

25 In a particular embodiment, the method further comprises a step of mass spectrometry characterization of the fraction of proteins of step (g).

In a particular embodiment, the method further comprises a step of selecting proteins that contain a protease domain. To determine what type of protease activity would be required for detyrosination, various inhibitors of cysteine, aspartic, serine, threonine proteases and metalloproteases may be tested.

In a particular embodiment, the fraction of proteins with a tubulin carboxypeptidase activity comprises at least one protein having at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99% amino acid sequence identity with the amino acid sequence selected from SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10 and SEQ ID N°11.

In a particular embodiment, the fraction of proteins with a tubulin carboxypeptidase activity is obtained from a brain extract, such as a brain extract from pigs, and the mass spectrometric data are aligned with human reference sequences, in order to identify corresponding human proteins.

Advantageously, such fraction of proteins comprises at least one protein selected from human Ubiquitin carboxyl-terminal hydrolase 14 (UBP14 - SEQ ID N°1), human Ubiquitin carboxyl-terminal hydrolase 5 (UBP5 - SEQ ID N°2), human Methionine aminopeptidase 2 (MAP2 - SEQ ID N°3), human Xaa-Pro aminopeptidase 1 (XPP1 - SEQ ID N°4), human Tripeptidyl-peptidase 2 (TPP2 - SEQ ID N°5), human Vasohibin-1 (VASH1 - SEQ ID N°6), human dihydropyrimidinase-related protein 1 (DPYL1 - SEQ ID N°7), human dihydropyrimidinase-related protein 2 (DPYL2 - SEQ ID N°8), human dihydropyrimidinase-related protein 3 (DPYL3 - SEQ ID N°9), human dihydropyrimidinase-related protein 4 (DPYL4 - SEQ ID N°10) and human dihydropyrimidinase-related protein 5 (DPYL5 - SEQ ID N°11).

In another embodiment, the fraction of proteins comprises at least one protein selected from the proteins listed in Table 1.

Advantageously, the fraction of proteins with a tubulin carboxypeptidase activity is further contacted with microtubules and the level of isolated tyrosine (Y) is measured, thereby confirming the tubulin carboxypeptidase activity of the fraction of proteins. For instance, the microtubules comprise synthetic microtubules and/or α -tubulins, with labeled C-terminal Y.

After a time of contact of the fraction of proteins with putative TCP activity with microtubules, under conditions suitable for the proteins with putative TCP activity to perform detyrosination of the microtubules and/or α -tubulin, the amount of isolated Y, or free Y, in the sample is measured. For instance, tubulin tyrosine ligase (TTL) enzyme incorporates radioactively labeled ^3H -tyrosine at the very C-terminus of detyrosinated soluble tubulin obtained from e.g. brain extracts. The radioactively labeled tubulin is incorporated in MT during a polymerization cycle to obtain radioactively labeled MTs. TCP activity contained by the biological sample will cut off the radioactively marked tyrosine which can be quantified by scintillation analysis. Alternatively, TCP activity can be monitored by comparing the ratio of detyrosinated versus tyrosinated tubulin before and after exposure to the biological sample by immunoblot analysis.

Several factors may affect the rate at which enzymatic reactions proceed: temperature, pH, enzyme concentration, substrate concentration, and the presence of any inhibitors or activators.

In some embodiments, it is possible to employ a buffer containing a nucleoside triphosphate, such as ATP, potassium chloride, magnesium chloride, and a reducing agent such as DTT in order to provide optimal conditions for the enzymes with putative TCP activity to detyrosinate the microtubules and/or α -tubulin.

The pH value is preferably in the range of 5 to 9, in order to provide suitable conditions for the enzymes with putative TCP activity to detyrosinate the microtubules and/or α -tubulin. More preferably, the pH value is between 5.5 and 8.5, even more preferably between 6 and 8.

A suitable reaction time for enzymes with putative TCP activity to detyrosinate the microtubules and/or α -tubulin may be in the range of 5 minutes to 10 hours, preferably 10 minutes to 5 hours, more preferably 1 hour to 3 hours.

In a particular embodiment, the concentration of fraction of proteins with a tubulin carboxypeptidase activity contacted with microtubules and/or α -tubulin is in the range of 0.1 μ M to 1 mM, preferably 0.25 μ M to 500 μ M, more preferably 0.5 μ M to 300 μ M, and even more preferably 1 μ M to 200 μ M, in order to provide optimal conditions for detyrosination of the microtubules and/or α -tubulin.

In a particular embodiment, the fraction of proteins with a tubulin carboxypeptidase activity is contacted with at least α -tubulin. In another particular embodiment, the fraction of proteins with a tubulin carboxypeptidase activity is contacted with a polypeptide corresponding to the C-terminus of α -tubulin. In another embodiment, the fraction of proteins with a tubulin carboxypeptidase activity is contacted with a mixture of microtubules and α -tubulin.

Advantageously, the microtubules and/or α -tubulin comprise synthetic microtubules/peptides and/or α -tubulins, wherein the α -tubulin comprises labeled C-terminal Y, so that step of measuring free Y may be easily implemented.

According to the invention, the tubulin carboxypeptidase activity of the fraction of proteins is confirmed if detectable isolated Y in the sample/microtubules and/or α -tubulin in the sample is observed and compared to a fraction of protein that lacks TCP activity (negative control).

By “*synthetic microtubules/peptides and/or α -tubulin*” it is intended a microtubule or α -tubulin that has been chemically constructed. The synthetic microtubules or α -tubulin may be artificially constructed by methods of synthetic biology, including solid phase peptide synthesis

(SPPS), prior thiol capture strategy, native chemical ligation (NCL). The term “*Synthetic microtubules and/or α -tubulin*” also encompasses natural microtubule or α -tubulin that has been treated to change its C-terminal amino acid by a labeled -Y.

According to the invention, the labeled -Y consists of a tyrosine that is labeled with a molecule or material that can produce a detectable (such as visually, electronically, radioactively, or otherwise) signal that indicates the presence and/or concentration of the tyrosine in a sample. Thereby, e.g., the presence, location and/or concentration of the tyrosine in a sample can be detected by detecting the signal produced by the detectable molecule or material. The labeled -Y can be detected directly or indirectly. In certain embodiments, the label, or detectable molecule or material, may react with a suitable substrate (e.g., a luciferin) to generate a detectable signal. In particular, the detectable label can be a fluorophore, an enzyme (peroxidase, luciferase), a radioisotope, a fluorescent protein, or a fluorescent dye. Other detectable molecule or material including chemiluminescent labels, electrochemiluminescent labels, bioluminescent labels, polymers, polymer particles, metal particles, haptens, and dyes.

In a particular embodiment, the level of isolated Y in the sample is compared to the level of isolated Y in a control sample comprising solely microtubules and/or α -tubulin. The control sample is free of the fraction of proteins with a tubulin carboxypeptidase activity, so that the difference between both amounts of isolated Y can be attributed to said compound.

Peptidic based inhibitors able to inhibit tubulin carboxypeptidase activity

According to the invention, the peptidic based inhibitors block or reduce the tubulin carboxypeptidase activity of an enzyme. A peptidic inhibitor can act with competitive, uncompetitive or noncompetitive inhibition. A peptidic inhibitor of the invention can bind reversibly or irreversibly, and therefore the term includes compounds that are suicide substrates of an enzyme with TCP activity.

The present invention relates to a method for selecting a peptidic based inhibitor able to inhibit a tubulin carboxypeptidase activity that has been selected and designed based on the natural C-terminal sequence of α -tubulin.

More particularly, the inventors have developed a method, wherein a peptidic inhibitor containing a peptidic moiety constituted of 1 to 20 amino acids, wherein the most C-terminal amino acid is selected from Y or F, is contacted with a biological extract from an animal, in order to identify and isolate a peptide having a tubulin carboxypeptidase activity.

Therefore, it is an object of the invention to provide a method for selecting a peptidic based inhibitor able to inhibit a tubulin carboxypeptidase activity among peptidic based inhibitor candidates that comprise a peptidic moiety constituted of 1 to 20 amino acids, said peptidic moiety having at the C-terminal position an amino acid selected from Y or F, wherein the method comprises a step (a) of contacting the peptidic based inhibitor candidate with a mixture containing both a fraction of protein with a tubulin carboxypeptidase activity and microtubules; and a step (b) of measuring the level of isolated Y and/or detyrosinated microtubules.

As used herein, the terms "*tubulin carboxypeptidase inhibitor*" or "*peptidic based inhibitor*" refers to a class of molecules that target and inhibit, at least partially, the activity of *proteins* having a tubulin carboxypeptidase activity, and thereby inhibit microtubule detyrosination.

The amino acid sequences defined herein use the one letter code as following : A : Ala (alanine) ; R : Arg (arginine) ; N : Asn (asparagine) ; D : Asp (aspartic acid); C : Cys (cysteine) ; Q : Gln (glutamine) ; E : Glu (glutamic acid); G : Gly (glycine); H : His (histidine); I : Ile (isoleucine); L : Leu (leucine); K : Lys (lysine); M : Met (methionine); F : Phe (phenylalanine); P : Pro (proline); S : Ser (serine); T : Thr (threonine); W : Trp (tryptophan); Y : Tyr (tyrosine); V : Val (valine).

The amino acid sequences may also comprise non-naturally-occurring amino acid such as azetidinecarboxylic acid, 2-aminoadipic acid, 3-aminoadipic acid, beta-alanine, aminopropionic acid, 2-aminobutyric acid, A-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminopimelic acid, 2,4-diaminoisobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, hydroxylysine, allo-hydroxylysine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, N-methylisoleucine, N-methylvaline, norvaline, norleucine, ornithine, selenocysteine, nitrotyrosine, dihydroxyphenylalanine, and pipecolic acid.

The term "*peptide*" refers herein to a polymer of amino acid residues linked together by peptide (amide) bonds. Said term also encompasses fragments of polypeptides. Said fragments have preferably biological activity. Said fragments may have a length of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450 or more amino acids.

The term "*peptidic moiety*" refers to a moiety containing at least one amino acid and at most 20 amino acids. When the peptidic moiety comprises two or more amino acids, said amino acids are linked together by peptide bonds and chemically modified or not.

According to the method of the invention for selecting a peptidic based inhibitor able to inhibit a tubulin carboxypeptidase activity, the peptidic based inhibitor candidate is contacted with a mixture containing both a fraction of protein with a tubulin carboxypeptidase activity and microtubules (step a), and the rate of inhibition of TCP activity is calculated by measuring the level of isolated Y and/or detyrosinated microtubules (step b).

In some embodiments, said method for selecting a peptidic based inhibitor able to inhibit a tubulin carboxypeptidase activity among peptidic based inhibitor candidates that comprise a peptidic moiety constituted of 1 to 20 amino acids, said peptidic moiety having at the C-terminal position an amino acid selected from Y or F, wherein the method comprises: (a) contacting the peptidic based inhibitor candidate with a mixture containing both a fraction of native or recombinant proteins with a tubulin carboxypeptidase activity and microtubules, which preferably comprise synthetic microtubules and/or α -tubulins, with labeled C-terminal Y; (b) measuring the level of isolated Y and/or detyrosinated microtubules.

In a particular embodiment, the reaction temperature is maintained in the range of 1 °C to 70 °C, preferably 5 °C to 65 °C, more preferably 10 °C to 60 °C, even more preferably 15 °C to 55 °C, most preferably 19 °C to 43°C, and for example 19°C to 37 °C in order to provide optimal conditions for the putative TCPase enzyme to detyrosinate the microtubules and/or α -tubulin.

The method of the invention may be implemented with a large kind of peptidic based inhibitors that share sequence identity or homology with the C-terminal amino acid sequence of α -tubulin.

More particularly, the peptidic based inhibitor of the invention comprises a peptidic moiety constituted of 1 to 20 amino acids, said peptidic moiety having at the C-terminal position an amino acid selected from Y or F.

According to the invention, in the three-dimensional conformation of the peptidic moiety the C-terminal Y or F is accessible to enzymes, and more particularly to proteins having a TCP activity.

In a preferred embodiment, the peptidic moiety is constituted of the 1 to 20 amino acid of the most C-terminal amino acid part of alpha-tubulin.

In a particular embodiment, the peptidic moiety is constituted of between 1 and 16 of the most C-terminal amino acids of the amino acid sequence Nter-X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-Cter (SEQ ID N°20), wherein

- X1, X2, X5, X7, X9 and X13 are hydrophobic amino acids, preferably selected from G, A or V,

- X3, X6, X8, X10, X11, X12, X14 and X15 are negatively charged amino acids, preferably selected from E or D,

5 - X4 is a polar uncharged side chains, preferably selected from S, T, N or Q, and

- X16 is a large hydrophobic amino acid, preferably selected from Y or F.

In general, "X" can denote any amino acid unless indicated otherwise herein.

The physicochemical groups are generally defined as following: the non-polar or hydrophobic amino acids including A, V, I, L, P, F, M, and W, but more narrowly the non-aromatic
10 hydrophobic amino acids as including A, V, I, L, P, and M; the uncharged polar group including G, S, T, C, Y, N and Q; the negatively charged polar group including E and D; and the positively charged polar group including R and K.

X16 refers to the ultimate C-terminal amino acid in the peptidic moiety. Preferably, X16 is Y. The other amino acids are optional. The peptidic moiety may comprise all or part of the amino
15 acids of SEQ ID N°12, with respect of the numeration, wherein X1, if present is the N-terminal amino acid in the peptidic moiety, and so on.

In a particular embodiment, the amino acid sequence of the peptidic moiety consists on Y (X16).

In another particular embodiment, the amino acid sequence of the peptidic moiety consists on
20 EDY.

In another particular embodiment, the amino acid sequence of the peptidic moiety consists on EEY.

In another particular embodiment, the amino acid sequence of the peptidic moiety consists on EAY.

25 In another embodiment, the amino acid sequence of the peptidic moiety comprises or consists on the amino acid sequence selected from SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16, SEQ ID N°17, SEQ ID N°18.

The peptidic inhibitor may inhibit irreversibly or reversibly a tubulin carboxypeptidase activity. As an example, a chemically modified amino acid that may irreversibly react with the cysteine contained by the protein with TCP activity, such as a catalytic triad, is considered to be irreversible. On the other hand a peptide, or chemically modified peptide, that does not
5 covalently react or form reversible bonds with the thiol group contained by the enzyme may be washed off and is considered as reversible. Iodoacetamide is an irreversible inhibitor of all cysteine peptidases, with the mechanism of inhibition occurring from alkylation of the catalytic cysteine residue.

According to the invention, the activity of the peptidic inhibitor may be modulated by
10 expanding the number of amino acid residues and/or by use of a reactive moiety, preferably selected from epoxysuccinyl (Eps), acyloxymethyl, aldehydes and ketones. Such reactive moiety that further functionalizes the peptide may be linked to the peptidic inhibitor by use of known methods in the art, such as, example given, methods of synthetic medicinal chemistry, synthesis of various intermediates, deuterated forms of the compounds and stereoisomers
15 thereof (Fig. 18).

For instance, the peptidic inhibitors is Eps-EEY. Such peptidic inhibitor act as a reversible inhibitor of TCPase activity.

The present invention thus provides a method to design plethora of peptidic inhibitors able to inhibit a tubulin carboxypeptidase activity.

20 Therapeutic use of peptidic based inhibitors

As well exposed above, deetyrosination of microtubules is associated with cancer progression, aberrant neuronal networks, weak neuronal remodeling, plasticity and/or adaptation. Accordingly, the use of such peptidic inhibitors may have a positive impact in the treatment of disorders involving microtubule deetyrosination. For instance, peptidic inhibitors of the present
25 invention may be used for increasing the microtubule dynamics and thereby impacting neuroregeneration.

The present invention thus relates to peptidic based inhibitor for use in the treatment of heart disorder, vascular disorder, cancers, neurodegenerative disorders, muscle disorders, infertility, ciliopathies, more generally a disorder involving altered microtubule deetyrosination in an
30 animal, preferably but not limited to a mammal, wherein the peptidic based inhibitor comprises a peptidic moiety constituted of 1 to 20 amino acids, said peptidic moiety having an amino acid selected from Y or F at the C-terminal position, and wherein the peptidic based inhibitor inhibits

at least partially a tubulin carboxypeptidase activity. In a particular embodiment, the peptidic based inhibitor comprises GVDSVEAEAEEGEEY (SEQ ID N°19). In another embodiment, the peptidic based inhibitor comprises GEEY.

Thus, peptidic inhibitors of the present invention are good candidate for treating neurodegenerative diseases, preferably selected from Alzheimer disease, Parkinson disease, psychiatric disorders, and neural disorders, neuronal regeneration disorders, cancers, preferably selected from colon cancer and neuroblastoma, muscle disorders such as muscular dystrophies, retinal degeneration, heart diseases, vascular disorders, infertility, and ciliopathies.

It is an object of the present invention to provide peptidic based inhibitor for use for treating neurodegenerative diseases. In a particular embodiment, the neurodegenerative disease is Alzheimer disease.

It is a further embodiment to provide peptidic based inhibitor for use for treating cancers.

It is a further embodiment to provide peptidic based inhibitor for use for treating muscular dystrophies, particularly Duchenne muscular dystrophy.

The invention additionally provides a pharmaceutical composition comprising a therapeutically effective amount of a peptidic inhibitors according of the invention.

By "*therapeutically effective amount*" is meant an amount of the peptidic inhibitor of the invention that elicits a desired therapeutic effect. The exact amount dose will depend on the purpose of the treatment and will be ascertainable by one skilled in the art using known techniques. As is known in the art, adjustments for age, body weight, general health, sex, diet, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

A pharmaceutical composition according to the present invention may further comprise one or more pharmaceutically acceptable carriers. In a specific embodiment, the term "*pharmaceutically acceptable*" means approved by a regulatory agency or other generally recognized pharmacopoeia for use in animals, and more particularly in humans.

Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as water, 5% dextrose, or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil, or injectable organic esters that are suitable for administration to a human or non-human subject. In some embodiments, a pharmaceutically acceptable carrier or composition is sterile. A pharmaceutical composition

can comprise, in addition to the active agent, physiologically acceptable compounds that act, for example, as bulking agents, fillers, solubilizers, stabilizers, osmotic agents, uptake enhancers, etc. Physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose, lactose, dextrans, polyols such as mannitol, antioxidants, such as ascorbic acid or glutathione, preservatives, chelating agents, buffers, or other stabilizers or excipients.

The choice of a pharmaceutically acceptable carrier(s) and/or physiologically acceptable compound(s) can depend for example, on the nature of the active agent, e.g., solubility, compatibility (meaning that the substances can be present together in the composition without interacting in a manner that would substantially reduce the pharmaceutical efficacy of the pharmaceutical composition under ordinary use situations) and/or route of administration of the composition.

Pharmaceutical compositions of the invention comprise a therapeutically effective amount of one or several peptidic inhibitors according to the invention and can be formulated in various forms, e.g. in solid, liquid, gaseous or lyophilized form and may be, inter alia, in the form of an ointment, a cream, transdermal patches, a gel, powder, a tablet, solution, an aerosol, granules, pills, suspensions, emulsions, capsules, syrups, liquids, elixirs, extracts, tincture or fluid extracts or in a form which is particularly suitable for topical or oral administration. A variety of routes are applicable for administration of the polypeptide of the invention, including, but not limited to, orally, topically, transdermal, subcutaneously, intravenously, intraperitoneally, intramuscularly or intraocularly. However, any other route may readily be chosen by the person skilled in the art if desired.

The pharmaceutical compositions can be used for the treatment of a wide variety of different diseases and disorders. Thus the invention also encompasses methods of treatment comprising administering a therapeutically effective amount of a peptidic inhibitors of the invention to a subject in need thereof. The subject is typically a mammal, e.g., a human. In some embodiments the subject is a non-human animal that serves as a model for a disease or disorder that affects humans. The animal model may be used, e.g., in preclinical studies, e.g., to assess efficacy and/or determine a suitable dose.

In some embodiments, a peptidic inhibitor of the invention is administered prophylactically, e.g., to a subject who does not exhibit signs or symptoms of the disease or disorder (but may be at increased risk of developing the disorder or is expected to develop the disease or disorder).

EXAMPLES

Example 1: Isolation of native TCP activity from brain extract

The method for purifying proteins having a tubulin carboxypeptidase activity of the invention has been performed on porcine brain extract. More particularly, microtubule associated proteins (MAPS) were isolated from the crude brain extract (Figure 2) as exposed below.

Material & method

PEM-buffer composition:

	<u>concentration</u>	<u>chemical</u>	<u>stock</u>
	50 mM	PIPES/NaOH, pH 6.8	400 mM, 4°C
10	1 mM	EGTA	100 mM, RT
	1 mM	MgCl ₂	1 M, RT

Experimental procedure

Pig brains were quickly removed from the skull and cooled down in ice-cold water by shaking and they were kept on ice. For each 10 g of brain material, 15 ml of PEM buffer containing 1 µl β-mercaptoethanol were added. The brains were pre-homogenised in a mixer and then transferred into a Potter homogeniser (on ice). The extract was spun for 1 h at 22,000 g at 2°C and the supernatant was removed carefully.

1st polymerization cycle:

The supernatant was supplemented with 1mM GTP and incubated at 37°C for 30 minutes while the solution was stirred smoothly. Following the incubation, sample was spun down at 22,000 g for 45 min. The pellet containing microtubules and Microtubule Associated Proteins (MAPs) was kept while the supernatant was discarded.

2nd and 3rd polymerization cycle:

The pellet was re-suspended in 0.1 vol. of the initial volume in ice-cold PEM buffer containing 0.1 mM GTP and re-homogenised in an ice-cold Potter homogeniser. Next, the suspension was

incubated on ice for 30 min and centrifuged for 30 min at 150,000 g at 2°C (41,000 rpm in a 50-2Ti rotor). The pellet was discarded.

The supernatant was adjusted to 1 mM GTP and incubated in pre-weighted centrifuge tubes for 30 min at 37°C and after spun for 30 min at 30-37°C, 50,000 g (24,000 rpm in a 50-2Ti rotor).

- 5 The supernatant was discarded while the pellet, which contains microtubules and MAPs was re-suspended in PEM buffer supplemented with 1mM GTP and subjected to 3rd polymerization cycle.

- Following the third cycle the pellet containing microtubules and MAPs was re-suspended and subjected to DEAE-Sephadex ion exchange chromatography. This step serves to separate the
10 tubulin (microtubules), which was found associated with the column while MAPs containing the TCP activity were found in the flow through.

- The flow through from the DEAE column was collected and the MAPs were concentrated. Differential ammonium sulphate precipitation was used. The majority of the TCP activity was recovered at 60% of saturated ammonium sulphate solution. This is a critical step
15 experimentally obtained to yield a MAP fraction containing native TCP activity.

- To further enrich for TCP activity an additional inventive step consisting of chromatography based on hydrophobic interactions was added. The MAP fraction recovered from ammonium sulphate precipitation was loaded on phenyl sepharose chromatography and eluted by gradually decreasing ammonium sulphate concentration in order to optimize the recovery of native TCP
20 activity from brain extracts. The presence of ammonium sulphate strongly increases surface tension in aqueous solutions and promotes hydrophobic interactions.

- Following the elution, the fraction with the highest TCP activity (Figure 3) was subjected to characterization by mass spectrometry. Importantly the fraction was analyzed in a detyrosination assay to confirm the presence of TCPase activity (Figure 4). Mass spectrometry
25 analysis of the enriched fraction (Figure 3) yielded a total of 584 identified proteins, as listed in Table 1 below, which were analyzed by functional homology search to specifically identify TCP candidates based on the presence of protease domain. TCP candidates are peptidases, enzymes that hydrolysis peptide bonds. In the context of the invention, proteases, proteinases and proteolytic enzymes are used interchangeably. To search for potential candidates that
30 contain a protease domain, the obtained list of peptides was analysed for Conserved Domain. This was performed using Conserved Domain Database (CDD) a curated database that annotates functional units in proteins (hosted by NCBI). The collection of domain models includes a set curated by NCBI, which utilizes 3D structure to provide insights into

sequence/structure/function relationships. In as such to short the list of proteins obtained after specific enrichment down to potential candidates having at least one potential protease domain, enquiries were performed. Besides, further description of the candidates was obtained using MEROPS database a second independent resource for information on peptidases (<http://merops.sanger.ac.uk/about/index.shtml>). Additionally, proteins with newly identified protease domain were also screened in literature. This resulted in a selection of 11 potential TCP candidates (SEQ ID N°1 to SEQ ID N°11), among which the family of proteins consisted of collapsin response mediator protein (CRMPs) has been characterized as example.

Table 1. List of microtubules associated proteins obtained after purification steps (>2 peptides per hit)

UNIPROT ID	PROTEINS	GENES	Mol weight [kDa]	Sequence coverage [%]
A2AGT5	Cytoskeleton-associated protein 5	Ckap5	226	16,3
A2AJI0	MAP7 domain-containing protein 1	Map7d1	93	3,4
D3Z2H9	Uncharacterized protein	Tpm3-rs7	29	23,8
E9PY16	ArfGAP with dual PH domains 1	Adap1	43	15,8
E9Q557	Desmoplakin	Dsp	333	0,7
E9Q912	RAP1, GTP-GDP dissociation stimulator 1	Rap1gds1	66	15,3
E9QAS7	Inositol polyphosphate-5-phosphatase A	Inpp5a	49	14,5
F8VPN4	Amylo-1,6-glucosidase, 4-alpha-glucanotransferase	Agl	174	1,4
O08532	Voltage-dependent calcium channel subunit alpha-2/delta-1	Cacna2d1	125	3,4
O08539	Myc box-dependent-interacting protein 1	Bin1	64	5,4
O08553	Dihydropyrimidinase-related protein 2	Dpysl2	62	49,7
O08599	Syntaxin-binding protein 1	Stxbp1	68	35,4
O08663	Methionine aminopeptidase 2	Metap2	53	13,2
O08749	Dihydrolipoyl dehydrogenase, mitochondrial	Dld	54	11
O08788	Dynactin subunit 1	Dctn1	142	17,7
O08848	60 kDa SS-A/Ro ribonucleoprotein	Trove2	60	5,4

O09061	Proteasome subunit beta type-1	Psmb1	26	33,3
O35098	Dihydropyrimidinase-related protein 4	Dpysl4	62	15,7
O35136	Neural cell adhesion molecule 2	Ncam2	93	4,8
O35226	26S proteasome non-ATPase regulatory subunit 4	Psmd4	41	14,4
O35286	Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	Dhx15	91	2,9
O35464	Semaphorin-6A	Sema6a	114	4,8
O35593	26S proteasome non-ATPase regulatory subunit 14	Psmd14	35	9,4
O35685	Nuclear migration protein nudC	Nudc	38	19,6
O35841	Apoptosis inhibitor 5	Api5	57	16,3
O35864	COP9 signalosome complex subunit 5	Cops5	38	17,7
O54829	Regulator of G-protein signaling 7	Rgs7	55	5,1
O55013	Trafficking protein particle complex subunit 3	Trappc3	20	10
O55100	Synaptogyrin-1	Syngr1	26	10,3
O55131	Septin-7	Sept7	51	17,2
O55234	Proteasome subunit beta type-5	Psmb5	29	20,8
O70194	Eukaryotic translation initiation factor 3 subunit D	Eif3d	64	4
O70310	Glycylpeptide N-tetradecanoyltransferase 1	Nmt1	57	22,8
O70311	Glycylpeptide N-tetradecanoyltransferase 2	Nmt2	60	8,3
O70435	Proteasome subunit alpha type-3	Psma3	28	30,6
O70493	Sorting nexin-12	Snx12	19	12,7
O88342	WD repeat-containing protein 1	Wdr1	66	30,5
O88447	Kinesin light chain 1	Klc1	61	6,7
O88485	Cytoplasmic dynein 1 intermediate chain 1	Dync1i1	71	12,7
O88487	Cytoplasmic dynein 1 intermediate chain 2	Dync1i2	68	7,2
O88543	COP9 signalosome complex subunit 3	Cops3	48	9
O88544	COP9 signalosome complex subunit 4	Cops4	46	14
O88569	Heterogeneous nuclear ribonucleoproteins A2/B1	Hnrnpa2b1	37	21,8
O88643	Serine/threonine-protein kinase PAK 1	Pak1	61	20,9

O88685	26S protease regulatory subunit 6A	Psmc3	50	27,6
O88735	Ensconsin	Map7	82	5,2
O88844	Isocitrate dehydrogenase [NADP] cytoplasmic	Idh1	47	23,9
O88935	Synapsin-1	Syn1	74	10,3
P00920	Carbonic anhydrase 2	Ca2	29	5,4
P01027	Complement C3	C3	186	2,9
P01869	Ig gamma-1 chain C region, membrane-bound form	Ighg1	43	5,6
P02088	Hemoglobin subunit beta-1	Hbb-b1	16	12,2
P03995	Glial fibrillary acidic protein	Gfap	50	15,8
P04370	Myelin basic protein	Mbp	27	8
P05063	Fructose-bisphosphate aldolase C	Aldoc	39	41
P05064	Fructose-bisphosphate aldolase A	Aldoa	39	35,4
P05132	cAMP-dependent protein kinase catalytic subunit alpha	Prkaca	41	29,1
P05202	Aspartate aminotransferase, mitochondrial	Got2	47	15,1
P06151	L-lactate dehydrogenase A chain	Ldha	36	8,7
P06745	Glucose-6-phosphate isomerase	Gpi	63	26
P07356	Annexin A2	Anxa2	39	34,5
P07901	Heat shock protein HSP 90-alpha	Hsp90aa1	85	15,3
P08113	Endoplasmin	Hsp90b1	92	23,2
P08249	Malate dehydrogenase, mitochondrial	Mdh2	36	26,9
P08551	Neurofilament light polypeptide	Nefl	62	35,5
P08553	Neurofilament medium polypeptide	Nefm	96	20,5
P09041	Phosphoglycerate kinase 2	Pgk2	45	25,9
P09405	Nucleolin	Ncl	77	3,3
P09411	Phosphoglycerate kinase 1	Pgk1	45	47,2
P0CG49	Polyubiquitin-B	Ubb	9	44,2
P10107	Annexin A1	Anxa1	39	7,8
P10126	Elongation factor 1-alpha 1	Eef1a1	50	37,7
P10630	Eukaryotic initiation factor 4A-II	Eif4a2	46	33,4

P10637	Microtubule-associated protein tau	Mapt	76	17,6
P10711	Transcription elongation factor A protein 1	Tcea1	34	26,9
P11103	Poly [ADP-ribose] polymerase 1	Parp1	113	7,6
P11247	Myeloperoxidase	Mpo	81	3,3
P11499	Heat shock protein HSP 90-beta	Hsp90ab1	83	17,1
P11798	Calcium/calmodulin-dependent protein kinase type II subunit alpha	Camk2a	54	6,9
P11983	T-complex protein 1 subunit alpha	Tcp1	60	10,4
P12367	cAMP-dependent protein kinase type II-alpha regulatory subunit	Prkar2a	45	12,2
P12382	ATP-dependent 6-phosphofructokinase, liver type	Pfkl	85	15,5
P12960	Contactin-1	Cntn1	113	11,9
P14152	Malate dehydrogenase, cytoplasmic	Mdh1	37	9
P14211	Calreticulin	Calr	48	11,5
P14685	26S proteasome non-ATPase regulatory subunit 3	Psmd3	61	16,8
P14824	Annexin A6	Anxa6	76	20,5
P14873	Microtubule-associated protein 1B	Map1b	270	11,5
P15105	Glutamine synthetase	Glul	42	8,3
P16330	2',3'-cyclic-nucleotide 3'-phosphodiesterase	Cnp	47	8,1
P16546	Spectrin alpha chain, non-erythrocytic 1	Sptan1	285	6
P16627	Heat shock 70 kDa protein 1-like	Hspa1l	71	23,4
P16858	Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	36	26,4
P17095	High mobility group protein HMG-I/HMG-Y	Hmga1	12	26,2
P17156	Heat shock-related 70 kDa protein 2	Hspa2	70	45
P17182	Alpha-enolase	Eno1	47	35,5
P17183	Gamma-enolase	Eno2	47	11,8
P17426	AP-2 complex subunit alpha-1	Ap2a1	108	11,4
P17427	AP-2 complex subunit alpha-2	Ap2a2	104	3
P17742	Peptidyl-prolyl cis-trans isomerase A	Ppia	18	23,2

P17751	Triosephosphate isomerase	Tpi1	32	14
P18760	Cofilin-1	Cfl1	19	25,9
P19246	Neurofilament heavy polypeptide	Nefh	117	5,8
P20029	78 kDa glucose-regulated protein	Hspa5	72	40,3
P20357	Microtubule-associated protein 2	Map2	199	15,9
P21550	Beta-enolase	Eno3	47	24,2
P24369	Peptidyl-prolyl cis-trans isomerase B	Ppib	24	30,1
P26040	Ezrin	Ezr	69	15,5
P26041	Moesin	Msn	68	39
P26043	Radixin	Rdx	69	38,6
P26443	Glutamate dehydrogenase 1, mitochondrial	Glud1	61	10
P26516	26S proteasome non-ATPase regulatory subunit 7	Psmd7	37	13,1
P26638	Serine--tRNA ligase, cytoplasmic	Sars	58	20,1
P27546	Microtubule-associated protein 4	Map4	117	3,6
P27773	Protein disulfide-isomerase A3	Pdia3	57	19,6
P28271	Cytoplasmic aconitate hydratase	Aco1	98	2,9
P28352	DNA-(apurinic or apyrimidinic site) lyase	Apex1	35	18,9
P28481	Collagen alpha-1(II) chain	Col2a1	142	1,6
P28650	Adenylosuccinate synthetase isozyme 1	Adssl1	50	24,9
P28652	Calcium/calmodulin-dependent protein kinase type II subunit beta	Camk2b	60	3,9
P28660	Nck-associated protein 1	Nckap1	129	11,8
P28663	Beta-soluble NSF attachment protein	Napb	34	17,8
P28738	Kinesin heavy chain isoform 5C	Kif5c	109	25
P28740	Kinesin-like protein KIF2A	Kif2a	80	19,7
P30416	Peptidyl-prolyl cis-trans isomerase FKBP4	Fkbp4	52	9
P31230	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	Aimp1	34	9,7
P31324	cAMP-dependent protein kinase type II-beta regulatory subunit	Prkar2b	46	29,1

P31938	Dual specificity mitogen-activated protein kinase kinase 1	Map2k1	43	24,2
P32883	GTPase KRas	Kras	22	21,2
P32921	Tryptophan--tRNA ligase, cytoplasmic	Wars	54	11,2
P34152	Focal adhesion kinase 1	Ptk2	124	2
P35235	Tyrosine-protein phosphatase non-receptor type 11	Ptpn11	68	25,8
P35700	Peroxiredoxin-1	Prdx1	22	27,1
P36916	Guanine nucleotide-binding protein-like 1	Gnl1	69	8,4
P37804	Transgelin	Tagln	23	17,4
P38647	Stress-70 protein, mitochondrial	Hspa9	73	12,8
P39053	Dynamin-1	Dnm1	98	19,7
P39054	Dynamin-2	Dnm2	98	7
P39749	Flap endonuclease 1	Fen1	42	8,5
P40124	Adenylyl cyclase-associated protein 1	Cap1	52	16,7
P40142	Transketolase	Tkt	68	17,3
P42669	Transcriptional activator protein Pur-alpha	Pura	35	15,3
P42932	T-complex protein 1 subunit theta	Cct8	60	13,1
P45591	Cofilin-2	Cfl2	19	18,7
P45878	Peptidyl-prolyl cis-trans isomerase FKBP2	Fkbp2	15	17,1
P46096	Synaptotagmin-1	Syt1	47	33
P46460	Vesicle-fusing ATPase	Nsf	83	14
P46471	26S protease regulatory subunit 7	Psmc2	49	4,8
P46660	Alpha-internexin	Ina	55	23,8
P46664	Adenylosuccinate synthetase isozyme 2	Adss	50	6,4
P47199	Quinone oxidoreductase	Cryz	35	5,7
P47708	Rabphilin-3A	Rph3a	75	4
P47753	F-actin-capping protein subunit alpha-1	Capza1	33	22,4
P47754	F-actin-capping protein subunit alpha-2	Capza2	33	51,7
P47757	F-actin-capping protein subunit beta	Capzb	31	20,9

P47857	ATP-dependent 6-phosphofructokinase, muscle type	Pfkm	85	17,7
P47934	Carnitine O-acetyltransferase	Crat	71	2,7
P48024	Eukaryotic translation initiation factor 1	Eif1	13	55,8
P48036	Annexin A5	Anxa5	36	6,3
P48722	Heat shock 70 kDa protein 4L	Hspa4l	94	8,5
P48758	Carbonyl reductase [NADPH] 1	Cbr1	31	8,3
P49182	Heparin cofactor 2	Serpind1	54	2,9
P49312	Heterogeneous nuclear ribonucleoprotein A1	Hnrnpa1	34	17,2
P49615	Cyclin-dependent-like kinase 5	Cdk5	33	44,9
P49722	Proteasome subunit alpha type-2	Psm2	26	42,7
P50516	V-type proton ATPase catalytic subunit A	Atp6v1a	68	29,5
P50518	V-type proton ATPase subunit E 1	Atp6v1e1	26	29,2
P50580	Proliferation-associated protein 2G4	Pa2g4	44	33,5
P51174	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	Acadl	48	6
P51432	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-3	Plcb3	139	1,6
P51859	Hepatoma-derived growth factor	Hdgf	26	14,8
P52196	Thiosulfate sulfurtransferase	Tst	33	8,8
P52480	Pyruvate kinase PKM	Pkm	58	35,6
P54071	Isocitrate dehydrogenase [NADP], mitochondrial	Idh2	51	22,6
P54227	Stathmin	Stmn1	17	14,8
P54823	Probable ATP-dependent RNA helicase DDX6	Ddx6	54	4,8
P55066	Neurocan core protein	Ncan	137	1,5
P55821	Stathmin-2	Stmn2	21	10,6
P56212	cAMP-regulated phosphoprotein 19	Arpp19	12	49,1
P56399	Ubiquitin carboxyl-terminal hydrolase 5	Usp5	96	2,4
P57759	Endoplasmic reticulum resident protein 29	Erp29	29	6,9
P58252	Elongation factor 2	Eef2	95	22,7

P59325	Eukaryotic translation initiation factor 5	Eif5	49	23,8
P60521	Gamma-aminobutyric acid receptor-associated protein-like 2	Gabarapl2	14	36,8
P60710	Actin, cytoplasmic 1	Actb	42	48
P60840	Alpha-endosulfine	Ensa	13	30,6
P60843	Eukaryotic initiation factor 4A-I	Eif4a1	46	17,5
P61082	NEDD8-conjugating enzyme Ubc12	Ube2m	21	16,4
P61089	Ubiquitin-conjugating enzyme E2 N	Ube2n	17	43,4
P61148	Fibroblast growth factor 1	Fgf1	17	20
P61161	Actin-related protein 2	Actr2	45	22,8
P61164	Alpha-centractin	Actr1a	43	28,7
P61202	COP9 signalosome complex subunit 2	Cops2	52	13,5
P61222	ATP-binding cassette sub-family E member 1	Abce1	67	23,7
P61329	Fibroblast growth factor 12	Fgf12	27	22,2
P61961	Ubiquitin-fold modifier 1	Ufm1	9	50,6
P61965	WD repeat-containing protein 5	Wdr5	37	13,5
P61982	14-3-3 protein gamma	Ywhag	28	33,2
P62082	40S ribosomal protein S7	Rps7	22	32,8
P62137	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	Ppp1ca	38	13
P62141	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	Ppp1cb	37	17,7
P62196	26S protease regulatory subunit 8	Psmc5	46	9,6
P62204	Calmodulin	Calm1	17	33,6
P62245	40S ribosomal protein S15a	Rps15a	15	23,8
P62334	26S protease regulatory subunit 10B	Psmc6	44	7,5
P62627	Dynein light chain roadblock-type 1	Dynlrb1	11	21,9
P62631	Elongation factor 1-alpha 2	Eef1a2	50	55,3
P62806	Histone H4	Hist1h4a	11	17,5
P62814	V-type proton ATPase subunit B, brain isoform	Atp6v1b2	57	5,1

P62827	GTP-binding nuclear protein Ran	Ran	24	19,9
P62852	40S ribosomal protein S25	Rps25	14	14,4
P62858	40S ribosomal protein S28	Rps28	8	30,4
P62881	Guanine nucleotide-binding protein subunit beta-5	Gnb5	44	8,4
P63001	Ras-related C3 botulinum toxin substrate 1	Rac1	21	16,7
P63005	Platelet-activating factor acetylhydrolase IB subunit alpha	Pafah1b1	47	39,8
P63017	Heat shock cognate 71 kDa protein	Hspa8	71	56,7
P63028	Translationally-controlled tumor protein	Tpt1	19	18,6
P63046	Sulfotransferase 4A1	Sult4a1	33	14,1
P63085	Mitogen-activated protein kinase 1	Mapk1	41	37,4
P63087	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	Ppp1cc	37	12,1
P63101	14-3-3 protein zeta/delta	Ywhaz	28	22,4
P63158	High mobility group protein B1	Hmgb1	25	21,9
P63242	Eukaryotic translation initiation factor 5A-1	Eif5a	17	34,4
P63280	SUMO-conjugating enzyme UBC9	Ube2i	18	16,5
P63328	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	Ppp3ca	59	3,5
P68033	Actin, alpha cardiac muscle 1	Actc1	42	27,3
P68037	Ubiquitin-conjugating enzyme E2 L3	Ube2l3	18	51,9
P68181	cAMP-dependent protein kinase catalytic subunit beta	Prkacb	41	22,8
P68254	14-3-3 protein theta	Ywhaq	28	11,8
P68368	Tubulin alpha-4A chain	Tuba4a	50	33,3
P68372	Tubulin beta-4B chain	Tubb4b	50	48,8
P68373	Tubulin alpha-1C chain	Tuba1c	50	43
P70122	Ribosome maturation protein SBDS	Sbds	29	40,4
P70236	Dual specificity mitogen-activated protein kinase kinase 6	Map2k6	37	20,1
P70296	Phosphatidylethanolamine-binding protein 1	Pebp1	21	17,6

P70336	Rho-associated protein kinase 2	Rock2	161	9,1
P70441	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	Slc9a3r1	39	7
P70670	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	Naca	221	2,6
P70695	Fructose-1,6-bisphosphatase isozyme 2	Fbp2	37	6,8
P80313	T-complex protein 1 subunit eta	Cct7	60	16,4
P80314	T-complex protein 1 subunit beta	Cct2	57	25
P80315	T-complex protein 1 subunit delta	Cct4	58	7,4
P80316	T-complex protein 1 subunit epsilon	Cct5	60	9,6
P80317	T-complex protein 1 subunit zeta	Cct6a	58	18,6
P80318	T-complex protein 1 subunit gamma	Cct3	61	25,5
P84078	ADP-ribosylation factor 1	Arf1	21	43,6
P84091	AP-2 complex subunit mu	Ap2m1	50	4,8
P97376	Protein FRG1	Frg1	29	15,5
P97390	Vacuolar protein sorting-associated protein 45	Vps45	65	7,2
P97427	Dihydropyrimidinase-related protein 1	Crmp1	62	21,7
P97807	Fumarate hydratase, mitochondrial	Fh	54	13
P99024	Tubulin beta-5 chain	Tubb5	50	48,9
P99026	Proteasome subunit beta type-4	Psmb4	29	8,7
Q00PI9	Heterogeneous nuclear ribonucleoprotein U-like protein 2	Hnrnpul2	85	2,6
Q01730	Ras suppressor protein 1	Rsu1	32	27,8
Q02053	Ubiquitin-like modifier-activating enzyme 1	Uba1	118	4
Q04447	Creatine kinase B-type	Ckb	43	13,6
Q05BC3	Echinoderm microtubule-associated protein-like 1	Eml1	90	18,7
Q06138	Calcium-binding protein 39	Cab39	40	24,9
Q08642	Protein-arginine deiminase type-2	Padi2	76	8,3
Q2NL51	Glycogen synthase kinase-3 alpha	Gsk3a	52	7,1
Q2PFD7	PH and SEC7 domain-containing protein 3	Psd3	115	5,2

Q3TGF2	Protein FAM107B	Fam107b	16	16
Q3THG9	Alanyl-tRNA editing protein Aarsd1	Aarsd1	61	4,4
Q3THK3	General transcription factor IIF subunit 1	Gtf2f1	57	6,7
Q3THK7	GMP synthase [glutamine-hydrolyzing]	Gmps	77	6,2
Q3TKT4	Transcription activator BRG1	Smarca4	181	1,4
Q3TXS7	26S proteasome non-ATPase regulatory subunit 1	Psmd1	106	9,4
Q3UGR5	Haloacid dehalogenase-like hydrolase domain-containing protein 2	Hdhd2	29	11,2
Q3UHL1	CaM kinase-like vesicle-associated protein	Camkv	55	20,1
Q3UHX2	28 kDa heat- and acid-stable phosphoprotein	Pdap1	21	27,1
Q3ULJ0	Glycerol-3-phosphate dehydrogenase 1-like protein	Gpd1l	38	4,6
Q3UM45	Protein phosphatase 1 regulatory subunit 7	Ppp1r7	41	10,2
Q3UMU9	Hepatoma-derived growth factor-related protein 2	Hdgfrp2	74	9,3
Q3UMY5	Echinoderm microtubule-associated protein-like 4	Eml4	110	2,8
Q3UV17	Keratin, type II cytoskeletal 2 oral	Krt76	63	2,7
Q3UX10	Tubulin alpha chain-like 3	Tuba13	50	6,3
Q3V1L4	Cytosolic purine 5'-nucleotidase	Nt5c2	65	4,8
Q4KMM3	Oxidation resistance protein 1	Oxr1	96	4,7
Q5M8N0	CB1 cannabinoid receptor-interacting protein 1	Cnrip1	19	5,5
Q5SQX6	Cytoplasmic FMR1-interacting protein 2	Cyfp2	146	12,8
Q5SSL4	Active breakpoint cluster region-related protein	Abr	98	2,2
Q60668	Heterogeneous nuclear ribonucleoprotein D0	Hnrnpd	38	32,1
Q60676	Serine/threonine-protein phosphatase 5	Ppp5c	57	24,2
Q60692	Proteasome subunit beta type-6	Psmb6	25	8
Q60864	Stress-induced-phosphoprotein 1	Stip1	63	23,4
Q60872	Eukaryotic translation initiation factor 1A	Eif1a	16	18,1
Q60875	Rho guanine nucleotide exchange factor 2	Arhgef2	112	4,3
Q60900	ELAV-like protein 3	Elavl3	40	4,4
Q60972	Histone-binding protein RBBP4	Rbbp4	48	16,2

Q61035	Histidine--tRNA ligase, cytoplasmic	Hars	57	14,9
Q61036	Serine/threonine-protein kinase PAK 3	Pak3	62	11,1
Q61142	Spindlin-1	Spin1	30	10,3
Q61166	Microtubule-associated protein RP/EB family member 1	Mapre1	30	21,3
Q61316	Heat shock 70 kDa protein 4	Hspa4	94	2,9
Q61425	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	Hadh	34	6,7
Q61548	Clathrin coat assembly protein AP180	Snap91	92	12,7
Q61553	Fascin	Fscn1	55	19,3
Q61644	Protein kinase C and casein kinase substrate in neurons protein 1	Paccin1	51	12,5
Q61646	Haptoglobin	Hp	39	4,9
Q61696	Heat shock 70 kDa protein 1A	Hspa1a	70	32,3
Q61753	D-3-phosphoglycerate dehydrogenase	Phgdh	57	7,3
Q61768	Kinesin-1 heavy chain	Kif5b	110	15,9
Q61879	Myosin-10	Myh10	229	3,8
Q62165	Dystroglycan	Dag1	97	3,1
Q62188	Dihydropyrimidinase-related protein 3	Dpysl3	62	24,7
Q62261	Spectrin beta chain, non-erythrocytic 1	Sptbn1	274	1,2
Q62420	Endophilin-A1	Sh3gl2	40	15,3
Q62446	Peptidyl-prolyl cis-trans isomerase FKBP3	Fkbp3	25	25,9
Q63844	Mitogen-activated protein kinase 3	Mapk3	43	15,5
Q63912	Oligodendrocyte-myelin glycoprotein	Omg	49	9,5
Q64152	Transcription factor BTF3	Btf3	22	32,4
Q641P0	Actin-related protein 3B	Actr3b	48	12,9
Q64467	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	Gapdhs	48	4,1
Q64514	Tripeptidyl-peptidase 2	Tpp2	140	3,7
Q64669	NAD(P)H dehydrogenase [quinone] 1	Nqo1	31	6,9

Q68FL6	Methionine--tRNA ligase, cytoplasmic	Mars	101	3,2
Q69ZS7	HBS1-like protein	Hbs1l	75	8,4
Q6A028	Switch-associated protein 70	Swap70	69	12,6
Q6DIC0	Probable global transcription activator SNF2L2	Smarca2	180	1,6
Q6NZB0	DnaJ homolog subfamily C member 8	Dnajc8	30	36
Q6P1B1	Xaa-Pro aminopeptidase 1	Xpnpep1	70	14,6
Q6PDI5	Proteasome-associated protein ECM29 homolog	Ecm29	204	5,9
Q6PDL0	Cytoplasmic dynein 1 light intermediate chain 2	Dync1li2	54	8,9
Q6PER3	Microtubule-associated protein RP/EB family member 3	Mapre3	32	35,6
Q6PGN3	Serine/threonine-protein kinase DCLK2	Dclk2	83	6,1
Q6WVG3	BTB/POZ domain-containing protein KCTD12	Kctd12	36	7,3
Q6ZPJ3	(E3-independent) E2 ubiquitin-conjugating enzyme UBE2O	Ube2o	141	1,8
Q6ZQ38	Cullin-associated NEDD8-dissociated protein 1	Cand1	136	12,1
Q6ZWX6	Eukaryotic translation initiation factor 2 subunit 1	Eif2s1	36	44,8
Q71LX4	Talin-2	Tln2	254	1
Q78JW9	Ubiquitin domain-containing protein UBFD1	Ubfd1	40	11,4
Q78PG9	Coiled-coil domain-containing protein 25	Ccdc25	24	17,3
Q78ZA7	Nucleosome assembly protein 1-like 4	Nap1l4	43	7,5
Q792Z1	MCG140784	Try10	26	8,1
Q7M6Y3	Phosphatidylinositol-binding clathrin assembly protein	Picalm	72	6,7
Q7TMB8	Cytoplasmic FMR1-interacting protein 1	Cyfip1	145	7,7
Q7TMK9	Heterogeneous nuclear ribonucleoprotein Q	Syncrip	70	8,2
Q7TMM9	Tubulin beta-2A chain	Tubb2a	50	50,8
Q7TNG5	Echinoderm microtubule-associated protein-like 2	Eml2	71	4,5
Q7TNV0	Protein DEK	Dek	43	6,6
Q7TQD2	Tubulin polymerization-promoting protein	Tppp	24	16,1
Q7TSJ2	Microtubule-associated protein 6	Map6	96	3,9

Q80TV8	CLIP-associating protein 1	Clasp1	169	10,2
Q80UG5	Septin-9	Sept9	66	4,3
Q80UM3	N-alpha-acetyltransferase 15, NatA auxiliary subunit	Naa15	101	9,9
Q80VP1	Epsin-1	Epn1	60	3,8
Q80XU3	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	Nucks1	26	19,7
Q810S1	Calcium uniporter regulatory subunit MCub, mitochondrial	Mcub	40	5,5
Q810U3	Neurofascin	Nfasc	138	6
Q8BFR5	Elongation factor Tu, mitochondrial	Tufm	50	5,8
Q8BFZ3	Beta-actin-like protein 2	Actbl2	42	22,1
Q8BG05	Heterogeneous nuclear ribonucleoprotein A3	Hnrnpa3	40	24,8
Q8BG32	26S proteasome non-ATPase regulatory subunit 11	Psmd11	47	32
Q8BGA3	Leucine-rich repeat transmembrane neuronal protein 2	Lrrtm2	59	5,4
Q8BGQ7	Alanine--tRNA ligase, cytoplasmic	Aars	107	2,4
Q8BGR9	Ubiquitin-like domain-containing CTD phosphatase 1	Ublcp1	37	20,1
Q8BGT8	Phytanoyl-CoA hydroxylase-interacting protein-like	Phyhipl	42	14,7
Q8BGY2	Eukaryotic translation initiation factor 5A-2	Eif5a2	17	19
Q8BH57	WD repeat-containing protein 48	Wdr48	76	11,2
Q8BJ37	Tyrosyl-DNA phosphodiesterase 1	Tdp1	69	5,1
Q8BJD1	Inter-alpha-trypsin inhibitor heavy chain H5	Itih5	107	3,2
Q8BK63	Casein kinase I isoform alpha	Csnk1a1	39	10,4
Q8BK64	Activator of 90 kDa heat shock protein ATPase homolog 1	Ahsa1	38	18,9
Q8BK67	Protein RCC2	Rcc2	56	14,8
Q8BKG3	Inactive tyrosine-protein kinase 7	Ptk7	118	2,8
Q8BKX1	Brain-specific angiogenesis inhibitor 1-associated protein 2	Baiap2	59	6,7
Q8BLJ3	PI-PLC X domain-containing protein 3	Plcxd3	36	10,3
Q8BMF3	NADP-dependent malic enzyme, mitochondrial	Me3	67	3,1

Q8BMJ2	Leucine--tRNA ligase, cytoplasmic	Lars	134	2,6
Q8BP47	Asparagine--tRNA ligase, cytoplasmic	Nars	64	5,5
Q8BRT1	CLIP-associating protein 2	Clasp2	141	9,3
Q8BU30	Isoleucine--tRNA ligase, cytoplasmic	lars	144	2,9
Q8BVI4	Dihydropteridine reductase	Qdpr	26	15,8
Q8BVQ5	Protein phosphatase methylesterase 1	Ppme1	42	8,8
Q8BVU5	ADP-ribose pyrophosphatase, mitochondrial	Nudt9	39	6,6
Q8BW96	Calcium/calmodulin-dependent protein kinase type 1D	Camk1d	43	16,1
Q8BWG8	Beta-arrestin-1	Arrb1	47	10,8
Q8BWR2	PITH domain-containing protein 1	Pithd1	24	28,4
Q8BWY3	Eukaryotic peptide chain release factor subunit 1	Etf1	49	10,1
Q8BWZ3	N-alpha-acetyltransferase 25, NatB auxiliary subunit	Naa25	112	6,8
Q8BYB9	Protein O-glucosyltransferase 1	Poglut1	46	11,7
Q8BZ98	Dynamin-3	Dnm3	97	8,8
Q8C1B1	Calmodulin-regulated spectrin-associated protein 2	Camsap2	164	1,6
Q8C1B7	Septin-11	Sept11	50	18,8
Q8C1W1	Vasohibin-1	Vash1	42	12,8
Q8C4Q6	Axin interactor, dorsalization-associated protein	Aida	35	16,4
Q8C5R8	Phosphoribosyl pyrophosphate synthetase 1-like 1	Prps1l1	35	11,6
Q8C8R3	Ankyrin-2	Ank2	426	1,2
Q8CBY8	Dynactin subunit 4	Dctn4	53	7,3
Q8CDN6	Thioredoxin-like protein 1	Txn1l	32	30,1
Q8CGC7	Bifunctional glutamate/proline--tRNA ligase	Eprs	170	2
Q8CGF7	Transcription elongation regulator 1	Tcerg1	124	3,9
Q8CHC4	Synaptojanin-1	Synj1	173	13
Q8CIB5	Fermitin family homolog 2	Fermt2	78	3,2
Q8CIN4	Serine/threonine-protein kinase PAK 2	Pak2	58	11,5
Q8JZK9	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	Hmgcs1	58	6,9
Q8JZQ9	Eukaryotic translation initiation factor 3 subunit B	Eif3b	91	3,9

Q8K0S0	Phytanoyl-CoA hydroxylase-interacting protein	Phyhip	38	8,2
Q8K0U4	Heat shock 70 kDa protein 12A	Hspa12a	75	9,6
Q8K1J6	CCA tRNA nucleotidyltransferase 1, mitochondrial	Trnt1	50	9,2
Q8K1M6	Dynamin-1-like protein	Dnm1l	83	11,7
Q8K2T1	NmrA-like family domain-containing protein 1	Nmral1	34	8,1
Q8K409	DNA polymerase beta	Polb	38	13,7
Q8QZT1	Acetyl-CoA acetyltransferase, mitochondrial	Acat1	45	9,9
Q8QZY1	Eukaryotic translation initiation factor 3 subunit L	Eif3l	67	8,2
Q8R001	Microtubule-associated protein RP/EB family member 2	Mapre2	37	21,2
Q8R050	Eukaryotic peptide chain release factor GTP-binding subunit ERF3A	Gspt1	69	9
Q8R0F6	Integrin-linked kinase-associated serine/threonine phosphatase 2C	Ilkap	43	18,1
Q8R0Y6	Cytosolic 10-formyltetrahydrofolate dehydrogenase	Aldh1l1	99	3,1
Q8R1B4	Eukaryotic translation initiation factor 3 subunit C	Eif3c	106	7,7
Q8R1Q8	Cytoplasmic dynein 1 light intermediate chain 1	Dync1li1	57	5,4
Q8R3R8	Gamma-aminobutyric acid receptor-associated protein-like 1	Gabarapl1	14	35
Q8R574	Phosphoribosyl pyrophosphate synthase-associated protein 2	Prpsap2	41	28,2
Q8R5C5	Beta-centractin	Actr1b	42	20,5
Q8R5H6	Wiskott-Aldrich syndrome protein family member 1	Wasf1	62	3,6
Q8VDD5	Myosin-9	Myh9	226	5
Q8VDM4	26S proteasome non-ATPase regulatory subunit 2	Psmd2	100	13,4
Q8VE37	Regulator of chromosome condensation	Rcc1	45	18,3
Q8VED9	Galectin-related protein	Lgalsl	19	25,6
Q8VEK3	Heterogeneous nuclear ribonucleoprotein U	Hnrnpu	88	11,4
Q8VHM5	Heterogeneous nuclear ribonucleoprotein R	Hnrnpr	71	6
Q91UZ1	Phosphoinositide phospholipase C	Plcb4	135	6,7
Q91V09	WD repeat-containing protein 13	Wdr13	54	7,8

Q91V12	Cytosolic acyl coenzyme A thioester hydrolase	Acot7	43	18,6
Q91V57	N-chimaerin	Chn1	53	6,1
Q91V89	Protein phosphatase 2, regulatory subunit B (B56), delta isoform	Ppp2r5d	69	4,2
Q91V92	ATP-citrate synthase	Acly	120	15,4
Q91VK1	Basic leucine zipper and W2 domain-containing protein 2	Bzw2	48	19,8
Q91VR5	ATP-dependent RNA helicase DDX1	Ddx1	82	8,4
Q91VR7	Microtubule-associated proteins 1A/1B light chain 3A	Map1lc3a	14	23,1
Q91VZ6	Stromal membrane-associated protein 1	Smap1	48	4,5
Q91WC0	Histone-lysine N-methyltransferase setd3	Setd3	67	3
Q91WQ3	Tyrosine--tRNA ligase, cytoplasmic	Yars	59	37,7
Q91XL9	Oxysterol-binding protein-related protein 1	Osbpl1a	108	7,2
Q91XM9	Disks large homolog 2	Dlg2	95	16,2
Q91YE3	Egl nine homolog 1	Egln1	43	5,2
Q91YJ3	Thymocyte nuclear protein 1	Thyn1	26	7,5
Q91YP2	Neurolysin, mitochondrial	Nln	80	6,4
Q91YR1	Twinfilin-1	Twf1	40	15,4
Q91ZJ5	UTP--glucose-1-phosphate uridylyltransferase	Ugp2	57	22
Q91ZW3	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	Smarca5	122	2
Q921M7	Protein FAM49B	Fam49b	37	38
Q921W0	Charged multivesicular body protein 1a	Chmp1a	22	12,8
Q921X9	Protein disulfide-isomerase A5	Pdia5	59	6,6
Q922B2	Aspartate--tRNA ligase, cytoplasmic	Dars	57	5,6
Q922D8	C-1-tetrahydrofolate synthase, cytoplasmic	Mthfd1	101	7,1
Q922F4	Tubulin beta-6 chain	Tubb6	50	26,8
Q922J3	CAP-Gly domain-containing linker protein 1	Clip1	156	12,9
Q923D2	Flavin reductase (NADPH)	Blvrb	22	21,4
Q924Y0	Gamma-butyrobetaine dioxygenase	Bbox1	45	6,5

Q99020	Heterogeneous nuclear ribonucleoprotein A/B	Hnrnpab	31	20
Q99104	Unconventional myosin-Va	Myo5a	216	1,1
Q99J08	SEC14-like protein 2	Sec14l2	46	5,2
Q99J36	THUMP domain-containing protein 1	Thumpd1	39	19,1
Q99J77	Sialic acid synthase	Nans	40	8,9
Q99JF8	PC4 and SFRS1-interacting protein	Psip1	60	14,8
Q99JI4	26S proteasome non-ATPase regulatory subunit 6	Psmd6	46	11,8
Q99JY9	Actin-related protein 3	Actr3	47	30,1
Q99K85	Phosphoserine aminotransferase	Psat1	40	7,8
Q99KB8	Hydroxyacylglutathione hydrolase, mitochondrial	Hagh	34	17,2
Q99KI0	Aconitate hydratase, mitochondrial	Aco2	85	27,2
Q99KJ8	Dynactin subunit 2	Dctn2	44	17,7
Q99KK2	N-acylneuraminate cytidyltransferase	Cmas	48	4,6
Q99L45	Eukaryotic translation initiation factor 2 subunit 2	Eif2s2	38	24,2
Q99LC8	Translation initiation factor eIF-2B subunit alpha	Eif2b1	34	9,2
Q99LD4	COP9 signalosome complex subunit 1	Gps1	53	5,5
Q99LF4	tRNA-splicing ligase RtcB homolog	Rtcb	55	24,2
Q99LU0	Charged multivesicular body protein 1b-1	Chmp1b1	22	6
Q99NF3	Centrosomal protein of 41 kDa	Cep41	41	7
Q99PT1	Rho GDP-dissociation inhibitor 1	Arhgdia	23	38,2
Q9CQ65	S-methyl-5'-thioadenosine phosphorylase	Mtap	31	10,2
Q9CQC6	Basic leucine zipper and W2 domain-containing protein 1	Bzw1	48	27
Q9CQH7	Transcription factor BTF3 homolog 4	Btf3l4	17	55,7
Q9CQJ6	Density-regulated protein	Denr	22	20,7
Q9CQV6	Microtubule-associated proteins 1A/1B light chain 3B	Map1lc3b	15	29,6
Q9CQV8	14-3-3 protein beta/alpha	Ywhab	28	21,1
Q9CR16	Peptidyl-prolyl cis-trans isomerase D	Ppid	41	26,5
Q9CR29	Coiled-coil domain-containing protein 43	Ccdc43	25	12,2

Q9CRB6	Tubulin polymerization-promoting protein family member 3	Tppp3	19	29
Q9CRC8	Leucine-rich repeat-containing protein 40	Lrrc40	68	2,8
Q9CRD2	ER membrane protein complex subunit 2	Emc2	35	10,1
Q9CS42	Ribose-phosphate pyrophosphokinase 2	Prps2	35	23,3
Q9CVB6	Actin-related protein 2/3 complex subunit 2	Arpc2	34	29,7
Q9CWJ9	Bifunctional purine biosynthesis protein PURH	Atic	64	11,3
Q9CX34	Protein SGT1 homolog	Sugt1	38	18,8
Q9CXU9	Eukaryotic translation initiation factor 1b	Eif1b	13	44,2
Q9CXW3	Calcyclin-binding protein	Cacybp	27	21,8
Q9CXW4	60S ribosomal protein L11	Rpl11	20	12,6
Q9CY64	Biliverdin reductase A	Blvra	34	7,1
Q9CYR6	Phosphoacetylglucosamine mutase	Pgm3	59	4,6
Q9CYT6	Adenylyl cyclase-associated protein 2	Cap2	53	4,8
Q9CZ30	Obg-like ATPase 1	Ola1	45	39,6
Q9CZ44	NSFL1 cofactor p47	Nsfl1c	41	7
Q9CZD3	Glycine--tRNA ligase	Gars	82	9,6
Q9CZT6	Protein CMSS1	Cmss1	32	7,6
Q9CZU6	Citrate synthase, mitochondrial	Cs	52	7,8
Q9CZW5	Mitochondrial import receptor subunit TOM70	Tomm70	68	8,3
Q9CZX8	40S ribosomal protein S19	Rps19	16	23,4
Q9D051	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	Pdhb	39	12,3
Q9D0I9	Arginine--tRNA ligase, cytoplasmic	Rars	76	5
Q9D0K2	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	Oxct1	56	7,9
Q9D0L8	mRNA cap guanine-N7 methyltransferase	Rnmt	53	5,6
Q9D0M1	Phosphoribosyl pyrophosphate synthase-associated protein 1	Prpsap1	39	22,8
Q9D0R2	Threonine--tRNA ligase, cytoplasmic	Tars	83	12,6

Q9D1J3	SAP domain-containing ribonucleoprotein	Sarnp	24	20,5
Q9D1P4	Cysteine and histidine-rich domain-containing protein 1	Chordc1	37	18,7
Q9D2M8	Ubiquitin-conjugating enzyme E2 variant 2	Ube2v2	16	60
Q9D2R0	Acetoacetyl-CoA synthetase	Aacs	75	11,8
Q9D358	Low molecular weight phosphotyrosine protein phosphatase	Acp1	18	12,7
Q9D6F9	Tubulin beta-4A chain	Tubb4a	50	48,9
Q9D708	Putative uncharacterized protein	S100a16	14	10,5
Q9D7G0	Ribose-phosphate pyrophosphokinase 1	Prps1	35	15,1
Q9D7H3	RNA 3'-terminal phosphate cyclase	RtcA	39	11,2
Q9D8B3	Charged multivesicular body protein 4b	Chmp4b	25	13,4
Q9D8N0	Elongation factor 1-gamma	Eef1g	50	22,2
Q9D8W5	26S proteasome non-ATPase regulatory subunit 12	Psmd12	53	16,9
Q9D8Y0	EF-hand domain-containing protein D2	Efhd2	27	15,8
Q9DB16	Calcium-binding protein 39-like	Cab39l	39	13,1
Q9DB27	Malignant T-cell-amplified sequence 1	Mcts1	21	21
Q9DBG3	AP-2 complex subunit beta	Ap2b1	105	9,2
Q9DBP5	UMP-CMP kinase	Cmpk1	22	23,5
Q9DCD0	6-phosphogluconate dehydrogenase, decarboxylating	Pgd	53	18
Q9DCD6	Gamma-aminobutyric acid receptor-associated protein	Gabarap	14	34,2
Q9DCL9	Multifunctional protein ADE2	Paics	47	5,9
Q9DCN2	NADH-cytochrome b5 reductase 3	Cyb5r3	34	8
Q9DD18	D-tyrosyl-tRNA(Tyr) deacylase 1	Dtd1	23	18,2
Q9EQF6	Dihydropyrimidinase-related protein 5	Dpysl5	62	15,6
Q9EQX4	Allograft inflammatory factor 1-like	Aif1l	17	21,3
Q9ERD7	Tubulin beta-3 chain	Tubb3	50	52,2
Q9ERE7	LDLR chaperone MESD	Mesdc2	25	26,3
Q9ERQ8	Carbonic anhydrase 7	Ca7	30	12,9

Q9ESN6	Tripartite motif-containing protein 2	Trim2	81	26,6
Q9JHQ5	Leucine zipper transcription factor-like protein 1	Lztfl1	35	7,4
Q9JHU4	Cytoplasmic dynein 1 heavy chain 1	Dync1h1	532	3,7
Q9JIF0	Protein arginine N-methyltransferase 1	Prmt1	42	6,2
Q9JJK2	LanC-like protein 2	Lancl2	51	8,4
Q9JJZ2	Tubulin alpha-8 chain	Tuba8	50	22,3
Q9JKK7	Tropomodulin-2	Tmod2	40	20,8
Q9JLM8	Serine/threonine-protein kinase DCLK1	Dclk1	84	12,3
Q9JLV5	Cullin-3	Cul3	89	17,8
Q9JM76	Actin-related protein 2/3 complex subunit 3	Arpc3	21	24,7
Q9JMA1	Ubiquitin carboxyl-terminal hydrolase 14	Usp14	56	17,6
Q9JMG1	Endothelial differentiation-related factor 1	Edf1	16	31,8
Q9JMG7	Hepatoma-derived growth factor-related protein 3	Hdgfrp3	22	32,2
Q9QUM9	Proteasome subunit alpha type-6	Psma6	27	31,3
Q9QUP5	Hyaluronan and proteoglycan link protein 1	Hapln1	40	7,6
Q9QUR7	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1	Pin1	18	37
Q9QX11	Cytohesin-1	Cyth1	46	5
Q9QXL2	Kinesin-like protein KIF21A	Kif21a	187	4,6
Q9QY36	N-alpha-acetyltransferase 10	Naa10	27	9,4
Q9QY76	Vesicle-associated membrane protein-associated protein B	Vapb	27	16
Q9QYB8	Beta-adducin	Add2	81	11,7
Q9QYC0	Alpha-adducin	Add1	81	6,9
Q9QYR6	Microtubule-associated protein 1A	Map1a	300	10,7
Q9QZ73	DCN1-like protein 1	Dcun1d1	30	10,8
Q9QZD9	Eukaryotic translation initiation factor 3 subunit I	Eif3i	36	12,6
Q9R0P4	Small acidic protein	Smap	20	21,5
Q9R0Q6	Actin-related protein 2/3 complex subunit 1A	Arpc1a	42	17,6
Q9R0Y5	Adenylate kinase isoenzyme 1	Ak1	22	12,9

Q9R1P1	Proteasome subunit beta type-3	Psmb3	23	34,1
Q9R1P3	Proteasome subunit beta type-2	Psmb2	23	12,9
Q9R1P4	Proteasome subunit alpha type-1	Psma1	30	21,7
Q9R1Q8	Transgelin-3	Tagln3	22	38,2
Q9R1R2	Tripartite motif-containing protein 3	Trim3	81	14,9
Q9WTN0	Geranylgeranyl pyrophosphate synthase	Ggps1	35	9,7
Q9WTX5	S-phase kinase-associated protein 1	Skp1	19	15,3
Q9WTX6	Cullin-1	Cul1	90	11,1
Q9WUA2	Phenylalanine--tRNA ligase beta subunit	Farsb	66	7,8
Q9WUA3	ATP-dependent 6-phosphofructokinase, platelet type	Pfkip	85	9,6
Q9WUA6	RAC-gamma serine/threonine-protein kinase	Akt3	56	4,6
Q9WUK2	Eukaryotic translation initiation factor 4H	Eif4h	27	16,9
Q9WUM3	Coronin-1B	Coro1b	54	3,7
Q9WUM4	Coronin-1C	Coro1c	53	13,7
Q9WV32	Actin-related protein 2/3 complex subunit 1B	Arpc1b	41	11
Q9WV55	Vesicle-associated membrane protein-associated protein A	Vapa	28	8,4
Q9WV60	Glycogen synthase kinase-3 beta	Gsk3b	47	19,5
Q9WVA3	Mitotic checkpoint protein BUB3	Bub3	37	15,6
Q9WVA4	Transgelin-2	Tagln2	22	27,1
Q9Z0H8	CAP-Gly domain-containing linker protein 2	Clip2	116	4,8
Q9Z0N2	Eukaryotic translation initiation factor 2 subunit 3, Y-linked	Eif2s3y	51	16,7
Q9Z0P5	Twinfilin-2	Twf2	39	5,2
Q9Z130	Heterogeneous nuclear ribonucleoprotein D-like	Hnrnpdl	34	10,6
Q9Z140	Copine-6	Cpne6	62	9,3
Q9Z172	Small ubiquitin-related modifier 3	Sumo3	12	19,1
Q9Z1B3	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1	Plcb1	138	19
Q9Z1B7	Mitogen-activated protein kinase 13	Mapk13	42	5,5

Q9Z1G3	V-type proton ATPase subunit C 1	Atp6v1c1	44	7,9
Q9Z1G4	V-type proton ATPase 116 kDa subunit a isoform 1	Atp6v0a1	96	2,4
Q9Z1N5	Spliceosome RNA helicase Ddx39b	Ddx39b	49	26,4
Q9Z1S5	Neuronal-specific septin-3	Sept3	40	17,7
Q9Z1Z2	Serine-threonine kinase receptor-associated protein	Strap	38	12,3
Q9Z2H5	Band 4.1-like protein 1	Epb41l1	98	3
Q9Z2U0	Proteasome subunit alpha type-7	Psm7	28	29
Q9Z2U1	Proteasome subunit alpha type-5	Psm5	26	27
Q9Z2Y8	Proline synthase co-transcribed bacterial homolog protein	Prosc	30	12

One important object of the invention is the possibility of testing candidate inhibitors in crude protein extracts containing native inherent TCP activity. By native inherent activity is understood the naturally obtained enzymatic activity contained within the biological sample and which has been obtained solely by described extraction method from a specific tissue, organ of biological sample. It is important to clarify that native activity is in a natural, unadorned or unchanged state. It has not been engineered nor adapted and reflects physiologically present activity in the studied biological sample such as but not limited to a specific tissue/organ. On the other hand, purified recombinant proteins of the different identified TCPase can also be tested in the dephosphorylation assay.

Example 2: Validation of the TCP activity

Before testing the different compounds of the fraction of proteins having a TCP activity (MAPs fraction), the TCP activity was assessed by use of a dephosphorylation assay (Figure 4).

Material & method

An assay involving radioactively labelled tyrosine (^3H -Y) was used to quantitatively determine the TCPase activity. Brain tubulin was isolated and purified and radioactively labelled with ^3H -Y by recombinant TTL. To do so, recombinant bacterially expressed purified TTL (>90% purity) was put in contact with micrograms of pig brain purified tubulin. The reaction sample was incubated for one hour at 37°C in presence of radioactively labelled ^3H -Y and ATP. Following incorporation of ^3H -Y to tubulin, a polymerization cycle was performed by adding GTP and incubating for 30min at 37°C . Next the samples are centrifuged and the obtained pellet was

washed twice with PEM buffer. The resulting radioactively labelled MTs were store at -80°C until further testing. The candidate TCPase protein was expressed and purified from bacteria using His-tagged purification strategy to at least 80% purity. After obtaining both purified recombinant TCPase candidate and radioactively labelled MTs, the proteins were put into
5 contact and various amounts of TCPase were presented to MTs. Removal of the radioactive tyrosine by the candidate detyrosinase was measured by quantification of radioactivity in both the soluble and insoluble fraction of the reaction using a liquid scintillator counter.

To further gain insights in the potential protease that could embody the TCPase activity, the isolated MAPs from crude brain extracts were exposed to a selection of specific Cysteine,
10 Aspartic, Metallo and Serine proteinase inhibitors were tested on the extract.

Results

As expected, when MAPs fraction was added to the microtubule sample, increased detyrosination could be observed by western blot (Δ 1-tubulin), showing that the MAPs fraction contains detyrosination activity (Figure 4). In line with previous observation, TCPase activity
15 was found in protein extracts obtained from pig brain confirming that brain has high endogenous TCPase activity, likely in part originating from neurones.

While Serine proteases inhibitors did hardly reduce native TCPase activity in brain MAPs, metallo proteases inhibitors, such as the EDTA and EGTA chelators, all led to significant inhibition up to 50% of the total TCPase activity contained in the brain MAPs. However,
20 cysteine protease inhibitor treatment with compounds as E64 and Iodoacetamide (irreversible cysteine inhibitor) led to a complete inhibition of TCPase activity (Figure 5). Of note, based on these data it seems likely that various specific proteins contain TCPase activity.

Example 3: Study of the CRMP family

As part of the identified proteins in Table I, the family of collapsing response mediator proteins
25 (CRMPs) was found. The CRMPs family has not yet been associated with regulation of TCPase activity.

In order to establish the involvement in regulation of TCP activity of these proteins, an assay was performed with a protein extract obtained from HEK293 ectopically expressing individual HA-tagged CRMP family members. All five members of the CRMP family were cloned into
30 pRK5-HA vector and equal amounts of plasmids were transfected into HEK293 cells in a 6-wells plate. Two days after transfection cells were collected in Laemmli lysis buffer and

subjected to immunoblotting analysis using a specific $\Delta 1$ -tubulin antibody. Overexpression of CRMP1 drastically increased tubulin detyrosination, whereas the other CRMPs showed no detyrosination activity (Figure 6).

To further validate the TCPase activity observed in the overexpression experiment for CRMP1, we performed immunofluorescence analysis on U2OS cells. Equal amounts of pRK5 plasmid containing all the 5 members of the CRMP family were transfected using a polyethylenimine derivative transfection reagent. Two days after transfection cells were ethanol fixed and subjected to immunofluorescence labelling of HA tag and $\Delta 1$ -tubulin (Figure 7). All ectopically expressed CRMP proteins are labeled with HA but only CRMP1 transfected cells showed increased $\Delta 1$ -tubulin staining.

An assay for evaluating knockdown of endogenous CRMP1 expression in U2OS cells by siRNA interference was also performed. U2OS cells were routinely cultured in the laboratory under standard conditions. Knockdown was obtained by transfection using INTERFERin (Polyplus) of specific RNAi sequences targeting CRMP1. Seventy two hours post-transfection, cells were collected and lysed in Laemmli buffer. Equal amounts of proteins were subjected to immunoblotting protocol. A decrease in posttranslational modification of tubulin as detyrosination and acetylation are shown to correlate with decrease in CRMP1 expression (Figure 8).

To further validate the loss of TCPase activity observed by knockdown of CRMP1 in U2OS cells, routinely cultured cells were plated in 6-wells plate, ethanol fixed and analysed by immunofluorescence labelling. As anticipated, knockdown of CRMP1 resulted in reduction of $\Delta 1$ -tubulin staining (Figure 9).

Interestingly, depletion of CRMP1 by transfection with specific RNAi sequences resulted in cell cycle arrest in the human U2OS cells. This was observed by western blot analysis of depleted cells. Knockdown of CRMP1 resulted in increase of p21 and p53 protein levels, a marker for cell cycle arrest (Figure 10A). This was further validated by flow cytometry analysis. Indeed, CRMP1 depleted cells showed an increase of cells with 2n bulk DNA content, indicative for a G1 arrest (Figure 10B-C). This is particularly interesting in the context of cancerous cells that have uncontrolled cell division.

Example 4: Inhibitor activity of peptidic based inhibitors

One important object of the invention is the possibility of testing candidate inhibitor in crude protein extracts containing native inherent TCP activity. By native inherent activity is

understood the naturally obtained enzymatic activity contained within the biological sample and which has been obtained solely by described extraction method from a specific tissue, organ of biological sample. According to the invention, “native activity” corresponds to natural, unadorned or unchanged state; it has not been engineered nor adapted and reflects physiologically present activity in the studied biological sample, such as but not limited to a specific tissue/organ.

The natural protruding alpha tubulin tail on the surface of the MT's was used as a base for the development of peptidic inhibitors. Among the various peptides that could be tested to validate the inhibitor activity of the peptidic inhibitors of the invention, a representative set of two peptides composed of EDY and EEY was evaluated.

Material & method

Recombinant bacterially expressed purified TTL (>90% purity) was put in contact with micrograms of pig brain purified tubulin. The reaction sample was incubated for one hour at 37°C in presence of radioactively labeled ³H-Y and ATP. Following incorporation of ³H-Y to tubulin, a polymerization cycle was performed by adding GTP and incubating for 30min at 37°C. Next, the samples were centrifuged and the obtained pellet was washed twice with PEM buffer. The resulting radioactively labeled MTs were store at -80°C until further testing. The isolated MAPs from crude brain extracts (as obtained in example 1) were contacted to the radioactively labeled MTs in absence or presence of different peptidic inhibitors or an increasing concentration of peptidic inhibitor. Release of radioactive tyrosine by native TCPase containing brain MAPs was measured by quantification of radioactivity in both the soluble and insoluble fraction of the reaction using a liquid scintillator counter.

Results

This method for selecting and designing peptidic inhibitors with different properties allows differential applications of the inhibitors based on selectivity and potency criteria. We observed that the three amino acid peptide EDY already partially blocked TCP activity in the MAPs fraction (Figure 11A). Most interestingly, the tripeptide EEY almost fully inhibited TCP activity in this setting (Figure 11A). To further pharmacologically describe the peptidic inhibitor, a dose-response curve analysis was performed (Figure 11B). The obtained inhibition reflected specific inhibition of TCPase by the peptidic inhibitor (Figures 11A and 11B).

Example 5: In cellulo inhibition of TCPase activity with a peptidic inhibitor

To further explore the *in cellulo* efficacy of EEY peptide to inhibit TCP activity in a relevant model, C2C12 muscle cells were cultured and differentiated.

Myogenesis is a complex phenomenon and mechanistically linked to detyrosination status of the microtubules. Duchenne muscular dystrophy (DMD) is a severe type of muscular dystrophy and some of the altered biochemical processes are mimicked in the C2C12 muscle cell model.

Material & method

C2C12 cells is an immortal cell line of mouse skeletal myoblasts originally derived from satellite cells from the thigh muscle cells. C2C12 cells were routinely grown at 37°C in a CO₂ incubator. Myogenic differentiation is initiated upon reaching confluence by switching the cells to medium containing 2% horse serum. Cells were collected at start of the myogenic differentiation and every two days over a period of 6 days for molecular analysis.

In addition, to assess the role of TCPase in a different model, analysis of TCPase expression in a neuronal differentiation process using neuroblastoma cell line (SH-SY5Y) was tested. SH-SY5Y cells were routinely cultured at 37°C in a CO₂ incubator prior to differentiation. Cells were resuspended in growing media and plated at low density in culture plates. The differentiation process was followed by light microscopy and clear neural phenotype could be observed at day 8. Post-mitotic SH-SY5Y cells displayed increasing number of outgrowth and neurites. Cells were collected during the neuronal differentiation process at day 0, 2, 3, 6, 7 and 10 for gene expression analysis by quantitative PCR. qPCR probes were designed using primer3 software and CRMP1 gene expression was analyzed.

Results

To further study the use of a TCPase inhibitor, C2C12 cells were treated with or without EEY peptide (Figure 12). The protein expression of Myosin was monitored by western blotting as control for muscle differentiation. Vinculin acts as loading control. Acetylation and detyrosination of the microtubules was assessed. Whereas acetylation increases during differentiation (Ac-Tubulin) no difference in the status could be observed in the treated cells. Interestingly, detyrosination levels were increased already at day after onset of myogenic differentiation. Besides, the presence of the TCPase inhibitor clearly inhibited detyrosination (Δ -1 Tubulin), further supporting the notion that the TCPase inhibitor is cell permeable and acts on intrinsic TCPase activity.

As expected a strong induction of DDC was measured during the neural differentiation process. DDC is a marker of dopaminergic neurons and validates the neural differentiation process during the experiment (Figure 13). As anticipated, the level of CRMP1 also increased as TCPase activity also increases during the process. This is in line with the critical role of TCPase activity in the maintenance of axonal projection. The SH-SY5Y differentiation process recapitulates many molecular mechanisms known to be dysregulated in Parkinson disease and other neurodegenerative disorders.

Example 6: Study of the detyrosination process of microtubules in muscular dystrophy

Myoblast cells were obtained from a healthy control (Ctrl) and from a patient diagnosed with Duchenne muscular dystrophy (DMD), a genetic disorder characterized by progressive muscle degeneration and weakness. DMD is caused by an absence of dystrophin, a protein that helps keep muscle cells intact.

The cells obtained from DMD patients have been sequenced and due to a genetic mutation, they lack dystrophin protein. After isolation and purification of the myoblast cells from the explants, the cells were cultured in a standard humidified tissue culture incubator at 37°C in presence of 5% CO₂. The cells were amplified in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 20% fetal bovine serum (FBS), 10% horse serum, purified growth factors and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). The serum-rich growth medium supports both proliferation and differentiation of myogenic cells. The cells were plated in a 6cm culture dish and grown to confluency prior to myogenic differentiation. Cells were collected at indicated steps by scrapping in PBS and mild centrifugation. The PBS buffer was removed and samples were snap frozen in liquid nitrogen and kept in -80°C freezer until analysis. All the samples contained a similar amount of cells. A denaturing Laemmli buffer (containing 2% SDS, 2,5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 0.125 M Tris HCl, pH adjusted to 6.8) was added and the samples were boiled at 95°C to further to denature the proteins present. After cooling, the samples were loaded on a 10% polyacrylamide gel and subjected to electrophoresis for separation and transferred to a nitrocellulose membrane (GE Healthcare). The Antibodies recognizing detyrosinated tubulin (deTyr-tub), beta tubulin (E7, hybridoma) and vinculin (Sigma) were used to detect protein levels. A secondary antibody coupled to HRP (Cell Signaling) was used for detection of the protein of interest.

The basal level of detyrosination was lower in DMD cells in the basal condition whereas after differentiation detyrosination was much higher (Fig 14.). The observed increase in tubulin detyrosination in cells originating from a DMD patient could represent a new therapeutic opportunity.

Example 7: Study of the detyrosination process of microtubules in neurodegenerative diseases

a) Tauopathies belong to a class of neurodegenerative diseases associated with the pathological aggregation of the microtubule-associated protein (MAP) known as Tau protein in neurofibrillary tangles in the human brain. Tangles results from hyperphosphorylation of Tau protein, causing the protein to dissociate from microtubules and to form insoluble aggregates. Altered detyrosination will lead to exposure of negatively charged glutamate residues.

The human cell line SH-SY5Y is a widely used model for studying the molecular events in the pathophysiology of Alzheimer, Parkinson disease and more generally neurodegenerative diseases. Using SH-SY5Y, it is possible to drive differentiation in order to obtain neuronal morphology with long, extensively branched neurites that express neurospecific markers. The cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) in presence of antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). The cells were routinely passaged and cultured in a standard humidified tissue culture incubator at 37°C in presence of 5% CO₂. Prior to differentiation cells were trypsinized and counted twice using an automated cell counter (Countess II; Thermo Scientific) and 0,8 x10⁵ were plated in a 6-wells plate (Nunc). Next day, cells were washed twice with PBS and B-27 (Gibco) supplemented medium containing all-trans-retinoic acid (RA: Sigma R 2625) at 10µM. Samples of SH-SY5Y cells ongoing neural differentiation were collected every day in a RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, at a pH of 7.4), and quantitation of total protein performed using BCA kit (Thermo Fisher Scientific). A 20 µg protein sample of a total cell extract was run on 10% SDS-PAGE, transferred to nitrocellulose, and probed with each antibody.

As observed by western blot analysis, tubulin detyrosination increases during neural differentiation. By reducing the level of detyrosination, displacement of Tau from the MT's may be hampered as such reducing intracellular aggregates. b) Recent evidences converge to the essential role of the microtubule-associated proteins known as Tau that builds up in the brain during the course of the disease but acting on microtubule modifications have so far been neglected.

To further understand the role of detyrosination in the pathophysiology of Alzheimer Disease skin fibroblasts from patients carrying familial Alzheimer's Disease mutations were obtained and induced pluripotent stem cell (iPSC) generated.

The cells were maintained at the neural progenitor stage and samples were collected every day in a RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, at a pH of 7.4), and quantitation of total protein performed using BCA kit (Thermo Fisher Scientific). A 20µg protein sample of a total cell extract was run on 10% SDS-PAGE, transferred to nitrocellulose, and probed with each antibody.

Western blot analysis showed a striking increase of tubulin detyrosination in one of the cell-line carrying a genetic mutation (Fig. 16). Whereas current pharmaceutical efforts target the phosphorylation status of Tau protein itself, this observation opens a complete new window for therapeutic intervention. Pharmacological inhibition of detyrosinase act by directly regulating the level of microtubule detyrosination and as such may restore Tau binding to microtubules as well as endosomal-lysosomal processing efficiency, which is known to be defective during neurodegeneration.

Overall inhibition of detyrosination will 1) restore axonal transport which is essential for the clearance of Tau aggregates and other aggregates, and 2) reduce the level of negatively charged amino acid at the microtubule surface (detyrosinated microtubules exposed a negatively charged glutamate) leading to improved trapping of hyperphosphorylated Tau proteins. The bulky hydrophobic aromatic residues such as tyrosine have the ability to obscure the negative charges of glutamates.

Example 8: Study of the detyrosination process of microtubules in cancers

Despite the major advances in therapeutic approaches and personalized medicine, the spread of primary tumors toward distant organs and the subsequent metastatic colonization is still responsible for 90% of cancer-associated mortality. Tumors arising from epithelial tissues represent the vast majority of life-threatening cancers because of their ability to metastasize in different secondary organs. Therefore, a pressing concern in tumor biology has been the elucidation of factors and mechanisms regulating the migratory activity of these cells, tumor vascularization and colonization.

Accumulating data point out that increased tumor aggressiveness is associated with misregulation of the tyrosination/detyrosination cycle of tubulin. Increased level of tubulin detyrosination has been observed during cell migration, intravasation and in colonization suggesting a key role of this modification in metastasis.

By using CHL-1 cells that is a human melanoma cell line and HEK cells that have been demonstrated the ability to form colonies in soft agar and tumors of different size with varying

frequencies in immunocompromized mice, we analyzed the use of a peptidic inhibitor to reduced taxol induced detyrosination. Cells were routinely cultured in a standard humidified tissue culture incubator at 37°C in presence of 5% CO₂ and plated in a 6-wells culture dish. The cells were treated for 2 hours with 10µM Taxol in absence or presence of 50µM peptidic inhibitor.

The cells were collected in a RIPA buffer (of 50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, at a pH of 7.4), and quantitation of total protein performed using BCA kit (Thermo Fisher Scientific). A 20µg protein sample of a total cell extract was run on 10% SDS-PAGE, transferred to nitrocellulose, and probed with each antibody.

Western blot analysis showed a striking decrease of taxol treated (2 hours) and consequent tubulin detyrosination in both CHL-1 and HEK cells (Fig. 17).

CLAIMS

1. A method for purifying proteins having a tubulin carboxypeptidase activity from a biological extract, comprising:

- 5 (a) centrifuging the biological extract at a temperature comprised between 0 and 10°C, preferably between 2 and 5°C, more preferably at 2°C;
- (b) recovering the supernatant from step (a) and proceeding to a first microtubule polymerization cycle by adding GTP and incubating the mixture at a temperature between 35 and 40°C, preferably at 37°C, +/- 2°C, then centrifuging;
- 10 (c) recovering the pellets of step (b), resuspending in ice-cold buffer, incubating at 4°C +/- 1°C, and proceeding to a second microtubule polymerization cycle by adding GTP and incubating the mixture at 37°C, +/- 2°C, then centrifuging;
- (d) recovering the pellets of step (c) resuspending in ice-cold buffer, incubating at 4°C +/- 1°C, and proceeding to a third microtubule polymerization cycle by adding GTP and incubating the
- 15 mixture at 37°C, +/- 2°C, then centrifuging;
- (e) resuspending the pellets of step (d) and submitting the mixture to an ion exchange chromatography and recovering the flow through;
- (f) precipitating the proteins of the flow through with a 60% saturated ammonium sulphate solution;
- 20 (g) submitting the precipitated fraction of step (f) to an hydrophobic chromatography and eluting by gradually decreasing ammonium sulphate concentration up to zero to recover the fraction of proteins with a tubulin carboxypeptidase activity.

2. The method for purifying proteins according to claim 1, wherein

- the first polymerization cycle comprises

- 25 (i) adding GTP and incubating the mixture at 37°C, +/- 2°C, for 30 minutes, +/- 10 minutes;

(ii) centrifuging at 22,000 g, +/- 1,000 g, at 37°C, +/- 2°C, for 45 minutes, +/- 10 minutes;

and/or

- the second polymerization cycle comprises:

5 (i) incubating the mixture on ice for 30 minutes, +/- 10 minutes;

(ii) centrifuging at 150,000g +/- 10,000 g, 30 minutes, +/- 10 minutes;

(iii) recovering the supernatant and adding GTP;

(iv) incubating the mixture at 37°C, +/- 2°C, for at 30 minutes, +/- 10 minutes:

10 (v) centrifuging at 50,000 g, +/- 1,000 g at a temperature comprised between 30°C and 37°C, for 30 minutes, +/- 10 minutes;

and/or

- the third polymerization cycle comprises :

(i) incubating the mixture on ice for 30 minutes, +/- 10 minutes;

(ii) recovering the supernatant and adding GTP;

15 (iii) incubating the mixture at 37°C, +/- 2°C, for at 30 minutes, +/- 10 minutes:

(iv) centrifuging at 50,000 g, +/- 1,000 g at a temperature comprised between 30°C and 37°C, for 30 minutes, +/- 10 minutes.

20 3. The method for purifying proteins of claim 1 or 2, further comprising a step of mass spectrometry characterization of the fraction of proteins of step (g) and/or further comprising a step of selecting proteins that contain a protease domain.

4. The method for purifying proteins of anyone of claims 1 to 3, wherein the biological sample is selected from eukaryote organisms extracts, preferably animal extracts, more preferably mammal extracts, such as brain extract, testis extract, and lung extract.

5. The method for purifying proteins of anyone of claims 1 to 4, wherein the fraction of proteins with a tubulin carboxypeptidase activity comprises at least one protein having at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99% amino acid sequence identity with the amino acid sequence selected from SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°4, SEQ ID N°5, SEQ ID N°6, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°10 and SEQ ID N°11.

6. The method for purifying proteins of anyone of claims 1 to 5, wherein the fraction of native or recombinant proteins with a tubulin carboxypeptidase activity is further contacted with microtubules and the level of isolated tyrosine (Y) is measured, thereby confirming the tubulin carboxypeptidase activity of the fraction of proteins, wherein the microtubules preferably comprise synthetic microtubules and/or α -tubulins, with labeled C-terminal Y.

7. A method for selecting a peptidic based inhibitor able to inhibit a tubulin carboxypeptidase activity among peptidic based inhibitor candidates that comprise a peptidic moiety constituted of 1 to 20 amino acids, said peptidic moiety having at the C-terminal position an amino acid selected from Y or F, wherein the method comprises:

(a) contacting the peptidic based inhibitor candidate with a mixture containing both a fraction of native or recombinant proteins with a tubulin carboxypeptidase activity and microtubules, which preferably comprise synthetic microtubules and/or α -tubulins, with labeled C-terminal Y;

(b) measuring the level of isolated Y and/or detyrosinated microtubules.

8. The method claim 7, wherein the level of isolated Y in the sample is compared to the level of isolated Y in a control sample comprising solely a protein extract obtained with a method for purifying proteins according to anyone of claims 1 to 6 and microtubules.

9. The method of claim 7 or 8, wherein the peptidic moiety of the peptidic based inhibitor candidate is constituted of between 1 and 20 amino acids of the most C-terminal amino acids of an α - tubulin.

10. The method of anyone of claims 7 to 9, wherein the peptidic moiety of the peptidic based inhibitor candidate is constituted of between 1 and 16 of the most C-terminal amino acids of the amino acid sequence Nter-X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-Cter, wherein

- X1, X2, X5, X7, X9 and X13 are hydrophobic amino acids, preferably selected from G, A or V,

- X3, X6, X8, X10, X11, X12, X14 and X15 are negatively charged amino acids, preferably selected from E or D,
- X4 is a polar uncharged side chains, preferably selected from S, T, N or Q, and
- X16 is a large hydrophobic amino acid, selected from Y or F.

5 11. The method of anyone of claims 7 to 10, wherein the peptidic moiety of the peptidic based inhibitor candidate has the amino acid sequence selected from Y, EDY, EAY and EEY.

12. The method of anyone of claims 7 to 11 wherein the peptidic based inhibitor candidate further comprises a reactive moiety, preferably selected from epoxysuccinyl, acyloxymethyl, aldehydes and ketones.

10 13. Peptidic based inhibitor for use in the treatment of a disorder involving altered microtubule detyrosination in an animal, wherein the peptidic based inhibitor comprises a peptidic moiety constituted of 1 to 20 amino acids, preferably 1 and 20 amino acids of the most C-terminal amino acids of an alpha- tubulin that has been chemically modified or not, said peptidic moiety having an amino acid selected from Y or F at the C-terminal position, and wherein the peptidic
15 based inhibitor inhibits at least partially a tubulin carboxypeptidase activity.

14. Peptidic based inhibitor for use according to claim 13, wherein the peptidic moiety is constituted of between 1 and 16 amino acids of the most C-terminal amino acids of the amino acid sequence Nter-X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-Cter, wherein

20 - X1, X2, X5, X7, X9 and X13 are hydrophobic amino acids, preferably selected from G, A or V,

- X3, X6, X8, X10, X11, X12, X14 and X15 are negatively charged amino acids, preferably selected from E or D,

- X4 is a polar uncharged side chains, preferably selected from S, T, N or Q, and

25 - X16 is a large hydrophobic amino acid, preferably selected from Y or F.

15. Peptidic based inhibitor for use according to claim 13 or 14, wherein the peptidic moiety has the amino acid sequence selected from Y, EDY, EAY and EEY.

16. Peptidic based inhibitor for use according to any one of claims 13 to 15, wherein the peptidic based inhibitor further comprises a reactive moiety, preferably selected from epoxysuccinyl, acyloxymethyl, aldehydes and ketones.

5 17. Peptidic based inhibitor for use according to any one of claims 13 to 16, wherein the peptidic based inhibitor inhibits irreversibly or reversibly a tubulin carboxypeptidase activity.

10 18. Peptidic based inhibitor for use according to any one of claims 13 to 17, wherein the disorder is selected from neurodegenerative diseases, preferably selected from Alzheimer disease, Parkinson disease, psychiatric disorders, and neural disorders, cancers, preferably selected from colon cancer and neuroblastoma, muscular dystrophies, heart diseases, vascular disorders, infertility, retinal degeneration and ciliopathies.

19. Peptidic based inhibitor for use according to claim 18, wherein the disorder is selected from neurodegenerative diseases, cancers and muscular dystrophies.

20. A pharmaceutical composition comprising a therapeutically effective amount of a peptidic based inhibitors according to anyone of claims 13 to 17.

1/11

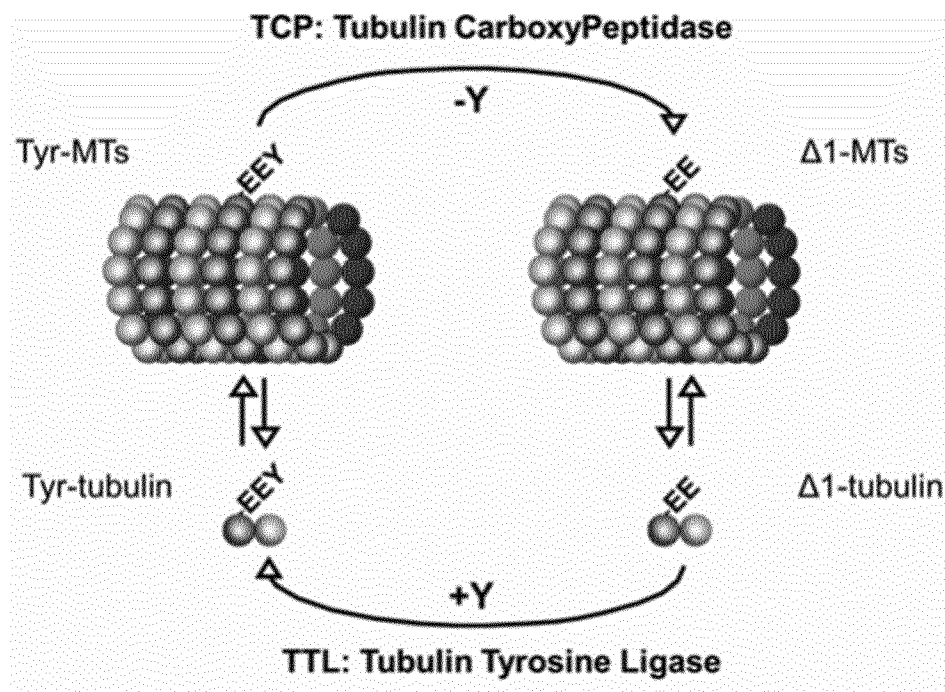


FIGURE 1

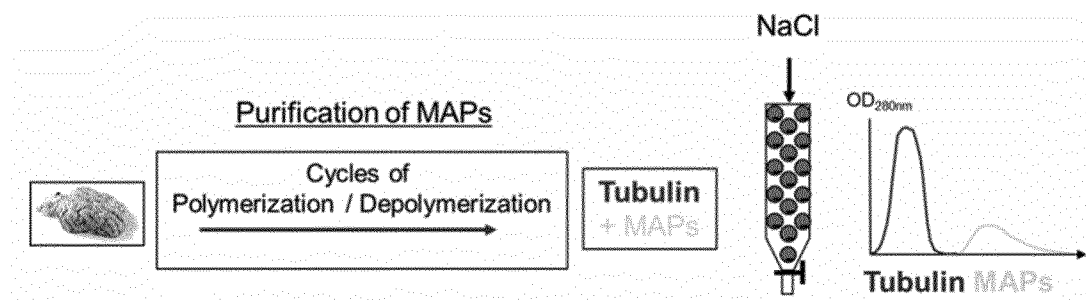


FIGURE 2

2/11

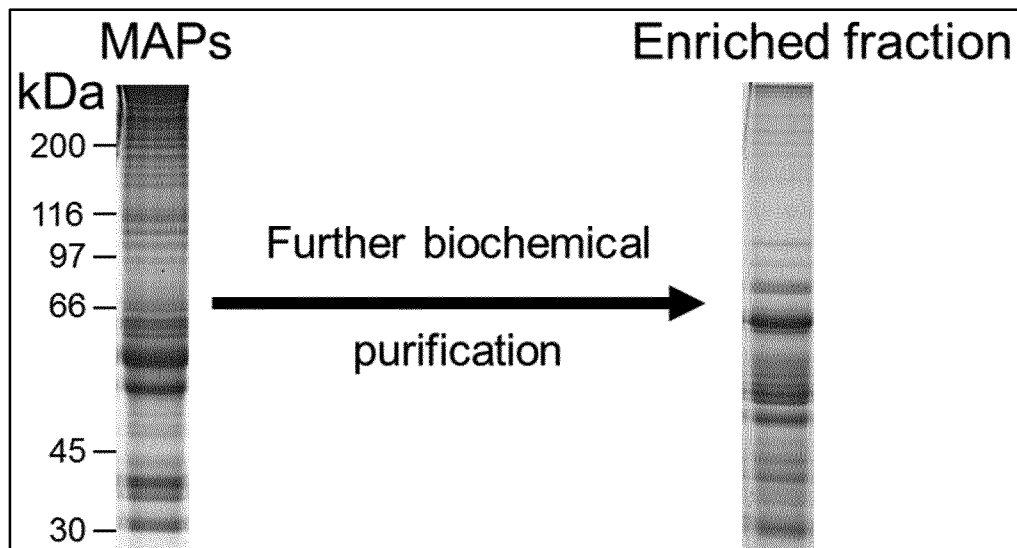


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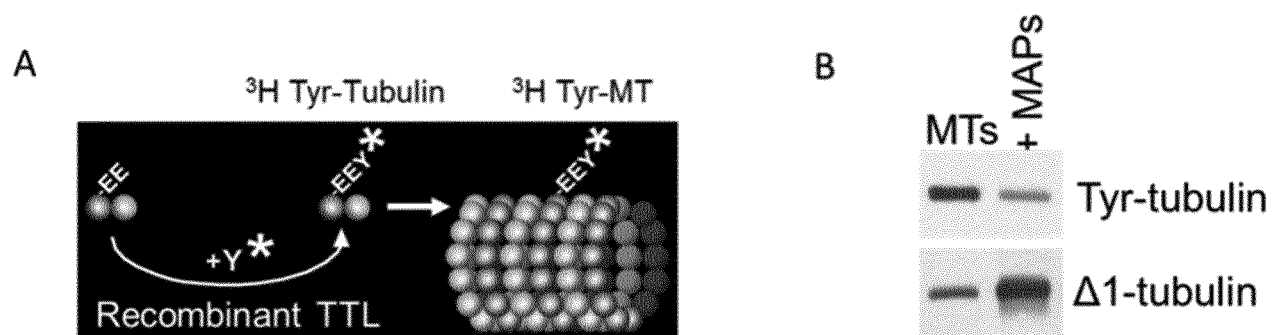


FIGURE 4

3/11

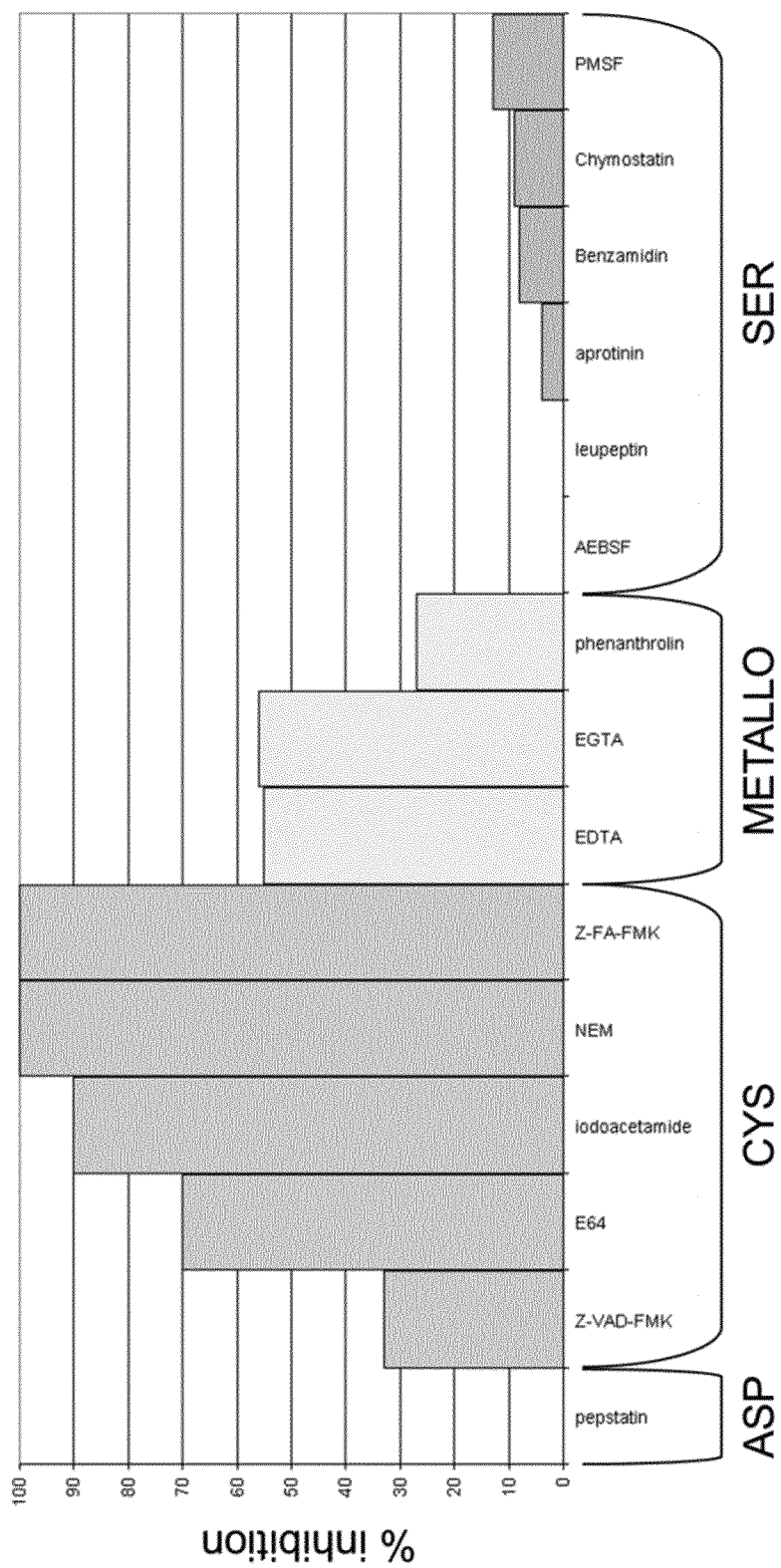


FIGURE 5

4/11

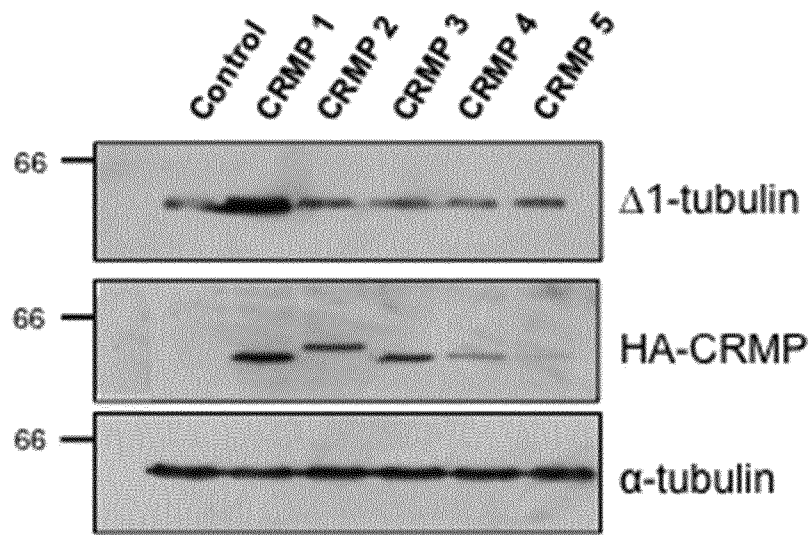


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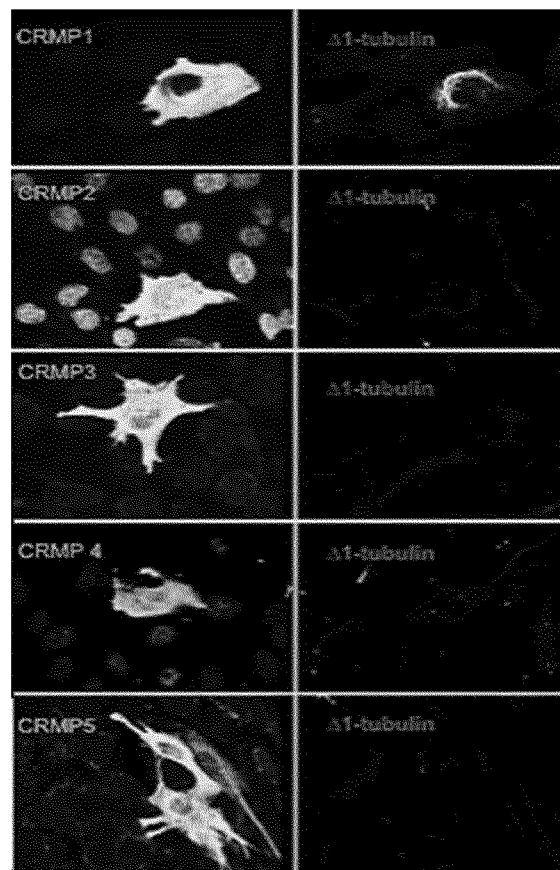


FIGURE 7

5/11

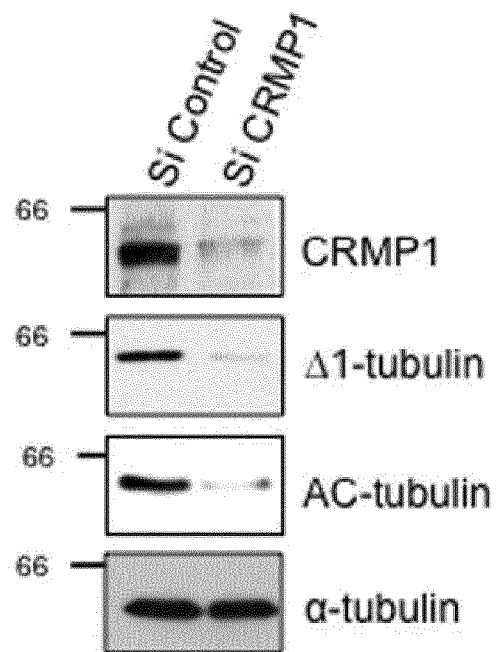


FIGURE 8

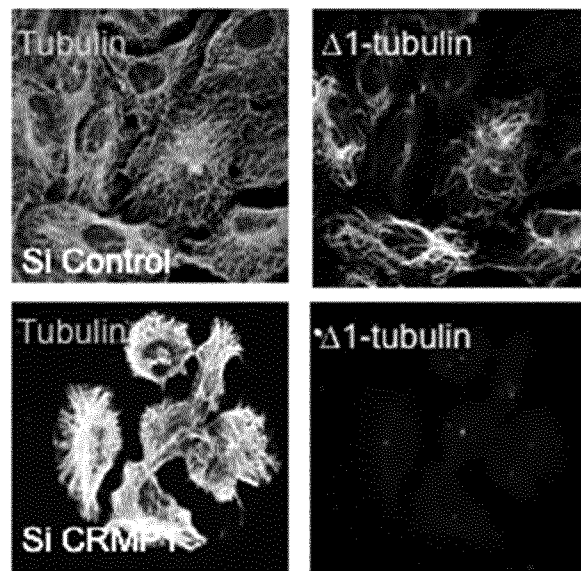


FIGURE 9

6/11

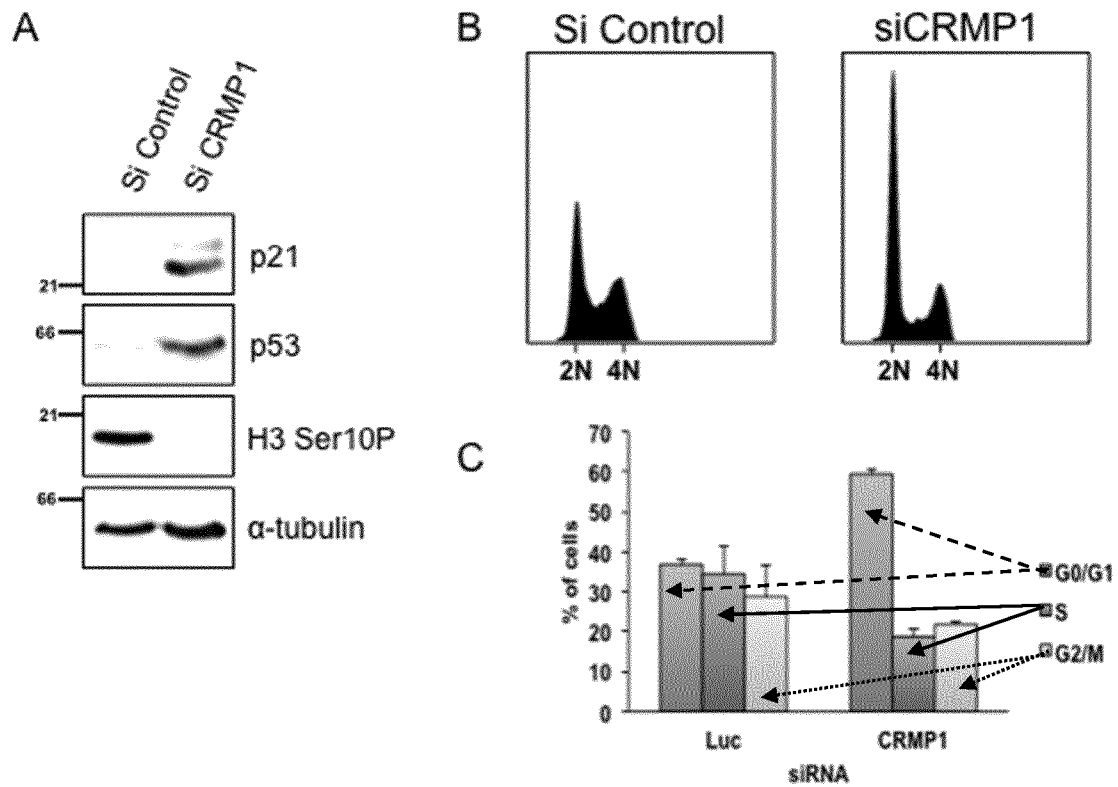
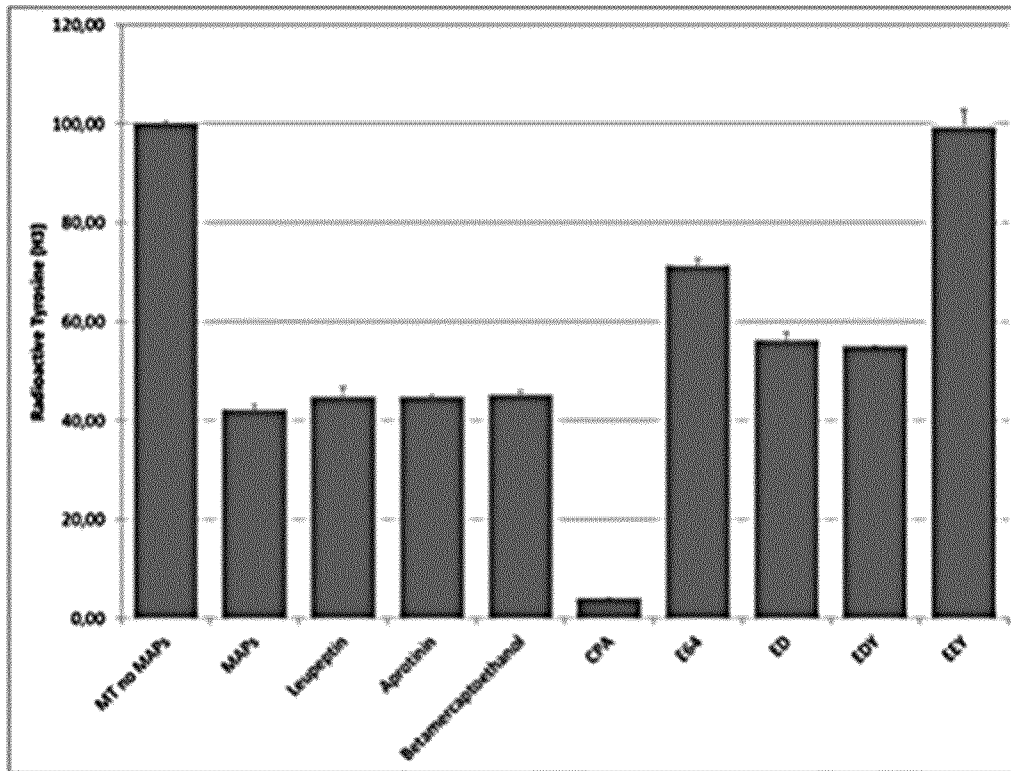


FIGURE 10

7/11

A



B

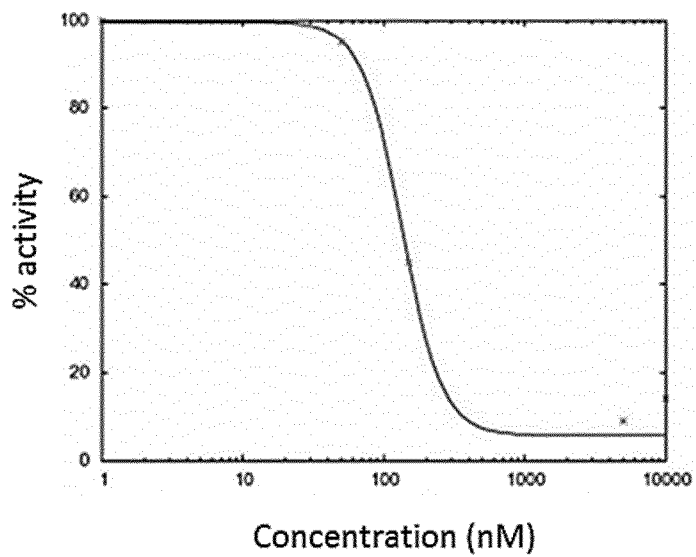


FIGURE 11

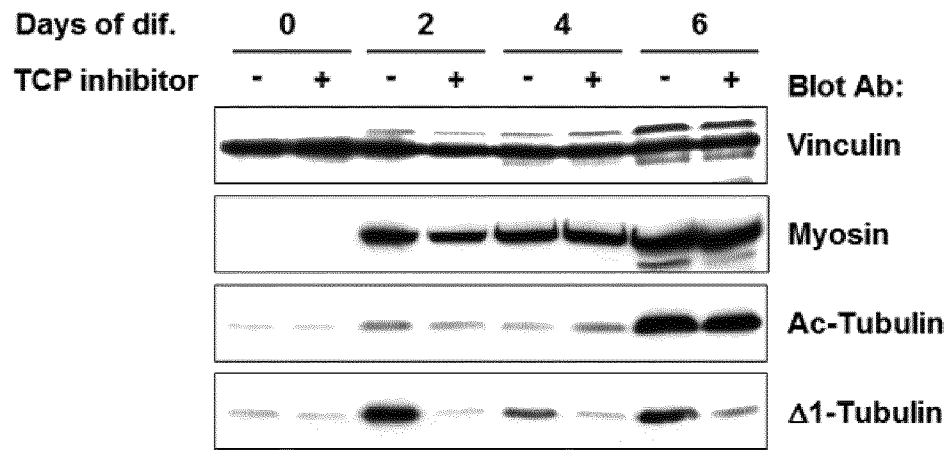


FIGURE 12

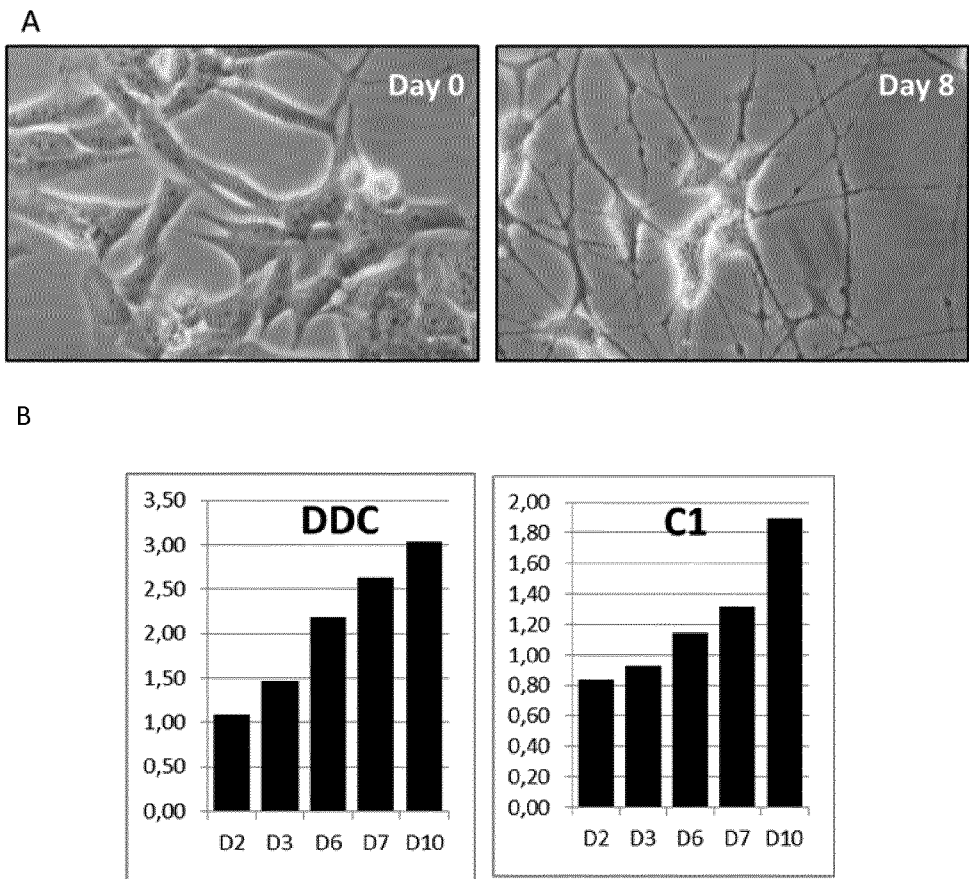


FIGURE 13

9/11

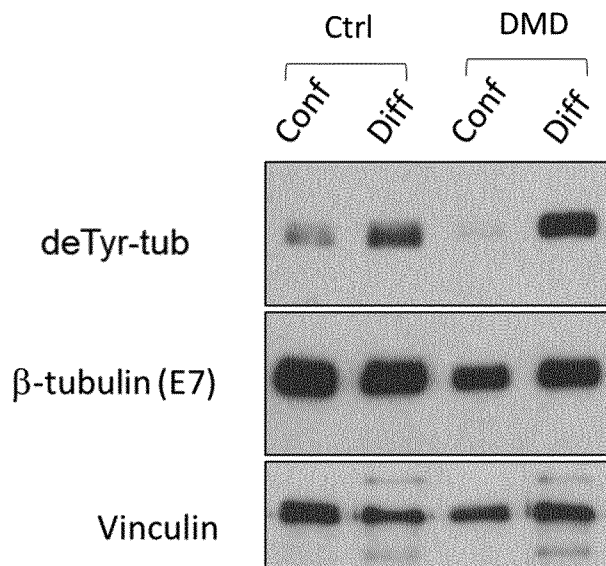


FIGURE 14

SH-SY5Y neural differentiation

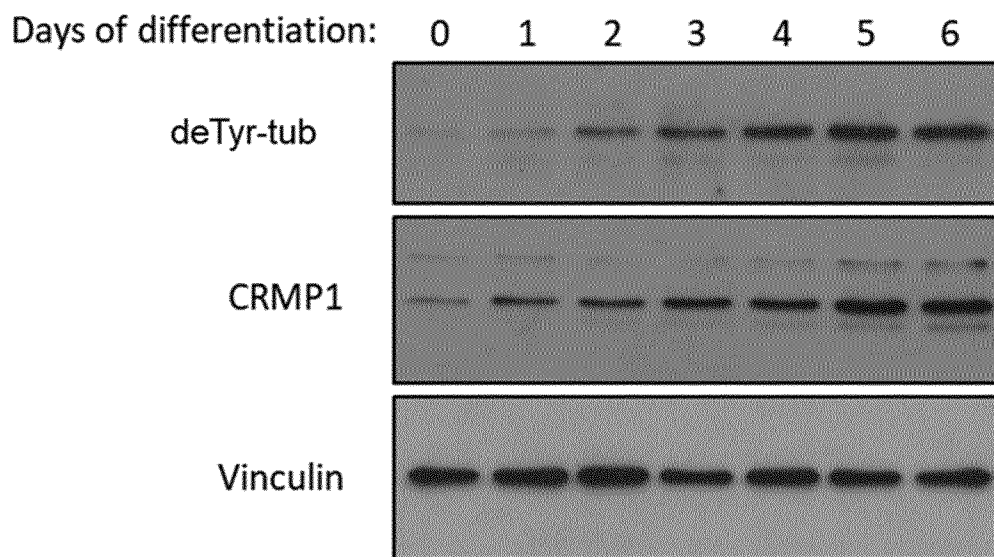


FIGURE 15

10/11

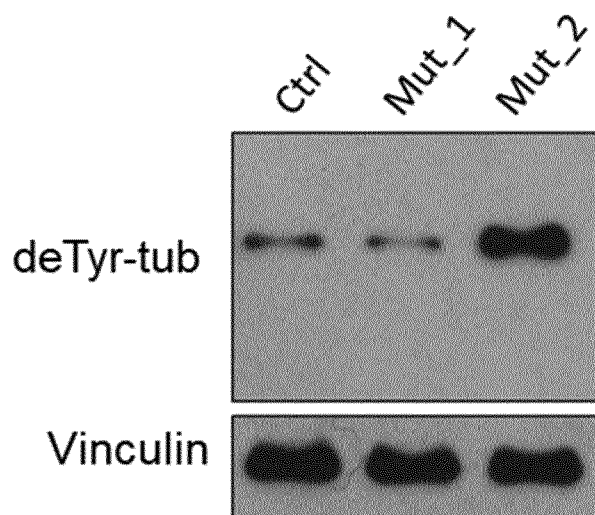


FIGURE 16

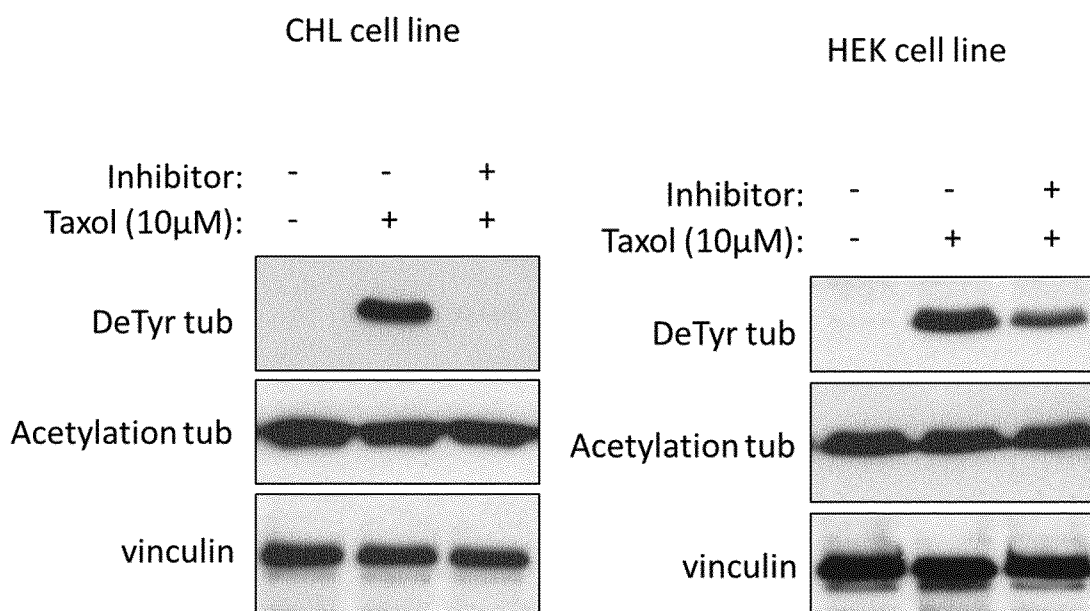


FIGURE 17

11/11

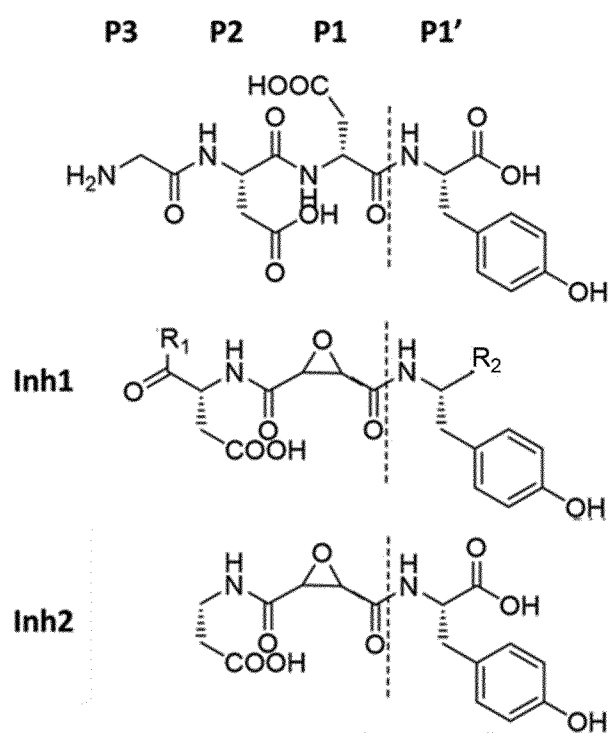


FIGURE 18

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/069496

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K7/06 C12N9/48 C12N9/00 C07K7/08
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K C12N

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

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

EPO-Internal, CHEM ABS Data, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A BOSSON ET AL.: "Cap-Gly proteins at microtubules plus ends: is EB1 detyrosination involved?", PLOS ONE, vol. 7, no. 3, E33490, 14 March 2012 (2012-03-14), XP002777257, Public Library of Science ISSN: 1932-6203, DOI: :10.1371/journal.pone.0033490 pubmed:22432029 the whole document ----- -/--	1-6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

6 September 2018

Date of mailing of the international search report

14/09/2018

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Masturzo, Pietro

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/069496

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	X FONROSE ET AL.: "Parthenolide inhibits tubulin carboxypeptidase activity", CANCER RESEARCH, vol. 67, no. 7, 1 April 2007 (2007-04-01), pages 3371-3378, XP055213047, AACR - American Association for Cancer Research ISSN: 0008-5472 the whole document -----	7-13
A	A BRUNET-SIMON ET AL.: "Onset of zygotic transcription and maternal transcript legacy in the rabbit embryo", MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 58, no. 2, January 2001 (2001-01), pages 127-136, XP002777258, WILEY-LISS, INC ISSN: 1040-452X table 1 -----	14-20
T	C AILLAUD ET AL.: "Vasohibin/SVBP are tubulin carboxypeptidases (TCPs) that regulate neuron differentiation", SCIENCE, vol. 358, 15 December 2017 (2017-12-15), pages 1448-1453, XP002777259, American Association for the Advancement of Science ISSN: 0036-8075 the whole document -----	1-19