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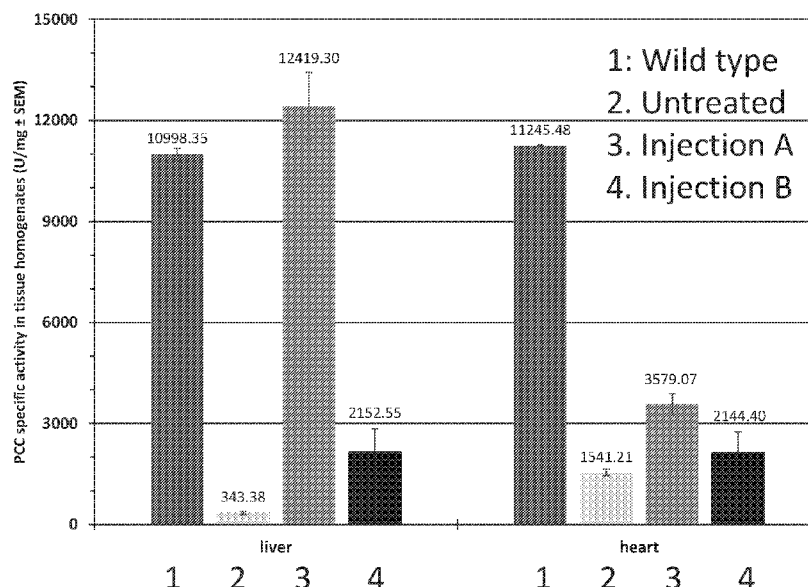
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(54) Title: PROPIONYL-COA CARBOXYLASE COMPOSITIONS AND USES THEREOF

Figure 7

EXP1C - liver & heart PCC specific activity, IP, 20 mg/kg TAT-PCCAB @ 0h & 3h



(57) **Abstract:** The invention provides compositions of recombinant human propionyl-CoA carboxylase (PCC). The PCCAB dodecamer and subunits, including PCCA and PCCB, conjugated to cell penetrating and mitochondria penetrating peptides, and pharmaceutical compositions of the foregoing are described herein. Also included are methods for treating conditions such as propionic acidemia (PA), propionic aciduria, propionyl-CoA carboxylase deficiency, and ketotic glycinemia using the compositions of the invention.



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PROPIONYL-CoA CARBOXYLASE COMPOSITIONS AND USES THEREOF**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority to U.S. Provisional 62/330,913 filed May 3, 2016, which is herein incorporated by reference in its entirety. This application is also related to International Application No. PCT/US2016/030504, entitled PROPIONYL-CoA CARBOXYLASE COMPOSITIONS AND USES THEREOF, with a priority date of May 3, 2016, the contents of which are herein incorporated by reference in their entirety.

SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The sequence listing file, entitled SEQ_LST_2089.1501PCT.txt, was created on May 3, 2017 and is 187,816 bytes in size. The information in electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present invention relates generally to compositions and methods for ameliorating deficits and deficiencies of propionyl-CoA carboxylase (PCC) including treating a spectrum of conditions such as propionic academia (PA), PA-related disorders, propionic aciduria, propionyl-CoA carboxylase deficiency, and/or ketotic glycinemia.

BACKGROUND OF THE INVENTION

[0004] Propionyl-CoA carboxylase (PCC) is a complex mitochondrial matrix protein that catalyzes the conversion of propionyl-CoA to D-methylmalonyl-CoA in the mitochondrial matrix. PCC is composed of nonidentical subunits, alpha (α) and beta (β). Human PCC is an $\alpha\beta_6$ heterododecamer (PCCAB) that is about 800 kDa (See, Chloupkova et al., 2000 *Mol Genet Metab.* 71:623-32, which is herein incorporated by reference in its entirety). The 72 kDa α subunit and the 56 kDa β subunit (see, Gravel et al., 1980 *Archives of Biochemistry & Biophysics.* 201:669-73; Kalousek et al., 1980 *Journal of Biological Chemistry.* 255:60-5, which are herein incorporated by reference in their entireties) are encoded by separate genes designated, *PCCA*, found on chromosome 13 (see, Lamhonwah et al., 1986 *Proc. Nat. Acad. Sci.* 83:4864-8, which is herein incorporated by reference in its entirety), and *PCCB*, found on chromosome 3 (see, Kraus et al., 1986 *Proc. Nat. Acad. Sci.* 83:2047-51), which is herein incorporated by reference in its entirety). The corresponding cDNAs have been sequenced (See, Kraus et al., 1986 *Proc. Nat. Acad. Sci.* 83:8049-53; Lamhonwah, et al., 1989 *Nucleic Acids Research.* 17:4396; Lamhonwah et al., 1994 *Genomics.* 19:500-505; Ohura et al., 1993 *J Inherit Metab Dis.* 16:863-7, which are herein incorporated by reference in their entireties).

The subunits are translated from the genes via mRNAs in the cytoplasm as larger precursors and imported into mitochondria. (See, Kraus *et al.*, 1986 *Proc. Nat. Acad. Sci.* 83:8049-53; Browner *et al.*, 1989 *Journal of Biological Chemistry*. 264:12680-5). N-terminal leader sequences are proteolytically removed, and the mature enzyme is assembled. The β -subunits form a central core hexameric core decorated on the outside by six non-interacting α -subunits. Biotin, bicarbonate, and ATP have binding sites on the α -subunit while propionyl CoA binds to the β -subunit. The crystal structure of a 780 kDa $\alpha_6\beta_6$ dodecamer of bacterial propionyl-CoA carboxylase has been determined to provide the three-dimensional structure of the enzyme. See, Huang *et al.*, *Nature*. 2010 Aug 19;466(7309):1001-5. doi: 10.1038/nature09302, which is herein incorporated by reference in its entirety.

[0005] Human mature dodecamer (PCCAB) may be expressed in *E. coli* from a single plasmid. *E. coli* covalently attaches the PCC cofactor biotin to produce a fully functional enzyme. The molecular chaperone, GroES/EL, is often co-expressed from a second plasmid to encourage proper PCCAB folding and assembly.

[0006] The alpha (α) subunit contains the sequence that accepts biotin (see, Kalousek *et al.*, 1980 *Journal of Biological Chemistry*. 255:60-5; Lamhonwah *et al.*, 1987 *Archives of Biochemistry & Biophysics*. 254:631-6; Leon-Del-Rio & Gravel 1994 *Journal of Biological Chemistry*. 269:22964-8, which are herein incorporated by reference in their entireties) and binds CO₂, Mg²⁺, ATP, and K⁺, which provide a means of regulation (see, Kalousek *et al.*, 1980 *Journal of Biological Chemistry*. 255:60-5). The β subunit binds propionyl-CoA (Fenton *et al.*, 2001 *The Online Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D., eds) pp. 2165-2204, McGraw-Hill, Inc., New York, which is herein incorporated by reference in its entirety). Mutations in either gene may result in PCC deficiency to cause propionic acidemia (PA). To date, 81 and 86 mutations have been identified in the PCCA and PCCB genes, respectively, from PA patients. (a public continuously updated list of all reported PCC mutations can be found at the Kraus lab webpage at the University of Colorado at Denver). Inherited metabolic disorders represent a therapeutic challenge and in recent years there is an increased search for new era treatments for metabolic disorders, such as gene therapy or enzyme replacement therapy (ERT).

[0007] The spectrum of PA ranges from neonatal-onset to late-onset disease. Propionic acidemia is an autosomal recessive disorder in which a defective form of PCC results in the accumulation of propionic acid, propionyl-CoA, 3-hydroxypropionate, propionyl carnitine, and methyl citrate, primarily in mitochondria of hepatocytes.

[0008] Neonatal-onset PA, the most common form of PA, is characterized by poor feeding, vomiting, and somnolence in the early days of life in a previously healthy infant, followed by lethargy, seizures, coma, and death. The condition is frequently accompanied by metabolic acidosis with anion gap, ketonuria, hypoglycemia, hyperammonemia, and cytopenias. Late-onset PA causes developmental regression, chronic vomiting, protein intolerance, failure to thrive, hypotonia, and occasionally basal ganglia infarction (resulting in dystonia and choreoathetosis) and cardiomyopathy (see, Shchelochkov et al., 2012 May 17 [Updated 2016 Oct 6] *GeneReviews*[®] [Internet] (Pagon RA, Adam MP, Ardinger HH, et al., editors. Seattle (WA): University of Washington, Seattle; 1993-2017, which is herein incorporated by reference in its entirety). The incidence of PA has been estimated to be in the range of 1:35,000-1:70,000, which is similar to the incidence of methylmalonic acidemia (see, Saudubray et al., 1989 *J Inherit Metab Dis.* 12:25-41; Chace et al., 2001 *Clinical Chemistry* 47:2040-44, which are herein incorporated by reference in their entireties). PA also results from a decrease in PCC activity from a lack of co-enzymes such as biotin. The incidence of PA carriers is about 5% in the Inuit population of Greenland, which is much higher than the incidence of most other autosomal recessive diseases (Ravn et al., 2000 *Am J Hum Genet.* 67:203-6, which is hereby incorporated by reference in its entirety). Biochemically, patients with PA have elevated levels of propionyl CoA, propionic acid, methylcitrate, beta-hydroxy-propionate, propionylglycine, tiglyglycine, and ketones. Ketones, such as butanone, may also be found in urine of these patients (Menkes et al., 1966 *The Journal of pediatrics.* 69:413-21).

[0009] PA is a potentially life-threatening disease. Currently there is no cure and the treatment is based on dietary management recommending low protein diet and limiting intake of propiogenic substrates. Additionally, the use of supplements such as L-carnitine is recommended. Liver transplantation is being utilized with limited success (see, Charbit-Henrion et al., *American Journal of Transplantation* 2015; 15: 786–791). Enzyme replacement therapy (ERT) is a therapeutic approach in which the deficient enzyme is replaced by recombinant active protein. ERT would represent a major improvement in treatment of patients if the enzyme or its subunits could be imported into the mitochondrial matrix.

[00010] A related PCC-deficiency condition, hyperammonemia originates secondarily from carbamoyl phosphate synthetase inhibition (Coude et al., 1979 *Journal of Clinical Investigation.* 64:1544-51; Stewart & Walser, 1980 *Journal of Clinical Investigation.* 66:484-92, which are herein incorporated by reference in their entireties). Further, ketoacidotic

episodes are frequently life threatening and one third of affected neonates die within the first few weeks of life (Fenton et al., 2001 *The Online Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D., eds) pp. 2165-2204, McGraw-Hill, Inc., New York, which is herein incorporated by reference in its entirety). The condition is currently treated by severe restrictions of the patient's protein intake; however, management of this type of treatment is often difficult (Wolf et al., 1981 *Journal of Pediatrics*. 99:835-46, which is hereby incorporated by reference in its entirety).

[00011] Currently, there is no cure for PA and other PCC-deficiency related conditions, and current treatment provides only partial alleviation of symptoms. However, specific delivery of PCC to the mitochondria presents challenges. There is a long-felt need in the art to develop a technique to develop pharmaceutical compositions and methods for delivering active PCC enzyme to the active site of intracellular mitochondria of afflicted patients to ameliorate deficits and deficiencies thereof.

SUMMARY OF THE INVENTION

[00012] The invention provides compositions and methods for ameliorating deficits and deficiencies of propionyl-CoA carboxylase (PCC) including treating a spectrum of conditions such as propionic academia (PA), PA-related disorders, propionic aciduria, PCC deficiency, and/or ketotic glycinemia.

[00013] In one embodiment, the pharmaceutical compositions comprise one or more proteins or protein subunits which recapitulate the function of the PCC enzyme. Such enzyme subunits include PCCA and/or PCCB.

[00014] In one aspect, the PCCA protein and/or PCCB protein comprises a mitochondrial leader sequence.

[00015] In another aspect, the PCCA protein and/or PCCB protein lack a mitochondrial leader sequence.

[00016] In some embodiments, the PCCA protein and/or PCCB proteins are genetically engineered proteins or variants thereof.

[00017] In another embodiment, the PCCA protein and/or PCCB protein is covalently linked to one or a plurality of cell penetrating peptides. One non-limiting example of such a cell penetration peptide is trans-activating transcriptional activator (TAT) or a tissue specific variant thereof.

[00018] In some embodiments, the cell-penetrating peptide is chemically added post-translation to the assembled dodecamer.

[00019] In certain embodiments, the PCCA and/or PCCB proteins are produced recombinantly. As such, the PCCA and/or PCCB proteins may be produced in prokaryotic or eukaryotic cells, more specifically yeast, mammalian, or *E. coli*.

[00020] Various embodiments of the invention herein provide a pharmaceutical composition comprising an isolated human PCCAB dodecamer having a post-translational modification comprising a cell penetrating peptide or mitochondria penetrating peptide.

[00021] In certain embodiments of the pharmaceutical composition, the PCCAB dodecamer does not include a purification tag. In certain embodiments, the PCCAB dodecamer comprises a PCCA subunit having the amino acid sequence of SEQ ID NO:41, or a fragment or a variant thereof sharing a sequence similarity of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50% to SEQ ID NO:41. In certain embodiments, the PCCAB dodecamer comprises a PCCA subunit having the amino acid sequence of SEQ ID NO:43, or a fragment or a variant thereof sharing a sequence similarity of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50% to SEQ ID NO:43. In certain embodiments, a nucleic acid sequence encoding the PCCAB is codon optimized for recombinant cell expression. In certain embodiments, a nucleic acid sequence encoding the PCCA subunit is SEQ ID NO:40, or a fragment or a variant thereof sharing a sequence similarity of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50% to SEQ ID NO:40. In certain embodiments, a nucleic acid sequence encoding the PCCB subunit is SEQ ID NO:42, or a fragment or a variant thereof sharing a sequence similarity of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50% to SEQ ID NO:42.

[00022] Various embodiments of the invention herein provide a pharmaceutical composition for treating PCC-deficiency, the composition comprising a therapeutically effective amount of an isolated human PCC enzyme or a variant thereof, the PCC enzyme or the variant therefore comprising at least one member selected from the group consisting of an isolated propionyl-CoA carboxylase alpha chain protein (PCCA) subunit, and an isolated propionyl-CoA carboxylase beta chain protein (PCCB) subunit, each independently or in combination with a pharmaceutically acceptable carrier, diluent or excipient.

[00023] In certain embodiments, the PCCA subunit comprises the amino acid sequence of SEQ ID NO:2 or a fragment thereof. In certain embodiments, the PCCB subunit comprises the amino acid sequence of SEQ ID NO:4 or a fragment thereof. In certain embodiments, the PCCA subunit comprises a mitochondrial leader sequence. Alternatively, the PCCA subunit does not comprise a mitochondrial leader sequence. In certain embodiments, the PCCB

subunit comprises a mitochondrial leader sequence. Alternatively, the PCCB subunit does not comprise a mitochondrial leader sequence. In certain embodiments, the PCCA subunit comprises at least one selected from the group consisting of SEQ ID NOs:48, 50, and 52. In certain embodiments, the PCCB subunit comprises at least one sequence selected from SEQ ID NOs:49 and 51. In certain embodiments, the PCCA subunit and/or the PCCB subunit comprises at least one mutation in a region of the protein relative to naturally occurring PCCA or PCCB to facilitate penetration of the PCCA subunit and/or the PCCB subunit into mitochondria. In certain embodiments, the PCCA subunit is covalently linked to one or a plurality of cell penetrating peptides or mitochondria penetrating peptides.

[00024] In certain embodiments, the PCCB subunit is covalently linked to one or a plurality of cell penetrating peptides or mitochondria penetrating peptides. In certain embodiments, the PCCA and the PCCB both are covalently linked to one or a plurality of cell penetrating peptides or mitochondria penetrating peptides. In certain embodiments, the cell penetrating peptide or mitochondria penetrating peptide is derived from a protein selected from the group consisting of a trans-activating transcriptional activator (TAT), mitochondria penetrating proteins (MPP), antennapedia, herpes simplex virus type 1 protein VP22, penetratin, transportan, amphipathic protein MPG, Pep-1, MAP, SAP, PPTGI, poly-Arginine sequence, hCT, SynB, Pvec, and a tissue specific variant thereof. In certain embodiments, the PCCA subunit is produced recombinantly. In certain embodiments, the PCCB subunit is produced recombinantly. For example, the PCCA subunit and/or the PCCB subunit are produced in prokaryotic cells. Alternatively, the PCCA subunit and/or the PCCB subunit are produced in eukaryotic cells. In an aspect of the embodiment, the prokaryotic cells are *E. coli*. In some embodiments, the eukaryotic cells are yeast or mammalian cells. In certain embodiments, the pharmaceutical composition further comprises a His-tag at the C-terminal end and/or the N-terminal end of the PCCA subunit or the PCCB subunit. In certain embodiments, the mitochondria penetrating peptide is an MPP1A or MPP2A. In certain embodiments, the cell penetrating peptide is less the 100 amino acids, less than 50 amino acids, less than 30 amino acids, and less than 10 amino acids in length. In certain embodiments, the pharmaceutical composition further comprises at least one pharmaceutically acceptable salt or excipient.

[00025] Various embodiments of the invention herein provide a method for reducing propionyl-CoA levels in a PCC deficient subject comprising administering a pharmaceutical composition described herein. In certain embodiments, the pharmaceutical composition is administered by intravenous injection (IV), subcutaneous injection (SC), or intraperitoneal

injection (IP). For example, the pharmaceutical composition is administered by intraperitoneal injection (IP).

[00026] Various embodiments of the invention herein provide a method for elevating propionyl-CoA carboxylase, in a subject in need thereof comprising administering to said individual a pharmaceutical composition described herein.

[00027] Various embodiments of the invention herein provide a method for treating or ameliorating a disease, disorder, or condition in a subject, the disease, disorder, or condition being associated with elevated propionyl CoA, propionic acid, methylcitrate, beta-hydroxy-propionate, propionylglycine, tiglic acid, and/or ketones comprising administering to an individual in need thereof a pharmaceutically effective amount of a pharmaceutical composition described herein.

[00028] In certain embodiments, the subject presents with at least one symptom selected from the group consisting of poor feeding, vomiting, and somnolence, lethargy, seizures, coma, metabolic acidosis, anion gap, ketonuria, hypoglycemia, hyperammonemia, cytopenias, developmental regression, chronic vomiting, protein intolerance, failure to thrive, hypotonia, basal ganglia infarction, dystonia, choreoathetosis, and cardiomyopathy. In certain embodiments, administering of the pharmaceutical composition occurs in a concentration of at least 0.5 μ M, at least 1 μ M, at least 2 μ M, at least 5 μ M, and at least 10 μ M. In certain embodiments, administering occurs at least once a day, at least twice a day, at least three times a day, and at least 4 times a day. In certain embodiments, administering is repeated for at least 2 days, at least 3 days, at least 4 days, at least 5 days, and at least 6 days.

[00029] Various embodiments of the invention herein provide a method of producing the PCCA subunit or the PCCB subunit described herein comprising recombinantly producing a PCCA precursor or a PCCB precursor including a cell penetrating peptide or mitochondria penetrating peptide.

[00030] In certain embodiments of the method, the PCCA precursor is encoded by the nucleic acid sequence of SEQ ID NO:1. In certain embodiments, the PCCB precursor is encoded by the nucleic acid sequence of SEQ ID NO:3.

[00031] Various embodiments of the invention herein provide a method of producing the pharmaceutical composition disclosed herein, the method comprising: transfecting cells with an expression vector encoding the PCCAB and at least one molecular chaperone to mediate formation of a PCCAB dodecamer, and conjugating a cell penetrating peptide or mitochondria penetrating peptide to the PCCAB dodecamer post-translationally, thereby producing the pharmaceutical composition.

[00032] In certain embodiments, the molecular chaperone is a GroEL or a GroES protein. In certain embodiments, the PCCAB dodecamer comprises a PCCA subunit and a PCCB subunit. In certain embodiments, the expression vector comprises a nucleic acid sequence of SEQ ID NO:40, or a fragment or a variant thereof sharing a sequence similarity of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50% to SEQ ID NO:40 encoding the PCCA subunit. In certain embodiments, the expression vector comprises a nucleic acid sequence of SEQ ID NO:42, or a fragment or a variant thereof sharing a sequence similarity of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50% to SEQ ID NO:42 encoding the PCCB subunit. In certain embodiments, the method further comprises formulating the pharmaceutical composition with at least one of a pharmaceutically acceptable salt or excipient. In certain embodiments, the PCCA subunit comprises an amino acid sequence beginning at residue 52 of SEQ ID NO:2. In certain embodiments, the PCCB subunit comprises an amino acid sequence beginning at residue 29 of SEQ ID NO:4.

BRIEF DESCRIPTION OF THE DRAWINGS

[00033] The foregoing and other objects, features and advantages will be apparent from the following description of particular embodiments of the invention, as illustrated in the accompanying drawings. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of various embodiments of the invention.

[00034] Figure 1 shows non-limiting examples of PCC protein expression constructs. Further a C-terminal or N-terminal His-tag may be conjugated to the expression construct as shown for certain constructs in this figure.

[00035] Figure 2 shows images of a fluorescence immunostaining of MPP2A-PCCAB import into human PCCA deficient fibroblasts. Panel A shows a merged image of panels B (nuclei), C (PCC), and D (mitochondria). In a color image, one would note a strong immunostaining of PCC in panel C (medium gray) and a similar pattern of immunostaining of mitochondria in panel D (dark gray). Further, in the color image, one would see in the merge of panel A (dark, medium, and light gray) that the PCC and mitochondrial stains co-localize, indicating import of PCC into the mitochondria of the patient fibroblast. Shown here in gray-scale, one can appreciate the similar staining patterns of the imported PCC shown in panel C and that of the mitochondria shown in panel D. In patient fibroblasts not exposed to MPP2A-PCCAB, no PCC staining is detectable.

[00036] Figure 3 is a histogram plot showing PCC activity after import of a TAT or MPP2A conjugated PCCAB construct into isolated mouse mutant mitochondria.

[00037] Figure 4 shows a histogram plot of dose response of PCC activity across patient cell lines.

[00038] Figure 5 shows a graph of plasma C3/C2 ratios after *in vivo* IP administration of a conjugate of the invention.

[00039] Figure 6 shows a line graph of PCC activity in mouse plasma over time after *in vivo* IP administration of TAT-PCCAB conjugate of the invention.

[00040] Figure 7 shows a histogram plot of PCC activity in heart and liver after *in vivo* IP administration of a conjugate of the invention.

[00041] Figure 8 shows a line graph of C3/C2 ratios after 20 mg/kg TATPCCB injection IP in A138T mice.

[00042] Figure 9 shows oxygen consumption of isolated mitochondria during TAT-PCC conjugate import monitored over a 1.5-hour period. Substrate was added after oxygen was consumed at time points 37, 68, and 86 minutes.

[00043] Figure 10 shows a time course of PCC activity (0-20 min) demonstrated the susceptibility of PCC to trypsin. The first group of samples (T0, T5, T10, and T20) were proteolyzed in the presence of a trypsin inhibitor. In the R samples, trypsin was added to the PCC enzyme and the trypsin inhibitor was used to stop the reaction in 0-20 minutes (R0, R5, R10, and R20). One unit of PCC activity is defined as pmol/min/mg protein.

[00044] Figure 11 shows a Western blot of PCC in mitochondrial lysates after import of increasing amounts of TAT-PCC into isolated mutant A138T PCCA mouse mitochondria and trypsin treatment for 25 minutes. Lane M is the molecular weight marker Precision Plus Protein standards (Bio-Rad, Hercules, CA). Lane E is 100ng of PCC enzyme; 1. WT mitochondria; 2. mutant mitochondria; 3. Import of 1 μ M TAT-PCC; 4. Import of 5 μ M TAT-PCC; and 5. Import of 10 μ M TAT-PCC.

[00045] Figure 12 shows a graph of PCC activity in patient fibroblasts #3380 and #3383 having imported MPP1A-PCCAB, MPP2A-PCCAB, and TAT-PCCAB.

[00046] Figure 13 shows PCCA deficient cells grown on microscopy slides. Import of 1 μ M TAT-PCCAB into the fibroblasts was performed for 1 hour at 37°C. After import, live cells were stained as follows: DAPI blue fluorescent dye (dark gray) was used to stain the nuclei in the far-left panel and MitoTracker CMXRos in the second from right panel. Following the cell fixation, staining was performed with anti PCCA antibody and then with green fluorescent (light gray) secondary antibody in the second panel from the left. A merged image of the staining is shown in the far-right panel.

[00047] Skilled artisans will appreciate that elements in the figures are illustrated for simplicity and clarity and are not necessarily to scale. For example, dimensions of particular elements in the figures can be exaggerated relative to other elements to help improve understanding of the embodiments of the present invention described herein

DETAILED DESCRIPTION OF THE INVENTION

[00048] Considering the above background of the art, the present invention provides advantages and advancements over the prior art.

[00049] Provided herein are pharmaceutical compositions and methods of treatment for treating patients with PA or other PCC-deficiency related conditions, for example, enzyme therapy. The spectrum of PA (also referred to as: propionyl-CoA carboxylase deficiency, PCC deficiency, ketotic glycinemia, hyperglycinemia with ketoacidosis and leukopenia, or ketotic hyperglycinemia), ranges from neonatal-onset to late-onset disease. Accordingly, certain embodiments of the present invention provide a method for treating or ameliorating a disease, disorder, or condition in a subject, the disease, disorder, or condition being associated with elevation of at least one selected from the group of propionyl CoA, propionic acid, methylcitrate, beta-hydroxy-propionate, propionylglycine, tiglylglycine, and ketones, the method including a step of administering to the subject a pharmaceutically effective amount of a composition comprising PCC proteins.

[00050] Therapeutic compositions as administered to a patient by methods herein reduce or alleviate at least one symptom or clinical manifestation of the disease, eliminate the disease, alleviate secondary diseases resulting from the occurrence of the primary disease, and prevent incidence of the disease.

I. COMPOSITIONS OF THE INVENTION

[00051] PCC is a biotin-dependent, mitochondrial matrix enzyme involved in organic acid metabolism in humans. The present inventors have explored the biochemistry and molecular genetics of propionic acidemia (PA) and its cause and treatment in arriving at the present invention. Embodiments of the invention address challenges of treatment of PA and other PCC-deficiency related conditions. The resulting pharmaceutical compositions and methods of the invention exploit cell-penetrating proteins, such as TAT, to import assembled PCC or individual PCC subunits into cells, particularly mitochondria, to correct the propionyl-CoA carboxylase enzyme deficiency.

[00052] PCC makes up a multimeric mitochondrial biotin-dependent enzyme. Human PCC enzyme is an $\alpha_6\beta_6$ heterododecamer having a molecular weight of about 800 kDa. The 72

kDa PCC-alpha subunit and the 56 kDa PCC-beta subunit are encoded by genes designated, *PCCA* and *PCCB*. The α subunit contains biotin carboxylase and biotin carboxyl carrier protein domains. The β subunit is responsible for carboxyltransferase activity of the enzyme.

[00053] According to the present invention, the PCC protein includes, but is not limited to, purified *PCCA* and *PCCB* proteins, chemically cleaved and recombinantly produced *PCCA* and *PCCB* proteins, and isolated *PCCA* and *PCCB* proteins associated with other proteins or peptides. More specifically, an isolated human PCC peptide, according to embodiments of the invention herein, is a protein or peptide removed from its natural milieu (*i.e.*, subject to human manipulation) and is combined with, for example, purified proteins, partially purified proteins, recombinantly produced proteins, and synthetically produced proteins. As such, the term "isolated" does not, in some cases, reflect the extent to which the protein has been purified.

Constructs

[00054] Starting with the native or wild type sequences, various compositions and conjugates may be designed and/or engineered. Such starting sequences include the DNA sequence for propionyl Coenzyme A carboxylase alpha (α) subunit protein (*PCCA*), designated SEQ ID NO:1, and its amino acid sequence for full-length human *PCCA*, having 702 amino acid residues, designated SEQ ID NO:2. The DNA sequence for propionyl Coenzyme A carboxylase beta subunit protein (*PCCB*) is designated SEQ ID NO:3, and an its amino acid sequence for full-length human *PCCB*, having 539 amino acid residues, is designated SEQ ID NO:4.

[00055] In certain embodiments, the invention provides a composition of matter comprising one or both of an isolated propionyl-CoA carboxylase alpha chain protein (*PCCA*) comprising the amino acid sequence of SEQ ID NO:2, and/or an isolated propionyl-CoA carboxylase beta chain protein (*PCCB*) comprising the amino acid sequence of SEQ ID NO:4 or functional fragments thereof. Such functional fragments may represent the mature protein as well as any portion thereof.

[00056] Wild type or native sequences encoding the PCC enzyme and its subunit precursors are given in Table 1. The N-terminus of the mature protein is underlined and bolded in both SEQ ID NO. 2 and SEQ ID NO. 4.

Table 1. PCC Nucleic acid and protein sequences

PCCA	ggcatcggggttctggctcgtgattggcggagctcctgcgctcccttctccacccctccggctgtgtgagaggtcagcaga
Nucleotide	ggggcggtctgcggggacaacaatggcgggggttctgggtcgggacagcaccgctggctcgtccggacggcgtgggcgg
NM_000282	tggccgccgagcagctgatcgtgagcgcggcgtcggaccctgaagcatgttctgtactattcaagacagtcttaatggt
X14608	gtcccgtaatcttggtcagtgggatatgacctaataaaaaacttttgataaaattctgttgctaatagaggagaaattgcatgt

propionyl-CoA carboxylase alpha chain, mitochondrial isoform a precursor SEQ ID NO:1	cgggttattagaacttgaagaagatgggcatlaagacagttgcatccacagtgatgttgatgctagtctgttcatgtgaaat ggcgggatgagctgtctgttggcccagctcccaccagtaaaagctacctaacaatggatgccatcatggaagccattlaaga aaaccaggggcccaagctgtacatccagggtatggattccttcagaaaacaagaatttggccagatgttggcagcagaagatg tcgttttcattggacctgacacacatgctattcaagccatggcgacaagattgaaagcaaatatttagctaaagaagcagaggt taatacaatccctggctttagtgagtagtcaggatgcagaagaagctgtcagaattgcaagggaattggctaccctgtcat gatcaaggccctcagcaggtgggtgggaaagcatgctgcatgttggatgatgaagagaccagggcgttttagattgt catctcaagaagctgcttctagtttggcgatgatagactactaataaataaatttattgataatccctgtcatatagaatccaggt tctaggtgataaacatgggaatgctttatggcttaataaagagagtgctcaattcagagaagaatcagaaggtgggtggagga agcaccaagcatttttggatgaggagactcgaagagcagatgggagaacaagctgtagctcttggcagagcagtaaatattc ctctgtgggaccgtggagttcctgttgactctaagaagaatttttatttcttggaaatgaatacagactccaggttgagcatcc tgctacagaatgcatctagctggcctgagcctagtcaggaaatgatccgtgttgaagggtaccctctcaggcacaacaagc tgalattcgcataacggctggcagtgatgtcgggttatgtctgagggaccctacaagcttttgggtttaccatctattggag attgtctcagtaaccaagcgtatcatctacctgtgtccgagtgagcagtgccatccaaccaggagatgattatgacattatt atgatctatgattcaaaactaatcacatattggctctgatagaactgaggcactgaagagaatggcagatgcactggataacta tgtattcagaggtgttacataatattgcatctcagagaggtgataatcaactacgctttgtaaaaggagacatcagcactaa atttctctccgatgtatcctgtatggctcaaggacacatgctaaccaagagtgagaagaaccagttattggcgaatagcatcat cattgttttggcattccagtaagagcacacatttcaagaaatcaagaatccctgtattataaacagacatagccaactgg gagctctcagtaaaatgcatgataaagtcataccgttagtgcatacaaatgggicagtggttctcgttgggaagtgatgggt cgaaactaatgtgaccagcacgtggaacctggcttcccttattgtctgcagcgttgatggcactcagaggactgtccagt gtcttctcagagaagcaggtggaacatgagcattcagtttctgtacagtgtaagaatgataatcctaaccagactggccga gaattgaacaaatattgtctggaagaaagtgactgaggacacaagcagtgctgtcgttcccgatccccgagtggtgtggc cgtctctgcaagcctgagagcgggtagcagaaggtcaagaatttgggtgattgaagccatgaaatgcagaatgatga cagctgggaaactggcacgggtgaactgtgactgtcagctgcaagctggagacacagttggagaaggaggtctgtctgtgagct ggaatgaaggattataaccttgcagtcacccaatttaattagccatttgcagatgctttcacacaaattgattcaagcattat acaggaaacccccgtgcagctacgtttacgtcgtcatttaccacagagtcagacaaatattctgccaaaaataccaatg gaaatttctcattgatataaatactgtacatagattgtacttctgtgtgagatccctagtgtcaaaatgaatcaataaaactgag catttctcaataaaaaaaaaaaaaa
PCCA Protein NP_000273 P05165 propionyl-CoA carboxylase alpha chain, mitochondrial isoform a precursor SEQ ID NO:2	MAGFWVGTAPLVAAGRRGRWPPQQLMLSAALRTLKHVLYYSRQCLMVSRNL GSVGYDPNEKTFDKILVANRGEIACRVIRTCKKMGIKTVAIHSDVDASSVHVK MADEAVCVGPAPTSKSYLNMDAIMEAIAKKTRAQAVHPGYGFLSENKEFARCLA AEDVVFIGPDTHAIQAMGDKIESKLLAKKAEVNTIPGFDGVVKDAEEAVRIARE IGYPVMIKASAGGGGKGMRIA WDDEETRDGFRLLSSQEAASSFGDDRLLIEKFID NPRHIEIQVLGDKHGNALWLNRECSIQRRNQKVVEEAPSIFLDAETRRAMGEQ AVALARAVKYSSAGTVEFLVDSKKNFYFLEMNTRLQVEHPVTECITGLDLVQE MIRVAKGYPLRHKQADIRINGVAVECRVY AEDPYKSFGLPSIGRLSQQYQPLHL PGVRVDSGIQPGSDISIYYDPMISKALITYGSDRTEALKRMADALDNYVIRGVTHN IALREVIINSRFVKGDISTKFLSDVYPDGFKGHMLTKSEKNQLLAIASSLFVAFQ LRAQHFQENSRRMPVIKPDIANWELSVKLHDKVHTVVASNNGSVFSVEVDGSKL NVTSTWNLASPLLSVSVDGTQRTVQCLSREAGGNMSIQFLGTVYKVNILTRLA AELNKFMLEKVTEDTSSVLRSPMPGVVAVSVKPGDAVAEGQEICVIEAMKM QNSMTAGKTGTVKS VHCQAGDTVGEGLLVELE
PCCB Nucleotide NM_000532 propionyl-CoA carboxylase beta chain, mitochondrial isoform 1 precursor SEQ ID NO:3	agcacatgcgtactcaggtgcgcgggtagggagcgcggcgacagcaaaaatggcggcggaftacgggtggcggcg tcggggcaaggctcagcgttctggcgagcgggtctccggcgccggcgcgcagccttgcagccagccacctctgttaacga acgcacgaaaacaagcgccggaccgcgctgctggagggggccaacgccgtattgacgcgcagcacaagcgaggaaa gctaacagccaggagagatcagctcttctgctgaccctggcagcgttctgtgagagcgacatgttggtaacacagatgtg cagatttggaaatggctgataagaataagtttctggagacagcgtgtgacagcagggccgaatcaatggaagattg gtttatgtcttcagtcaggtttacagttttggagcagctgtcagagcagcatgcccaaaagatctgcaaaatcatggacca ggccataacgggtggggctccagtgattgggctgaatgactctgggggagcagcagatccaagaaggagtgaggctttggct ggctatgcagacatcttctgaggaaatgttacggcatccggagtcacccctcagatttctgtatcatggcccatgtgctgtgg ggccgtctactccccagccctaacagacttcacgttcagtgtaaaaggacacctctacctgttcacactggccctgatgtgtg aagctgtaccaatgaggatgtaccagaggagctcgtgtgtgccaagaccacaccacatgtcaggtgtggccacaca gagctttgaaaatgatgtgatgcttgtgaatcctgggatttctcaactacctgccccctgagcagtcaggaccggctccc gtccgtgagtgccacagatccagtgaccgtctgttctgtgagcttgacacaattgtcccttggaaatcaaccaaacctacaaca tgggtggacatcacactctgttggatgagcgtgaatttttgagatcatgcccattatgccaaacatcattgtgttttgca agaatgaatgggaggactgttgaattgtggcaaccaacctaaaggtggcctcaggatgcttggaatgaatcattctgtgaaag gggctcgtttgtcagattctgtgatgcattcaatttcactacatcatttgtgtgtccctggcttctactggcagacagacag gaatacggggcattacccggatggtgccaagcttctacgcatgtgtgaggcaactgtacccaaggtcagatgcattcacc aggaagccatgagggtgcctatgatgtcagctctaaagcaccttgtgtgataccaactatgctggccaccgcaga gattgcagtcagggagcaaggcgtgtggagatcatctcaagggcagatgagaatgtggaagctgtcaggcagagtag atcagagaatttgcacaccttccctgcagcagtcgagggtttgtgagatcatcatcaaccttcttccacacgtgcccgaa

	tctgctgtgacctggatgcttggccagcaagaaggtacaacgtccttggagaaaacatgcaaataftccattgtaacaaatca aaggaaaagaaaccaagaactgaattactgtctgcccaftcacatcccattcctgaccttggcaatcatgaaacctgggaatcca aatagttggataacttagaataactaagtttattaaattctagaagaatctcaaaaaaaaaa
PCCB Protein NP_000523 P05166 propionyl-CoA carboxylase beta chain, mitochondrial isoform 1 precursor SEQ ID NO:4	MAAALRVAAVGARLSVLASGLRAAVRSLCSQATSVNERIENKRRRTALLGGGQ RRIDAQHKRGKLTARERISLLDPGSFVESDMFVEHRCADFGMAADKNKFPD SVVTGRGRINGRLVYVFSQDFTVFGGSLSGAHAQKICKIMDQAITVGAPVIGLN DSGGARIQEGVESLAGYADIFLRNVTASGVIPQISLIMGPCAGGAVYSPALTDFT FMVKDTSYLFITGPDVVKSVTNEDVTQEELGGAKTHTTMSGVAHRAFENDVD ALCNLRDFFNYLPLSSQDPAPVRECHDPSDRLVPELDTIVPLESTKAYNMVDIIH SVVDEREFFEIMPNYAKNIIVGFARMNGRTVGIVGNQPKVASGCLDINSSVKGA RFVRFCDAFNIPLITFVDVPGFLPGTAQEYGGIIRHGAKLLYAFAEATVPKVTVIT RKAYGGAYDVMSSKHLCGDTNYAWPTAEIAVMGAKGAVEIIFKGHENVEAAQ AEYIEKFANFPFAAVRGFVDDIIQPSSTRARICCDLDVLASKKVQRPWRKHANIP L

II. CONJUGATES

[00057] Various embodiments provide PCC proteins or subunits conjugated to a cell penetrating peptide. Cell-penetrating peptides and mitochondria penetrating peptides are short peptides (typically less than 30 amino acids) that facilitate cellular uptake of various molecules. Cell penetrating and mitochondria penetrating peptides are tools for non-invasive cellular import of cargo and have been successfully applied for *in vitro* and *in vivo* delivery of therapeutic molecules, *e.g.*, small chemical molecules, nucleic acids, proteins, peptides, liposomes, and particles.

[00058] ERT for mitochondrial enzymes requires transport of the cargo through the plasma membrane as well as through the outer and inner mitochondrial membranes. First, delivery of protein is limited by their ability to penetrate the cell membrane. A cell penetrating and mitochondria penetrating peptide can be linked to a molecule through covalent bonds or non-covalent bonds, and are coupled to the PCC peptides using standard methods of bioconjugation. Mitochondria is made up of two membrane system. Whereas the mitochondrial outer membrane is similar to the plasma membrane in terms of protein to lipid constitution (1:1), there are no proteoglycans present on the surface of mitochondria although the phospholipid, cardiolipin, imparts a net negative charge to the membrane. The inner mitochondrial membrane displays a higher protein to lipid ratio (3:1) compared to the plasma and outer mitochondria membranes (see, Gohil et al., J. Cell Biol. Vol. 184 No. 4 469–472, 2009, which is herein incorporated by reference in its entirety). Apart of from proteins encoded by the mitochondrial genome most mitochondrial proteins need to be delivered into this organelle following their translation on cytoplasmic ribosomes. They are then transported with the help of translocases and chaperones. The mitochondrial targeting sequence (MTS) is recognized by a receptor in the translocase of the outer membrane. After a protein arrives in the mitochondrial matrix, a protease removes its N-terminal matrix-targeting sequence.

[00059] HIV-1 trans-activating transcriptional activator (TAT) domain and its variants are used most frequently used for many different types of cargo. See, Frankel et al., *Cell*. 1988 Dec 23;55(6):1189-93, which is herein incorporated by reference in its entirety. The minimal peptide sequence of TAT protein responsible for cellular uptake is YGRKKRRQRRR (SEQ ID NO:5), which contains six arginine and two lysine residues and therefore possesses a high net positive charge at physiological pH levels. The TAT domain has been used to deliver lipoamide dehydrogenase (LAD) to mitochondria in fibroblasts from patients suffering from LAD deficiency. Furthermore, this transduced enzyme was observed to be able to replace the defective enzyme in a large multisubunit complex to restore enzymatic function to near-normal levels. LAD is the third catalytic subunit (E3) of three multicomponent enzymatic in the mitochondrial matrix. Previously, C6orf66 assembly factor that restores Complex I activity in patient cells was also successfully replaced. This demonstrated a possibility for repair of multicomponent complex proteins using a TAT fusion protein strategy (see, Marcus et al., *Mol Med*. 2013; 19(1): 124–134, which is herein incorporated by reference in its entirety). TAT-frataxin has also been announced as a drug candidate to increase lifespan and cardiac function in a conditional Friedreich's ataxia mouse model.

[00060] For the enzyme of interest herein, single subunit PCCA or PCCB import has been described (see, Damavandi et al., *Mol Genet Metab Rep*. 2016 Sep; 8: 51–60, which is herein incorporated by reference in its entirety). The import of PCC heterododecamer ($\alpha\beta 6$) using the TAT transduction domain was also analyzed herein.

[00061] Pharmaceutical compositions herein include purified PCCAB conjugated to a cell penetrating peptide, such as TAT, or mitochondria-penetrating peptide (MPP) by maleimide-mediated thioether bond formation with available cysteine residue(s) of the PCCAB.

Alternatively, a cell penetrating peptide is conjugated to PCCAB by amide bond formation between an NHS ester and lysine residues.

[00062] In certain embodiments, the TAT peptide comprises the amino acid sequence YGRKKRRQRRR (SEQ ID NO:5). Alternatively, in another embodiment, the TAT peptide has the amino acid sequence GRKKRRQRRRPQ (SEQ ID NO:6) or a fragment thereof. In yet another embodiment, the TAT peptide comprises the amino sequence CFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQ (SEQ ID NO:20) or a fragment thereof. Alternatively, the TAT peptide comprises the amino acid sequence Maleoyl-beta-AGYGRKKRRQRRR (SEQ ID NO:21) or a fragment thereof, or the amino acid sequence GYGRKKRRQRRR (SEQ ID NO: 22) or a fragment thereof.

[00063] Additional examples of cell penetrating peptides known in the art include: homeodomain transcription factors such as Antennapedia (RQIKIYFQNRRMKWKK, SEQ ID NO:7), herpes simplex virus type 1 protein VP22 (DAATATRGRSAASRPTERPRAPARSASRRRPVD, SEQ ID NO:8), HIV trans-activating transcriptional activator (YGRKKRRQRRR, SEQ ID NO:5), penetratin (RQIKIWFQNRRMKWKK, SEQ ID NO:9), transportan (GWTLSAGYLLGKINLKALAALAKK IL, SEQ ID NO:10); amphipathic proteins such as MPG (GALFLGFLGAAGSTMGAWSQPKKKRKV, SEQ ID NO:11), Pep-1 (KETWWETWWTEWSQPKKKRKV, SEQ ID NO:12), MAP (KALAKALAKALA, SEQ ID NO:13), SAP (VRLPPPVRLLPPPVRLLPPP, SEQ ID NO:14), PPTGI (GLFRALLRLLRSLWRLLLRA, SEQ ID NO:15); and peptides such as poly-Arginine sequences (*e.g.*, RRRRRRRR, SEQ ID NO:16), hCT (LGTYTQDFNKTFPQTAIGVG AP, SEQ ID NO:17), SynB (RGGRLSYSRRRFSTSTGR, SEQ ID NO:18), and Pvec (LLIILRRRIRKQAHAAHSK, SEQ ID NO:19). Cell penetrating proteins are discussed, for example, in: Fang et al., 2013 *PLOS ONE* 8(3):e57318; Ruoslahti et al., 2009 *J Cell Biology* 188(6):759-68; Foged & Nielsen, 2008 *Expert Opin. Drug Deliv.* 5(1):105-17; and Treat et al., 2012 *ACS Macro Lett.* 1(1):100-04, which are herein incorporated by reference in their entireties.

[00064] In certain embodiments, the MPP1A peptide comprises the amino acid sequence Cha-DArg-Cha-Lys-Cha-DArg-Cha-Lys (Cha-R-Cha-K-Cha-R-Cha-K) (SEQ ID NO:23). In certain embodiments, the MPP2A peptide comprises the amino acid sequence Cha-DArg-Cha-Lys (Cha-R-Cha-K) (SEQ ID NO:24).

[00065] In certain embodiments, the MPP1A peptide consists essentially of the amino acid sequence Cha-DArg-Cha-Lys-Cha-DArg-Cha-Lys (Cha-R-Cha-K-Cha-R-Cha-K) (SEQ ID NO:23). In certain embodiments, the MPP2A peptide consists essentially of the amino acid sequence Cha-DArg-Cha-Lys (Cha-R-Cha-K) (SEQ ID NO:24).

[00066] In certain embodiments, the MPP1A peptide is the amino acid sequence Cha-DArg-Cha-Lys-Cha-DArg-Cha-Lys (Cha-R-Cha-K-Cha-R-Cha-K) (SEQ ID NO:23). In certain embodiments, the MPP2A peptide is the amino acid sequence Cha-DArg-Cha-Lys (Cha-R-Cha-K) (SEQ ID NO:24).

[00067] PCC derivatives are within the scope of the present invention. Examples of PCC derivatives or variants, include, but are not limited to, genetically engineered modifications including nucleic acid and/or amino acid modifications or chemical modifications. For example, modifications that mask potential immunogenic epitopes on the surface of a protein

and/or hinder access to the protein for proteolytic enzymes are of interest. Other modifications of interest are those that advantageously alter the physio-chemical properties of the PCC peptide, thus modifying its biodistribution, stability, and solubility without significantly detracting from its potency. Such derivatives may be chemically modified PCC protein compositions in which PCC protein is linked to a polymer. The polymer selected is typically water-soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as the physiological environment. The polymer may be of any molecular weight, and may be branched or unbranched. Included within the scope of PCC protein polymers is a mixture of polymers. In specific embodiments, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable.

[00068] Further examples of cell penetrating peptides include homeodomain transcription factors such as Antennapedia, VP22, TAT, penetratin, and transportan; amphipathic molecules such as MPG, Pep-1, MAP, SAP and PPTG1; poly-Arginine sequences; hCT; SynB; and Pvec. Examples of these ligands are antibodies, antigens, receptors and receptor ligands. Manipulating the chemical formula of the lipid portion of the delivery vehicle can modulate the extracellular or intracellular targeting of the delivery vehicle. For example, a chemical can be added to the lipid formula of a liposome that alters the charge of the lipid bilayer of the liposome so that the liposome fuses with particular cells having particular charge characteristics. In specific embodiments, liposomes of the present invention include those liposomes commonly used in, for example, protein delivery methods known to those of skill in the art.

[00069] Complexing a liposome with a protein of the examples herein provide nucleic acid and/or amino acid modifications or PCC chemical modifications that mask potential immunogenic epitopes on the surface of a protein and/or hinder access to the protein for proteolytic enzymes. Additional modifications analyzed herein include those that advantageously alter the physio-chemical properties of PCC, to modify biodistribution, stability, and solubility without significantly detracting from its potency. Further examples analyze the effect of chemical modifications to deliver the proteins to cells, and optimization of the TAT peptide sequence or other cell-penetrating peptides.

[00070] In certain embodiments, the amino acid sequence of the mitochondrial targeting leader corresponds to the first 51 amino acids of a full-length PCCA subunit (of SEQ ID NO:2) and the first 28 amino acids of a full-length PCCB subunit (of SEQ ID NO:4).

[00071] Both mature subunits may be modified covalently with TAT or mitochondrial targeting peptide. The subunits including the leader sequences may then be expressed with TAT preceding the leader.

[00072] In other aspects, the PCCA protein and/or PCCB protein comprises a mitochondrial leader sequence. In yet other aspects, the PCCA protein and/or PCCB protein lack a mitochondrial leader sequence. In various embodiments, the PCCA protein and/or PCCB proteins are genetically engineered proteins or variants thereof.

[00073] In another embodiment, the PCCA protein and/or PCCB protein is covalently linked to one or a plurality of cell penetrating proteins. As used herein, a "cell penetrating protein" or "cell penetrating peptide" is an amino-acid based polypeptide which facilitates or fosters the transport of a biomolecule across any cell membrane. A non-limiting example of such a cell penetration protein is trans-activating transcriptional activator (TAT) or a tissue specific variant thereof. In some embodiments, the cell-penetrating protein is chemically added post-translation of the PCCA or PCCB peptide.

[00074] In certain aspects, a PCC protein or conjugate of the present invention comprises an amino acid sequence that is less than 100% identical to SEQ ID NO:2 and/or SEQ ID NO:4, and in specific embodiments having 75% sequence identity, 80% sequence identity, 85% sequence identity, 90% sequence identity, 91% sequence identity, 92% sequence identity, 93% sequence identity, 94% sequence identity, 95% sequence identity, 96% sequence identity, 97% sequence identity, 98% sequence identity, or 99% sequence identity, to SEQ ID NO:2 and/or SEQ ID NO:4.

[00075] In one embodiment, the PCC protein derivative will have a single cell-penetrating or mitochondria-penetrating molecule at the amino terminus. In particular embodiments, the PCC protein enzyme conjugated to the cell-penetrating or the mitochondria penetrating molecule provided by an embodiment has an average of about 1 to about 10, more particularly 2 to 5 and more particularly 3 to 5 cell-penetrating or mitochondria-penetrating molecules covalently attached to each PCC enzyme subunit in the composition.

III. PRODUCTION AND PURIFICATION

Production

[00076] Use of recombinant DNA technologies to improve control of expression of transfected nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within the host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications was explored in the examples.

Additionally, the promoter sequence may be genetically engineered to improve the level of expression as compared to the native promoter.

[00077] Recombinant techniques useful for controlling the expression of nucleic acid molecules include, but are not limited to, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (*e.g.*, promoters, operators, enhancers), substitutions or modifications of translational control signals (*e.g.*, ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid molecules to correspond to the codon usage of the host cell, and deletion of sequences that destabilize transcripts.

[00078] Methods well-known to those skilled in the art are used to construct expression vectors and recombinant bacterial cells according to embodiments of this invention. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, *in vivo* recombination techniques, and PCR techniques. For example, techniques described in Maniatis et al., 1989, MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, New York; Ausubel et al., 1989, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates and Wiley Interscience, New York, and PCR Protocols: A Guide to Methods and Applications (Innis et al., 1990, Academic Press, San Diego, CA), which are herein incorporated by reference in their entireties.

[00079] In certain embodiments, the PCCA and/or PCCB proteins are produced recombinantly. The PCCA and/or PCCB proteins may be produced in prokaryotic or eukaryotic cells, more specifically yeast, mammalian, or *E. coli* cells.

Chromatographic separation

[00080] Chromatographic separation comprises an ion exchange chromatography column for purification. In one embodiment, the ion exchange chromatography column is an anion exchanger. Various types of anion exchange resins can be used, DEAE-cellulose, DEAE-cellulose DE 52, and DEAE-Sepharose-FF. According to one embodiment, the anion exchange resin is DEAE-Sepharose-FF.

[00081] Additional chromatographic steps provided in certain embodiments of the methods of this invention for purifying PCC from a PCC-containing solution include use of a monomeric avidin column. Avidin columns are useful for non-denaturing affinity purification of biotinylated molecules.

[00082] Chromatography matrices useful in the method of the invention are materials capable of binding biochemical compounds, preferably proteins, nucleic acids, and/or

endotoxins, wherein the affinity of said biochemical compounds to said chromatography matrix is influenced by the ion composition of the surrounding solution (buffer).

[00083] Controlling the ion composition of said solution allows to use the chromatography materials of the invention either in subtractive mode (PCC passes through said chromatography matrix and at least certain contaminants bind to said chromatography matrix) or, preferably, in adsorptive mode (PCC binds to the chromatography matrix).

[00084] In particular embodiments, the method for purification comprises the step of homogenizing host cells, particularly recombinant cells and in certain embodiments, recombinant cells producing mammalian, preferably human, PCC proteins, wherein said recombinant construct encodes a PCC protein that is a naturally occurring or a genetically engineered variant thereof, and particularly wherein said construct has been optimized for recombinant cell expression. In particular embodiments, said recombinant cells are prokaryotic cells, particularly bacterial cells or eukaryotic cells, particularly yeast or mammalian cells. In certain particular embodiments, the bacterial cells are *E. coli* cells and the PCC sequence has been engineered in the recombinant expression construct to be optimized for expression in said cells; a specific embodiment of such a nucleic acid sequence optimized for PCC expression in *E. coli* is set forth in the plasmid pPCCAB of the examples, which is also described in Kelson et al., 1996 Human Molecular Genetics. 5:331-37. In said methods, cells are harvested, e.g. by centrifugation, and optionally stored at -80°C. Homogenization of host cells is performed by disrupting the cells using physical, chemical, or enzymatic means or by a combination thereof. Advantageously, for purification from bacterial sources, homogenization is performed by disrupting the cell wall of said bacterial host by sonication. Alternatively or additionally, homogenizing is performed by destabilizing the bacterial cell wall of the host by exposure to a cell wall degrading enzyme such as lysozyme.

[00085] The methods of the invention can further comprise a clarified PCC homogenate, wherein cell debris is removed from the homogenate by either filtration or centrifugation. In certain embodiments, clarifying is performed by centrifuging the homogenate at an effective rotational speed. The centrifugation time depends inter alia on the volume of the homogenate, which is determined empirically to obtain a sufficiently solid pellet. To obtain an essentially cell debris-free clarified homogenate, a combination of centrifugation and filtration can be performed on the homogenate.

[00086] Methods to measure protein expression levels of the PCC protein according to the invention include, but are not limited to Coomassie blue or silver staining of protein in a

separation media, such as gel electrophoresis, Western blotting, immunocytochemistry, other immunologic-based assays; and assays based on a property of the protein including but not limited to, enzyme assays, ligand binding or interaction with other protein partners.

IV. PHARMACEUTICAL COMPOSITIONS AND METHODS OF USE

[00087] Various embodiments of the invention provide a method of correcting a PCC-deficiency related disease or condition in a cell including the steps of contacting the cell with a preparation of isolated human PCC at a concentration sufficient for the cell to take up a therapeutically effective amount of PCC, such that the preparation contains at least one selected from the group of an isolated propionyl-CoA carboxylase PCCA protein comprising a functional portion or variant of the amino acid sequence of SEQ ID NO:2 or an isolated propionyl-CoA carboxylase PCCB protein comprising a functional portion or variant of the amino acid sequence of SEQ ID NO:4.

[00088] Such pharmaceutical compositions are superior to those in the art as they traffic efficiently to the mitochondria and have sufficient stability and duration of action to effect therapeutically relevant outcomes.

Localization to mitochondria

[00089] Given the function of the PCC enzyme, localization to the mitochondria is important. The present invention shows by confocal microscopy importation and colocalization of immunofluorescently labeled TAT-PCCAB with a mitochondria-specific marker, MITOTRACKER®.

[00090] Concomitant measurement of metabolite levels in mutant cell extracts were observed to have normalized propionyl-carnitine (C3) to acetyl-carnitine (C2) ratios. Additionally, import of MPP2a-modified PCCAB into isolated mitochondria from PCC-deficient mouse liver, followed by trypsin treatment of the mitochondria, was observed to result in active PCC in cases where the enzyme was protected from trypsin. PCC activity in the mitochondrial extract far exceeded the original residual mutant activity and was higher than activity in an extract prepared from wild type mitochondria.

[00091] Consequently, PCC imported into the cells by the processes described herein provided reproducible data demonstrating that the diagnostic C3/C2 ratio (propionyl-/acetyl-carnitine) in cell extracts of the PCC-treated cells significantly decreased in comparison to untreated controls.

[00092] Further, individual PCC subunits having the TAT peptide followed by the mitochondrial targeting leader peptide were imported into patient fibroblasts. TAT-prePCCB

was observed by confocal fluorescence microscopy to be targeted to mitochondria as shown in Figure 2.

Therapeutic uses

[00093] In a further aspect, the invention provides a method for treating PCC deficiency in an individual in need thereof, comprising administering a therapeutically effective amount of a pharmaceutical composition of isolated human PCC to the individual in need thereof, wherein the isolated human PCC comprises one or both of an isolated propionyl-CoA carboxylase alpha chain protein (PCCA) conjugate comprising the amino acid sequence of SEQ ID NO:2 or functional fragment thereof, and/or an isolated propionyl-CoA carboxylase beta chain protein (PCCB) conjugate comprising the amino acid sequence of SEQ ID NO:4 or functional fragment thereof.

[00094] In yet another aspect, the invention provides pharmaceutical composition comprising a therapeutically effective amount of isolated human PCC wherein the PCC comprises one or both of an isolated propionyl-CoA carboxylase alpha chain protein (PCCA) comprising the amino acid sequence of SEQ ID NO:2 or functional fragment thereof, and/or an isolated propionyl-CoA carboxylase beta chain protein (PCCB) comprising the amino acid sequence of SEQ ID NO:4 or functional fragment thereof, and a pharmaceutically acceptable carrier, diluent or excipient.

[00095] In some embodiments, the pharmaceutical composition is administered by intravenous injection (IV), subcutaneous injection (SQ), or intraperitoneal injection (IP).

[00096] The pharmaceutical composition may comprise an amount of PCC protein wherein 0.01 mg/kg – 20 mg/kg is administered to an individual in need thereof.

[00097] In some embodiments, the dose may be administered as a single daily dose, a weekly dose, a monthly dose, a yearly dose.

[00098] In some embodiments, the dose is administered as a split dose whereby a total daily dose is divided into equal or unequal amounts and administered over the course of the same day.

[00099] In some embodiments, the dose is 0.1 mg/kg to 0.5 mg/kg; 0.1 mg/kg to 2 mg/kg; about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 7 mg/kg; from 2-10 mg/kg; from 3-15 mg/kg; more than 10 mg/kg, more than 20 mg/kg.

[00100] In another embodiment, the invention provides a method for treating or ameliorating a disease, disorder, or condition, associated with elevated propionyl CoA, propionic acid, methylcitrate, beta-hydroxy-propionate, propionylglycine, tiglic acid, and ketones comprising administering to an individual in need thereof a pharmaceutically

effective amount of a pharmaceutical composition of PCC. In one embodiment, the disease, disorder, or condition associated with elevated propionyl CoA, propionic acid, methylcitrate, beta-hydroxy-propionate, propionylglycine, tiglic acid, and ketones is poor feeding, vomiting, and somnolence, lethargy, seizures, coma, metabolic acidosis, anion gap, ketonuria, hypoglycemia, hyperammonemia, cytopenias, developmental regression, chronic vomiting, protein intolerance, failure to thrive, hypotonia, basal ganglia infarction, dystonia, choreoathetosis, and cardiomyopathy.

[000101] In some embodiments, the pharmaceutical composition is administered by intravenous injection, subcutaneous injection, or intraperitoneal injection.

[000102] In another embodiment, the invention provides a method for treating or ameliorating a disease, disorder, or condition, associated with elevated propionyl CoA, propionic acid, methylcitrate, beta-hydroxy-propionate, propionylglycine, tiglylglycine, and ketones comprising administering to an individual in need thereof a pharmaceutically effective amount of a pharmaceutical composition of PCC. In one embodiment, the disease, disorder, or condition associated with elevated propionyl CoA, propionic acid, methylcitrate, beta-hydroxy-propionate, propionylglycine, tiglylglycine, and ketones is poor feeding, vomiting, and somnolence, lethargy, seizures, coma, metabolic acidosis, anion gap, ketonuria, hypoglycemia, hyperammonemia, cytopenias, developmental regression, chronic vomiting, protein intolerance, failure to thrive, hypotonia, basal ganglia infarction, dystonia, choreoathetosis, and cardiomyopathy.

Formulations

[000103] The compositions of the present invention may be formulated in any manner suitable for delivery. The formulation may be, but is not limited to, nanoparticles, poly(lactic-co-glycolic acid)(PLGA) microspheres, lipidoids, lipoplex, liposome, polymers, carbohydrates (including simple sugars), cationic lipids and combinations thereof.

[000104] In one embodiment, the formulation is a nanoparticle which may comprise at least one lipid. The lipid may be selected from, but is not limited to, DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, PLGA, PEG, PEG-DMG and PEGylated lipids. In another aspect, the lipid may be a cationic lipid such as, but not limited to, DLin-DMA, DLin-D-DMA, DLin-MC3-DMA, DLin-KC2-DMA and DODMA.

[000105] Formulation may be in standard saline solutions or any suitable buffer.

V. DEFINITIONS

[000106] At various places in the present specification, substituents of compounds of the present disclosure are disclosed in groups or in ranges. It is specifically intended that the

present disclosure include each and every individual subcombination of the members of such groups and ranges.

[000107] *About*: As used herein, the term “about” means +/- 10% of the recited value.

[000108] *Activity*: As used herein, the term “activity” refers to the condition in which things are happening or being done. Compositions of the invention may have activity and this activity may involve one or more biological events.

[000109] *Administered in combination*: As used herein, the term “administered in combination” or “combined administration” means that two or more agents are administered to a subject at the same time or within an interval such that there may be an overlap of an effect of each agent on the patient. In some embodiments, they are administered within about 60, 30, 15, 10, 5, or 1 minute of one another. In some embodiments, the administrations of the agents are spaced sufficiently closely together such that a combinatorial (*e.g.*, a synergistic) effect is achieved.

[000110] *Amelioration*: As used herein, the term “amelioration” or “ameliorating” refers to a lessening of severity of at least one indicator of a condition or disease. For example, in the context of neurodegeneration disorder, amelioration includes the reduction of neuron loss.

[000111] *Animal*: As used herein, the term “animal” refers to any member of the animal kingdom. In some embodiments, “animal” refers to humans at any stage of development. In some embodiments, “animal” refers to non-human animals at any stage of development. In certain embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, and worms. In some embodiments, the animal is a transgenic animal, genetically-engineered animal, or a clone.

[000112] *Antibody*: As used herein, the term “antibody” is referred to in the broadest sense and specifically covers various embodiments including, but not limited to monoclonal antibodies, polyclonal antibodies, multi-specific antibodies (*e.g.* bispecific antibodies formed from at least two intact antibodies), and antibody fragments (*e.g.*, diabodies) so long as they exhibit a desired biological activity (*e.g.*, “functional”). Antibodies are primarily amino acid based molecules but may also comprise one or more modifications (including, but not limited to the addition of sugar moieties, fluorescent moieties, chemical tags, *etc.*). Non-limiting examples of antibodies or fragments thereof include V_H and V_L domains, scFvs, Fab, Fab', F(ab')₂, Fv fragments, diabodies, linear antibodies, single chain antibody molecules, multispecific antibodies, bispecific antibodies, intrabodies, monoclonal antibodies, polyclonal

antibodies, humanized antibodies, codon-optimized antibodies, tandem scFv antibodies, bispecific T-cell engagers, mAb2 antibodies, chimeric antigen receptors (CAR), tetravalent bispecific antibodies, biosynthetic antibodies, native antibodies, miniaturized antibodies, unibodies, maxibodies, antibodies to senescent cells, antibodies to conformers, antibodies to disease specific epitopes, or antibodies to innate defense molecules.

[000113] *Approximately*: As used herein, the term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term “approximately” or “about” refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context and except where such number would exceed 100% of a possible value.

[000114] *Associated with or conjugated to*: As used herein, the terms “associated with,” “conjugated,” “linked,” “attached,” and “tethered,” when used with respect to two or more moieties, means that the moieties are physically associated or connected with one another, either directly or via one or more additional moieties that serves as a linking agent, to form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, *e.g.*, physiological conditions. An “association” need not be strictly through direct covalent chemical bonding. It may also suggest ionic or hydrogen bonding or a hybridization based connectivity sufficiently stable such that the “associated” entities remain physically associated. Conjugation may be via covalent linkage.

[000115] *Bacterial cell*: The term “bacterial cell” as used herein refers to bacteria that produces a mammalian, preferably human, PCC protein *inter alia* using recombinant genetic methods including progeny of said recombinant cell. The PCC protein is a naturally occurring or a genetically engineered variant.

[000116] *Bifunctional*: As used herein, the term “bifunctional” refers to any substance, molecule or moiety which is capable of or maintains at least two functions. The functions may affect the same outcome or a different outcome. The structure that produces the function may be the same or different.

[000117] *Biocompatible*: As used herein, the term “biocompatible” means compatible with living cells, tissues, organs, or systems posing little to no risk of injury, toxicity, or rejection by the immune system.

[000118] *Biologically active*: As used herein, the phrase “biologically active” refers to a characteristic of any substance that has activity in a biological system and/or organism. For

instance, a substance that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active.

[000119] *Complementary and substantially complementary:* As used herein, the term “complementary” refers to the ability of polynucleotides to form base pairs with one another. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands. Complementary polynucleotide strands can form base pairs in the Watson-Crick manner (e.g., A to T, A to U, C to G), or in any other manner that allows for the formation of duplexes. As persons skilled in the art are aware, when using RNA as opposed to DNA, uracil rather than thymine is the base that is considered to be complementary to adenosine. However, when a U is denoted in the context of the present invention, the ability to substitute a T is implied, unless otherwise stated. Perfect complementarity or 100% complementarity refers to the situation in which each nucleotide unit of one polynucleotide strand can form hydrogen bonds with a nucleotide unit of a second polynucleotide strand. Less than perfect complementarity refers to the situation in which some, but not all, nucleotide units of two strands can form hydrogen bonds with each other. For example, for two 20-mers, if only two base pairs on each strand can form hydrogen bonds with each other, the polynucleotide strands exhibit 10% complementarity. In the same example, if 18 base pairs on each strand can form hydrogen bonds with each other, the polynucleotide strands exhibit 90% complementarity.

[000120] *Compound:* Compounds of the present disclosure include all of the isotopes of the atoms occurring in the intermediate or final compounds. “Isotopes” refers to atoms having the same atomic number but different mass numbers resulting from a different number of neutrons in the nuclei. For example, isotopes of hydrogen include tritium and deuterium.

[000121] The compounds and salts of the present disclosure can be prepared in combination with solvent or water molecules to form solvates and hydrates by routine methods.

[000122] *Conditionally active:* As used herein, the term “conditionally active” refers to a mutant or variant of a wild-type polypeptide, wherein the mutant or variant is more or less active at physiological conditions than the parent polypeptide. Further, the conditionally active polypeptide may have increased or decreased activity at aberrant conditions as compared to the parent polypeptide. A conditionally active polypeptide may be reversibly or irreversibly inactivated at normal physiological conditions or aberrant conditions.

[000123] *Conserved:* As used herein, the term “conserved” refers to nucleotides or amino acid residues of a polynucleotide sequence or polypeptide sequence, respectively, that are those that occur unaltered in the same position of two or more sequences being compared.

Nucleotides or amino acids that are relatively conserved are those that are conserved amongst more related sequences than nucleotides or amino acids appearing elsewhere in the sequences.

[000124] In some embodiments, two or more sequences are said to be “completely conserved” if they are 100% identical to one another. In some embodiments, two or more sequences are said to be “highly conserved” if they are at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be “highly conserved” if they are about 70% identical, about 80% identical, about 90% identical, about 95%, about 98%, or about 99% identical to one another. In some embodiments, two or more sequences are said to be “conserved” if they are at least 30% identical, at least 40% identical, at least 50% identical, at least 60% identical, at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be “conserved” if they are about 30% identical, about 40% identical, about 50% identical, about 60% identical, about 70% identical, about 80% identical, about 90% identical, about 95% identical, about 98% identical, or about 99% identical to one another. Conservation of sequence may apply to the entire length of a polynucleotide or polypeptide or may apply to a portion, region, or feature thereof.

[000125] *Control Elements*: As used herein, “control elements”, “regulatory control elements” or “regulatory sequences” refers to promoter regions, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites (“IRES”), enhancers, and the like, which provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control elements need always be present as long as the selected coding sequence is capable of being replicated, transcribed, and/or translated in an appropriate host cell.

[000126] *Controlled Release*: As used herein, the term “controlled release” refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome.

[000127] *Delivery*: As used herein, “delivery” refers to the act or manner of delivering a particle, compound, substance, entity, moiety, cargo, or payload.

[000128] *Delivery Agent*: As used herein, “delivery agent” refers to any substance which facilitates, at least in part, the *in vivo* delivery of compound or pharmaceutical composition to targeted cells.

[000129] *Detectable label*: As used herein, “detectable label” refers to one or more markers, signals, or moieties which are attached, incorporated, or associated with another entity that is readily detected by methods known in the art including radiography, fluorescence, chemiluminescence, enzymatic activity, absorbance, and the like. Detectable labels include radioisotopes, fluorophores, chromophores, enzymes, dyes, metal ions, ligands such as biotin, avidin, streptavidin and haptens, quantum dots, and the like. Detectable labels may be located at any position in the peptides or proteins disclosed herein. They may be within the amino acids, the peptides, or proteins, and located at the N- or C- termini.

[000130] *Digest*: As used herein, the term “digest” means to break apart into smaller pieces or components. When referring to polypeptides or proteins, digestion results in the production of peptides.

[000131] *Disease*: The term, “disease” refers to deviation from the normal health of a patient and includes a state when disease symptoms are present, as well as conditions in which a deviation (*e.g.*, infection, gene mutation, genetic defect, *etc.*) has occurred, yet symptoms are not yet manifested (*e.g.*, a predisease condition).

[000132] *Dosing regimen*: As used herein, a “dosing regimen” is a schedule of administration or physician determined regimen of treatment, prophylaxis, or palliative care.

[000133] *Engineered*: As used herein, embodiments of the invention are “engineered” when they are designed to have a feature or property, whether structural or chemical, that varies from a starting point, wild type, or native molecule.

[000134] *Effective Amount*: As used herein, the term “effective amount” of an agent is an amount sufficient to effect beneficial or desired results, for example, clinical results, and, as such, an “effective amount” depends upon the context in which it is being applied. For example, in the context of administering an agent that treats PCC deficiency, an effective amount of an agent is, for example, an amount sufficient to achieve treatment, as defined herein, of PCC deficiency, as compared to the response obtained without administration of the agent.

[000135] *Epitope*: As used herein, an “epitope” refers to a surface or region on a molecule that is capable of interacting with a biomolecule. For example, a protein may contain one or more amino acids, *e.g.*, an epitope, which interacts with an antibody, *e.g.*, a biomolecule. In some embodiments, when referring to a protein or protein module, an epitope may comprise a linear stretch of amino acids or a three-dimensional structure formed by folded amino acid chains.

[000136] *Expression*: As used herein, “expression” of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (*e.g.*, by transcription); (2) processing of an RNA transcript (*e.g.*, by splicing, editing, 5' cap formation, and/or 3' end processing); (3) translation of an RNA into a polypeptide or protein; and (4) post-translational modification of a polypeptide or protein.

[000137] *Feature*: As used herein, a “feature” refers to a characteristic, a property, or a distinctive element.

[000138] *Formulation*: As used herein, a “formulation” includes at least one pharmaceutical compound or active agent and a delivery agent.

[000139] *Fragment*: A “fragment,” as used herein, refers to a portion. For example, fragments of proteins may comprise polypeptides obtained by digesting full-length protein isolated from cultured cells.

[000140] *Functional*: As used herein, a “functional” biological molecule is a biological molecule in a form in which it exhibits a property and/or activity by which it is characterized.

[000141] *Gene expression*: The term “gene expression” refers to the process by which a nucleic acid sequence undergoes successful transcription and in most instances translation to produce a protein or peptide. For clarity, when reference is made to measurement of “gene expression”, this should be understood to mean that measurements may be of the nucleic acid product of transcription, *e.g.*, RNA or mRNA or of the amino acid product of translation, *e.g.*, polypeptides or peptides. Methods of measuring the amount or levels of RNA, mRNA, polypeptides and peptides are well known in the art.

[000142] *Homology*: As used herein, the term “homology” refers to the overall relatedness between polymeric molecules, *e.g.* between polynucleotide molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be “homologous” to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical or similar to each other. The term “homologous” necessarily refers to a comparison between at least two sequences (polynucleotide or polypeptide sequences). In accordance with the invention, two polynucleotide sequences are considered to be homologous if the polypeptides they encode are at least about 50%, 60%, 70%, 80%, 90%, 95%, or even 99% for at least one stretch of at least about 20 amino acids. In some embodiments, homologous polynucleotide sequences are characterized by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. For polynucleotide sequences less than 60 nucleotides in length, homology is determined by the ability to encode

a stretch of at least 4–5 uniquely specified amino acids. In accordance with the invention, two protein sequences are considered to be homologous if the proteins are at least about 50%, 60%, 70%, 80%, or 90% identical for at least one stretch of at least about 20 amino acids.

[000143] *Heterologous Region*: As used herein the term “heterologous region” refers to a region which would not be considered a homologous region.

[000144] *Homologous Region*: As used herein the term “homologous region” refers to a region which is similar in position, structure, evolution origin, character, form or function.

[000145] *Identity*: As used herein, the term “identity” refers to the overall relatedness between polymeric molecules, *e.g.*, between polynucleotide molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two polynucleotide sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleotide sequences can be determined using methods such as those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; each of which is incorporated herein by reference in its entirety. For example, the percent identity between two nucleotide sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4:11-17), which has been incorporated into the ALIGN program (version 2.0) using a

PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleotide sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix. Methods commonly employed to determine percent identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., SIAM J Applied Math., 48:1073 (1988); which is incorporated herein by reference in its entirety. Techniques for determining identity are codified in publicly available computer programs. Exemplary computer software to determine homology between two sequences include, but are not limited to, GCG program package, Devereux, J., *et al.*, *Nucleic Acids Research*, 12(1), 387 (1984)), BLASTP, BLASTN, and FASTA Altschul, S. F. *et al.*, *J. Molec. Biol.*, 215, 403 (1990)).

[000146] *Inhibit expression of a gene:* As used herein, the phrase “inhibit expression of a gene” means to cause a reduction in the amount of an expression product of the gene. The expression product can be an RNA transcribed from the gene (*e.g.*, an mRNA) or a polypeptide translated from an mRNA transcribed from the gene. Typically, a reduction in the level of an mRNA results in a reduction in the level of a polypeptide translated therefrom. The level of expression may be determined using standard techniques for measuring mRNA or protein.

[000147] *In vitro:* As used herein, the term “*in vitro*” refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, in a Petri dish, *etc.*, rather than within an organism (*e.g.*, animal, plant, or microbe).

[000148] *In vivo:* As used herein, the term “*in vivo*” refers to events that occur within an organism (*e.g.*, animal, plant, or microbe or cell or tissue thereof).

[000149] *Isolated:* As used herein, the term “isolated” refers to a substance or entity that has been separated from at least some of the components with which it was associated (whether in nature or in an experimental setting). Isolated substances may have varying levels of purity in reference to the substances from which they have been associated. Isolated substances and/or entities may be separated from at least about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or more of the other components with which they were initially associated. In some embodiments, isolated agents are more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is “pure” if it is substantially free of other components.

[000150] *Substantially isolated:* By “substantially isolated” is meant that a substance is substantially separated from the environment in which it was formed or detected. Partial

separation can include, for example, a composition enriched in the substance of the present disclosure. Substantial separation can include compositions containing at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, or at least about 99% by weight of the compound of the present disclosure, or salt thereof. Methods for isolating compounds and their salts are routine in the art.

[000151] *Linker*: As used herein “linker” refers to a molecule or group of molecules which connects two molecules, such as a V_H chain and V_L chain of an antibody. A linker may be a nucleic acid sequence connecting two nucleic acid sequences encoding two different polypeptides. A linker may be amino acid based. The linker may or may not be translated. The linker may be a cleavable linker.

[000152] *Modified*: As used herein “modified” refers to a changed state or structure of a molecule of the invention. Molecules may be modified in many ways including chemically, structurally, and functionally.

[000153] *Naturally Occurring*: As used herein, “naturally occurring” or “wild-type” or “native” means existing in nature without artificial aid, or without involvement of the hand of man.

[000154] *Non-human vertebrate*: As used herein, a “non-human vertebrate” includes all vertebrates except *Homo sapiens*, including wild and domesticated species. Examples of non-human vertebrates include, but are not limited to, mammals, such as alpaca, banteng, bison, camel, cat, cattle, deer, dog, donkey, gayal, goat, guinea pig, horse, llama, mule, pig, rabbit, reindeer, sheep water buffalo, and yak.

[000155] *Open reading frame*: As used herein, “open reading frame” or “ORF” refers to a sequence which does not contain a stop codon in a given reading frame.

[000156] *Operably linked*: As used herein, the phrase “operably linked” refers to a functional connection between two or more molecules, constructs, transcripts, entities, moieties, or the like.

[000157] *Patient*: As used herein, “patient” refers to a subject who may seek or be in need of treatment, requires treatment, is receiving treatment, will receive treatment, or a subject who is under care by a trained professional for a particular disease or condition.

[000158] *Peptide*: As used herein, “peptide” is less than or equal to 50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

[000159] *Pharmaceutically acceptable*: The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms

which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[000160] *Pharmaceutically acceptable excipient or carrier:* The phrase “pharmaceutically acceptable excipient,” as used herein, refers any ingredient other than the compounds described herein (for example, a vehicle capable of suspending or dissolving the active compound) and having the properties of being substantially nontoxic and non-inflammatory in a patient. Excipients may include, for example: anti-adherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes (colors), emollients, emulsifiers, fillers (diluent), film formers or coatings, flavors, fragrances, glidants (flow enhancers), lubricants, preservatives, printing inks, sorbents, suspending or dispersing agents, sweeteners, and waters of hydration. Exemplary excipients include, but are not limited to: butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, crosslinked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E, vitamin C, and xylitol. For example, a pharmaceutically acceptable carrier may be a controlled release formulation that slowly releases the pharmaceutical composition into a patient or culture.

[000161] *Pharmaceutically acceptable salts:* The present disclosure also includes pharmaceutically acceptable salts of the compounds described herein. As used herein, “pharmaceutically acceptable salts” refers to derivatives of the disclosed compounds wherein the parent compound is modified by converting an existing acid or base moiety to its salt form (e.g., by reacting the free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, acetic acid, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzene sulfonic acid, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate,

lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. The pharmaceutically acceptable salts of the present disclosure include the conventional non-toxic salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. The pharmaceutically acceptable salts of the present disclosure can be synthesized from the parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in *Remington's Pharmaceutical Sciences*, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, *Pharmaceutical Salts: Properties, Selection, and Use*, P.H. Stahl and C.G. Wermuth (eds.), Wiley-VCH, 2008, and Berge *et al.*, *Journal of Pharmaceutical Science*, 66, 1-19 (1977), each of which is incorporated herein by reference in its entirety.

[000162] *Pharmacokinetic*: As used herein, "pharmacokinetic" refers to any one or more properties of a molecule or compound as it relates to the determination of the fate of substances administered to a living organism. Pharmacokinetics is divided into several areas including the extent and rate of absorption, distribution, metabolism and excretion. This is commonly referred to as ADME where: (A) Absorption is the process of a substance entering the blood circulation; (D) Distribution is the dispersion or dissemination of substances throughout the fluids and tissues of the body; (M) Metabolism (or Biotransformation) is the irreversible transformation of parent compounds into daughter metabolites; and (E) Excretion (or Elimination) refers to the elimination of the substances from the body. In rare cases, some drugs irreversibly accumulate in body tissue.

[000163] *Physicochemical*: As used herein, "physicochemical" means of or relating to a physical and/or chemical property.

[000164] *Preventing*: As used herein, the term “preventing” refers to partially or completely delaying onset of an infection, disease, disorder and/or condition; partially or completely delaying onset of one or more symptoms, features, or clinical manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying onset of one or more symptoms, features, or manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying progression from an infection, a particular disease, disorder and/or condition; and/or decreasing the risk of developing pathology associated with the infection, the disease, disorder, and/or condition.

[000165] *Prophylactic*: As used herein, “prophylactic” refers to a therapeutic or course of action used to prevent the spread of disease.

[000166] *Prophylaxis*: As used herein, a “prophylaxis” refers to a measure taken to maintain health and prevent the spread of disease.

[000167] *Protein of interest*: As used herein, the terms “proteins of interest” or “desired proteins” include those provided herein and fragments, mutants, variants, and alterations thereof.

[000168] *Purified*: As used herein, “purify,” “purified,” “purification” means to make substantially pure or clear from unwanted components, material defilement, admixture or imperfection. “Purified” refers to the state of being pure. “Purification” refers to the process of making pure.

[000169] *Recombinant cell*: The term “recombinant cell” as used herein refers to suitable cells (including progeny of such cells) from any species (prokaryotic or eukaryotic) into which a recombinant expression construct capable of expressing a nucleic acid encoding PCC peptide has been introduced. The construct is preferably a human PCC protein or genetically engineered variant thereof.

[000170] *Recombinant expression construct*: The term “recombinant expression construct” as used herein refers to a nucleic acid having a nucleotide sequence of a mammalian, preferably human, PCC protein, and sequences sufficient to direct the synthesis of PCC protein in cultures of cells into which the recombinant expression construct is introduced and the progeny thereof.

[000171] *Region*: As used herein, the term “region” refers to a zone or general area. In some embodiments, when referring to a protein or protein module, a region may comprise a linear sequence of amino acids along the protein or protein module or may comprise a three-dimensional area, an epitope, and/or a cluster of epitopes. In some embodiments, regions comprise terminal regions. As used herein, the term “terminal region” refers to regions

located at the ends or termini of a given agent. When referring to proteins, terminal regions may comprise N- and/or C-termini. N-termini refer to the end of a protein comprising an amino acid with a free amino group. C-termini refer to the end of a protein comprising an amino acid with a free carboxyl group. N- and/or C-terminal regions may therefore comprise the N- and/or C-termini as well as surrounding amino acids. In some embodiments, N- and/or C-terminal regions comprise from about 3 amino acid to about 30 amino acids, from about 5 amino acids to about 40 amino acids, from about 10 amino acids to about 50 amino acids, from about 20 amino acids to about 100 amino acids and/or at least 100 amino acids. In some embodiments, N-terminal regions may comprise any length of amino acids that includes the N-terminus, but does not include the C-terminus. In some embodiments, C-terminal regions may comprise any length of amino acids, which include the C-terminus, but do not comprise the N-terminus.

[000172] In some embodiments, when referring to a polynucleotide, a region may comprise a linear sequence of nucleic acids along the polynucleotide or may comprise a three dimensional area, secondary structure, or tertiary structure. In some embodiments, regions comprise terminal regions. As used herein, the term “terminal region” refers to regions located at the ends or termini of a given agent. When referring to polynucleotides, terminal regions may comprise 5' and 3' termini. 5' termini refer to the end of a polynucleotide comprising a nucleic acid with a free phosphate group. 3' termini refer to the end of a polynucleotide comprising a nucleic acid with a free hydroxyl group. 5' and 3' regions may there for comprise the 5' and 3' termini as well as surrounding nucleic acids. In some embodiments, 5' and 3' terminal regions comprise from about 9 nucleic acids to about 90 nucleic acids, from about 15 nucleic acids to about 120 nucleic acids, from about 30 nucleic acids to about 150 nucleic acids, from about 60 nucleic acids to about 300 nucleic acids and/or at least 300 nucleic acids. In some embodiments, 5' regions may comprise any length of nucleic acids that includes the 5' terminus, but does not include the 3' terminus. In some embodiments, 3' regions may comprise any length of nucleic acids, which include the 3' terminus, but does not comprise the 5' terminus.

[000173] *RNA or RNA molecule:* As used herein, the term “RNA” or “RNA molecule” or “ribonucleic acid molecule” refers to a polymer of ribonucleotides; the term “DNA” or “DNA molecule” or “deoxyribonucleic acid molecule” refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally, *e.g.*, by DNA replication and transcription of DNA, respectively; or be chemically synthesized. DNA and RNA can be single-stranded (*i.e.*, ssRNA or ssDNA, respectively) or multi-stranded (*e.g.*, double stranded,

i.e., dsRNA and dsDNA, respectively). The term “mRNA” or “messenger RNA”, as used herein, refers to a single stranded RNA that encodes the amino acid sequence of one or more polypeptide chains.

[000174] *Sample*: As used herein, the term “sample” or “biological sample” refers to a subset of its tissues, cells or component parts (*e.g.* body fluids, including but not limited to blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid, and semen). A sample further may include a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs. A sample further refers to a medium, such as a nutrient broth or gel, which may contain cellular components, such as proteins or nucleic acid molecule.

[000175] *Signal Sequences*: As used herein, the phrase “signal sequences” refers to a sequence which can direct the transport or localization of a protein.

[000176] *Similarity*: As used herein, the term “similarity” refers to the overall relatedness between polymeric molecules, *e.g.* between polynucleotide molecules (*e.g.* DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of percent similarity of polymeric molecules to one another can be performed in the same manner as a calculation of percent identity, except that calculation of percent similarity takes into account conservative substitutions as is understood in the art.

[000177] *Stable*: As used herein “stable” refers to a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and preferably capable of formulation into an efficacious therapeutic agent.

[000178] *Stabilized*: As used herein, the term “stabilize”, “stabilized,” “stabilized region” means to make or become stable.

[000179] *Subject*: As used herein, the term “subject” or “patient” refers to any organism to which a composition in accordance with the invention may be administered, *e.g.*, for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates, and humans) and/or plants.

[000180] *Substantially*: As used herein, the term “substantially” refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and

chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term “substantially” is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[000181] *Substantially equal*: As used herein as it relates to time differences between doses, the term means plus/minus 2%.

[000182] *Substantially simultaneously*: As used herein and as it relates to plurality of doses, the term means within 2 seconds.

[000183] *Suffering from*: An individual who is “suffering from” a disease, disorder, and/or condition has been diagnosed with or displays one or more symptoms of a disease, disorder, and/or condition.

[000184] *Susceptible to*: An individual who is “susceptible to” a disease, disorder, and/or condition has not been diagnosed with and/or may not exhibit symptoms of the disease, disorder, and/or condition but harbors a propensity to develop a disease or its symptoms. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition (for example, cancer) may be characterized by one or more of the following: (1) a genetic mutation associated with development of the disease, disorder, and/or condition; (2) a genetic polymorphism associated with development of the disease, disorder, and/or condition; (3) increased and/or decreased expression and/or activity of a protein and/or nucleic acid associated with the disease, disorder, and/or condition; (4) habits and/or lifestyles associated with development of the disease, disorder, and/or condition; (5) a family history of the disease, disorder, and/or condition; and (6) exposure to and/or infection with a microbe associated with development of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[000185] *Synthetic*: The term “synthetic” means produced, prepared, and/or manufactured by the hand of man. Synthesis of polynucleotides or polypeptides or other molecules of the present invention may be chemical or enzymatic.

[000186] *Targeted Cells*: As used herein, “targeted cells” refers to any one or more cells of interest. The cells may be found *in vitro*, *in vivo*, *in situ* or in the tissue or organ of an organism. The organism may be an animal, preferably a mammal, more preferably a human and most preferably a patient.

[000187] *Therapeutic Agent*: The term “therapeutic agent” refers to any agent that, when administered to a subject, has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[000188] *Therapeutically effective amount*: As used herein, the term “therapeutically effective amount” means an amount of an agent to be delivered (*e.g.*, nucleic acid, drug, therapeutic agent, diagnostic agent, prophylactic agent, *etc.*) that is sufficient, when administered to a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition. In some embodiments, a therapeutically effective amount is provided in a single dose. In some embodiments, a therapeutically effective amount is administered in a dosage regimen comprising a plurality of doses. Those skilled in the art will appreciate that in some embodiments, a unit dosage form may be considered to comprise a therapeutically effective amount of a particular agent or entity if it comprises an amount that is effective when administered as part of such a dosage regimen.

[000189] *Therapeutically effective outcome*: As used herein, the term “therapeutically effective outcome” means an outcome that is sufficient in a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

[000190] *Total daily dose*: As used herein, a “total daily dose” is an amount given or prescribed in a 24 hr period. It may be administered as a single unit dose.

[000191] *Transfection*: As used herein, the term “transfection” refers to methods to introduce exogenous nucleic acids into a cell. Methods of transfection include, but are not limited to, chemical methods, physical treatments, and cationic lipids or mixtures.

[000192] *Treating*: As used herein, the term “treating” refers to partially or completely alleviating, ameliorating, improving, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of a particular infection, disease, disorder, and/or condition. For example, “treating” cancer may refer to inhibiting survival, growth, and/or spread of a tumor. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition and/or to a subject who exhibits only early signs of a disease, disorder, and/or condition for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

[000193] *Unmodified*: As used herein, “unmodified” refers to any substance, compound or molecule prior to being changed in any way. Unmodified may, but does not always, refer to

the wild type or native form of a biomolecule. Molecules may undergo a series of modifications whereby each modified molecule may serve as the “unmodified” starting molecule for a subsequent modification.

[000194] *Vector*: As used herein, a “vector” is any molecule or moiety which transports, transduces or otherwise acts as a carrier of a heterologous molecule. Vectors of the present invention may be produced recombinantly. In non-limiting examples, such sequences may comprise any one or more of the following sequences: a polynucleotide sequence encoding a polypeptide or multi-polypeptide, whose sequence may be wild-type or modified from wild-type and which sequence may encode full-length or partial sequence of a protein, protein domain, or one or more subunits of a protein; a polynucleotide comprising a modulatory or regulatory nucleic acid which sequence may be wild-type or modified from wild-type; and a transgene that may or may not be modified from wild-type sequence. These sequences may serve as either the “donor” sequence of one or more codons (at the nucleic acid level) or amino acids (at the polypeptide level) or “acceptor” sequences of one or more codons (at the nucleic acid level) or amino acids (at the polypeptide level).

[000195] The details of one or more embodiments of the invention are set forth in the accompanying description. Other features, objects and advantages of the invention will be apparent from the description. In the description, the singular forms also include the plural unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In the case of conflict, the present description will control.

[000196] The present invention is further illustrated by the following non-limiting examples.

VI. EXAMPLES

Example 1. Experimental procedures

A. Cell culture

[000197] Skin fibroblast cultured cells used were from two patients bearing mutations in either the PCCA or the PCCB subunit as well as from a wild type healthy control. The cells were grown in a humidified atmosphere with 5% CO₂ at 37°C and maintained in Minimum Essential Medium (HyClone, Logan, UT) supplemented with 15% of Fetal clone III serum (HyClone, Logan, UT) 100ug/ml penicillin and 100ug/ml streptomycin, 100μM MEM non-essential amino acids (HyClone, Logan, UT).

B. Isolation of mitochondria

[000198] Mitochondria were obtained from the liver of A138T mice (PCC deficient) using a differential centrifugation protocol. The liver from these mice have 2% of wild type PCC activity. Freshly dissected livers were minced finely before using a motor driven TEFLON™ and glass Potter Elvehjem homogenizer, 6-9 strokes at 1000RPM. The homogenization buffer contained 220mM D-mannitol, 70mM sucrose, 2mM Hepes pH 7.4 and 0.5mg/mL BSA (HMS+). The first centrifugation of a 15% homogenate in HMS+ buffer was performed for 1 minute at 3000g Beckman Avanti J-25) at 4°C to remove nuclei and cell debris. The supernatant was centrifuged 2 minutes for 18,750g at 4°C in order to obtain the mitochondria pellet. The resulting pellet was resuspended in HMS+ buffer and 0.035% digitonin. Digitonin improves mitochondrial recovery by selectively disrupting lipid membranes enriched in sterols to improve purity of the mitochondrial preparations and increase the yield. After 5 minutes of centrifugation at 18,900g the mitochondria were washed 3 times in HMS buffer without BSA before the import. Alternatively, after 5 minutes of centrifugation at 12,500 RPM, the mitochondria were washed twice in HMS buffer without BSA to prepare for import.

C. Mitochondria oxygen consumption

[000199] The mitochondrial pellet was resuspended in 2.5mL of respiration medium MiR05 0.5 mM EGTA, 3 mM MgCl₂*6H₂O, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM Sucrose, and 1 g/l fatty acid free BSA.

[000200] An Oroboros Oxygraph 2K was used to measure Oxygen consumption was measured in a medium containing the actively respiring mitochondria. Trypsin was applied to one of the mitochondria samples in a ratio of 1:100 trypsin/enzyme after incubation for 5 minutes of import and another sample after 25 minutes of import at 37°C. The mitochondrial pellet was resuspended in 2.5 mL of respiration medium: MiR05 0.5 mM EGTA, 3 mM MgCl₂*6H₂O, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM Sucrose, and 1 g/L fatty acid free BSA. Measurement of oxygen consumption in isolated mitochondria was in a closed chamber for approximately 1 hour. When oxygen was completely consumed, the chamber was opened and the process was repeated 3 times.

D. PCC conjugation reaction

[000201] For import into mitochondria and fibroblasts, conjugated TAT-PCC, MPP1A-PCC, MPP2A-PCC was used. To form these conjugates, maleoyl-beta-Ala-TAT (Kerastase) was dissolved in a neutral buffer like PBS. The reaction of peptide maleoyl—beta-Ala-TAT with 1μM PCC was performed overnight in 20 mM Hepes, pH 7.0, 500mM KCl at a ratio of

2:1 (TAT:PCC). The excess peptide was removed on a Biorad Bio-Spin 6 column, for import into mitochondria equilibrated in HMS buffer (220mM D-mannitol, 70mM sucrose, 2mM Hepes pH 7.4) or PBS for import into fibroblasts.

[000202] The preparation of MPP1A-PCCAB failed prior to importing into isolated mitochondria because after desalting the spin column there was not enough material for the experiment.

E. Mitochondrial lysates

[000203] The mitochondrial pellet was resuspended in fresh lysis buffer (50mM TrisHCl pH 8.0, 1mM DTT, 1mM EDTA pH 8.0, inhibitors (Sigma P8340). Use 3x the volume of the buffer as compared to the volume of mitochondrial pellet. The mitochondria were homogenized in the lysis buffer by pipetting. Sonication was performed using the following settings: twice for 10 seconds at power 3, pulse 1 second on and 0.5 sec off using a microtip probe. The supernatant was collected in a fresh tube after pelleting in a centrifuge (20,000g, 4°C) for 15 min. Protein concentration was determined by Bradford assay.

F. Import reaction into mitochondria

[000204] The import of TAT-PCC at the desired concentration was performed at 27°C for 30 minutes. For import of MPP2A-PCC conjugate, the reaction was performed at 27°C for 1 hour. The excess peptide was removed on a Biorad Bio-Spin 6 column equilibrated with HMS buffer. The mitochondria were centrifuged for 5 minutes for 12,500 RPM in 4°C.

[000205] Trypsin was applied at a ratio of 1:100 (w/w) for 25 minutes to digest any adsorbed PCC on the outside of the outer mitochondrial membrane to ensure that the mitochondrial lysate represented only PCC that had been imported to the inside of the organelle. The reaction was stopped with soybean trypsin inhibitor at a ratio of 1:1 (w/w). The mitochondria were washed in HMS buffer three times, each time centrifuged for 10 minutes for 18,900g in 4°C.

[000206] The time course of proteolysis by the trypsin was followed by measuring PCC activity.

G. PCC enzyme assay

[000207] The reaction mixture contained 50mM Tris-HCl, pH 8.0, 2mM ATP, 125mM KCl, 10mM MgCl₂, 3mM propionyl-CoA, 0.5mg/ml bovine serum albumin, PCC enzyme (0.1µg of purified PCC, 150µg for mitochondria lysates and fibroblast lysates), and 10 mM [14C]bicarbonate in a final volume of 50µl, and was incubated at 37 °C for 15 minutes. The reaction was terminated by adding 50µl of 10% trichloroacetic acid. The mixture was centrifuged at 13,000 g for 5 minutes and 50µl of supernatant was dried in a scintillation vial

in a heating block at 80°C for 50 minutes. The dry residue was dissolved in 0.15ml of H₂O, and 4ml of OPTI-Fluor scintillation fluid (PerkinElmer Life Sciences) was added. The samples were counted in a Beckman LS 3801 scintillation counter. A blank containing the assay mixture without propionyl-CoA was subtracted.

[000208] Protein content was determined by a Bradford assay using bovine serum albumin as a standard. One unit of PCC activity is defined as 1pmol of bicarbonate per minute per mg of protein at 37°C.

H. Western Blotting

[000209] Proteins were resolved on 10% sodium dodecyl sulfate polyacrylamide electrophoresis gels and transferred onto Immun-Blot PVDF membrane (Bio-Rad). For all mitochondrial lysates samples were loaded with 50µg of protein/lane or 100ng/lane of purified PCC. Western blot analysis was performed using anti-PCCA (Abcam, Cambridge, UK) and anti-PCCB (Abcam, Cambridge, UK) antibodies at 1:1000 dilution to identify the proteins.

I. Antibodies

[000210] For chemiluminescence, Supersignal West Pico (Thermo Science) developed membrane polyclonal mouse Anti-PCCA 1000x Abnova #H00005095-B01P to recognize the PCCA 72kDa subunit after 2 minutes of exposure in a quantity higher than 100ng. For fluorescent imaging system, Typhoon™ (GE Life Sciences), the Alexa 647, Alexa Fluor 647 or Cy5 (dye with similar wavelength) are the preferred options as secondary antibodies. Quantities of purified PCC enzyme higher than 100ng were confirmed to be visible at the position of an expected size corresponding to 72kDa. For chemiluminescence, Supersignal West Pico (Thermo Science) also developed membrane Polyclonal mouse Abcam ab89784 Anti-PCCA to recognize the PCCA 72kDa subunit after a 90 second exposure in a quantity higher than 100ng.

[000211] Polyclonal rabbit Abcam ab154254 Anti-PCCA recognized quantities higher than 100ng in the correct size for PCCA 72kDa, and had a slightly stronger signal than Polyclonal mouse Abcam ab89784 Anti-PCCA but showed a second band around 50kDa. The fluorescent imaging system Typhoon™ with Alexa 647 as a secondary antibody detected, similarly as on chemiluminescent developed Western blot, a second band around 50kDa.

[000212] For chemiluminescence, Supersignal West Pico (Thermo Science) developed membrane polyclonal mouse Anti-PCCB Abcam ab70416, polyclonal mouse anti-1000x to recognize the PCCB 58kDa subunit after 2 minutes of exposure in a quantity higher than 100ng. Quantities of purified PCC enzyme higher than 175ng were visible in scans at a size

corresponding to 58kDa. Typhoon™ scan with Alexa647 secondary antibody provided a low signal for 50ng.

[000213] Polyclonal mouse PCCA Abnova H00005095-B01P and polyclonal mouse PCCB Abcam ab70416 were used for immunostaining. Alternatively, polyclonal mouse PCCA Abnova H00005095-B01P, polyclonal mouse PCCB Abcam ab70416, polyclonal rabbit PCCA Abcam ab154254, polyclonal mouse PCCA Abcam ab89784, or polyclonal rabbit PCCAB Krauslab (positively recognizes PCCA or PCCB in both the chemiluminescent and the fluorescent detection systems) may be used.

J. Import into patient fibroblasts

[000214] Fibroblast cells were grown in 150cm² flasks. When the cells reached 90% confluency the medium was removed with PBS and replaced by 1μM TAT-PCC, MPP1A-PCC, or MPP2A-PCC diluted in PBS at a concentration of 0.13mg/mL. After 1 hour incubation at 37°C, the cells were washed with PBS, trypsinized, pelleted and kept at -80°C until use.

K. Fibroblast cell lysates

[000215] Cells are harvested using 0.25% trypsin=2.5mg/mL (Ratio 1:1 protease/protein). The pellet of cells is washed 3x in PBS, each time resuspended in 20mL PBS and spin 800g for 10 min, finally transferred to a small Eppendorf tube.

[000216] The cell pellet was resuspended in the lysis buffer (50mM Tris HCl pH 8.0, 1mM DTT, 1mM EDTA pH 8.0, protease inhibitors (Sigma P8340). Three times the volume of the buffer as compared to the volume of cell pellet was used. Cells were homogenized in the lysis buffer by pipetting. The cells were sonicated twice for 10 seconds at power 3 and pulsed for 1 second on/0.5 sec off using a microtip probe. The supernatant was collected after a 15 minute centrifugation at 20,000 g and 4°C. Protein concentration was determined by the Bradford assay using 20x or 40x dilutions.

L. Confocal microscopy

[000217] Fibroblast cells were grown in a complete MEM media on 8-chamber tissue culture slides (Falcon) to 70% confluency and incubated with 1μM TAT-PCC for 1.5 hours. The cells were then washed with PBS before staining. First, Mitotracker Red CMXRos was used to stain mitochondria live cells. Next, cells were fixed with 4% formaldehyde for 10 minutes and permeabilized by methanol. The cells were blocked with 2% BSA + 5% goat serum in PBS for 30 minutes at room temperature, then were washed 3-5 times with PBS. For specific staining, Ab89784 anti-PCCA mouse or anti-PCCB (Abcam, Cambridge, UK) were used as the primary antibodies, and anti-mouse IgG ATTO 488 (Sigma) was used as a

secondary fluorescent antibody. DAPI staining was used to visualize the nuclei. The cells were washed a final time with PBS. A mounting medium was added to the cells, and a coverslip was sealed with nail polish over each chamber on the slide. The cells were washed a final time with PBS. A mounting medium was added to the cells, and a coverslip was sealed with nail polish over each chamber on the slide.

Example 2. Design of constructs, vectors and conjugates

[000218] Examples of the wild type or native starting nucleic acid sequences encoding human PCC and the amino acid sequences encoded thereby are shown in Table 1 above.

[000219] Various constructs were engineered, with or without leader sequences, targeting sequences, or tag sequences. A brief list and overview description of those constructs are given in Table 2. Various construct designs are also given in Figure 1.

Table 2. Examples of constructs for expression of PCC enzyme

Constructs Expressed
PCCA
PCCA with or without a mitochondrial targeting leader sequence
PCCA with or without a mitochondrial targeting leader sequence with cell penetrating peptide
PCCA with or without a mitochondrial targeting leader sequence with post-translationally linked cell penetrating peptide
PCCB
PCCB with or without a mitochondrial targeting leader sequence
PCCB with or without a mitochondrial targeting leader sequence with cell penetrating peptide
PCCB with or without a mitochondrial targeting leader sequence with post-translationally linked cell penetrating peptide
PCCA:PCCB
PCCA:PCCB without a mitochondrial targeting leader sequence with post-translationally linked cell penetrating peptide

A. Cell penetrating proteins

[000220] Cell penetrating proteins or peptides linked or conjugated to the constructs and expressed in the vectors described include those detailed in Table 3.

Table 3. Cell penetrating proteins

CPP Name	Sequence	SEQ ID NO
TAT peptide variant	YGRKKRRQRRR	5
TAT peptide variant	GRKKRRQRRRPQ	6
Antennapedia	RQIKIYFQNRMRMKWKK	7
herpes simplex virus type 1 protein VP22	DAATATRGRSAASRPTERPRAPARSASRPRRPVD	8
penetratin	RQIKIWFQNRMRMKWKK	9
transportan	GWTLSAGYLLGKINLKALAALAKKIL	10
amphipathic protein MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	11
Pep-1	KETWWETWWTEWSQPKKKRKV	12
MAP	KALAKALAKALA	13
SAP	VRLPPPVRLLPPVRLPPP	14
PPTG1	GLFRALLRLLRSLWRLLLRA	15
poly-Arginine sequence	RRRRRRRR	16
hCT	LGTYTQDFNKTFPQTAIGVGAP	17

SynB	RGGRLSYSRRRFSTSTGR	18
Pvec	LLIILRRRIKQAHHSK	19
TAT peptide variant	CFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQ	20
TAT peptide variant	Maleoyl-beta-AGYGRKKRRQRRR	21
TAT peptide variant	GYGRKKRRQRRR	22
Mitochondrial penetrating peptide (MPP1A)	Cha-DArg-Cha-Lys-Cha-DArg-Cha-Lys (Cha-R-Cha-K-Cha-R-Cha-K) [Cha is cyclohexylalanine and DArg is the D amino acid]	23
Mitochondrial penetrating peptide (MPP2A)	Cha-DArg-Cha-Lys (Cha-R-Cha-K) [where Cha is cyclohexylalanine and DArg is the D amino acid]	24

B. Vectors

[000221] Vectors necessary to produce the PCC variants and their conjugates were also designed. Those vectors are given in Table 4 along with descriptions of their length, encoded subunit identity, the nature of any conjugated signal sequence, the promoter as well as the regions for PCR expansion. The PCC subunit may include a cell penetrating peptide (CPP) region.

Table 4. Vectors and plasmid components

Vector	SEQ ID NO	Length	Subunit (region)	CPP (region)	Promoter (region)	Primer (region)
pET28-C-TATprePCCA	25	7453	C-TAT-prePCCA (191-2437)	TAT (197-223)	T7 promoter (104-120); T7 terminator (2502-2548)	673 (104-123); 674 (2487-2505)
pET28-C-TATprePCCB	26	6886	C-TAT-prePCCB (191-1870)	TAT (197-223)	T7 promoter (104-120); T7 terminator (1935-1981)	673 (104-123); 674 (1920-1938)
pET47-NP-TAT-prePCCA	27	7264	NP-TAT-prePCCA (159-2432)	TAT (216-242)	T7 promoter (71-87); T7 terminator (2512-2559)	
pET47-NP-TAT-prePCCB	28	6697	PCCB (159-1865)	TAT (216-242)	T7 promoter (71-87); T7 terminator (1945-1992)	
pETDS1-PCCAB	29	9353	PCCA (71-2107); PCCB (2259-3797)		T7 promoter (2173-2189); T7 terminator (3906-3953)	71180-3 (2148-2167); 71179-3 (complement 2148-2167); 69337-3 (complement 3892-3910); 69214-3 (9291-9306)
pUC57KAN-TATprePCCA	30	4796	TAT-prePCCA (432-2648)	TAT	Lac promoter (complement 2755-2784)	669 (379-395); 672 (complement 2706-2727)
pUC57KAN-TATprePCCB	31	4243	PCCB (438-2087)	TAT	Lac promoter (complement 2202-2231)	669 (379-395); 672 (complement 2153-2174)

[000222] In certain embodiments of pET28-C-TATprePCCA (SEQ ID NO:25), a leader sequence spans the region 230-376 and a His tag spans the region 2171-2134. In certain embodiments of pET28-C-TATprePCCB (SEQ ID NO:26), a leader sequence spans the region 230-307 and a His tag spans the region 1850-1867. In certain embodiments pET47-NP-TAT-prePCCA (SEQ ID NO:27), the leader sequence spans the region 249-395, a His tag

spans the region 165-182, and a 3C cleavage sequence spans the region 192-214. In certain embodiments pET47-NP-TAT-prePCCB (SEQ ID NO:28), the leader sequence spans the region 249-326, a His tag spans the region 165-182, and a 3C cleavage sequence spans the region 192-214. In some embodiments, region 210-212 of SEQ ID NO:27 or SEQ ID NO:28 was mutated from GGA (Gly) to GGG (Gly) to change the *SanDI* restriction site to a *Apal* restriction site. In some embodiments of the complement sequence of SEQ ID NO:27 (6384-6386) and SEQ ID NO:28 (5817-5819), GGC (Gly) codon mutated to GGT (Gly) codon to remove the *Apal* restriction site.

[000223] In certain embodiments of pETDS1-PCCAB (SEQ ID NO: 29), a ribosome binding site spans the region 58-63, and an S-tag spans 3810-3854.

C. *PCC variants and conjugates*

[000224] Several PCC enzyme and enzyme subunit conjugates were produced for the studies disclosed herein. A list of those constructs is given in Table 5. The vector name is also given in the table.

Table 5. PCC variants and subunit conjugates

Description	SEQ ID NO	Length of Sequence	Vector Name
C-TAT-prePCCA DNA	32	2247	pET28-C-TATprePCCA
C-TAT-prePCCA Protein	33	748	pET28-C-TATprePCCA
C-TAT-prePCCB DNA	34	1680	pET28-C-TATprePCCB
C-TAT-prePCCB Protein	35	559	pET28-C-TATprePCCB
NP-TAT-prePCCA DNA	36	2274	pET47-NP-TAT-prePCCA
NP-TAT-prePCCA Protein	37	757	pET47-NP-TAT-prePCCA
NP-TAT-prePCCB DNA	38	1707	pET47-NP-TAT-prePCCB
NP-TAT-PCCB Protein	39	568	pET47-NP-TAT-prePCCB
PCCA DNA	40	2037	pETDS1-PCCAB
PCCA Protein	41	678	pETDS1-PCCAB
PCCB DNA	42	1539	pETDS1-PCCAB
PCCB Protein	43	512	pETDS1-PCCAB
TAT-prePCCA DNA	44	2217	pUC57KAN-TATprePCCA
TAT-prePCCA Protein	45	738	pUC57KAN-TATprePCCA
TAT-prePCCB DNA	46	1650	pUC57KAN-TATprePCCB
TAT-prePCCB Protein	47	549	pUC57KAN-TATprePCCB
TAT-prePCCA Protein	48	727	pET28-C-TATprePCCA
TATprePCCB Protein	49	538	pET28-C-TATprePCCB
NP-TATprePCCA Protein	48	727	pET47-NP-TAT-prePCCA
NP-TATprePCCB Protein	49	538	pET47-NP-TAT-prePCCB
PCCA Protein	50	677	pETDS1-PCCAB
PCCB Protein	51	511	pETDS1-PCCAB
TAT-prePCCA Protein	52	727	pUC57KAN-TATprePCCA
TAT-prePCCB Protein	51	511	pUC57KAN-TATprePCCB

[000225] In certain embodiments of C-TATprePCCA, a mitochondrial leader sequence spans the region of 743-748 and the mature PCCA chain spans the region of 64-740 of SEQ ID NO:33. In certain embodiments of C-TATprePCCB, a mitochondrial leader sequence

spans the region of 40-551, a 6x His tag spans the region of 554-559, and the mature PCCB chain spans the region of 40-551 fo SEQ ID NO:35. In certain embodiments of NP-TATprePCCA, a mitochondrial leader sequence spans the region of 31-79, a 6x His tag spans the region of 3-8, a HRV3C protease binding site spans the region of 12-19, and the mature PCCA chain spans the region of 80-757 of SEQ ID NO:37. In certain embodiments of NP-TATprePCCB, a 6x His tag spans the region of 3-8, a HRV3C protease binding site spans the region of 12-19, a TAT peptide spans the region of 20-28, a mitochondrial leader sequence spans the region of 31-56, and the mature PCCB chains spans the region of 57-268 of SEQ ID NO:39.

Example 3. Production and Purification of constructs: primers

A. Primer sets

[000226] The vectors described in Example 1 were used in the studies described herein. Primer sets were designed to these vectors and are given in Table 6.

Table 6. Primers

Primer Name	SEQ ID NO (5' to 3')	Length	Description
1083	53	22	Forward; SphI site, Tm=60.9°C
1084	54	21	Reverse; SphI site, Tm=60.8°C
1069	55	33	Forward; NcoI site, Tm=66.5°C
1070	56	30	Reverse; NotI site, Tm=66.5°C
1071	57	28	Forward; NcoI site, Tm=63.0°C
1072	58	28	Reverse; XhoI site, Tm=63.4°C
1073	59	29	Forward; ApaI site, Tm=64.1°C
1074	60	32	Reverse; XhoI site, Tm=64.6°C
1075	61	34	34-mers, 62 AA deletion, Tm = 80.4°C
1076	62	34	34-mers, 62 AA deletion, Tm = 80.4°C
1085	63	33	Forward; NcoI site, Tm=66.6°C
1070	64	28	Reverse; NotI site, Tm=66.5°C
1077	65	35	Forward; NdeI site, Tm=63.9°C
1078	66	35	Reverse; XhoI site, Tm=63.8°C
1079	67	28	Forward; NdeI site, Tm=64.1°C
1078	68	35	Reverse; XhoI site, Tm=63.8°C
1086	69	26	Reverse; XhoI site, Tm=62.9°C
1087	70	27	Forward; ApaI site, Tm=64.0°C
1088	71	35	Reverse; XhoI site, Tm=63.8°C
71180-3	72	20	DuetUP2 Primer
71179-3	73	20	DuetDOWN1 Primer
69337-3	74	19	T7 Terminator Primer
69214-3	75	16	pET Upstream Primer
673	76	20	T7 promoter primer for sequencing
674	77	19	T7 terminator primer for sequencing
669	78	17	Forward; M13FWD20
672	79	22	Reverse; M13REV20

Example 4. Production of constructs: vectors and host cells (bioprocessing)

[000227] A new construct for expression of PCCAB (*i.e.* native PCC dodecamer consisting of 6 PCCA and 6 PCCB subunits) was prepared to deliver a higher yield of enzyme than the

wild-type to support development of an enzyme replacement therapy (ERT). The construct used in the examples contains a codon-optimized sequence of human PCC for heterologous expression in an *E. coli* host; a stronger promoter for increased productivity and allowing tighter regulation; and stabilization of plasmid for better retention and antibiotic-free expression.

[000228] Plasmid constructs were developed for expression of propionyl-CoA carboxylase enzyme in *E. coli*. In certain embodiments, constructs were codon optimized for expression in *E. coli*. Parental nucleic acid constructs which were codon optimized include those of SEQ ID NO:1 or 3 or portions thereof. These were codon optimized with or without the encoded mitochondrial targeting leader amino acid sequence, and individual PCC subunits with and without a cell-penetrating peptide, such as trans-activating transcriptional activator (TAT) peptide, *e.g.*, YGRKKRRQRRR (SEQ ID NO:5).

A. Promoters

[000229] Since both subunits are needed in equimolar amount, use of the same promoter was preferable. Novagen's DUET™ system was used for co-expression of multiple proteins (or subunits of a multimeric protein). The systems were based on T7 promoter and IPTG induction, and the systems differed in plasmid backbone for copy number, antibiotic resistance, and/or compatibility with co-transformed plasmids. pET-DUET, (Novagen) was used in the examples, which was most similar to Novagen's other pET vectors.

B. Codon optimization

[000230] GENSCRIPT® produced the codon-optimized sequence used herein. The sequence was optimized for each subunit *i.e.* including a penetrating peptide (TAT) sequence, a mitochondrial leader sequence, and the coding sequence itself.

C. Plasmid stabilization and design of vector: pETDS1

[000231] There are several alternative systems available for plasmid stabilization and ATB-free expression which are based on the principle of Delphi Genetic's STABY® system. The basis of the system is that the antidote gene (*ccdA*) is carried on the plasmid DNA under the control of a constitutive promoter used with T7-based expression. The principle provides that the toxic gene (*ccdB*) is permanently carried on the chromosome of the bacteria.

[000232] Expression of the toxic gene is under the control of a promoter strongly repressed in the presence of the plasmid. When the plasmid is lost, the antidote is degraded and production of the toxin is induced, causing cell death. The result is that 100% of cells contain the plasmid, from which expression of the product of interest is initiated without the need of ATB.

[000233] An antidote gene (ccdA) with its own regulatory elements from Delphi Genetics pStaby 1.2 vector was PCR amplified and inserted into a SphI site of Novagen's pET-DUET-1, producing pETDS1 or pETDS2 based on direction of insertion. pETDS1 was used for further cloning.

D. Bacterial production

[000234] Several bacterial expression hosts were considered for testing. All the prepared plasmids could be used with a DE3-containing host, such as *E. coli* BL21 (DE3, with appropriate ATB for plasmid selection. Clone of BL21, *E. coli* C43, was successfully used for expression of toxic or complex proteins. For stabilized plasmids (i.e. those carrying STABY® cassette), ATB was not needed, if *E. coli* was used. This strain is a BL21 (DE3) clone containing a toxic cassette to function in conjunction with an antidote carried on and expressed from the plasmid.

E. Production scheme: single subunit replacement

[000235] Since PCC-deficient patients generally have only one subunit affected (either PCCA or PCCB), expression of an individual subunit for ERT development was explored. Expression of an individual subunit including TAT and mitochondrial leader was expected to result in an insoluble protein. To purify the protein from an insoluble fraction (inclusion bodies), 6x His tag on either a C-terminus or an N-terminus was added. The 6x His tag was used with IMAC chemistry for purification under native or denaturing conditions. Constructs based on pET28 (for a permanent C-terminal tag) or pET47 (for a removable N-terminal tag) vectors with a coding region and adjacent regulatory elements were engineered.

[000236] These constructs for individual subunits yielded protein for transport, i.e. already expressed as a single polypeptide with a penetrating peptide (TAT) and mitochondria-targeting leader. This simplified and reduced cost of the whole preparation.

[000237] For PCCAB, constructs were prepared for both: ready-to-use (similar to individual subunits constructs) and post-purification modification with TAT. These constructs did not contain an additional purification tag.

F. Chaperones

[000238] Co-expression of molecular chaperones was observed to improve yield of PCCAB. Several molecular chaperones expressed from a separate, co-transformed, and compatible plasmid were tested. These plasmids were from the Takara Chaperone plasmid set, and the pG-KJE8 and pGro7 yielded the best results. The experiments conducted herein utilize pGro7.

[000239] Co-expression of molecular chaperones with PCCAB in forms of TAT-ed precursors or as mature polypeptides in a preferred expression host (SE1) showed that total amount of expressed of PCCAB is relatively high and most of stays in soluble fraction (in case of native matured constructs). Co-expression of GroEL/ES, but not DnaK-DnaJ-GrpE molecular chaperones was found to be crucial for 3-4-fold higher PCC specific activity and probably the yield as well (1,200,000 versus 350,000 pmol/min/mg). Therefore, expression of PCC from pETDS1-PCCAB construct along with pGro7 in SE1 is way to go for purification of native PCCAB.

Example 5. Purification of PCC and variants thereof

[000240] For purifications, doubly transformed cells were selected on LB media containing 50 mg/ml ampicillin and 50 mg/ml chloramphenicol. Bacterial cultures grown to confluence overnight were diluted 1/100 and used to inoculate 0.5 L aliquots of LB media which were grown with shaker aeration at 37°C in the presence of ampicillin (300 mg/ml), chloramphenicol (30 mg/ml), and biotin (5 mM) to a turbidity of about 0.4 at 600 nm prior to induction with 1 mM IPTG (BRL). The induced cells were allowed to grow for 2–24 hours before collection. Cells were harvested on ice, collected by centrifugation (10,000 g for 10 minutes), washed with phosphate-buffered saline (PBS), and resuspended in 100 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1 mM DTT, and 1 mg/ml lysozyme followed by stirring for 1 hour at 4°C. The lysate was sonicated twice for 5 minutes at 50% duty with a power setting of 3–4 using a model W225 sonicator (Heat-Ultrasonics, Inc.). Cell lysates were cleared by centrifugation at 15,000 g for 15 minutes and the supernatant (soluble fraction) was collected. The pellet (insoluble fraction) was resuspended in the original volume of Laemmli sample buffer and dissolved by boiling for 5 min.

[000241] The procedure was optimized for native PCCAB purification to provide enough pure native PCCAB for import studies, and to purify some of the 6xHis-tagged TAT conjugated PCCA or PCCB precursor from insoluble fraction using immobilized metal affinity chromatography (IMAC) under denaturing conditions.

[000242] For native PCCAB purification, the capture resin, DEAE cellulose, was replaced with EMD's FRACTOGEL® DEAE tentacle resin, which has a higher capacity than GE's DEAE Sepharose.

[000243] For the initial purification, the conditions for the capture column from the previous example with slightly adjusted conditions for an affinity column (Pierce's Monomeric avidin Sepharose). A different formulation buffer was used for the final protein elution on Sephadex G-25 column. The level of PCC enzyme activity was analyzed for the

above fractions gathered by various techniques. Table 7 provides the amount of recovery of PCC enzyme activity from common protein purification techniques utilizing a capture column. For this purification, PCC enzyme activity was measured in each fraction to determine the percent of enzyme recovered as compared to the wild-type. Results of the assay are shown in Table 7.

Table 7. *E. coli* SE1 (DE3) + pETDS1-PCCAB + pGro7 expressed in 12x1L LB

Fraction	Specific Activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Bradford (mg/ml)	Volume (ml)	TP (mg)	Total Activity ($\mu\text{mol}/\text{min}$)	Yield Activity (%)
CRUDE EXTRACT	1001835	10.7	430	4601	4.61E+09	100
DEAE UNBOUND	210868.6	0.5	1160	580	1.22E+08	2.65
DEAE ELUTION	3159633	3.5	296	1036	3.27E+09	71.01
DEAE STRIP	168194.8	3.7	420	1554	2.61E+08	5.67
AVIDIN FT	297820.2	2.5	350	875	2.61E+08	5.65
AVIDIN ELUTION	20340689	1.5	55	82	1.68E+09	36.41
G-25	20703722	1.3	60	78	1.61E+09	35.03
PCCAB	20578569	22	3.048	67	1.38E+09	29.94

[000244] After optimization of the column size, the DEAE fractogel resin was significantly improved. Phosphate concentration in elution buffer (to 100mM). The yield from DEAE capture step was in an acceptable range. Capture column step using Fractogel DEAE resin was significantly improved after the change in the size of the column.

[000245] Activity remained in the flow through, despite scaling up of the affinity column and formulation with concentration. The amount of activity is within the acceptable range, and is a significant increase over previous purification. The strip from affinity column in PCC assay cannot be analyzed as the pH of strip buffer is 2.8 which likely damages the enzyme; therefore, the strip fraction was not collected. The biotin concentration in an elution buffer was increased to improve purification.

A. SDS-PAGE

[000246] SDS-PAGE was used for assessment of total cellular proteins, soluble fraction and insoluble fraction. These results indicate that PCC and the subunits thereof were preferably expressed in at least 10-20% of total cellular protein (TCP) and were soluble for subsequent native purification from cell lysates.

Example 6. Production of Conjugates

[000247] In examples herein, PCC proteins or subunits were linked or conjugated to a molecule that permits cell entry of the PCC protein.

A. Synthesis of conjugates

[000248] This example identifies peptide candidates for addition of an N-terminal maleimide suitable to prepare a complex with PCC enzyme. Synthesis of the peptides was performed by GenScript.

[000249] PCC sulfhydryls were chemically conjugated to the maleimide group of TAT to couple the peptide to the enzyme. In certain embodiments, TAT comprises the amino acid sequence Maleoyl-beta-Ala-Gly-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg (SEQ ID NO:21). MPP1A comprises the amino acid sequence, Cha (cyclohexylalanine)-DArg-Cha-Lys-Cha-DArg-Cha-Lys (SEQ ID NO:23) and MPP2A comprises the amino acid sequence Cha-DArg-Cha-Lys (SEQ ID NO:24). Success of import into mitochondria and cells was evaluated by enzyme activity measurement and Western blot.

[000250] Maleoyl-beta-Ala-TAT was dissolved in neutral buffer, *i.e.*, PBS. The mixture was reacted with PCC overnight in room temperature. Maleoyl-beta-Ala-TAT was present in excess of PCC in a ratio of 2:1. Unconjugated peptide and conjugate were separated for further analysis.

[000251] Two reaction mixtures to produce conjugated PCCAB-TAT and PCCAB-MPP were made. One mixture was used for import as is, and the second mixture was purified using a Spin column (PD SpinTrap G-25 preparation). Table 8 provides the components of each reaction mixture: 1 μ M PCC concentration and a 2x excess of TAT peptide (KeraFast Maleoyl-beta-Ala-TAT Cat. # EAA001).

Table 8. Reaction mixture

Components	Volume (μ l)	Conc. final	Stock	μ g in reaction	Nmols in reaction
PCCAB 20mM Hepes, 100mM KCl pH7.4	39	5 mg/ml	18 mg/ml	702	5
KeraFast Maleoyl-beta-Ala-TAT	17.7	0.126 mg/ml	1 mg/ml	17.7	10
200mM KP pH6.5	35	50 mM	200 mM		
Water	48.3				
Total volume (μ l)	140				

[000252] The conjugate of PCC and TAT was prepared in 140 μ L volume for GE Spin Trap. A sample of one of the reaction mixtures produced in Example 5 was suspended by vortexing. The bottom closure of the column was removed, and the column was placed in an appropriately sized collection tube. The storage solution was removed by centrifugation for 1 minute at 800g using Beckman GS-15R swinging bucket rotor F2402H. The column was

equilibrated by addition of 400 μ l equilibration buffer PBS and centrifugation for 1 minute at 800g. This step was repeated four additional times. After each centrifugation, the flow-through was discarded and the collection tube replaced. A new clean collection tube was used for sample collection. The sample (100–180 μ l) was applied slowly to the middle of the packed bed.

[000253] To confirm that conjugate TAT-PCCAB was produced in the above reaction, TAT labeled with FITC was reacted with PCCAB.

[000254] Maleoyl-beta-Ala-TAT FITC at a concentration of 0.126 mg/ml was contacted in a 2:1 excess to PCCAB at a concentration of 5mg/mL. The reaction was incubated 1 hour at room temperature, and samples for native and SDS-PAGE were prepared. For Typhoon scanning, red laser 633nm was used to detect Alexa 647 and green 532nm laser was used to detect FITC. The FITC label was visible by scanning with Typhoon scan. The fluorescent antibody Alexa 647 was used as a secondary antibody for Mouse anti PCCA Abnova 1:1000 and Mouse anti PCCB Abcam 70416 1:1000.

B. Additional synthesis methods for TAT conjugates

[000255] Alternatively to post-translation modification to produce PCCA or PCCB conjugated to TAT, a sequence encoding a precursor of the PCCA or PCCB subunit was cloned into an expression plasmid preceded by the TAT peptide sequence. The expressed protein was reconstituted from inclusion bodies and added to the MEM medium in which hamster cells were grown on a microscope slide. The cells were fixed and stained with anti-PCCA or anti-PCCB antibody. Subsequently, a secondary fluorescent antibody was used. Presence of PCC was detected throughout the cells.

[000256] Inclusion bodies pET47-NP-TATprePCCA 5.5mg construct (55ml) and pET47-NP-TATprePCCB 6.8mg construct (68ml) were extracted and purified according to the Novagen protocol and solubilized in N-Lauroylsarcosine to a 0.06% final concentration.

[000257] Table 9 contains the different conditions for chemical refold of PCCA and PCCB for 4 different constructs. Samples 3, 5, 7-8, 10, and 12 were dialyzed in HMS. Hepes-mannitol-sucrose buffer, which is known in the art, were used during mitochondrial import.

Table 9. Suspensions of individual PCC subunits

#	Conditions of chemical refold of solubilized PCCA and PCCB subunits used for import
1	C-TAT-prePCCA (C-A) construct 10% glycerol pH7.3 (NO dialysis)
2	C-TAT-prePCCA (C-A) construct 4.2 mg/ml dextran sulfate pH7.3 (NO dialysis)
3	C-TAT-prePCCA (C-A) construct 4.2 mg/ml dextran sulfate pH7.3 (dialyzed into HMS buffer)
4	NP-TAT-prePCCA (NP-A) construct Alpha-cyclodextrin pH7.3 (NO dialysis)
5	NP-TAT-prePCCA (NP-A) construct Alpha-cyclodextrin pH7.3 (dialyzed into HMS buffer)
6	NP-TAT-prePCCA (NP-A) construct 0.5M arginine pH7.3 (NO dialysis)
7	NP-TAT-prePCCA (NP-A) construct 0.5M arginine pH7.3 (dialyzed into HMS buffer)

8	C-TAT-prePCCB (C-B) construct 0.4M NDSB pH7.3 (dialyzed into HMS buffer)
9	NP-TAT-prePCCB (NP-B) construct 10% glycerol pH7.3 (NO dialysis)
10	NP-TAT-prePCCB (NP-B) construct 10% glycerol pH7.3 (dialyzed into HMS buffer)
11	NP-TAT-prePCCB (NP-B) construct 0.5M arginine pH7.3 (NO dialysis)
12	NP-TAT-prePCCB (NP-B) construct 0.5M arginine pH7.3 (dialyzed into HMS buffer)
13	NP-TAT-prePCCA (NP-A) construct + NP-TAT-prePCCB (NP-B) construct 0.5M arginine pH7.3 (NO dialysis)
14	NP-TAT-prePCCA (NP-A) construct + NP-TAT-prePCCB (NP-B) construct 0.5M arginine pH7.3 (dialyzed into HMS buffer)
15	Control PCCAB import 1 μ M

[000258] To determine whether conjugation was effective for the PCCAB oligomer, SDS-PAGE and native-PAGE analysis were performed.

[000259] Chloroform/methanol precipitation was used to purify C-TATprePCCA by precipitating 205 μ g and 102.5 μ g in a total volume of 100 μ l 6M guanidine buffer (added to 100 μ l). The obtained pellet was dissolved in 205/102.5 μ l of SDS sample buffer to an assumed concentration 1mg/ml. Sonication was necessary to disperse the pellet. After boiling, samples were separated on SDS-PAGE.

C. TAT conjugation to PCCAB

[000260] Although the difference in size between PCCAB enzyme and conjugate PCCAB TAT-FITC was nearly undistinguishable (a conjugate with one TAT would be about 2 kDa larger, *i.e.*, TAT MW = 1785 Da, FITC MW = 389 Da), results of the SDS-PAGE and the native gel indicated that TAT-FITC conjugate was present on both subunits of PCCAB. Therefore, the conjugate withstood boiling for the for SDS-PAGE sample preparation. Using the anti-PCCA and anti-PCCB antibodies above on a Western blot revealed PCCA and PCCB subunits having the correct size. Although SDS-PAGE results were clearer, a Typhoon scan detected the fluorescent signal of Alexa 647 and FITC. The FITC signal detected in the native gel was not as strong as the signal in SDS-PAGE. Therefore, after import and uptake by mitochondria, TAT was likely hidden in a complex protein. The Typhoon scan was performed with the red laser settings of 633nm, 670 short pass filter, 600 PMT (photomultiplier voltage) for Alexa 647 specific for the anti-PCCB and the anti-PCCA antibodies, and laser settings of 532nm, 526 short pass filter, 700 PMT (photomultiplier voltage) for FITC. Biorad Catalog 162-0264 Immune-Blot[®] Low Fluorescence PVDF membrane was used to transfer results on the gel.

[000261] The Typhoon scan for FITC provided evidence that PCCA and PCCB with a mitochondrial leader sequence are inside of mitochondria. In the mitochondria, those subunits were present without a mitochondrial leader sequence, which had been cleaved during processing.

[000262] Low fluorescence Immunoblot PVDF membrane produced lower background signal during the Typhoon scan. Therefore, examples herein use low fluorescence Immunoblot PVDF membrane with the Typhoon scan protocol.

[000263] TAT and MPP proteins were then conjugated to PCC or a subunit thereof and prepared for import into mitochondria and human fibroblasts.

[000264] The isolation of mitochondria was performed separately for each import study.

Example 7. Quality of mitochondria: respiration capacity

[000265] To analyze the quality of isolated mitochondria, an isolation of mitochondria from PCC-/- A138T mice was performed. During an import and trypsinization of the mitochondria, respiration capacity was analyzed on an Oroboros Oxygraph 2K.

[000266] To ensure that the mitochondria were intact and functional during import, oxygen consumption by the mitochondria was monitored while performing the import and trypsinization (Figure 9). An aliquot of the mitochondria suspension was tested in the Oroboros Oxygraph 2K. Measurement of oxygen consumption in isolated mitochondria was performed in a closed chamber for approximately one and half hours. Whenever the oxygen was used up, the chamber was opened and the process was repeated 3 times.

[000267] Results showed changes in oxygen concentration over the three iterations of the process. A sharp decrease of oxygen concentration was observed; therefore, mitochondria respired during import of PCCAB.

A. Confirming the presence of mitochondria

[000268] As a control for the mitochondrial fraction, voltage-dependent anion channels (VDAC), a class of porin ion channel protein specific to mitochondria located on the outer mitochondrial membrane, were used in a Western blot to confirm presence of mitochondria in the fraction. A 31 kDa band, which is the molecular weight for VDAC1, was present in wild type and A138T PCC-/- samples as detected by Abcam 154856 rabbit monoclonal antibody.

Example 8. Isolated mitochondria import studies

[000269] Due to challenges associated with importing PCC (entire enzyme or subunits) into fibroblasts, mitochondria-penetrating peptides (MPP) and/or TAT peptides were conjugated to the PCC enzyme subunits or portions of the subunits as described in Example 5 for their ability to be imported into isolated mitochondria and to determine efficacy of these peptides for PCC enzyme replacement therapy (ERT). Import of various forms of PCC subunits, with and without mitochondrial leader peptides, and/or with and without cell-penetrating peptides were examined in: isolated rat liver mitochondria, human control samples, human PA patient derived cells, and PA mouse models. Pharmacokinetic and pharmacodynamic

characterization of the PCC import and levels of toxic metabolites characteristic of PA disease were also analyzed.

[000270] MPPs were developed as mitochondrial transporters, as they are synthetic cell-permeable peptides that are able to enter mitochondria. Efficient uptake of MPPs was observed in a variety of cell types, and organelle specificity is attained with sequences that possess specific chemical properties. MPPs are cationic and lipophilic; this combination of characteristics facilitates permeation of the hydrophobic mitochondrial membrane (see, Horton *et al*, *Chemistry & Biology*, 15, 375-382, 2008, which is herein incorporated by reference in its entirety).

[000271] PCC proteins and subunits were conjugated to three distinct peptides suitable for cargo delivery: TAT, MPP1A and MPP2A.

[000272] For initial screening of the peptides, MPP1A, MPP2A, or TAT were each conjugated to PCC, PCC subunits, or subregions of the PCCA or PCCB subunits were imported into mitochondria and fibroblasts. A 2:1 ratio of MPPs to PCC, PCC subunits or subregions of the PCCA or PCCB subunits was combined in an overnight reaction.

A. Trypsin proteolysis of PCC

[000273] Since trypsin was used in all import experiments to digest any adsorbed PCC to the outside of the outer mitochondrial membrane to ensure that the mitochondrial lysate represented only PCC that had been imported to the inside of the organelle, the susceptibility of the enzyme to digestion by trypsin was first assessed.

[000274] The digestion with trypsin at 1:100 (w/w) ratio was performed for 5, 10, and 20 minutes in HMS buffer used for mitochondrial import. The time course of proteolysis was followed by measuring PCC activity. There were no changes in PCC activity in the first group of samples (T) where the trypsin inhibitor was added before the trypsin.

[000275] In the second group (R) of samples, trypsin was enzymatically active causing the PCC activity to decrease in time. After 5 minutes of trypsin action only 37% of PCC activity was remaining, and after 20 minutes of treatment only 7% PCC activity remained (Figure 10).

[000276] The digestion products were analyzed by Western blot. Results showed intact α and β subunits of the correct sizes in the first set of samples with trypsin inhibitor preventing the proteolysis but showed increasing amounts of degraded bands in the trypsinized PCC samples.

Example 9. Import of 3 μ M TAT and MPP2A conjugates into isolated mitochondria

[000277] Selected MPP peptides and TAT were tested for their ability to deliver propionyl-CoA carboxylase into mitochondria. PCC activity was measured in mitochondrial lysates. The effectiveness of different amounts of PCCAB for import into mitochondria were compared.

[000278] Import of 3 μ M TAT-PCCAB into mitochondria was observed to increase PCC activity 12-fold compared to PCC activity in mutant A138T mitochondria lysate. Import of either TAT-PCCAB or MPP2A-PCCAB was observed to increase the PCC activity above the level of enzymatic activity of the control wild type.

[000279] After successful mitochondrial import, 3 μ M of TAT-PCCAB (about 14,000 pmol/min/mg) increased the PCC activity 12-fold compared to the specific activity of mutant A138T mitochondria lysate (about 1,000 pmol/min/mg). For 3 μ M import of shorter peptide conjugate MPP2A-PCCAB (about 18,000 pmol/min/mg), the activity of PCC was observed to increase 14-fold compared to the mutant A138T mitochondria lysate activity. The data are shown in Figure 3.

A. *Western blot*

[000280] Lysates of mitochondria from livers of mutant A138T mice and lysates of mitochondria from livers of mutant A138T mice after import of TAT-PCC, MPP1A-PCC, and MPP2A-PCC were compared to control PCC enzyme on a Western blot. Primary antibodies for Western blot were Abcam anti-PCCA 80784 and anti-PCCB 70416 diluted 1:1000.

[000281] Results of the Western blot provided evidence that lysate of mutant A138T mouse liver contained PCC, especially the PCC alpha subunit. The amount of PCC in A138T lysates after the import of TAT-PCC and MPP2A-PCC into the isolated mitochondria was observed to be significantly greater than the amount of PCC in the control A138T-MITO mitochondria lysate.

B. *Effects of trypsinization on PCC activity*

[000282] Formulated TAT-PCC conjugate had been previously observed to be stable during the freeze thaw process. To get a better understanding of how the TAT-PCC delivery mechanism affects the ability of the conjugate to be delivered across the mitochondrial membrane as a functional enzyme, 1 μ M TAT-PCC was incubated with isolated mutant A138T PCC^{-/-} mouse liver mitochondria at 27°C for 30 minutes followed by trypsinization for time periods of 5 and 25 minutes at 37°C. As a control, enzymatic activity was measured in mitochondrial lysates of wild type liver without performing the import. Table 10 shows PCC activity of mitochondrial lysates after import of TAT-PCC for 30 minutes in samples: 1.

WT mitochondria, 2. A138T PCC^{-/-} mouse mitochondria, 3. Import of 1 μ M TAT-PCCAB into A138T PCC^{-/-} mouse mitochondria, 4. Import of 1 μ M TAT-PCCAB into A138T PCC^{-/-} mouse mitochondria followed by 5 minutes trypsinization, and 5. Import of 1 μ M TAT-PCCAB into A138T PCC^{-/-} mouse mitochondria and 25 minutes of trypsin.

Table 10. PCC activity for trypsinized samples

Sample	PCC specific activity [U/mg]
1	3253.00
2	258.40
3	3359.68
4	3471.00
5	3839.00

[000283] The mutant liver mitochondrial lysate activity was significantly lower, *i.e.*, about 8% of wild type activity. After TAT-PCCAB delivery into mutant mitochondria, the PCC activity reached the level of the wild type liver mitochondria PCC activity. The length of trypsinization did not affect the activity.

[000284] Western blot confirmed the presence of the delivered fusion protein. In wild type mouse mitochondria fraction, both subunits of PCC enzyme were observed. The PCC alpha subunit, however was missing in the lysate of the mutant mitochondrial fraction from the A138T PCC^{-/-} mouse. Nevertheless, both alpha and beta PCC subunits were observed in the same mitochondria after 1 μ M PCCAB import was performed for 30 minutes. Additionally, PCC alpha and beta subunits were observed in both 5 minutes and 25 minutes trypsin treated samples indicating that the PCC enzyme was inside of the mitochondria protected from the protease.

[000285] PCC activity was measured in control A138T mitochondria and the three mitochondria lysates in which import was performed. Import of PCCAB into mitochondria was performed in each lysate for 30 minutes at 27°C. After the import, trypsin in a ratio 1:100 was added and one sample that had been incubated 5 minutes, was added to a different sample that had been incubated 25 minutes at 37°C. Specific activities in each 10 μ L of the four samples were analyzed.

[000286] PCC was detected in wild type mitochondria. The second import which was allowed to incubate for a longer time was the most efficient of the import samples, and activity was highest of the four samples. Therefore, a longer import time may improve the import.

[000287] PCC activity increased more than 10-fold in mitochondria after PCCAB import compared to specific activity of PCC in the control mitochondrial lysate. PCC activity

after trypsin treatment was observed to remain similar to activity levels in samples not treated with trypsin.

Example 10: Dose response of import into isolated mitochondria

[000288] The effects of increasing concentrations of TAT-PCCAB on import into isolated mutant mitochondria were analyzed.

[000289] SDS-PAGE was performed to determine success of import in the samples of A138TMITO+Import PCCAB 1 μ M 0.140 μ g/mL, A138TMITO+Import PCCAB 2 μ M 0.140 μ g/mL, A138TMITO+Import PCCAB 5 μ M 0.140 μ g/mL, and 1:100 Trypsin treated 25 minutes A138TMITO+Import PCCAB 1 μ M 0.140 μ g/ml, as compared to wild type mitochondria lysate, A138TMITO, and PCC FITC-TAT Dilute 500x.

[000290] In the mitochondrial fraction from wild type mice, both subunits of PCC enzyme were detected. PCC alpha subunit was faintly visible in the lysate of mutant mitochondrial fraction A138T PCC-/- mouse. PCC alpha and beta subunits were in the same mitochondrial lysates after 1 μ M PCCAB import, slightly increased in 2 μ M and 5 μ M import. PCC alpha and beta subunit were present in the three import samples after 5 and 25 minutes of trypsin treatment samples indicating that PCC enzyme is inside of mitochondria protected until the preparation of lysate because trypsin treatment would cleave most of the PCC enzyme in solution. Therefore, increase in concentration of imported PCCAB enzyme increases the amount of PCCAB detected in mitochondria.

[000291] The specific PCC activity increased in mitochondrial lysates when increasing the concentration of PCC for import as shown in Table 11.

Table 11: PCC specific activity for increasing doses

Concentration	Specific activity [U/mg of protein]
Wild type control	6703.78
No import	884.4
1 μ M	6432.51
2 μ M	11515.15
5 μ M	17516.63

[000292] For 1 μ M import of PCCAB the activity (between 6000 and 7000 pmol/min/mg) was observed to increase 7-fold compared to enzyme activity (less than 1000 pmol/min/mg) in mutant A138T mitochondria lysate with no import, likewise 2 μ M import increased activity (about 12000) 13-fold, and 5 μ M import increased activity (almost 18000) 20-fold, exceeding PCC activity in wild type mitochondria about 2.2-fold. This was measured in samples after trypsin treatment for time periods of 5 and 25 minutes. Therefore, specific PCC activity increases in mitochondrial lysates when increasing the concentration of PCC for import.

[000293] Western blot was used to detect both subunits of PCC in the wild type and isolated mutant mouse mitochondria fractions, which is shown in Figure 11. The PCC alpha subunit was nearly undetectable in the lysate of the mutant mitochondrial fraction from the A138T PCC^{-/-} mouse, but the PCC alpha and beta subunits were easily detectable in the mutant mitochondria following import of 1 μ M PCCAB. The amounts of each subunit increased following import of 2 μ M and 5 μ M PCCAB. Subsequent treatment with trypsin for 25 minutes was expected to digest the unimported PCC enzyme in the incubation mixture. Both the alpha and beta subunits in all three import samples were observed after 25 minutes of trypsin treatment. Higher concentration of PCC enzyme during import led to an increase of the amount of PCC detected. Therefore, a higher concentration of PCC enzyme during import was observed to lead to an increase of the amount of PCC detected. Lane E represents PCC FITC-TAT, the wild-type control.

Example 11. Import studies in isolated mitochondria: Individual subunit import with TAT

[000294] Import of individual subunits PCCA or PCCB with TAT were analyzed to determine whether the conjugates would enter the mitochondria of cells. Import of 1 μ M TAT-PCCAB was repeated as described above as a control.

[000295] A 1 μ M import of TAT-PCCA and TAT-PCCB was performed with each solubilized subunit, the suspension of mitochondria in the above listed buffer or HMS buffer was incubated 60 minutes at 27°C. For import of PCCA and PCCB, a solution of 400 μ l HMS (no BSA) and 1 μ M TAT PCCAB (130 μ g/mL) was used.

[000296] For import of PCCA (e.g. NP-TAT prePCCA) and PCCB (e.g. C-TAT prePCCB), the solution of inclusion bodies resuspended in N-lauroyl sarcosyl 0.06% were diluted 20x from starting concentration 20 mg/ml to achieve the approximately 1 μ M import.

[000297] To remove TAT-PCC not imported into the mitochondria, trypsin was added to the suspension in a ratio 1:100 for 25 minutes, and the trypsin reaction was stopped with trypsin inhibitor from soybean in ratio 1:1. Lysates were prepared as previously described.

[000298] No significant difference in PCC activity was observed between import of PCCA or PCCB subunits. The positive control of FITC-TAT- PCCAB had a 17-fold increase in activity compared to the A138T mitochondrial lysate. The specific activity of FITC-TAT-PCCAB was observed to be over 9,000 pmol/min/mg, while neither the control nor any of the import of individual subunits exceeded a specific activity of greater than 1000 pmol/min/mg.

[000299] Consistent with previous studies with whole cells contacted with N-Lauroyl sarcosyl, the solution dissolved mitochondria and structures of the mitochondria were not

distinguishable after staining. The overlay was unclear although the stain for PCCA was positive.

Example 12. Import studies of individual subunits in patient fibroblasts

[000300] Import was performed on patient fibroblast cells #3380 (PCCA deficient) grown on microscope slides to approximately 70% confluency, which were then incubated 20 minutes for import. Immediately after import, immunostaining was performed to confirm success using the protocol in Example 1.

[000301] Samples were stained with MITOTRACKER® CMX 2000x dye specific to mitochondria. The primary antibody was polyclonal mouse Anti PCCA Abcam Ab89784 antibody 200x, and the secondary antibody was AttoM488 1000x. Samples were also stained with DAPI, which is specific to DNA.

[000302] Fibroblast cell line #3380 after TAT-prePCCA import was stained with DAPI, MITOTRACKER® CMX, and Anti PCCA antibody Abcam Ab89784. The samples were stained as follows: Stain Mitotracker only; Stain Anti PCCA antibody only; and DAPI, MITOTRACKER® CMX and Anti PCCA antibody Abcam Ab89784.

[000303] In fibroblast cells of #3380 20 minutes after import of NP-TATprePCCA, a strong fluorescent signal in green of anti-PCCA antibody was observed. The overlap with red MITOTRACKER® CMX and green anti-PCCA antibody was also visible in another sample. Therefore, the inclusion bodies were successfully imported into mitochondria of cells.

[000304] The same immunostaining protocol was used on patient fibroblasts #3380 after import of 5 μ M TAT-PCCAB. The import was successful with NP-TAT prePCCA. When diluted 20x, concentration of protein was 20 mg/mL according to a Bradford assay.

[000305] PCC activity was measured in cell lysates and immunofluorescence was observed for these fibroblast cells.

A. Import of 3 μ M MPP1A, MPP2A, and TAT conjugates

[000306] PCC activity in fibroblasts after import was measured in the cell lysate following import of 3 μ M MPP1A-PCCAB and MPP2A-PCCAB into patient fibroblasts #3380 patient. The import of MPP conjugates MPP1A and MPP2A into cells was performed for 1.5 hour at 37°C. Cells were harvested using 0.25% trypsin=2.5mg/mL. The pellet of cells was washed 3 times in PBS, and each time resuspended in 20 mL PBS. After centrifugation at 800 g for 10 minutes, the mixture was transferred to a small Eppendorf tube.

[000307] Lysis buffer containing 50mM TrisHCl pH 8.0, 1 mM DTT, 1 mM EDTA pH 8.0, inhibitors (Sigma P8340) was commercially obtained. Three times the volume of the buffer as compared to the volume of cell pellet was used. Cells in the lysis buffer was homogenized

by pipetting. The suspension was sonicated twice for 10 seconds at power 3 pulsing 1 second on, 0.5 second off using a microtip. Cells were spun in a cooled microcentrifuge (20,000 g, 4°C) for 15 minutes. Supernatant was transferred into a fresh tube. Protein concentration was measured by Bradford assay using 20x or 40x dilutions.

[000308] 3 μ M MPP2A-PCCAB and 3 μ M MPPIA-PCCAB were separately incubated with cells for 1.5 hour in 37°C. To confirm successful import of 3 μ M MPP2A-PCCAB or 3 μ M MPPIA-PCCAB into patient fibroblasts #3380, immunofluorescence was performed. A control was created by staining of patient fibroblast #3380, PCCA deficient cells, without import.

[000309] Results from immunofluorescence after import of MPP2A-PCC into patient fibroblasts #3380 provided a strong green fluorescent stain of PCC. The side to side view with MITOTRACKER® staining provided comparison of the stains. An overlay of the results indicated that some green and red areas were superimposing, which is evidence of import into mitochondria. PCC was not detectable in the #3380 patient fibroblast cells, as expected. See Figure 2.

[000310] The same protocol was repeated for PCCB deficient patient fibroblasts #3383. After the import of MPP2A-PCC into #3383 patient fibroblasts, a strong green fluorescent staining of PCC was observed. An overlay of the immunofluorescence study is shown in Figure 2. The side to side view with MITOTRACKER® staining provided comparison of both stains. An overlay of results for this sample indicated that green and red areas are superimposing, which indicates import into mitochondria. Results after import of MPPIA-PCC to patient fibroblasts #3383 did not provide an overlay, indicating that MPPIA-PCC was not successfully imported the mitochondria of the fibroblasts.

[000311] The patient fibroblast cell lines #3380 and #3383 were observed to have a very low PCC activity as shown in Figure 12. For 3 μ M import into the cells of the shorter peptide conjugate MPP2A-PCCAB, PCC activity was observed to increase 14-fold compared to PCC activity in the mutant A138T mitochondria lysate. A significant increase of PCC activity in cell lysates was observed after the import of 3 μ M TAT-PCC conjugate, similar to additional results gathered herein. Further, MPPIA-PCC (about 100 pmol/min/mg in #3380 and about 5000 in #3383) resulted in a much higher activity than MPP2A-PCC (less than 500 pmol/min/mg in #3380 and about 600 in #3383). Import of PCC by MPP2A-PCCAB was confirmed using immunostaining. The staining did not indicate successful import by MPPIA-PCCAB.

Example 13. Staining to confirm import in patient fibroblasts

[000312] Pure PCC was linked to a FITC-labeled TAT peptide. Fibroblasts were grown in complete MEM media. 1 μ M PCC was imported into #3380 fibroblasts (two mutations in PCCA) for 1 hour at 37°C as described in Example 1. The day before staining, the fibroblasts in 50-80% confluence were plated in duplicate on an 8 chamber slide as follows: #3380 import TAT-PCC Mitotracker CMX 2000x with no primary antibody and secondary rabbit Atto488 1000x; #3380 import TAT-PCC with Mitotracker CMX 2000x, primary anti-PCCA Ab154254 Rabbit (200x), and secondary ATTO488 rabbit 1000x; #3380 import TAT-PCC with Mitotracker CMX 2000x, Ab70416 Anti-PCCB mouse (200x), and secondary Atto488 mouse 1000x; and #3380 Mitotracker CMX 2000x, Ab70416 Anti-PCCB mouse (200x) 200x, and secondary mouse Atto647 1000x.

[000313] To analyze 1 μ M TAT-PCCAB imported into patient fibroblasts #3380, immunofluorescent analysis was performed at 60X magnification (confocal microscope Olympus FV-1000 ALMC core facility). Staining results shown in Figure 13 show PCCA deficient cells. After import, live cells were stained as follows: MitoTracker CMXR in the second from right panel. Following the cell fixation, staining was performed with anti PCCA antibody and then with green fluorescent (light gray) secondary antibody in the second panel from the left and DAPI blue fluorescent dye (dark gray) was used to stain the nuclei in the far-left panel. A merged image of the staining is shown in the far-right panel.

[000314] Green fluorescent (light gray) signal of anti-PCCA was strong. For patient #3380 fibroblast cell line after import of 1 μ M FITC-TAT-PCC. The merged panel in Figure 13 shows import of the TAT-PCCAB into the mitochondria.

Example 14. Import studies in patient fibroblasts: PCCAB conjugates

[000315] Maleoyl-beta-Ala-TAT (KeraFAST®) was combined with the purified $\alpha 6\beta 6$ PCC. Two different PA patient fibroblast cell lines were transfected with the PCC and TAT protein mixture. Each cell line reproducibly demonstrated enzyme activity in the range of full restoration of enzymatic activity of normal control fibroblasts to about ten times the activity of normal control fibroblasts. For *in vitro* import of 1 μ M TAT-PCCAB and MPP2A-PCCAB into patient fibroblast #3380, the reaction of PCC enzyme with maleoyl-beta-Ala-TAT was incubated overnight. One reaction was used for the import as is, and the second was purified on a spin column G-25 to remove excess of maleoyl-beta-Ala-TAT peptide.

[000316] The import into cells was performed for 1 hour at 37°C. TAT-PCCAB complex was diluted in PBS and applied to a 150cm² flask of confluent cells. Each of conjugate PCC-TAT is added to 5 mL complete MEM media and applied to a 150cm² flask with patient #3380 fibroblast cells 80-90% confluent. Cells are incubated for 1 hour at 37°C.

[000317] After import, cells were harvested using 0.25% trypsin. The pellet of cells was washed 3x in PBS, each time resuspended in 20mL PBS and centrifuged at 800 g for 10 minutes, then transferred to a small Eppendorf tube.

[000318] Cells were harvested, and PCC activity was measured in cell lysate. PCC activity was analyzed in normal control fibroblast #5142 and in patient fibroblasts #3380 and #3383, and in each type of fibroblasts after import of conjugate TAT-PCCAB.

[000319] Patient fibroblasts #3380 were observed to have low PCC activity. After import of 1 μ M TAT-PCCAB conjugate, the activity of PCC was 34 times higher than in the control sample without treatment and doubled compared to the control fibroblasts. There was not a significant difference of PCC activity between import of conjugate TAT-PCCAB that was loaded on G25 spin column to separate free TAT and the conjugate that was applied after reaction without additional treatment. Samples analyzed in the assay were as follows: patient fibroblast #3380, patient fibroblast #3380 after import of conjugate TAT-PCCAB purified on G25, patient fibroblast #3380 after import of conjugate TAT-PCCAB, and control fibroblast #5142.

[000320] The activity in fibroblast lysate extracts was measured for 150 μ g of protein in a 100 μ l assay. Protein concentration was calculated using a Bradford protein assay.

[000321] Cells were homogenized in the lysis buffer by pipetting. The cell suspension was sonicated twice for 10 seconds at power 3, pulse 1 sec on, 1 sec off using a microtip. Volumes were too small to use a power setting greater than 3. Cells were centrifuged in a cooled microcentrifuge at 20,000 g and 4°C for 15 minutes. The supernatant was transferred to a fresh tube. 20 μ l of the 2x Reaction mixture was combined with the cells. The 30mM propionylCoA (final 3mM) was added at a volume of 5 μ l, or 5 μ l of water was added for a blank.

[000322] The sample or dilution buffer for the blank were added at a volume of 15 μ l, and the mixture was transferred at 37°C. The combined mixture was incubated for 2 minutes prior to starting the reaction. The adjusted ¹⁴C sodium bicarbonate, which starts the reaction, was added in a volume of 10 μ l. The reaction was incubated at 37°C for 15 minutes under the hood. The reaction was stopped by mixing with 50 μ l of ice-cold 10% TCA, and further transfer to ice. The tubes were centrifuged at max speed for 5 minutes in the hood. 50 μ l of the supernatant was transferred into a labeled glass scintillation vial. Unreacted CO₂ was evaporated in a dry block at 80°C for 20-30 minutes. The dry pellet was dissolved in 150 μ l of ddH₂O. Scintillation liquid was added at a volume of 4ml for counting.

[000323] Specific activity of adjusted [^{14}C] sodium bicarbonate was calculated by mixing 10 μl of the adjusted [^{14}C] sodium bicarbonate mixture and 4990 μl of ddH $_2\text{O}$. In a scintillation vial, 10 μl of this 500x dilution was separated. The scintillation liquid was added at a volume of 4ml, and counted with the samples. Results are shown in Table 12.

Table 12. PCC Activity Studies

Sample	Assay counts [cpm]	Back-ground adj. counts [cpm]	Assayed enzyme [μl]	Time [min]	Labeled Methyl-malonyl-CoA [$\mu\text{mol/min}$]	Total protein [$\text{ng}/\mu\text{l}$]	PCC activity (Specific Activity) [$\mu\text{mol/min/mg}$]
blank	186	0	10	5		0	
PCCAB Hepes 18 mg/ml	13340	13154	10	5	1977.30	10	19773017.7
PCCAB Hepes 18mg/ml	15726	15540	10	5	2335.96	10	23359639.2
CE prepared RC	24511	24325	10	5	3656.52	500	731304.0
CE prepared RC	21328	21142	10	5	3178.05	500	635610.7
activity1 PCCAB+TAT	10235	10049	10	5	1510.56	10	15105.599.4
activity1 PCCAB+TAT	11346	11160	10	5	1677.56	10	16775648.3
activity2 eluate PCCAB TAT G25	6393	6207	10	5	933.03	10	9330326.9
activity2 eluate PCCAB TAT G25	5222	5036	10	5	757.01	10	7570086.4
3380 TAT PCCAB G25	12647	12461	15	15	624.38	10000	4162.5
3380 TAT PCCAB G25	13413	13227	15	15	662.76	10000	4418.4
3380 TAT+PCCAB	16450	16264	15	15	814.93	10000	5432.9
3380 TAT+PCCAB	12698	12512	15	15	626.93	10000	4179.5
3380 patient	519	333	15	15	16.69	10000	111.2
3380 patient	587	401	15	15	20.09	10000	134.0
5142 control fibroblast	6173	5987	15	15	299.99	10000	1999.9
5142 control fibroblast	6729	6543	15	15	327.85	10000	2185.6

[000324] There is no significant difference of PCC activity between import of conjugate TAT-PCCAB that was loaded on spin column to separate free TAT and the conjugate that was applied after a reaction without additional treatment. Exposure of the PCC-deficient fibroblasts to TAT-PCCAB led to a large increase of PCC activity from less than 1% to about 400% of the enzyme activity of control fibroblasts.

Example 15. Import studies in patient fibroblasts: Dose response to PCCAB conjugates

[000325] In another experiment, the import was performed at different concentrations: 1 μM , 5 μM , and 10 μM , of PCC using the technique in Example 1 for the import of 1 μM TAT-PCCAB into patient fibroblast #3380. Patient fibroblasts #3380 had mutation in the PCCA subunit and #3383 had a mutation in the PCCB subunit, and as a consequence both cell lines have low PCC activity.

[000326] Each sample was combined with a reaction mixture containing 50mM Tris-HCl, pH 8.0, 2mM ATP, 250mM KCl, 10mM MgCl $_2$, 3mM propionyl-CoA, 0.5mg/ml bovine serum albumin, PCC enzyme (150 μg of cell lysate), and 10mM [^{14}C] bicarbonate for a final

volume of 50 μ L, and incubated at 37°C for 15 minutes. The reaction was terminated with 50 μ L of 10% trichloroacetic acid. The mixture was centrifuged at 13,000g for 5 minutes, and 50 μ L of supernatant was dried in a scintillation vial in a heating block at 80 °C for 30 minutes. The dry residue was dissolved in 0.15ml of H₂O, and 4ml of OPTI-Fluor scintillation fluid (PerkinElmer Life Sciences) was added. The samples were counted by a Beckman LS 3801 scintillation counter. A blank containing the assay mixture without propionyl-CoA was subtracted from the measured values.

[000327] After the import of 1 μ M, 5 μ M and 10 μ M TAT-PCCAB conjugate, the PCC activity increased in response to the increasing TAT-PCCAB concentration.

[000328] For example, in patient fibroblasts #3380, the 1 μ M import had a specific activity of about 4000 pmol/min/mg, the 5 μ M import had a specific activity of about 13,500 pmol/min/mg, and the 10 μ M had a specific activity of about 17,000 pmol/min/mg. The results are shown in Figure 4.

Example 16. Import studies in patient fibroblasts: PCCA and PCCB deficient fibroblasts

[000329] In order to examine the ability of TAT to deliver PCC *in situ*, PCC import into fibroblast cells from propionic acidemia patients. One patient was homozygous for two different mutations in the PCCA subunit and the second one was PCCB deficient. Fibroblast cells were incubated for 1 hour at 37°C with different amounts of TAT-PCC. The cells were harvested with 0.25% trypsin (ratio 1:1 protease/protein). The PCC activity was tested in cell lysates. Table 13 shows C, control cell line; 1, PCCA deficient fibroblast lysate; 2 – 4, Import of 1 μ M, 5 μ M, and 10 μ M TAT-PCC into PCCA deficient cells for one hour; 5, PCCB deficient fibroblasts; and 6 -8, import of 1 μ M, 5 μ M, and 10 μ M TAT-PCC into PCCB deficient cells for one hour.

Table 13. PCC activity in PCCA or PCCB deficient fibroblasts

Import into PCCA deficient fibroblasts		Import into PCCA deficient fibroblasts	
Sample	Specific Activity	Sample	Specific Activity
Control	1558.57	Control	1558.57
PCCA deficient	132.65	PCCB deficient	111.78
1 μ M	3825.93	1 μ M	2074.47
5 μ M	13378.79	5 μ M	9211.81
10 μ M	17164.47	10 μ M	13775.69

[000330] Both PCCA and PCCB deficient skin fibroblasts had less than 3% of control fibroblast activity. The conjugated TAT-PCCAB was successfully imported into patient cells with either defective PCCA or PCCB. The activity at the highest 10 μ M TAT-PCC

concentration in the incubation mixture exceeded the control activity 11- and 9- times for the PCCA and PCCB deficient cells, respectively.

Example 17. Import studies *in vivo* mouse studies

[000331] A wild type mouse control and liver from PCC^{-/-} A138T mice were analyzed to confirm that TAT-PCCAB was successfully imported into cells' mitochondria and affects metabolite levels in mouse plasma. The import was performed for two hours into isolated mitochondria from the PCC deficient mice only. Trypsin was applied to one of the mitochondrial samples in a ratio of 2:1 trypsin/enzyme. Each type of sample was analyzed using Western blot and PCC activity was measured.

[000332] PCC was observed in the sample of wild type mouse mitochondrial lysate. Import of PCC was also observed; therefore, PCCA is potentially only found in this sample, as PCC was not detected in the other samples. TLCK was used to inhibit trypsin action.

[000333] Both subunits of PCC enzyme were observed in the lane corresponding to the wild type mouse mitochondrial fraction. PCC alpha subunit was missing in the lysate of mutant mitochondrial fraction A138T PCC^{-/-} mouse. PCC alpha and beta subunit in the same mitochondria remained present after 1 μ M PCCAB import. Treatment with trypsin for 25 minutes generally cleaves PCC enzyme in solution. PCC alpha and beta subunit were present in the samples treated for 25 minutes with trypsin indicating that PCC enzyme is inside of the mitochondria and is protected until preparation of lysate.

A. Mouse model

[000334] To prepare for injection of PCC into mice, the enzyme stability was tested at 37°C in plasma of PCC^{-/-} mice (mouse model is described in Guenzel et al, vol. 21 no. 7, 1316-1323 (July 2013)). Deletion of *Pcca* in mice causes similar symptoms as PA in humans. *Pcca*^{-/-} mice generally die within 36 hours of birth which does not allow much time to test intravenous therapies. The examples herein used an adult hypomorphic model of PA that is an A138T mutant as described in Guenzel et al. These *Pcca*^{-/-} mice retain 2% of the PCC enzyme activity of the wild type and survive to adulthood. Further, A138T mice have elevated levels of propionyl-carnitine, methylcitrate, glycine, alanine, lysine, ammonia, and markers associated with cardiomyopathy, which is similar to levels of these compounds in PA patients.

B. Enzyme activity

[000335] Blood from PCC^{-/-} mice was collected in anti-coagulant treated tubes. After centrifugation for 10 minutes at 200g, the plasma was transferred to a clean tube. Stability of

PCC in plasma was measured at time points 0, 20, 40, 60, 90, and 180 minutes, remaining at 37°C.

[000336] Plasma was placed in a 37°C water bath for 10 minutes to preincubate. PCCAB enzyme, in 20 mM Hepes buffer pH 7.4, 10mM KCl (18 mg/ml) was added to the plasma to a final dilution of 180x to reach an enzyme concentration of 100 ng/μL. A 108ng/μl PCC to 330μL plasma + 2μL of PCC (18mg/mL) sample was prepared, then the 2μl of protease inhibitor further diluted the sample.

[000337] The plasma and enzyme mixture was incubated in water bath at 37°C. At each timepoint: 0, 20, 40, 60, 90, 180 minutes, a sample of 28μL of plasma was added to 2μL of protease inhibitor incubated on ice (protease inhibitor cocktail (Sigma 8340) to prevent proteolytic degradation). A Western blot and PCC enzyme activity assay were performed on a sample from each timepoint and a sample of 18mg/mL PCCAB. The Western blot was performed with anti PCCAB antibody purified, diluted 200x and secondary anti-rabbit diluted 5000x to analyze the presence of PCCAB at each timepoint. For the enzyme assay, a sample having a concentration of 100ng/μL of PCC was diluted 10x. A 10μL aliquot was used in each assay as 100ng of enzyme was needed for detection by the antibodies. Activity was analyzed as described in Example 1. Results are provided below in Table 14.

Table 14. PCC enzyme activity and percent of activity of wild type

Sample	PCC Specific activity (pmol/min/mg)	% Activity
PCCAB in Hepes	17426052	100
T0	17682011	100
T20	16774404	95
T40	15870620	90.5
T60	15606268	89
T90	23042768.53	N/A
T180	13488736.53	77

[000338] PCC activity decreases gradually in time, about 10% decrease was observed in PCC enzyme activity after 1 hour incubation and up to 23% decrease in PCC activity after 3 hours of incubation, PCCAB was diluted to a concentration of 100ng/μL in plasma at 37°C. Results were confirmed by Western blot showing that PCC enzyme appeared to be relatively stable over 3 hours, 77% of PCC activity is still measured after 3 hours in plasma at 37°C. There were no degradation products observed on Western blot.

[000339] PCC activity was observed to decrease gradually over time. An about 10% decrease in PCC enzyme activity was observed after 1 hour of incubation and an up to 23% decrease in PCC activity was observed after 3 hours of incubation.

[000340] PCC enzyme was observed to be relatively stable over the course of 3 hours, as 77% of PCC activity is measured after 3 hours in plasma at 37°C. Further, no degradation of products was observed on the Western blot.

Example 18. Import studies: Activity *in vivo*

[000341] In order to prepare for injecting TAT-PCCAB into mice, enzyme stability was analyzed at 37°C in mPCC-/- hPCC A138T+/+ (A138T) mouse plasma. This experiment simulated *in vitro* conditions for TAT-PCCAB in plasma during *in vivo* mouse experiments. Initial analysis was carried out only for 3 hours as the TAT-PCCAB was hypothesized to be attached and/or imported to the cell by then. However, activity in plasma was observed 24 hours after IV injection suggesting that import was not occurring or was slower than previously thought. Therefore, plasma stability testing was repeated for a longer period of time and the results are provided by Table 15.

Table 15. Specific activity over 72 hours

Time point	PCC specific activity [x10 ⁶ pmol/min/mg of protein]
-1	30.1
0	29.93
3	26.09
6	25.9
24	19.04
48	18.66
72	8.53

[000342] PCC enzyme was observed to be stable in plasma at 37°C. Results indicate that PCC activity decreases gradually over time. For example, an about 40% decrease in PCC activity after 24 hours of incubation was observed.

A. Diurnal variation

[000343] To establish whether TAT-PCCAB is active in a cell's mitochondria and affects metabolites in mouse plasma, hypomorphic PCCA mice (knock-out for mouse PCCA and containing the transgene for human PCCA A138T mutant; further abbreviated PCCA A138T) were bled at time T0 before a first injection for metabolites, for example, two days prior to injection or immediately prior to the injection.

[000344] There were 3 groups of mice each consisting of 4 animals: 1 untreated/uninjected control group, and 2 treated/injected groups each receiving either 10 mg/kg or 20 mg/kg. The untreated control group was bled throughout a day to find out diurnal variation of metabolites and establish the best timing for injecting/bleeding. Bleedings of the treated mice were performed 15 minutes after the first injection (T1), 24 hours after the first injection and prior

to the second injection (T24), 24 hours after the second injection (T48), and 72 hours after the last injection (T96).

[000345] Natural variation of propionic acidemia (PA)-relevant metabolites, propionyl-carnitine (C3) and acetyl-carnitine (C2), was monitored in a control (untreated) group of 4 mice by collecting plasma samples at different time of day: 8:30, 11:00, 13:00 and 15:00.

[000346] There is a natural fluctuation in PA-relevant metabolites in plasma. As observed in the results in Table 16, the C3/C2 ratio was the lowest around 13:00, and levels of MC were lowest around 11:00.

[000347] Therefore, plasma was sampled at 13:00 from experimental mice going forward.

Table 16. C3/C2 ratio for diurnal variation (n=4)

Time of day sample was taken	C3/C2
8:30	3.35
11:00	3.12
13:00	1.89
15:00	2.51

[000348] Results provided that the C3/C2 (propionyl-/acetyl-carnitine) ratio was lowest at 13:00, which indicates that it is an advantageous time for plasma sample collection.

B. Dose selection

[000349] Acylcarnitines (C3 and C2) and methylcitrate (MC) were measured in plasma samples collected from mice treated with 2 doses of TAT-PCCAB in two groups: 10 and 20 mg/kg as outlined above. Results are shown in Table 17.

Table 17. C3/C2 ratio in plasma after 2 doses of TAT-PCCB

Time [hours after injection]	C3/C2 ratio for 10 mg/kg	C3/C2 ratio for 20 mg/kg
0	3.96	3.11
24	2.61	3.41
48	2.48	2.47
96	2.10	1.90

[000350] Metabolic marker MC (methylcitrate) did not show significant variation throughout the day. Both treated groups were observed to experience a decline in C3/C2 ratio for 96 hours with no additional effect observed for the group receiving 20 mg/kg. Therefore, 10 mg/kg was determined to be a sufficient dosage for examples herein.

[000351] For reference, C3/C2 ratio in healthy heterozygous mice is 0.1 μ M, respectively. As observed in the results above, PA-relevant metabolites were not significantly altered in plasma of the A138T PA mouse model. A slight trend in lowering both C3/C2 ratio and MC plasma levels was observed but does not seem relevant due to relatively high variation in the

trend. Similar to PCC activity measurement, a 2 times higher dose did not result in a 2-fold decrease in C3/C2 ratio.

Example 19. Import studies: Activity *in vivo* after IV, IP and SQ administration

[000352] PCCA A138T mice were split into 4 groups each consisting of 4 or 2 animals: 4 mice were injected by IV and 3 groups of 2 mice each were injected IV, IP, or SQ with TAT-PCCAB. The first injection was administered at 13:00, and a second injection was administered 24 hours later. For bleeding the IV injected mice (total of 6), mice were subdivided into 2 groups of 2+1 and bled as follows: Group 1 - 15 minutes, 24 hours, 48 hours, 72 hours, 96 hours, and 168 hours after the first injection; Group 2 - 4 hours, 28 hours, 48 hours, 72 hours; 96 hours, and 168 hours after the first injection. The IP and SQ injected mice were bled as follows: 4 hours, 24 hours, 48 hours, 72 hours, 96 hours, and 168 hours after the first injection.

[000353] This example was performed using a similar protocol to the previous example. Additional extra time points and injection routes were analyzed. A138T mice were injected with a single dose of 20 mg/kg. There were four groups: IV group injected (n=4), IV group (n=2), SQ group (n=2), and IP group (n=2). Each mouse received two injections 24 hours apart. Plasma samples were collected differently for each study group as follows:

[000354] IV - subgroup 1 (n=2 veterinary tech + 1 laboratory technician): 0.25, 24, 48, 72, 96 and 168 hours after the 1st injection

[000355] IV - subgroup 2 (n=2 veterinary tech + 1 laboratory technician): 4, 28, 48, 72, 96 and 168 hours after the 1st injection

[000356] SQ and IP - 4, 24, 48, 72, 96, and 168 hours after the 1st injection

[000357] Results were provided in Table 18.

Table 18. C3/C2 ratio after IP, IV, or SQ administration

Time [hour(s)]	IP	IV	SQ	Time [hour(s)]	IP	IV	SQ
0.25	0.67	2.13	NA	72	1.70	2.07	2.37
4	NA	2.85	2.97	96	2.16	2.16	2.71
24	1.96	1.96	2.47	168	2.49	2.32	2.94
48	2.34	2.29	2.49				

[000358] Levels of MC were not observed to be affected in IP group, yet variations occurred in levels of MC of the SQ-treated animals. Therefore, MC was not chosen as a metabolic marker for examples herein.

[000359] The C3/C2 ratio was observed to be significantly decreased only in the IP-injected group. C3/C2 levels were altered for less than 24 hours which indicates that a higher dosage and/or increased administration occurrence would prolong the desired effect.

[000360] Acylcarnitines (C3 and C2) were measured in all plasma samples collected from mice treated with 2 doses of TAT-PCCAB (20 mg/kg) administered via IV, SQ, or IP.

[000361] While IV or SQ administration of TAT-PCCAB had essentially no impact on the C3/C2 ratio, an IP administration resulted in a significant improvement in C3/C2 ratio within four hours after the first IP injection (from more than 25-fold elevated to only 6-fold elevated compared to a healthy heterozygous A138T mice C3/C2 ratio). This result has significant implications for future mouse experimental design as well as TAT resistance to proteolysis.

[000362] In previous examples, IP administration of TAT-PCCAB resulted in a significant improvement of C3/C2 ratio 4 hours after the first injection. At the time for the second injection (*i.e.* 24 hours after the first one), the effect was no longer present.

Example 20. Import studies: Activity sustainability *in vivo* after IP administration

[000363] To confirm that TAT-PCCAB delivery by an IP route results in sustainable and reproducible decrease of C3/C2 ratio, PCCA A138T mice were split into 2 groups each consisting of 3-4 animals (depending on enzyme availability) with injections made by two different individuals. Two injections were administered 3 hours apart at 7:00 and 10:00. Plasma samples were collected as follows: before the 2nd injection at 10:00 (T3), 6 hours after the first injection at 13:00 (T6) and 8 hours after the first injection at 15:00 (T8). Urine sample were collected 6 hours after the first injection at 13:00. After the final bleeding, mice were sacrificed and flushed with PBS. The liver, heart, and brain were harvested and frozen in liquid nitrogen for PCC activity measurement in tissues.

[000364] To further analyze the effect of IP TAT-PCCAB injection, hypomorphic PCCA mice (*i.e.* knock-out for mouse PCCA, but carrying transgene for human PCCA A138T mutant) were bled before the first injection for metabolites in time T0. Two IP injections were administered 3 hours apart: at 7:00 (Injection A) and 3 hours later at 10:00 (Injection B). Dose was the same as used in previous examples: 20 mg/kg. Mice were split into two groups: injected by two different individuals. Plasma samples were collected as follows: before the 2nd injection at 10:00 (T3), 6 hours after the first injection at 13:00 (T6), and 8 hours after the first injection at 15:00 (T8). After the final bleeding, mice were sacrificed, flushed with PBS and liver, heart and brain were harvested and frozen in liquid nitrogen. Metabolites in plasma and PCC activity were measured in plasma as well as tissue homogenates. The results are shown in the graphs of Figures 5-7.

[000365] In Figure 5, C3/C2 ratio decreased for up to 9 hours with peak PCC activity seen at approximately 6 hours (Figure 6).

[000366] Figure 7 illustrates a degree of activity in both liver and heart above untreated controls.

Example 21. Import studies: *In vivo* pharmacokinetics (PK) analyses

[000367] Examples herein analyze changes in pharmacokinetics (PK) and pharmacodynamics (PD) of metabolites after a single IP injection.

[000368] Increase of PCC activity was observed in plasma and tissues, including heart and liver, of mice injected with TAT-PCCAB compared to untreated controls, which correlates with improvement of the C3/C2. IV injection was observed to yield approximately 2-fold higher PCC specific activities in plasma compared to previous examples.

[000369] The length of the effect of IP administered TAT-PCCAB and PK of TAT-PCCAB importation into the cells was analyzed to determine the length of time that TAT-PCCAB persists in the mice following IP administration. PCCA A138T mice were split into 2 sub-groups each consisting of 4 animals to split bleedings and maximize the number of timepoints over the course of a day. A single dose of TAT-PCCAB was administered IP at 8:00. Plasma samples were collected as follows: Group A – 2 hours (T2), 4 hours (T4) and 8 hours (T8) after injection; Group B – 3 hours (T3), 6 hours (T6), and 9 hours (T9) after injection. All mice were also bled 24 hours (T24) after injection. Control group C (injected IP with PBS only) was bled at the same intervals. Mice were sacrificed, and each liver was harvested. The groups are organized in Tables 19-21 below. Table 19 provides details of Group A having plasma samples taken at T2, T4, and T8 after injection.

Table 19. Group A

Mouse ID	Genotype	M/F	Age (weeks)	Weight (g)	Injection (μl)	Injection (μl)
157	-/- A138T	M	11.1	26.0	126.8	130
159	-/- A138T	M	11.1	24.0	117.1	120
124	-/- A138T	F	14.6	18.9	92.2	90
125	-/- A138T	F	14.6	20.4	99.5	100
Minimum needed for dose				440		

[000370] Table 20 provides details of Group B having plasma samples taken at T3, T6, and T9 after injection.

Table 20. Group B

Mouse ID	Genotype	M/F	Age (weeks)	Weight (g)	Injection (μl)	Injection (μl)
122	-/- A138T	M	14.6	23.4	114.1	120
150	-/- A138T	M	14.1	24.5	119.5	120
160	-/- A138T	F	11.1	19.4	94.6	100
162	-/- A138T	F	11.1	17.3	84.4	90
Minimum needed for dose				430		

[000371] Table 21 provides details of the control group (Group C) that was injected with PBS only.

Table 21. Group C

Mouse ID	Genotype	M/F	Age (weeks)	Weight (g)	Injection (μl)
144	-/- A138T	F	15.1	16.7	81.5
145	-/- A138T	F	15.1	22.7	110.7
148	-/- A138T	F	15.1	18.6	90.7
149	-/- A138T	F	15.6	23.6	115.1
Minimum needed for dose				430	

[000372] Amounts and ratios of propionylCoAcarnitine (C3) and acetylCoAcarnitine (C2) for each mouse are shown in Table 22.

Table 22. C3/C2 ratios over 24 hours

SAMPLE_ID	C2 μM	C3 μM	C3/C2 ratio	C3/C2 ratio	STD	SEM
1D-A117-T0	10.931	13.505	1.24	2.38	0.18	0.10
1D-A121-T0	7.702	17.31	2.25			
1D-A124-T0	10.262	26.565	2.59			
1D-A125-T0	11.154	25.813	2.31			
1D-B122-T0	10.202	19.383	1.90	2.36	0.51	0.26
1D-B150-T0	6.911	20.58	2.98	Groups A and B together:		
1D-B160-T0	6.79	17.526	2.58	2.37	0.38	0.14
1D-B162-T0	10.858	21.588	1.99			
1D-A124-T2	8.969	20.165	2.25	2.26	0.96	0.48
1D-A125-T2	10.14	18.633	1.84			
1D-A157-T2	11.46	15.722	1.37			
1D-A159-T2	8.769	31.508	3.59			
1D-B122-T3	10.286	22.327	2.17	2.21	0.16	0.08
1D-B150-T3	10.223	24.295	2.38			
1D-B160-T3	11.186	25.47	2.28			
1D-B162-T3	10.258	20.653	2.01			
1D-A124-T4	12.805	15.521	1.21	1.23	0.03	0.02
1D-A125-T4	15.567	32.701	2.10			
1D-A157-T4	10.114	26.118	2.58			
1D-A159-T4	19.508	24.494	1.26			
1D-B122-T6	10.337	20.926	2.02	1.29	0.10	0.07
1D-B150-T6	18.912	23.086	1.22			
1D-B160-T6	12.895	29.222	2.27			
1D-B162-T6	15.306	20.86	1.36			
1D-A124-T8	18.729	14.889	0.79	0.87	0.12	0.07
1D-A125-T8	30.818	24.64	0.80			
1D-A157-T8	12.731	22.247	1.75			
1D-A159-T8	24.737	25.059	1.01			
1D-B122-T9	10.696	22.194	2.07	0.78	0.21	0.15
1D-B150-T9	23.006	21.408	0.93			
1D-B160-T9	32.309	20.462	0.63			
1D-B162-T9	11.592	17.683	1.53			
1D-A124-T24	13.385	14.372	1.07	2.43	1.02	0.51
1D-A125-T24	7.356	26.221	3.56			
1D-A157-T24	8.522	21.594	2.53			
1D-A159-T24	10.294	26.235	2.55			
1D-B122-T24	11.038	15.913	1.44	2.01	0.60	0.30
1D-B150-T24	8.93	24.896	2.79	Groups A and B together:		

ID-B160-T24	9.869	21.036	2.13	2.22	0.81	0.29
ID-B162-T24	10.606	17.599	1.66			
ID-C144-T0	10.439	27.261	2.61	2.06	0.39	0.20
ID-C145-T0	13.44	22.851	1.70			
ID-C148-T0	10.347	20.859	2.02			
ID-C149-T0	10.736	20.428	1.90			
ID-C144-T2	11.631	26.111	2.24	2.55	0.55	0.28
ID-C145-T2	11.876	32.033	2.70			
ID-C148-T2	12.005	24.069	2.00			
ID-C149-T2	7.131	23.226	3.26			
ID-C144-T4	10.813	26.318	2.43	2.19	0.68	0.34
ID-C145-T4	11.604	27.444	2.37			
ID-C148-T4	15.85	19.121	1.21			
ID-C149-T4	8.983	24.777	2.76			
ID-C144-T8	11.354	27.598	2.43	1.88	0.46	0.23
ID-C145-T8	14.602	29.585	2.03			
ID-C148-T8	15.089	20.639	1.37			
ID-C149-T8	14.279	23.933	1.68			
ID-C144-T24	11.394	22.722	1.99	2.33	0.28	0.14
ID-C145-T24	11.223	24.992	2.23			
ID-C148-T24	9.257	24.612	2.66			
ID-C149-T24	10.996	26.712	2.43			

[000373] The mean of the C3/C2 ratios for the treated and untreated group and the standard error of the mean (SEM) values are shown in Table 23. Note that samples were not taken from the untreated group at 3 (T3), 6 (T6), and 9 (T9) hours.

Table 23. Mean of C3/C2 ratios in treated and untreated groups

time [h]	TAT-PCCAB	SEM	PBS	SEM
0	2.37	0.14	2.06	0.20
2	2.26	0.48	2.55	0.28
3	2.21	0.08	NA	NA
4	1.23	0.02	2.19	0.34
6	1.29	0.07	NA	NA
8	0.87	0.07	1.88	0.23
9	0.78	0.15	NA	NA
24	2.43	0.51	2.33	0.14

[000374] As evidenced by these results, four hours following a single injection of TAT-PCCAB, the C3/C2 ratio decreased for a period of 9 hours. After 9 hours, the ratio began to increase. The mice having a single IP injection experienced a steady and significant decrease in C3/C2 ratio compared to controls within 9 hours post injection with the lowest ratio. Within 24 hours, the C3/C2 ratios returned to starting levels. The results are shown in Figure 8.

VII. EQUIVALENTS AND SCOPE

[000375] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with

the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[000376] In the claims, articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or the entire group members are present in, employed in, or otherwise relevant to a given product or process.

[000377] It is also noted that the term “comprising” is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term “comprising” is used herein, the term “consisting of” is thus also encompassed and disclosed.

[000378] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[000379] In addition, it is to be understood that any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the invention (*e.g.*, any antibiotic, therapeutic or active ingredient; any method of production; any method of use; etc.) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[000380] It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the invention in its broader aspects.

[000381] Publications, patents, and patent applications cited herein are hereby expressly incorporated by reference in their entireties.

[000382] While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with reference to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

CLAIMS

1. A pharmaceutical composition comprising an isolated human PCCAB dodecamer having a post-translational modification comprising a cell penetrating peptide or mitochondria penetrating peptide.
2. The pharmaceutical composition of claim 1, wherein the PCCAB dodecamer is not conjugated to a purification tag.
3. The pharmaceutical composition of claim 1 or 2, wherein the PCCAB dodecamer comprises a PCCA subunit comprising the amino acid sequence of SEQ ID NO:41, or a fragment or a variant thereof sharing a sequence similarity with SEQ ID NO:41 of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.
4. The pharmaceutical composition of any one of claims 1-3, wherein the PCCAB dodecamer comprises a PCCB subunit having the amino acid sequence of at least one sequence selected from the group consisting of: SEQ ID NO:43, or a fragment or a variant thereof sharing a sequence similarity with SEQ ID NO:43 of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.
5. The pharmaceutical composition of claims 3 or 4, wherein a nucleic acid sequence encoding the PCCA subunit and/or the PCCB subunit is codon optimized for recombinant cell expression.
6. The pharmaceutical composition of any one of claims 3-5, wherein a nucleic acid sequence encoding the PCCA subunit is SEQ ID NO:40, or a fragment or a variant thereof sharing a sequence similarity with SEQ ID NO:40 of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.
7. The pharmaceutical composition of any one of claims 4-6, wherein a nucleic acid sequence encoding the PCCB subunit is SEQ ID NO:42, or a fragment or a variant thereof sharing a sequence similarity with SEQ ID NO:42 of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.

8. A pharmaceutical composition for treating PCC-deficiency, the composition comprising a therapeutically effective amount of an isolated human PCC enzyme or a variant thereof, the PCC enzyme or the variant therefore comprising at least one member selected from the group consisting of an isolated propionyl-CoA carboxylase alpha chain protein (PCCA) subunit, and an isolated propionyl-CoA carboxylase beta chain protein (PCCB) subunit, each independently or in combination with a pharmaceutically acceptable carrier, diluent, or excipient.
9. The pharmaceutical composition of claim 8, wherein the PCCA subunit comprises the amino acid sequence of SEQ ID NO:2, or a fragment or a variant thereof sharing a sequence similarity with SEQ ID NO:2 of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.
10. The pharmaceutical composition of claim 8, wherein the PCCB subunit comprises the amino acid sequence of SEQ ID NO:4, or a fragment or a variant thereof sharing a sequence similarity with SEQ ID NO:4 of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.
11. The pharmaceutical composition of any one of claims 3-9, wherein the PCCA subunit comprises a mitochondrial leader sequence.
12. The pharmaceutical composition of any one of claims 8, wherein the PCCA subunit does not comprise a mitochondrial leader sequence.
13. The pharmaceutical composition of any one of claims 4-10, wherein the PCCB subunit comprises a mitochondrial leader sequence.
14. The pharmaceutical composition of any one of claims 8, wherein the PCCB subunit does not comprise a mitochondrial leader sequence.
15. The pharmaceutical composition of any one of claims 8-9 or 11-12, wherein the PCCA subunit comprises at least one selected from the group consisting of SEQ ID NOs:48, 50, and 52.

16. The pharmaceutical composition of any one of claims 8, 10, or 13-14, wherein the PCCB subunit comprises at least one sequence selected from SEQ ID NOs:49 and 51.
17. The pharmaceutical composition of any one of claims 8-16, wherein the PCCA subunit and/or the PCCB subunit comprises at least one mutation in a region of the protein relative to naturally occurring PCCA or PCCB to facilitate penetration of the PCCA subunit and/or the PCCB subunit into mitochondria.
18. The pharmaceutical composition of any one of claims 8-9, 11-12, 15, or 17, wherein the PCCA subunit is covalently linked to one or a plurality of cell penetrating peptides or mitochondria penetrating peptides.
19. The pharmaceutical composition of any one of claims 8, 10, 13-14, or 16-17, wherein the PCCB subunit is covalently linked to one or a plurality of cell penetrating peptides or mitochondria penetrating peptides.
20. The pharmaceutical composition of any one of claims 8-19, wherein the PCCA and the PCCB both are covalently linked to one or a plurality of cell penetrating peptides or mitochondria penetrating peptides.
21. The pharmaceutical composition of claims 6-7 or 18-20, wherein the cell penetrating peptide or mitochondria penetrating peptide is derived from a protein selected from the group consisting of a trans-activating transcriptional activator (TAT), mitochondria penetrating proteins (MPP), antennapedia, herpes simplex virus type 1 protein VP22, penetratin, transportan, amphipathic protein MPG, Pep-1, MAP, SAP, PPTG1, poly-Arginine sequence, hCT, SynB, Pvec, and a tissue specific variant thereof.
22. The pharmaceutical composition of any one of claims 3-9, 11-12, 15, 17, or 20-21, wherein the PCCA subunit is produced recombinantly.
23. The pharmaceutical composition of any one of claims 4-8, 10, 13-14, 16-17, or 19-21, wherein the PCCB subunit is produced recombinantly.

24. The pharmaceutical composition of claims 22-23, wherein the PCCA subunit and/or the PCCB subunit is produced in prokaryotic cells.
25. The pharmaceutical composition of claims 22-23, wherein the PCCA subunit and/or the PCCB subunit are produced in eukaryotic cells.
26. The pharmaceutical composition of claim 24, wherein the prokaryotic cells are *E. coli*.
27. The pharmaceutical composition of claim 25, wherein the eukaryotic cells are yeast or mammalian cells.
28. The pharmaceutical composition of any one of claims 8-27, further comprising a His-tag at the C-terminal end and/or the N-terminal end of the PCCA subunit or the PCCB subunit.
29. The pharmaceutical composition of claim 18-28, wherein the mitochondria penetrating peptide is a MPP1A or MPP2A.
30. The pharmaceutical composition of any one of claims 18-29, wherein the cell penetrating peptide is less than 100 amino acids, less than 50 amino acids, less than 30 amino acids, and less than 10 amino acids.
31. The pharmaceutical composition of any one of claims 1-30, further comprising at least one pharmaceutically acceptable salt or excipient.
32. A method for reducing propionyl-CoA levels in a PCC deficient subject comprising administering the pharmaceutical composition of any one of claims 1-31.
33. The method of claim 32, wherein the pharmaceutical composition is administered by intravenous injection (IV), subcutaneous injection (SC), or intraperitoneal injection (IP).
34. The method of claim 32, wherein the pharmaceutical composition is administered by intraperitoneal injection (IP).

35. A method for elevating propionyl-CoA carboxylase, in a subject in need thereof comprising administering to said individual the pharmaceutical composition of any one of claims 1-31.
36. A method for treating or ameliorating a disease, disorder, or condition in a subject, the disease, disorder, or condition being associated with elevated propionyl CoA, propionic acid, methylcitrate, beta-hydroxy-propionate, propionylglycine, tiglic acid, and/or ketones comprising administering to an individual in need thereof a pharmaceutically effective amount of the pharmaceutical composition of any of claims 1-31.
37. The method of claim 36, wherein the subject presents with at least one symptom selected from the group consisting of poor feeding, vomiting, and somnolence, lethargy, seizures, coma, metabolic acidosis, anion gap, ketonuria, hypoglycemia, hyperammonemia, cytopenias, developmental regression, chronic vomiting, protein intolerance, failure to thrive, hypotonia, basal ganglia infarction, dystonia, choreoathetosis, and cardiomyopathy.
38. The method of any one of claims 32-37, wherein administering of the pharmaceutical composition occurs in a concentration of at least 0.5 μ M, at least 1 μ M, at least 2 μ M, at least 5 μ M, and at least 10 μ M.
39. The method of any one of claims 32-38, wherein administering occurs at least once a day, at least twice a day, at least three times a day, and at least 4 times a day.
40. The method of any one of claims 31-38, wherein the administering step is repeated for at least 2 days.
41. A method of producing the PCCA subunit or the PCCB subunit of any one of claims 1-31 comprising recombinantly producing a PCCA precursor or a PCCB precursor including a cell penetrating peptide or mitochondria penetrating peptide.
42. The method of claim 41, wherein the PCCA precursor is encoded by the nucleic acid sequence of SEQ ID NO:1.

43. The method of any one of claims 41-42, wherein the PCCB is encoded by the nucleic acid sequence of SEQ ID NO:3.
44. A method of producing the pharmaceutical composition of any one of claims 1-7, the method comprising:
 - (a) transfecting cells with an expression vector encoding PCCAB and at least one molecular chaperone, and
 - (b) conjugating a cell penetrating peptide or mitochondria penetrating peptide to the PCCAB dodecamer post-translationally, thereby producing the pharmaceutical composition of any one of claims 1-7.
45. The method of claim 44, wherein the molecular chaperone is a GroEL or a GroES protein.
46. The method of claim 44 or 45, wherein the PCCAB comprises a PCCA subunit and a PCCB subunit.
47. The method of any one of claims 44-46, wherein the expression vector comprises a nucleic acid sequence encoding the PCCA subunit comprising SEQ ID NO:40, or a fragment or a variant thereof sharing a sequence similarity with SEQ ID NO:41 of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.
48. The method of any one of claims 44-47, wherein the expression vector comprises a nucleic acid sequence encoding the PCCB subunit comprising SEQ ID NO:42, or a fragment or a variant thereof sharing a sequence similarity with SEQ ID NO:43 of at least 99%, at least 95%, at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.
49. The method of any one of claims 44-48, further comprising formulating the pharmaceutical composition with at least one of a pharmaceutically acceptable salt or excipient.
50. The method of any of claims 44-46, wherein the PCCA subunit comprises an amino acid sequence beginning at residue 52 of SEQ ID NO:2.

51. The method of any of claims 44-46, wherein the PCCB subunit comprises an amino acid sequence beginning at residue 29 or SEQ ID NO:4.

Figure 1

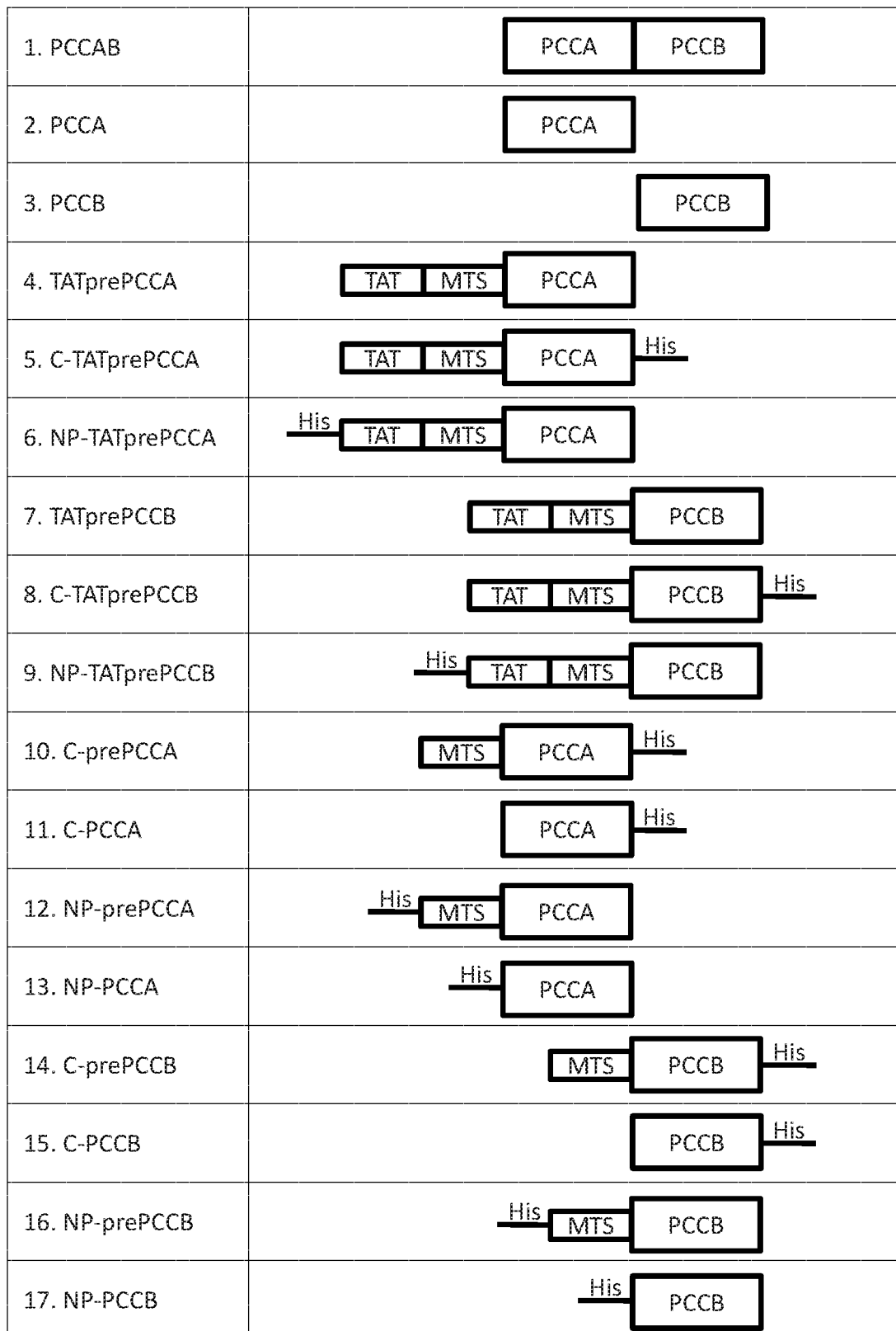


Figure 2

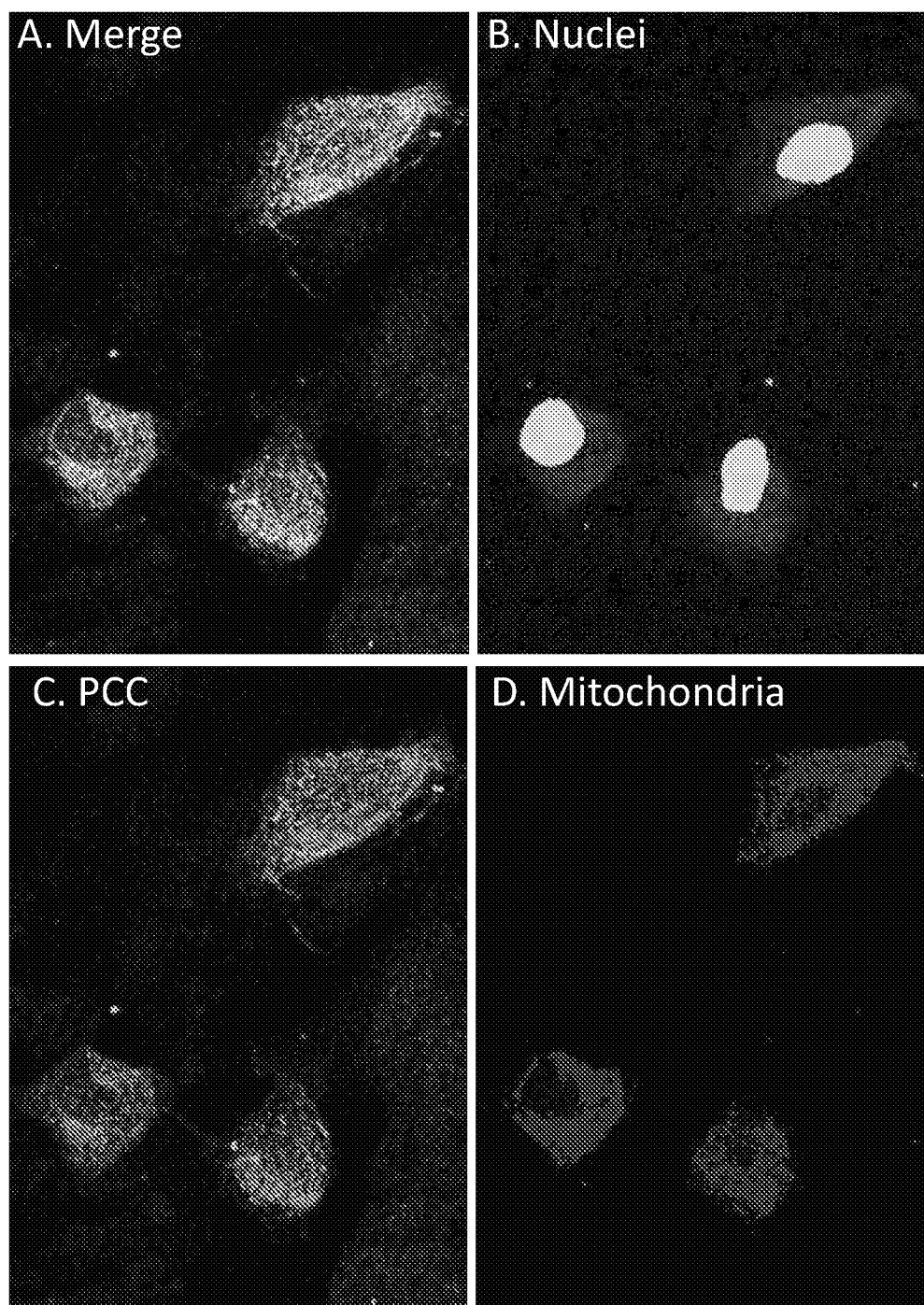


Figure 3

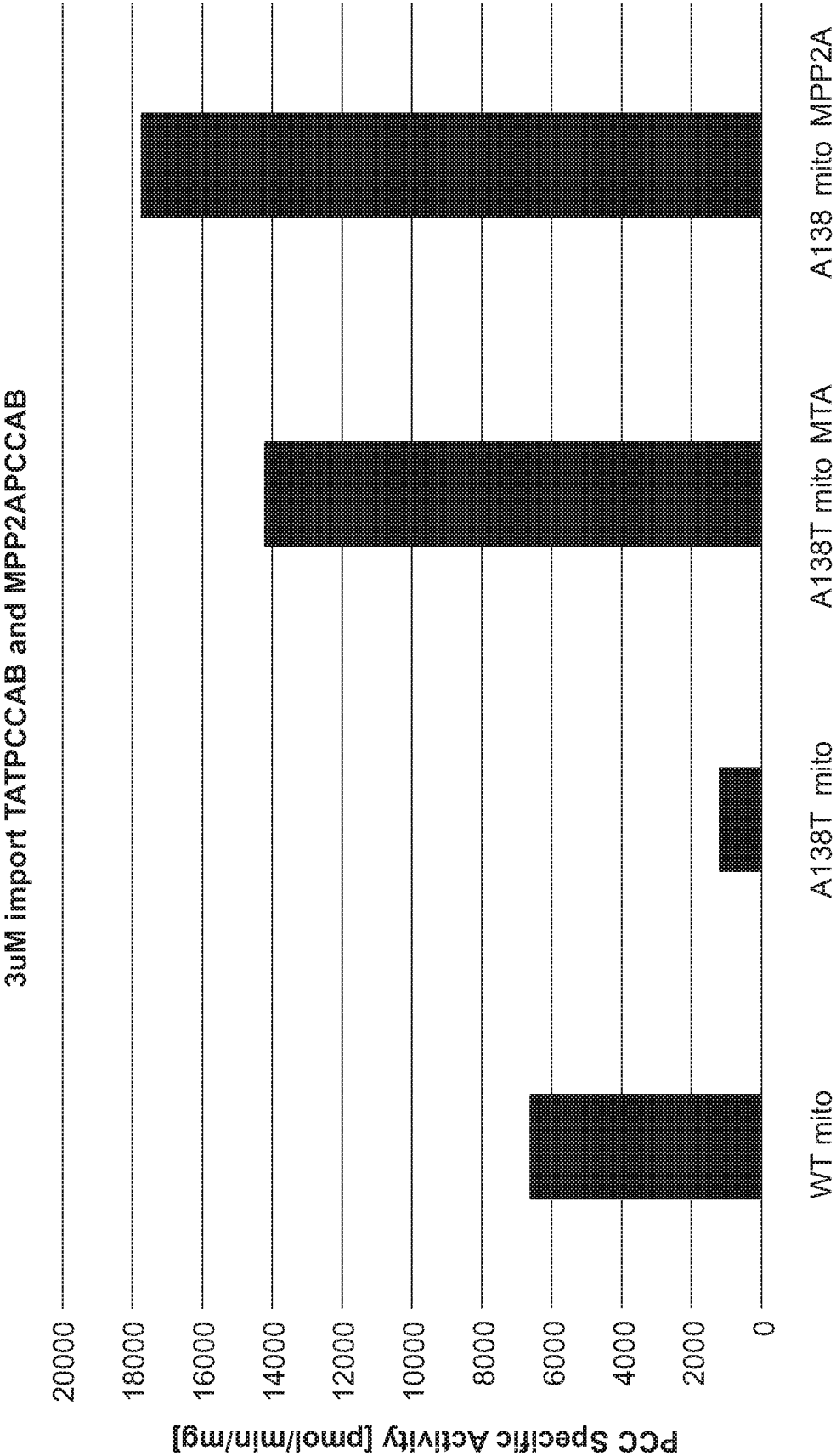


Figure 4

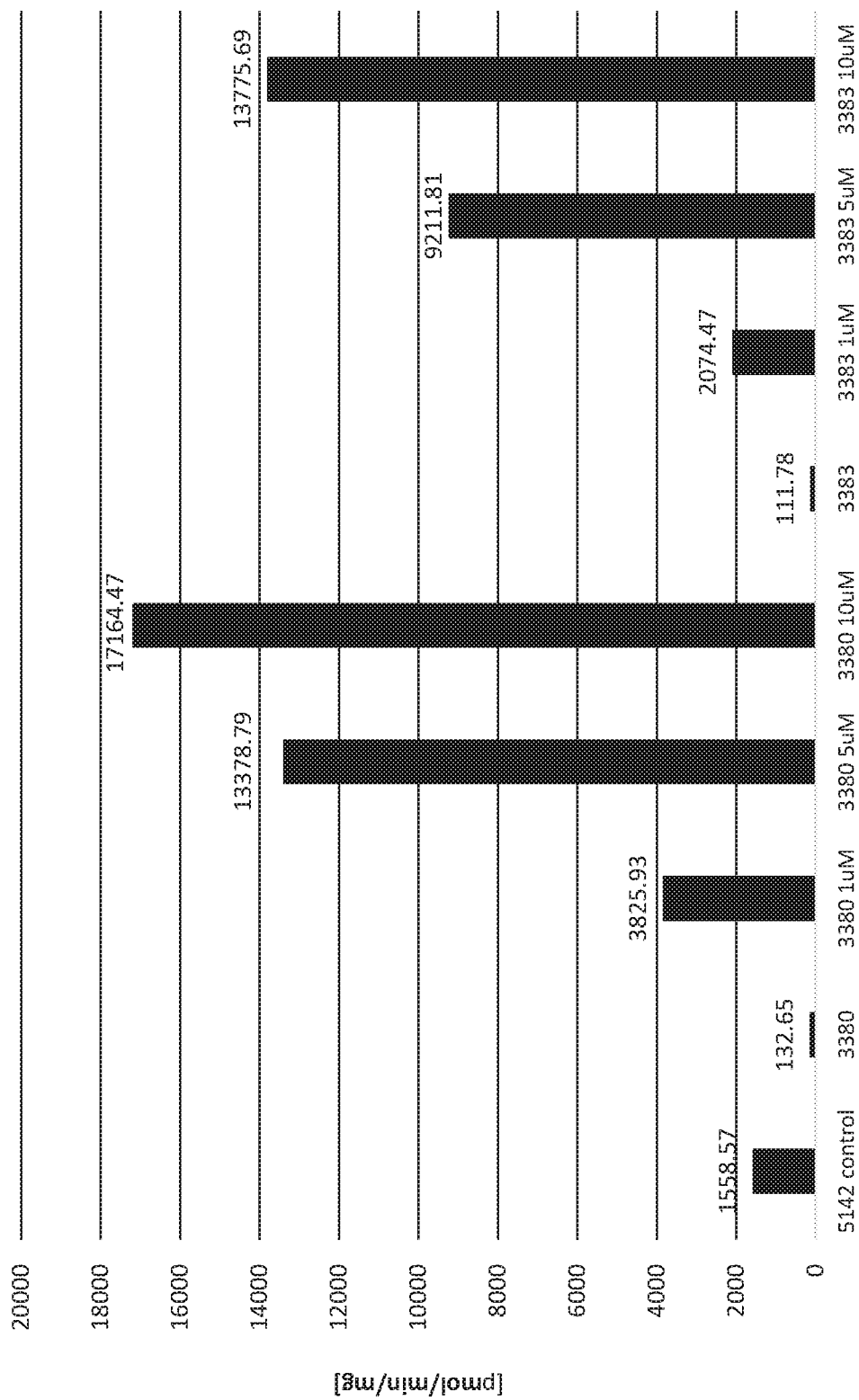


Figure 5

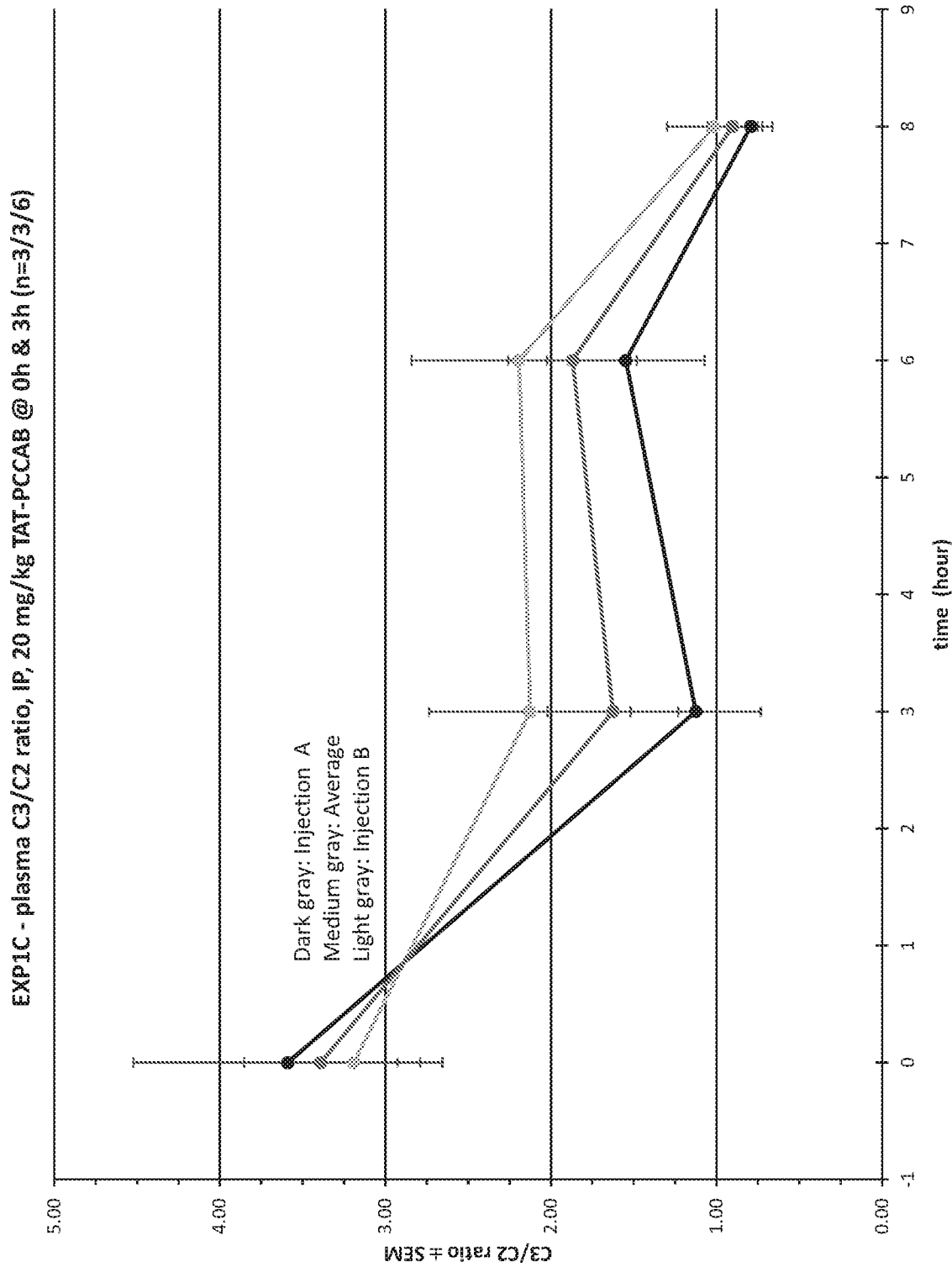


Figure 6

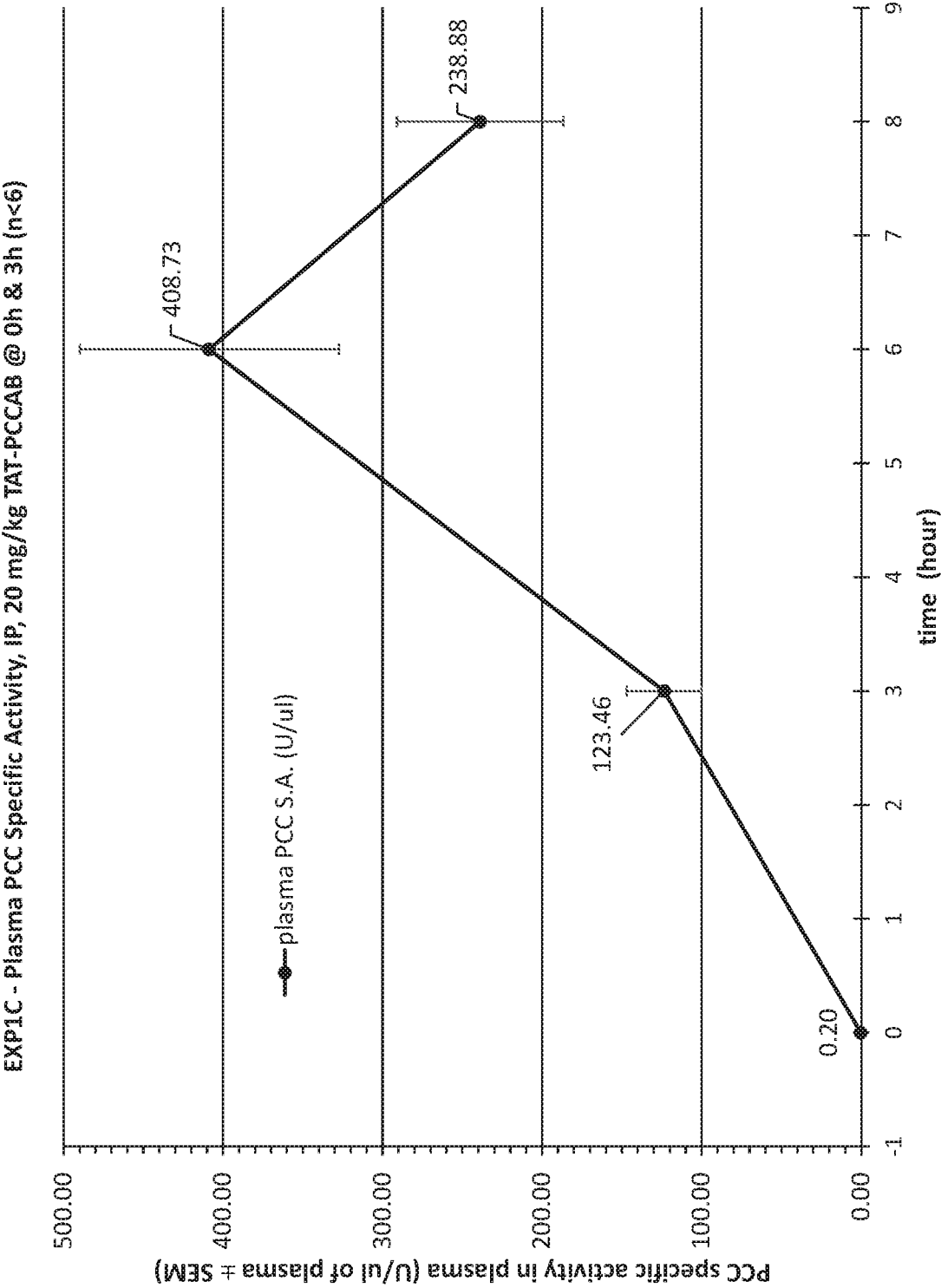


Figure 7

EXP1C - liver & heart PCC specific activity, IP, 20 mg/kg TAT-PCCAB @ 0h & 3h

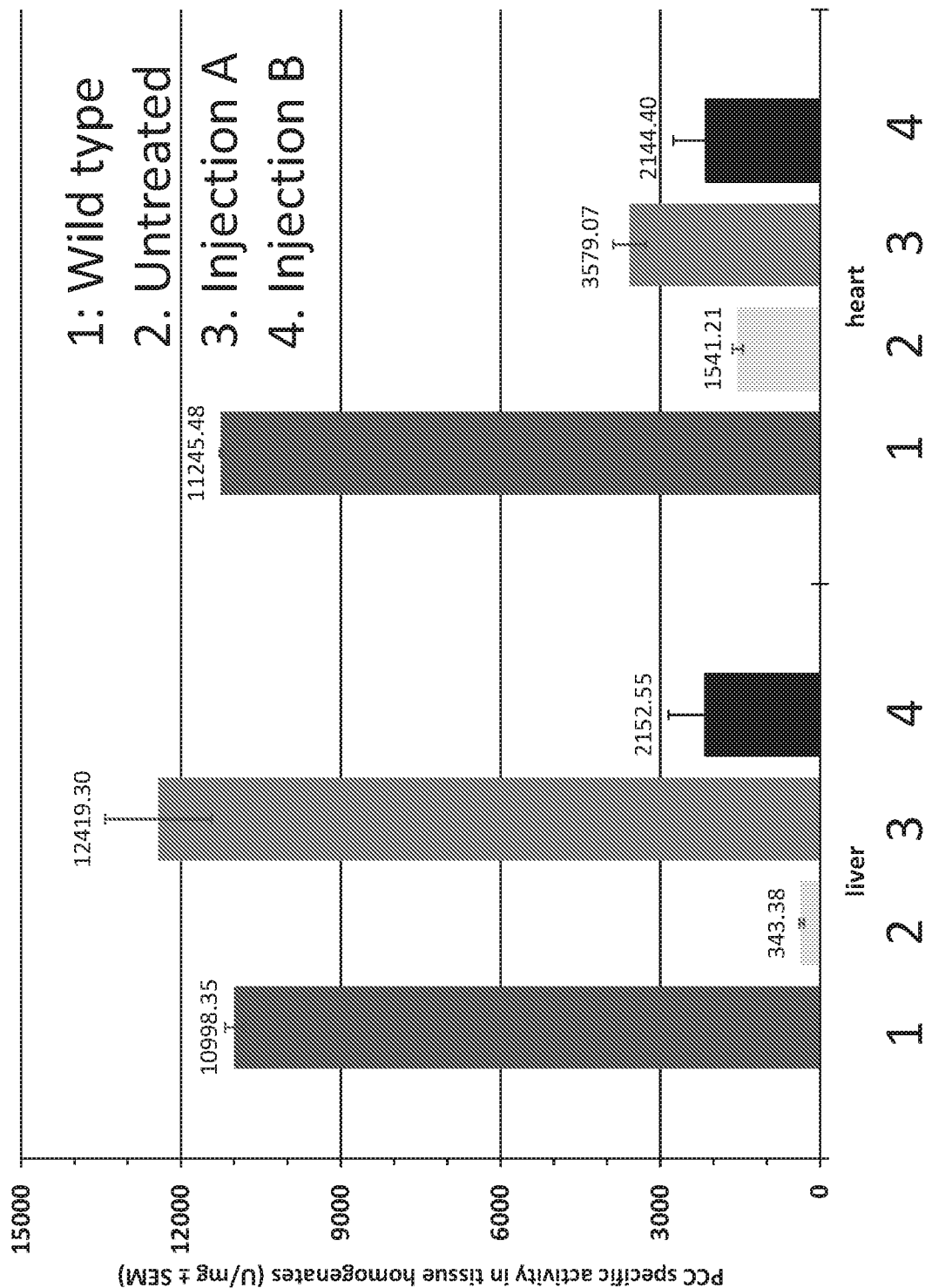


Figure 8

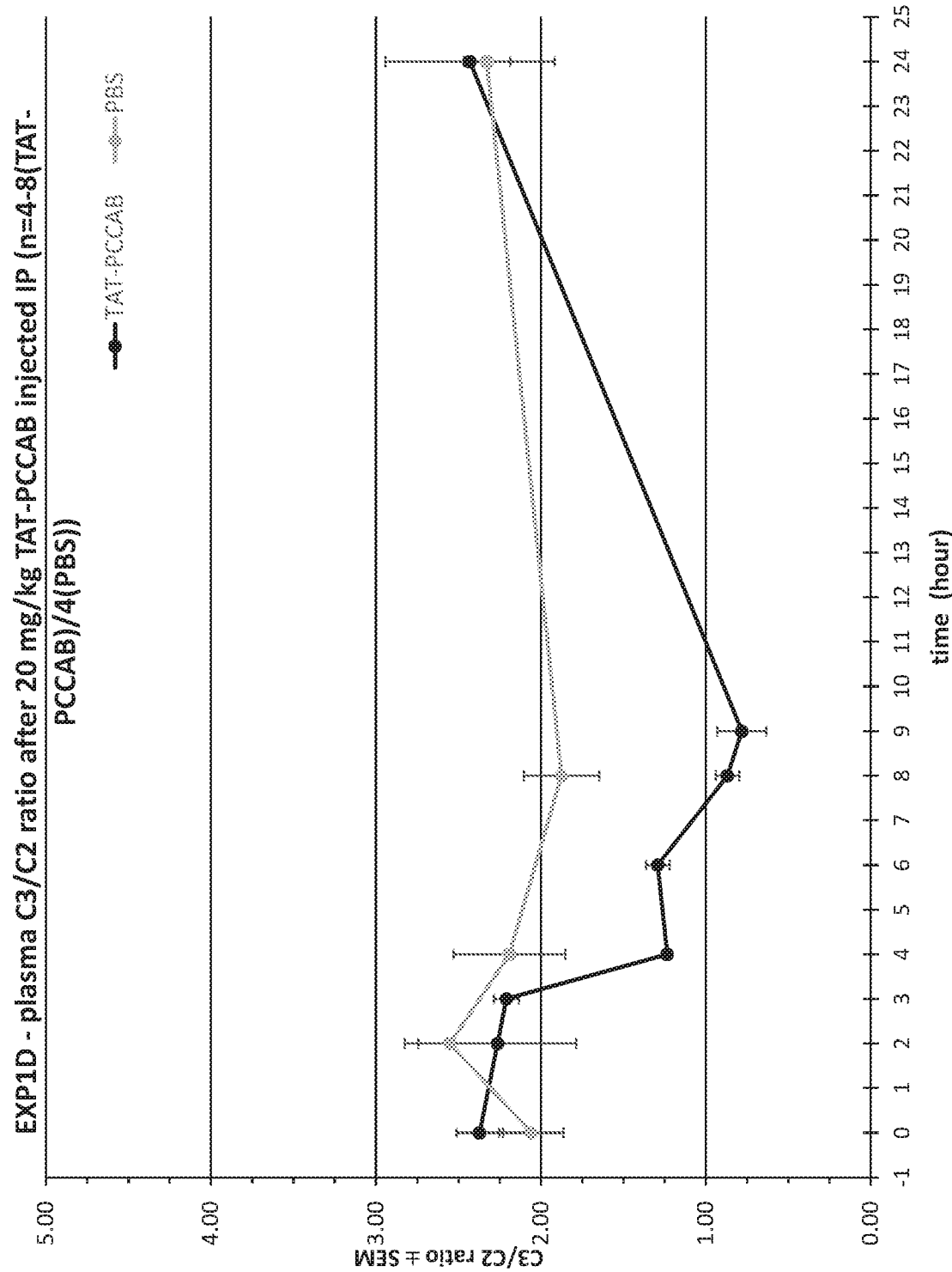


Figure 9

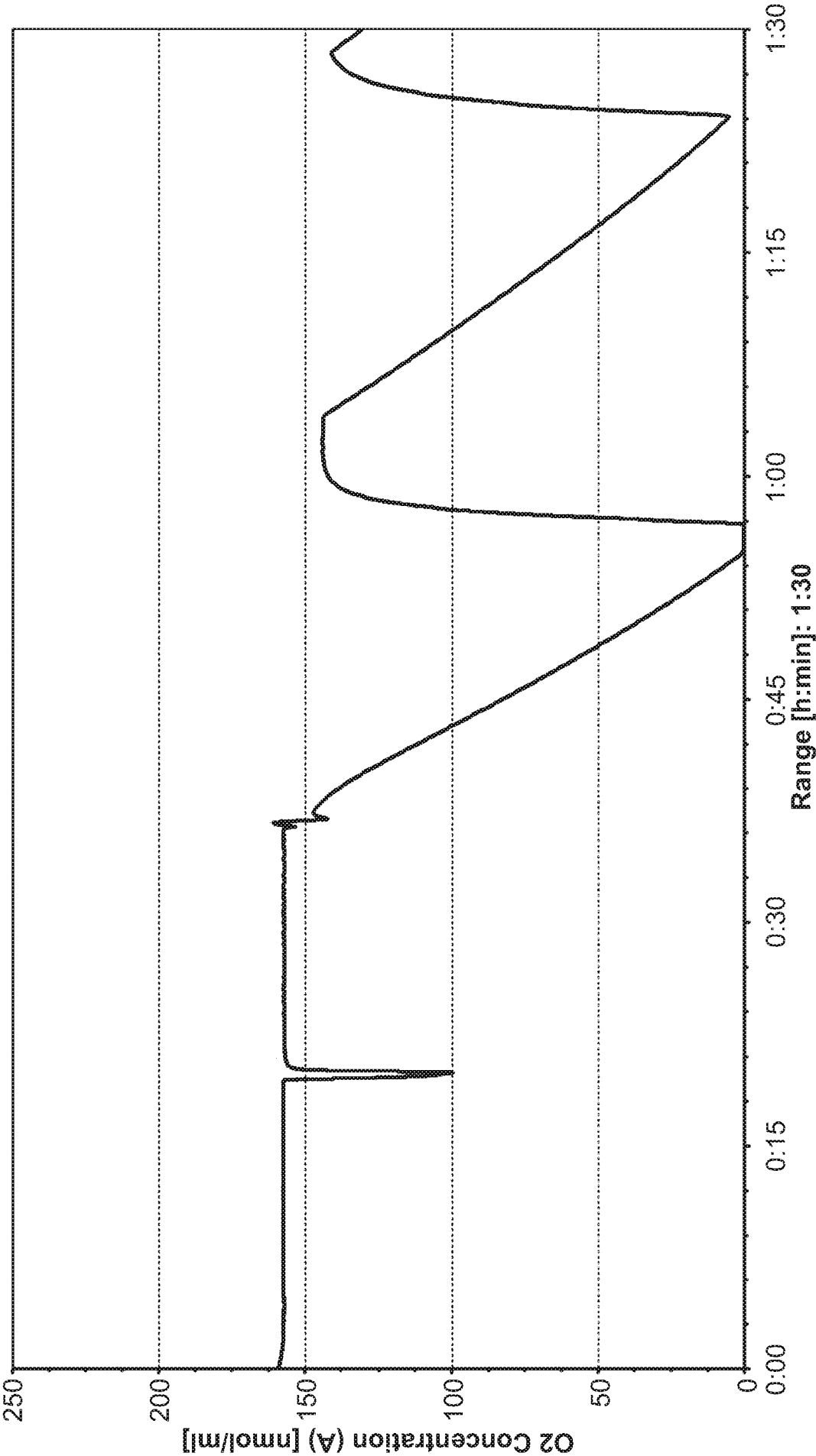


Figure 10

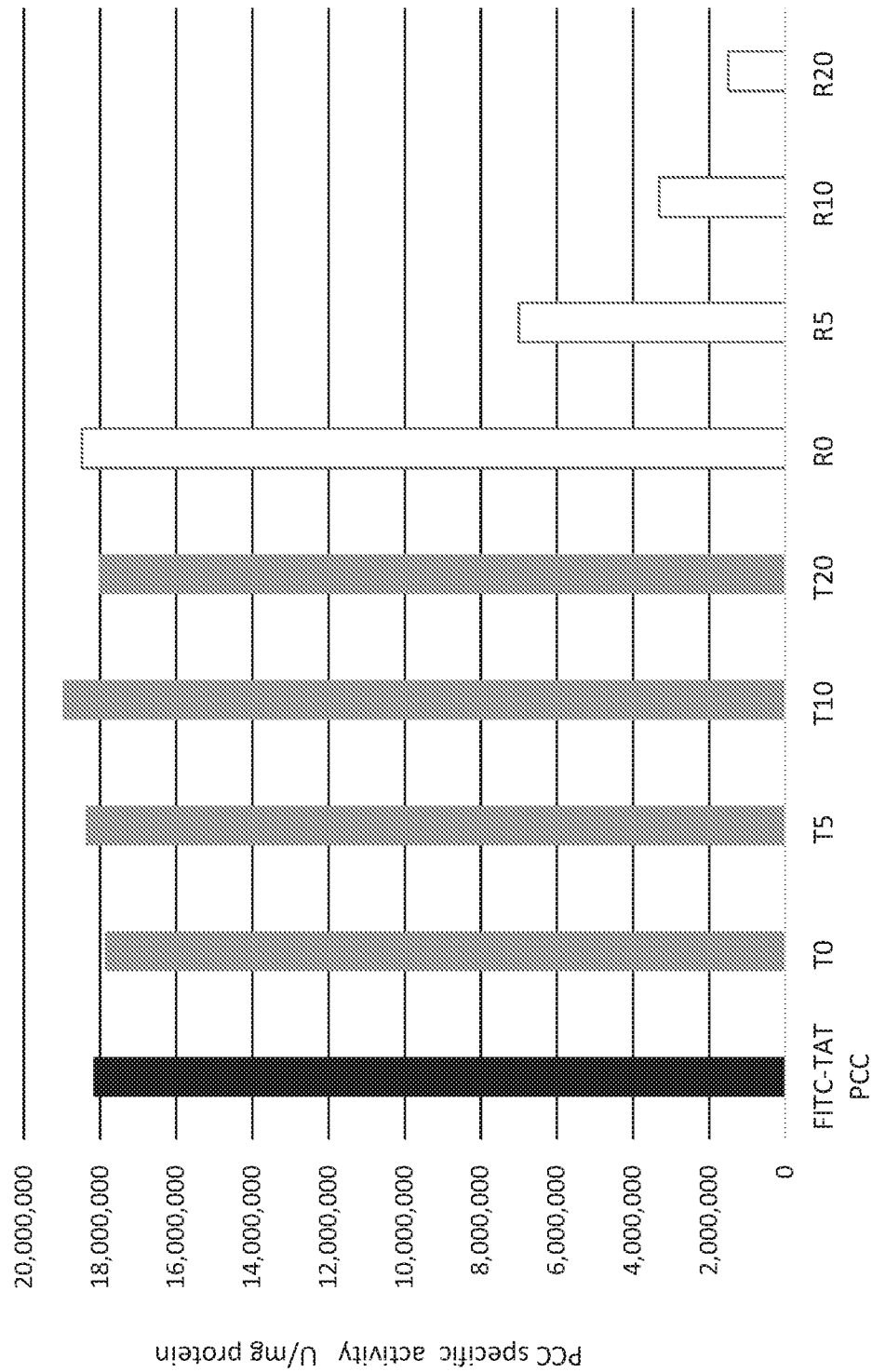


Figure 11

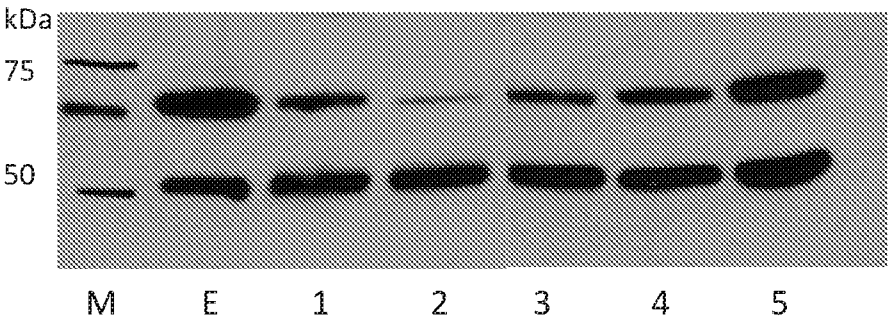


Figure 12

Mitochondria penetration peptides import

TAT-PCC and MPP1A-PCC and MPP2A import in patients fibroblast

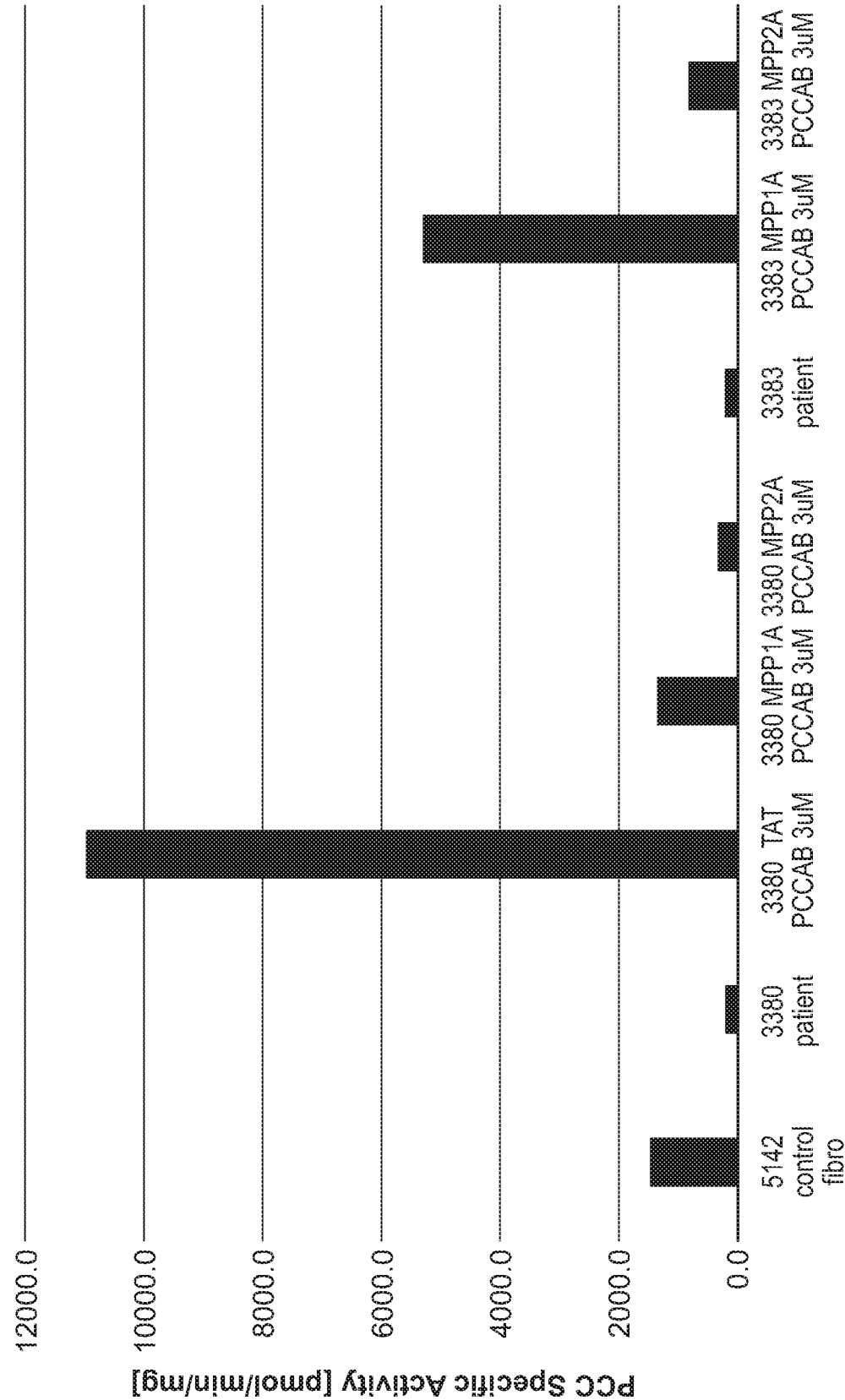
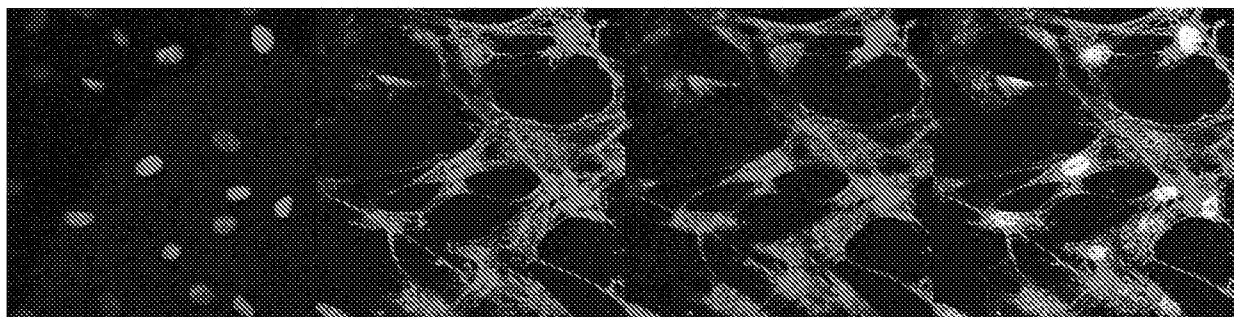


Figure 13



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/030904

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/00; C07K 14/47; C12N 9/00; C12N 15/11; C12N 15/12 (2017.01)

CPC - A61K 38/00; A61K 38/43; C07K 14/47; C12N 15/11; C12N 15/52 (2017.02)

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)

See Search History document

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

USPC - 514/12; 530/350; 536/23.2 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/0101874 A1 (GHOSH et al) 27 May 2004 (27.05.2004) entire document	8-10, 12, 14
A	CHLOUPKOVA et al. "Propionic acidemia: analysis of mutant propionyl-CoA carboxylase enzymes expressed in Escherichia coli," Hum Mutat, 01 June 2002 (01.06.2002), Vol. 19, Pgs. 629-640. entire document	1-3, 8-10, 12, 14
A	US 2015/0343095 A1 (GEMVAX & KAELE CO., LTD) 03 December 2015 (03.12.2015) entire document	1-3, 8-10, 12, 14
A	KELSON et al. "Chaperonin-mediated assembly of wild-type and mutant subunits of human propionyl-CoA carboxylase expressed in Escherichia coli," Hum Mol Genet, 01 March 1996 (01.03.1996), Vol. 5, Pgs. 331-337. entire document	1-3, 8-10, 12, 14
A	WO 2015/017519 A1 (PHASERX, INC.) 05 February 2015 (05.02.2015) entire document	1-3, 8-10, 12, 14

☐ 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

17 July 2017

Date of mailing of the international search report

01 AUG 2017

Name and mailing address of the ISA/US

Mall Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/030904

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

- a. ☒ forming part of the international application as filed:
☒ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOS: 2, 4, and 41 were searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/030904

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-7, 11, 13, 15-25, 27-51
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.