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(71) Applicant: **THE REGENTS OF THE UNIVERSITY OF MICHIGAN** [US/US]; 1600 Huron Parkway, 2nd Floor, Ann Arbor, Michigan 448109 (US).

(72) Inventors: **ARVAN, Peter**; 1600 Huron Parkway, 2nd Floor, Ann Arbor, Michigan 48109 (US). **LIU, Ming**; 1600 Huron Parkway, 2nd Floor, Ann Arbor, Michigan 48109 (US).

(74) Agent: **STAPLE, David W.**; 2275 Deming Way, Ste 310, Middleton, Wisconsin 53562 (US).

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(54) Title: STABILIZING PROINSULIN MONOMERS

(57) Abstract: Provided herein are compositions and methods for the protection of the proinsulin dimerization surface, and the treatment of poor insulin production, pancreatic beta cell failure, and diabetes therewith. In particular, misfolded proinsulin is prevented from propagating its deleterious effects by inhibiting the dimerization of proinsulin in general and misfolded proinsulin specifically.

STABILIZING PROINSULIN MONOMERS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present invention claims the priority benefit of U.S. Provisional Patent Application 62/328,910, filed April 28, 2016, which is incorporated by reference in its entirety.

STATEMENT REGARDING FEDERAL FUNDING

This invention was made with government support under grant numbers DK048280 and DK088856 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD

Provided herein are compositions and methods for the stabilization of proinsulin monomers for the treatment of poor insulin production, pancreatic beta cell failure, and diabetes therewith. In particular, misfolded proinsulin is prevented from propagating its deleterious effects by inhibiting the dimerization of proinsulin in general and misfolded proinsulin specifically.

BACKGROUND

Classically, dimerization/oligomerization in the endoplasmic reticulum (ER) promotes anterograde intracellular transport of secretory proteins (refs. 1, 2; incorporated by reference in their entireties), but in some cases may form the basis for ER retention, leading to disease (ref. 3; incorporated by reference in its entirety). In patients with Mutant *INS*-gene-induced Diabetes of Youth (MIDY), insulin deficiency is initiated by an attack of misfolded mutant proinsulin on bystander wild-type (WT) proinsulin in the ER (refs. 4,5; incorporated by reference in their entireties). The ER is the site of dimerization of proinsulin (ref. 6; incorporated by reference in its entirety).

Diabetes mellitus type 2 is a long term metabolic disorder that is characterized by high blood sugar and insulin resistance. Pancreatic β-cells increase insulin production/secretion to compensate for the insulin resistance, to and maintain normoglycemia. Stress stemming from β-cell overactivity typically leads to β-cell dysfunction, failure, and death of β-cells.

SUMMARY

Provided herein are compositions and methods for the stabilization of proinsulin monomers, and the treatment of poor insulin production, pancreatic beta cell failure, and diabetes therewith. In particular, misfolded proinsulin is prevented from propagating its deleterious effects by protecting the dimerization surface of proinsulin in general and misfolded proinsulin specifically.

In some embodiments, provided herein are methods of treating diabetes (e.g., type II diabetes, MIDY, etc.) in a subject comprising protecting the proinsulin dimerization surface. In some embodiments, protecting the dimerization surface of proinsulin comprises administering a pharmaceutical composition to the subject. In some embodiments, the pharmaceutical composition binds to the dimerization surface of proinsulin polypeptides and may inhibit the formation of dimers. In some embodiments, the pharmaceutical composition binds to the dimerization surface of misfolded proinsulin polypeptides. In some embodiments, the pharmaceutical composition binds to the dimerization surface of properly folded proinsulin polypeptides. In some embodiments, the pharmaceutical composition is a small molecule, peptide, peptide mimetic, or antibody or fragment thereof. In some embodiments, the pharmaceutical composition is a small molecule that may be chemically related to camptothecin, 7-methoxycamptothecin, 11-methoxycamptothecin, 7- CH_3Cl-camptothecin, irinotecan, pinafide, and derivatives thereof.

In some embodiments, provided herein are pharmaceutical compositions comprising a proinsulin dimerization surface protector (PDSP). In some embodiments, the protector is a small molecule, peptide, peptide mimetic, or antibody or fragment thereof. In some embodiments, the protector binds to the dimerization surface. In some embodiments, the protector is a small molecule selected from camptothecin, 7-methoxycamptothecin, 11-methoxycamptothecin, 7- CH_3Cl-camptothecin, irinotecan, pinafide, and derivatives thereof.

In some embodiments, provided herein are proinsulin dimerization surface protectors for use in treating diabetes.

In some embodiments, provided herein is the use of a proinsulin dimerization surface protector for the manufacture of a medicament for treating diabetes.

Additional embodiments within the scope herein are described in the Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Proinsulin B-chain tyrosine-16 plays an important role in proinsuin dimerization. **A.** Ribbon diagram shows two insulin molecules in dimer configuration. Along the B-chain helix, the aromatic Tyr(B16) is nearly central to the dimerization interface, protruding from one (pro)insulin monomer towards the opposing (pro)insulin monomer. **B.** From the insulin crystal structure (Protein Data Bank code 2R34) residues at the dimerization surface, identified via the protein-protein interface comparison server (PROTORP) were calculated for their fractional contribution to the accessible surface area (ASA) of each residue along the dimerization surface. **C.** and **D.** 293T cells co-transfected with the plasmids encoding proinsulin(PI)-WT or hProCpepSFGFP-C(A7)Y or C(A7)Y/Y(B16)D at a DNA ratio of 1:1. The cells were labeled with ^{35}Met/Cys for 30 min, lysed with co-immunoprecipitation buffer, and split in half. One half was immunoprecipitated with anti-insulin, the other half was co-immunoprecipitated with anti-GFP, and the proinsulin co-immunoprecipitated with hProCpepGFP mutants was analyzed in the panel C and quantified from 3 independent experiments in the panel D. **E.** 293T cells were co-transfected with the plasmids encoding human PI-WT and mouse PI-WT or mutants as indicated. The secretion of human PI-WT in the presence of mouse WT or mutant proinsulin was measured using human PI-specific RIA. **F.** 293T cells were co-transfected with the plasmids encoding untagged PI-WT and Myc-tagged PI-WT or mutants as indicated. At 48 h post-transfection, the cells were labeled with ^{35}S-Met/Cys for 30 min with 0 or 2 h chase as indicated. The chase media ("M") were collected and cells ("C") were lysed. The biosynthesis and secretion of WT and mutant proinsulin were analyzed by NuPage under reducing conditions.

Fig. 2. Mutating Tyr(B16) in proinsulin-C(A7)Y partially restores insulin production from co-expressed proinsulin-WT and alleviates beta cell ER stress. **A.** Rat insulinoma INS1 cells were transfected with plasmids encoding ProCpepSFGFP-WT or mutants. 48 h post-transfection, the cells were fixed, permeabilized, immunoblotted with anti-insulin, and visualized by confocal microscope. Both ProCpepSFGFP-WT and Y(B16)D were co-localized with endogenous insulin. The cells expressing ProCpepSFGFP-C(A7)Y (arrow) showed a decrease of endogenous insulin. However, in the cells expressing ProCpepSFGFP-C(A7)Y/Y(B16)D (arrow head), endogenous insulin was not affected. **B.** MIN6 cells were co-transfected with plasmids encoding human proinsulin-WT with either mouse proinsulin-WT or mutants. At 48 h post-transfection, human insulin content in the transfected cells was measured using human insulin specific RIA. **C.** MIN6 cells were triple-transfected with

plasmids encoding BiP-firefly Luciferase and CMV-renilla luciferase plus proinsulin-WT or mutants. 48 h post-transfection, luciferase activities in transfected cells were measured using dual-luciferase assay.

Fig. 3. A PDSP docks with the proinsulin dimerization interface, alleviates trans-dominant negative effect of C(A7)Y, and increases insulin production from Akita islets. **A.** 293T cells were co-transfected with plasmids encoding human proinsulin-WT and mouse proinsulin-C(A7)Y. At 30 h post-transfection, the cells were incubated individually with one of a series of putative PDSPs for 16 h. Secreted human proinsulin-WT was measured by human proinsulin specific RIA. **B.** 3D conformer of 11-MCPT from Pubchem Compound. **C.** and **D.** 11-MCPT docks with the insulin dimerization surface predicted by the Protein-Protein Interaction Server. **E.** and **F.** The islets isolated from *Akita* mice were pretreated with 3 μg/mL 11-MCPT for 24 h. The islets were then pulse-labeled with ^{35}S-Met/Cys for 12 min with 0 or 2 h chase as indicated. The newly synthesized proinsulin and processed insulin were analyzed in panel E, and quantified in panel F. **G.** and **H.** The islets isolated from *Akita* mice were incubated with 3 μg/mL 11-MCPT for 24 h. The steady state levels of proinsulin and insulin content in the islets were then analyzed by anti-insulin western blotting in panel G, and quantified in panel H.

Fig. 4. A PDSP alleviates the development and progression of diabetes in *Akita* mice. **A.** 6-8 week old male diabetic *Akita* mice were randomly separated into two groups based on their body weight and blood glucose. The mice were given 11-MCPT (1mg/kg body weight) or vehicle (5% DMSO in saline) daily by intraperitoneal injection for 28 days. The fasting blood glucose levels were monitored once a week. **B.** and **C.** On the 21st day of 11-MCPT treatment, the mice were fasted overnight followed by orally administrated with dextrose (1g/kg body weight). Blood glucose (Panel B) and serum insulin (Panel C) were measured at 0 and 120 min after oral dextrose. **D.** After 28 days of 11-MCPT treatment, the mouse pancreases from each group were dissected, fixed, and immune-blotted with anti-insulin and anti-BiP. **E.** Islets were isolated from *Akita* mice treated with 11-MCPT (1mg/kg body weight) or vehicle for 7 days. The isolated islets were immediately fixed and processed for transmission electron microscope. **F-H.** Islets were isolated from *Akita* mice treated with 11-MCPT (1mg/kg body weight) or vehicle for 28 days. The steady state levels of proinsulin and insulin content in the islets were then analyzed by anti-insulin western-blotting in panel F, and quantified in panel G. The ratio of insulin to proinsulin is shown in panel H.

Fig. 5. Proinsulin-Y(B16)D and Y(B16)A are well folded and secreted. **A.** 293T cells were transfected with empty vector (EV) or plasmids encoding proinsulin wild-type (WT) or mutants as indicated. At 48 h post-transfection, newly synthesized proinsulin molecules were labeled with ^{35}S-Met/Cys for 30 min. The biosynthesis and folding of WT or mutant proinsulin were analyzed using tris-tricine-urea-SDS-PAGE under both non-reducing and reducing conditions. **B.** Native proinsulin with correct disulfide pairing recovered under non-reducing conditions and total synthesized proinsulin recovered under reducing conditions was quantified and calculated. The recovery of native proinsulin in the cells expressing proinsulin-WT was set up as 100%. **C.** and **D.** 293T cells were transfected and labeled as in the panel A. After labeling, the cells were chased 0 or 2 h. The chase media ("M") were collected and cells ("C") were lysed. The biosynthesis and secretion of mutant proinsulins were analyzed by NuPage under reducing conditions in C, and quantified in D.

Fig. 6. Proinsulin-C(A7)Y/Y(B16)D were misfolded and retained in the ER. **A.** 293T cells expressing proinsulin-WT or mutants were labeled with ^{35}S-Met/Cys for 30 min. The folding of proinsulin was analyzed by tris-tricine-urea-SDS-Page under both non-reducing and reducing conditions. **B.** 293T cells were transfected and labeled as in the panel A. After labeling, the cells were chased 0 or 2 h. The chase media ("M") were collected and cells ("C") were lysed. The biosynthesis and secretion of mutant proinsulins were analyzed by NuPage under reducing conditions.

Fig. 7. The PDSP, 11-MCPT, did not affect insulin level in islets from wild-type mice. **A.** and **B.** After overnight recovery, the islets isolated from wild-type mice were incubated with 3 μg/mL 11-MCPT for 24 h. The steady state levels of proinsulin and insulin content in the islets were then analyzed by anti-insulin western blotting in the panel A, and quantified in the panel B.

Fig. 8. Misfolded mutant proinsulin affecting bystander WT proinsulin, and a target to improve proinsulin ER export and insulin production. Proinsulin forms dimers in the ER. In the beta cells that co-express wild-type (WT) proinsulin and misfolded mutant proinsulin, the mutant abnormally interacts with co-expressed bystander WT proinsulin, forming heterodimers and protein aggregates, through which not only affects normal folding and ER export of WT proinsulin but also induces ER stress and beta cell death. Proinsulin dimerization surface (PDS) is the initiation site that misfolded mutant proinsulin uses to attack bystander proinsulin, leading to beta cell failure and diabetes. Targeting PDS with PDSPs limits interactions of misfolded proinsulin and bystander proinsulin, decreasing trans-

dominant effect of misfolded proinsulin, increasing proinsulin export, alleviating ER stress, increasing insulin production, and therefore retarding progression of beta cell failure and diabetes.

Fig. 9. Misfolded *Akita* mutant proinsulin bearing the B16D substitution, which diminishes proinsulin dimerization, diminishes the dominant-negative effect of the misfolded proinsulin on co-expressed bystander proinsulin-WT. Cells (293T) transfected to express Myc-tagged proinsulin-WT along with either untagged proinsulin-WT or untagged *Akita* mutant proinsulin or untagged *Akita* mutant proinsulin bearing the B16D substitution, were metabolically labeled with radioactive amino acids for 30 min followed by lysis of the cells at time 0 ("No Chase") or time 3 hr. During the 3 hour "Chase", the cells were incubated either in the absence or presence of 10 μM MG132, which inhibits proteasomal degradation. All forms of proinsulin were recovered by immunoprecipitation with an anti-insulin antibody and analyzed using tris-tricine-urea-SDS-PAGE under both non-reducing conditions (upper panel) and reducing conditions (lower panel). Note that more Myc-tagged proinsulin-WT is recovered when the misfolded *Akita* mutant proinsulin carries the B16D substitution.

Fig. 10. The dominant-negative behavior of misfolded *Akita* mutant proinsulin on wildtype (WT) proinsulin, which leads to diabetes, is ameliorated when the WT proinsulin carries the B16D substitution that diminishes proinsulin dimerization. Cells (293T) transfected to express untagged proinsulin-WT or proinsulin-B16D along with either Myc-tagged proinsulin-WT or Myc-tagged *Akita* mutant proinsulin. Each co-transfection expresses only two constructs leading to bands shown at the bottom of the gel. At 40 hour post-transfection, transfected cells were metabolically labeled with radioactive amino acids for 30 min, followed by lysis of the cells ("C") at time 0 or time 3 hr, as indicated. After 3 hr, secretion of proinsulin to the medium ("M") was also analyzed. Both cell lysates ("C") and secretion into the media ("M") were immunoprecipitated with anti-insulin followed by analysis using 4-12% NuPage gel with phosphorimaging to see the cellular or secreted proinsulin. Note that more proinsulin-WT bearing the B16D substitution can be secreted despite co-expression with misfolded *Akita* mutant proinsulin.

Fig. 11. 7-methoxy camptothecin derivatives.

Fig. 12. 7- CH_3Cl-CPT increases secretion of proinsulin-WT co-expressed with misfolded *Akita* mutant proinsulin. Cells (293T) were co-transfected with Myc-tagged proinsulin-WT and either untagged proinsulin-WT or untagged *Akita* mutant proinsulin. At the 24 hour post-transfection, the transfected cells were treated with candidate compounds

(3μg/mL, structures shown above, but 7-methoxy camptothecin was not examined in this experiment) for 16 hours. At the end of this time, the media were collected and the cells were lysed. The cell lysates (left panel) and media (right panel) were analyzed by immunoblotting with anti-Myc antibody (the lowest band on the gel is the specific Myc-tagged proinsulin-WT). Note that whereas misfolded *Akita* mutant proinsulin severely inhibits the secretion of Myc-tagged proinsulin-WT in all samples, the presence of 7-chloromethyl-camptothecin partially reverses the inhibition of secretion of proinsulin-WT.

Fig. 13. 7- CH_3Cl-CPT increases insulin content in Akita islets. Islets were isolated from *Akita* mice and were recovered overnight in 11.1 mM glucose RPMI medium containing 10% fetal bovine serum. The islets were then incubated with 3 μg/mL candidate compound 7- CH_3Cl-CPT or DMSO (control) for 24 hours. The islet lysates were resolved using 4-12% NuPage under reducing conditions, electrotransferred to nitrocellulose, and immunoblotted with anti-insulin. Each sample was run in duplicate; the samples that had been treated with the 7-chloromethyl-camptothecin recovered more protein in the insulin (B-chain) band.

DEFINITIONS

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments described herein, some preferred methods, compositions, devices, and materials are described herein. However, before the present materials and methods are described, it is to be understood that this invention is not limited to the particular molecules, compositions, methodologies or protocols herein described, as these may vary in accordance with routine experimentation and optimization. It is also to be understood that the terminology used in the description is for the purpose of describing the particular versions or embodiments only, and is not intended to limit the scope of the embodiments described herein.

Unless otherwise defined, 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. However, in case of conflict, the present specification, including definitions, will control. Accordingly, in the context of the embodiments described herein, the following definitions apply.

As used herein and in the appended claims, the singular forms “a”, “an” and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example,

reference to "a peptide amphiphile" is a reference to one or more peptide amphiphiles and equivalents thereof known to those skilled in the art, and so forth.

As used herein, the term "comprise" and linguistic variations thereof denote the presence of recited feature(s), element(s), method step(s), etc. without the exclusion of the presence of additional feature(s), element(s), method step(s), etc.

As used herein, the term "consists of" and linguistic variations thereof denote the presence of recited feature(s), element(s), method step(s), etc. without the exclusion of the presence of additional feature(s), element(s), method step(s), etc. Conversely, the term "consisting of" excludes any unrecited feature(s), element(s), method step(s), etc. except for ordinarily-associated impurities.

As used herein, the phrase "consisting essentially of" denotes the recited feature(s), element(s), method step(s), etc. and any additional feature(s), element(s), method step(s), etc. that do not materially affect the basic nature of the composition, system, or method.

As used herein, the term "diabetes" refers to the set of diseases and conditions known collectively as "diabetes mellitus," including "type 1 diabetes," "type 2 diabetes," "gestational diabetes" (during pregnancy), "Mutant INS-gene-induced Diabetes of Youth" (MIDY), and other states that cause hyperglycaemia. The term is used for disorders in which the pancreas produces and/or secretes insufficient amounts of active/properly-folded insulin, and/or in which the cells of the body fail to respond appropriately to insulin (e.g., "insulin resistance") thus preventing cells from absorbing glucose. As a result of the different, untreated forms of diabetes, glucose builds up in the blood.

"Type 1 diabetes," also called "insulin-dependent diabetes mellitus" ("IDDM") and "juvenile-onset diabetes," is caused by B-cell destruction and/or the inabiulity of the pancreas to produce active insulin, usually leading to absolute insulin deficiency.

"Type 2 diabetes," also known as "non-insulin-dependent diabetes mellitus" (NIDDM) and "adult-onset diabetes," is associated with predominant insulin resistance and thus relative insulin deficiency and/or a predominantly insulin secretory defect with insulin resistance.

"Mutant INS-gene-induced Diabetes of Youth" ("MIDY") is associated with insulin deficiency initiated by an attack of misfolded mutant proinsulin on bystander wild-type (WT) proinsulin in the ER.

As used herein, the term "subject" broadly refers to any animal, including but not limited to, human and non-human mammals (e.g., primates, rodents, dogs, cats, cows, horses,

sheep, etc.). As used herein, the term "patient" typically refers to a subject that is suffering from and/or being treated for a disease or condition (e.g., diabetes, etc.).

As used herein, the term "antibody" refers to a whole antibody molecule or a fragment thereof (e.g., Fab, Fab', F(ab')2, Fv, scFv, Fd, diabodies, and other antibody fragments that retain at least a portion of the variable region of an intact antibody. See, e.g., Hudson et al. (2003) Nat. Med. 9:129-134; herein incorporated by reference in its entirety); it may be a polyclonal or monoclonal antibody, chimeric, a humanized, etc.

As used herein, when an antibody or other entity "specifically recognizes" or "specifically binds" an antigen or epitope, it preferentially recognizes the antigen in a complex mixture of proteins and/or macromolecules, and binds the antigen or epitope with affinity which is substantially higher than to other entities not displaying the antigen or epitope. In this regard, "affinity which is substantially higher" means affinity that is high enough to enable detection of an antigen or epitope which is distinguished from entities using a desired assay or measurement apparatus. Typically, it means binding affinity having a binding constant (K_a) of at least 10^7 M^{-1} (e.g., $>10^7$ M^{-1}, $>10^8$ M^{-1}, $>10^9$ M^{-1}, $>10^{10}$ M^{-1}, $>10^{11}$ M^{-1}, $>10^{12}$ M^{-1}, $>10^{13}$ M^{-1}, etc.). In certain such embodiments, an antibody is capable of binding different antigens so long as the different antigens comprise that particular epitope. In certain instances, for example, homologous proteins from different species may comprise the same epitope.

The term "epitope" refers to any polypeptide determinant capable of specifically binding to an immunoglobulin or a T-cell or B-cell receptor. In certain embodiments, an epitope is a region of an antigen that is specifically bound by an antibody. In certain embodiments, an epitope may include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl groups. In certain embodiments, an epitope may have specific three dimensional structural characteristics (e.g., a "conformational" epitope) and/or specific charge characteristics. An epitope is defined as "the same" as another epitope if a particular antibody specifically binds to both epitopes. In certain embodiments, polypeptides having different primary amino acid sequences may comprise epitopes that are the same. In certain embodiments, epitopes that are the same may have different primary amino acid sequences. Different antibodies are said to bind to the same epitope if they compete for specific binding to that epitope.

As used herein, the term "wild-type," refers to a gene or gene product (e.g., protein) that has the characteristics (e.g., sequence) of that gene or gene product isolated from a

naturally occurring source, and is most frequently observed in a population. In contrast, the term "mutant" refers to a gene or gene product that displays modifications in sequence when compared to the wild-type gene or gene product. It is noted that "naturally-occurring mutants" are genes or gene products that occur in nature, but have altered sequences when compared to the wild-type gene or gene product; they are not the most commonly occurring sequence. "Synthetic mutants" are genes or gene products that have altered sequences when compared to the wild-type gene or gene product and do not occur in nature. Mutant genes or gene products may be naturally occurring sequences that are present in nature, but not the most common variant of the gene or gene product, or "synthetic," produced by human or experimental intervention.

The term "effective dose" or "effective amount" refers to an amount of an agent, e.g., a PDSP, which results in a desired biological outcome (e.g., protection of the proinsulin dimerization surface).

As used herein, the terms "administration" and "administering" refer to the act of providing a therapeutic, prophylactic, or other agent to a subject for the treatment or prevention of one or more diseases or conditions. Exemplary routes of administration to the human body are through space under the arachnoid membrane of the brain or spinal cord (intrathecal), the eyes (ophthalmic), mouth (oral), skin (topical or transdermal), nose (nasal), lungs (inhalant), oral mucosa (buccal), ear, rectal, vaginal, by injection (e.g., intravenously, subcutaneously, intratumorally, intraperitoneally, etc.) and the like.

As used herein, the term "treat," and linguistic variations thereof, encompasses therapeutic measures, while the term "prevent" and linguistic variations thereof, encompasses prophylactic measures, unless otherwise indicated.

As used herein, the terms "co-administration" and "co-administering" refer to the administration of at least two agent(s) or therapies to a subject (e.g., a PDSP and one or more therapeutic agents). In some embodiments, the co-administration of two or more agents or therapies is concurrent. In other embodiments, a first agent/therapy is administered prior to a second agent/therapy. Those of skill in the art understand that the formulations and/or routes of administration of the various agents or therapies used may vary. The appropriate dosage for co-administration can be readily determined by one skilled in the art. In some embodiments, when agents or therapies are co-administered, the respective agents or therapies are administered at lower dosages than appropriate for their administration alone. Thus, co-administration is especially desirable in embodiments where the co-administration

of the agents or therapies lowers the requisite dosage of a potentially harmful (e.g., toxic) agent(s), and/or when co-administration of two or more agents results in sensitization of a subject to beneficial effects of one of the agents via co-administration of the other agent.

As used herein, the term "pharmaceutical composition" refers to the combination of an active agent (e.g., PDSP) with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

The term "pharmaceutically acceptable" as used herein, refers to compositions that do not substantially produce adverse reactions, e.g., toxic, allergic, or immunological reactions, when administered to a subject.

As used herein, the term "pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers including, but not limited to, phosphate buffered saline solution, water, emulsions (e.g., such as an oil/water or water/oil emulsions), and various types of wetting agents, any and all solvents, dispersion media, coatings, sodium lauryl sulfate, isotonic and absorption delaying agents, disintgrants (e.g., potato starch or sodium starch glycolate), and the like. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see, e.g., Martin, Remington's Pharmaceutical Sciences, 15th Ed., Mack Publ. Co., Easton, Pa. (1975), incorporated herein by reference in its entirety.

As used herein, the term "derivative" (e.g., "camptothecin derivative") refers to a compound that is structurally related to a references compound (e.g., camptothecin), but comprises one or more structural modifications (e.g., substitutions). In some embodiments, a derivative maintains the functional activity of the reference compound (e.g., protection of the dimerization surface of proinsulin monomers) or exhibits an enhancement of the functional activity of the reference compound.

As used herein, the term "substituted" refers to the modification of a position on a molecule with one or more additional group(s). Non-limiting examples of substituents include, for example: halogen, hydroxy, oxo (=O), thioxo (=S), cyano (-CN), nitro ($-NO_2$), imino (=N-H), oximo (=N-OH), hydrazino (=N-NH_2), $-R^b-OR^a$, $-R^b-OC(O)-R^a$, $-R^b-OC(O)-OR^a$, $-R^b-OC(O)-N(R^a)_2$, $-R^b-N(R^a)_2$, $-R^b-C(O)R^a$, $-R^b-C(O)OR^a$, $-R^b-C(O)N(R^a)_2$, $-R^b-O-R^c-C(O)N(R^a)_2$, $-R^b-N(R^a)C(O)OR^a$, $-R^b-N(R^a)C(O)R^a$, $-R^b-N(R^a)S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tOR^a$ (where t is 1 or 2), and $-R^b-S(O)_tN(R^a)_2$ (where t is 1 or 2); and alkyl, alkenyl, alkynyl, each of which may be optionally substituted by halogen, oxo (=O), thioxo (=S), cyano (-CN), nitro ($-NO_2$),

imino (=N-H), oximo (=N-OH), hydrazine (=N-NH_2), $-R^b-OR^a$, $-R^b-OC(O)-R^a$, $-R^b-OC(O)-OR^a$, $-R^b-OC(O)-N(R^a)_2$, $-R^b-N(R^a)_2$, $-R^b-C(O)R^a$, $-R^b-C(O)OR^a$, $-R^b-C(O)N(R^a)_2$, $-R^b-O-R^c-C(O)N(R^a)_2$, $-R^b-N(R^a)C(O)OR^a$, $-R^b-N(R^a)C(O)R^a$, $-R^b-N(R^a)S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tOR^a$ (where t is 1 or 2), $-R^b-S(O)_tN(R^a)_2$ (where t is 1 or 2), carbocycle and heterocycle; wherein each R^a is independently selected from hydrogen, alkyl, alkenyl, alkynyl, carbocycle and heterocycle, wherein each R^a, valence permitting, may be optionally substituted with halogen, oxo (=O), thioxo (=S), cyano (-CN), nitro ($-NO_2$), imino (=N-H), oximo (=N-OH), hydrazine (=N-NH_2), $-R^b-OR^a$, $-R^b-OC(O)-R^a$, $-R^b-OC(O)-OR^a$, $-R^b-OC(O)-N(R^a)_2$, $-R^b-N(R^a)_2$, $-R^b-C(O)R^a$, $-R^b-C(O)OR^a$, $-R^b-C(O)N(R^a)_2$, $-R^b-O-R^c-C(O)N(R^a)_2$, $-R^b-N(R^a)C(O)OR^a$, $-R^b-N(R^a)C(O)R^a$, $-R^b-N(R^a)S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tOR^a$ (where t is 1 or 2) and $-R^b-S(O)_tN(R^a)_2$ (where t is 1 or 2); and wherein each R^b is independently selected from a direct bond or a straight or branched alkylene, alkenylene, or alkynylene chain, and each R^c is a straight or branched alkylene, alkenylene or alkynylene chain. In some embodiments, substituent groups may be selected from, but are not limited to: alkyl, alkenyl, alkynyl, cycloalkyl, aryl, heteroaryl, heterocycloalkyl, hydroxyl, alkoxy, mercaptyl, cyano, halo, carbonyl, thiocarbonyl, isocyanato, thiocyanato, isothiocyanato, nitro, perhaloalkyl, perfluoroalkyl, and amino, including mono- and di-substituted amino groups, and the protected derivatives thereof.

DETAILED DESCRIPTION

Provided herein are compositions and methods for protection of the dimerization surface of proinsulin monomers, and the treatment of poor insulin production, pancreatic beta cell failure, and diabetes therewith. In particular, misfolded proinsulin is prevented from propagating its deleterious effects by protecting the dimerization surface of proinsulin in general and misfolded proinsulin specifically.

Protecting the dimerization interface diminishes dominant negative effects of mutant proinsulin and retard progression of diabetes caused by misfolded proinsulin. Specifically, it was found that proinsulin B-chain tyrosine 16, Tyr(B16), played a critical role in proinsulin dimerization. A proinsulin variant Y(B16)D does not impair intracellular transport of proinsulin-WT, but limits abnormal interactions between WT and mutant proinsulin, permitting the ER export of WT proinsulin, alleviating ER stress, and increasing active insulin production. Using *in silico* screening, Proinsulin Dimerization Surface Protectors

(PDSPs) have been identified that dock at proinsulin dimerization interface and prevent the downstream negative effects of dimerization of misfolded proinsulin with active proinsulin molecules. Experiments conducted during development of embodiments herein indicate that two small molecule PDSPs (11-methoxycamptothecin (11-MCPT) and pinafide (2-[2-(1-Pyrrolidyl)ethyl]-5-nitro-1H-benz[de]isoquinoline-1,3(2H)-dione)) are capable of binding at the dimerization site to prevent dimerization and prevent propagation of the negative effects of misfolding. In cells co-expressing proinsulin-WT and a mutant causing MIDY, it was found that 11-MCPT significantly increased proinsulin ER export and level of mature insulin in islets isolated from *Akita* mice, which carry an MIDY mutant in one of their Ins2 alleles. Administration of 11-MCPT to *Akita* mice increased insulin in the circulation, improving blood glucose, and retarding progression of diabetes. These data demonstrate that PDSPs, by limiting interactions between misfolded proinsulin and WT proinsulin molecules, are useful as therapeutic agents to limit the extent of proinsulin misfolding, improve insulin production, and protect patients from pancreatic beta cell failure and diabetes.

Proinsulin misfolding with endoplasmic reticulum (ER) stress and decreased pancreatic beta cell mass are hallmarks of pancreatic beta cell failure and diabetes (ref. 7; incorporated by reference in its entirety). In MIDY, affected patients are heterozygotes carrying proinsulin misfolding-inducing mutations in one of their two *INS* gene alleles (refs. 8-12; incorporated by reference in their entireties). Although one normal *INS* allele is ordinarily enough to maintain normoglycemia (ref. 13,14; incorporated by reference in their entireties), MIDY patients typically develop severe insulin-deficient diabetes (ref. 8-10,12; incorporated by reference in their entireties). An underlying mechanism initiating this insulin deficiency is that misfolded mutant proinsulin interacts with proinsulin-WT in the ER, impairing the ER export of proinsulin-WT, decreasing bioactive insulin production (refs. 4,5,15; incorporated by reference in their entireties). In some embodiments, preventing these intermolecular proinsulin interactions limits attack of potentially active insulin by misfolded mutant proinsulin, allowing active, bystander proinsulin-WT to exit from the ER.

In some embodiments, provided herein are methods and compositions for treating diabetes (e.g., type II diabetes, MIDY, etc.) by inhibiting the dimerization of proinsulins, and thereby allowing export of monomeric proinsulin. In some embodiments, by protecting the dimerization surface of proinsulin monomers, the propagation of the effect of misfolded proinsulin onto properly-folded and potentially active proinsulins through dimerization

thereto is inhibited. In some embodiments, any suitable methods and/or compositions for protecting the dimerization surface of proinsulin are within the scope herein.

In some embodiments, dimerization of misfolded proinsulin to properly-folded proinsulin is inhibited. In such embodiments, an inhibitor binds to a feature on the dimerization surface of misfolded proinsulin, but not properly-folded proinsulin, thereby preventing dimerization of the misfolded proinsulin (e.g., to other misfolded proinsulins as well as to properly-folded proinsulins), while allowing dimerization of properly-folded proinsulins.

In such embodiments, an inhibitor binds to a feature on the dimerization surface of both misfolded and properly-folded proinsulin, thereby inhibiting the dimerization of all proinsulins (e.g., misfolded, properly-folded, or therebetween).

In some embodiments, an inhibitor binds to misfolded proinsulin (e.g., at the dimerization surface or elsewhere), but not properly-folded proinsulin, and sequesters the bound proinsulin from dimerization. In some embodiments, the inhibitor prevents dimerization through the location of its binding (e.g., to the dimerization surface); by altering the structure of the dimerization surface; by binding to another location on proinsulin that is important for dimerization; and/or by having a bulky structure (e.g., antibody) that, when bound to proinsulin, isn't compatible with dimerization.

In some embodiments, an agent binds to misfolded, but not properly-folded proinsulin, and promotes the dimerization of two bound/misfolded proinsulins to each other. In some embodiments, an agent binds to misfolded, but not properly-folded proinsulin, and promotes the oligomerization of of multiple bound/misfolded proinsulins to each other. In some embodiments, by promoting the dimerization and/or oligomerization of misfolded proinsulins to each other, dimerization of misfolded proinsulin to properly-folded proinsulin is inhibited.

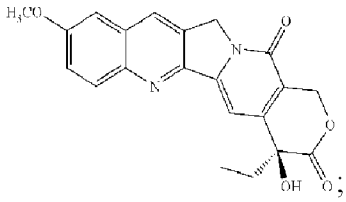
In some embodiments, an agent binds to properly-folded, but not misfolded proinsulin, and promotes the dimerization of two bound/properly-folded proinsulins to each other. In some embodiments, by promoting the dimerization of properly-folded proinsulins to each other, dimerization of misfolded proinsulin to properly-folded proinsulin is inhibited.

In some embodiments, provided herein are compositions that bind to the dimerization surface of proinsulin and/or inhibit dimerization of proinsulin. In some embodiments, such compositions associate with, interact with, and/or bind directly to the dimerization surface of proinsulin. In other embodiments, compositions interact with another portion of proinsulin,

but cause a structural change that disfavors/inhibits/prevents dimerization. In some embodiments, compositions comprise peptides, peptide mimetics, polypeptide, antibodies, antibody fragments, small molecules, nucleic acids, etc.

In some embodiments, a composition for inhibiting proinsulin dimerization and/or binding to the dimerization surface is a small molecule compound. Exemplary PDSP compounds that find use in embodiments herein include:

11-methoxycamptothecin (11-MCPT)



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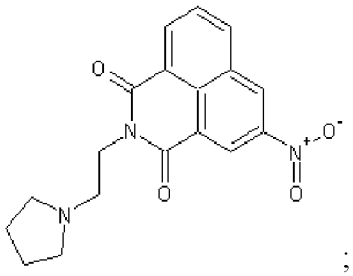
pinafide (2-[2-(1-Pyrrolidyl)ethyl]-5-nitro-1H-benz[de]isoquinoline-1,3(2H)-dione))



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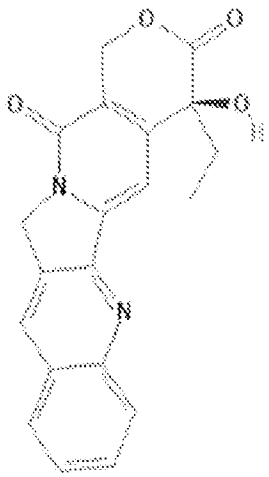
Camptothecin



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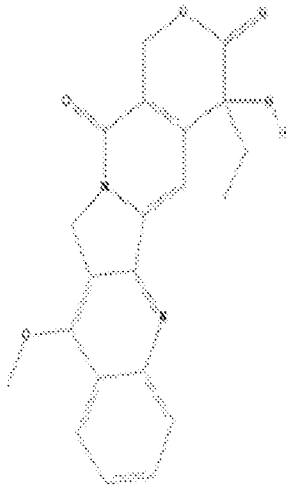
7-methoxycamptothecin



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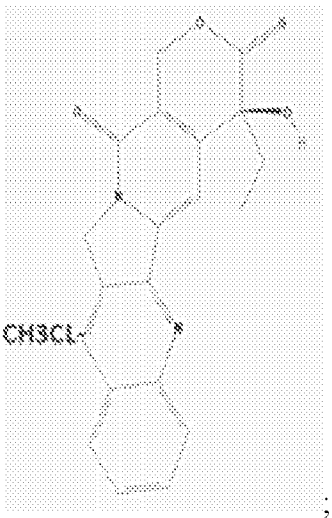
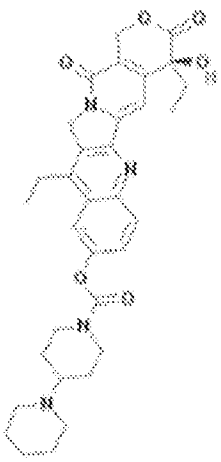
7- CH_3Cl-camptothecin



; and

irinotecan



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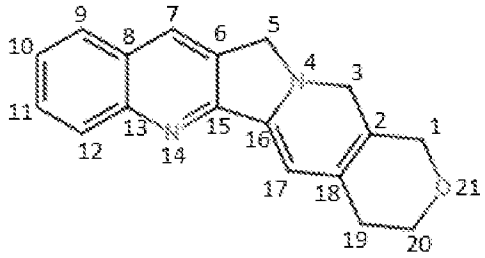
In some embodiments, a composition for inhibiting proinsulin dimerization and/or binding to the dimerization surface is a camptothecin derivative (e.g., 7-methoxycamptothecin, 11-methoxycamptothecin, 7- CH_3Cl-camptothecin, etc.). In some embodiments, a camptothecin derivative comprises a camptothecin core structure with one or more additional substituents at one or more of positions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and/or 21. Suitable substituents are any organic substituents understood in the field, for example, those described herein. In some embodiments, the camptothecin derivative comprises formula (I):



; comprising one or more substituents at any of positions 1-21. In some embodiments, substituents are selected from: halogen, hydroxy, oxo (=O), thioxo (=S), cyano (-CN), nitro ($-NO_2$), imino (=N-H), oximo (=N-OH), hydrazino (=N-NH_2), $-R^b-OR^a$, $-R^b-OC(O)-R^a$, $-R^b-OC(O)-OR^a$, $-R^b-OC(O)-N(R^a)_2$, $-R^b-N(R^a)_2$, $-R^b-C(O)R^a$, $-R^b-C(O)OR^a$, $-R^b-C(O)N(R^a)_2$, $-R^b-O-R^c-C(O)N(R^a)_2$, $-R^b-N(R^a)C(O)OR^a$, $-R^b-N(R^a)C(O)R^a$, $-R^b-N(R^a)S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tOR^a$ (where t is 1 or 2), and $-R^b-S(O)_tN(R^a)_2$ (where t is 1 or 2); and alkyl, alkenyl, alkynyl, each of which may be optionally substituted by halogen, oxo (=O), thioxo (=S), cyano (-CN), nitro ($-NO_2$), imino (=N-H), oximo (=N-OH), hydrazine (=N-NH_2), $-R^b-OR^a$, $-R^b-OC(O)-R^a$, $-R^b-OC(O)-OR^a$, $-R^b-OC(O)-N(R^a)_2$, $-R^b-N(R^a)_2$, $-R^b-C(O)R^a$, $-R^b-C(O)OR^a$, $-R^b-C(O)N(R^a)_2$, $-R^b-O-R^c-C(O)N(R^a)_2$, $-R^b-N(R^a)C(O)OR^a$, $-R^b-N(R^a)C(O)R^a$, $-R^b-N(R^a)S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tOR^a$ (where t is 1 or 2), $-R^b-S(O)_tN(R^a)_2$ (where t is 1 or 2), carbocycle and heterocycle; wherein each R^a is independently selected from hydrogen, alkyl, alkenyl, alkynyl, carbocycle and heterocycle, wherein each R^a, valence permitting, may be optionally substituted with halogen, oxo (=O), thioxo (=S), cyano (-CN), nitro ($-NO_2$), imino (=N-H), oximo (=N-OH), hydrazine (=N-NH_2), $-R^b-OR^a$, $-R^b-OC(O)-R^a$, $-R^b-OC(O)-OR^a$, $-R^b-OC(O)-N(R^a)_2$, $-R^b-N(R^a)_2$, $-R^b-C(O)R^a$, $-R^b-C(O)OR^a$, $-R^b-C(O)N(R^a)_2$, $-R^b-O-R^c-C(O)N(R^a)_2$, $-R^b-N(R^a)C(O)OR^a$, $-R^b-N(R^a)C(O)R^a$, $-R^b-N(R^a)S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tR^a$ (where t is 1 or 2), $-R^b-S(O)_tOR^a$ (where t

is 1 or 2) and $-R^b-S(O)_tN(R^a)_2$ (where t is 1 or 2); and wherein each R^b is independently selected from a direct bond or a straight or branched alkylene, alkenylene, or alkynylene chain, and each R^c is a straight or branched alkylene, alkenylene or alkynylene chain. In some embodiments, substituent groups may be selected from, but are not limited to: alkyl, alkenyl, alkynyl, cycloalkyl, aryl, heteroaryl, heterocycloalkyl, hydroxyl, alkoxy, mercaptyl, cyano, halo, carbonyl, thiocarbonyl, isocyanato, thiocyanato, isothiocyanato, nitro, perhaloalkyl, perfluoroalkyl, and amino, including mono- and di-substituted amino groups, and the protected derivatives thereof. In some embodiments, one or more of positions 1-21 are modified (e.g., from C, O, or N) to C, O, S, or N. Exemplary substituted camptothecin molecules and methods for preparation and design thereof are described in, for example, U.S. Pat. No. 4,604,463; U.S. Pat. No. 8,168,648; U.S. Pat. No. 8,685,997; U.S. Pat. No. 6,194,579; U.S. Pat. No. 5,900,419; U.S. Pat. No. 4,39,9276; incorporated by reference in their entireties.

The experiments conducted during development of embodiments herein demonstrate that protection of the proinsulin dimerization surface, whether by small molecule inhibitor or genetic mutation of the proinsulin dimerization surface, treats diabetes, increases active insulin production/secretion, and decreases loss of beta cells. Therefore, embodiments herein are not limited to these exemplary compounds. Rather, any compound, other agent, method, or mechanism for inhibiting the dimerization of misfolded proinsulin with bystander (or properly folded) proinsulin finds use in the embodiments herein. In some embodiments, compounds within the scope herein comprise modified versions, conjugates, and/or derivatives of camptothecin, 7-methoxycamptothecin, 11-methoxycamptothecin, 7- CH_3Cl-camptothecin, irinotecan, and pinafide. In some embodiments, compounds not structurally-related to camptothecin, 7-methoxycamptothecin, 11-methoxycamptothecin, 7- CH_3Cl-camptothecin, irinotecan, pinafide, and derivatives thereof find use herein. In some embodiments, compositions such as peptides, antibodies, etc. that inhibit proinsulin dimerization are within the scope herein.

Provided herein are pharmaceutical compositions comprising a PDSP (e.g., 11-MCPT, 7-MCPT, pinafide, etc.), alone or in combination with at least one other agent, such as a stabilizing compound, and may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water.

As is well known in the medical arts, dosages for any one patient depends upon many

factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and interaction with other drugs being concurrently administered.

Depending on the condition being treated, these pharmaceutical compositions may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in the latest edition of "Remington's Pharmaceutical Sciences" (Mack Publishing Co, Easton Pa.). Suitable routes may, for example, include oral or transmucosal administration; as well as parenteral delivery, including intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraperitoneal, or intranasal administration.

For injection, pharmaceutical compositions may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. For tissue or cellular administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

In other embodiments, the pharmaceutical compositions are formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral or nasal ingestion by a patient to be treated.

Pharmaceutical compositions include compositions wherein the active ingredients (e.g., 11-MCPT, 7-MCPT, pinafide, etc.) are contained in an effective amount to achieve the intended purpose. For example, an effective amount of a PDSP may be that amount that restores a normal (non-diseased) rate of insulin mediated glucose metabolism. Determination of effective amounts is well within the capability of those skilled in the art, especially in light of the disclosure provided herein.

In addition to the active ingredients (e.g., 11-MCPT, 7-MCPT, pinafide, etc.) pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations that can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known (e.g., by means of conventional mixing, dissolving, granulating, dragee-

making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes).

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, etc; cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; and gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, (e.g., dosage).

Pharmaceutical preparations for oral administration include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients mixed with a filler or binders such as lactose or starches, lubricants such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycol with or without stabilizers.

Compositions comprising a PDSP formulated in a pharmaceutical acceptable carrier may be prepared, placed in an appropriate container, and labeled for treatment of an indicated

condition. Conditions indicated on the label may include treatment of obesity, diabetes, insulin resistance, or weight loss.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder in 1 mM-50 mM histidine, 0.1%-2% sucrose, 2%-7% mannitol at a pH range of 4.5 to 5.5 that is combined with buffer prior to use.

In some embodiments, a therapeutically effective dose may be estimated initially from cell culture assays and/or animal models (particularly murine models). A therapeutically effective dose refers to that amount of a PDSP that ameliorates symptoms of the disease state or unwanted condition. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. Data obtained from these cell culture assays and additional animal studies can be used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration. The exact dosage is chosen by the individual clinician in view of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Additional factors which may be taken into account include the severity of the disease state; age, weight, and gender of the patient; diet, time and frequency of administration, drug combination (s), reaction sensitivities, and tolerance/response to therapy. Long acting pharmaceutical compositions might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Typical dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature (See, U.S. Pat. Nos. 4,657,760;

5,206,344; 5,225,212; WO2004/097009, or WO2005/075465, each of which are herein incorporated by reference).

In some embodiments, the therapeutics herein are combined or used in combination with other agents useful in the treatment of diabetes. Or, by way of example only, the therapeutic effectiveness of one of the compounds described herein may be enhanced by administration of an adjuvant (e.g., by itself the adjuvant may only have minimal therapeutic benefit, but in combination with another therapeutic agent, the overall therapeutic benefit to the patient is enhanced).

Such other agents, adjuvants, or drugs, may be administered, by a route and in an amount commonly used therefor, simultaneously or sequentially with a compound as disclosed herein. When a therapeutic is used contemporaneously with one or more other drugs, a pharmaceutical composition containing such other drugs in addition to the compound disclosed herein may be utilized, but is not required.

In some embodiments, a therapeutic described in embodiments herein is combined with an anti-obesity and/or an anti-diabetes therapy. For example, in some embodiments a therapeutic described in embodiments herein is provided with a meglitinide, e.g., to stimulate the release of insulin. Exemplary meglitinides are repaglinide (Prandin) and nateglinide (Starlix). In some embodiments, a therapeutic described in embodiments herein is provided with a sulfonylurea, e.g., to stimulate the release of insulin. Exemplary sulfonylureas are glipizide (Glucotrol), glimepiride (Amaryl), and glyburide (DiaBeta, Glynase).

In some embodiments, a therapeutic described in embodiments herein is provided with a dipeptidyl peptidase-4 (DPP-4) inhibitor, e.g., to stimulate the release of insulin and/or to inhibit the release of glucose from the liver. Exemplary dipeptidyl peptidase-4 (DPP-4) inhibitors are saxagliptin (Onglyza), sitagliptin (Januvia), and linagliptin (Tradjenta). In some embodiments, a compound described herein is provided with a biguanide, e.g., to inhibit the release of glucose from the liver and/or to improve sensitivity to insulin. An exemplary biguanide is metformin (Fortamet, Glucophage). In some embodiments, a therapeutic described in embodiments herein is provided with a thiazolidinedione, e.g., to improve sensitivity to insulin and/or to inhibit the release of glucose from the liver. Exemplary thiazolidinediones include but are not limited to rosiglitazone (Avandia) and pioglitazone (Actos). In some embodiments a compound described herein is provided with an alpha-glucosidase inhibitor, e.g., to slow the breakdown of starches and some sugars. Exemplary alpha-glucosidase inhibitors include acarbose (Precose) and miglitol (Glyset). In some

embodiments, a compound as described herein is provided with an injectable medication such as an amylin mimetic or an incretin memetic, e.g., to stimulate the release of insulin. An exemplary amylin mimetic is pramlintide (Symlin); exemplary incretin mimetics include exenatide (Byetta) and liraglutide (Victoza). In some embodiments a compound described herein is provided with insulin. The technology is not limited to any particular form of insulin, but encompasses any form of insulin. In some embodiments, the therapeutic described in embodiments herein are used with an insulin injection. In some embodiments, more than one additional therapy (e.g., drug or other biologically active composition or compound), e.g., two, three, four or more therapies are used. In other embodiments, the therapeutics herein are administered without administering insulin and/or replace the need for insulin therapy in a subject.

In some embodiments, a PDSP is co-administered with one or more additional therapeutic agents or medical interventions. In some embodiments, co-administration involves co-formulation of two or more agents together into the same medicament. In other embodiments, the agents are in separate formulations but are administered together, either simultaneously or in sequence (e.g., separated by one or more minutes, hours, days, etc.). In some embodiments, where a synergistic or additive benefit is achieved, the co-administered agent may be provided at a lower dose than would normally be administered if that agent were being used in isolation to treat the disease or condition.

The technology provided herein also includes kits for use in the instant methods. Kits of the technology comprise one or more containers comprising a PDSP or a pharmaceutically acceptable salt thereof, and/or a second agent, and in some variations further comprise instructions for use in accordance with any of the methods provided herein. The kit may further comprise a description of selecting an individual suitable treatment. Instructions supplied in the kits of the technology are typically written instructions on a label or package insert (e.g., a paper insert included with the kit), but machine-readable instructions (e.g., instructions carried on a magnetic or optical storage disk) are also contemplated. In some embodiments, the kit is a package containing a sealed container comprising any one of the preparations described above, together with instructions for use. The kit can also include a diluent container containing a pharmaceutically acceptable diluent. The kit can further comprise instructions for mixing the preparation and the diluent. The diluent can be any pharmaceutically acceptable diluent. Well known diluents include 5% dextrose solution and physiological saline solution. The container can be an infusion bag, a sealed bottle, a vial, a

vial with a septum, an ampoule, an ampoule with a septum, an infusion bag, or a syringe. The containers can optionally include indicia indicating that the containers have been autoclaved or otherwise subjected to sterilization techniques. The kit can include instructions for administering the various solutions contained in the containers to subjects.

The technology also relates to methods of treatment with a PDSP. According to another aspect of the technology, a method is provided for treating a subject in need of such treatment with an effective amount of a PDSP or a salt thereof. For example, some subjects in need of compositions according to the technology have diabetes, insulin resistance, obesity, etc. The method involves administering to the subject an effective amount of a PDSP in any one of the pharmaceutical preparations described above, detailed herein, and/or set forth in the claims. The subject can be any subject in need of such treatment. In the foregoing description, the technology is in connection with a PDSP or salts thereof. Such salts include, but are not limited to, bromide salts, chloride salts, iodide salts, carbonate salts, and sulfate salts.

In some embodiments, provided herein are methods of treatment comprising: administering a pharmaceutically effective amount of a PDSP, alone or in combination with another agent, to a subject having a condition in need of treatment. In some embodiments, the administration causes one or more of: a reduction in or elimination of one or more symptoms of the condition, prevention of increased severity of one or more symptoms of the condition, and/or reduction, prevention, or elimination of further diseases or conditions.

In some embodiments, the methods provided comprise testing a subject for a disease or condition followed by administering a PDSP, a derivative thereof, or a pharmaceutically acceptable salt thereof, alone or in combination with other agents. In some embodiments, methods comprise administering to a subject a PDSP, alone or in combination with other agents, followed by testing the subject for a disease or a condition. In some embodiments, methods comprise testing a subject for a disease or condition followed by administering a PDSP, alone or in combination with other agents, followed by a second round of testing for a disease or condition (e.g., to monitor the effect of the treatment). In some embodiments, methods comprise testing a subject for a disease or condition followed by administering a PDSP, followed by a second round of testing for a disease or condition and a second administration of a PDSP, with this second administration being modified in dose, duration, frequency, or administration route in a manner dependent upon the results of the prior testing. In some embodiments, a subject is tested to assess the presence, the absence, or the level of a

disease, e.g., by assaying or measuring a biomarker, a metabolite, a physical symptom, an indication, etc., to determine the risk of or the presence of the disease and thereafter the subject is treated with a PDSP based on the outcome of the test. In some embodiments, a patient is tested, treated, and then tested again to monitor the response to therapy. In some embodiments, cycles of testing and treatment may occur without limitation to the pattern of testing and treating (e.g., test/treat, test/treat/test, test/treat/test/treat, test/treat/test/treat/test, test/treat/treat/test/treat/treat, etc.), the periodicity, or the duration of the interval between each testing and treatment phase.

In some embodiments, the technology provided comprises use of a PDSP in the manufacture of a medicament for the treatment of a condition. In some embodiments, the technology provides a PDSP for the treatment of a condition.

EXPERIMENTAL

Example 1

Proinsulin initiates dimerization in the ER (ref. 6; incorporated by reference in its entirety), yet dimerization is not required for ER export of proinsulin (ref. 16; incorporated by reference in its entirety). The interface between proinsulin or insulin monomers involves only a subset of insulin B-chain residues (ref. 17; incorporated by reference in its entirety). Analysis of the insulin dimer interface highlights Tyr(B16) as a disproportionately large contributor to the contact surface as this aromatic site chain emerging from the B-chain helix of one insulin subunit protrudes into a concave pocket on the B-chain of an opposing subunit (Fig. 1A,B). To demonstrate the contribution of this residue to interactions between the Akita mutant proinsulin-C(A7)Y and proinsulin-WT, Y(B16)D and Y(B16)A variants of proinsulin-WT or proinsulin-C(A7)Y were prepared.

Using tris-tricine-urea-SDS-PAGE under both non-reducing and reducing conditions, it was demonstrated that both Y(B16)D and Y(B16)A variants of proinsulin-WT showed native disulfide bonding (Fig. 5A,B) and normal secretion (Fig. 5C,D). By contrast, introduction of the C(A7)Y missense mutation into GFP-tagged proinsulin blocks proinsulin folding and secretion and, in trans, impairs secretion of co-expressed proinsulin-WT (ref. 18,19; incorporated by reference in their entireties). However, when the Y(B16)D variant was either introduced or not introduced into the GFP-tagged proinsulin-C(A7)Y the amount of co-immunoprecipitated proinsulin-WT was significantly decreased by the Y(B16)D substitution (Fig. 1C-D). Accompanying this diminished interaction, when Y(B16)D was

introduced into mouse *Akita* proinsulin-C(A7)Y, the dominant-negative blockade of ER export of co-expressed human proinsulin-WT was no longer detected (Fig. 1E).

Abrogating the dominant-negative blockade of proinsulin-WT could not be explained by a direct rescue of the folding defect of proinsulin-C(A7)Y, because the double mutant was still misfolded (Fig. 6A) and was still retained intracellularly (Fig. 6B). Indeed, when untagged proinsulin-WT was co-expressed with either Myc-tagged proinsulin-WT, Myc-tagged proinsulin-C(A7)Y, or Myc-tagged proinsulin-C(A7)Y/Y(B16D), it was clear that the Myc-tagged proinsulin-C(A7)Y/Y(B16)D exhibited intracellular retention like *Akita* proinsulin, but unlike in the presence of proinsulin-C(A7)Y, the co-expressed untagged proinsulin-WT continued to be secreted normally (Fig. 1F see lanes 8-9, 11-12, 17-18). Moreover, unlike pancreatic beta cells expressing proinsulin-C(A7)Y, the cells expressing the proinsulin-C(A7)Y/Y(B16)D double mutant did not lose detectable insulin immunofluorescence derived from endogenous proinsulin-WT (Fig. 2A), and did not lose detectable immunoreactive processed human insulin derived from an expressed cDNA encoding human proinsulin-WT (Fig. 2B). Thus, proinsulin-WT could undergo successful intracellular transport (and processing to insulin) in the presence of co-expressed misfolded proinsulin-C(A7)Y/Y(B16)D. As a consequence, for the cells with improved ER export of proinsulin-WT, was a significant reduction in ER stress measured by a BiP promoter-luciferase reporter, compared to cells expressing proinsulin-C(A7)Y (Fig. 2C). Together, these results indicate that limiting abnormal interactions between misfolded proinsulin and bystander proinsulin in the ER allows active bystander insulin to proceed through the secretory pathway, decreasing proinsulin accumulation in the ER, alleviating ER stress, and increasing insulin production.

Experiments were conducted during development of embodiments herein to demonstrate that the proinsulin dimerization interface is a druggable target for treating diabetes involving proinsulin misfolding. *In silico* molecular docking of the dimerization interface was performed to screen a set of 139,735 drug-like small molecules with precisely known structures, as putative Proinsulin Dimerization Surface Protectors (PDSPs). The top 40 scoring compounds were then ordered from the National Cancer Institute Open Chemical Repository for cell based functional testing. Molecular docking indicates that Pinafide and 11-methoxycamptocethixcin (11-MCPT, Fig. 3B) fit at the dimerization interface of insulin monomers (See Fig. 3C for 11-MCPT) and impair proinsulin dimerization potential (See Fig. 3D for 11-MCPT). Experiments indicate that the presence of either compound significantly

increased secretion of proinsulin-WT in the presence of co-expressed proinsulin-C(A7)Y in HEK293T cells (Fig. 3A).

To examine the effect of PDSPs in primary beta cells, islets from *Akita* mice that express one MIDY mutant *Ins2* allele plus one WT *Ins2* and two WT *Ins1* alleles were isolated. Despite being only one of four alleles, the product of the MIDY mutant allele is sufficient to impair intracellular transport of much of the bystander proinsulin-WT, bringing about insulin-deficient diabetes refs. 5,15; incorporated by reference in their entireties). Treatment of islets with the 11-MCPT did not affect proinsulin or insulin levels in islets of wild-type control mice (Fig. 7). However, PDSP-treatment of *Akita* islets, with no significant effect on proinsulin translation (Fig. 3E, F) markedly increased newly synthesized proinsulin and fully processed mature insulin by 2 h of chase (Fig. 3E, quantified in Fig. 3F). Consequently in PDSP-treated islets, steady state levels of proinsulin and insulin increased (Fig. 3G, quantified in 3H). Together, these results in pancreatic islets demonstrate that PDSPs alleviate dominant-negative behavior of misfolded mutant proinsulin to allow proinsulin-WT to be stabilized and exported from the ER, so that it is be processed and stored as mature insulin.

To evaluate the clinical benefit of treatment with a PDSP on the progression of mouse diabetes caused by misfolded proinsulin, 11-MCPT was administered to *Akita* males beginning at 6-8 weeks of age when the mice started to develop frank diabetes (ref. 20; incorporated by reference in its entirety). During a 28 d course of treatment in vehicle-treated *Akita* mice, fasting blood glucose progressively increased, whereas in PDSP-treated *Akita* mice within 7 days fasting blood glucose was lower than vehicle-treated Akita mice and it remained lower throughout the course of treatment (Fig. 4A). Two-hour glucose tolerance test also showed marked improvement in PDSB-treated mice (Fig. 4B). This improvement appeared to result from increased serum insulin levels both under fasting and glucose-stimulated states (Fig. 4C). Parallel with improved blood insulin levels, pancreatic immunofluorescent insulin content in PDSP-treated *Akita* mice was increased compared to vehicle-treated *Akita* mice (Fig. 4D). Remarkably, at the ultrastructural level, the feature swollen ER of *Akita* beta cells was dramatically decreased (although still persistent) along with an obvious increase of mature dense-core insulin secretory granules (Fig. 4E). Importantly in PDSP-treated *Akita* mice, although pancreatic proinsulin levels at steady state were comparable to that of vehicle-treated *Akita* mice (Fig. 4F), processed mature insulin was significantly increased (Fig. 4G, ratio quantified in 4H). Altogether, these data in *Akita* mice

demonstrate that PDSPs improve proinsulin ER export, decrease ER stress, increase mature insulin production and secretion, helping to alleviate insulin deficiency and diabetes.

ER stress in pancreatic beta cells has been implicated in the development and progression of type 1, type 2, and some monogenetic diabetes (refs. 21-24; incorporated by reference in their entireties). As the most abundant protein in the ER of beta cells, the predisposition of proinsulin to misfolding is not only an important potential driver of beta cell ER stress (ref. 25-28; incorporated by reference in their entireties) but exacerbates the problem by propagating misfolding onto newly synthesized bystander proinsulin molecules (refs. 4,5,15; incorporated by reference in their entireties). Experiments conducted during development of embodiments herein demonstrate that the proinsulin dimerization interface is a site initiating abnormal interactions between misfolded proinsulin and bystander proinsulin-WT in the ER (Fig. 8, left panel). Targeting the proinsulin dimerization interface by PDSPs is effective for limiting the ability of misfolded proinsulin to propagate misfolding to bystanders, increasing insulin production (Fig. 8, right panel), and reducing beta cell secretory failure and diabetes.

Example 2

Experiments conducted during development of embodiments herein demonstrate that mutation of proinsulin Tyr-B16 (e.g. Y(B16)D) at the proinsulin dimerization interface alleviates the dominant-negative effect of misfolded *Akita* proinsulin on co-expressed wildtype proinsulin bystander molecules. Additional experiments demonstrate that misfolded *Akita* proinsulin could dominantly affect the secretion of co-expressed bystander proinsulin-Y(B16)D. The dominant negative effect was smaller than the dominant-negative effect exerted on co-expressed proinsulin that has a wildtype dimerization surface. In fact, if proinsulin molecules (misfolded *Akita* proinsulin and the bystander partner) both have B16D mutations, the dominant-negative was still further decreased (Fig. 9).

Misfolded *Akita* proinsulin accelerates the degradation of co-expressed wildtype bystander proinsulin. However, when the wildtype bystander proinsulin was co-expressed with proinsulin-Y(B16)D/*Akita*, the degradation of wildtype bystander proinsulin was decreased (Fig. 10). Proinsulin-Y(B16)D/*Akita* has less effect on the secretion of co-expressed proinsulin-WT at the steady state.

Several 7-methoxy camptothecin derivatives were produced (7-MCPT; Fig. 11), and testing revealed that 7-chloromethyl-camptothecin most-effectively increased the secretion of

proinsulin-WT co-expressed with misfolded *Akita* proinsulin in transfected 293T cells (fig. 12). Western blot experiments also demonstrated that 7-CH3CL-camptothecin increased the insulin content in isolated Akita islets (Fig. 13).

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CLAIMS

1. A method of treating diabetes in a subject comprising protecting the dimerization surface of proinsulin polypeptides.

2. The method of claim 1, wherein protecting the dimerization surface of proinsulin polypeptides comprises administering a pharmaceutical composition to the subject.

3. The method of claim 2, wherein the pharmaceutical composition binds to the dimerization surface of proinsulin polypeptides to inhibit the formation of dimers.

4. The method of claim 3, wherein the pharmaceutical composition binds to the dimerization surface of misfolded proinsulin polypeptides.

5. The method of claim 3, wherein the pharmaceutical composition binds to the dimerization surface of properly proinsulin polypeptides.

6. The method of claim 2, wherein the pharmaceutical composition is a small molecule, peptide, or antibody.

7. The method of claim 1, wherein the pharmaceutical composition is a small molecule selected from camptothecin, 7-methoxycamptothecin, 11-methoxycamptothecin, 7-CH_3Cl-camptothecin, irinotecan, pinafide, and derivatives thereof.

8. A pharmaceutical composition comprising a protector of the proinsulin dimerization surface.

9. The pharmaceutical composition, wherein the inhibitor is a small molecule, peptide, or antibody.

10. The pharmaceutical composition, wherein the protector binds to the dimerization surface.

11. The pharmaceutical composition, wherein the protector is a small molecule selected from 11-methoxycamptothecin, pinafide, and derivatives thereof.

12. A protector of the proinsulin dimerization surface for use in treating diabetes.

13. Use of a protector of the proinsulin dimerization surface for the manufacture of a medicament for treating diabetes.

FIG. 1

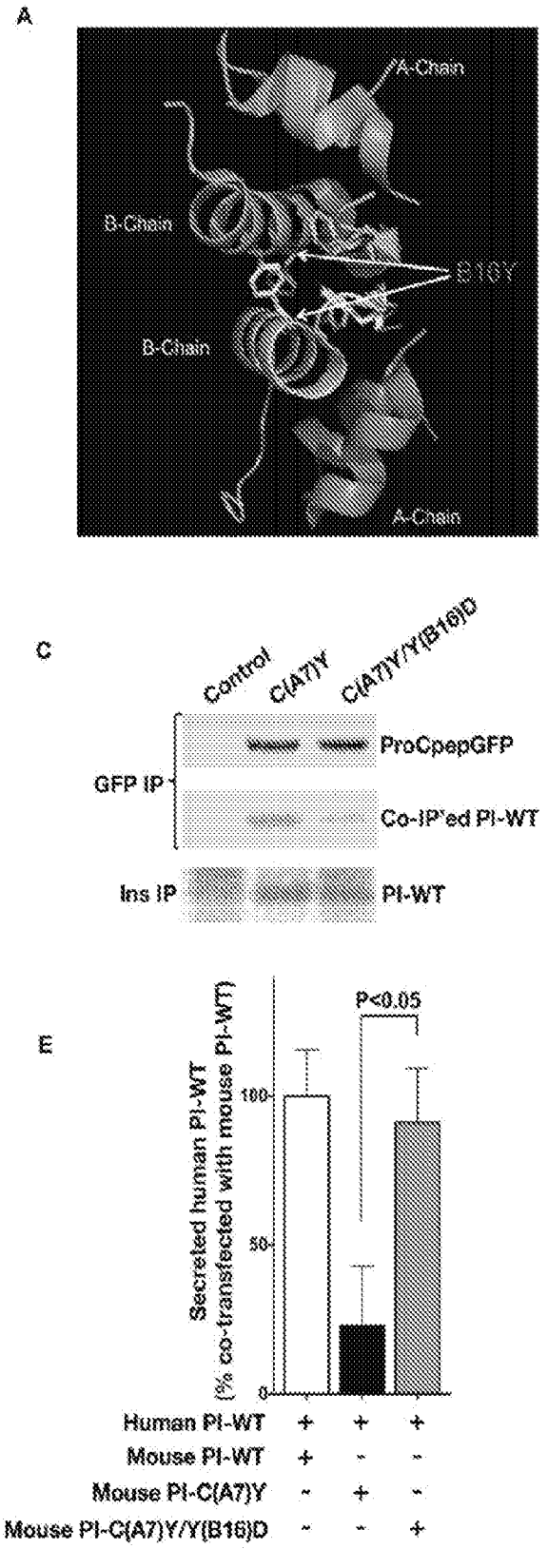
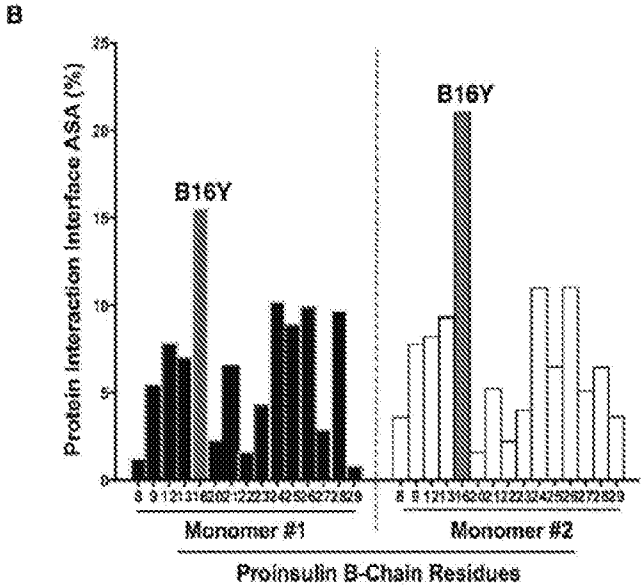
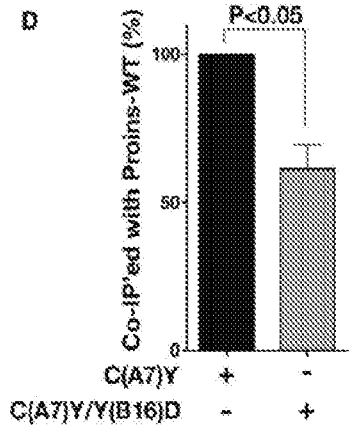
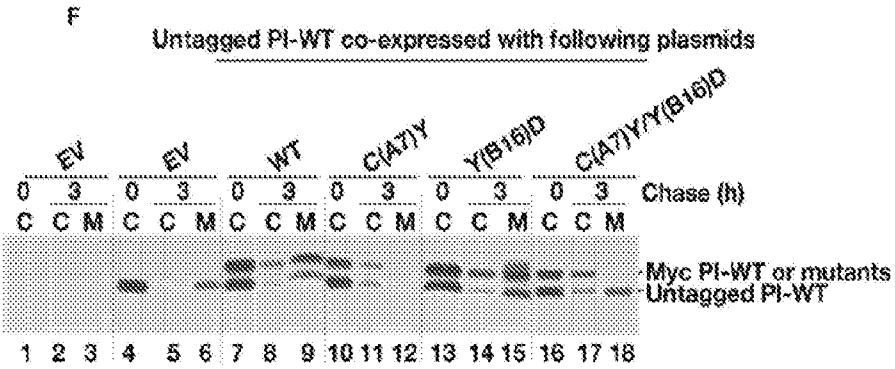

A
A-Chain
B-Chain
B16Y
B-Chain
A-Chain
C
Control
C(A7)Y
C(A7)Y/Y(B16)D
GFP IP
ProCpepGFP
Co-IP'ed PI-WT
Ins IP
PI-WT
E
P<0.05
Secreted human PI-WT
(% co-transfected with mouse PI-WT)
100
50
0
Human PI-WT + + +
Mouse PI-WT + - -
Mouse PI-C(A7)Y - + -
Mouse PI-C(A7)Y/Y(B16)D - - +

FIG. 1 (cont.)







Sun JH, et al, Fig. 1

FIG. 2

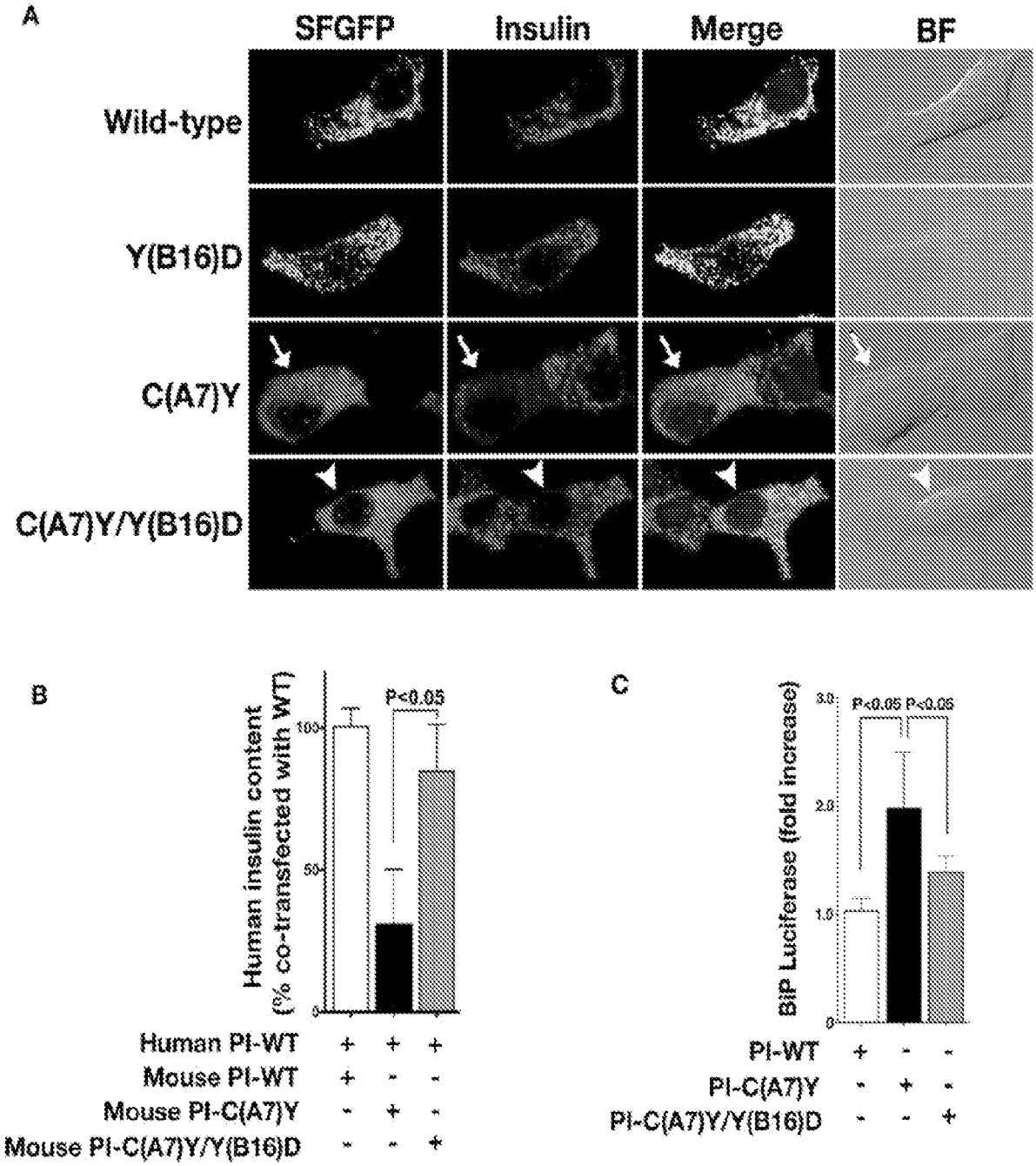

A
SFGFP
Insulin
Merge
BF
Wild-type
Y(B16)D
C(A7)Y
C(A7)Y/Y(B16)D
B
Human insulin content
(% co-transfected with WT)
P<0.05
100
50
0
Human PI-WT + + +
Mouse PI-WT + - -
Mouse PI-C(A7)Y - + -
Mouse PI-C(A7)Y/Y(B16)D - - +
C
BiP Luciferase (fold increase)
P<0.05 P<0.05
3.0
2.0
1.0
0
PI-WT + - -
PI-C(A7)Y - + -
PI-C(A7)Y/Y(B16)D - - +

FIG. 3

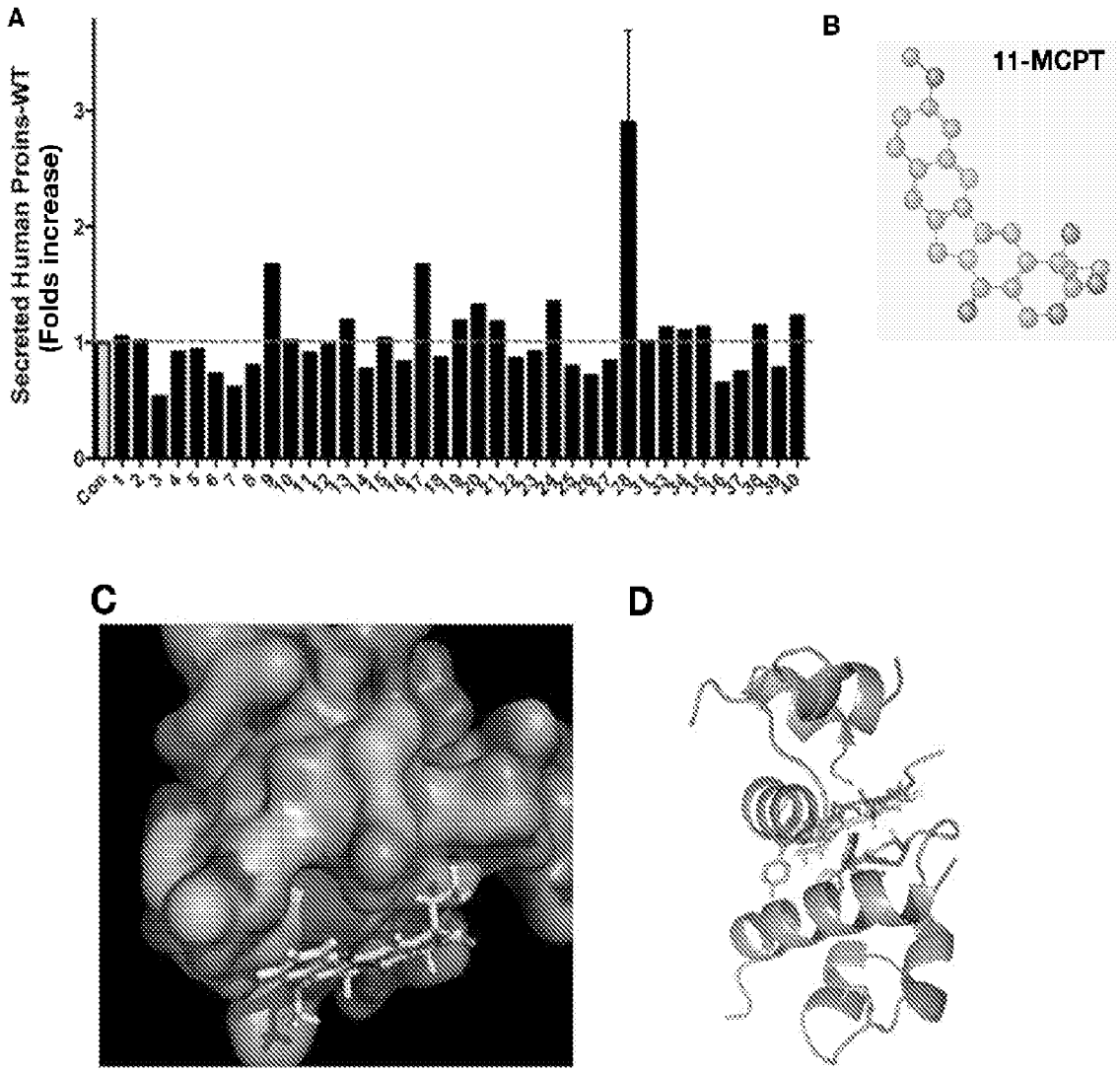
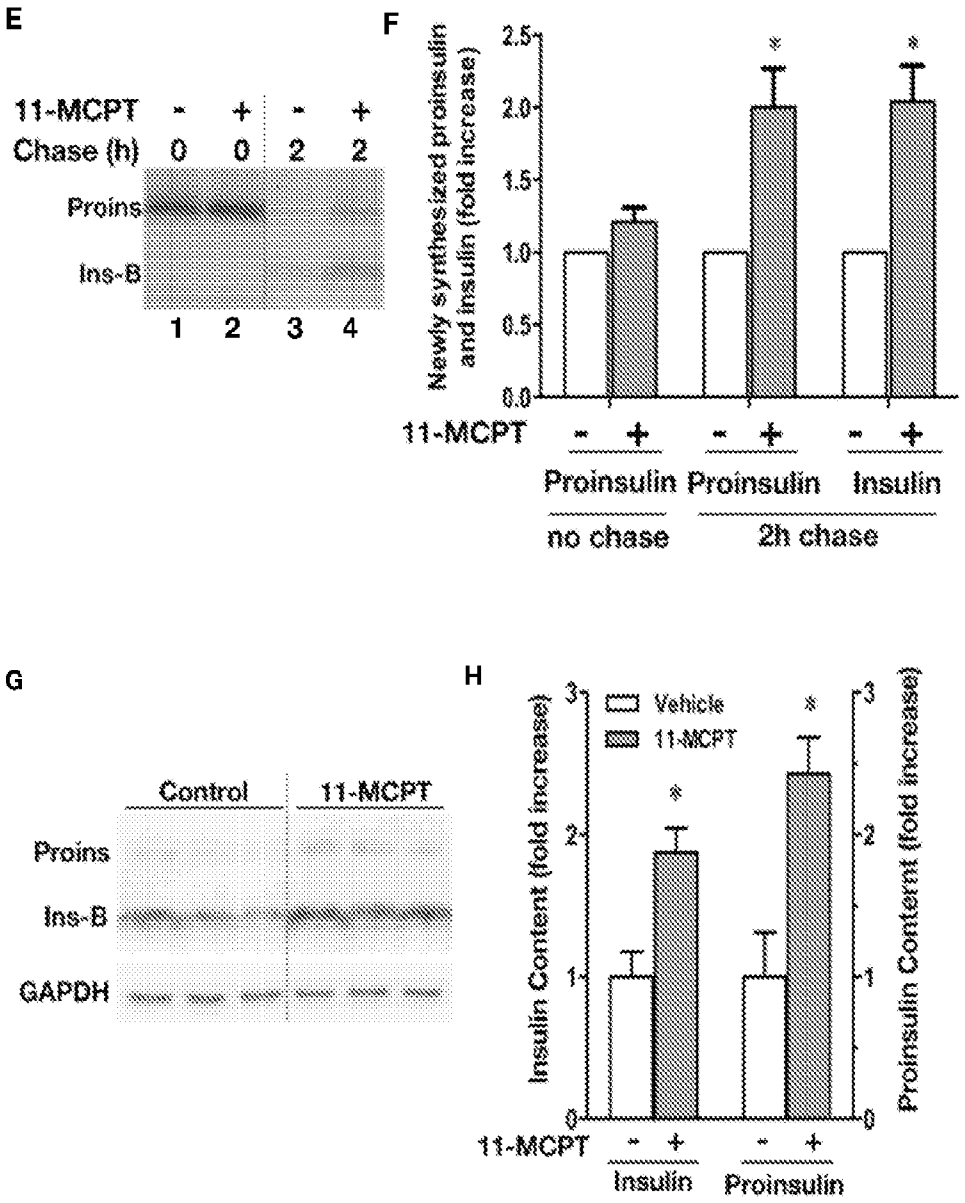


FIG. 3 (cont.)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11-MCPT - + - +
Chase (h) 0 0 2 2
Proins
Ins-B
1 2 3 4
F
Newly synthesized proinsulin and insulin (fold increase)
2.5
2.0
1.5
1.0
0.5
0.0
11-MCPT - + - + - +
Proinsulin Proinsulin Insulin
no chase 2h chase
G
Control 11-MCPT
Proins
Ins-B
GAPDH
H
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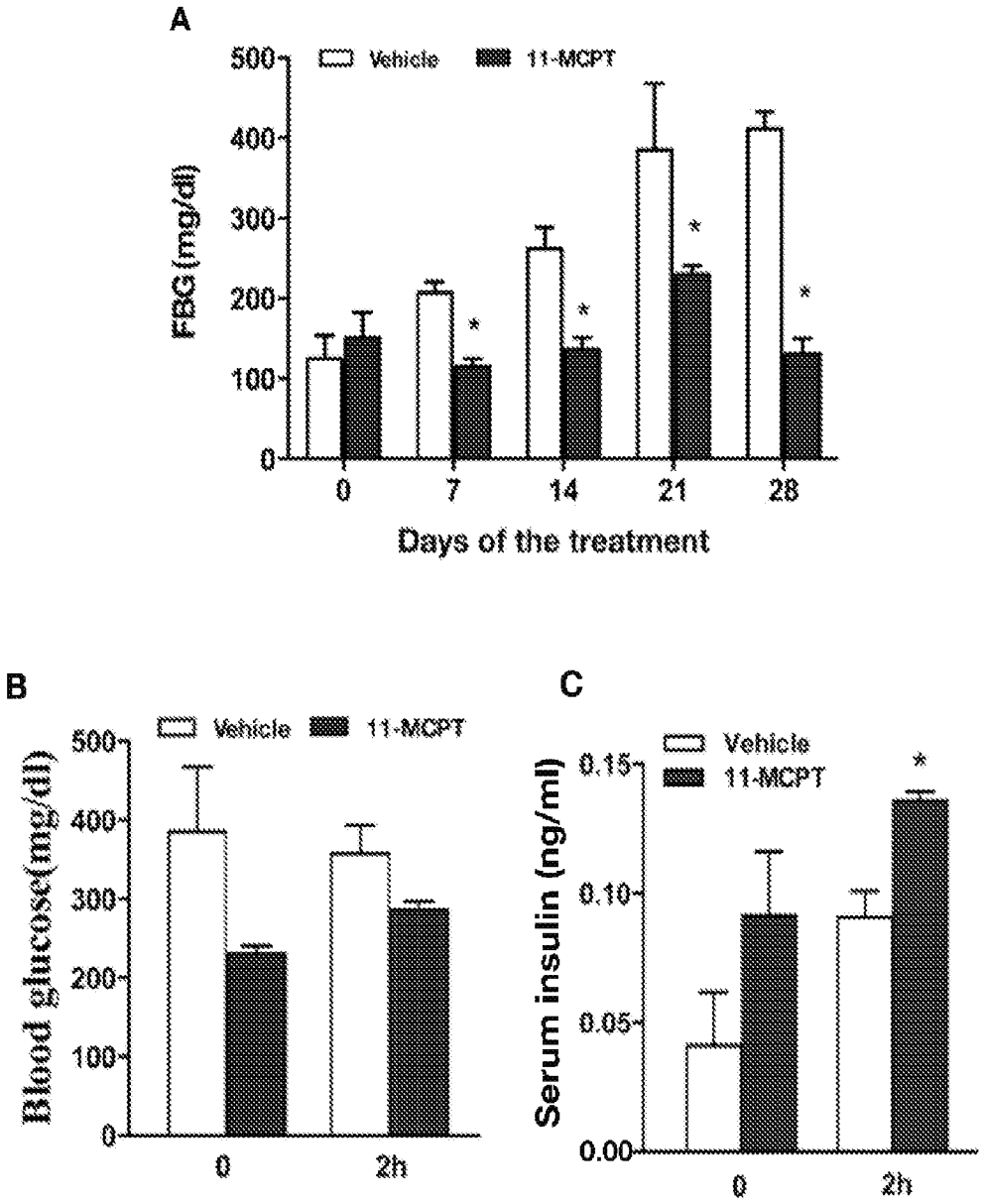
Proinsulin Content (fold increase)
Vehicle
11-MCPT
11-MCPT - + - +
Insulin Proinsulin

FIG. 4


A
Vehicle
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FBG (mg/dl)
500
400
300
200
100
0
0
7
14
21
28
Days of the treatment
B
Vehicle
11-MCPT
Blood glucose(mg/dl)
500
400
300
200
100
0
0
2h
C
Vehicle
11-MCPT
Serum insulin (ng/ml)
0.15
0.10
0.05
0.00
0
2h

FIG. 4 (cont.)

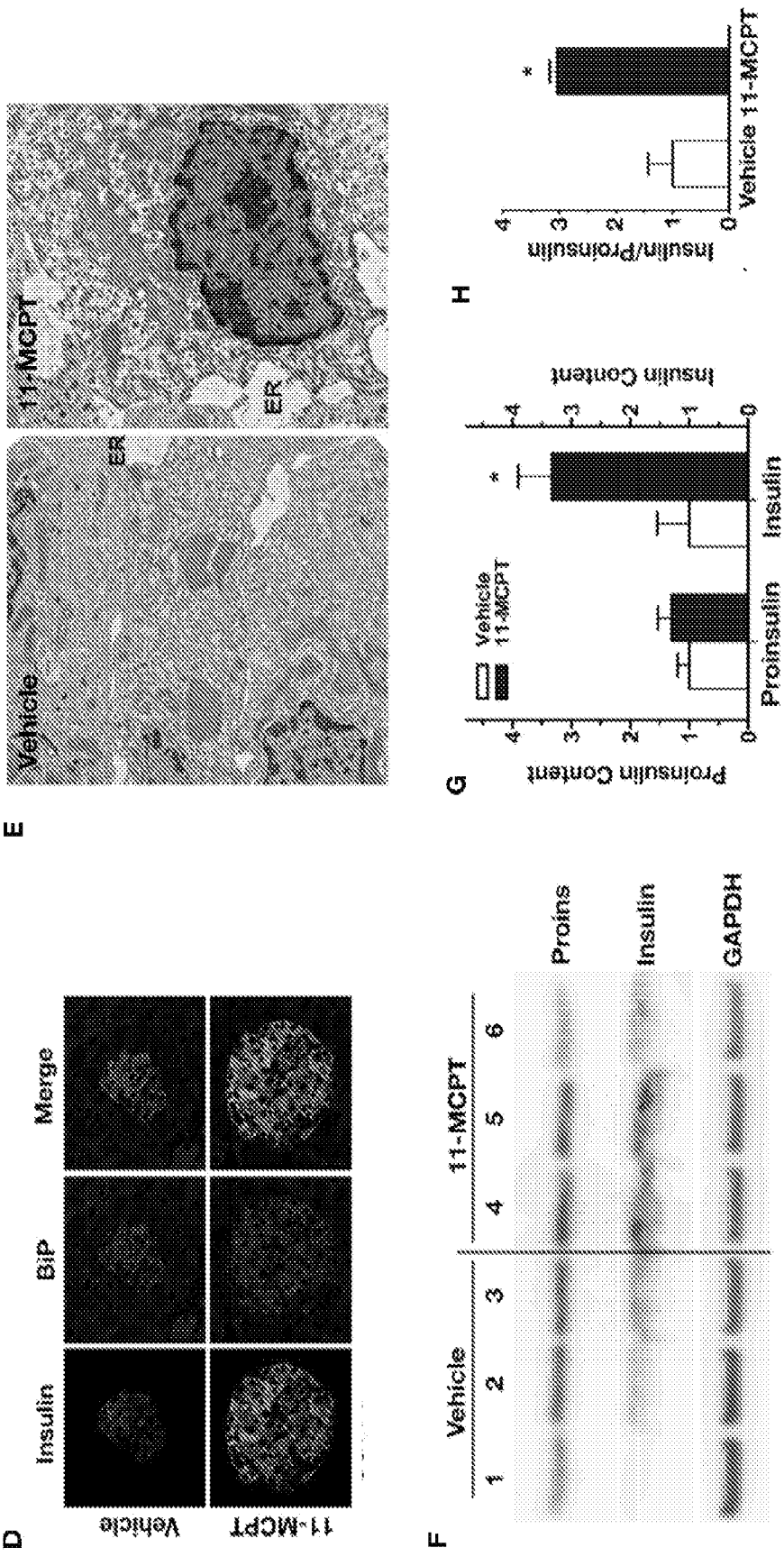

D
Vehicle
11-MCPT
Insulin
BiP
Merge
E
Vehicle
11-MCPT
ER
ER
G
Vehicle
11-MCPT
Proinsulin Content
Insulin Content
Proinsulin
Insulin
H
Insulin/Proinsulin
Vehicle
11-MCPT
F
Vehicle
11-MCPT
1
2
3
4
5
6
Proins
Insulin
GAPDH

FIG. 5

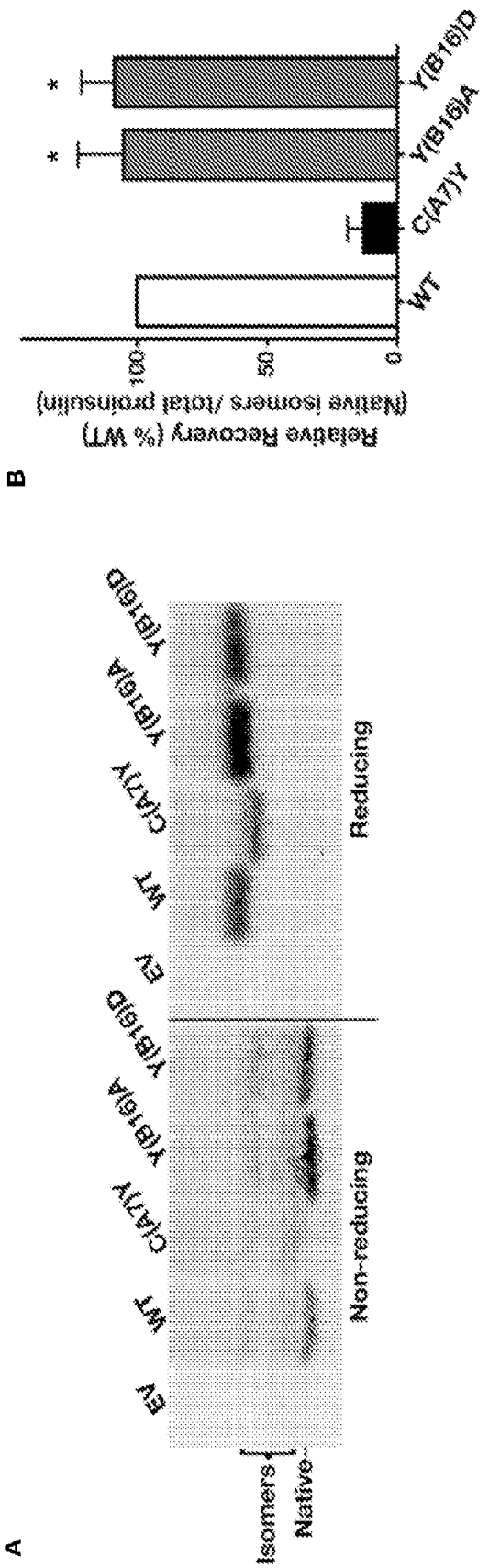
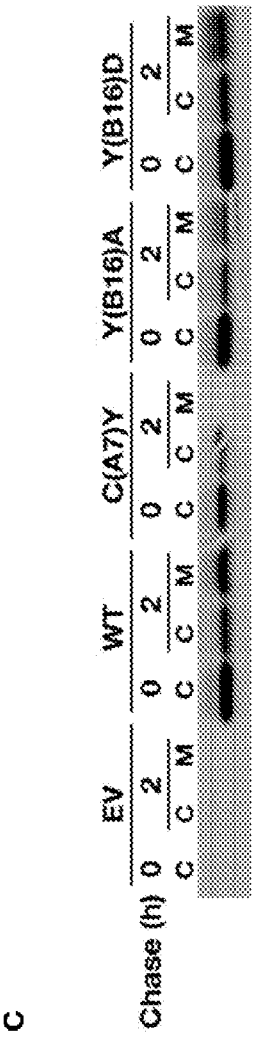

B
Relative Recovery (% WT)
(Native isomers /total protinsulin)
100
50
0
WT
C(A7)Y
Y(B16)A
Y(B16)D
*
*
A
Isomers
Native
EV
WT
C(A7)Y
Y(B16)A
Y(B16)D
EV
WT
C(A7)Y
Y(B16)A
Y(B16)D
Non-reducing
Reducing

FIG. 5 (cont.)


C
Chase (h)
EV
WT
C(A7)Y
Y(B16)A
Y(B16)D
0
2
C
M

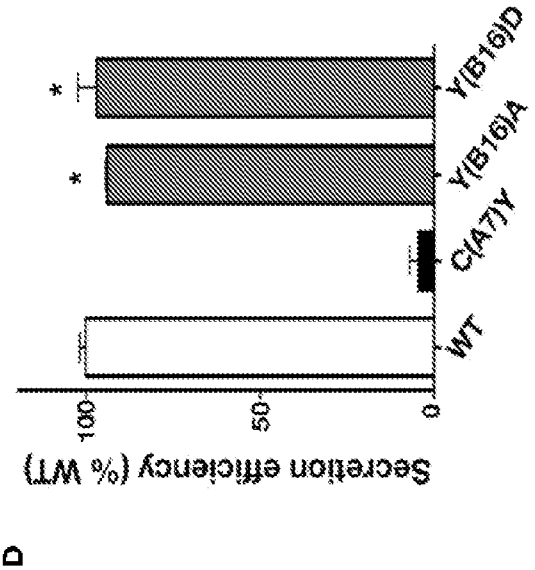

D
Secretion efficiency (% WT)
100
50
0
WT
C(A7)Y
Y(B16)A
Y(B16)D
*
*

FIG. 6

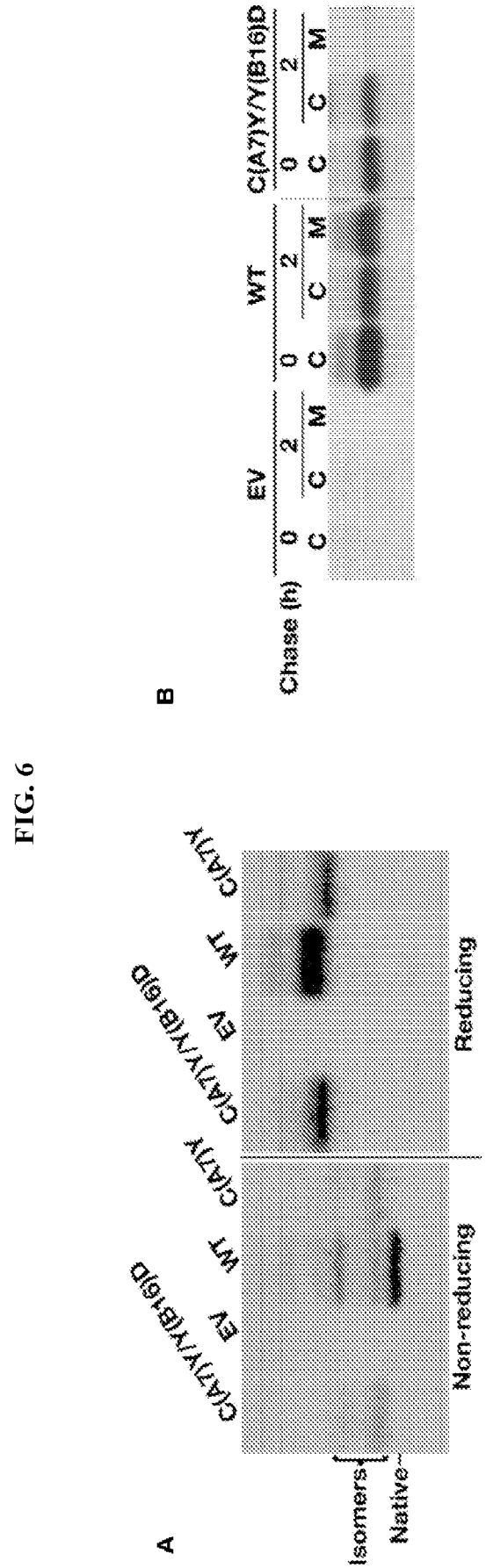

A
Isomers
Native
Non-reducing
Reducing
C(A7)Y/Y(B16)D
EV
WT
C(A7)Y
C(A7)Y/Y(B16)D
EV
WT
C(A7)Y
B
Chase (h)
0
2
0
2
C
C
M
C
C
M
EV
WT
C(A7)Y/Y(B16)D
0
2
0
2
C
C
M
C
C
M

FIG. 7

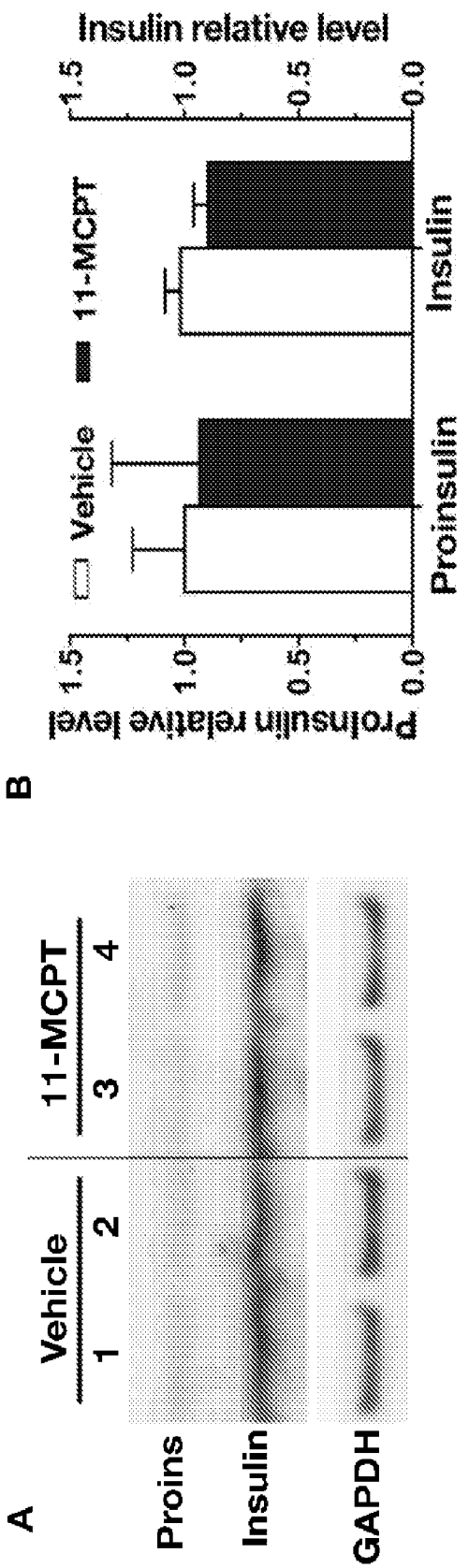

A
Vehicle
1
2
11-MCPT
3
4
Proins
Insulin
GAPDH
B
Proinsulin relative level
1.5
1.0
0.5
0.0
Vehicle
11-MCPT
Proinsulin
Insulin relative level
1.5
1.0
0.5
0.0
Insulin

FIG. 8

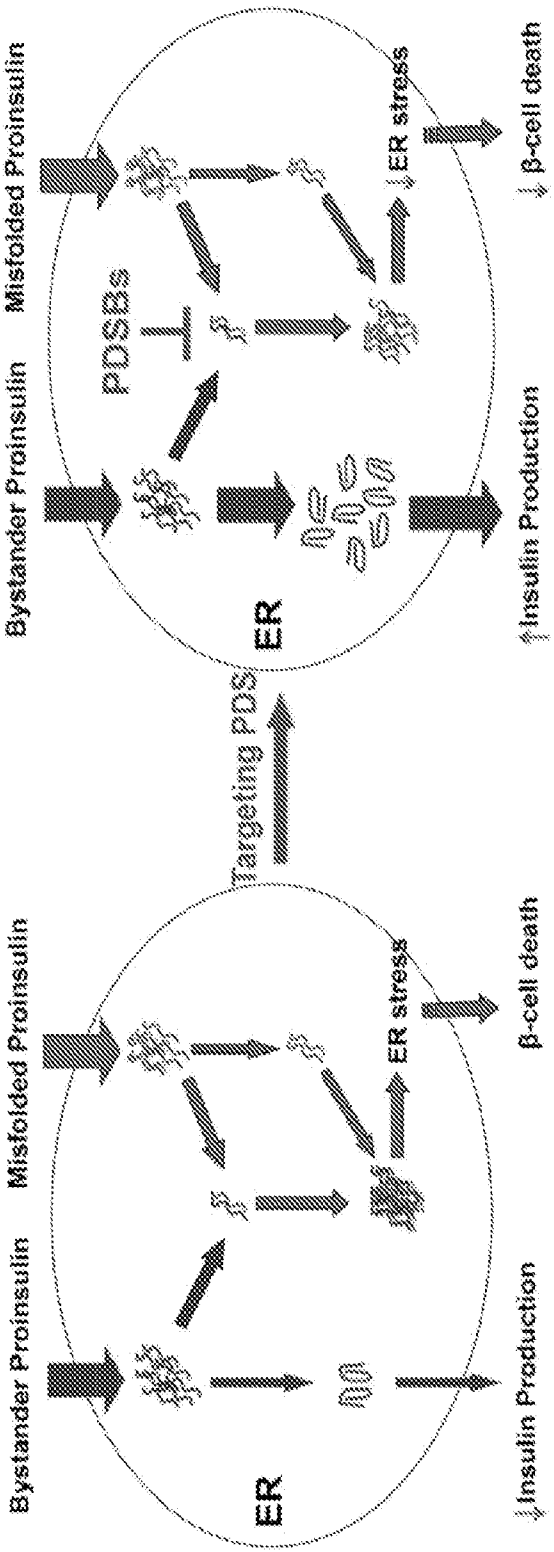

Bystander Proinsulin
Misfolded Proinsulin
PDSBs
ER
↑Insulin Production
↓ER stress
↓β-cell death
Targeting PDS
Bystander Proinsulin
Misfolded Proinsulin
ER
↓Insulin Production
ER stress
β-cell death

FIG. 9

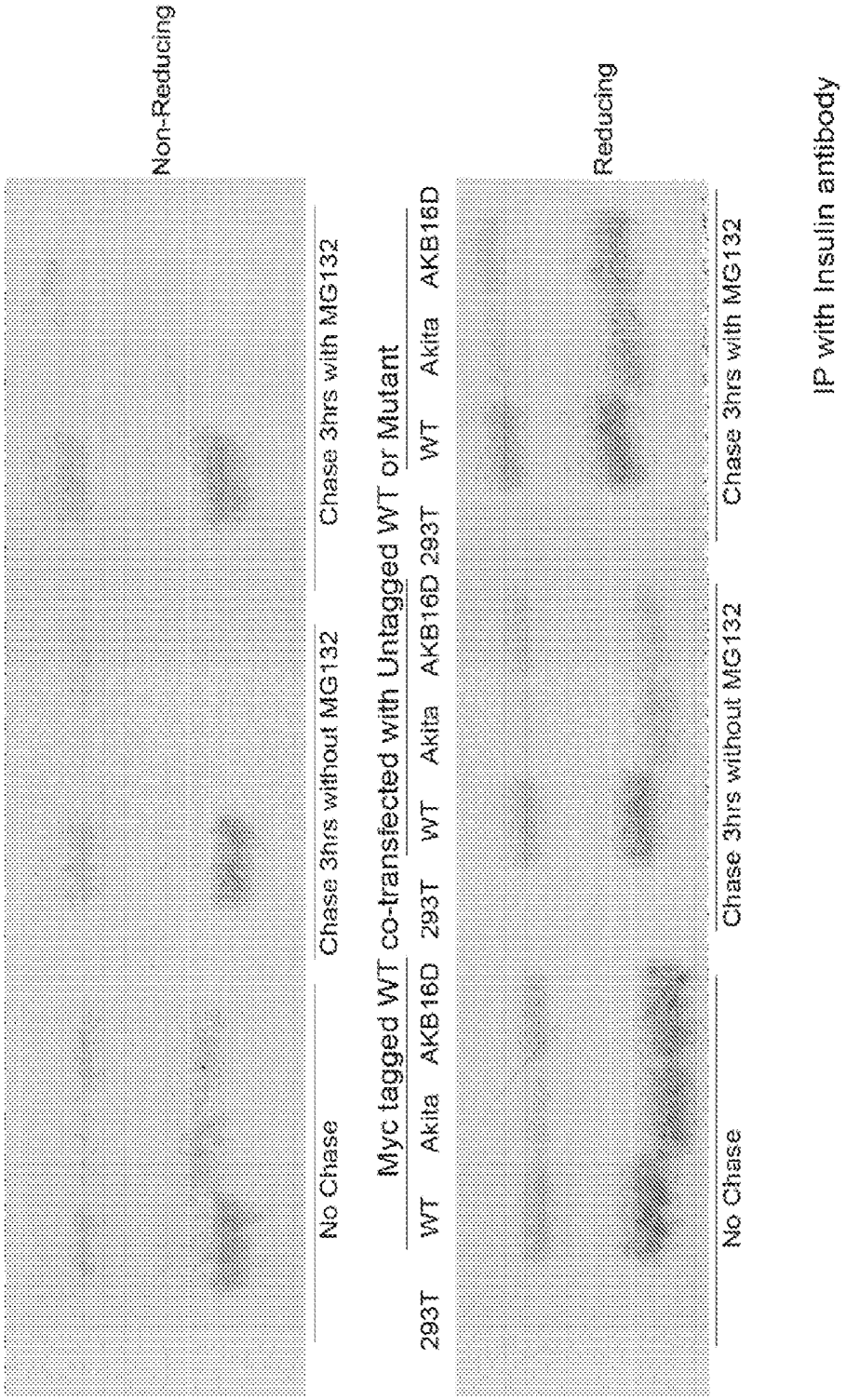

Non-Reducing
Reducing
Myc tagged WT co-transfected with Untagged WT or Mutant
293T
WT
Akita
AKB16D
293T
WT
Akita
AKB16D
No Chase
Chase 3hrs without MG132
Chase 3hrs with MG132
IP with Insulin antibody

FIG. 10

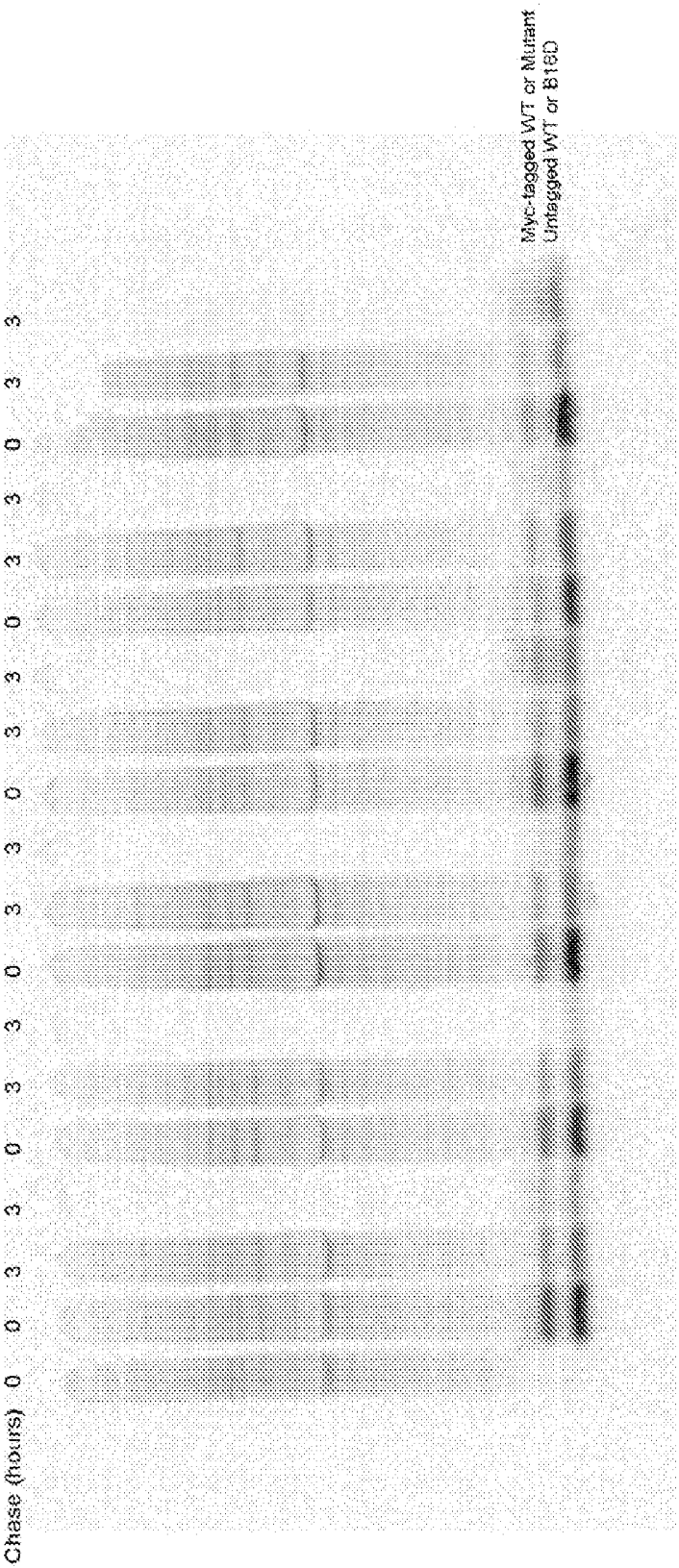

293T
Chase (hours) 0
untagged mouse WT +myc-WT or Mut
hWT
hAKITA
hAK-B16D
C C M C C M C C M
0 3 3 0 3 3 0 3 3
untagged mouse B16D +Myc-WT or Mut
hWT
hAKITA
hAK-B16D
C C M C C M C C M
0 3 3 0 3 3 0 3 3
Myc-tagged WT or Mutant
Untagged WT or B16D

FIG. 11

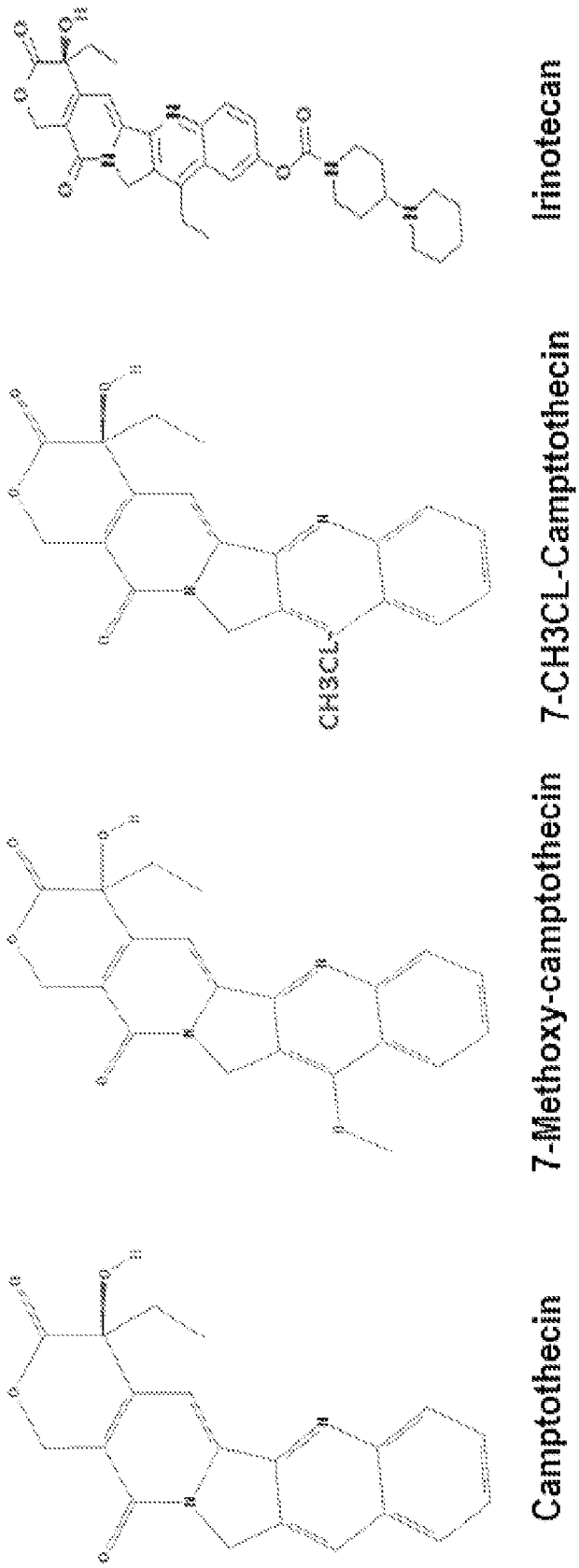

CH3CL
Camptothecin
7-Methoxy-camptothecin
7-CH3CL-Camptothecin
Irinotecan

FIG. 12

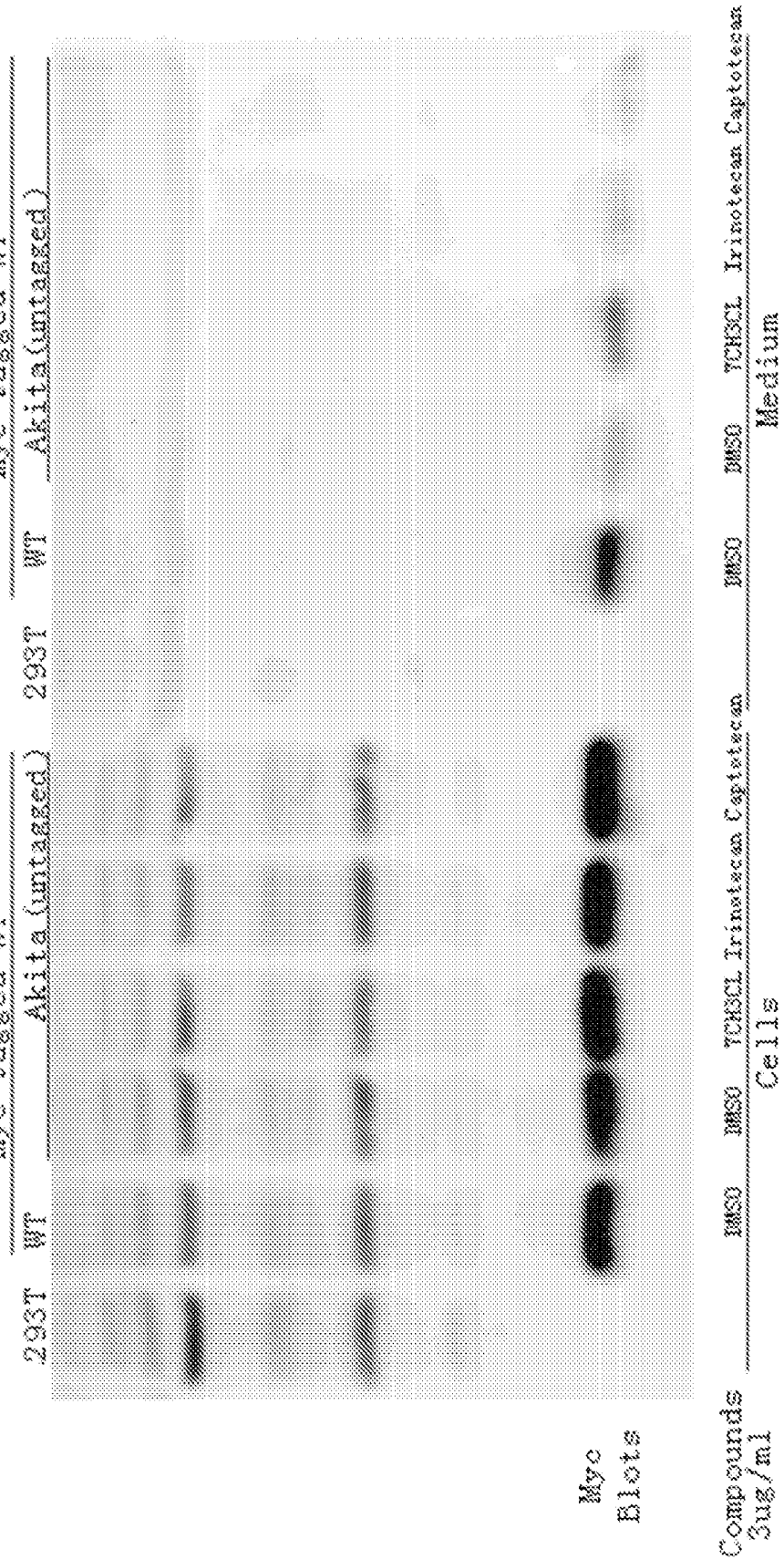
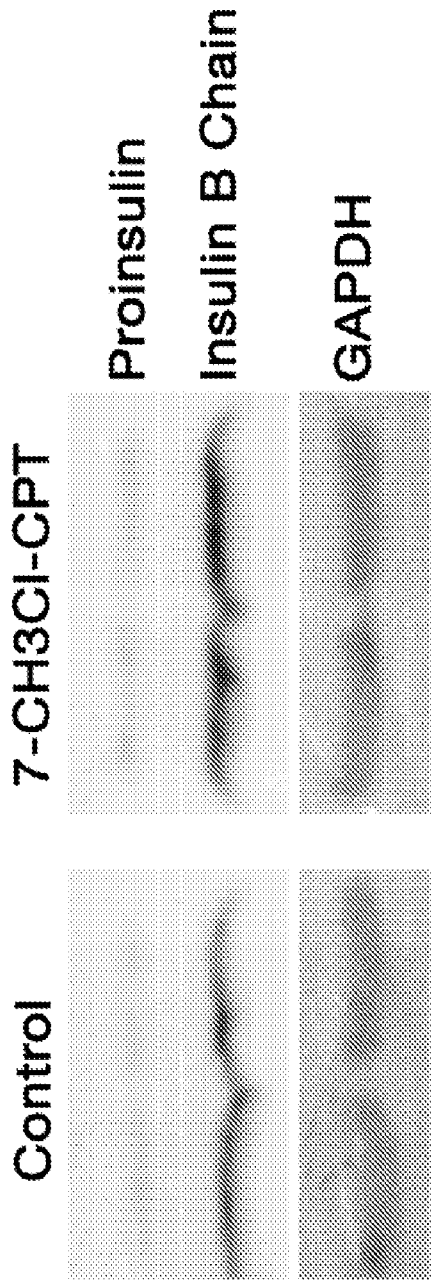

293T WT Myc-tagged WT Akita(untagged)
293T WT Myc-tagged WT Akita(untagged)
Myc Blots
Compounds 3ug/ml
DMSO DMSO TCH3CL Irinotecan Captotecan
Cells
DMSO DMSO TCH3CL Irinotecan Captotecan
Medium

FIG. 13



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/30214

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07D 491/147; A61P 3/10; C07K 14/62 (2017.01)
CPC - C07D 491/147; C07K 14/62

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
See Search History Document

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 7,994,186 B2 (NAKAZAWA et al.) 09 August 2011 (09.08.2011) col 2, ln 63-66	8-11
Y	WINTER et al. 'Catalytic Activity and Chaperone Function of Human Protein-disulfide Isomerase Are Required for the Efficient Refolding of Proinsulin', THE JOURNAL OF BIOLOGICAL CHEMISTRY, 2002, Vol. 277, No. 1, pp. 310-317. pg 311, col 1, para 2; pg 312, col 2, para 1-2; Fig 1; pg 313, col 1, para 3 to pg 314, col 1, para 1; Fig 3; Fig 4	1-7, 12-13
Y	US 2007/0162985 A1 (MOSE LARSEN et al.) 12 July 2007 (12.07.2007) para [0022], [0170]-[0173], [0179], Table Ib; Table 2	1-7, 12-13
Y	US 2010/0015046 A1 (GOVINDAN et al.) 21 January 2010 (21.01.2010) para [0002], [0018], [0060], [0119]; claim 1	7
A	CSORBA et al. Abnormal proinsulin congeners as autoantigens that initiate the pathogenesis of Type 1 diabetes, Medical Hypotheses, 2005, Volume 64, Issue 1, Pages 186-191. abstract; Fig 3	1-7, 12-13
A	LIU et al. 'Mutant INS-Gene Induced Diabetes of Youth: Proinsulin Cysteine Residues Impose Dominant-Negative Inhibition on Wild-Type Proinsulin Transport', PLoS ONE 2010, 5(10): e13333, pages 1-13. abstract; pg 2, col 1, para 2	1-7, 12-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"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
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Date of the actual completion of the international search	Date of mailing of the international search report
07 July 2017	14 AUG 2017

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774